LIGHTING APPROACHES TO IMPROVE GROWTH IN CONTROLLED ENVIRONMENT SYSTEMS

by

RUQAYAH HAMIDA BHUIYAN

(Under the Direction of Marc W. van Iersel)

ABSTRACT

Controlled environment agriculture (CEA) is often used to realize year-round production in the horticultural sector. In CEA systems, supplemental lighting can account for a large percentage of operating costs (15 - 30%). The research aims to decrease production cost and increase profits for growers. Results from our first study indicate that 'Little Gem' and 'Green Salad Bowl' lettuce tolerate fluctuating light levels, suggesting that regulating supplemental light in response to real-time electricity prices is feasible for CEA. Our results in the far-red lighting study indicate that lettuce biomass and size increase as the amount of supplemental far-red light increases, suggesting supplemental far-red is effective in CEA. The lack of far-red effects on *LsXTH8* expression indicate that other genes may play a role in the leaf expansion effects seen under far-red light. More research is needed to determine which cell wall loosening or cell division genes are involved.

INDEX WORDS: assimilation, chlorophyll, *Lactuca sativa*, light-emitting diodes, photosynthesis, photosynthetic photon flux density, variable electricity prices, far-red, leaf expansion

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DEDICATION

I dedicate this thesis to my family. Every one of them had an integral role in my development as a person. To my best friends in the whole world, my brothers Tawfiq, Tawhid, Ridwan, and Safwan Bhuiyan. They have made me a stronger person and have encouraged me to chase every dream I had and will have. To my mother Sherryann Bhuiyan, a constant comfort, and a refuge during my times of need and struggle, and during my times of total elation. To my father Haider Bhuiyan for instilling a sense of hard work and the pursuit of knowledge as the key to life. To my two sisters-in-laws, Bushra Alfaraj and Tiffany Eberhard. They have taught me to be a better woman and to never compromise who I am. To the loves of my life, Yunus Oswald and Isa James Bhuiyan. Thank you for coming into my life and bringing this family so much joy. Finally, to Dr. Paul Thomas. The man who expanded my horizon and managed to deal with all my craziness. I “got it done” PT and had you by my side the whole time!

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

Introduction and Literature Review

Introduction

Horticulture is a vast subject matter that enters the realm of science but is truly an art form. Horticultural crops include fruits, flowers, vegetables, and ornamental plants. There are many forms of horticulture, from farms to nurseries, greenhouses to plant factories, and flower shops to retail/ wholesale businesses. The horticultural industry contributes greatly to the U.S. economy and accounts for $13.8 billion in floricultural sales, $703 million in food crop sales (grown under protection), and $370 million in the sales for vegetable and strawberry transplants (USDA, 2019). To facilitate the production of these crops, most are started or grown completely, especially in the case of high value crops, in some form of controlled environment systems. Controlled environment agriculture (CEA) allows for the optimization of plant growth in areas where the natural environment would otherwise not allow. It can also extend the production season for growers, ensuring freshness for market, production security, and profitability (Sipos et al., 2020).

In CEA systems, natural light and ambient temperatures are often the limiting factor. To compensate for this, supplemental lighting and heaters are used to create ideal growing environments. CO_2 enrichment is often used to increase the efficiency of supplemental lighting, thereby increasing photosynthesis and growth, making the use of those lights more economical (Both et al., 1997; Ferentinos et al., 2000). However, supplemental lighting can be costly. It can

account for 30% of total operating costs in greenhouses (van Iersel and Gianino, 2017) and 40–50% in plant factories, either to provide the light or to remove the heat generated by the light fixtures (Watanabe, 2011; Zeidler and Schubert, 2014). To mitigate those costs, light emitting diodes (LED) can be used. This form of supplemental light has high upfront cost but uses less energy and is more easily controlled (van Iersel and Gianino, 2017). The flexibility of LEDs can give growers the ability to use supplemental lights only when needed which can reduce operating costs and increase profitability.

To aid growers in getting the benefits of supplemental lighting, more research is needed to understand how plants respond to changes in their lighting environment. Plants are resilient organisms and can respond to changes in their environment within seconds. Altering how much light a plant receives, what type of light, and when that light is provided requires a deeper understanding of photosynthesis and plant growth. This thesis outlines two altered growing environments and analyzes growth and physiological responses to those environments.

Literature Review

Controlled Environment Agriculture

Growing plants in a controlled environment system allows for a very precise method of crop production, often leading to near-perfect commodities for the market. In greenhouses and plant factories, growers can control the amount of light a plant receives, the temperature, CO_2 concentration, and humidity (Albright et al., 2000). Commonly-grown crops in controlled environment systems include leafy greens, such as lettuce, mustards, and kales, as well as vining crops such as tomatoes and strawberries. Field-grown tomatoes (1.85 pounds per ft^2) have an 82% lower production rate per square foot compared to greenhouse-grown tomatoes (10.59 pounds per ft^2), while field-grown lettuce (0.69 pounds per ft^2) has a 92% lower production rate per square foot compared to greenhouse-grown lettuce (8.71 pounds per ft^2) (Agrilist, 2017). Controlled environments allow for higher yields and year-round production despite day-to-day and seasonal fluctuations in light level and temperature. This is of particular importance in higher latitudes where the fluctuations in light level, DLI, and temperature are more severe (Faust and Logan, 2018).

Photosynthesis

Light energy in the context of photosynthesis is measured as photosynthetic photon flux density (PPFD), most simply described as the number of photons landing on a square meter surface per second with units of $\mu mol \cdot m^{-2} \cdot s^{-1}$. Higher PPFDs usually increase photosynthesis asymptotically and lead to higher biomass production. The reactions that occur in plants under light are very rapid. Photosynthesis has two main sets of reactions: light reactions and carbon reactions. The light reactions convert light energy into electron transport and ultimately chemical

energy. The products of these reactions are used in the carbon reaction. When carbon metabolism is limited, this downregulates electron transport and can affect overall photosynthetic rates.

Light is absorbed by the plant, via the photosynthetic pigments chlorophyll and carotenoids, in the form of photons. Those photons excite electrons, and that excitation energy can be used by two types of reaction centers, photosystems I and photosystem II, to drive electron transport. The biochemical processes that occur via linear electron flow form a proton gradient (pH change) across the thylakoid membrane and result in the production of ATP. Electrons moving in linear electron transport via ferredoxin result in the production of NADPH. The electrochemical gradient of protons drives ATP synthase which produces ATP. ATP and NADPH are then used in the carbon reactions of photosynthesis, in the Calvin- Benson cycle (CBC) (Ruban, 2015). Not all absorbed light energy is used in photochemistry and this will be discussed later in this review.

Large fluctuations in PPFD or the type of photosynthetically active radiation (PAR) may affect CBC activity. The products of the light reactions determine the rate of activation of key CBC enzymes and the biochemical reactions within that cycle. Fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoribulokinase require thioredoxin, produced from reduced ferredoxin, for the reduction of regulatory disulfides and are all activated by products of the light reactions (Michelet et al., 2013). These enzymes can limit photosynthesis by limiting the regeneration of ribulose 1,5-bisphosphate (RuBP). Rubisco is inactive in the dark, during which metabolites bind to its active site. It is dependent on Rubisco activase to remove those metabolites in order to start functioning again in the light (Zhang and Portis, 1999). Rubisco activase activity depends on ATP (Portis Jr,

A. R.,1995; Kleczkowski, 1994) and relies on a high stroma pH, which results from hydrogen transport from the stroma into the lumen in the light reactions.

Horticultural Lighting

Conventional forms of horticultural lighting, such as high pressure sodium, metal halide lamps, or fluorescent lamps, often require more energy in comparison to light emitting diodes (LEDs). LEDs have a higher efficacy. They require less energy, operating at cooler temperatures, and lose less energy from the generation of heat. LEDs also come in a range of different colors and the intensity of light can be controlled. LEDs also allow growers to control the type of spectra the plants receive during growth, which can help in maximizing growth (Schratz et al., 2016). The studies in this thesis utilized the dimmability of LEDs to better understand growth response to changes in the lighting environment.

Imaging: Projected Canopy Size

The light reactions of photosynthesis generate essential energy molecules, ATP and NADPH, necessary for the carbon reactions of photosynthesis. However, excess light energy can be harmful to the photosynthetic apparatus, especially Photosystem II (PSII), leading to photoinhibition (Pinnola, 2019). Damage occurs in the D1 proteins of reaction centers when the light energy exceeds the processing ability (Pinnola 2019). Nonphotochemical quenching of chlorophyll fluorescence (NPQ, qE) aids plants in the dissipation of light energy as heat (Roach and Kreiger-Liszkay 2012) and chlorophyll fluorescence (a small fraction of total energy, 1-2%) provides a path of energy dissipation. Chlorophyll fluorescence from the plants can be used in imaging as a nondestructive method of tracking plant growth, and to determine the incident light, the amount of light being received by the plant at a given moment. Imaging proved useful for Elkins and van Iersel (2020) in response to different PPFD and photoperiod treatments, all with

the same daily light integral. There was a positive correlation between projected canopy size (calculated from chlorophyll fluorescent imaging) and final dry mass at various time points. This provided important information on canopy photosynthesis and early treatment effects.

Fluctuating Light Systems

There is extensive research on sunflecks and fluctuating light levels in a natural environment. Many understory plants have adapted to low light levels and have evolved to respond to high light for short periods of time. Fluctuating lights occur naturally in many understory environments due to changes in weather pattern (wind, rain, etc.), at different times of the year, and due to the movement of leaves, branches, and the sun. Sunflecks are a form of irradiance that can provide up to 8 mins of valuable light energy to understory plants. Other forms of fluctuating irradiance are sun patches (more than 8 mins), sun gap (60 mins), and clearings (120 mins) (Kaiser et al. 2018). However, little is known on the effects of fluctuating light levels in a controlled environment operation, greenhouse, or plant factory.

The cost of providing lighting can be high. In the case of variable electricity prices, providing most of the light when electricity prices are low can reduce costs. Dynamic algorithms that control supplemental lighting in response to variable sunlight conditions (Seginer et al., 2006) could be used along with real time pricing in the greenhouse industry. Such algorithms have been described (Clausen et al., 2015; Harbick et al., 2016; Sørensen et al., 2016), but it is not clear if they have been implemented in such operations.

We hypothesized that plants that receive a constant PPFD will produce more biomass than those grown under fluctuating light levels. To understand potential growth reductions caused by fluctuating light levels, we quantified the effects of fluctuating lights on the photosynthetic physiology, morphology, and growth of ‘Little Gem’ and ‘Green Salad Bowl’

lettuce. Results suggest an ability of lettuce to tolerate a wide range of fluctuating light levels that can be adjusted in response to variable electricity pricing.

Far Red and Leaf Morphology

Photosynthetically active radiation (PAR) is defined as the spectral range between 400 and 700 nm that plants utilize for photosynthesis. However, this range leaves out far-red and ultraviolet light, potentially integral to increasing photosynthetic rates. Far-red light is between 700-800 nm. It is detected by plants via a photoreceptor family, phytochrome (P). Phytochrome has an active state (Pfr) and an inactive state (Pr) (Kalaitzoglou et al., 2019). Understory plants are typically exposed to light with a high fraction of far-red light and phytochrome allows them to sense that they are shaded. This can induce shade avoidance responses that can alter leaf morphology and plant development. One way that plants respond to far-red light is by increasing leaf expansion and/or plant height. Increase in canopy size can increase canopy photosynthesis which can lead to increases in biomass accumulation.

Far-red light not only alters plant morphology, it also can directly affect photosynthesis. In combination with shorter wavelengths of light, far-red can increase photosynthetic rates (Emerson et al., 1957). Zhen and Bugbee (2020) reported an increase in gross photosynthesis by 6.7-20% with an addition of 40-140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of supplemental far-red (background white light, 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Similar results were observed with the addition of 10-35% of white light on top of the background PPFD of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. This indicates that far-red photons may have the same photosynthetic effectiveness as shorter wavelength photons (Zhen and Bugbee, 2020).

Many researchers have observed growth effects due to supplemental far-red light. One way that far-red light effects on the state of phytochrome are measured is the phytochrome

photostationary state (PSS). This is defined as the ratio of Pfr to total P. Increased dry weight and fruit yield was reported in tomatoes with increasing far-red level (low PSS) (Kalaitzoglou et al., 2019). Zou et al. (2019) observed an increase in dry weight by 39% and 25% with the addition of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of supplemental far-red during the day or at end of day, respectively. Leaf area also increased by 49% when far-red was applied during the day and by 27% at end of day (Zou et al., 2019). Legendre and van Iersel (2020), the inspiration for this study, also observed increases in leaf area, projected canopy size, and leaf expansion (length and width) with increasing far-red light.

Little is known about the genetic factors that play a role in controlling leaf morphological responses to far-red light. The two main modes of leaf growth are cell division and cell expansion. It has been reported that far-red light can increase rates of cell division in young leaves, but only as the red to far-red ratio increase (far-red light decreases) (Lee et al., 2015). There are several cell expansion/ cell wall loosening enzymes that may play a role in leaf expansion, in particular Xyloglucan endotransglucosylase/ hydrolase (XTH). *LsXTH* belong to a gene family, among which some members are expressed in high abundance in developing leaves that have not yet reached maturity (Wagstaff et al., 2010). Far-red light can increase leaf elongation and projected canopy size of lettuce (Legendre and van Iersel, 2020) and *LsXTH* has been shown to be involved in cell wall loosening and leaf elongation in lettuce. We thus hypothesized *LsXTH* may be upregulated in response to increasing far-red light. This relationship is analyzed in this thesis.

Literature Cited

Agrylist. (2017). State of Indoor Farming. 39 pp.

Albright, L., Both, A., and Chiu, A. (2000). Controlling greenhouse light to a consistent daily integral. *Transactions of the ASAE* 43**,** 421-431. doi: 10.13031/2013.2721.

Both, A.-J., Albright, L., and Langhans, R. (1997). Coordinated management of daily PAR integral and carbon dioxide for hydroponic lettuce production. II Modelling Plant Growth, Environmental Control and Farm Management in Protected Cultivation 456, 45-52.

Clausen, A., Maersk-Moeller, H.M., Soerensen, J.C., Joergensen, B.N., Kjaer, K.H., and Ottosen, C.O. (2015). "Integrating Commercial Greenhouses in the Smart Grid with Demand Response based Control of Supplemental Lighting", in: *International Conference on Industrial Technology and Management Science (ITMS 2015).*

Elkins, C., and van Iersel, M.W. (2020). Longer Photoperiods with the Same Daily Light Integral Increase Daily Electron Transport through Photosystem II in Lettuce. *Plants* 9**,** 1172. doi: 10.3390/plants9091172.

Emerson, R., Chalmers, R., and Cederstrand, C. (1957). Some factors influencing the long-wave limit of photosynthesis. Proc. Natl. Acad. Sci. U.S.A. 43, 133–143. doi: 10.1073/pnas.43.1.133

Faust, J.E., and Logan, J. (2018). Daily light integral: A research review and high-resolution maps of the United States. *HortScience* 53**,** 1250-1257. doi: 10.21273/hortsci13144-18.

Ferentinos, K., Albright, L., and Ramani, D. (2000). SE—Structures and Environment: Optimal Light Integral and Carbon Dioxide Concentration Combinations for Lettuce in Ventilated Greenhouses. Journal of agricultural engineering research 77, 309-315.

Harbick, K., Albright, L.D., and Mattson, N.S. (2016). "Electrical savings comparison of supplemental lighting control systems in greenhouse environments", in: *2016 ASABE Annual International Meeting*: American Society of Agricultural and Biological Engineers), 1. doi: 10.13031/aim.20162460478.

Kaiser, E., Walther, D., and Armbruster, U. (2020). Growth under fluctuating light reveals large trait variation in a panel of arabidopsis accessions. *Plants* 9**,** 316. doi: 10.3390/plants9030316.

Kalaitzoglou, P., Van Ieperen, W., Harbinson, J., Van Der Meer, M., Martinakos, S., Weerheim, K., et al. (2019). Effects of Continuous or End-of-Day Far-Red Light on Tomato Plant Growth, Morphology, Light Absorption, and Fruit Production. Front Plant Sci 10, 322. doi: 10.3389/fpls.2019.00322.

Kleczkowski, L.A. (1994). Inhibitors of photosynthetic enzymes/carriers and metabolism. *Annual review of plant biology* 45**,** 339-367. doi: 10.1146/annurev.pp.45.060194.002011.

Lee, M.-J., Park, S.-Y., and Oh, M.-M. (2015). Growth and cell division of lettuce plants under various ratios of red to far-red light-emitting diodes. Horticulture, Environment, and Biotechnology 56, 186-194.

Legendre, R., and Van Iersel, M.W. (2021). Supplemental Far-Red Light Stimulates Lettuce Growth: Disentangling Morphological and Physiological Effects. Plants (Basel) 10. doi: 10.3390/plants10010166.

Michelet, L., Zaffagnini, M., Morisse, S., Sparla, F., Pérez-Pérez, M.E., Francia, F., et al. (2013). Redox regulation of the Calvin–Benson cycle: something old, something new. *Frontiers in Plant Science* 4. doi: 10.3389/fpls.2013.00470.

Pinnola, A. (2019). The rise and fall of Light-Harvesting Complex Stress-Related proteins as photoprotection agents during evolution. *Journal of Experimental Botany* 70**,** 5527-5535. doi: 10.1093/jxb/erz317.

Portis Jr, A.R. (1995). The regulation of Rubisco by Rubisco activase. Journal of Experimental Botany, 1285-1291.

Roach, T., and Krieger-Liszkay, A. (2012). The role of the PsbS protein in the protection of photosystems I and II against high light in Arabidopsis thaliana. *Biochim Biophys Acta* 1817**,** 2158-2165. doi: 10.1016/j.bbabio.2012.09.011.

Ruban, A.V., 2015. Evolution under the sun: Optimizing light harvesting in photosynthesis. J. Exp. Bot. 66:7–23.

Schratz, M., Gupta, C., Struhs, T.J., and Gray, K. (2016). A New Way to See the Light: Improving Light Quality with Cost-Effective LED Technology. IEEE Industry Applications Magazine 22, 55-62. doi: 10.1109/mias.2015.2459089.

Seginer, I., Albright, L.D., and Ioslovich, I. (2006). Improved strategy for a constant daily light integral in greenhouses. *Biosystems engineering* 93**,** 69-80. doi: 10.1016/j.biosystemseng.2005.09.007.

Sipos, L., Boros, I.F., Csambalik, L., Székely, G., Jung, A., and Balázs, L. (2020). Horticultural lighting system optimalization: A review. *Scientia Horticulturae* 273. doi: 10.1016/j.scienta.2020.109631.

Sørensen, J.C., Kjær, K.H., Ottosen, C.-O., and Jørgensen, B.N. (2016). "DynaGrow-Multi-Objective Optimization for Energy Cost-efficient Control of Supplemental Light in Greenhouses", in: *8th International Joint Conference on Computational Intelligence (IJCCI 2016)*), 41-48.

USDA. 2019. Census of Horticultural Specialties (2017). Natl. Agr. Stat. Serv., Washington, DC

van Iersel, M.W., and Gianino, D. (2017). An adaptive control approach for light-emitting diode lights can reduce the energy costs of supplemental lighting in greenhouses. *HortScience* 52**,** 72-77. doi: 10.21273/hortsci11385-16.

Wagstaff, C., Clarkson, G.J., Zhang, F., Rothwell, S.D., Fry, S.C., Taylor, G., et al. (2010). Modification of cell wall properties in lettuce improves shelf life. *J Exp Bot* 61**,** 1239-1248. doi: 10.1093/jxb/erq038.

Watanabe, H. (2011). "Light-controlled plant cultivation system in Japan-development of a vegetable factory using LEDs as a light source for plants", in: *VI International Symposium on Light in Horticulture 907*), 37-44. doi: 10.17660/ActaHortic.2011.907.2.

Zeidler, C., and Schubert, D. (2014). "From Bioregenerative Life Support Systems for Space to Vertical Farming on Earth - The 100% Spin-off", in: *Life in Space for Life on Earth Symposium*).

Zhang, N., and Portis, A.R. (1999). Mechanism of light regulation of Rubisco: A specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. *Proceedings of the National Academy of Sciences* 96(16)**,** 9438-9443. doi: 10.1073/pnas.96.16.9438.

Zhen, S., and Bugbee, B. (2020). Far-red photons have equivalent efficiency to traditional photosynthetic photons: Implications for redefining photosynthetically active radiation. *Plant Cell Environ* 43**,** 1259-1272. doi: 10.1111/pce.13730.

Zou, J., Zhang, Y., Zhang, Y., Bian, Z., Fanourakis, D., Yang, Q., et al. (2019). Morphological and physiological properties of indoor cultivated lettuce in response to additional far-red light. *Scientia Horticulturae* 257**,** 108725.

CHAPTER 2

Only Extreme Fluctuations in Light Levels Reduce Lettuce Growth Under Sole Source Lighting[1]

[1]Bhuiyan, Ruqayah and van Iersel, Marc W. Reprinted here with permission of publisher.

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Abstract

The cost of providing lighting in greenhouses and plant factories can be high. In the case of variable electricity prices, providing most of the light when electricity prices are low can reduce costs. However, it is not clear how plants respond to the resulting fluctuating light levels. We hypothesized that plants that receive a constant photosynthetic photon flux density (PPFD) will produce more biomass than those grown under fluctuating light levels. To understand potential growth reductions caused by fluctuating light levels, we quantified the effects of fluctuating PPFD on the photosynthetic physiology, morphology, and growth of 'Little Gem' and 'Green Salad Bowl' lettuce. Plants were grown in a growth chamber with dimmable white LED bars, alternating between high and low PPFDs every 15 minutes. The PPFDs were ~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, with a photoperiod of 16 hours and a DLI of ~11.5 $mol \cdot m^{-2} \cdot d^{-1}$ in all treatments. CO_2 was ~ 800 $\mu mol \cdot mol^{-1}$. Plants in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment had ~69% lower $A_{n,30}$ (net assimilation averaged over15 minutes at high and 15 minutes at low PPFD) than plants grown at a PPFD of 320/80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (or treatments with smaller PPFD fluctuations). The low $A_{n,30}$ in the 400/0, and to a lesser extent the 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment was caused by low net assimilation at 360 and 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants grown at 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ also had fewer leaves and lower chlorophyll content compared to those in other treatments. The four treatments with the smallest PPFD fluctuations produced plants with similar numbers of leaves, chlorophyll content, specific leaf area, dry mass, and leaf area. Chlorophyll content, $A_{n,30}$, and dry mass were positively correlated with each other. Our results show that lettuce tolerates a wide range of fluctuating PPFD without negative effects on growth and development. However, when fluctuations in PPFD are extreme (400/0 or 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$), chlorophyll levels and $A_{n,30}$ are low, which can explain the low poor growth in

these treatments. The ability of lettuce to tolerate a wide range of fluctuating light levels suggests that PPFD can be adjusted in response to variable electricity pricing.

Introduction

Increased year-round demand for fresh fruits and vegetables has increased the need for productive and profitable controlled environment growing operations, such as greenhouses and plant factories. Among the most popular crops for controlled environment agriculture are various leafy greens, including lettuce (Agrylist, 2017). Because of large day-to-day and seasonal fluctuations in the daily light integral (DLI) from sunlight (Albright et al., 2000), consistent, year-round greenhouse production of lettuce may require supplemental lighting from Fall through Spring. This is especially important at higher latitudes, where seasonal fluctuations in DLI from sun are greatest (Faust and Logan, 2018). However, the light environment in greenhouses is often poorly controlled (van Iersel and Gianino, 2017) and the variable light environment makes greenhouse production less predictable. The capital and operating costs of supplemental lighting are high (Albright et al., 2000). Lighting accounts for up to 30% of total operating costs in greenhouses (van Iersel and Gianino, 2017) and 40–50% in plant factories, either to provide the light or to remove the heat generated by the light fixtures (Watanabe, 2011; Zeidler and Schubert, 2014). Reducing the cost of lighting in controlled environment agriculture can reduce operating costs and increase profitability. One potential approach to decrease the cost of supplemental lighting is the use of photovoltaic greenhouses, where part of the greenhouse roof is covered with solar panels (Emmott et al., 2015; Cossu et al., 2017). However, the resulting shading of the greenhouse crop can reduce yields (Cossu et al., 2020). In addition, photovoltaic panels generate most electricity when there is ample sunlight, so there is a

disconnect between the availability of electricity from photovoltaic panels and the need for supplemental lighting. Although the power generated by photovoltaic panels can be stored in batteries, this is expensive.

One obvious option for reducing electricity costs is to take advantage of variable electricity prices. The Light and Shade System Implementation (LASSI) algorithm can account for variable electricity prices and was shown to reduce electricity costs of greenhouse production by 8 – 37% as compared to threshold lighting control, where lights are controlled based on PPFD readings. The magnitude of the cost savings depended on location and which threshold control algorithm LASSI was compared to (Harbick et al., 2016). Sørensen et al. (2016) used a multi-objective evolutionary algorithm in their DynaGrow control system to optimize greenhouse temperature, CO_2, and supplemental lighting, based on the greenhouse environment, electricity price forecasts, and weather forecasts. DynaGrow successfully reduced energy use and cost, while resulting in similar quality plants as a standard lighting control approach. Based on this prior work, accounting for energy prices in control algorithms for supplemental light can reduce energy costs. However, Kjær et al. (2011) showed that an irregular greenhouse light environment resulted in poor flowering of *Campanula*, which could be prevented by assuring that the photoperiod was the same each day.

How fluctuating light levels affect photosynthetic physiology in controlled environments is not clear. Leaves in outdoor canopies experience changes in PPFD in the form of sunflecks, lasting anywhere from a few seconds to a few minutes, and shadeflecks, due to cloud cover, which can last hours (Knapp and Smith, 1987). The occurrence of sunflecks is dependent on movement of the sun and/or leaves higher in the canopy. Understory plants have adapted to the occurrence of sunflecks and have developed photosynthetic machinery to facilitate efficient use

of this high PPFD (Chazdon and Pearcy, 1991). When plants are exposed to high light after periods of low light or darkness, it can take 10-40 minutes for leaves to acclimate and reach steady state photosynthesis and is dependent on the duration and timing of those sunflecks (Chazdon and Pearcy, 1986;1991). Vice versa, (Kromdijk et al., 2016) showed that downregulation of photoprotective mechanisms as sunlit leaves are suddenly shaded can be slow, reducing photosynthesis of those shaded leaves. Upregulating the expression of genes encoding violaxanthin de-epoxidase, zeaxanthin epoxidase and PSII subunit S allowed plants to respond more quickly to sudden reductions in sunlight increased dry matter production of tobacco (*Nicotiana tabacum*) by 15%.

Vialet-Chabrand et al. (2017) compared the photosynthetic physiology and growth of *Arabidopsis thaliana* under four different lighting treatments, constant high or low PPFD during the entire photoperiod versus natural fluctuations in PPFD, resulting in the same DLI. Plants grown with a greater DLI had a higher light-saturated rate of photosynthesis, but whether that DLI was provided with constant or fluctuating PPFD had little impact on the photosynthetic physiology. However, fluctuating PPFD resulted in thinner leaves, decreased leaf area and shoot biomass, and increased specific leaf area (SLA), as compared to constant PPFD with the same DLI. This reduction in growth under fluctuating PPFD was at least partly explained by a greater daily net carbon gain (photosynthesis minus respiration) under constant as compared to fluctuating PPFD (Vialet-Chabrand et al., 2017). These differences in daily net carbon gain are likely cause by multiple factors. First, under fluctuating PPFD conditions, plants are required to constantly acclimate to a changing light environment, which can reduce photosynthetic efficiency and growth (Kromdijk et al., 2016). Secondly, because of the asymptotic shape of photosynthesis-light response curves (Vialet-Chabrand et al., 2017), the total photosynthesis over

the course of a day, given a specific DLI, is achieved under constant PPFD conditions (Sims and Pearcy, 1993). Likewise, the daily electron transport rate, the photosynthetic process most directly impacted by light, with a specific DLI as PPFD fluctuations decrease (Weaver and van Iersel, 2019).

Our objective was to quantify the photosynthesis and growth of lettuce in response to fluctuating PPFD levels. We hypothesized that plant biomass would decrease as the magnitude of PPFD fluctuations increased, because of the effect of such fluctuations on photosynthesis and carbon gain. By quantifying the effects of fluctuating PPFD on plant physiological parameters and crop growth, we aimed to determine whether it is possible to take advantage of variable electricity prices to provide light to controlled environment agriculture crops.

Materials and Methods

Growing conditions

The study was conducted in a 54 m^3 walk-in growth chamber. The chamber contained three racks with three shelves each. Each shelf was divided into two 0.74 m^2 growing areas. Each growing area was outfitted with two dimmable LED bars (SPYDRx with Physiospec indoor spectrum, Fluence Bioengineering, Austin, TX, USA). Environmental conditions were monitored with a temperature/humidity probe (HMP50, Vaisala, Helsinki, Finland) and a CO_2 sensor (GMC20, Vaisala, Vantaa, Finland) connected to a datalogger (CR6, Campbell Scientific, Logan, UT, USA), which calculated the vapor pressure deficit (VPD) from the temperature and relative humidity measurements. The datalogger controlled CO_2 levels by opening a valve connected to a compressed CO_2 cylinder for 0.1 s, whenever the measured CO_2 dropped below 800 $\mu mol \cdot mol^{-1}$. CO_2 enrichment was used because it can make supplemental lighting more

economical by increasing photosynthesis and growth more than supplemental lighting by itself (Both et al., 1997; Ferentinos et al., 2000). Excess water vapor was removed using a dehumidifier (FAD704DWD13, Electrolux, Charlotte, NC, USA). The temperature was 19.7 ± 0.8 °C, CO_2 concentration was 797 ± 47 µmol·mol^{-1}, and the VPD was 0.99 ± 0.17 kPa (mean ± SD).

Plant material

Lettuce 'Green Salad Bowl' and 'Little Gem' were seeded into 10-cm square pots filled with peat-perlite substrate (Fafard 2P; Sun Gro Horticulture, Agawam, MA, USA). Seedlings were thinned to one plant per pot at 6 days after seeding. Plants were sub-irrigated as needed using a water-soluble fertilizer solution with a nitrogen concentration of 100 mg·L^{-1} (Peters Excel 15-5-15 CalMag Special, ICL, Summerville, SC, USA). The experimental unit was a group of 15 plants of one cultivar, with three replications, and six treatments (PPFD fluctuations). The plants were grown over a 6-week period.

Treatments

Plants were grown under six different fluctuating lighting treatments with the photosynthetic photon flux density (PPFD) switching from high to low PPFD every 15 minutes throughout the photoperiod. The PPFDs in the different treatments were approximately 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 µmol·m^{-2}·s^{-1}, with a photoperiod of 16 hr. The daily light integral in all treatments was ~11.5 mol·m^{-2}·d^{-1}. The actual PPFD was not exactly equal to the target PPFD and measured using a spectroradiometer (SS-110, Apogee, Logan, UT) (Table 1). Measurements were taken at 9 cm height from the ebb-and-flow tray, 3 cm above the soil line at the center of each 15-unit tray.

Data collection and analysis

Canopy images of trays with 15 plants were taken weekly after seedling emergence (16, 23, 30, and 37 days after planting (DAP). We used a monochrome camera (CM3-U3-31S4M-CS, Flir, Wilsonville, OR, USA) outfitted with a 680 nm long-pass filter (Midwest Optics, Palatine, IL, USA) mounted inside a light-proof grow tent. Plants were illuminated with a blue LED (225 ultrathin grow light, Yescom USA, City of Industry, CA, USA). The camera took images of the fluorescence emitted by the leaves, excited by the blue light, resulting in greyscale images, with the canopy light and the background dark. The projected canopy size for each tray of plants was determined using threshold separation in ImageJ (Narayanan et al., 2019).

Gas exchange data was collected on one plant per experimental unit at 35-37 DAP to determine the photosynthesis of plants within each treatment using a portable leaf gas exchange system (CIRAS-3, PP Systems, Inc., Amesbury, MA). The youngest fully-expanded leaf was used for these measurements. The leaf gas exchange system was programmed to run for 45 minutes; 15 mins of low PPFD (as an acclimation period), followed by 15 min of high and 15 min of low PPFD. Built in white LEDs were programed to set the target PPFDs in the leaf cuvette. Cuvette temperature, CO_2 concentration, and VPD were similar to conditions in the growth chamber. The net assimilation data for each 15-min period were averaged ($A_{n,15}$), as were the data from the 30-min period, which included 15 min of both high and low PPFD ($A_{n,30}$). Stomatal conductance was measured as well.

'Green Salad Bowl' was harvested at 40 DAP and 'Little Gem' was harvested at 43 DAP. The chlorophyll content index (CCI) (Opti-Sciences, CCM-200plus, Hudson, NH), number of leaves, length and width of the longest leaf, total leaf area, and shoot dry weight were measured on the three plants in the center of each tray. Specific leaf area was calculated as leaf area/shoot

dry weight. Dry mass measurements were collected from the 12 remaining border plants for calculating total dry mass.

Experimental design and statistical analysis

The study was set up as a randomized complete block with three replications and a split-plot (cultivar). Data was analyzed using both linear and non-linear regression (SigmaPlot 11, Systat Software, Inc., San Jose, CA).

Results

Crop Growth and Morphology

Projected canopy size at 16 DAP was low and not affected by PPFD fluctuations for either cultivar. At all subsequent times, PPFD fluctuations did affect projected canopy size, with 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluctuations resulting in the smallest canopy size in both cultivars. In 'Green Salad Bowl', the 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment resulted in slightly lower projected canopy size than treatments with smaller PPFD fluctuations at 23 and 30 DAP, but no longer at 37 DAP (Figure 2.1).

Projected canopy size of 'Green Salad Bowl' was more sensitive to PPFD fluctuations than that of 'Little Gem'; at 37 DAP, projected canopy size of 'Little Gem' was 32% lower with 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluctuations than in the other treatments, while for 'Green Salad Bowl', this reduction was 64%. In treatments with PPFD fluctuations of 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or less, 'Little Gem' had a ~12.5% smaller projected canopy than 'Green Salad Bowl' at 37 DAP (Figure 2.1). This is consistent with the growth habits of these two cultivars; 'Green Salad Bowl' is a loose-leaf lettuce, while 'Little Gem' forms a small head.

In both cultivars, there was an asymptotic increase in leaf number, length, width, and chlorophyll content index. ‘Green Salad Bowl’ plants averaged 6.7 leaves in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment, compared to 12.3 leaves in the other treatments (Figure 2.2). For ‘Little Gem,’ plants in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment averaged 11.6 leaves, increasing to 14.3 leaves in the 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment and 17.4 leaves in the other treatments (Figure 2.2). Leaf length of ‘Green Salad Bowl’ averaged 12.5 cm in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment, compared to 19.4 cm in the other treatments. ‘Little Gem’ plants in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment averaged a leaf length of 10.5 cm, increasing to 14.4 cm in the 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 16.6 cm for the other treatments (Figure 2.2). Leaf width for ‘Green Salad Bowl’ averaged 5.8 cm in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment, increasing to 13.1 cm in the 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments and 15.1 cm in all other treatments. For ‘Little Gem,’ the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment resulted in a leaf width of 7.0 cm, increasing to 8.6 cm in the other treatments (Figure 2.2).

‘Green Salad Bowl’ had an ~67% lower chlorophyll content index than ‘Little Gem’. Plants grown under the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment had an ~65% and ~75% lower chlorophyll content index as compared to the other treatments in ‘Green Salad Bowl’ and ‘Little Gem,’ respectively. ‘Green Salad Bowl’ had larger but fewer leaves than ‘Little Gem’ and the number of leaves increased more gradually, from ~12 to 18, for ‘Little Gem than for Green Salad Bowl’ (~7 to 12 leaves), as PPFD fluctuations decreased (Figure 2.2, Supplementary figure 2.1).

On average, ‘Little Gem’ had an ~8% larger leaf area than ‘Green Salad Bowl’ (Figure 2.3), which contrasts with the substantially larger projected canopy size of ‘Green Salad Bowl’. This is likely related to the compact and head-forming ‘Little Gem’ having smaller but more leaves (Figure 2.2), which overlap each other more than the leaves of the loose-leaf ‘Green Salad Bowl’ lettuce.

Leaf area and total dry mass of both cultivars increased asymptotically as the lower PPFD increased from 0 to 200 µmol·m^{-2}·s^{-1}. 'Green Salad Bowl' plants in the 400/0 µmol·m^{-2}·s^{-1} and 360/40 µmol·m^{-2}·s^{-1} treatments, had a ~90% and ~28% lower dry mass and an ~83% and ~30% lower leaf area compared to the other treatments (Figure 2.3). 'Little Gem' plants in the 400/0 µmol·m^{-2}·s^{-1} and 360/40 µmol·m^{-2}·s^{-1} treatments, had a ~70% and ~22% lower dry mass and an ~59% and ~16% lower leaf area compared to the other treatments. 'Green Salad Bowl' had a ~12% lower dry mass and ~28% lower leaf area than 'Little Gem' (Figure 2.3).

Specific leaf area decreased exponentially as the lower PPFD increased from 0 to 200 µmol·m^{-2}·s^{-1}, suggesting thinner leaves with large PPFD fluctuations in both lettuce cultivars. The specific leaf area of 'Green Salad Bowl' plants in the 360/40 µmol·m^{-2}·s^{-1} treatment was ~28%, and in those with smaller PPFD fluctuations ~37%, lower than in the 400/0 µmol·m^{-2}·s^{-1} treatment (Figure 2.3). 'Little Gem' specific leaf area in the 360/40 µmol·m^{-2}·s^{-1} treatment was ~23%, and in the other treatments ~27% lower than in the 400/0 µmol·m^{-2}·s^{-1} treatment. 'Little Gem' had a ~18% lower specific leaf area than 'Green Salad Bowl' (Figure 2.3).

Leaf Assimilation Rates

Net assimilation rates of 'Green Salad Bowl' lettuce in most treatments increased rapidly as the PPFD was changed from low to high. However, plants in the 400/0 µmol·m^{-2}·s^{-1} treatment, and to a lesser extent the 360/40 µmol·m^{-2}·s^{-1} treatment, showed a more gradual initial increase in A_n (for about 5 min) following exposure to high PPFD. Net assimilation did not reach a steady state during the 15 min at high PPFD in the 400/0 and 360/40 µmol·m^{-2}·s^{-1} treatments, but instead kept increasing slowly (Figure 2.4). This suggests that the plants may have been trying to acclimate to the high PPFD but were not able to fully do so before the PPFD was lowered again. In all other treatments, stable A_n was reached within two minutes at high PPFD.

After switching from high to low PPFD, A_n stabilized quickly in all treatments. The 200/200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment resulted in consistent A_n over the 30 min period, ranging between 8.1 and 8.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Figure 2.4).

The $A_{n,15}$ of 'Green Salad Bowl' lettuce increased linearly, from ~-1 to 14 $\mu mol \cdot m^{-2} \cdot s^{-1}$, as PPFD increased from 0 to 320 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and decreased rapidly at even higher PPFDs. At 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, $A_{n,15}$ averaged only ~4 $\mu mol \cdot m^{-2} \cdot s^{-1}$, ~9.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ lower than at a PPFD of 320 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Figure 2.5), indicating that the extreme PPFD fluctuations in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment seriously impaired the photosynthetic physiology.

The $A_{n,30}$ of 'Green Salad Bowl' lettuce increased asymptotically as the lower PPFD increased from 0 to 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (and the high PPFD decreased from 400 to 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$), with little or no difference among the 320/80, 280/120, 240/160, and 200/200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments (Figure 2.6). The linear relationship between $A_{n,15}$ at PPFDs from 0 to 320 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Figure 2.5) explains the lack of differences $A_{n,30}$ among these four treatments (Figure 2.6).

The $A_{n,30}$ in the 360/40 and 400/0 treatments was ~27% and 69% lower compared to the other treatments with smaller PPFD fluctuations. The rapid decrease in $A_{n,15}$ at a PPFD above of 320 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Figure 2.5) explains the low $A_{n,30}$ in the two treatments with the greatest PPFD fluctuations.

The $A_{n,30}$ data follow the same trends as the dry mass and leaf area data (Figure 2.3). There was a strong positive correlation between the $A_{n,30}$ and shoot dry mass of 'Green Salad Bowl' lettuce, largely due to the low $A_{n,30}$ and dry mass in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment (Figure 2.6). Since A_n underlies dry mass production, this correlation is not surprising. The $A_{n,30}$ of 'Green Salad Bowl' lettuce also was positively correlated with the leaf chlorophyll content index (Figure 2.6), suggesting that the low A_n and dry mass of plants grown under a PPFD of

400/0 μmol·m^{-2}·s^{-1} were at least partly due to the low chlorophyll levels in the leaves of these plants.

Specific leaf area of 'Green Salad Bowl' lettuce was negatively correlated with both $A_{n,30}$ and CCI (Figure 2.7). High specific leaf area suggests thinner leaves with fewer and/or smaller mesophyll cells, where most of the carbon assimilation occurs. As the specific leaf area decreased from ~780 cm^2·g^{-1} (in the 400/0 μmol·m^{-2}·s^{-1} treatment) to 460 cm^2·g^{-1}, $A_{n,30}$ increased from 1.9 to 7.6 μmol·m^{-2}·s^{-1} and CCI increased from 1.7 to 5.4 (Figure 2.7).

Discussion

The Importance of Canopy Size

Projected canopy size (PCS) is a good indicator of the amount of light a canopy intercepts (Klassen et al., 2004) and of morphological changes in response to environmental conditions, in this case fluctuations in PPFD. When taken over a growing period, it provides information on growth rates from seed to maturity. In the 400/0 μmol·m^{-2}·s^{-1} treatment for both cultivars, the plants had a lower PCS than those in other treatments throughout the growing period from 23 DAP until the end of the study (Figure 2.1). A lower projected canopy size reduces the amount of incident light, canopy photosynthesis, and growth (Klassen et al., 2004). Projected canopy sizes in all other treatments were similar, indicating that lettuce canopy development tolerates wide fluctuations in PPFD.

The PCS of 'Green Salad Bowl' was more sensitive to large PPFD fluctuations than that of 'Little Gem'. At 30 and 37 DAP, 'Green Salad Bowl' had a larger PCS than 'Little Gem in treatments with relatively small PPFD fluctuations (200/200 to 320/80 μmol·m^{-2}·s^{-1}), while 'Green Salad Bowl' had a smaller PCS in the 400/0 μmol·m^{-2}·s^{-1}. This indicates that genetic

factors play a role in determining both PCS, as well as cultivar responses to fluctuating PPFD. ‘Green Salad Bowl’ produces larger leaves than ‘Little Gem’, a small head-forming lettuce (Figure 2.2). The importance of PCS in determining crop growth is evident from the positive correlation between PCS at 23, 30, and 37 DAP and final dry mass (Figure 2.8). Our results suggest that measurements of PCS during the growing cycle can provide an early indication of final dry mass production in response to different lighting treatments. Similar correlations between PCS and final dry mass were reported by Elkins and van Iersel (2020) in response to different PPFD and photoperiod treatments, all with the same daily light integral. Differences in growth among lettuce cultivars are also strongly correlated with differences in canopy size early in the growing cycle (Kim and van Iersel, 2019).

The effects of fluctuating light levels on PCS were consistent with effects on leaf number, length, width, and total leaf area in both cultivars (Figure 2.2, 2.3). These treatment effects tended to be larger in ‘Green Salad Bowl’ than in ‘Little Gem’. The reductions in these morphological parameters in response to the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and to a lesser extent in the 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments, may be the result the low $A_{n,30}$ (Figure 2.6) and the resulting limited carbohydrate supply for new growth. Plants in 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment had a higher SLA than those in treatments with smaller PPFD fluctuations, possibly in an attempt to produce as much leaf as possible with the limited carbohydrate supply. Smaller leaf area and reduced leaf number in response to a fluctuation light levels (900 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ every four minutes, compared to a constant PPFD of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$) has also been reported in *Arabidopsis thaliana* (Kaiser, Walther and Armbruster, 2020).

Fluctuating light and photosynthesis

Since plants in our study were exposed to fluctuating PPFD, their photosynthetic processes had to constantly respond to those changing conditions. Steady state A_n is typically achieved within 5-10 minutes of exposure to high PPFD (Kalaji et al., 2014). In our study, steady-state A_n was achieved within 2 min after exposure to a high PPFD in the 240/160, 280/120, and 320/80 μmol·m^{-2}·s^{-1} treatments (Figure. 2.4). This fast response to a change from low to high PPFD suggests that the photosynthetic apparatus in those plants was adequately activated under low PPFD to allow for a rapid response to an increase in PPFD. However, when PPFD was increased from 0 to 400 μmol·m^{-2}·s^{-1} or from 40 to 360 μmol·m^{-2}·s^{-1}, A_n initially increased rapidly, followed by a more gradual increase during the remainder of the 15-min period, never reaching a steady state (Figure 2.4), suggesting that activation of the photosynthetic apparatus in response to a rapid change in PPFD depends on the magnitude of the change in PPFD. Sims and Pearcy (1993) grew the understory species *Alocasia macrorrhiza* with sunflecks for 10-12 minutes every hour (PPFD of ~280 μmol·m^{-2}·s^{-1} sunflecks alternating with ~16 μmol·m^{-2}·s^{-1} during the remainder of the hour) and without sunflecks. Plants in both treatments receiving a similar DLI. Induction of full photosynthetic activity in response to a sunfleck required ~40 minutes, consistent with our observation that plants in the 400/0 and 360/40 μmol·m^{-2}·s^{-1} treatments did not achieve steady-state photosynthesis during the 15 minutes at high PPFD. Exposing plants to sunflecks reduced leaf carbon gain, dry mass (by 89%) and increased SLA (Sims and Pearcy, 1993), similar to our findings in the 400/0 and 360/40 μmol·m^{-2}·s^{-1} treatments.

Surprisingly, $A_{n,15}$ decreased as PPFD increased from 320 to 400 μmol·m^{-2}·s^{-1} (Figure. 2.5). This indicates that large PPFD fluctuations negatively affect the photosynthetic

performance of lettuce leaves. Leaf A_n depends on light harvesting, subsequent electron transport in the light reactions of photosynthesis, and the ability of Calvin cycle enzymes to use the products of the light reactions to assimilate CO_2. Pigments in the thylakoid membrane of chloroplasts absorb light energy (photons) and that energy is used to drive electron transport. This results in the reduction of ferredoxin, followed by the reduction of $NADP^+$ to NADPH (Pinnola, 2019) and the formation of a hydrogen gradient across the thylakoid membrane. This hydrogen gradient facilitates the synthesis of ATP. The rate of the light reactions depends on how much light is absorbed by photosynthetic pigments. The CCI was lower in the 400/0 and 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments as compared to the treatments with smaller light fluctuations (Figure 2.6). A low CCI is associated with low leaf absorptance (Bauerle et al., 2004) and would thus be expected to result in low electron transport rates, which may result in low rates of NADPH and ATP production. This is supported by Wei et al. (2020), who reported that fluctuating light inhibits photosystem I and II activity through upregulation of non-photochemical quenching in rice (*Oryza sativa*). This resulted in decreased electron transport and lower ATP synthase activity. Fluctuating light also interfered with stacking of the thylakoid membrane. Thus, fluctuating PPFDs can have a strong impact on the light reactions of photosynthesis.

The low chlorophyll levels in the treatments with large PPFD fluctuations may be due to light-dependent nature of chlorophyll biosynthesis. A key step in chlorophyll biosynthesis is the conversion of protochlorophyllide to chlorophyllide, the immediate precursor to chlorophyll a and chlorophyll b. This process that is both NADPH- and light-dependent (via the enzyme protochlorophyllide oxidoreductase, POR) (Reinbothe and Reinbothe, 1996). The activation of POR is unique in that its activation depends on the absorption of photons by its substrate

protochlorophyllide. This induces a conformational change in the enzyme, activating it. Further complicating the effect of light on POR activity is that plants have multiple POR genes. In *Arabidopsis thaliana*, PORA is expressed in the dark and its expression is strongly inhibited in the light, through a phytochrome mediated process. PORB and PORC, on the other hand have low expression levels in the dark, and expression of PORC is upregulated in the light, through phytochrome-interacting factors (Gabruk and Mysliwa-Kurdziel, 2015). Thus, both the transcript levels and activity of POR are light-dependent, and it seems plausible that production of chlorophyll cannot proceed normally when leaves are exposed to constant large light fluctuations, consistent with the low CCI in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment (Figure 2.2). The idea that the low $A_{n,15}$ at PPFDs of 360 and 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was due at least in part due to poor light absorptance is supported by the positive correlation between CCI and $A_{n,30}$ (Figure 2.6). The low CCI in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment may also have been caused partly by leaf morphological effects. Plants in the 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment had a high SLA, i.e. low biomass per unit leaf area. Since CCI is an indicator of the amount of chlorophyll per unit leaf area, a high SLA is likely associated a low CCI. We did indeed find strong negative correlations between SLA and both CCI and $A_{n,30}$ (Figure 2.7), consistent with prior findings (Brodersen and Vogelmann, 2010).

Large fluctuations in PPFD may also affect Calvin cycle activity. The activation of key Calvin cycle enzymes and the biochemical reactions of the Calvin cycle themselves depend on products of the light reactions. Specifically, activation of fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoribulokinase requires thioredoxin, produced from reduced ferredoxin, for the reduction of regulatory disulfides (Michelet et al., 2013). Low activity of these enzymes can limit

photosynthesis by limiting the regeneration of ribulose 1,5-bisphosphate (RuBP). In addition, Rubisco activase is light-dependent, since it relies on a high stroma pH, which results from hydrogen transport from the stroma into the lumen in the light reactions. Rubisco activase activity depends on NADPH and thus on the light reactions (Kleczkowski, 1994). Rubsico is inactive in the dark because of the binding of metabolites to its active site and depends on Rubisco activase to remove those metabolites (Zhang and Portis, 1999).

Thus, light is not only required to drive the light reactions, but also controls the production and activity of chlorophyll and Calvin cycle enzymes. Although our data do not shed light on which enzymatic processes may have been affected by large PPFD fluctuations, it seems likely that such fluctuations interfere with the development of photosynthetic machinery and normal CO_2 assimilation. The low $A_{n,15}$ at PPFDs of 360 and 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ resulted in low $A_{n,30}$ in the 360/40 and especially 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments. That low A_n was likely partly responsible for the relatively poor growth in the 360/40 and 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments, since $A_{n,30}$ was strongly and positively correlated with shoot dry mass (Figure 2.6).

Stomatal conductance was also greatly affected by the light treatments (Supplementary figure 2.2), with conductance decreasing with increasing PPFD fluctuations. Interestingly, conductance was not very responsive to the PPFD fluctuations themselves and remained stable during 15 minutes at high, followed by 15 minutes at low PPFD. The results may suggest that the low $A_{n,30}$ in the 400/0 and 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments may have been partly due to the low stomatal conductance in these treatments. However, that appears unlikely, given that the leaf internal CO_2 concentration was 568 to 738 $\mu mol \cdot mol^{-1}$ and not affected by treatment. These relatively high leaf internal CO_2 concentrations are unlikely to seriously limit CO_2 assimilation.

The differences in stomatal conductance thus seem to have been the result, rather than the cause, of the differences in $A_{n,30}$.

Concluding Remarks

Our results indicate that lettuce can tolerate a wide range of fluctuating light levels. A constant PPFD is not needed to maintain proper growth and development of 'Little Gem' and 'Green Salad Bowl'. Extreme fluctuations, 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and to a lesser extent the 360/40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments, resulted in plants with fewer and smaller leaves, lower chlorophyll content, and lower assimilation rates compared to those in all other treatments. However, results with smaller PPFD fluctuations indicate that growers can take advantage of variable electricity prices to provide light in controlled environment operations. This can aid growers in reducing operating costs and increase profitability.

Literature Cited

Agrylist. (2017). State of Indoor Farming. 39 pp.

Albright, L., Both, A., and Chiu, A. (2000). Controlling greenhouse light to a consistent daily integral. *Transactions of the ASAE* 43**,** 421-431. doi: 10.13031/2013.2721.

Bauerle, W.L., Weston, D.J., Bowden, J.D., Dudley, J.B., and Toler, J.E. (2004). Leaf absorptance of photosynthetically active radiation in relation to chlorophyll meter estimates among woody plant species. *Scientia Horticulturae* 101**,** 169-178. doi: 10.1016/j.scienta.2003.09.010.

Both, A.-J., Albright, L., and Langhans, R. (1997). Coordinated management of daily PAR integral and carbon dioxide for hydroponic lettuce production. *II Modelling Plant Growth, Environmental Control and Farm Management in Protected Cultivation 456***,** 45-52.

Brodersen, C.R., and Vogelmann, T.C. (2010). Do changes in light direction affect absorption profiles in leaves? *Functional Plant Biology* 37**,** 403-412. doi: 10.1071/FP09262.

Chazdon, R.L., and Pearcy, R.W. (1986). Photosynthetic responses to light variation in rainforest species. II. Carbon gain and photosynthetic efficiency during lightflecks. *Oecologia***,** 524-531.

Chazdon, R.L., and Pearcy, R.W. (1991). The importance of sunflecks for forest understory plants. *Bioscience* 41**,** 760-766.

Clausen, A., Maersk-Moeller, H.M., Soerensen, J.C., Joergensen, B.N., Kjaer, K.H., and Ottosen, C.O. (2015). "Integrating Commercial Greenhouses in the Smart Grid with Demand Response based Control of Supplemental Lighting", in: *International Conference on Industrial Technology and Management Science (ITMS 2015).*

Cossu, M., Ledda, L., Urracci, G., Sirigu, A., Cossu, A., Murgia, L., et al. (2017). An algorithm for the calculation of the light distribution in photovoltaic greenhouses. *Solar Energy* 141**,** 38-48. doi: 10.1016/j.solener.2016.11.024.

Cossu, M., Yano, A., Solinas, S., Deligios, P.A., Tiloca, M.T., Cossu, A., et al. (2020). Agricultural sustainability estimation of the European photovoltaic greenhouses. *European Journal of Agronomy* 118**,** 126074.

Elkins, C., and van Iersel, M.W. (2020). Longer Photoperiods with the Same Daily Light Integral Increase Daily Electron Transport through Photosystem II in Lettuce. *Plants* 9**,** 1172. doi: 10.3390/plants9091172.

Emmott, C.J., Röhr, J.A., Campoy-Quiles, M., Kirchartz, T., Urbina, A., Ekins-Daukes, N.J., et al. (2015). Organic photovoltaic greenhouses: a unique application for semi-transparent PV? *Energy & environmental science* 8**,** 1317-1328.

Faust, J.E., and Logan, J. (2018). Daily light integral: A research review and high-resolution maps of the United States. *HortScience* 53**,** 1250-1257. doi: 10.21273/hortsci13144-18.

Ferentinos, K., Albright, L., and Ramani, D. (2000). SE—Structures and Environment: Optimal Light Integral and Carbon Dioxide Concentration Combinations for Lettuce in Ventilated Greenhouses. *Journal of agricultural engineering research* 77**,** 309-315.

Gabruk, M., and Mysliwa-Kurdziel, B. (2015). Light-dependent protochlorophyllide oxidoreductase: phylogeny, regulation, and catalytic properties. *Biochemistry* 54**,** 5255-5262. doi: 10.1021/acs.biochem.5b00704.

Harbick, K., Albright, L.D., and Mattson, N.S. (2016). "Electrical savings comparison of supplemental lighting control systems in greenhouse environments", in: *2016 ASABE*

Annual International Meeting: American Society of Agricultural and Biological Engineers), 1. doi: 10.13031/aim.20162460478.

Kaiser, E., Walther, D., and Armbruster, U. (2020). Growth under fluctuating light reveals large trait variation in a panel of arabidopsis accessions. *Plants* 9**,** 316. doi: 10.3390/plants9030316.

Kalaji, H.M., Schansker, G., Ladle, R.J., Goltsev, V., Bosa, K., Allakhverdiev, S.I., et al. (2014). Frequently asked questions about in vivo chlorophyll fluorescence: practical issues. *Photosynthesis research* 122**,** 121-158. doi: 10.1007/s11120-014-0024-6.

Kim, C., and van Iersel, M.W. (2019). "Morphological and Physiological Screening for Growth Differences Among 11 Lettuce Cultivars", in: *Annual Conference of the American-Society-for-Horticultural-Science*: AMER SOC HORTICULTURAL SCIENCE, 113 S WEST ST, STE 200, ALEXANDRIA, VA 22314-2851 USA), S89-S90.

Kjær, K.H., Ottosen, C.-O., and Jørgensen, B.N. (2011). Cost-efficient light control for production of two campanula species. *Scientia horticulturae* 129**,** 825-831. doi: 10.1016/j.scienta.2011.05.003.

Klassen, S., Ritchie, G., Frantz, J., Pinnock, D., and Bugbee, B. (2004). Real-time imaging of ground cover: Relationships with radiation capture, canopy photosynthesis, and daily growth rate. *Digital imaging and spectral techniques: applications to precision agriculture and crop physiology* 66**,** 1-14. doi: 10.2134/asaspecpub66.c1.

Kleczkowski, L.A. (1994). Inhibitors of photosynthetic enzymes/carriers and metabolism. *Annual review of plant biology* 45**,** 339-367. doi: 10.1146/annurev.pp.45.060194.002011.

Knapp, A., and Smith, W. (1987). Stomatal and photosynthetic responses during sun/shade transitions in subalpine plants: influence on water use efficiency. *Oecologia* 74**,** 62-67.

Kromdijk, J., Głowacka, K., Leonelli, L., Gabilly, S.T., Iwai, M., Niyogi, K.K., et al. (2016). Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science* 354**,** 857-861. doi: 10.1126/science.aai8878.

Michelet, L., Zaffagnini, M., Morisse, S., Sparla, F., Pérez-Pérez, M.E., Francia, F., et al. (2013). Redox regulation of the Calvin–Benson cycle: something old, something new. *Frontiers in Plant Science* 4. doi: 10.3389/fpls.2013.00470.

Narayanan, M., van Iersel, M.W., and Haidekker, M. (2019). "Chlorophyll Fluorescence Imaging: A Novel, Simple and Non-Destructive Method for Canopy Size Imaging", in: *2019 ASHS Annual Conference*: ASHS).

Pinnola, A. (2019). The rise and fall of Light-Harvesting Complex Stress-Related proteins as photoprotection agents during evolution. *Journal of Experimental Botany* 70**,** 5527-5535. doi: 10.1093/jxb/erz317.

Reinbothe, S., and Reinbothe, C. (1996). The regulation of enzymes involved in chlorophyll biosynthesis. *European Journal of Biochemistry* 237**,** 323-343. doi: 10.1111/j.1432-1033.1996.00323.x.

Seginer, I., Albright, L.D., and Ioslovich, I. (2006). Improved strategy for a constant daily light integral in greenhouses. *Biosystems engineering* 93**,** 69-80. doi: 10.1016/j.biosystemseng.2005.09.007.

Sims, D., and Pearcy, R. (1993). Sunfleck frequency and duration affects growth rate of the understorey plant, Alocasia macrorrhiza. *Functional Ecology***,** 683-689. doi: 10.2307/2390189.

Sørensen, J.C., Kjær, K.H., Ottosen, C.-O., and Jørgensen, B.N. (2016). "DynaGrow-Multi-Objective Optimization for Energy Cost-efficient Control of Supplemental Light in

Greenhouses", in: *8th International Joint Conference on Computational Intelligence (IJCCI 2016)*), 41-48.

van Iersel, M.W., and Gianino, D. (2017). An adaptive control approach for light-emitting diode lights can reduce the energy costs of supplemental lighting in greenhouses. *HortScience* 52**,** 72-77. doi: 10.21273/hortsci11385-16.

Vialet-Chabrand, S., Matthews, J.S., Simkin, A.J., Raines, C.A., and Lawson, T. (2017). Importance of fluctuations in light on plant photosynthetic acclimation. *Plant Physiology* 173**,** 2163-2179. doi: 10.1104/pp.16.01767.

Watanabe, H. (2011). "Light-controlled plant cultivation system in Japan-development of a vegetable factory using LEDs as a light source for plants", in: *VI International Symposium on Light in Horticulture 907*), 37-44. doi: 10.17660/ActaHortic.2011.907.2.

Weaver, G., and van Iersel, M.W. (2019). Photochemical characterization of greenhouse-grown lettuce (Lactuca sativa L.'Green Towers') with applications for supplemental lighting control. *HortScience* 54**,** 317-322. doi: 10.21273/HORTSCI13553-18.

Wei, Z., Duan, F., Sun, X., Song, X., and Zhou, W. Leaf photosynthetic and anatomical insights into mechanisms of acclimation in rice in response to long-term fluctuating light. *Plant, Cell & Environment* In press. doi: https://doi.org/10.1111/pce.13954.

Zeidler, C., and Schubert, D. (2014). "From Bioregenerative Life Support Systems for Space to Vertical Farming on Earth - The 100% Spin-off", in: *Life in Space for Life on Earth Symposium*).

Zhang, N., and Portis, A.R. (1999). Mechanism of light regulation of Rubisco: A specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f.

Proceedings of the National Academy of Sciences 96(16)**,** 9438-9443. doi: 10.1073/pnas.96.16.9438.

Table and Figures

Table 1. Target PPFDs (mean ± sd; n=3) and actual measured PPFDs for each fluctuating lighting treatment. Data was collected at canopy level.

Target high and low PPFD ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	High PPFD ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Low PPFD ($\mu mol \cdot m^{-2} \cdot s^{-1}$)
200/200	211 ± 5	211 ± 5
240/160	249 ± 4	167 ± 4
280/120	283 ± 5	123 ± 3
320/80	341 ± 8	86 ± 2
360/40	367 ± 19	41 ± 2
400/0	420 ± 16	0.2 ± 0.1

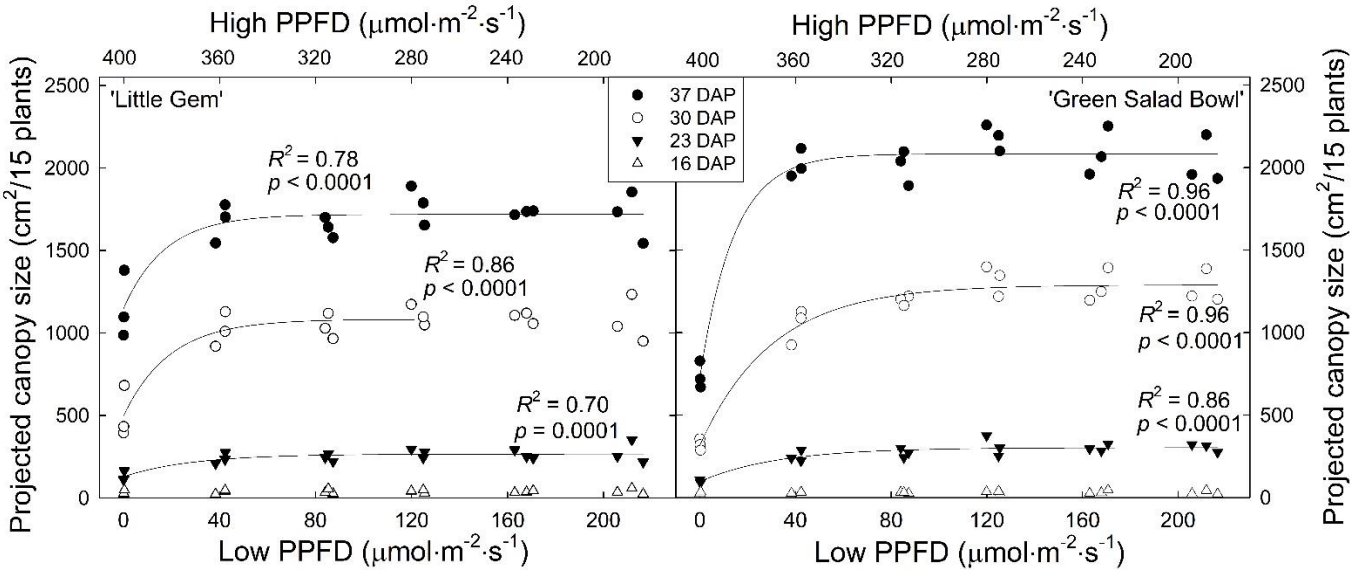


Figure 2.1. Projected canopy size of 'Little Gem' and 'Green Salad Bowl' lettuce (*Lactuca sativa*) at 16, 23, 30, and 37 days after planting (DAP), measured on experimental units consisting of 15 plants. Plants were grown under fluctuating photosynthetic photon flux density (PPFD), with PPFD changing every 15 min between high and low intensities (~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 μmol·m^{-2}·s^{-1}). Identical symbols represent the three replications of each treatment.

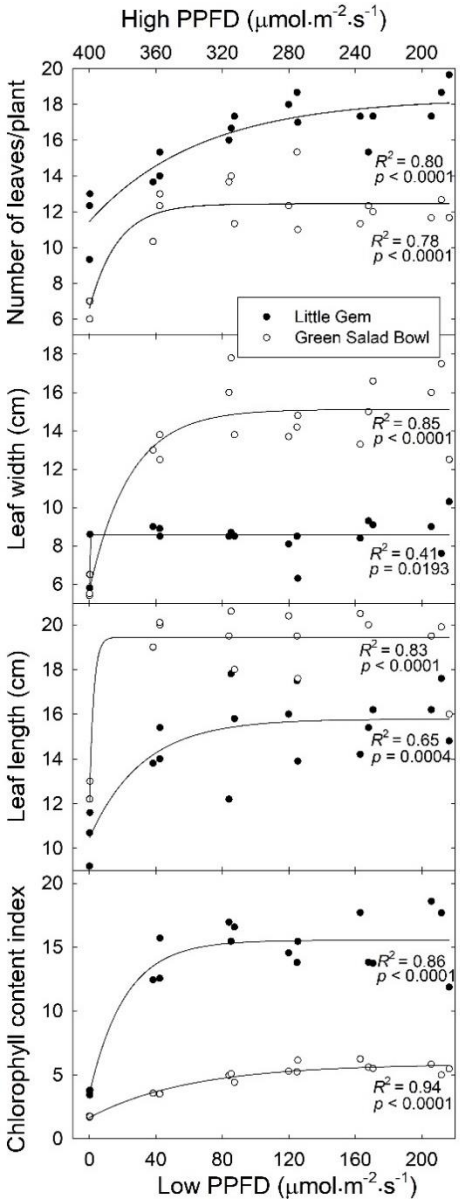


Figure 2.2. Number of leaves per plant, leaf width, leaf length, and chlorophyll content index as a function of treatment (x-axis indicates lower *PPFD*). Symbols (three replications per treatment) represent cultivars 'Green Salad Bowl' (open symbols) and 'Little Gem' (closed symbols) of lettuce (*Lactuca sativa*). Measurements are from the three center plants from each tray. Plants were grown under fluctuating photosynthetic photon flux density (*PPFD*), with *PPFD* changing every 15 min between high and low intensities (~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

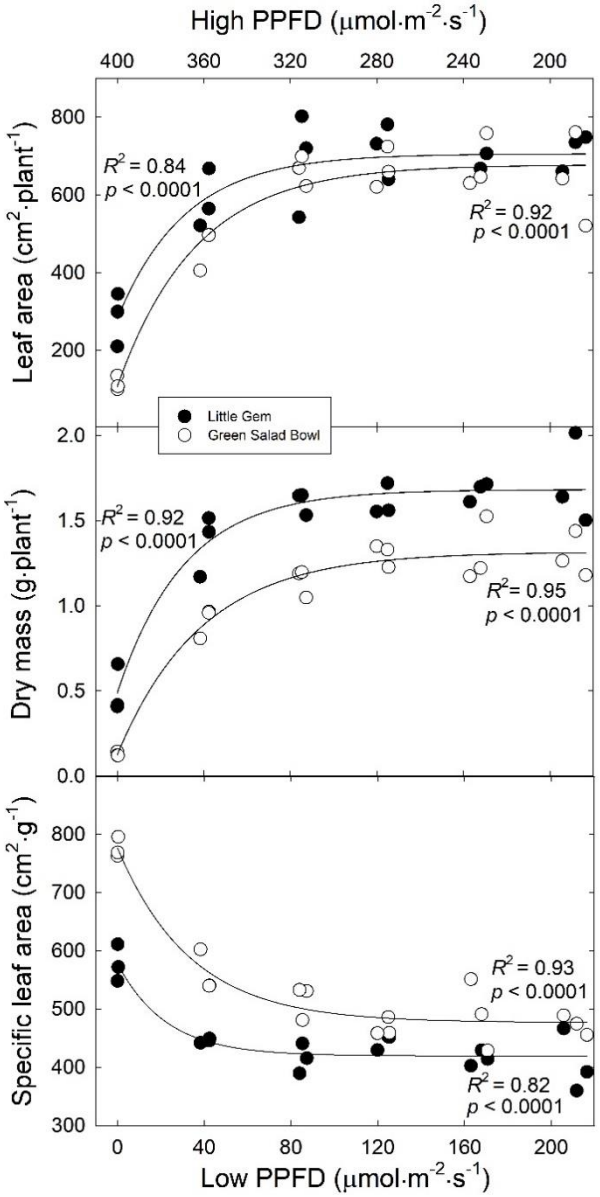


Figure 2.3. Leaf area per plant, dry mass per plant, and specific leaf area as a function of the treatments (x-axis indicates lower PPFD). Symbols (three replications per treatment) represent cultivars 'Green Salad Bowl' (open symbols) and 'Little Gem' (closed symbols) of lettuce (*Lactuca sativa*). Measurements from the three center plants from each tray. Plants were grown under fluctuating photosynthetic photon flux density (PPFD), with PPFD changing every 15 min between high and low intensities (~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

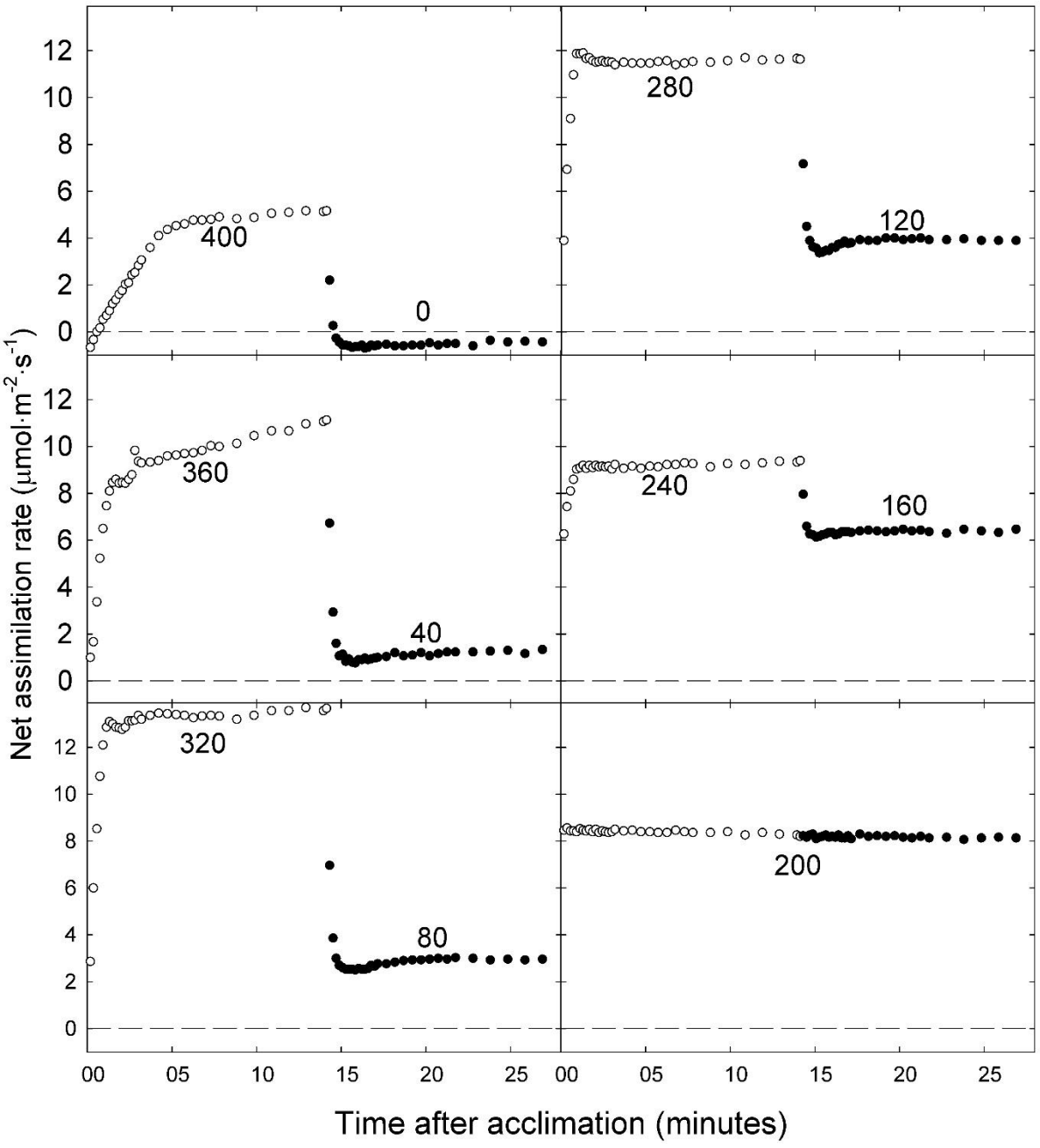


Figure 2.4. Net photosynthetic rate of 'Green Salad Bowl' lettuce (*Lactuca sativa*) during a 15-minute high photosynthetic photon flux density (PPFD) period followed by a 15-minute low PPFD period (400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 μmol·m^{-2}·s^{-1}). Open circles represent high PPFD, and closed circles low PPFD. Values in each graph indicate the PPFD.

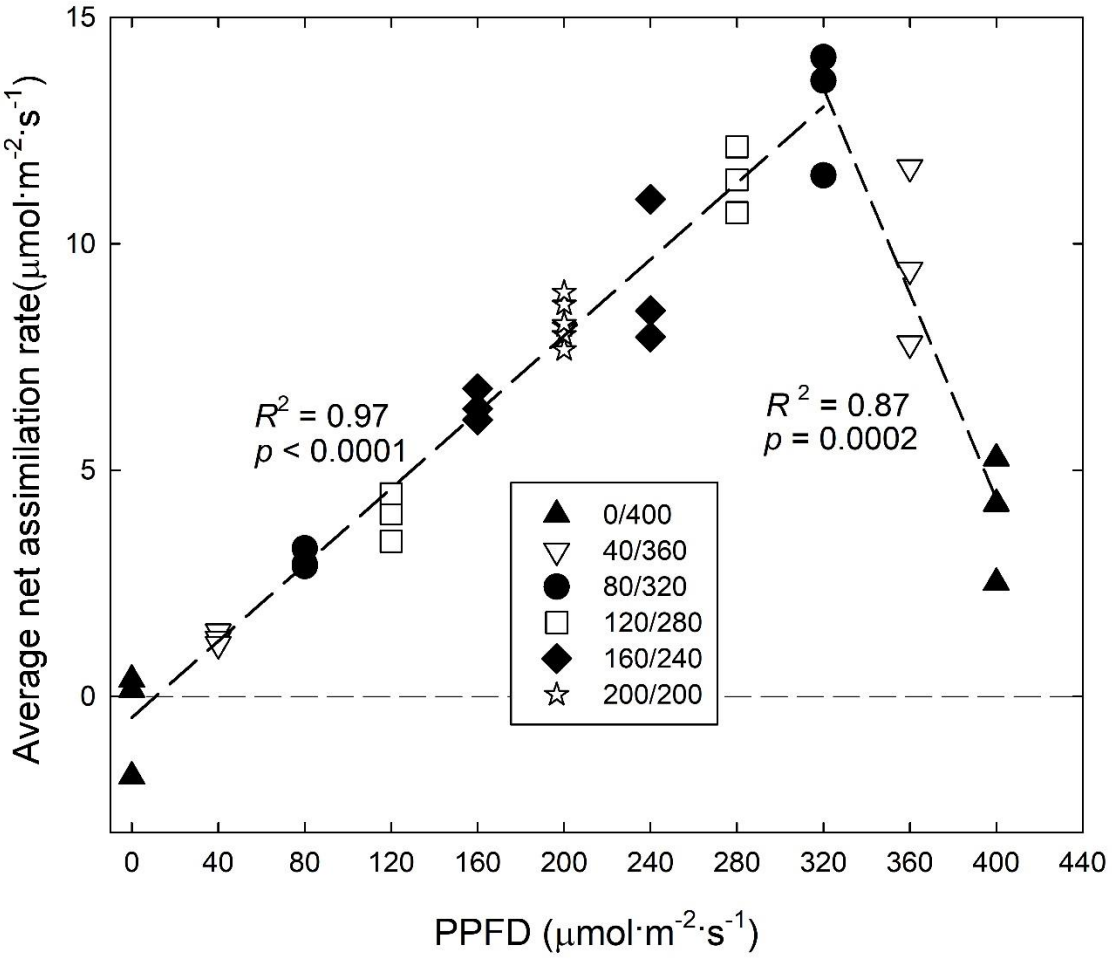


Figure 2.5. Average net assimilation rate over 15 minutes ($A_{n,\,15}$) of 'Green Salad Bowl' lettuce (*Lactuca sativa*) as a function of photosynthetic photon flux density (PPFD). Photosynthesis was measured for 15 min under high PPFD, followed by 15 min under low PPFD (see Fig. 2.4). Plants were grown under fluctuating PPFD, changing every 15 min between high and low PPFD (~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 μmol·m^{-2}·s^{-1}).

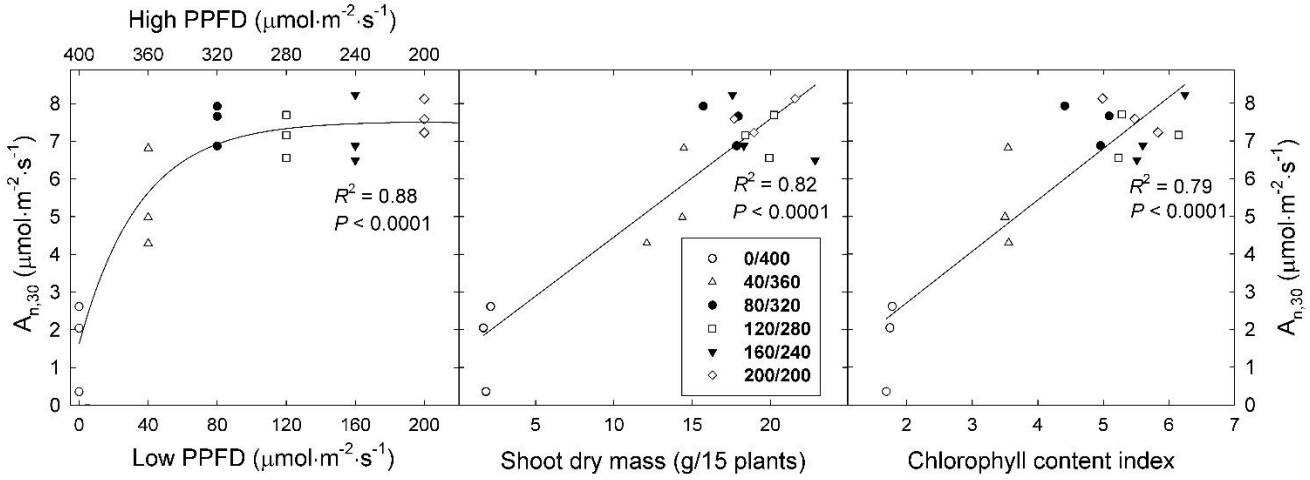


Figure 2.6. Average net assimilation rate over 30 minutes ($A_{n,\,30}$) of 'Green Salad Bowl' lettuce (*Lactuca sativa*) as a function of fluctuating photosynthetic photon flux density (PPFD), total dry mass, and chlorophyll content index. Symbols represent data from each lighting treatment (three replications per treatment). Plants were grown under fluctuating PPFD, changing every 15 min between high and low PPFD (~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 μmol·m^{-2}·s^{-1}).

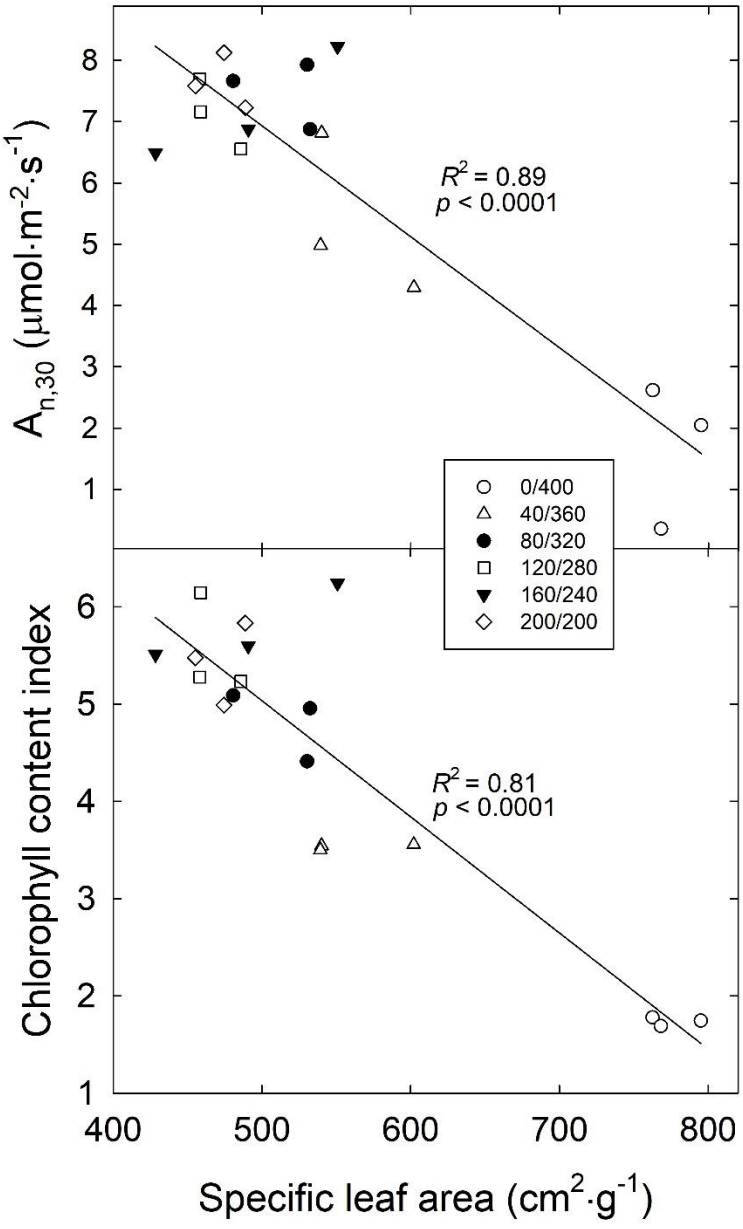


Figure 2.7. Average net photosynthesis over a 30-minute period ($A_{n,30}$) and chlorophyll content index of 'Green Salad Bowl' lettuce (*Lactuca sativa*) as a function of specific leaf area. Symbols represent each treatment, with three replications per treatment. Plants were grown and measured under fluctuating PPFD, changing every 15 min between high and low PPFD (~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 µmol·m^{-2}·s^{-1}).

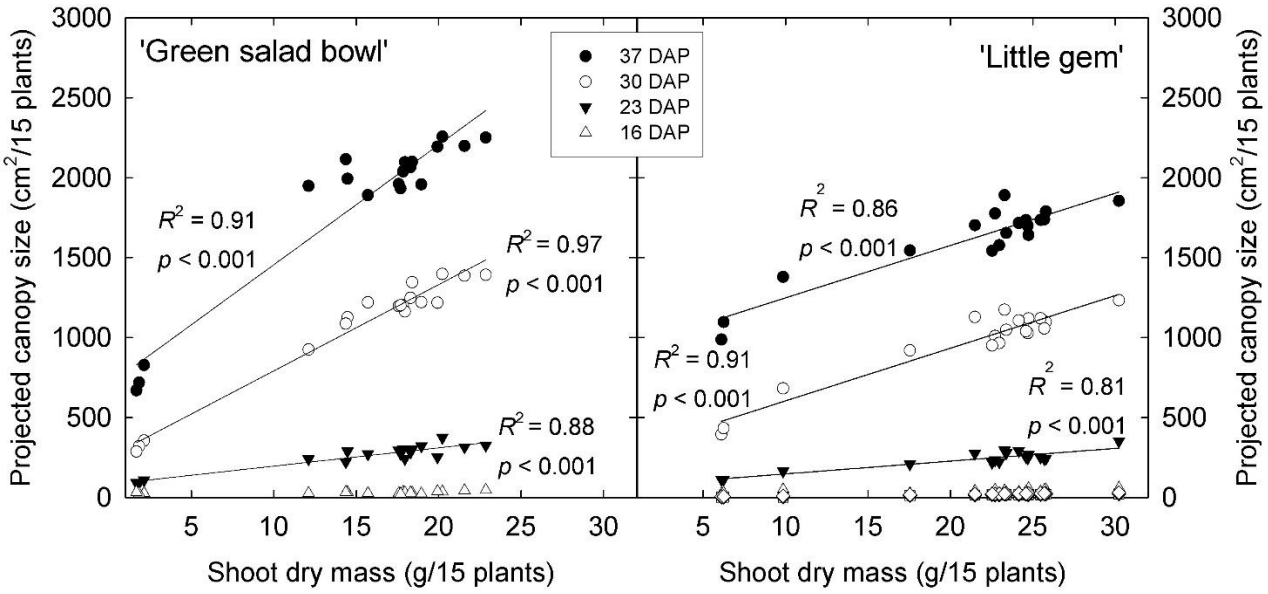
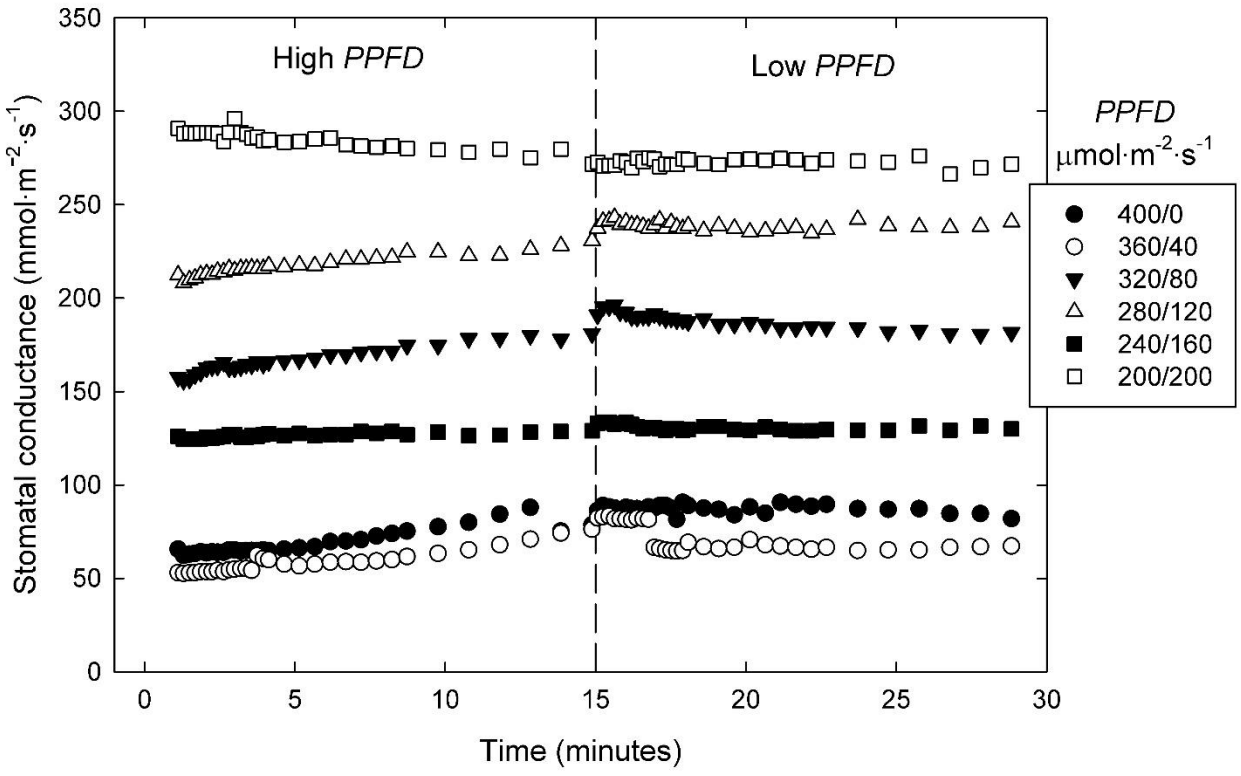


Figure 2.8. Projected canopy size of 'Green Salad Bowl' and 'Little Gem' lettuce (*Lactuca sativa*) at 16, 23, 30, and 37 days after planting (DAP) versus shoot dry mass of 15 plants. Symbols represent DAP. Plants were grown under fluctuating *PPFD*, with *PPFD* changing every 15 min between high and low intensities (~ 400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 µmol·m^{-2}·s^{-1}).

Supplementary figure 2.1. Images of 'Little Gem' (left and middle top) and 'Green Salad Bowl' (right and middle bottom) lettuce (*Lactuca sativa*) grown at PPFDs of 200/200 and 400/0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments, fluctuating at 15-minute intervals.



Supplementary figure 2.2. Stomatal conductance of 'Green Salad Bowl' lettuce (*Lactuca sativa*) during a 15-minute high photosynthetic photon flux density (*PPFD*) period followed by a 15-minute low *PPFD* period (~400/0, 360/40, 320/80, 280/120, 240/160, and 200/200 µmol·m^{-2}·s^{-1}).

CHAPTER 3

Far-red light increases lettuce growth: morphological, physiological and molecular responses

Bhuiyan, Ruqayah and van Iersel, Marc W. To be submitted for publication in *HortScience*

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Abstract

Horticultural lighting is used to stimulate plant growth in controlled environment systems and typically focuses on photosynthetically active radiation (PAR). PAR is defined as the spectral range between 400 and 700 nm. Far-red light (700- 800 nm) falls outside this range and is often left out of horticultural lighting fixtures in controlled environment systems. Previous studies have identified the morphological response of several species to far-red light, typically leading to increases in leaf expansion and plant height. However, it is not clear what genetic factors play a role in cell wall modifications in response to facilitate elongation in response to far-red light. We hypothesized that plant biomass and plant size would increase as the amount of supplemental far-red light increased. We also hypothesized that far-red light may have an impact on the elongation of younger leaves, leading to a larger canopy size thus increasing canopy photosynthesis early in the growing cycle, and that *LsXTH* expression (a cell wall modification enzyme) would increase with increasing far-red intensity. We quantified the effects of far-red light on plant morphological and physiological parameters and growth. 'Green salad bowl' lettuce (*Lactuca sativa*) was grown in a growth chamber with white LED lights and far-red light bars. The various far-red light intensities were 5, 15, and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$, with a photoperiod of 16 hours, a PPFD of ~ 317$\mu mol \cdot m^{-2} \cdot s^{-1}$ and a DLI of ~18 $mol \cdot m^{-2} \cdot d^{-1}$ in all treatments. CO_2 concentrations were the same as ambient air. Plants in the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ had ~12% and ~18% lower projected canopy sizes than in the 30 and 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red treatments. Plants under 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of far-red averaged a leaf area of 746 cm^2, while plants in the 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment averaged 899 cm^2. There was a negative correlation between leaf number and a positive correlation between leaf length and leaf width and far-red light intensity. No difference was observed between the treatments in leaf net assimilation, specific leaf area, or light use

efficiency. There were also no significant differences in *LsXTH8* transcript levels among the treatments, however significant differences were observed between old, mature, and young leaves. The increases in leaf expansion and growth in response to increasing far-red intensity suggests that far-red is valuable in controlled environment systems. More research is needed to identify what key cell modifying enzymes are involved in the leaf elongation response to far-red light.

Introduction

In a natural environment plants compete for resources such as light, nutrients, water, and space. Understory plans and high density crops especially compete for available light. Plants have evolved to adapt to such conditions and utilize receptors to respond rapidly to changes in their lighting environment (Keuskamp et al., 2010). In such cases, photoreceptors are key to determining how much light is available and the spectral distribution. In greenhouses, horticultural lighting is used to optimize plant growth in areas of higher latitudes, where seasonal fluctuations in DLI from sun are greatest (Faust and Logan, 2018), while plant factories rely entirely on electric lighting. For the provided light to be effective in stimulating plant growth, most of the provided light needs to be photosynthetically active. The most common definition of photosynthetically active radiation (PAR) only includes the spectral range between 400 and 700 nm (McCree, 1972). However, this range leaves out far-red and ultraviolet light, which can be integral in increasing photosynthetic rates. Far-red light has wavelengths between 700-800 nm and is often not included in horticultural light fixtures, because it is outside of the PAR range. However, far-red light can be equally effective at driving photosynthesis as increasing the number of PAR photons (Zhen and Bugbee, 2020), and potentially cost less for growers.

Far-red light not only has photosynthetic activity, it also affects the morphology of plants. Typical plant responses to a high ratio of far-red to red light include increase in leaf expansion, leaf width and length, and plant height. Larger leaves increase the amount of light a plant receives and alter photosynthetic rates (Zou et al., 2019). In combination with shorter wavelengths of light, far-red may increase photosynthetic rates (Emerson et al., 1957), which can drive biomass production. However, increased leaf elongation in response to far-red light also can result in a higher specific leaf area (SLA, leaf area/leaf dry weight), which can decrease photosynthesis due to decreases in leaf thickness. In previous studies, SLA did not change in response to increases in far-red light (Legendre and van Iersel, 2021). However, the canopy size did increase with increasing far-red light. Whole-plant photosynthesis in that case was dependent on the expanding canopy size, capturing more light (Legendre and van Iersel, 2021).

Far-red, often present in shaded areas due to high absorptance of red light by the upper canopy, is detected by plants via the photoreceptor family, phytochrome. There are two main forms of phytochrome, the active form (Pfr) and the inactive form (Pr) (Kalaitzoglou et al., 2019). Far-red light can be measured as a photon flux density or by calculating the ratio of Pfr to total P (phytochrome photostationary state, PSS). Plants that detect a high ratio of far-red to red light are often below the canopy and are shaded by other plants, which can induce shade avoidance responses (Sager et al., 1988). The subsequent changes in morphology and physiology, resulting from changes in gene expression, aid the plant in more effective acquisition of resources (Keuskamp et al., 2010). Shoot cellular expansion drives elongation responses and is facilitated by cell wall loosening. Cell wall enzymes like pectinesterases and pectin lyases are involved in cell wall loosening. Enzymes like xyloglucan endotransglycosylase/hydrolase (XTH) are of particular importance, because they can be involved in cell wall loosening as well as cell

strengthening. XTH genes encode for proteins that have two catalytic activities. The first is a nonhydrolytic cleavage and ligation of the xyloglucan chains within the cell wall (xyloglucan endotransglucosylase [XET]) and the other is an irreversible chain shortening of xyloglucan endo-hydrolase (XEH) (Rose et al., 2002). XTH consists of a large gene family, 33 members in *Arabidopsis thaliana* (Rose et al., 2002), that drive cell expansion by regulating turgor pressure without compromising the cell structure (Palmer and Davies, 1996). *LsXTH5*, *LsXTH8*, *LsXTH16*, and *LsXTH28* have high expression in young and developing leaves in lettuce, with the highest expression from *LsXTH8* and *LsXTH28* in young and mature leaves (Wagstaff et al., 2010).

This study was inspired by the findings of Legendre and van Iersel (2020). As far-red light increased, leaf area, leaf width, and length also increased. Higher amounts of far-red stimulated more growth, which increased the amount of intercepted light. That drove canopy photosynthesis and biomass accumulation (Legendre and van Iersel, 2021; Lee et al., 2015, Lee et al., 2016). The addition of far-red light in horticultural light fixtures can result in increased growth. Although the effect of far-red light on plant morphology is well-known, the genetic basis for those response is not clear. This study focused on confirming prior results and analyzing what genes may be involved in these growth responses due to far-red light.

Our objective was to quantify the photosynthetic, growth, and genetic responses in lettuce to various levels of far-red light, 5, 15, and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$. We hypothesized that plant biomass and plant size would increase as the amount of supplemental far-red light increased. Far-red may stimulate expansion in younger leaves, leading to a larger canopy size. Increased expansion of younger leaves can increase canopy photosynthesis early in the growing cycle. We hypothesized that *LsXTH* expression would increase with increasing far-red intensity. By quantifying the

effects of far-red on plant physiological parameters and crop growth, we aimed to prove that far-red is integral to plant growth in controlled environment systems.

Materials and Methods

Growing conditions

The study was conducted in a 0.8 m × 1.8 m growth chamber (E15, Conviron, Winnipeg, MB, Canada). The chamber was divided into three 0.8 m × 0.6 m sections. Each section was outfitted with two cool-white LED panels (Cool white 225 LED ultrathin grow light panel, Yescom USA, City of Industry, CA, USA) hung 0.6 m above the growth chamber floor. Two sections were also outfitted with custom-built far-red LEDs bars (peak at 735 nm with a full width at half maximum of 25 nm). The spectrum of the two different LED lights is shown in Supplementary figure 1, 39% blue (400–500 nm), 40% green (500–600 nm), 19% red (600–700 nm), and 2% far-red (700–800 nm). The temperature in the growth chamber was 24.7 ± 1.1°C, the VPD was 1.50 ± 0.50 kPa (mean ± SD), and CO_2 concentration was the same as the ambient air.

Plant material

'Green Salad Bowl' lettuce (*Lactuca sativa*) was seeded into 10-cm square pots filled with peat-perlite substrate (Fafard 2P; Sun Gro Horticulture, Agawam, MA, USA). Pots were thinned to one plant per pot at 7 days after planting. Plants were overhead irrigated as needed using a water-soluble fertilizer solution with a nitrogen concentration of 100 $mg \cdot L^{-1}$ (Peters Excel 15-5-15 CalMag Special, ICL, Summerville, SC, USA). The experimental unit was one plant, with each treatment containing 12 pseudo-replications. The plants were grown under different far-red treatments over a 5-week period. To facilitate canopy imaging, the substrate was

sprayed with a mixture of H_2O_2 and peroxyacetic acid (ZeroTol 2.0, Biosafe Systems, East Hartford, CT, USA) once a week to prevent the growth of algae. A 1:200 dilution of ZeroTol 2.0 to water was used.

Treatments

Plants were grown under three different far-red treatments, 5, 15, and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$, with all treatments getting 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of far-red light from the LED panels and the remaining far-red coming from the far-red LEDs. All plants were grown at similar photosynthetic photon flux density (PPFD) (317 ± 53 $\mu mol \cdot m^{-2} \cdot s^{-1}$), with a photoperiod of 16 hr. The daily light integral in all treatments was ~18 $mol \cdot m^{-2} \cdot d^{-1}$. Since the light distribution was not perfectly uniform and it was measured at the location of each plant using a spectroradiometer (SS-110, Apogee, Logan, UT), these measurements were taken 3 cm above the soil line at the center of each pot.

Data collection and analysis

Canopy images of each plant were taken twice a week after seedling emergence [8, 12, 15, 19, 23, 26, 30, 33 days after planting (DAP)]. A multispectral digital imaging system (TopView, Aris, Eindhoven, The Netherlands) outfitted with a 695 nm long-pass filter in front of a monochrome camera was used. Blue actinic light (peak at 450 nm) was used to induce chlorophyll fluorescence in a light-secure chamber and the camera took pictures of the fluorescence emitted by the plants (Legendre and van Iersel, 2020). This produced a greyscale image of the canopy. ImageJ software was used to calculate projected canopy size (PCS) using threshold separation (Narayanan et al., 2019).

A portable leaf gas exchange system (CIRAS-3, PP Systems, Inc., Amesbury, MA) was used to measure the net assimilation rate of plants within each treatment. Gas exchange data was

collected on the youngest fully expanded leaf from six plant per experimental treatment at 32 DAP. Plants were dark-acclimated, for 15 minutes, and then exposed to 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light from the LEDs built into the leaf cuvette. Cuvette temperature, CO_2 concentration, and VPD were similar to conditions in the growth chamber.

Lettuce plants were harvested at 34 DAP. The number of leaves, length and width of the longest leaf, total leaf area, and shoot dry weight were measured on six randomly selected plants from each treatment. Specific leaf area was calculated as leaf area/shoot dry weight. Leaf overlap ratio was calculated as total leaf area / projected canopy size at 33 DAP. The remaining six plants were used for gene expression analysis.

The light use efficiency and total incident light were calculated following protocol from Legendre and van Iersel (2021). PCS for every plant was estimated for each day. Incident photon flux for each day was calculated by multiplying the PCS by the PPFD + far-red photon flux density. Cumulative incident photon flux was calculated by summing those daily values. The shoot dry weight was divided by total incident photon flux to calculate the light use efficiency (LUE; g of biomass/mol of light) as a measure of how efficiently incident photon flux is used to produce biomass (Legendre and van Iersel, 2021).

Identification of *LsXTH8* and *LsACTIN* and primer design

LsXTH8 gene for this study was selected due to its transcript abundance during leaf development (Wagstaff et al. 2010). Primers for *LsXTH8* and *LsACTIN* were designed using (ApE- A Plasmid Editor (http://jorgensen.biology.utah.edu/wayned/ape/) software. Primer specificity was checked using Primer-BLAST program from National Center for Biotechnology Information (NCBI). We unsuccessfully tried to quantify transcript levels of *LsXTH28* as well. Based on the melting curve analysis, with *LsXTH28* primers, two peaks were observed. It is

possible that one of the peaks were *LsXTH28*-gene specific peak and the other was either a primer dimer or non-specific product and was therefore not used for quantification.

RNA extraction and cDNA synthesis

Leaf tissue samples from three developmental stages were collected from six plants for each of the three treatments in 50 mL falcon tubes and immediately frozen in liquid nitrogen. The three developmental stages were young, mature, and old leaves. Young leaves were defined as closest to the meristem and partially expanded. Mature leaves were defined as a fully expanded leaf located midway from the meristem and oldest leaf. Old leaves were defined as a fully expanded leaf furthest from the meristem. A total of 54 leaf samples were collected and stored in a -80 °C freezer until further processing. For RNA extraction, samples were ground into a fine powder using a mortar and pestle under liquid nitrogen. RNA was extracted using the TRIzol method adapted from Rio et al., (2010). Briefly, 100 mg sample was homogenized in 1 mL of TRIzol. The supernatant was extracted and washed two times with 400 µL chloroform to remove residual phenol. Next, RNA was precipitated using 200 µL isopropanol. The RNA pellet was washed with 500 µL of 70% ethanol. Subsequently the RNA pellet was dried and resuspended in 10 µL of Diethylpyrocarbonate (DEPC) water. Samples were analyzed using agarose gel electrophoresis to ensure that all RNA displayed high quality. Further, RNA quality was assessed on a Nanodrop (Thermo Scientific Nanodrop 8000 Spectrophotometer, Waltham, MA) to ensure the 260/280 absorbance values were between 1.9 and 2.1. cDNA was synthesized using 1 µg of mRNA from each sample. Samples were treated with DNAase for 34 minutes at 37 °C. Next, reverse transcription was carried out by following the manufacturer's protocol (Promega, Madison, WI, USA). Finally, samples were diluted 5-fold using DEPC water to 100 µl.

For the qRT-PCR reactions, two primers for *LsXTH8* and *LsACTIN* were used with every cDNA. A reaction of 12 µL was set-up containing 0.02 µM (*LsXTH8*) and 0.15 µM (*LsACTIN*) of the forward and reverse primer pairs, 1 µL of diluted cDNA, and 6 µL of SYBR PowerUP SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The qRT-PCR reactions were set up using a AriaMx Real-time PCR System (Agilent Technologies, CA). qRT-PCR cycles were as following the following parameters: 50 °C for 2 min, 95 °C for 5 min, followed by 95 °C for 15 s, and 60 °C (LsXTH8)/63 °C (*LsACTIN*) for 1 min repeated for 40 cycles. Melting curve analysis was performed using the program of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s. Primer efficiency was calculated for every reaction using LinRegPCR (v. 11.0; Ruitjers et al., 2009). Fold-change in transcript abundance was calculated using the relative quantities (RQ) which were corrected for primer efficiency. The RQ values were normalized using the reference gene, *LsACTIN* (NRQ), using the value from old leaves in the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment. The NRQ values were log_2 transformed prior to statistical analyses.

Experimental design and statistical analysis

The study was set up as a randomized design with twelve pseudo-replications at each far-red intensity. Data from each plant, excluding plants for gene transcript analysis, was analyzed using both linear and non-linear regression, as appropriate based on observed responses (SigmaPlot 11, Systat Software, Inc., San Jose, CA). Far-red light received by each pseudo-replication within a treatment differed and the individual far-red light intensity for each plant were used in the regressions. Relative gene transcript levels were analyzed using two-way ANOVA followed by Tukey's HSD test, using JMP Pro (version 15.0.0, SAS Institute, Cary, NC) to test for differences in transcript levels in response to far-red intensity, leaf age, and their

interaction. For the analysis of transcript levels, differences in far-red light intensity among pseudo-replicates were ignored.

Results

Growth and Morphology

Projected canopy size at 12 and 19 DAP was not affected by far-red treatment, averaging 0.5 and 13.6 $cm^2 \cdot plant^{-1}$, respectively. At both subsequent times, increasing far-red light increased projected canopy size (Figure 3.1). At 26 DAP projected canopy size in the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red treatment was ~12% and ~18% lower than in the 30 and 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red treatments, respectively. At 33 DAP, projected canopy size in the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red treatment was ~20% lower than in 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment and ~17% lower than in the 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment (Figure 3.1). The overlap ratio between the treatments were very similar, ranging on average 1.58 in the 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to 1.71 in the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

There was a negative correlation between leaf number and far-red level. The number of leaves$\cdot plant^{-1}$ decreased from ~17.5 at a far-red light intensity of 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to ~ 14.5 leaves in the 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment (P=0.0038, Figure 3.2). There was a linear increase in leaf width with increasing far-red level, from 11.4 cm at a far-red level of 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to 13.5 cm in the 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment (Figure 3.2). Leaf length increased non-linearly from an average 14.8 cm with the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of far-red to 18.0 cm in the 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment. Leaf area followed a similar trend as leaf length. Leaf area of plants with 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of far-red averaged 746 cm^2 as compared to 899 cm^2 in the 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment (Figure 3.2).

Photosynthesis and Incident Light

Shoot dry mass increased asymptotically from 2.04 to 2.6 g/plant as far-red level increased, a 27% increase. No trends in specific leaf area or net assimilation rates in response to far-red level were noted (Figure 3.3).

Incident light increased asymptotically as far-red level increased, from ~6.4 mol·plant^{-1} with 5 µmol·m^{-2}·s^{-1} of far-red light to, increasing to ~8.5 mol·plant^{-1} in the 15 and 30 µmol·m^{-2}·s^{-1} far-red treatments (Figure 3.4). The plants in the 5 µmol·m^{-2}·s^{-1} far-red treatments had ~24% lower incident light compared to the plants in the 15 and 30 µmol·m^{-2}·s^{-1} treatments. No notable trend was seen in light use efficiency as far-red level increased. Light use efficiency averaged 0.31 g·mol^{-1} (Figure 3.4).

Gene Expression

No significant differences were observed between far-red treatments or the far-red treatment x leaf age interaction, but that there were significant differences among all three leaf ages: young, mature and old. Young leaves had significantly higher expression levels of *LsXTH8* compared to matures leaves, and mature leaves had significantly higher expression levels of *LsXTH8* compared to old leaves ($P < 0.01$) (Figure 3.5).

Discussion

Far-red light and canopy size

Projected canopy size (PCS) is a sensitive, non-destructive measure of morphological changes in response to far-red levels. Projected canopy size measurements can be taken throughout a growing cycle and provide information on growth rate. In the 5 µmol·m^{-2}·s^{-1} far-red treatment, the plants had a lower PCS than those in the 15 and 30 µmol·m^{-2}·s^{-1} treatments from

26 DAP until the end of the study (Figure 3.1). We hypothesized that far-red light would induce leaf expansion in seedlings and during early growth, however no differences in PCS were seen among the treatments until 26 DAP (Figure 3.1). Legendre and van Iersel (2021), on the other hand, observed a positive correlation between PCS, leaf length, leaf width, and leaf area with increasing far-red light at 16 DAP. PCS early in the growing cycle can also be a good predictor of final dry mass. Correlations have been reported between early PCS and final dry mass by Elkins and van Iersel (2020a) in response to different PPFD and photoperiod treatments (all with the same daily light integral). However, in our results PCS was only correlated with final dry mass on the last day PCS was measured (Supplementary figure 3.2), suggesting that PCS only during the mid-growth phase may also be a predictor for final dry mass.

PCS of plants in the 15 and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ increased with increasing far-red intensity at 26 and 33 DAP. Total incident light drives overall canopy photosynthesis and growth (Klassen et al., 2004). Plants with larger canopies can capture more light. This leads to increases in growth and the development of more canopy (Weaver and van Iersel, 2020). Our results are consistent with a study that stimulated growth in lettuce with the addition of various levels of far-red light. Legendre and van Iersel (2021) found longer, wider leaves with increasing far-red light. Both leaf length and width were positively correlated with PCS and incident light. Larger canopies captured more light, presumably increasing whole-plant photosynthesis, but the light use efficiency of those plants was not affected by far-red light intensity. This is consistent with our results, no differences were seen in light use efficiency, despite intercepted light increasing with increasing far-red light (Figure 3.4).

Leaf morphology

Greater leaf expansion was observed in both the 15 and 30 μmol·m^{-2}·s^{-1} far-red treatments compared to the 5 μmol·m^{-2}·s^{-1} treatment. Increasing far-red light increased both leaf width and leaf length (Figure 3.2), consistent with the results of Legendre and van Iersel (2021). However, there was a negative correlation between leaf number and far-red level (Figure 3.2), which also has been reported in other species (Park and Runkle 2017). Lee et al. (2015) observed the similar results in lettuce. As red to far-red ratios increased, less far-red light, so did the number of leaves. This indicates that lighting spectrum and cultivar do not play a role in leaf number, and that instead the amount of far-red light does.

Leaf expansion is a shade avoidance response induced by low red to far-red ratios (Elkins and van Iersel, 2020b; Lee et al., 2015, Lee et al., 2016; Legendre and van Iersel 2020). Legendre and van Iersel (2021) observed similar results using a gradient of far-red intensity (4.9-28.0 μmol·m^{-2}·s^{-1}). As far-red intensity increased, and the ratio of red to far-red decreased, leaf width and leaf length increased (Legendre and van Iersel 2021).

Leaf area increased asymptotically with increasing far-red light intensity. Plants in the 5 μmol·m^{-2}·s^{-1} treatment had 17% lower leaf area than plants grown with higher far-red intensity (Figure 3.2). A previous study reported increases in leaf area when 50 μmol·m^{-2}·s^{-1} of far-red light was applied during the entire day (49% increase) or at the end of the day (60 minutes of far-red without PPFD, 27% increase) (Zou et al., 2019). Zou et al. (2019) observed differences between their treatments, however their leaf areas were smaller than ours, reporting an average of 808 cm^2·plant^{-1} in the all-day far-red treatment and 691 cm^2·plant^{-1} in the end-of-day treatment. Our plants in the 15 and 30 μmol·m^{-2}·s^{-1} treatments averaged leaf areas between ~741-993 cm^2·plant^{-1}. This may be due to the length of our study. Their plants were harvested at

15 days after transplant (while our plants were harvested 35 DAP), with the 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ end-of-day far-red treatment showing similar responses to our 15 and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red treatments. Zou et al. (2019) might have observed larger leaf areas if the growing cycle had been extended for another 20 days. Their results indicate that providing far-red light by itself is likely effective in inducing phytochrome responses. Far-red applied at end-of-day, after the photoperiod, can be effective in inducing morphological changes, as seen by Zou et al. (2019).

Far-red indues conformational changes in the state of phytochrome, active (Pfr) and inactive (Pr). At low red to far-red ratios, Pfr is converted to Pr, and different phytochromes regulate plant responses to changing light environments. PHYB is the main regulator mediating shade avoidance responses at low red to far-red ratios (Keuskamp et al., 2010). It binds to PIF (Phytochrome Interacting Factors) proteins that induce changes in gene expression in the nucleus (Keuskamp et al., 2010; Sakamoto and Nagatani, 1996). Those changes affect hormonal responses and can induce leaf expansion.

Photosynthesis and biomass accumulation

Dry mass followed a similar trend as leaf area. Plants in the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ had lower dry mass compared to the plants in the 15 and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatments (Figure 3.3). Dry mass in the 15 and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red treatments was 27% higher than that in the 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ treatment, resulting from a <10% increase in total photon flux. Considering that it takes less energy to generate far-red photons compared to photons in the PPFD range (Kusuma et al., 2020), the large increase in dry mass in response to a relatively small amount additional of far-red light indicates the importance of including far-red light in the light spectrum used for sole-source lighting (no sunlight) in controlled environment systems. Previously, the effects of far-red light have been studied under only red LEDs (Lee et al., 2015) or in combination with red and

blue LEDs (2:8 ratio) (Lee et al., 2016). Plants receiving supplemental far-red light had significantly higher shoot dry mass compared to the control in both experiments (just red LEDs or just red/blue LEDs) (Lee et al., 2015; Lee et al., 2016). Dry mass accumulation depends on photosynthesis and thus incident light. As the amount of light a plant intercepts increases, so does the canopy photosynthesis, leading to increases in biomass accumulation (Klassen et al., 2004). Zou et al. (2019) reported a 39% and 25% increase in total dry mass (leaf, roots, and stem) with increased supplemental far-red (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) during the day or at the end-of-day, respectively, consistent with our results.

No significant trend was observed in specific leaf area and net assimilation rates in response to far-red light intensity. We anticipated that SLA and net assimilation rates would be correlated, but no correlations between SLA, net assimilation rate, and far-red light intensity were found. Zou et al. (2019) observed a significant increase in SLA with the addition of supplemental far-red light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) during the photoperiod or at the end-of-day compared to the control (no far-red light). The addition of far-red light also increased instantaneous leaf photosynthesis by 7-10% (Zou et al, 2019). Measurement in this study for leaf photosynthesis were taken at 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and did not include the various far-red light levels and thus were not representative of leaf photosynthetic rates under the lighting conditions the plants were grown under. Increases in leaf photosynthesis in response to increasing far-red light (0-90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of far-red light) were seen previously in greenhouse grown lettuce (Zhen and van Iersel, 2017).

Leaf size and specific leaf area are important in determining canopy size. This influences the total amount of light received by the plants, which is important for canopy photosynthesis. At the whole plant level, the differences in dry mass per plant suggest that far-red played a role in

whole-plant photosynthesis and leaf morphology, especially with the 27% higher dry mass in the 15 and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ far-red treatments in our study. Canopy photosynthesis drives biomass accumulation. Zhen and Bugbee (2020) reported an increase in canopy gross photosynthesis by 6.7-20% with an addition of 40-140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of supplemental far-red light (background white light, 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Far-red photons increased canopy photosynthesis as effectively as adding the same photon flux density from white light. This suggests that far-red photons have the same photosynthetic efficacy as PAR photons (Zhen and Bugbee, 2020).

Gene expression

Far-red light plays a role in mediating shade avoidance responses, including leaf elongation, in lettuce and other greenhouse crops. It is unclear if increased leaf expansion is due to increased cell division and/or cell expansion. Lee et al. (2015) reported cell division was induced with the addition of small amounts of far-red and was highest under red light only. The highest rates of cell division occurred in young leaves (Lee et al., 2015). Lee et al. (2016) reported no increase in cell division in response to far-red treatments and attributed that to the addition of blue light, diluting the far-red effect on cell division. If in fact far-red induces cell division, it is most likely in younger leaves. At some point though, far-red may be inducing cell expansion in maturing leaves. Xyloglucan endotransglucosylase/ hydrolase (XTH) is a cell-wall modifying enzyme that may play a role in leaf expansion and cell wall loosening. It is expressed in high abundance in developing leaves, which expand rapidly (Wagstaff et al., 2010). Our results indicate that expression of *LsXTH8* is not upregulated when far-red levels are increased from 5 to 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$. However, we did see *LsXTH8* expression decreasing as leaves age, consistent with the results of Wagstaff et al., (2010). As mentioned before, XTH consists of a 33 member gene family in *Arabidopsis thaliana*. It is not clear how may XTH genes are present in

lettuce but may be other members of this gene family that contribute to cell wall expansion and leaf elongation in response to far-red light. Wagstaff et al. (2010) reported *LsXTH28* as having high expression in young and mature leaves as compared to old leaves, and could possibly be upregulated at high levels of far-red. We tried to quantify this, but had difficulty designed the appropriate primer.

Other genes may play a role in regulating morphological responses to far-red light. More research is needed to determine which cell wall loosening or cell division genes and enzymes are involved when far-red levels are increased. B-type cyclins and KRPs (Kip related proteins) may be involved in cell division. These two genes display opposite patterns of transcript abundance. In younger leaves, B-type Cyclins are upregulated, while in older leaves KRPs are upregulated. If far-red induces cell division in young leaves, B-type Cyclins should be upregulated, while KRPs would be down-regulated. Expansins may also be activated in response to high far-red, and trigger cell wall changes that influence cell expansion (Eklof and Brumer, 2010).

Conclusions

Our results indicate that plant biomass and plant size of lettuce increase as the amount of supplemental far-red light increases. This growth-promoting effect of far-red light was associated with increased leaf width and length, as well as PCS. These morphological changes helped the plants intercept more light, likely increasing canopy photosynthesis. The genetic basis of the morphological response to far-red light was not found. *LsXTH8* expression did not increase with increasing far-red light; however, there may be other genes that can influence leaf expansion in response to far-red light. To increase the growth of plants in controlled environment systems, supplemental far-red light, at 15 or 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$, is effective. Since it requires less energy to

produce far-red photons as compared to PPFD, including far-red in the spectrum for sole source lighting can potentially save growers money by decreasing the energy required to drive adequate growth and allow for earlier harvests.

Literature Cited

Elkins, C., and van Iersel, M.W. (2020). Longer Photoperiods with the Same Daily Light Integral Increase Daily Electron Transport through Photosystem II in Lettuce. *Plants* 9**,** 1172. doi: 10.3390/plants9091172.

Elkins, C., and van Iersel, M.W. (2020). Supplemental Far-red Light-emitting Diode Light Increases Growth of Foxglove Seedlings Under Sole-source Lighting. *HortTechnology* 30**,** 564-569.

Emerson, R., Chalmers, R., and Cederstrand, C. (1957). Some factors influencing the long-wave limit of photosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* 43**,** 133.

Faust, J.E., and Logan, J. (2018). Daily light integral: A research review and high-resolution maps of the United States. *HortScience* 53**,** 1250-1257. doi: 10.21273/hortsci13144-18.

Kalaitzoglou, P., Van Ieperen, W., Harbinson, J., Van Der Meer, M., Martinakos, S., Weerheim, K., et al. (2019). Effects of Continuous or End-of-Day Far-Red Light on Tomato Plant Growth, Morphology, Light Absorption, and Fruit Production. *Front Plant Sci* 10**,** 322. doi: 10.3389/fpls.2019.00322.

Keuskamp, D.H., Sasidharan, R., and Pierik, R. (2010). Physiological regulation and functional significance of shade avoidance responses to neighbors. *Plant Signal Behav* 5**,** 655-662. doi: 10.4161/psb.5.6.11401.

Klassen, S., Ritchie, G., Frantz, J., Pinnock, D., and Bugbee, B. (2004). Real-time imaging of ground cover: Relationships with radiation capture, canopy photosynthesis, and daily growth rate. *Digital imaging and spectral techniques: applications to precision agriculture and crop physiology* 66**,** 1-14. doi: 10.2134/asaspecpub66.c1.

Kusuma, P., Pattison, P.M., and Bugbee, B. (2020). From physics to fixtures to food: current and potential LED efficacy. Horticulture research 7, 1-9.

Lee, M.-J., Park, S.-Y., and Oh, M.-M. (2015). Growth and cell division of lettuce plants under various ratios of red to far-red light-emitting diodes. *Horticulture, Environment, and Biotechnology* 56**,** 186-194.

Lee, M.-J., Son, K.-H., and Oh, M.-M. (2016). Increase in biomass and bioactive compounds in lettuce under various ratios of red to far-red LED light supplemented with blue LED light. *Horticulture, Environment, and Biotechnology* 57**,** 139-147.

Legendre, R., and van Iersel, M.W. (2021). Supplemental Far-Red Light Stimulates Lettuce Growth: Disentangling Morphological and Physiological Effects. *Plants (Basel)* 10. doi: 10.3390/plants10010166.

Mayorga-Gómez, A., and Nambeesan, S.U. (2020). Temporal expression patterns of fruit-specific α-EXPANSINS during cell expansion in bell pepper (Capsicum annuum L.). *BMC Plant Biology* 20**,** 1-12.

McCree, K.J. (1972). Test of current definitions of photosynthetically active radiation against leaf photosynthesis data. Agricultural meteorology 10, 443-453.

Narayanan, M., van Iersel, M.W., and Haidekker, M. (2019). "Chlorophyll Fluorescence Imaging: A Novel, Simple and Non-Destructive Method for Canopy Size Imaging", in: *2019 ASHS Annual Conference*: ASHS). https://ashs.confex.com/ashs/2019/meetingapp.cgi/Paper/30905.

Palmer, S.J., and Davies, W.J. (1996). An analysis of relative elemental growth rate, epidermal cell size and xyloglucan endotransglycosylase activity through the growing zone of ageing maize leaves. *Journal of experimental botany* 47**,** 339-347.

Park, Y., and Runkle, E.S. (2017). Far-red radiation promotes growth of seedlings by increasing leaf expansion and whole-plant net assimilation. *Environmental and Experimental Botany* 136**,** 41-49. doi: 10.1016/j.envexpbot.2016.12.013.

Rio, D.C., Ares, M., Jr., Hannon, G.J., and Nilsen, T.W. (2010). Purification of RNA using TRIzol (TRI reagent). *Cold Spring Harb Protoc* 2010**,** pdb prot5439. doi: 10.1101/pdb.prot5439.

Rose, J.K., Braam, J., Fry, S.C., and Nishitani, K. (2002). The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: current perspectives and a new unifying nomenclature. *Plant and Cell Physiology* 43**,** 1421-1435.

Ruijter, J., Ramakers, C., Hoogaars, W., Karlen, Y., Bakker, O., Van Den Hoff, M., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic acids research 37, e45-e45.

Sager, J., Smith, W., Edwards, J., and Cyr, K. (1988). Photosynthetic efficiency and phytochrome photoequilibria determination using spectral data. Transactions of the ASAE 31, 1882-1889.

Sakamoto, K., and Nagatani, A. (1996). Nuclear localization activity of phytochrome B. *The Plant Journal* 10**,** 859-868.

Wagstaff, C., Clarkson, G.J., Zhang, F., Rothwell, S.D., Fry, S.C., Taylor, G., et al. (2010). Modification of cell wall properties in lettuce improves shelf life. *J Exp Bot* 61**,** 1239-1248. doi: 10.1093/jxb/erq038.

Weaver, G., and van Iersel, M.W. (2020). Longer photoperiods with adaptive lighting control can improve growth of greenhouse-grown 'Little Gem' lettuce (Lactuca sativa). *HortScience* 55**,** 573-580.

Zhen, S., and Bugbee, B. (2020). Far-red photons have equivalent efficiency to traditional photosynthetic photons: Implications for redefining photosynthetically active radiation. *Plant Cell Environ* 43**,** 1259-1272. doi: 10.1111/pce.13730.

Zhen, S., and van Iersel, M.W. (2017). Far-red light is needed for efficient photochemistry and photosynthesis. *J Plant Physiol* 209**,** 115-122. doi: 10.1016/j.jplph.2016.12.004.

Zou, J., Zhang, Y., Zhang, Y., Bian, Z., Fanourakis, D., Yang, Q., et al. (2019). Morphological and physiological properties of indoor cultivated lettuce in response to additional far-red light. *Scientia Horticulturae* 257**,** 108725.

Figures

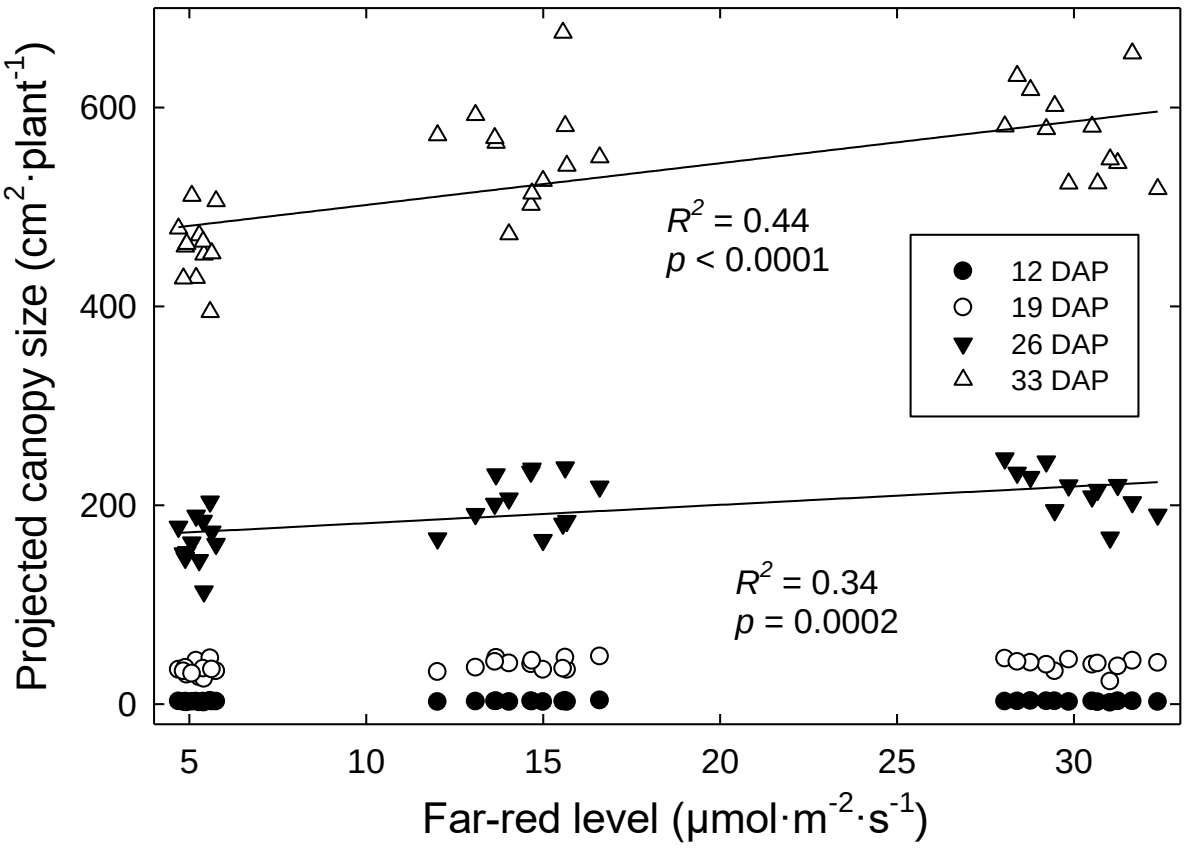


Figure 3.1. Projected canopy size of 'Green Salad Bowl' lettuce (*Lactuca sativa*) at 12, 19, 26, and 33 days after planting (DAP), measured on 6 pseudo-replications. Plants were grown under different far-red intensities, ~ 5, 15, and 30 µmol·m^{-2}·s^{-1} with a constant photosynthetic photon flux density. Identical symbols represent the twelve pseudo-replications of each treatment.

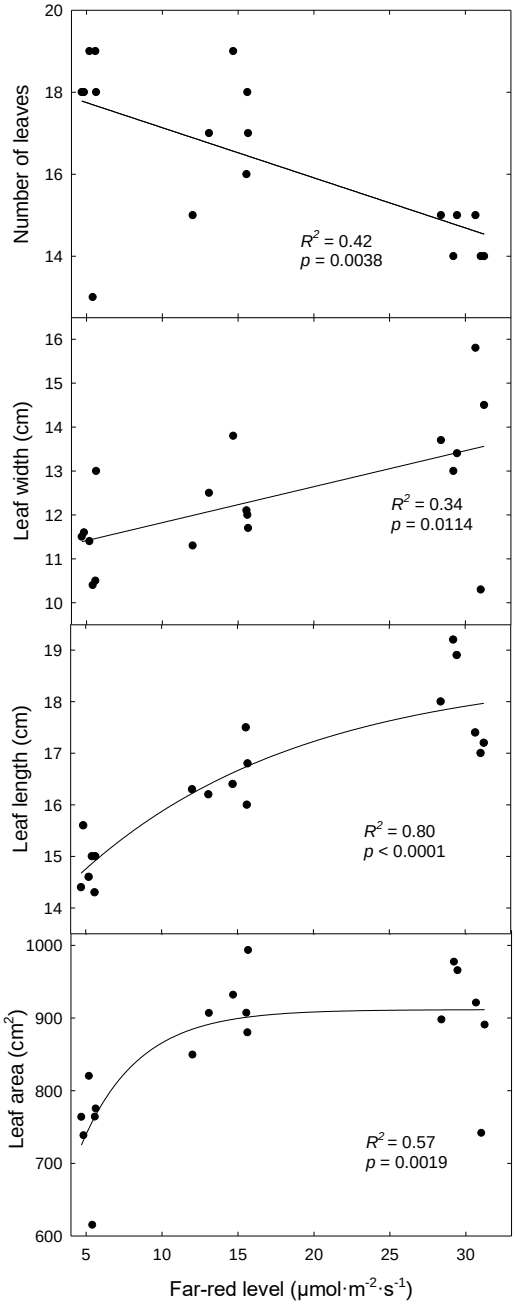


Figure 3.2. Number of leaves per plant, leaf width, leaf length, and leaf area of 'Green Salad Bowl' lettuce (*Lactuca sativa*) as a function of far-red photon flux density (six pseudo-replications per treatment). Plants were grown under different far-red intensities (~5, 15, and 30 µmol·m^{-2}·s^{-1}) with a similar photosynthetic photon flux density.

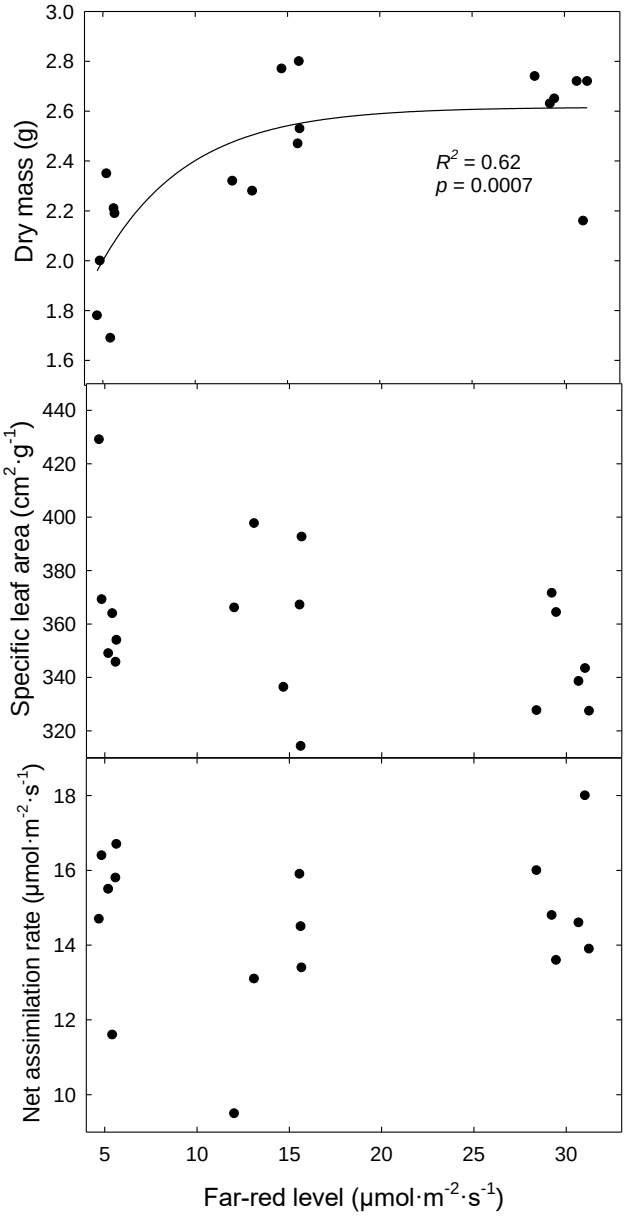


Figure 3.3. Dry mass, specific leaf area, and net assimilation of 'Green Salad Bowl' lettuce (*Lactuca sativa*) as a function of far-red photon flux density (six pseudo-replications per treatment). Plants were grown under different far-red intensities (~5, 15, and 30 μmol·m^{-2}·s^{-1}) with a similar photosynthetic photon flux density.

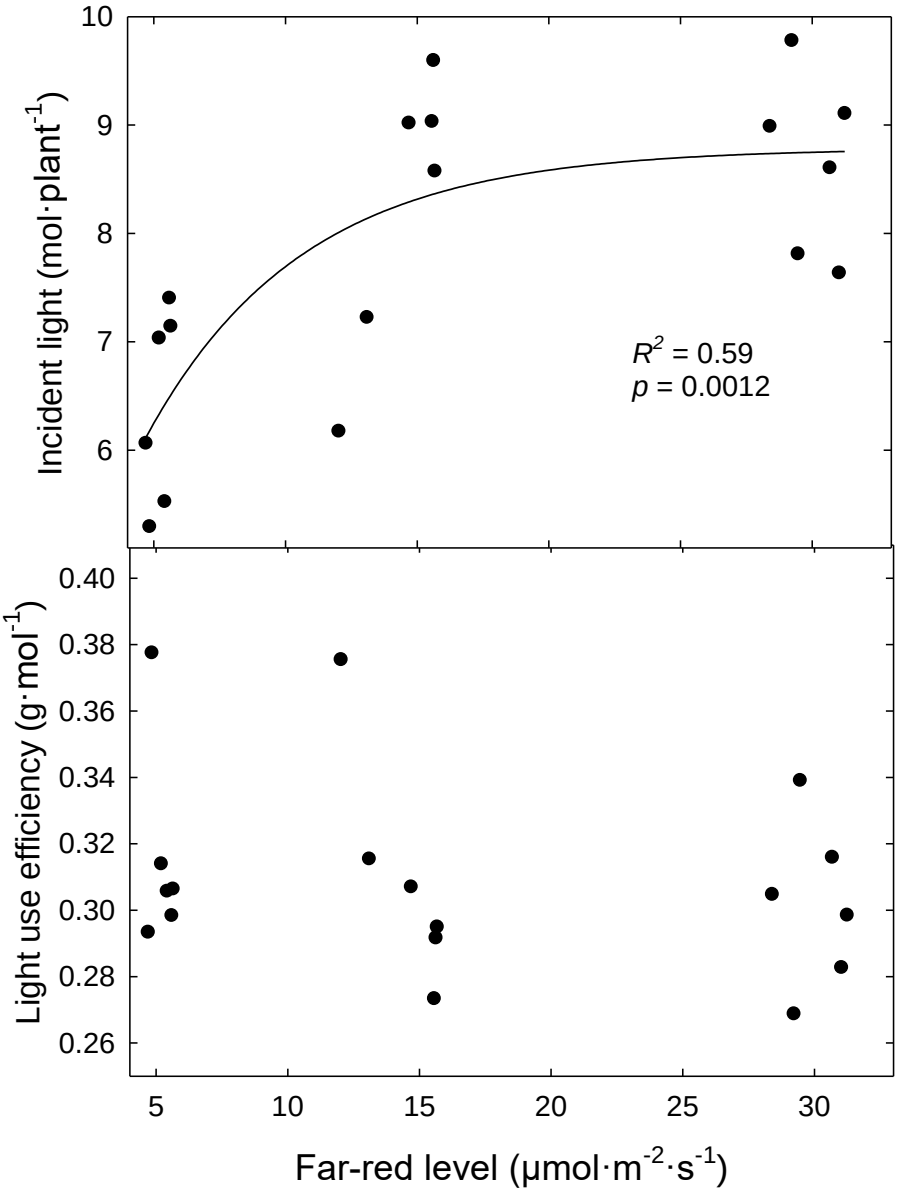


Figure 3.4. Incident light and light use efficiency as a function of 'Green Salad Bowl' lettuce (*Lactuca sativa*) as a function of far-red photon flux density (six pseudo-replications per treatment). Plants were grown under different far-red intensities ~5, 15, and 30 µmol·m^{-2}·s^{-1} with a constant photosynthetic photon flux density.

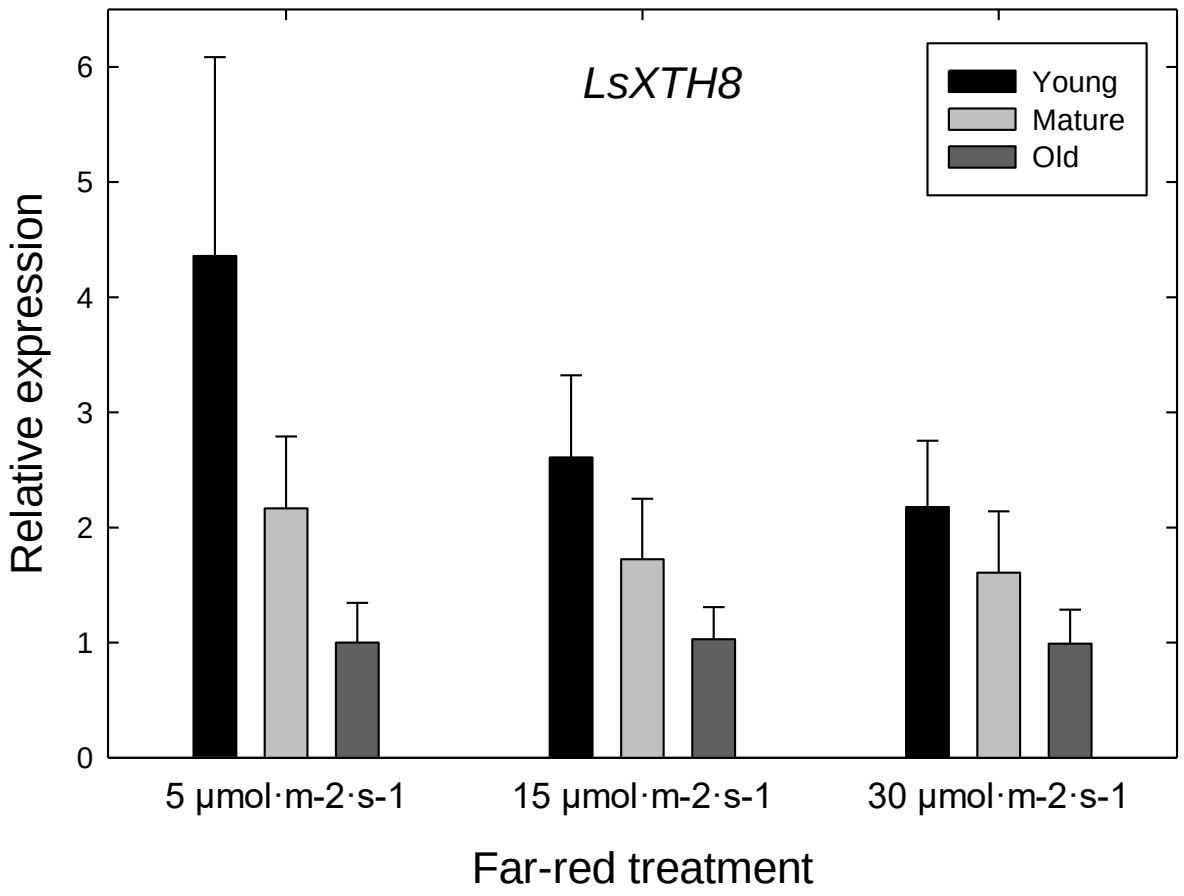
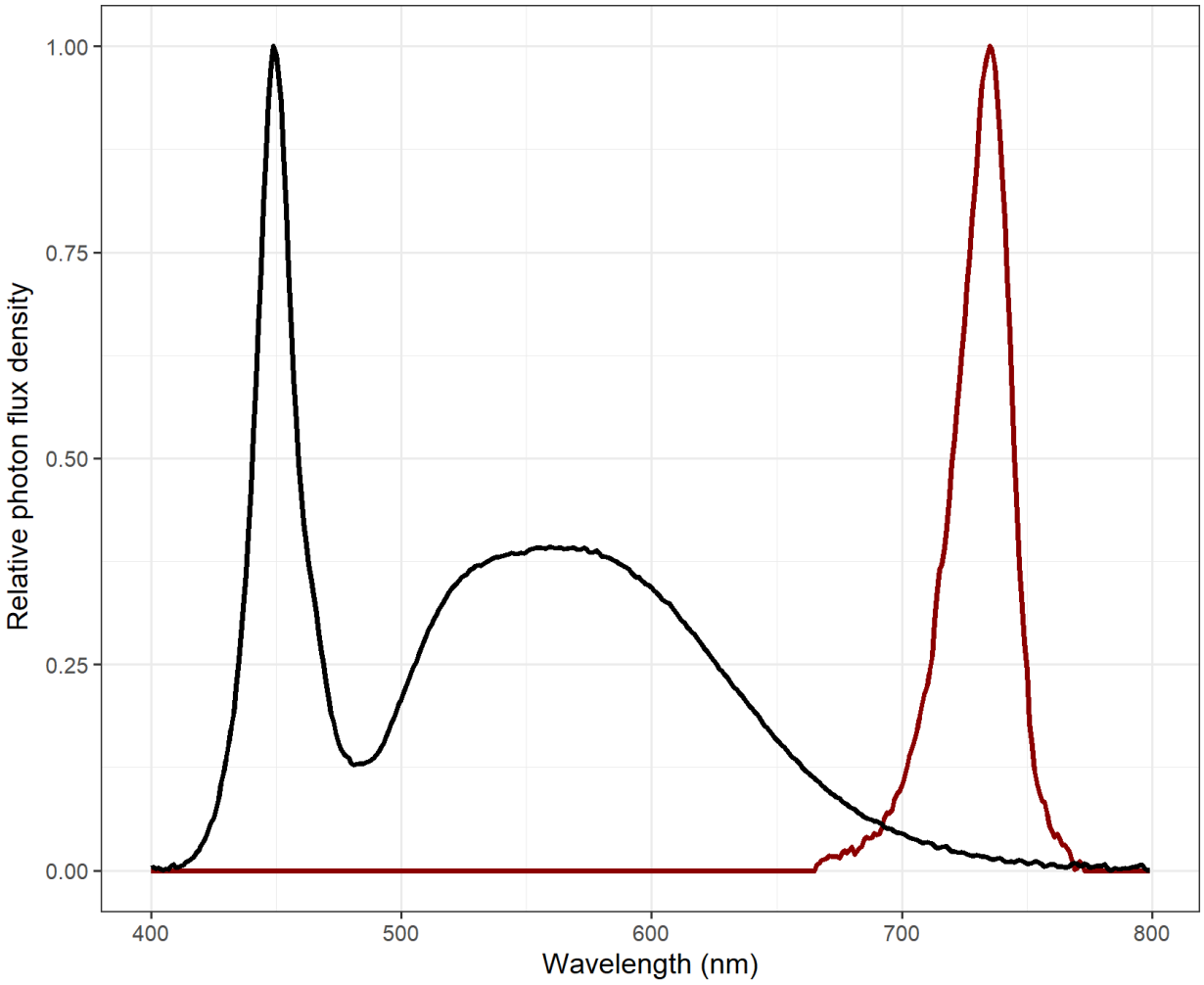
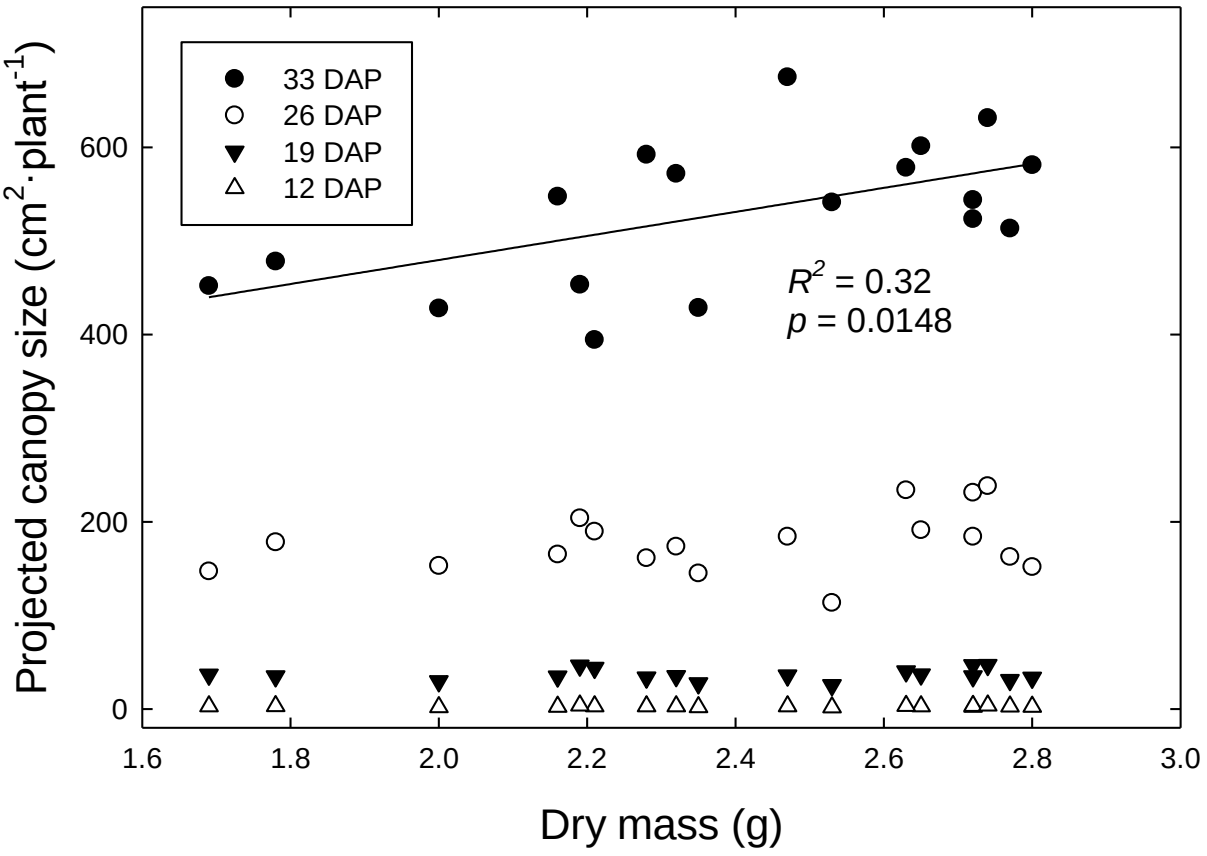


Figure 3. 5. Relative transcript levels of LsXTH8 gene in 'Green Salad Bowl' lettuce (*Lactuca sativa*) in young, mature, and old leaves, normalized to actin. Plants were grown at far-red photon flux densities of ~5, 15, and 30 µmol·m^{-2}·s^{-1} and similar photosynthetic photon flux density (317 ± 53 µmol·m^{-2}·s^{-1}). Bars indicate the mean of six pseudo-replications (±SE). No significant effect was observed between far-red or far-red x leaf age, but that there were significant differences among all three leaf ages ($P < 0.01$).



Supplementary figure 3.1. Relative photon flux density of light spectrums of the cool-white LED panels (black) and supplemental far-red LEDs (red) used to provide light during the experiments. (from Legendre and van Iersel, 2021)



Supplementary figure 3.2. Projected canopy size as a function of dry mass (x-axis indicates dry mass). Symbols (six pseudo-replications per treatment) represent 'Green Salad Bowl' lettuce (*Lactuca sativa*). Plants were grown under different far-red intensities ~5, 15, and 30 µmol·m^{-2}·s^{-1} with a constant photosynthetic photon flux density. Identical symbols represent the days after planting.

CHAPTER 4

Conclusions

Controlled environment systems are often required for year-round production. The demand for specialty food crops, landscaping plants, and cut flowers drive the importance of maintaining year-round and seasonal production. In CEA systems, natural light and ambient temperatures are often the limiting factor. Supplemental lighting and heaters are used to create ideal growing environments. The research in this thesis outline solutions that can aid growers in decreasing production cost and increasing profits.

Results from our first study indicate that 'Little Gem' and 'Green Salad Bowl' lettuce tolerate fluctuating light levels, as long as the fluctuations are not extreme. This is consistent with previous findings and suggests that regulating supplemental light in response to real-time electricity prices is feasible for controlled environment agriculture. Our research was limited to two lettuce cultivars, which generally behaved similarly. Follow-up research on spreading (e.g., strawberry) and vine crops (e.g. tomato, bell peppers, and cucumbers) is needed to determine how other crops respond. In addition, we only tested fluctuations at 15-minute intervals and how plants respond to different intervals is not clear. Although we did not answer all questions related to fluctuating lights, this research indicates that there is potential to reduce the electricity costs associated with supplemental lighting in response to real-time electricity price fluctuations. Dynamic algorithms that control supplemental lighting in response to variable sunlight conditions could be updated to incorporate real-time pricing and implemented in the greenhouse

industry. Such algorithms have been described but it is not clear if they have been implemented in commercial greenhouses.

Our results in the far-red lighting study indicate that plant biomass and plant size of lettuce increases as the amount of supplemental far-red light increases. Increases in growth indicate that supplemental far-red is effective in controlled environment systems. The morphological changes in response to far-red light helped the plants intercept more light, likely driving canopy photosynthesis. The lack of effects on *LsXTH8* expression indicate that other genes may play a role in the leaf expansion effects seen under far-red light. More research is needed to determine which cell wall loosening or cell division genes and enzymes are involved. B-type cyclins and KRPs (Kip related proteins) are candidates for cell division. If far-red induces cell division in young leaves, B-type Cyclins should be upregulated, while KRPs would be down-regulated. Additionally, other XTH genes and Expansin may also be activated in response to high far-red. Both genes have large gene families that may contain several cell wall expansion and loosening enzymes.

Since far-red light requires less energy to produce per photon compared to PPFD, including far-red in the spectrum for sole source lighting can potentially save growers money by decreasing the energy required to drive adequate growth.