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Research Article

Effects of Green Light Supplementation with Red and Blue Combinations of LED Light Spectrums On The Growth of Chlamydomonas Reinhardtii (Chlorophyta)

Murat TELLİ [a,*], Dina Nabil Mohammad ALJAMILI [a]

[a] *Department of Biology, Faculty of Arts and Sciences, Bolu Abant İzzet Baysal University, Bolu, TURKEY*

** Corresponding author's e-mail address: tellim@ibu.edu.tr*

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ABSTRACT

Light management strategy regarding optimum spectral composition is a critical factor in microalgae cultivation to improve biomass and biosynthesis of valuable bioactive compounds. Recent advance in LED light technology provides unparallel opportunity to test effects of specific wavelength on physiological response of algae. In this study, we investigated effects of white, monochromatic and combination of red (628 nm) and blue (462 nm) light in the ratio of 1: 1; 2: 1 and 1: 2 at the total light intensity of 40 µmol photons m^{-2} s^{-1} on the growth of *Chlamydomonas reinhardtii*. Moreover, effects of green light (518 nm) supplementation on growth of algae, green light (518 nm) was added gradually into the combination of red:blue (1:2) at the light intensity of 3, 6, 9, 12 and 15 µmol photons m^{-2} s^{-1} as an expense of red and blue light intensity at the ratio of 1:2. Results reveal that growth rate of *C. reinhadhtii* was found in the order of red:blue (1: 2) > red:blue (2: 1) > red:blue (1: 1) > red > white> blue. Green light supplementation applied as 3 µmol photons m^{-2} s^{-1} resulted in statistically significant higher optical density and dry weight than R:B (1: 2) used as control group in the experiment. Chlorophyll-a concentrations were found significantly higher in all green light supplementation than control group. Seems that 3 µmol photons m^{-2} s^{-1} supplementation of green light together with red:blue combination results in a significant promotion on growth rate, chlorophyll-a and dry weight of *C. reinhardtii*.

Keywords: *Chlamydomonas reinhardtii, Green Light Supplementation, Red Light, Blue Light, LED, Growth Rate*

Mavi ve Kırmızı Işık Spektrumları İle Birlikte Yeşil Işık Katkısının *Chlamydomonas Reinhardtii* (Chlorophyta) Büyümesine Olan Etkileri

ÖZET

Mikroalg kültürlerinde uygulanacak optimum ışıklandırma stratejilerinin belirlenmesi, biyoaktif moleküllerin biyosentezi ve biokütle artışı sağlamada önemli bir parametredir. LED ışık teknolojilerinde son yıllarda yaşanan ilerlemeler, belirli ışık spektrumlarının ve bunların farklı karışımlarının mikro alglerin fizyolojik tepkilerine olan etkilerini araştırmak için önemli fırsatlar sağlamaktadır. Bu çalışmada, beyaz ışık, kırmızı (628 nm) ve mavi (462 nm) ışık spektrumları tek başına ve sırasıyla 1:1; 2:1 ve 1:2 oranlarında toplam ışık şiddeti 40 µmol foton m^{-2} s^{-1} olacak şekilde *Chlamydomonas reinhardtii* mikroalg türünün büyümesine olan etkileri araştırılmıştır. Ayrıca, yeşil

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ışık katkısının mikroalg büyümesine olan etkileri, kırmızı:yeşil (1:2) ışık karışımında 3, 6, 9, 12 ve 15 µmol foton m^{-2} s^{-1} şiddetindeki mavi ışık şiddeti azaltılıp yerine yeşil ışık katkısı sağlanarak araştırılmıştır. Elde edilen sonuçlara göre, *C. reinhadhtii* mikroalg türünün büyüme oranları büyükten küçüğe doğru sırasıyla kırmızı:mavi (1: 2) > kırmızı:mavi (2: 1) > kırmızı:mavi (1: 1) > kırmızı > beyaz> mavi ışık olarak bulunmuştur. Yeşil ışık katkısında ise, 3 µmol foton m^{-2} s^{-1} şiddetinde mavi ışıkla yer değiştirilen yeşil ışığın, mikroalg büyümesinde ve biokütle artışında kontrol grubu olarak kullanılan kırmızı:mavi (1:2) grubuna göre istatistiksel olarak kayda değer bir artış sağladığı bulunmuştur. Yeşil ışık katkısının uygulandığı tüm deneysel gruplarda kontrol grubuna oranla klorofil konsantrasyonunda kayda değer bir artış gözlemlenmiştir. Bu çalışmada 3 µmol foton m^{-2} s^{-1} şiddetindeki yeşil ışık katkısının kırımızı:mavi ışık karışımı ile birlikte mikroalg büyümesinde, klorofil-a konsantrasyonunda ve kuru ağılık eldesinde kayda değer bir artışa neden olduğu bulunmuştur.

Anahtar Kelimeler: *Chlamydomonas reinhardtii, Yeşil Işık Katkısı, Kırmızı Işık, Mavi Işık, Büyüme, LED*

I. INTRODUCTION

In recent past decades, microalgae have emerged as a promising alternative and renewable energy resources in different industrial sectors of biotechnology. Developing in bioreactor systems applied for mass cultivation of microalgae enable to extract high value of bioactive compounds such as protein, fatty acids, lipids, vitamins, phycobilins and carotenoids in industrial scale for production of biofuel, food additive component, nutraceuticals, and pharmaceuticals ingredients [1]-[4].

The design of effective illumination systems integrated into photobioreactors is one of the key parameters for lower operating cost of algae production. Although, using natural sunlight has been widely used as the most cost-effective illumination source, it is highly fluctuated in light intensity and quality over day depending on regional and seasonal variations. Algae cultures are often subjected to light limiting during early and late hours of daytime or photo-inhibition condition in mid of day [5]. Light emitting diodes (LEDs) with narrow light spectra, high conversion rate and low heat emission provide effective and controllable illumination systems compared to fluorescent and halogen lambs for photobioreactors [6]. Recent advance in LED light technology enable to researchers to use a variety of LEDs emitting from far red to purple to investigate the physiological effects of specific wavelength and their combinations on photosynthetic efficiency of algae. Therefore, species specific optimization of LED light illumination has become an essential research effort to reap maximum yield from microalgae cultures.

Previous studies showed that spectral composition of red, and blue light have a significant impact on regulation of photosynthesis, energy conversion, morphology, cell cycle and biochemical composition of microalgae [7-9]. Baer et al. [10] reported in their study utilizing different combination of red and blue light on different microalgae species that blue light spectrum enhances the function of photosystem I while red light enhances photosystem II. In *Nannochloropsis sp*., blue light promotes biomass productivity and lipid biosynthesis [11]. Astaxanthin accumulation is enhanced under high irradiance of blue light illumination in *Haematococcus pluvialis*. [12]. Li et al. [13] shows lipid and carbohydrate synthesis are promoted under red-orange light combination, and protein synthesis under monochromatic blue light in *C. reinhardtii*. It has been reported that combination of red and blue light spectrums results in higher biomass, primary and secondary products of algae species than monochromatic light [6]. A complex antagonistic and synergetic interaction especially between phytochrome and cryptochrome photoreceptors regulating physiological response might be an explanation for attained higher biomass under red and blue light combinations [14].

However, green light as a supplementary light source in combination with red and blue light has not received attention due to its low absorption rate from chlorophyll. Studies associated with monochromatic green light applications on algae and plants has recorded the lowest biomass and cell proliferation compared to monochromatic blue and red light [10]. The only record associated with algae is published by Kalmaoğlu [15]. showed that green light supplantation significantly promotes growth rate of *Haematococcus pluvialis*. Moreover, Kim et al. [16]. addressing a potential increase in lettuce growth under 24 % green light supplementation in combination with red and blue spectrums. However, there is no comprehensive literature on supplemental of green light effects on physiological and molecular mechanisms of plant and algae species. It has been considered that higher light penetration rate of green light into plant canopy may promote light harvesting rate of plants in lower canopy [16]-[17].

In the present study, we aimed to investigate optimum spectral light quality to improve growth rate of *C. reinhardtii* by using monochromatic and dichromatic blue and red LED light spectrum in different proportion. Moreover, we used gradually increased green light supplementation into red: blue light combinations to investigate its putative additive effects on growth parameters of *C. reinhardtii*.

II. MATERIAL AND METHODS

A. STRAINS and CULTURE CONDITIONS

C. reinhardtii was obtained from the culture collection of algae at UTEX (Utex number: 89, Massachusetts, USA) The axenic stock culture was maintained in a 250 mL conical flask containing 200 mL of modified BG-11 medium [18]. The medium consisted of $NaNO_3$, 1.5 g; K_3HPO_4, 0.4 g; $MgSO_4*7H_2O$, 75 mg; $CaCl_2*2H_2O$, 36 mg; $C_6H_8O_7$, 6 mg; Ammonium Fe (III) citrate, 0.006 g; Na_2 EDTA*$2H_2O$, 1 mg; Na_2CO_3, 20 mg; H_3BO_3, 2.9 mg; $MnCl_2*4H_2O$, 1.8 mg; $ZnSO_4*7H_2O$, 0.22 mg; $Na_2MoO_4*2H_2O$, 0.39 mg; $CuSO_4*5H_2O$, 0.08 mg; $Co(NO_3)*6H_2O$, 0.05 mg Thiamine-HCl, 0.1 mg per liter of deionized water. The algal cultures were illuminated from the bottom of conical flask at a light intensity of 40 µmol photons m^{-2} s^{-1} white LED light sourced at of 25 ± 0.5 °C in a cycle of 18 h light and 6 h dark. Aeration was supplied by air bubbling through a sterile membrane filter of 0.2 µm in pore size at a rate of 250 mL min^{-1}, supplemented with 5 % CO2 to maintain the pH between 7.2 and 7.5.

B. EXPERIMENTAL CONDITIONS:

Two separate experiments (named as light quality and green light supplementation) were conducted. In light quality experiment we investigate effects of white, monochromatic and combination of red (peak value 628 nm) and blue (peak value 462 nm) light in the ratio of 1:1; 2:1 and 1:2 at the total light intensity of 40 µmol photons m^{-2} s^{-1}. In green light supplantation experiment, green light (peak value 518 nm) was supplemented gradually into the combination of red: blue (1: 2) at the light intensity of 3, 6, 9, 12 and 15 µmol photons m^{-2} s^{-1} as an expense of red and blue light intensity at the ratio of 1:2. Applied LED light conditions are given in Table 1. LED light systems is composed of 12V RGB 50/50 LED strips connected to an Arduino based computer control system. Light combinations were adjusted by changing electric currency of each R-G-B connections on LED strips using a modified Arduino software (Figure 1). Light intensities were measured by OHSP-350 portable light meter (Hopoocolor, China) from the center of the culture flask. Treatments were isolated by light proof dark plexiglass and replicated 3 times.

***Figure 1**. Arduino based computer controlled addressable RGB LED light system used for the experiments.*

***Table 1**. Applied LED light intensities and combinations in the experiments.*

Light Quality Experiment	Light intensity µmol photons m^{-2} s^{-1}	Green Light Supplementation Experiment	Light intensity of R:G:B µmol photons m^{-2} s^{-1}
White	40	R:B (1:2)	13:0:27
Blue	40	R:G:B -1	12:3:25
Red	40	R:G:B -2	11:6:23
R:B (1:1)	20:20	R:G:B -3	10:9:21
R:B (2:1)	27:13	R:G:B -4	9:12:19
R:B (1:2)	13:27	R:G:B -5	8:15:21

C. GROWTH ANALYSES

The growth rate algae cultures were monitored by measuring optical density measured at 664 nm using VWR (UV-6300 PC model) double beam UV visible spectrophotometer. Biomass was measured by dry mass. 10 mL culture were filtered through pre-dried and pre-weighted 0.2-µm cellulose nitrate membrane filters (Sartorius) and dried at 60°C over- night. Chlorophyll-a concentrations were measured spectrophotometrically according to [19] with slight modifications: 4mL homogenized sample was centrifuged for 5 min at 5000 rpm, and the remaining pellet was re-suspended in 1 mL of absolute methanol and incubated overnight in dark at 4 °C after vigorous vortex. Then, the solution was centrifuged for 5 min at 5000 rpm, and the supernatant quantified spectrophotometrically at a wavelength of 665 and 652 nm. Chlorophyll-a was calculated as µg/ml using the formula 16.72* A_{665} - 9.16*.A_{652} where A_{665} and A_{652} absorbance values at 665 and 652 nm respectively.

III. RESULTS

Growth rate of *C. reinhardtii* measured as optical density under monochromatic blue and red-light source did not sow significant differences then white light. There are significant higher growth rates measured under all red: blue combination of 1:1, 2.1 and 1:2 compared to white light (Figure 2 (a), Table 2 (a)). However, there is no statistically significant differences between red: blue combinations of 1:2 and 2:1. That might indicate, red or blue dominated fractions of combinations do not result in a significant difference in *C. reinhardtii*.

Table 2. *Bonferroni p-value of Post-hoc Tukey HSD test for (**a**) growth rate, (**b**) chlorophyll-a and (**c**) dry weight mg mL^{-1} of C. reinhardtii in different LED light quality. (statistically significance p<0.05*, p<0.01**).*

Light Combinations (a) Growth	**WHITE**	**RED**	**BLUE**	**R:B (1:1)**	**R:B (2:1)**	**R:B (1:2)**
WHITE		1.620	3.352	**0.03***	**0.00****	**0.00****
RED			0.159	0.805	**0.00****	**0.00****
BLUE				**0.003****	**0.00****	**0.00****
R:B (1:1)					**0.029***	0.221
R:B (2:1)						4.347
(b) Chlorophyll-a	WHITE	RED	BLUE	R:B (1:1)	R:B (2:1)	R:B (1:2)
WHITE		**0.00****	**0.045***	6.362	4.351	11.290
RED			**0.000****	**0.000****	**0.000****	**0.000****
BLUE				**0.021***	**0.013***	**0.029***
R:B (1:1)					11.770	9.340
R:B (2:1)						6.719
(c) Dry Weight	WHITE	RED	BLUE	R:B (1:1)	R:B (2:1)	R:B (1:2)
WHITE		0.229	0.115	0.899	0.498	0.653
RED			0.899	0.576	**0.012***	**0.019***
BLUE				0.347	**0.005****	**0.009****
R:B (1:1)					0.183	0.283
R:B (2:1)						0.899

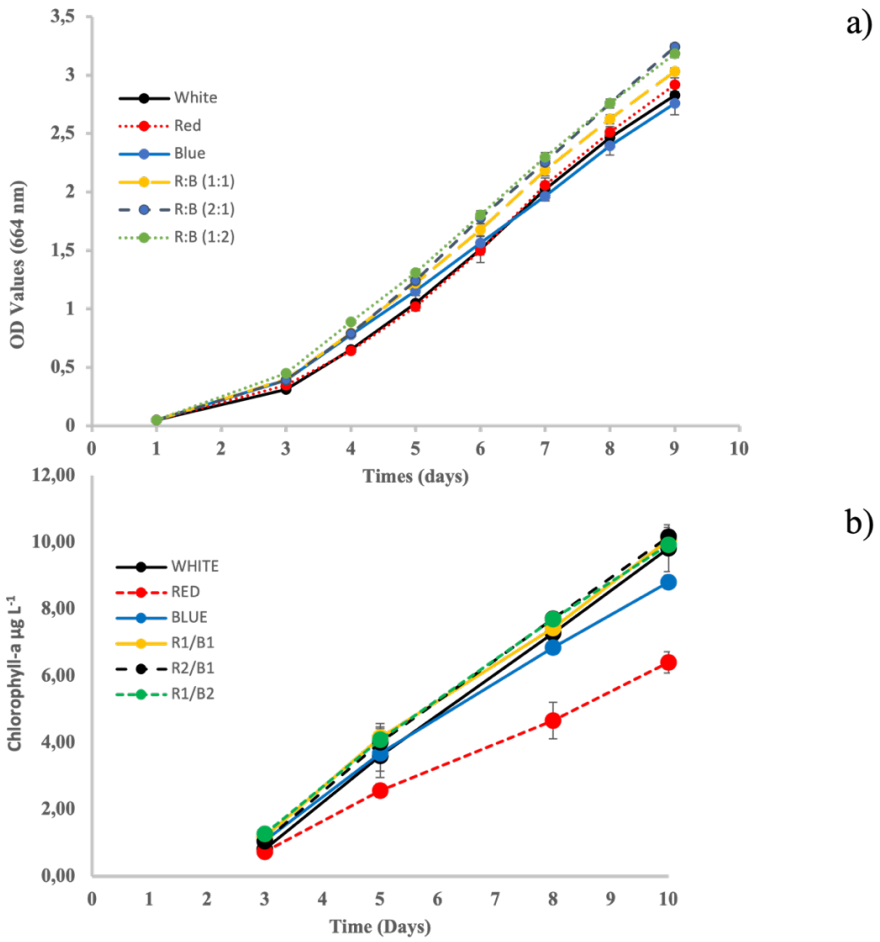

Figure 2. *(**a**) Optical density changing and (**b**) chlorophyll-a concentration of C. reinhardtii cultures in different LED light quality.*

Chlorophyll-a concentrations show a consistent pattern with growth rates that the lowest chlorophyll-a concentration was found in monochromatic red and blue light applications (Figure 2 (b); Table 2 (b)). However, the differences between white and red: blue combinations are not statistically significant. Associated with dry weight, it is not promoted by red: blue light combinations. Monochromatic blue and red light result in a significant decrease in dry weight compared to red: blue combinations of 1:2 and 2:1 ratios (Table 3 and 2 (c)).

***Table 3**. Dry weight of C. reinhardtii with standard deviations measured at the end of the light quality and green light supplementation experiments.*

Light Quality Experiment	Dry Weight (mg mL^{-1})	Standard Deviation	Green Light Supplementation Experiment	Dry Weight (mg mL^{-1})	Standard Deviation
White	0.175	0.04	R:B (1:2)	0.183	0.07
Blue	0.105	0.04	R:G:B -1	0.275	0.05
Red	0.120	0.04	R:G:B -2	0.207	0.06
R:B (1:1)	0.213	0.08	R:G:B -3	0.230	0.03
R:B (2:1)	0.213	0.02	R:G:B -4	0.263	0.06
R:B (1:2)	0.200	0.03	R:G:B -5	0.233	0.03

Based on the highest growth rate measured on light quality applications, we choose R:B (1:2) to test if green light supplementation promote growth rate of *C. reinhardtii*. Growth rate given in Figure 3 (a) show that green light supplemented as 3, 6, 9, 12 and 15 µmol photons m^{-2} s^{-1} as an expense of red and blue light intensity at the ratio of 1:2 respectively promote growth rate compared to R:B (1:2). Although, results are not statistically significant except for R:G:B-1 (3 µmol photons m^{-2} s^{-1} supplementation), decreasing red and blue light quantity up to 15 µmol photons m^{-2} s^{-1} did not result in decrease in growth rate (Table 4 (a)). Chlorophyll-a concentrations between green light supplementation treatments did not show statistically differentiation, but all supplemented groups are significantly higher chlorophyll-a concentration than R:B (1:2) used as control group in the experiment (Figure 3 (b) and Table 4 (b)). Dry weight results have also consistent pattern with growth rates that only R:G:B-1 (3 µmol photons m^{-2} s^{-1} supplementation) is statically higher dry weight compared to R/B (1:2) (Table 3 and 4 (c)). Seems that 3 µmol photons m^{-2} s^{-1} supplementation of green light promote growth rate, chlorophyll-a and dry weight of *C. reinhardtii*.

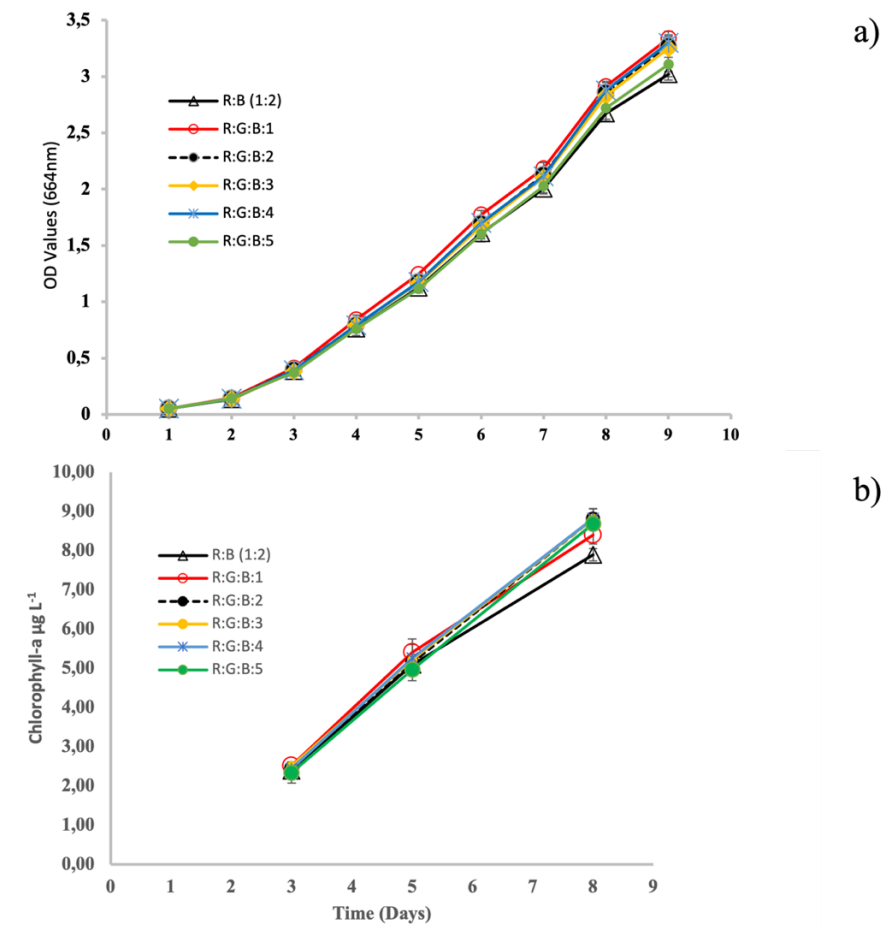

Figure 3. *(**a**) Optical density changing and (**b**) chlorophyll-a concentration of C. reinhardtii cultures in different green light supplementation.*

Table 4. *Bonferroni p-value of Post-hoc Tukey HSD test for growth rate (**a**), chlorophyll-a (**b**) and dry weight mg mL-1 (**c**) of C. reinhardtii in green light supplementation experiment. (*statistically significance for p<0.05, **statistically significance for p<0.01).*

Light Combinations (a) Growth	**R:B (1:2)**	**R:G:B -1**	**R:G:B -2**	**R:G:B -3**	**R:G:B -4**	**R:G:B -5**
R:B (1:2)		**0.016***	0.073	0.012	0.039	0.859
R:G:B -1			0.899	0.859	0.899	0.102
R:G:B -2				0.899	0.899	0.385
R:G:B -3					0.899	0.488
R:G:B -4						0.227
(b) Chlorophyll-a	R:B (1:2)	R:G:B -1	R:G:B -2	R:G:B -3	R:G:B -4	R:G:B -5
R:B (1:2)		0.089	**0.006****	**0.001****	**0.001****	**0.005****
R:G:B -1			0.611	0.224	0.194	0.560
R:G:B -2				0.899	0.899	0.899
R:G:B -3					0.899	0.899
R:G:B -4						0.899
(c) Dry Weight	R:B (1:2)	R:G:B -1	R:G:B -2	R:G:B -3	R:G:B -4	R:G:B -5
R:B (1:2)		**0.045***	0.899	0.840	0.419	0.798
R:G:B -1			0.115	0.272	0.672	0.305
R:G:B -2				0.899	0.714	0.899
R:G:B -3					0.899	0.899
R:G:B -4						0.899

IV. DISCUSSION

Previous studies regarding optimum spectral light quality strategies to improve algae growth showed that blue and red-LED light combinations promote microalgae biomass compared with monochromatic or full spectrum of white light [20], [23]. However, results of related studies are not consistent and show species dependent pattern about optimal red and blue rations. For example, red:blue ratios have been reported for as 1:3 for *Haematococcus lacustris* by Tran et al. [23]. 1:1 for *Phaeodactylum tricornutum* and *Isochrysis galbana* and *Nannochloropsis salina* by Ra et al. [22], 1:3 for *Dunaliella salina* by Fu et al. [24], 3:7 for *Arthrospira platensis* by Lima et al. [25]. Results in the present study are consistent with the literature. Growth rate of *C. reinhadhtii* was found in the order of red:blue - 1:2 > red:blue - 2:1 > red:blue - 1:1 > red > white> blue. Although red: blue combinations result in high optical density of algae cultures, red or blue dominated fraction rates seems not make a statistical significant differences between 2:1 and 1:2 of red: blue combinations (Table 2). Interestingly, under 1:1 ratio of red: blue combination growth rate is significantly decreased than 1:2 and 2:1 combination. Red dominated fraction rates may enhance excitation energy of PSII that might over-simulate PSI by promoting electron transfer to PSI over plastoquinone, cyt b_6f and plastocyanin complex. In blue dominated fraction rates, PSI may over-simulated directly by blue light together with electron transfer from PSII. Therefore, it might be logical to conclude that either in red or blue light fractionally dominated combination, reaction center of PSI is over-stimulated and increase photosynthetic activity compared to equal fractional rate of red and blue light combination in *C. reinhadhtii* [14], [10]. Chlorophyll ratios were also found significantly higher in red: blue combinations and white lights than monochromatic red and blue light in *C. reinhadhtii*. There are also significant differences between blue and red light. It has been reported that chlorophyll and carotenoid biosynthesis is mainly controlled by phototropin photoreceptors regulating blue light mediated changes in green algae of *Scenedesmus obliquus* and *Chlorella vulgaris* [26]. That might also be a case for blue light in *C. reinhardtii*. The same patter was observed for dry weight between monochromatic and dichromatic light source, red: blue combinations resulted in significant higher dry weight (Table 2 and 3).

The second experiment was performed to test if green light supplementation promote growth rate of *C. reinhadhtii*. As a general concept, green light spectrum is not absorbed and mostly reflected by plants and microalgae. Indeed, monochromatic green light applications results in the lowest photosynthetic activity in both plant and algae species. Therefore, green light has not received attention and mostly ignored in the literature. However recent publications showed that green light might have an important role regulating light harvesting, photosynthesis, and carbon assimilation in plant species [27], [16]. Terashima et al. [28] and Nishio [29] reported that depending on species, only 10-50 % of green light is reflected by chloroplast, the rest is absorbed by plant pigments. It is predicted that green light might have a vital role in CO_2 assimilation, signaling cascades regulation response of plant to changing in environmental conditions and promoting growth rate [27]. Green light might be used not only as photon source for photosynthesis but also as an information for signaling of light availability to acclimate shade conditions in lower canopy and control response of red and blue spectrum. However, the aforementioned studies only addressed higher plant species and there is no record on an algae species how to response to green light supplementation. Only study performed by Kalmaoğlu [15] on green algae *Haematococcus pluvialis* showed that algae species might have a similar function with higher plant in response to green light supplementation. Kalmaoğlu [15] reported that 20% green light supplementation with red and blue light combination result in around 20% increase in growth rate and upregulate expression of CO_2 assimilation gene Ribusco and light harvesting complex gene of PsbS in PSI.

In this study, green light supplementation was applied with red: blue (1:2) combinations that was recorded as highest growth rate of *C. reinhardtii*. We used a gradient green light supplementation dose of 3, 6, 9, 12 and 15 µmol photons m^{-2} s^{-1} as an expense of red and blue light intensity at the ratio of 1:2. Light intensity was kept constant at 40 µmol photons m^{-2} s^{-1} for all treatments against the bias

sourced from applied photon flux differences. Results show 10% increase in growth rate in 3 μmol photons m^{-2} s^{-1} green light supplementation than red: blue (1:2) combinations. This results show a dose dependent green light enhancement effects together with red: blue combinations in growth rate of *C. reinhardtii*. Moreover, although OD values are not statistically significant except for R:G:B-1, none of treatments show decreasing in growth rate compared to red: blue combinations even the supplementation rate was 15 μmol photons m^{-2} s^{-1} that composed of %38 of total light intensity. That might indicate a compensation capacity of green light supplementation against decreasing light intensity of red: blue combination in growth rate of *C. reinhardtii*. However, it is still an unsolved mechanism, how plant and algae harvest green light spectrum.

Carotenoids pigments such as xanthophylls having absorbance range extend into green region (400-550 nm) have been argued as a one of possible mechanism to harvest green light spectrum [30]. Xanthophylls is a key element of photoprotective mechanisms by dissipating excess energy into non-photochemical quenching under high irradiance. It has also transfer some of this excess energy to chlorophyll to be used in photosynthesis [31], [32]. Therefore, it might be logical to argue that xanthophylls pigments may have a role in harvesting green light spectrum to enhance growth rate in algae. The other argument as a putative electron acceptor mechanism form green light region is non-photosynthetic photoreceptors cryptochromes and tryptophan that absorb UV-A and blue light [33-35]. Sellaro et al. [35] showed that flavin adenine dinucleotide (FAD) light absorbing chromophore of cryptochrome may also absorbed green light region and transfer electron energy between flavin molecules during the redox reactions. However, it is not clear that if cryptochrome or uncharacterized another receptor regulate electron capture from green light and transfer the energy to be used in growth or developmental process in plant or algae.

V. CONCLUSION

LED light technology provide unparallel opportunity in plant and microalgae research to reveal effects of spectral light quality in photosynthesis and developmental process. That improve our knowledge to design species specific optimally balanced spectral LED system for higher growth in microalgae and plant. Results in this study show parallel with Kalmaoğlu [15] and Kim et al. [16] showing that dose dependent green light supplementation together with red:blue combinations can be used to increase growth rate of microalgae. Therefore, green light needs to be considered to use in horticultural LED light systems in indoor greenhouse applications for both plant and microalgae production.

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