# IMPROVING COLOR AND PHENOLIC CONCENTRATION OF LEAFY GREENS AND MICROGREENS WITH END-OF-PRODUCTION LIGHTING AND COOLING TREATMENT

By

Devin S. Brewer

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## ABSTRACT

Modern controlled environment agriculture (CEA) systems allow for the precise manipulation of environmental parameters such light intensity, quality, and mean daily temperature (MDT). This allows for use of end-of-production (EOP) cooling, light intensity, or quality strategies to improve crop quality parameters such as foliage color, mineral nutrients, or phenolic compound synthesis. The objective of Expt. 1 was to produce red leaf lettuce under the environmental conditions that Tarr et al. (2023) reported to maximize yield and quantify the influence of providing 150 μmol·m$^{-2}$ ·s $^{-1}$ of EOP sole-source lighting providing a light ratio (%) of either 100:00 blue:red (B:R), 75:25 B:R, or 50:50 B:R during the final 6-8 days of production on anthocyanin, nutrition, coloration, and yield of red leaf lettuce (*Latuca sativa*) 'Barlach', 'Rouxai', and 'Thurinus'. Anthocyanin concentration decreased similarly across treatments for each cultivar. In Expt. 2 we exposed red leaf lettuce to EOP treatment consisting of 150 μmol·m$^{-2}$ ·s $^{-1}$ of 75:25 B:R light and decreased the MDT to either 8, 14, or 20 °C during the final 6-8 days of production. EOP cooling of 8 or 14 °C increased anthocyanin concentration of each cultivar, however decreased fresh mass. In Expt. 3 we grew beet 'Bulls Blood' (*Beta vulgaris*), mustard 'Miz America' (*Brassica juncea*), pac choi 'Red Pac' (*Brasica rapa* var *chinensis*), cabbage 'Red Jewel' *(Brassica oleracea*), and daikon radish 'Shunkyo' (*Raphanus sativus*) microgreens at a MDT of 20 °C and light intensity of 150 μmol·m$^{-2}$ ·s$^{-1}$ then exposed them to EOP temperatures of 8, 14 or 20 °C for the final 3 days of production. Coloration of red species and shelf life of 'Bulls Blood', 'Miz America', and 'Shunkyo' improved when exposed to 8 °C

DEDICATED TO DANIEL BREWER
THANK-YOU FOR TEACHING ME HOW TO LIVE A KIND AND JOYOUS LIFE.
THOUGH NOW GONE FROM MY SIDE, YOUR EVERGREEN SPIRIT IS ALWAYS IN MY HEART.

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# LITERATURE REVIEW

## LITERATURE REVIEW: END-OF-PRODUCTION TREATMENT TO IMPROVE ANTHOCYANIN CONTENT, NUTRITION, AND COLORATION OF LEAFY GREENS AND MICROGREENS IN CONTROLLED ENVIRONMENT

### Introduction

Lettuce (*Lactuca sativa*) is a substantial horticultural crop in the United States with a total wholesale value of $3.5 billion (USDA, 2020a). Most lettuce is grown domestically with 95% of production occurring on irrigated fields located in California and Arizona (USDA, 2020a). Lettuce produced in controlled environments (CE) such as greenhouses and indoor farms has increased by 27% from a reported area of 402,270 $m^2$ to 513,847 $m^2$. Likewise, from 2014 to 2019, total sales of lettuce produced in CEs increased 28% from $55.5 million in 2014 to $71.1 million in 2019 (USDA, 2015; USDA, 2020b). Increasing demand for lettuce is driven by its health properties, which can be attributed to its supply of vitamins, minerals, and phenolic compounds such as anthocyanins and carotenoids (Llorach et al., 2008).

Microgreens are a relatively new specialty crop category that is referred to as "an exotic genre of edible greens or sprouts" (Mir et al., 2017). Seedlings are typically harvested within 20 days of emergence and after the first true leaves have unfolded (Xiao et al., 2014). The shoot is harvested for consumption as a salad or garnish, while the roots are discarded (Kou et al., 2013). Given that microgreen shoots are harvested from an array of vegetable and herb species, there is a broad range of colors, textures, and flavors available (Xiao et al., 2012; Pinto et al., 2015).

Due to the relatively low up-front investment, minimal resource demand, rapid turnover, and price premium of up to $60 to $100 per kilogram, the potential exists for year-round indoor production of microgreens (Enssle, 2020). Additionally, microgreens are considered a functional food, as they contain significantly higher concentrations of health promoting or disease preventing properties including vitamins, minerals, and phenolic compounds than their mature

counterparts (Xiao et al., 2015; Zhang et al., 2021). As a result, a niche market has emerged, driven by restaurants and upscale markets (Mir et al., 2017).

Currently there are challenges associated with food safety and shelf life of microgreens. Upon germination, seedlings release a mixture of carbohydrates and peptides that encourage bacterial growth around the rhizosphere (Turner et al., 2020). This issue is exacerbated because microgreens are particularly vulnerable to bacterial internalization as casparian strips have not yet formed and are not able to act as a defensive structure. The lack of this defensive structure allows bacteria to easily migrate from the roots into the xylem (Warriner et al., 2003). Microgreens also suffer from a short shelf life due to their relatively high respiration rate (Chandra et al., 2012). After harvest microgreens must be kept cold, between 1-5 °C, along with high humidity to mitigate respiration and achieve a shelf life of up to 14 days (Kou et al., 2013).

Anthocyanins are phenolic compounds that are associated with red and purple coloration in the foliage of red-leaf lettuce, kale, microgreens, and flowers (Li and Kubota, 2009; Samuoliené et al., 2012; Goins et al., 1998). Plants synthesize phenolic compounds, including anthocyanins, to counteract oxidative damage; synthesis may be induced by wounding, pathogen infection, chilling injury, nutrient deficiency, excessive exposure to wavelengths of 400-700nm or photosynthetically active radiation (PAR), and moderate exposure to wavelengths of 285-400nm or ultraviolet (UV) radiation (Pollastri and Tattini, 2011). Of the environmental factors that promote the production of anthocyanins, light quality and intensity are of particular interest because they can be altered in CEs using LED fixtures (Carvalho and Folta, 2016).

Beyond influencing foliage and flower color, anthocyanins demonstrate greater antioxidant activity than both vitamin C and E in vitro (Kim et al., 2007). While the mechanism of action for the antioxidative effect of anthocyanins is still uncertain, there are many in vivo and

in vitro studies demonstrating this effect and it is suggested that greater antioxidant activity through consumption of plants containing anthocyanins may provide health benefits such as improved gut-health, retinal protection, and hypolipidemia while reducing the likelihood or severity of cancer and neurodegeneration due to ageing (Blesso., 2019; Wallace et al., 2016; Zhu., 2018).

Leafy greens are also an excellent source of carotenoids, including lutein, zeaxanthin, and beta-carotene (Aramrueang et al., 2019). Carotenoids are a class of naturally occurring pigments that provide yellow, orange, and red hues to many fruits and vegetables (Edge et al., 1997). Furthermore, these compounds have been shown to have valuable health benefits, such as reducing the risk of age-related macular degeneration and cataracts, as well as decreasing the risk of certain types of cancer (Kaulmann and Bohn, 2014). Factors such as light intensity, temperature, and nutrient availability have potential to affect carotenoid accumulation in plants. Understanding the factors that influence anthocyanin and carotenoid accumulation in plants is important for improving the nutritional content and quality of crops, and for developing strategies to enhance the health benefits of plant-based foods.

**Controlled Environment Agriculture**

Controlled environment agriculture (CEA) refers to an approach centered on manipulating plant growth and development through the use of advanced horticultural techniques and modern technology (Gomez et al., 2019; Hodges et al., 1968). Through the manipulation of key environmental factors such as light intensity and quality, air velocity, temperature, relative air humidity, vapor pressure deficit, and carbon dioxide ($CO_2$) concentration, an environment which allows producers to obtain maximum plant growth, quality, and input resource efficiency can be achieved (Ahmed, et al., 2020; Kitaya et al., 1998; Lee et al., 2014; Hodges et al., 1968).

In recent years, interest in CEA has grown due to concerns of overpopulation, reduced arable land, climate change, and food security (Goodman and Minner, 2019; Sundström et al., 2014).

One such technological innovation is the use of light-emitting diode (LED) fixtures. An LED is a semiconductor diode that allows the control of spectral composition (light quality) as well as light intensity (Yeh and Chung, 2009). Furthermore, LEDs offer enhanced photoelectric conversion efficiency than high-pressure sodium (HPS) lamps; commercially available LED fixtures typically convert >75% of the electricity into photosynthetically active radiation (PAR) whereas traditional HPS lighting only converts 30% of the energy requirement into PAR, emitting another 30% as radiant heat (Mitchell et al., 2012; Kusuma et al. 2020). As a result, HPS lighting operates at temperatures >200 °C and focuses this radiant heat emission directly onto plants, often requiring the light source be moved further from the crop to avoid heat stress. Comparatively, LEDs operate at cooler temperatures and waste heat is generally diffused above and away from plants. This allows LEDs to be placed closer to crops with reduced risk of temperature stress (Bourget, C. 2008).

In an experiment by Runkle and Meng (2014), it was shown that a 14-watt LED proving a red (R), white (W), and far-red (FR) radiation with a R:FR ratio of 0.82 produced comparable results to a 150-watt HPS and 150-watt incandescent fixture when used to grow various long day ornamental crops in a greenhouse setting.

An economic review on LED lighting by Bugbee (2017) focused on testing 3 LED fixtures from Philips Lighting found that these modern fixtures had the greatest efficacy, of any type of fixture, to date at 1.94, 2.44, and 2.46 µmol per joule compared to the previous best efficacy of any light fixture 1.7 µmol per joule. However, the initial cost of the Phillips fixtures was 5-10 times greater than the initial cost of HPS fixtures. The study went on to calculate that it

would take 5-10 years to recuperate the cost of the initial investment in the Phillips fixtures assuming a kWh price of 0.10 and that fixtures would be used for 16 h each day. Another economic analysis comparing the use of LEDs or HPS lamps in a greenhouse setting demonstrated that LEDs could reduce production costs in the long term, with the cost of HPS lighting overtaking the cost of LED lighting in the 7th year of production (Devesh Singh et al., 2015). These advantages seem apparent to the industry given the increasing rate of LED utilization as the technology continues to become more efficient and the initial cost decreases (Lopez and Runkle, 2017).

**Light Quality**

The light environment consists of light quality and intensity, and photoperiod. Together, these factors provide plants with their main source of energy and regulate most physiological processes (Shahak, 2004; Saito, 2020). Light quality refers to the various wavelengths that compose the spectrum of light, with different wavelengths correlating with different colors and categories of light. PAR can be defined as radiation that plants utilize in photosynthesis and includes wavelengths from 400 to 700 nm, consisting of B (400 to 500 nm), green (G, 500-600 nm), and R (600 to 700 nm) (Faust, 2011). Plants are also capable of perceiving UV-B (280 to 315 nm), UV-A (315 to 400 nm), and FR (700 to 750 nm) wavelengths as well (Kohler, 2020). Furthermore, light quality is associated with unique physiological and morphological effects which vary among plant species and cultivars (Bantis et al., 2018, Samuolienė et al., 2013). Sunlight delivers the full spectrum of radiation mentioned above; however, adequate growth and photosynthesis can be achieved using only combinations of B and R light (Bian et al., 2018, Mitchell et al., 2015, Viršilė et al., 2017). As a result, many commercial LED fixtures provide

only red B and R light due to their high photosynthetic efficiency and photon efficacy (Nelson and Bugbee, 2014, Park and Runkle, 2018).

B light is a relatively high energy waveband capable of driving the photosynthetic reaction and a small amount must be present in the spectrum when growing plants under sole-source lighting fixtures to achieve functional photosynthesis and prevent excess stem elongation in some species (Runkle, 2017). B light is essential for chlorophyll biosynthesis, stomatal opening, enzyme synthesis, maturation of chloroplasts, and signaling of cryptochromes (Tibbitts et al., 1983; Chen and Chory, 2011). However, providing a high percentage of B light has several repercussions such as undesirable plant morphology, decreased yield, flower inhibition of short-day plants, eye injury risk, and a poor color rendering index (Dougher and Bugbee, 2004; Wargent et al., 2009). On the other hand, including an appropriate percentage of B light in the spectrum has beneficial effects; B light inhibits stem and leaf extension and results in a more compact plant, is efficiently converted from electricity, promotes flowering of long-day plants at a moderate intensity, enhances red coloration by promoting the accumulation of anthocyanins, and influences the production of carotenoids and other phytochemicals (Li and Kubota, 2009; Meng and Runkle, 2023).

R light is another key waveband capable of driving the photosynthetic reaction and is highly effective at regulating the growth and development of plants. Chlorophyll absorbs most B and R light it intercepts as opposed to reflecting or transmitting it, and thus, R light is among the most effective and efficient wavebands to stimulate plant growth (Ohashi-Kaneko et al., 2007; Lichtenthaler, and Buschmann, 2001; Schuerger et al., 1997).

Research comparing the growth and anthocyanin content of lettuce grown under various ratios (%) of B and R light (0:100, 10:90, 20:80, 30:70, and 100:0 B:R) found that fresh and dry

weight under 0:100 R light was 2.1-2.2 times greater than under 100:0 B:R light. However, anthocyanin content of plants under the 100:0 B was 9.2 times greater compared to those under the 0:100 B:R light (Lee et al., 2014). In a separate study, red leaf lettuce 'Rouxai' grown under 0:100 B:R light displayed increased L*, a value ranging from 0-100 where 0 is black and 100 is white. Increased b*, a value that indicates blue/yellow with positive numbers indicating yellow and negative indicating blue. However, decreased a*, a value that indicates red/green with negative numbers indicating green and positive numbers indicating red. Together these changes signal lighter, greener, and more yellow foliage when grown under 0:100 B:R light compared to spectral compositions that included B light (Meng and Runkle., 2020).

FR light is currently classified as the waveband from 700 to 750 nm in length, which is outside the current definition of PAR (400 to 700 nm) due to its poor absorption by leaves and low quantum yield when applied as a monochromatic lighting treatment (Zhen and van Iersel, 2017; McCree, 1972). However, some contend that the waveband of PAR should be extended to include wavelengths from UV-A to FR (e.g., 350 to 750 nm), thus the term extended-PAR (ePAR) has been created to represent these wavelengths as well. This is because wavelengths in these outer wavebands are minimally capable of driving photosynthesis alone; however, they may enhance photosynthesis or have morphological effects when supplemented to a light spectrum (Pazuki et al., 2017). For example, FR supplementation to shorter wavelength radiation (e.g., R light) can synergistically increase photochemistry and photosynthesis. This is because FR preferentially excites photosystem I (PSI) while shorter-wavelength radiation preferentially excites photosystem II (PSII) and balanced excitation of PSI and PSII increases the efficiency of photochemistry (Hogewoning et al., 2012; Myers, 1971). FR supplementation also has morphological effects such as increased biomass, stem length, and leaf width and has been

reported to increase dry mass and fruit yield in tomato (*Solanum lycopersicum*) (Zhang et al., 2019; Kalaitzoglou et al., 2019). An experiment conducted by He et al. (2021) using sole-source LED fixtures compared the effect of R and W light with R, W, and supplemental FR light on lettuce growth and found that anthocyanin content decreased under supplementing FR light. Furthermore, FR supplementation with or without the inclusion of UV-A resulted in the lightest, most green, and yellow plants according to changes in chromametric L*, a*, and b*.

Many researchers have conducted studies investigating the effect of constant light intensity and quality on the nutrition, phenolic accumulation, quality, and yield of lettuce and other leafy green plants, however, much less research has been conducted on the effects of end-of-production (EOP) light intensity and quality. EOP treatment is a strategy in which environmental parameters such as light intensity and/or quality are altered near the end of the cropping cycle, inducing stress responses to enhance desired characteristics such as phenolic compound synthesis. Additionally, EOP lighting treatments may reduce production costs associated with high photosynthetic photon flux densities (PPFDs), while avoiding undesirable effects from various light mentioned above (Gómez and Jimenez, 2020).

**Light Intensity**

Light intensity is the photon flux density of PAR that a plant receives in any given moment (Faust, 2011). The photosynthetic rate will increase as the PPFD increases until the species-specific light saturation point is reached; thereafter, the plant can no longer use any additional energy and the photosynthetic rate will begin to decrease (Faust, 2011). Across all plants, increased photosynthetic rate is associated with enhanced growth rate and dry mass accumulation (Bian et al., 2015; Chin and Chong, 2012). Research focused specifically on lettuce supports this and found that lettuce biomass increased up to 270% and production time

decreased by 30% through supplementing 100 μmol·m$^{-2}$·s$^{-1}$ in low light greenhouse conditions (Gaudreau et al., 1994). Furthermore, Woltering (2016) concluded that butterhead lettuce grown under optimal light intensities demonstrated a prolonged shelf-life.

Increasing PPFD has been shown to increase phenolic and flavonoid content, including anthocyanins (Oh et al., 2009; Zhou et al., 2009). Anthocyanins protect plants from excessive light exposure by absorbing UV and visible light as well as through antioxidant activity (Agati and Tattini, 2010). Similar to anthocyanins, carotenoids absorb excess energy from light and protect the plant from oxidative damage by scavenging free radicals (Frank and Brudvig, 2004). However, under low-light conditions, carotenoids assist photosynthetic functionality by harvesting light at wavelengths chlorophyll cannot (K. Strzałka et al., 2003). A study by Given et al. (2023) found that producing butterhead lettuce 'Rex' under increasing PPFD from 60 to 600 μmol·m$^{-2}$ ·s $^{-1}$ resulted in decreased carotenoid content including neoxanthin, violaxanthin, lutein, and total carotenoid content. Past research indicates macro and micronutrient content (N, P, K S, Ca, Mg, B, Cu, Fe, Mn, and Zn) in purple kohlrabi (*B. oleracea var. gongylodes* L.), mizuna (*B. rapa* L. *var. japonica*), and mustard (*B. juncea* L.) microgreens was greater under a PPFD of 105 μmol·m$^{-2}$·s$^{-1}$, when compared to those harvested at a higher PPFD of 315 μmol·m$^{-2}$·s$^{-1}$ (Gerovac et al., 2016).

Light intensity can also impact foliage color, leaf number, and leaf size. Research by Kelly et al. (2020) found that increasing DLI, through photoperiod extension or greater PPFD, resulted in decreased L* and b* of lettuce 'Rouxai' and increased a*. Together these changes indicate darker plants that are redder and bluer (less green and yellow) in color. A separate study found that leafy greens grown under a PPFD of 600 μmol·m$^{-2}$·s$^{-1}$ had harder, stiffer leaves with unpleasant textures compared to those grown under 200 μmol·m$^{-2}$·s$^{-1}$ (Meinen et al., 2018).

**Temperature**

Temperature is the primary influence on the rate of biochemical reactions within a plant. The rate of biochemical reactions determines the speed at which leaf, stem, and flower cells divide and mature. Biochemical reactions begin to take place at the base temperature ($T_b$) and increase linearly until the optimal temperature ($T_{opt}$) is reached, after which the rate of reaction will either plateau or decline. These cardinal temperatures are species and cultivar specific but generally follow a similar pattern among all species of plants (Heins et al., 1998).

Lettuce can be produced as a field crop between day temperatures of 17 to 28 °C and night temperatures of 3 to 12 °C, however in controlled environments is recommended to be grown at day/night temperatures of 25 and 18 °C, respectively (Wurr et al., 1992; Marsh, 1987; Tarr et al., 2023). Increasing the temperature to 25 °C increased lettuce biomass, photosynthetic rate, and leaf area. Furthermore, lettuce grown at 25 °C was of higher quality than those grown at 15 °C, which were described as thick and leathery (Marsh, 1987; Seigner et al., 1991).

Research by He et al. (2020) compared anthocyanin accumulation in purple head Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis*) grown at 12 °C or 25 °C and found that anthocyanin content and accumulation was significantly enhanced by low temperature. This supports findings that anthocyanin content is negatively correlated with high temperatures and that, generally, low or fluctuating temperatures significantly stimulate the accumulation of anthocyanins (Lovdal et al., 2010). However, anthocyanin accumulation is not necessary in order for a plant to develop freezing tolerance (Leyva et al., 1995). Therefore, it is believed that increased anthocyanin content in response to lower temperatures is an acclimation mechanism meant to alleviate stress in photosynthetic cells by screening excess photosynthetic photons (Boldt, 2013).

Research focused on algal species has found that carotenoid synthesis and accumulation increase with rising temperature (Juneja et al., 2013). However, heat and wounding stress events have been shown to negatively impact carotenoid content, indicating that there is likely a $T_{opt}$ for carotenoid accumulation (García-Plazaola et al., 2016). Furthermore, low temperature stress has also been shown to enhance carotenoid content in bell peppers (*Capsicum annuum*) (León-Chan et al., 2017).

**Vapor-Pressure Deficit**

Vapor-pressure deficit (VPD) is the difference between the amount of water vapor in the air and the potential vapor the air can hold when fully saturated and is measured in kilopascal (kPa) of evaporative demand. A higher VPD indicates greater evaporative demand (low relative humidity) and lower VPD indicates lesser evaporative demand. VPD is a more accurate measurement than relative humidity because its value is independent of temperature while relative humidity varies with temperature (Wollaeger and Runkle, 2015).

In high VPD environments, a plants' stoma will close to reduce water loss and avoid dehydration but as a result, the plant will have reduced carbon dioxide ($CO_2$) uptake and consequently a decreased photosynthetic rate (Amitrano et al., 2021). In low VPD environments it is possible for defective stoma to form, unable to properly close if VPD increases and reducing water-use efficiency due to excessive respiration. A stable VPD of 0.5 to 1.0 kPa is recommended for finishing plants to avoid issues with $CO_2$ uptake and stomatal aperture (Fanourakis et al., 2011). An experiment by Amitrano et al. (2021) comparing lettuce grown at a VPD of either 0.69 kPa or 1.76 kPa reported that lettuce grown under low VPDs had greater biomass, leaf area, number of leaves, and chlorophyll fluorescence ($F_v/F_m$) ratio. However, lettuce grown under the high VPD possessed increased levels of phytochemicals, especially in

the red leaf cultivar ‘Capitata’. Despite this, lettuce under the high VPD was described as “lighter and more vivid” with decreased L* and b*.

**Carbon Dioxide**

The atmospheric concentration of $CO_2$ is currently around 420 μmol·mol$^{-1}$ but may fall as low as 200 μmol·mol$^{-1}$ in tightly sealed greenhouses that are filled with crops during winter (Mortesen, 1987). Reduced $CO_2$ conditions will decrease the photosynthetic rate and decrease the economic value of supplemental lighting (Runkle, 2015). Likewise elevated $CO_2$ substantially increases the photosynthetic rate, growth, and biomass accumulation in $C_3$ plants by decreasing $O_2$ inhibition of photosynthesis (Gruda, 2005; Drake et al., 1997). This effect is enhanced in crops grown for their leaves as photosynthesis primarily occurs in plant leaves and, as a result, the majority of additional biomass accumulates in the leaves (Hicklenton, 1988). Similar to the light-saturation point, there is a species-specific $CO_2$ saturation point, beyond this level supplementing additional $CO_2$ will no longer increase the rate of photosynthesis (Dusenge et al., 2018).

In addition to increasing yields, elevated $CO_2$ can reduce harvest time, elevate the light saturation point and $T_{opt}$, and reduce post-harvest respiration and ethylene production (Behboudian et al., 1995). High $CO_2$ conditions improve the nutritional quality of lettuce by increasing phenolic content (Pérez-López et al., 2018). One possible explanation for this is that the additional carbon availability allows the plant to produce a surplus of carbohydrates, which are then allocated to secondary metabolites such as phenolics (Zhang et al., 2017; Becker and Kläring, 2016). Another theory is that less antioxidant molecules are consumed due to reduced stress in an elevated $CO_2$ atmosphere (Sgherri et al., 2017; Faust 2011).

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## SECTION II

## QUANTIFYING THE INFLUENCE OF BLUE OR BLUE+RED END-OF-PRODUCTION SOLE-SOURCE LIGHTING ON RED LEAF LETTUCE

# Quantifying the Influence of Blue or Blue +Red End-of-Production Sole-Source Lighting on Red Leaf Lettuce (*Lactuca sativa*)

Devin Brewer [1], Kellie Walters[2], Jennifer Bolt[3], and Roberto G. Lopez[1*]

[1] Department of Horticulture, Michigan State University, 1066 Bogue Street, East Lansing, MI 48824-1325, USA

[2] Plant Sciences Department, University of Tennessee, 2505 E.J. Chapman Drive, Knoxville, TN 48824, USA

[3] USDA-ARS Application Technology Research Unit, 2801 W. Bancroft Street Mail Stop 604, Toledo, OH 43606, USA

***** Corresponding author. Tel.: +1 517-353-0342. E-mail address: rglopez@msu.edu (R.G. Lopez).

**Abstract**

Plants synthesize anthocyanins to counteract oxidative damage from environmental stressors, such as excessive light intensity or exposure to certain wavebands of photosynthetically active radiation (PAR) 400-700nm and/ or ultraviolet (UV) 315-400nm radiation. In controlled environments (CE), light intensity and quality can be altered and possibly used to manipulate foliage color by increasing the anthocyanin concentration of crops prior to harvest. Our objectives were to quantify how end-of-production (EOP) sole-source lighting consisting of various combinations of blue (B) and red (R) light affect development, quality, yield, anthocyanin, carotenoid, and nutritional concentrations, of red leaf lettuce. Seeds of red leaf lettuce (*Latuca sativa*) cultivars 'Barlach', 'Rouxai', and 'Thurinus' were sown in a growth chamber with a mean daily temperature (MDT) set point of 22 °C, carbon dioxide ($CO_2$) concentration of 500 µmol·mol$^{-1}$, and a total photon flux density (TPFD) of 180 µmol·m$^{-2}$·s$^{-1}$ provided by light-emitting diodes (LEDs). After 11 d, seedlings were transplanted into deep flow hydroponic tanks in the same growth chamber with a $CO_2$ concentration of 800 µmol·mol$^{-1}$, day/night temperature set point of 28/21 °C (MDT of 26 °C) and under LEDs that provided a PPFD of 300 µmol·m$^{-2}$·s$^{-1}$ with a light ratio (%) of 19:39:39:3 blue:green:red:far-red for 17 h·d$^{-1}$. During the last 6-8 days of production, plants were either left in the same conditions or transferred to growth chambers with a light ratio (%) of either 100:00, 75:25, or 50:50 blue:red (B:R) light and a photosynthetic photon flux density of 150 µmol·m$^{-2}$·s$^{-1}$. The shoot fresh mass (SFM) and shoot dry mass (SDM) of 'Rouxai' and 'Thurinus', but not 'Barlach', were negatively influenced by EOP lighting. Compared to plants not placed under EOP lighting, SFM of 'Rouxai' was 16, 17, and 21% lower and SDM was 25, 27, and 29% lower under 100:00 B:R,75:25 B:R, and 50:50 B:R treatments, respectively. Similarly, placing 'Thurinus' under the

100:00, 75:25, and 50:50 B:R treatments resulted in 19 and 17%, 30 and 27%, and 28 and 25% decrease in SFM and SDM, respectively. Regardless of the EOP treatment, lightness (L*) of 'Barlach' and 'Thurinus' increased compared to untreated plants. By day 6 of the EOP treatment, L* of 'Thurinus' under the 100:00, 75:25, and 50:50 B:R treatments was 13%, 9%, and 12% greater than the control, respectively. EOP sole-source lighting had no effect on the mineral nutrient content of 'Barlach'. However, 'Thurinus' exposed to 100:00 B:R EOP lighting contained 22, 22, 47, 35, 24, 10, 40, 31, 25, 50, and 38% greater N, P, Ca, Mg, S, B, Cu, Fe, Mn, Mo, and Zn, respectively, compared to the control. 'Barlach', 'Rouxai', and 'Thurinus' accumulated between 52 and 68%, 55 and 57%, and 33 and 43% lower anthocyanin concentration, respectively, than plants not placed under EOP sole-source lighting. Our results indicate that light intensity may have a greater effect than light quality on inducing anthocyanin accumulation in red leaf lettuce.

Keywords: anthocyanin, controlled-environment agriculture, leafy greens, coloration, end-of-production, light quality

**Introduction**

Consumer demand for fresh, local, pesticide-free, and year-round leafy greens has spurred the growth of greenhouse and indoor controlled-environment (CE) production. Within CEs, photosynthesis, growth, yield, and quality can be maximized by manipulating environmental parameters similar to that achieved under sunlight by using combinations of primarily blue (B) and red (R) light (Bian et al., 2018, Viršilė et al., 2017). As a result, many commercial horticultural light-emitting diode (LED) fixtures emit primarily B and R light due to their high photosynthetic efficiency and photon efficacy (Nelson and Bugbee, 2014, Park and Runkle, 2018).

In the United States (U.S.) from 2014 to 2019, total sales of lettuce (*Lactuca sativa* L.) produced in CEs increased by 28% from $56 to 71 million (USDA, 2015; USDA, 2020b). The increased demand for lettuce and especially red-leaf varieties is primarily driven by their health properties, which can be attributed to a large supply of fiber and phenolic compounds such as anthocyanins and carotenoids (Nicolle et al., 2004; Llorach et al., 2008).

Anthocyanins are essential for the diversity of foliage and flower colors including red, blue, and purple pigmentation in microgreens, basil (*Ocimum basilicum*), kale (*Brassica oleracea* var. *sabellica*), and lettuce, a metric consumers consider when purchasing produce (Li and Kubota, 2009; Samuoliené et al., 2012; Goins et al., 1998). Plants synthesize anthocyanins as a protective pigment to counteract oxidative damage. Synthesis may be induced by wounding, pathogen infection, exposure to excessive photon flux density and/or UV radiation, low temperature, and phosphorus nutrient deficiency (Boldt et al., 2013). However, anthocyanin production may be inhibited by environmental conditions such as low light intensities and high mean daily temperatures (MDT) (Kleinhenz et al., 2003).

Anthocyanin producing pathways are regulated by a protein complex made up of genes from the MYB, bHLH, and WD40 families. Proteins in this complex bind to promoters of structural genes, regulating their transcription (Gonzalez et al., 2007; Xu et al.,2015). This complex is predominantly responsible for regulating anthocyanin production and accumulation, and many abiotic stresses regulate anthocyanins mainly through activating or inactivating this complex (Das et al., 2012).

Carotenoids are a class of yellow, orange, and red pigments within plants that contribute to coloration in many fruits and vegetables (Edge et al., 1997). Similar to anthocyanins, these compounds provide valuable health benefits, such as reducing the risk of age-related macular

degeneration, cataracts, and certain types of cancer (Kaulmann and Bohn, 2014). Carotenoid accumulation can be influenced by light intensity and quality, temperature, and nutrient availability. Of the environmental factors that induce the accumulation of anthocyanins and carotenoids light quality and intensity can be easily altered in CEs with adjustable intensity LED fixtures capable of providing different light qualities (Folta et al., 2016).

Light quality refers to the various wavelengths of the light spectrum; these wavelengths correlate to different colors and categories of light. Sunlight delivers a spectrum including visible, UV, and infrared light. However, plants primarily utilize PAR consisting of B (400-500 nm), green (G, 500-600 nm), and R (600-700 nm) for photosynthesis (Runkle, 2006). In addition, they perceive and are affected by UV-B (280-315 nm), UV-A (315-400 nm), and far-red (FR,700-750 nm) light (Kohler et al., 2020). Furthermore, each waveband is associated with unique physiological and morphological effects that can vary among plant species and cultivars (Bantis et al., 2018, Samuolienė et al., 2013).

Numerous studies have investigated the effects of sole-source light intensity and quality on the growth, nutritional content, and yield of lettuce and other leafy greens. However, less attention has been given to end-of-production (EOP) lighting treatments. EOP supplemental and sole-source lighting are novel strategies in which environmental parameters such as light intensity and/or quality are altered near the end of the cropping cycle, inducing stress responses to enhance desired characteristics including biomass, coloration, mineral nutrition, carotenoid, and anthocyanin accumulation (Gómez and Jiménez, 2020). For instance, exposing greenhouse and indoor grown lettuce to short durations of EOP supplemental light providing different intensities of UV-A, B and/or R light can improve coloration of red leaf lettuce (Owen and Lopez, 2015; Gómez and Jiménez, 2020).

Research conducted by Tarr et al., (2023) found that among the environmental conditions tested, lettuce yield was greatest at a MDT of 26 °C, $CO_2$ concentration of 800 μmol·mol$^{-1}$, and light intensity of 300 μmol·m$^{-2}$·s$^{-1}$ but foliage color was a greyish green instead of the burgundy red desired by consumer. Currently there is a lack of research on the effectiveness of EOP sole-source lighting providing ratios of B and R light to improve anthocyanin and mineral nutrient content and the extent to which EOP treatments may affect production costs for indoor farms. Therefore, the objectives of this study were 1) to produce red leaf lettuce under the environmental conditions that Tarr et al. (2023) reported to maximize yield and quantify the influence of sole-source EOP lighting providing a lower light intensity of blue or different ratios of blue + red light on anthocyanin and nutritional content, coloration, plant quality, and yield of red leaf lettuce. We hypothesized that EOP lighting would increase lettuce anthocyanin and carotenoid concentration, coloration, and plant quality, without sacrificing yield. Furthermore, the combination of B:R light will be more effective at improving anthocyanin concentration, coloration, and plant quality than monochromatic B.

**Materials and Methods**

*Plant material and propagation conditions*

On 28 Oct. 2021, 16 Dec. 2021, and 15 Feb. 2022 seeds of red oakleaf 'Rouxaï', Salanova butterhead 'Barlach', and cos lettuce 'Thurinus' (Rijk Zwaan USA; Salinas, CA) were sown into 200-cell (2.5 cm × 2.5 cm) rockwool plugs (AO 25/40 Starter Plugs; Gordan, Milton, ON, Canada). The varieties were chosen due to their suitability to be grown hydroponically and resistance to the physiological disorder tip burn. Rockwool plugs (AO 25/40 Starter Plugs; Gordan, Milton, ON, Canada) were presoaked in deionized (DI) water with a pH of 4.4 to 4.5 adjusted using diluted (1:31) 95 to 98% sulfuric acid (J.Y. Baker, Inc.; Phillipsburg, NJ). Trays

were then covered with translucent plastic domes for three days to maintain high humidity during germination. Next, trays were placed in one of three walk-in growth chambers (Hotpack environmental room UWP 2614-3; SP Scientific, Warminster, PA) with an MDT of 22±SD ºC, carbon dioxide ($CO_2$) concentration of 500±SD μmol·mol$^{-1}$, relative humidity (RH) of 60±SD %, and a vapor-pressure deficit (VPD) of 1.1±SD kPa. LED fixtures (GreenPower LED production module 3.0; Phillips, Amsterdam, Netherlands) provided a total photosynthetic photon flux density (TPFD) of 180 μmol·m$^{-2}$·s$^{-1}$ and a light ratio (%) of 19:39:39:3 blue:green:red:far-red for 24 h. After three days, the photoperiod was reduced to 20 h·d$^{-1}$ until seedlings were transplanted on day 11. Seedlings were sub-irrigated with DI water supplemented with water-soluble fertilizer providing (in mg·L$^{-1}$): 125 N, 18 P, 138 K, 73 Ca, 47 Mg, 1.56 Fe, 0.52 Mn, 0.36 Zn, 0.21 B, 0.21 Cu, 35 S, and 0.01 Mo (12N–4P–16K RO Hydro FeED; JR Peters, Inc., Allentown, PA). The pH and electrical conductivity of the solution were adjusted to 5.6 and 1.6 mS·cm$^{-1}$, respectively, using a pH/EC probe (HI 991301 pH/TDS/Temperature Monitor; Hanna Instruments, Smithfield, RI).

*Hydroponic systems*

On day 11 of each replication 36 seedlings of each cultivar were transplanted 20 cm apart into three 250 L, 0.9-m-wide by 1.8-m-long deep-flow hydroponic systems (Active Aqua premium high-rise flood table; Hydrofarm, Petaluma, CA) with one tank in each walk-in growth chamber (Hotpack environmental room UWP 2614-3; SP Scientific). Each hydroponic tank was covered with a floating 4-cm-thick extruded polystyrene foam sheet (R-3 Square Edge Rigid Foam Board Insulation Sheathing, Owens Corning, Toledo, OH). The foam sheets had 4-cm diameter holes drilled in them to accommodate plastic net baskets that held rockwool plugs and seedlings in contact with the nutrient solution. DI water supplemented with water-soluble

fertilizer providing ($mg \cdot L^{-1}$): 150 N, 22 P, 166 K, 87 Ca, 25 Mg, 1.9 Fe, 0.62 Mn, 0.44 Zn, 0.25 B, 0.25 Cu, and 0.01 Mo (Jack's 12N–4P–16K; JR Peters, Inc.), and 0.31 $g \cdot L^{-1}$ magnesium sulfate (Pennington Epsom Salt; Madison, GA). The pH and EC were measured and adjusted daily to maintain an EC of 1.7 ± 0.05 $mS \cdot cm^{-1}$ by adding DI water or concentrated nutrient solution as needed, while the pH was adjusted to 5.6 ± 0.05 using potassium bicarbonate and sulfuric acid. A dissolved oxygen concentration of 10.0 ± 0.03 $mg \cdot L^{-1}$ was maintained using air pumps (Active Aqua 70 $L \cdot min^{-1}$ commercial air pump; Hydrofarm) connected to air stones (Active Aqua air stone round 10.2 cm × 2.5 cm; Hydrofarm).

*Growth chamber environmental conditions*

Following the protocol of Tarr et al. (2023), the air day/night temperature (17 /7 h) 28/21 °C (MDT 26 °C) was measured every 5 seconds by a resistance temperature detector (Platinum RTD RBBJL-GW05A-00-M 36B; SensorTec, Inc., Fort Wayne, IN) and logged by a C6 controller (Environmental Growth Chambers, Chagrin Falls, OH). A light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was provided for 17 $h \cdot d^{-1}$ by LED fixtures (GreenPower LED production module 3.0; Phillips, Amsterdam, Netherlands) achieving a daily light integral of 18.4 $mol \cdot m^{-2} \cdot d^{-1}$ with the LED fixtures mounted 10-12 cm above the crop canopy. Water and leaf temperature and PPFD were monitored using a thermistor (ST-100; Apogee Instruments, Logan, UT), infrared thermocouple (OS36-01-T-80F; Omega Engineering, INC. Norwalk, CT), and quantum sensor (LI-190R; LI-COR Biosciences, Lincoln, NE), respectively, every 15 seconds and means were logged each hour by a CR-1000 datalogger (Campbell Scientific, Logan, UT). The $CO_2$ concentration was monitored and maintained at 800 $\mu mol \cdot mol^{-1}$ during the day with compressed $CO_2$ injection with a $CO_2$ sensor (GM86P; Vaisala, Helsinki, Finland), and logged by a C6 Controller (Environmental Growth Chambers) every 5 seconds. A VPD of 1.03 kPa was

maintained by having a day and night RH set point of 70 and 55%, respectively (Table I-1). Overhead fans were utilized to maintain an average air velocity of 0.7 $m^{-3} \cdot s^{-1}$ at plant height.

After 24 days, 'Thurinus' was placed under LEDs providing 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (GreenPower LED production module 3.0; Phillips, Amsterdam, Netherlands) of a ratio (%) of 100:0, 75:25 or 50:50 B:R light for 6 to 8 days depending on the cultivar (Table I-1). This process was repeated at day 30 for both 'Barlach' and 'Rouxai'. All other environmental conditions remained unchanged. Plants placed under the control treatment weren't exposed to the EOP treatment, receiving the same light intensity (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and quality until harvest.

*Growth data collection and analysis*

Foliage coloration measurements were conducted using a tristimulus colorimeter (Chroma Meter CR-400; Konica Minolta Sensing, Inc., Chiyoda, Tokyo) on 13 plants of 'Rouxai', 'Barlach', and 'Thurinus' from each treatment on days 24, 26, 28, and 30 for 'Thurinus', days 30, 32, 34, and 36 for 'Rouxai' and days 30, 32, 34, 36, and 38 for 'Barlach'. The relative chlorophyll concentration (RCC) of the most recent fully expanded leaf from each sample plant was estimated using a SPAD chlorophyll meter (MC-100 Chlorophyll Meter; Apogee Instruments, Logan, UT). A separate leaf was dark acclimated for >15 minutes using the manufacturer-supplied clips and then exposed to 3,500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of R light (peak wavelength 650 nm) to saturate photosystem II and the fluorescence was measured, averaged, and reported as $F_v/F_m$ by a portable chlorophyll fluorescence meter (Handy Plant Efficiency Analyzer; Hanstech Instruments Ltd. Norfolk, U.K.).

'Thurinus', 'Rouxaï', and 'Barlach' were harvested 30, 36, and 38 days after sowing, respectively. Shoot fresh mass (g), length and width (cm) of the sixth most recent fully expanded leaf, and leaf number (when >5 cm) was measured on 13 plants of each cultivar per treatment.

Plant height from the basal leaves to the apical meristem, and the width at the widest point and perpendicular from the widest point as well as the presence of tip burn was recorded. To provide an integrated measurement of plant size, the growth index (GI) was calculated (GI = {plant height + [(diameter 1 + diameter 2)/2]}/2) (Krug et al., 2010). After obtaining the shoot fresh mass of each plant, every other leaf was separated to reach approximately 40 g of biomass. The biomass was then placed into a polyethylene sampling bag and flash frozen by submerging the sample in liquid N. Frozen samples were then stored in a –80 °C freezer until being freeze-dried. The remaining plant material from each sample was placed in a forced-air drier maintained at 75 ºC for a minimum of 3 days. Freeze dried and oven dried samples were then weighed, added together, and recorded.

*Mineral nutrient analysis*

Each air-dried sample was ground individually using a mortar and pestle. The samples were then shipped to the USDA-ARS for tissue elemental analysis. Foliar nitrogen (N) was determined by measuring 2.5 mg of dry tissue into tin capsules (Costech Analytical, Valencia, CA) and analyzing with a CHN analyzer (vario MICRO cube; Elementar, Hanau, Germany). Remaining elements were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES). For all macronutrients and micronutrients except N, 0.25 g of dry tissue was placed in a Teflon vessel and 5 mL of nitric acid was added. Samples were then heated in a programmable microwave (MARS 6; CEM Corp., Matthews, NC) by increasing the temperature to 200 °C over 15 min., maintaining 200 °C for 15 min., and then cooling to room temperature. After reaching room temperature, 1.5 mL of hydrogen peroxide was added, solutions were reheated to 200 °C and maintained for 5 min. After cooling, 12 mL of 18 MΩ

water was added, and the solutions were filtered (Whatman #2). Then a 1.3 mL aliquot of solution was diluted with 8.7 mL 18 MΩ water and analyzed using ICP-OES.

*Anthocyanin isolation and quantification*

Total anthocyanins were extracted and analyzed at the University of Tennessee as reported by Darby et al. (2023). Freeze-dried tissue samples were homogenized in a ceramic mortar and pestle with liquid nitrogen. One hundred mg of each sample was saturated with 5 mL of 95% ethanol/1.5 N HCl (85:15, v:v). Samples were placed on an orbital shaker at 200 RPM for 15 min, then stored in the dark at 4 °C for 24 h. Sample contents were filtered into a 25 mL Erlenmeyer flask and a 200-µL aliquot of extractant was pipetted into a 96-well assay plate. Samples were analyzed using a Biotek PowerWave XS Microplate Reader (Agilent Technologies, Santa Clara CA). The optical density was measured at 530 nm and total anthocyanin concentrations were calculated based on the calibration curve of cyanidin-3-o-glucoside chloride (MilliporeSigma, Burlington MA).

*Carotenoid Isolation and Quantification*

The carotenoids α-carotene, β carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin were extracted and analyzed from freeze-dried ground tissue using the method described by Darby et al. (2023) and derived from Kopsell et al. (2012). In brief, under red light, chilled samples were extracted in purified water and tetrahydrofuran with an internal carotenoid standard being used to quantify sample loss during homogenization. Samples were homogenized and tetrahydrofuran was used for a second extraction followed by homogenization. The resulting solution was centrifuged, and the eluent was dried down on a stream evaporator. After the addition of acetone, samples were filtered, and an aliquot was stored for subsequent identification and quantification on a 1200 Agilent Series HPLC unit equipped with a diode array detector. A reverse phase C30

column was used with a mobile phase composed of methyl tert-butyl ether, methanol, and triethylamine.

**Results**

*Shoot fresh and dry mass*

We observed that the SFM and SDM of 'Rouxai' and 'Thurinus' were reduced when exposed to 6 d of EOP sole-source lighting (Table II-2). For example, SFM of 'Rouxai' was 16% (28.7 g), 17% (29.4 g), and 21% (34.6 g) lower under 100:00,75:25, and 50:50 B:R treatments, respectively, than the control. Likewise, SDM of 'Rouxai' placed under the 100:00, 75:25, and 50:50 B:R treatments was 25% (1.6 g), 27% (1.7 g), and 29% (1.9 g) lower than the control, respectively (Table II-2). Similarly, placing 'Thurinus' under the 100:00, 75:25, and 50:50 B:R treatments resulted in 19% (26.6 g) and 17% (0.9 g), 30% (43.0 g) and 27% (1.5 g), and 28% (42.7 g) and 25% (1.4 g) decrease in SFM and SDM, respectively (Table II-2). However, SFM and SDM of 'Barlach' wasn't altered by any EOP treatment (Table II-2).

*Plant morphology*

Leaf number generally decreased as B light percentage increased. 'Barlach', 'Rouxai', and 'Thurinus' placed under the 100:00 B:R treatment unfolded 4, 7, and 4 fewer leaves compared to plants under the 50:50 B:R treatment and 4, 5 and 5 fewer leaves compared to the control (Table II-2). Despite this, the GI of 'Barlach' and 'Thurinus' was greatest under the 100:00 B:R treatment, 13% and 8% greater than the control. However, GI of each cultivar was similar to the control when placed under the 75:25 or 50:50 B:R treatments (Table II-2).

*Pigmentation*

EOP treatment and duration influenced foliage lightness (L*) of 'Barlach' and 'Thurinus' (Fig. II-1). On day 4, L* of 'Barlach' exposed to the 100:00 B:R treatment had increased 13%

compared to the control, indicating a lighter color than both the pretreatment measurement and the control. On day 6 L* of 'Barlach' exposed to the 100:00 B:R, 75:25 B:R, and 50:50 B:R treatments was 15%, 14%, and 15% greater than that of the control. By day 8, the difference between 'Barlach' exposed to the 100:00 B:R, 75:25 B:R, and 50:50 B:R and control treatment grew to 18%, 20%, and 20%, respectively. On day 2, L* of 'Thurinus' exposed to the 100:00 B:R treatment was 7% greater than the control. By day 6, L* of 'Thurinus' under the 100:00, 75:25, and 50:50 B:R treatments was 13%, 9%, and 12% greater, respectively, than the control.

*Mineral nutrient content*

EOP treatment resulted in both increases or decreases in macro and micronutrient concentration in 'Rouxai' and 'Thurinus' dependent on spectrum (Fig. II-2, II-3). For instance, 'Rouxai' exposed to the 100:00 B:R treatment possessed 10% and 28% greater nitrogen (N) and molybdenum (Mo) and 17%, 18%, and 11% lower potassium (K), magnesium (Mg), and manganese (Mn) concentration than the control, respectively. Under the 75:25 treatment 'Rouxai' had a 7% and 27% greater N and Mo and 14% lower K and Mg concentration, than the control. When exposed to the 50:50 B:R treatment 'Rouxai' accumulated 8% and 30% greater N and Mo concentration, but 16% and 11% less K and Mg concentration.

Compared to plants under the control, 'Thurinus' exposed to 100:00 B:R EOP lighting contained 22, 22, 47, 35, 24, 10, 40, 31, 25, 50, and 38% greater N, P, calcium (Ca), Mg, sulfur (S), boron (B), copper (Cu), iron (Fe), manganese (Mn), Mo, and zinc (Zn) concentration, respectively. Similarly, the 75:25 B:R treatment possessed 22, 18, 36, 21, 19, 7, 18, 28, 11, 47, and 33% more N, P, Ca, Mg, S, B, Cu, Fe, Mn, Mo, and Zn concentration, respectively than the control. 'Thurinus' exposed to the 50:50 B:R treatments contained 16, 22, 48, 32, 22, 9, 25, 28,

47, and 36% greater N, P, Ca, Mg, S, B, Cu, Fe, Mn, Mo, and Zn concentration, respectively, compared to the control (Fig. II-2, II-3).

*Anthocyanin and carotenoids*

Plants placed under EOP sole-source lighting had lower anthocyanin concentration than the control (Fig. II-4). 'Barlach' contained 68, 52, and 63% lower anthocyanin concentration under the 100:00, 75:25, and 50:50 B:R treatments, respectively, compared to the control. Likewise 'Rouxai' placed under the 100:00, 75:25, and 50:50 B:R treatments had 59, 57, and 55% lower anthocyanin concentration than the control, respectively. Similarly, 'Thurinus' possessed 39, 43, and 33% lower anthocyanin concentration than the control when placed under the 100:00, 75:25, and 50:50 B:R treatments, respectively.

EOP treatment altered carotenoid composition of 'Rouxai' and 'Thurinus' but not 'Barlach'. (Table II-3). 'Rouxai' exposed to 100:00 B:R had 37, 43, 36, 50, 30, and 35% greater violaxanthin, neoxanthin, lutein, αcarotene, βcarotene, and total carotenoid concentration, respectively, compared to the 50:50 B:R treatment (Table II-3). Likewise 'Thurinus' exposed to 100:00 B:R accumulated 30, 37, 13, 50, 24, and 23% more violaxanthin, neoxanthin, lutein, alpha carotene, beta carotene, and total carotenoid concentration, respectively, compared to the 50:50 B:R treatment (Table II-3). Zeaxanthin of each cultivar wasn't altered by EOP cooling (Table II-3).

**Discussion**

Given that there were significant differences in the SFM and SDM of 'Rouxai' and 'Thurinus' among EOP treatments, we can confidently conclude that it was a result of the reduced light intensity during the EOP treatments (Table II-2). These results are congruent with outcomes from other studies such as one completed by Zhou et al. (2022) in which growing

romaine lettuce at a PPFD of 100 and 200 μmol·m$^{-2}$·s$^{-1}$ resulted in significantly lower fresh mass than plants grown under a PPFD of 350, 500, or 600 μmol·m$^{-2}$·s$^{-1}$ and MDT of either 20 or 30 °C. Likewise, Kelly et al. (2020) reported that SFM and SDM of 'Rouxai' was 51% and 31% greater when grown at a PPFD of 270 μmol·m$^{-2}$·s$^{-1}$ compared to a PPFD of 150 μmol·m$^{-2}$·s$^{-1}$.

Surprisingly, the SFM and SDM of 'Barlach' was not affected by the reduction in light intensity or change in light quality, despite a longer EOP treatment duration (Table II-2). This could indicate that 'Barlach' has a lower light saturation point than the other cultivars. Another possibility is that the growth rate of 'Barlach' slows between days 30 to 38. This would align with findings from Choi et al. (2000) in which SFM and relative growth rate of butterhead lettuce 'Omega' was greatest at 30/25 °C, compared to 20/15 °C, during the first 25 d, but by 35 d there was no longer a difference.

For 'Barlach' and 'Thurinus', L* increased similarly across all EOP treatments when compared to the control, indicating that light intensity had a greater effect on foliage color than light quality (Fig. II-1). This result aligns with the findings of Foster et al. (2018) in which red leaf lettuce 'Outredgeous' was grown under PPFDs of 250 μmol·m$^{-2}$·s$^{-1}$ and 500 μmol·m$^{-2}$·s$^{-1}$ and found that plants grown under the higher PPFD appeared darker and more red. Furthermore, research by Owen et al. (2015) reported that EOP supplemental lighting providing 100 μmol·m$^{-2}$·s$^{-1}$ of 50:50 B:R light was more effective at decreasing L* and increasing a* of red leaf lettuce varieties 'Magenta', 'Ruby Sky', and 'Cherokee' than supplemental lighting providing 100 μmol·m$^{-2}$·s$^{-1}$of 100:00 and 00:100 B:R light or 25 or 50 μmol·m$^{-2}$·s$^{-1}$ of 50:50 B:R light. This may indicate that at higher light intensities, or above a certain light threshold, light quality affects foliage coloration. EOP sole-source lighting increased some macro- and micro-nutrient concentration in 'Rouxai' and 'Thurinus' but decreased it in others. 'Rouxai' possessed

increased N and Mo and decreased K and Mg concentrations under each treatment when compared to the control. Under the 100:00 B:R treatment 'Rouxai' also displayed reduced Mn concentration (Figs II-2, II-3). This is unexpected; macro- and micro-nutrient concentrations generally increased under both decreased light intensity and increasing percentage of B light (Kospell and Sams, 2013; Gerovac et al., 2016). A possible explanation for this difference is that the current study only provided EOP lighting during the final 6 days of production and growth rate of 'Rouxai' may have decreased by that point, limiting the effect on elemental composition of the entire plant (Choi et al., 2000). 'Thurinus' placed under any EOP treatment possessed greater concentrations of N, P, Ca, Mg, S, B, Cu, Mn, Mo, and Zn (Figs II-2, II-3). This is expected; reduced light intensity increases macro and micro nutrition content due to dilution of nutrients under increased light intensity and supplemented $CO_2$ concentration (Gerovac et al., 2016; Kuehny et al., 1991). However, there were differences between treatments as well, which indicates that in addition to light intensity, light quality influences the accumulation of elements. As stated earlier, increasing the % of B light generally increased macro- and micro-nutrient concentration, and were greatest under the 100:00 B:R treatment. However, there were no differences in mineral nutrient concentration of 'Barlach' indicating a cultivar-specific response, which in combination with the lack of change in SFM or SDM of 'Barlach', suggests that growth rate may slow down at cultivar-specific stages of a plant life cycle, reducing the effect of EOP treatments.

Given that plants synthesize anthocyanins to counteract oxidative damage, synthesis may be induced by high light intensity or certain wavelengths of PAR as well as UV (Pollastri et al., 2011; Lev-Yadun and Gould, 2009). We observed that regardless of the EOP B:R ratio implemented, each cultivar possessed a lower anthocyanin concentration compared to the control

(Fig II-4). Furthermore, there were no significant differences in anthocyanin concentration between light quality treatments. This indicates that light intensity may have a greater effect on anthocyanin synthesis than light quality, and that at lower-light intensities, altering the light quality within PAR is not effective at promoting anthocyanin synthesis.

In ‘Rouxai’ and ‘Thurinus’ carotenoid concentration was greatest under the 100:00 treatment compared to 75:25 B:R and 50:50 B:R, however there were no differences between treatments for ‘Barlach’. Carotenoids are a plant pigment that both functions as an antenna for harvesting B light as well as protecting plants from photo oxidative damage via thermal dissipation (Stange & Flores, 2012; Jahns & Holzwarth, 2012). Furthermore, a recent study by Givens et al., (2023) found that increasing light intensity from 60 to 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ leading to harvest generally decreased βcarotene, lutein, neoxanthin, violaxanthin, and total carotenoid concentration. Therefore, it is expected that increased percent B light in the spectrum and a reduced light intensity during the EOP phase resulted in increased carotenoid concentration.

In conclusion, reducing the light intensity during the EOP phase was more influential than changes in light quality and resulted in reduced biomass of ‘Rouxai’ and ‘Thurinus’, lighter foliage in ‘Barlach’ and ‘Thurinus’, and reduced anthocyanin concentration in all cultivars. However, EOP treatment did increase mineral nutrient and carotenoid concentration of ‘Rouxai’ and ‘Thurinus’.

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## APPENDIX A: SECTION II FIGURES AND TABLES

Table II-1. Mean (± SD) day and night air, canopy, and hydroponic water temperature; carbon dioxide (CO2) concentration; photosynthetic photon flux density (PPFD) during production (Prod.) and end-of-production (EOP) sole-source (SS) lighting providing blue (B) and red (R) light; and vapor-pressure deficit (VPD) during 30 days of indoor deep-flow hydroponic production for red leaf lettuce (Lactuca sativa) 'Barlach', 'Rouxaï RZ', and 'Thurinus'.

| | EOP SS | Temperature (°C) | | | | $CO_2$ | Prod. PPFD | EOP PPFD | VPD |
|---|---|---|---|---|---|---|---|---|---|
| Replication | lighting (B:R) | Air day | Air night | Canopy | Water | ($\mu mol \cdot mol^{-1}$) | ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | (kPa) |
| 1 | 100:00 | 26.9 ± 0.3 | 22.8 ± 0.4 | 26.5 ± 3.2 | 25.6 ± 3.2 | 790.7 ± 84.2 | 297.8 ± 4.7 | 149.7 ± 13.0 | 1.02 ± 0.09 |
| | 75:25 | 27.3 ± 0.1 | 22.0 ± 0.4 | 28.7 ± 2.9 | 25.6 ± 1.3 | 791.7 ± 82.0 | 293.2 ± 5.1 | 152.3 ± 13.4 | 1.04 ± 0.10 |
| | 50:50 | 27.4 ± 0.1 | 22.9 ± 0.4 | 27.9 ± 2.9 | 25.9 ± 1.1 | 782.5 ± 90.4 | 296.8 ± 8.2 | 154.8 ± 10.7 | 1.00 ± 0.13 |
| 2 | 100:00 | 26.9 ± 0.4 | 22.4 ± 0.4 | 26.0 ± 3.1 | 26.0 ± 3.2 | 806.1 ± 45.0 | 292.5 ± 12.5 | 148.6 ± 17.4 | 1.07 ± 0.07 |
| | 75:25 | 27.6 ± 0.3 | 22.5 ± 0.4 | 28.7 ± 3.0 | 25.6 ± 1.4 | 805.8 ± 39.8 | 293.2 ± 19.8 | 153.8 ± 4.5 | 1.06 ± 0.17 |
| | 50:50 | 27.6 ± 0.1 | 22.4 ± 0.4 | 27.9 ± 2.9 | 25.9 ± 1.2 | 796.3 ± 42.9 | 294.9 ± 17.8 | 150.0 ± 8.9 | 1.04 ± 0.11 |
| 3 | 100:00 | 28.1 ± 0.6 | 21.7 ± 0.3 | 28.0 ± 3.3 | 25.3 ± 1.6 | 804.9 ± 22.7 | 296.4 ± 17.1 | 152.7 ± 13.5 | 1.05 ± 0.08 |
| | 75:25 | 27.8 ± 0.1 | 22.0 ± 0.4 | 28.3 ± 2.8 | 25.9 ± 1.2 | 796.5 ± 24.7 | 292.3 ± 16.2 | 155.3 ± 5.6 | 1.01 ± 0.15 |
| | 50:50 | 27.1 ± 0.4 | 21.9 ± 0.4 | 26.4 ± 3.2 | 25.4 ± 3.3 | 806.4 ± 18.0 | 297.8 ± 4.7 | 154.1 ± 4.8 | 1.01 ± 0.10 |

Table II-2. Influence of end-of-production (EOP) sole-source lighting treatments providing a light ratio (%) of 100:00, 75:25, and 50:50 blue:red (B:R) light on leaf no.; shoot fresh and dry mass; growth index; chlorophyll fluorescence ($F_v/F_m$); and total chlorophyll content of red leaf lettuce (*Lactuca sativa*) 'Barlach', 'Rouxai', and 'Thurinus'. Data represent the mean of three replications and cultivars with 13 samples. Analyses of variance for the effects of light quality and intensity and their interaction are included beside each cultivar mean. Different letters within columns signify significantly different means according to Tukey's honestly significant difference (HSD) test ($P < 0.05$).

| Treatment | Leaf (no.) | Fresh mass (g) | Dry mass (g) | Growth index | $F_v/F_m$ | Total chlorophyll ug/mL |
|---|---|---|---|---|---|---|
| | | | **'Barlach'** | | | |
| Control | 60.6 a | 180.4 a | 6.3 a | 18.4 b | 0.8391 a | --- |
| 100:00 | 56.7 b | 173.4 a | 5.9 a | 21.2 a | 0.8364 a | 11.4 a |
| 75:25 | 58.9 ab | 171.7 a | 5.8 a | 19.8 b | 0.8468 a | 5.0 b |
| 50:50 | 60.7 a | 170.7 a | 5.8 a | 19.3 b | 0.8381 a | 5.8 b |
| | | | **'Rouxai'** | | | |
| Control | 28.1 ab | 178.7 a | 6.4 a | 21.6 a | 0.8351 a | --- |
| 100:00 | 23.0 c | 150.0 b | 4.8 b | 21.7 a | 0.8403 a | 8.6 a |
| 75:25 | 26.3 b | 149.3 b | 4.7 b | 20.8 a | 0.8429 a | 4.9 b |
| 50:50 | 29.9 a | 144.1 b | 4.5 b | 20.6 a | 0.8414 a | 3.8 b |
| | | | **'Thurinus'** | | | |
| Control | 24.9 a | 142.9 a | 5.6 a | 26.1 b | 0.8281 b | --- |
| 100:00 | 19.8 b | 116.3 ab | 4.6 ab | 28.2 a | 0.8363 a | 16.3 a |
| 75:25 | 21.3 b | 99.9 b | 4.2 b | 25.0 b | 0.8373 a | 8.9 b |
| 50:50 | 24.6 a | 100.2 b | 4.3 b | 26.0 b | 0.8349 a | 11.3 ab |

Table II-3. Influence of end-of-production (EOP) sole-source lighting providing a light ratio (%) of 100:00, 75:25, or 50:50 blue:red (B:R) light on violaxanthin (µg/mL); neoxanthin (µg/mL); lutein (µg/mL); α carotene (µg/mL); β carotene (µg/mL); and total carotenoid content (µg/mL) of red leaf lettuce cultivars (Lactuca sativa) 'Barlach', 'Rouxai', and 'Thurinus'. Means followed by different letters within each element are significantly different based on Tukey's honestly significant difference test (α = 0.05).

| Treatment B:R | Violaxanthin | Neoxanthin | Lutein | α carotene | β carotene | Total carotenoids |
|---|---|---|---|---|---|---|
| | | | (ug/mL) | | | |
| | | | **'Barlach'** | | | |
| 100:00 | 0.5 a | 0.5 a | 0.8 a | 0.07 a | 1.7 a | 3.7 a |
| 75:25 | 0.4 a | 0.4 a | 0.7 a | 0.01 a | 1.6 a | 3.1 a |
| 50:50 | 0.4 a | 0.3 a | 0.7 a | 0.01 a | 1.5 a | 3.0 a |
| | | | **'Rouxai'** | | | |
| 100:00 | 0.8 a | 0.7 a | 1.1 a | 0.02 a | 2.3 a | 4.9 a |
| 75:25 | 0.6 b | 0.4 b | 0.8 b | 0.01 b | 1.6 b | 3.3 b |
| 50:50 | 0.5 b | 0.4 b | 0.7 b | 0.01 b | 1.6 b | 3.2 b |
| | | | **'Thurinus'** | | | |
| 100:00 | 1.0 a | 0.8 a | 1.3 a | 0.02 a | 2.5 a | 5.5 a |
| 75:25 | 0.8 b | 0.6 ab | 0.9 b | 0.01 a | 1.9 b | 4.3 b |
| 50:50 | 0.7 b | 0.5 b | 1.0 b | 0.01 a | 1.9 b | 4.2 b |

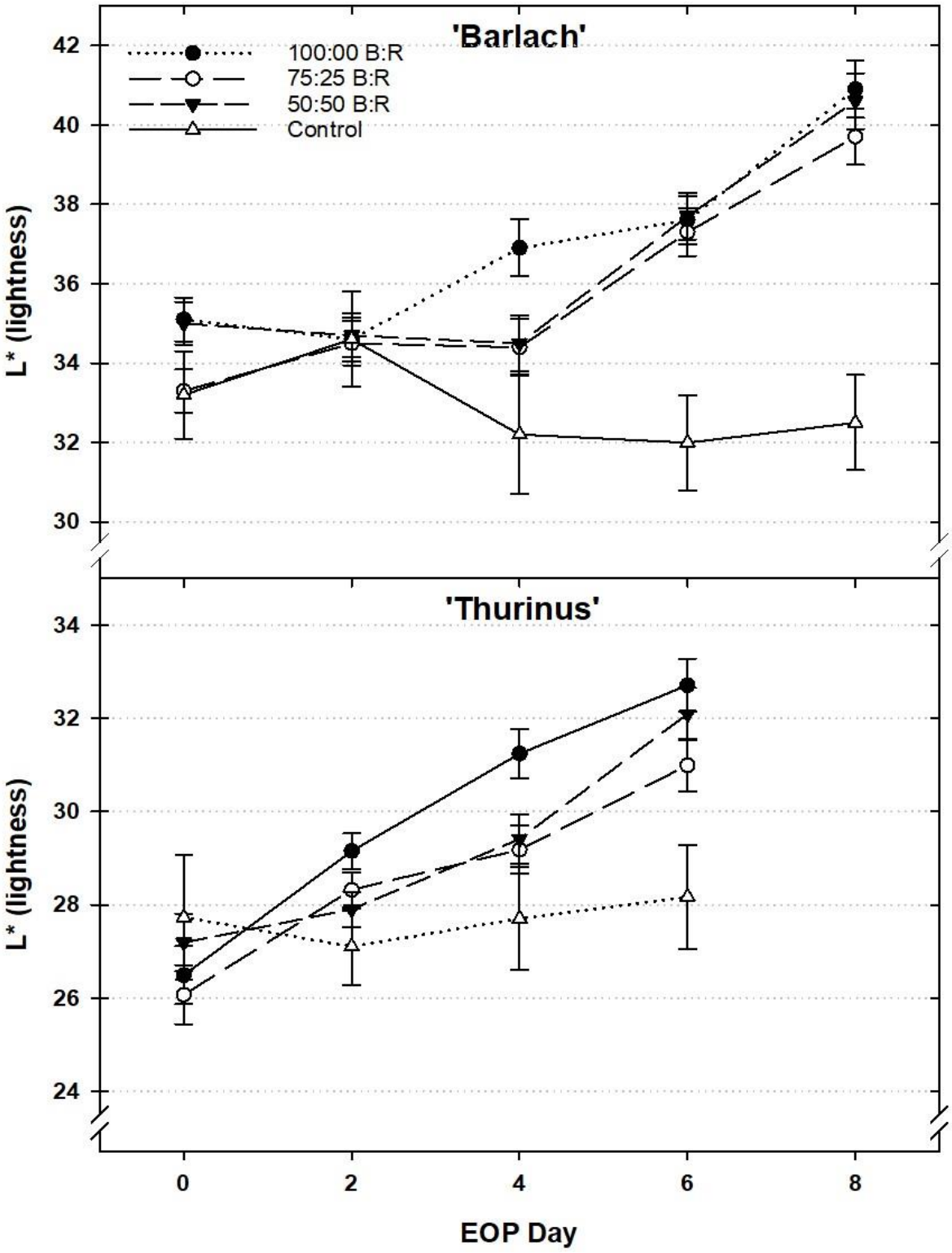


Figure II-1. Effect of end-of-production (EOP) sole-source lighting providing a light ratio of 100:00, 75:25, and 50:50 blue:red (B:R) light on L* of red leaf lettuce (Lactuca sativa) 'Barlach' on day 0, 2, 4, 6 and 8 and 'Thurinus on day 0, 2, 4, and 6. Error bars show standard error.

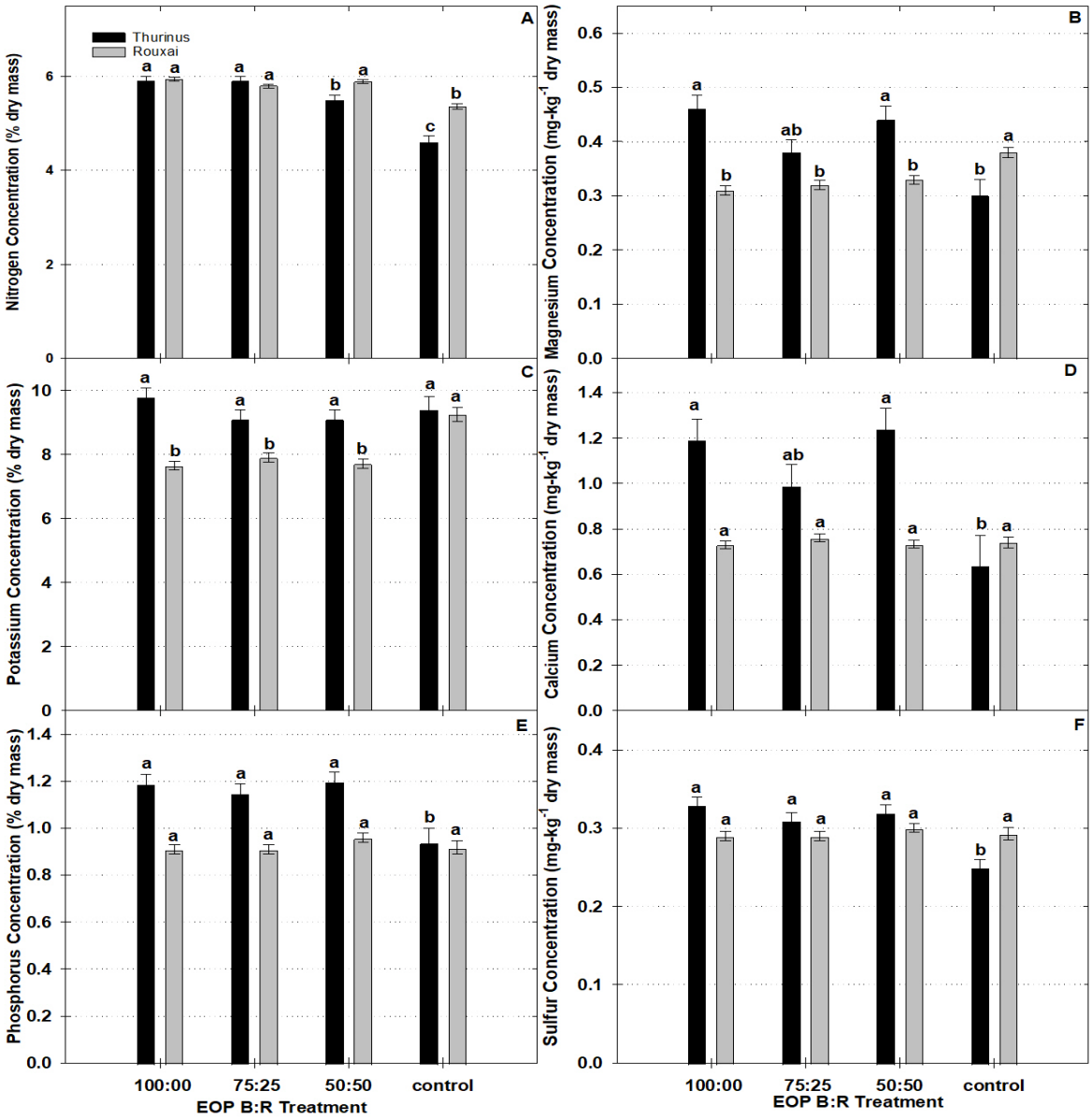


Figure II-2. Concentrations of macronutrients (nitrogen, potassium, phosphorus, magnesium, calcium, and sulfur) in leaf tissues of red leaf lettuce (*lactuca sativa*) 'Barlach' and 'Thurinus' under end-of-production (EOP) sole-source lighting providing a light ratio (%) of 100:00, 75:25, or 50:50 blue:red (B:R) light. Means followed by different letters within each cultivar are significantly different based on Tukey's honestly significant difference test ($\alpha = 0.05$). Error bars show standard error.

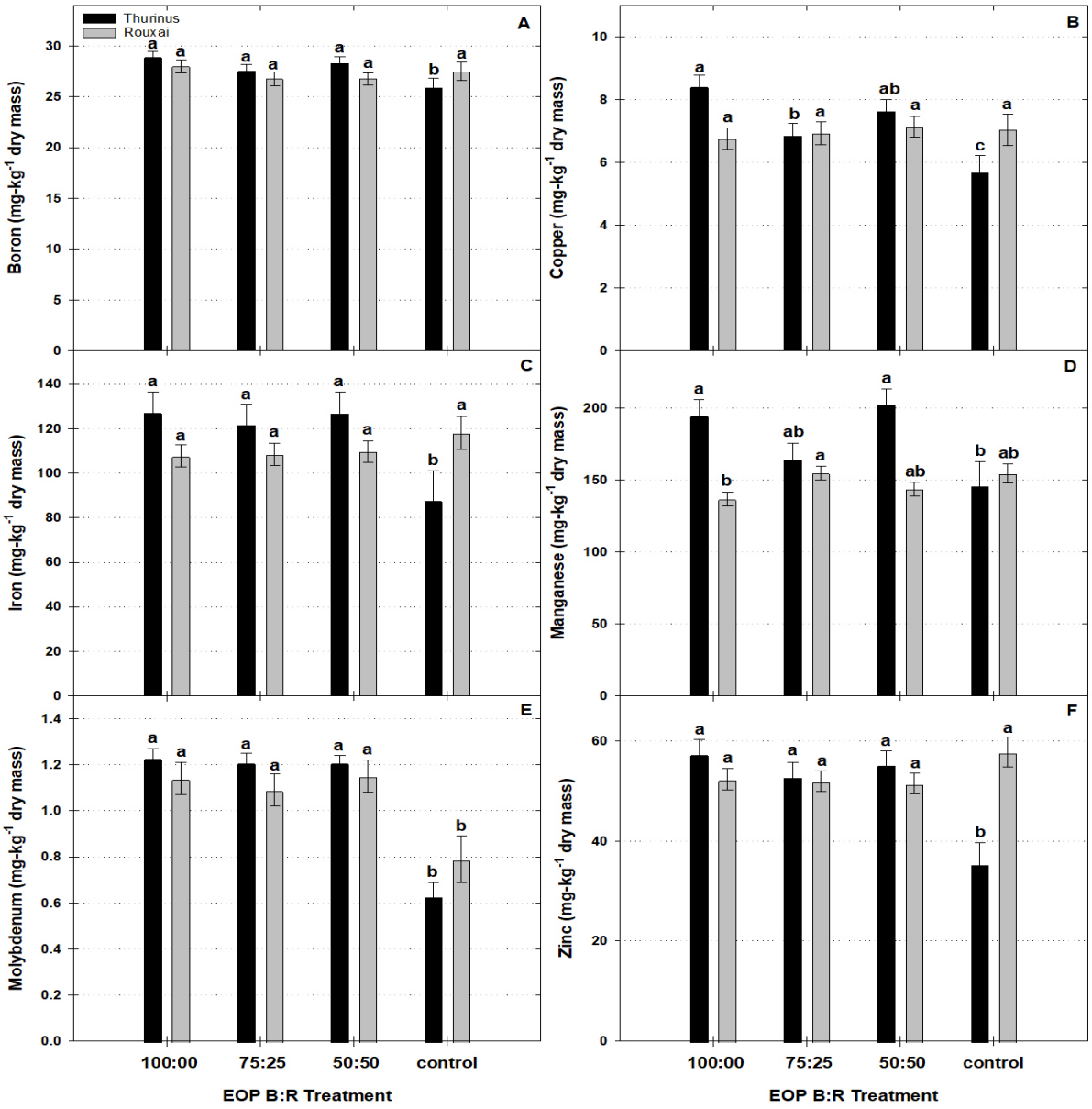


Figure II-3. Concentrations of micronutrients (boron, iron, molybdenum, copper, manganese, and zinc) in leaf tissues of red leaf lettuce (*lactuca sativa*) 'Barlach' and 'Thurinus' under end-of-production (EOP) sole-source lighting providing a light ratio (%) of 100:00, 75:25, or 50:50 blue:red (B:R) light. Means followed by different letters within each cultivar are significantly different based on Tukey's honestly significant difference test ($\alpha = 0.05$). Error bars show standard error.

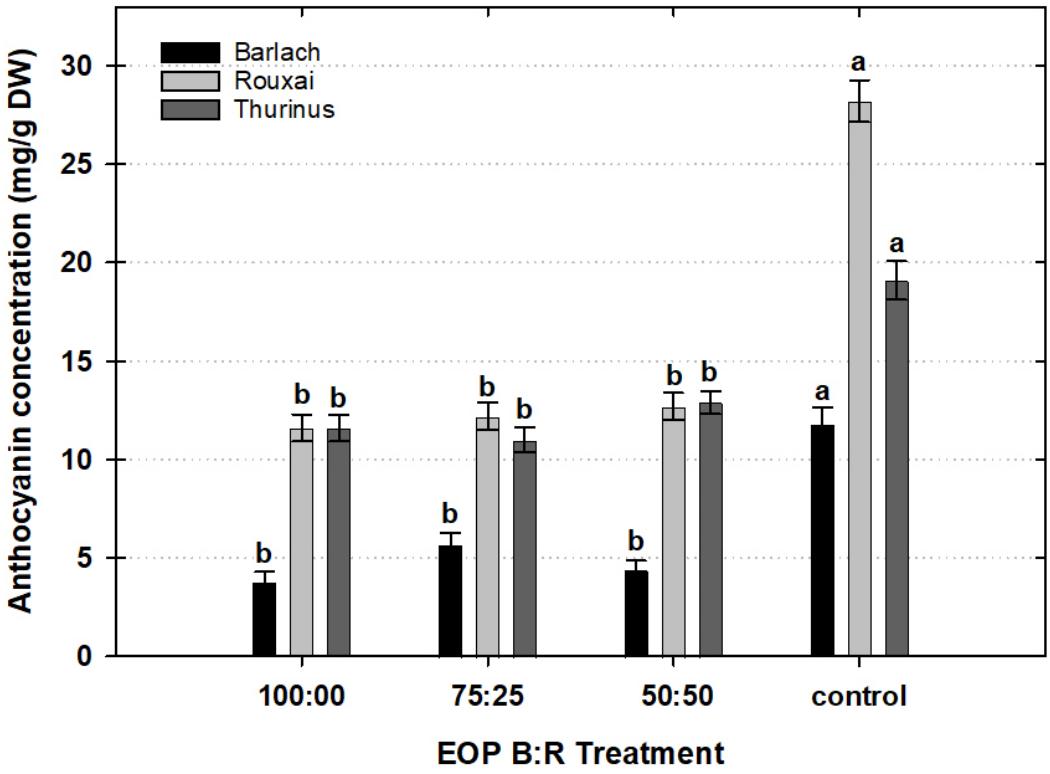


Figure II-4. Influence of end-of-production (EOP) sole-source lighting providing a light ratio (%) of 100:00, 75:25, or 50:50 blue:red (B:R) light on anthocyanin concentration of red leaf lettuce (*Lactuca sativa*) 'Barlach', 'Rouxai', and 'Thurinus'. Means followed by different letters within each cultivar are significantly different based on Tukey's honestly significant difference test ($\alpha = 0.05$). Error bars show standard error.

## SECTION III

## INFLUENCE OF END-OF-PRODUCTION BLUE+RED SOLE-SOURCE LIGHTING AND COOLING ON FOLIAGE COLOR, YIELD, AND NUTRITION OF RED LEAF LETTUCE

Influence of End-of-Production Blue+Red Sole-Source Lighting and Cooling on Foliage Color, Yield, and Nutrition of Red Leaf Lettuce (*Lactuca sativa*)

Devin Brewer [1], Kellie Walters[2], Jennifer Bolt[3], and Roberto G. Lopez[1*]

[1] Department of Horticulture, Michigan State University, 1066 Bogue Street, East Lansing, MI 48824-1325, USA

[2] Plant Sciences Department, University of Tennessee, 2505 E.J. Chapman Drive, Knoxville, TN 48824, USA

[3] USDA-ARS Application Technology Research Unit, 2801 W. Bancroft Street Mail Stop 604, Toledo, OH 43606, USA

***** Corresponding author. Tel.: +1 517-353-0342. E-mail address: rglopez@msu.edu (R.G. Lopez).

**Abstract**

Plants synthesize anthocyanins to counteract oxidative damage from environmental stressors, such as chilling injury. In controlled environments (CE), temperature can be manipulated and possibly used to increase anthocyanin concentration and thus improve foliage color of crops prior to harvest. Our objectives were to 1) quantify how end-of-production (EOP) cooling influences yield, growth, development, quality including foliage color and mineral nutrient, carotenoid, and anthocyanin concentrations of red leaf lettuce. Seeds of red leaf lettuce (*Latuca sativa*) 'Barlach', 'Rouxai', and 'Thurinus' were sown in a growth chamber with a mean daily temperature (MDT) set point of 22 °C, carbon dioxide ($CO_2$) concentration of 500 µmol·mol$^{-1}$, and a photosynthetic photon flux density (PPFD) of 180 µmol·m$^{-2}$·s$^{-1}$ provided by light-emitting diodes (LEDs). After 11 d, seedlings were transplanted into deep flow hydroponic tanks in the same growth chamber with a $CO_2$ concentration of 800 µmol·mol$^{-1}$, day/night temperature set point of 28/21 °C (MDT of 26 °C) and under LEDs that provided a PPFD of 300 µmol·m$^{-2}$·s$^{-1}$ for 17 h·d$^{-1}$. During the last 6-8 days of production, plants were either left in the same conditions or transferred to growth chambers with a constant MDT of either 8, 14, or 20 °C and under a ratio (%) of 75:25 blue (400-500nm):red (600-700nm) (B:R) light and a PPFD of 150 µmol·m$^{-2}$·s$^{-1}$. EOP cooling negatively influenced the shoot fresh mass (SFM) and shoot dry mass (SDM) of 'Barlach', 'Rouxai', and 'Thurinus'. Compared to uncooled plants, the SFM and SDM of 'Barlach', 'Rouxai', and 'Thurinus' in the 14 °C EOP cooling treatment were reduced by 27 and 17%, 25 and 20%, and 51 and 52%, respectively. The chromametric a* value of each cultivar increased, indicating a change from green to red, under all EOP treatments. By day 6 of EOP treatment, a* of 'Barlach' under the EOP 14 °C treatment increased from -4.18 to -1.66, while the a* of uncooled plants decreased from -5.06 to -6.97. By day 2, a* of 'Rouxai' and 'Thurinus'

at 14 °C increased from -1.7 to 0.06 and -0.99 to 1.08, respectively. EOP cooling generally increased mineral nutrient concentration. The tissue concentration of Mg, Mn, and Zn in 'Barlach' and 'Rouxai' increased by 23, 20, and 21%, and 26, 21, and 13%, respectively, at 14 °C. Plants exposed to EOP cooling had greater anthocyanin concentrations; 'Barlach', 'Rouxai', and 'Thurinus' possessed 62, 53, and 59% greater anthocyanin concentration at 14 °C compared to the control. We observed the highest concentration of violaxanthin, neoxanthin, lutein, α-carotene, β-carotene, and total carotenoids in both cultivars at 14 °C and the lowest under the control treatment. From the results of this study, we recommend exposing red leaf lettuce plants to 2 d EOP cooling at 14 °C prior to harvest to improve coloration and nutritional concentration while minimizing impact on yield. Our hypothesis was that the lowest end-of-production air temperature treatment would improve anthocyanin and carotenoid concentration, coloration, and mineral nutrient concentration of red leaf lettuce.

Keywords: anthocyanin, controlled-environment agriculture, leafy greens, coloration, end-of-production, cooling

**Introduction**

Temperature influences the rate of biochemical reactions within a plant. The rate of those reactions determines the time required for leaves, stems, roots, and flowers to develop. Biochemical reactions begin to take place and development occurs just above the base temperature ($T_b$) and increase linearly until the optimal temperature ($T_{opt}$) is reached, after which the rate of reaction and development rapidly decline and cease at the maximum temperature ($T_{max}$). These cardinal temperatures are species and cultivar specific but generally follow a similar pattern among plant species (Erwin et al., 1995).

The general recommendation is to grow lettuce (*Latuca sativa*) at day temperatures of 17 to 28 ℃ and night temperatures of 3 to 12 ℃ (Wurr et al., 1992; Marsh, 1987). However, recent research within controlled environments (CE), has shown that increasing the mean daily temperature (MDT) beyond 23 ℃ increased shoot fresh and dry mass, leaf number, and growth index of lettuce 'Rouxai' (Tarr et al., 2023). Additionally, Tarr et al. (2023) reported that increasing the ADT from 23 to 26 ℃ for red-leaf lettuce 'Rouxai' resulted in light green dull foliage instead of the desired red coloration. It has also been reported that lettuce 'Ostinata' grown at 25 ℃ was of higher quality than that grown at 15 ℃, the latter of which was described as thick and leathery (Marsh, 1987; Seigner et al., 1991).

Consumers prefer more colorful, nutritious, and vitamin rich vegetables (Llorach et al., 2008). Anthocyanins are the primary pigment associated with the vibrant red and purple foliage color of red-leaf lettuce and microgreens (Li and Kubota, 2009; Samuoliené et al., 2012) and provide many health benefits such as reducing the likelihood or severity of cancer and neurodegeneration due to aging (Blesso, 2019; Wallace et al., 2016; Zhu., 2018). They are synthesized for an array of functions including mitigating excessive light, adapting to chilling stress, recovering from herbivory damage, and functioning as osmoregulators during drought or salinity stress (Boldt et al., 2013). Anthocyanin content is negatively correlated with high temperatures and generally, sub-optimal or large day and night temperature fluctuations stimulate their accumulation (Lovdal et al., 2010). However, sub-optimal or fluctuating temperatures can result in reduced yield and crop quality (Marsh, 1987; Seigner et al., 1991; Dale, 1965).

Similarly, carotenoids contribute to the yellow, orange, red and purple coloration in plants and provide health benefits such as prevention of age-related macular degeneration and

decreasing the risk of some types of cancer (Maoka, 2020) Carotenoid synthesis and accumulation generally increase with rising temperature, however low temperature stress has also been shown to enhance carotenoid content in bell peppers (*Capsicum annuum*) (Juneja et al., 2013; León-Chan et al., 2017).

This presents an opportunity for CE lettuce producers to grow their crops under environmental conditions that are favorable to rapid growth and development and then expose them to cooler temperatures conducive to anthocyanin, carotenoid, and mineral nutrient accumulation at the end-of-production (EOP) without sacrificing yield.

To our knowledge, there is little to no scientific information available on the effectiveness of exposing plants to indoor EOP cooling to improve coloration, increase anthocyanin, carotenoid, and mineral nutrient concentration, or the extent to which EOP cooling affects growth, development, and yield. Therefore, our objective was to identify the most effective EOP cooling temperature and duration for enhancing anthocyanin concentration and nutritional quality without compromising plant quality or yield. Our hypothesis was that the lowest end-of-production air temperature treatment would improve anthocyanin and carotenoid concentration, coloration, and mineral nutrient concentration of red leaf lettuce.

**Materials and Methods**

*Plant material and propagation conditions*

Seeds of red oakleaf 'Rouxaï', Salanova butterhead 'Barlach', and cos 'Thurinus' lettuce (Rijk Zwaan USA; Salinas, CA) were sown into 200-cell (2.5 cm × 2.5 cm) rockwool plugs (AO 25/40 Starter Plugs; Gordan, Milton, ON, Canada) on 13 June 2022, 28 Oct. 2021, 16 Dec. 2021, and 15 Feb. 2022. These varieties were selected due to their foliage color, suitability to be grown hydroponically, and resistance to the physiological disorder tip burn. Deionized (DI) water

adjusted to a pH of 4.4 to 4.5 with diluted (1:31) 95 to 98% sulfuric acid (J.Y. Baker, Inc.; Phillipsburg, NJ) was used to presoak plugs. Increased humidity was achieved by covering trays with translucent plastic domes for three days. Trays were then placed in one of three walk-in growth chambers (Hotpack environmental room UWP 2614-3; SP Scientific, Warminster, PA) with setpoints of an ADT of 22 ºC, relative humidity (RH) of 60% to maintain a vapor pressure deficit (VPD) of 1.0 kPA, and carbon dioxide ($CO_2$) concentration of 500 μmol·mol$^{-1}$. Light-emitting diode (LED) fixtures (GreenPower LED production module 3.0; Phillips, Amsterdam, Netherlands) were utilized to provide a light ratio (%) of 19:39:39:3 blue (400–500 nm):green (500–600 nm):red (600–700 nm):far red (700–800 nm) (B:G:R:FR) and a PPFD of 180 μmol·m$^{-2}$·s$^{-1}$ for 24 h daily. After a three-day germination stage, the photoperiod was reduced to 20 h until seedlings were transplanted on day 11. Seedlings were sub-irrigated with DI water supplemented with water-soluble fertilizer providing (in mg·L$^{-1}$): 125 N, 18 P, 138 K, 73 Ca, 47 Mg, 1.56 Fe, 0.52 Mn, 0.36 Zn, 0.21 B, 0.21 Cu, 35 S, and 0.01 Mo (12N–4P–16K RO Hydro FeED; JR Peters, Inc., Allentown, PA). The pH and electrical conductivity of the solution was measured using a pH/EC probe and adjusted to 5.6 and 1.6 mS·cm$^{-1}$, respectively, each day (HI 991301 pH/TDS/Temperature Monitor; Hanna Instruments, Smithfield, RI).

*Hydroponic systems*

On day 11 of each replication, 36 seedlings of each cultivar were transplanted 20-cm apart into three 250 L, 0.9-m-wide by 1.8-m-long deep-flow hydroponic systems (Active Aqua premium high-rise flood table; Hydrofarm, Petaluma, CA) with one tank in each walk-in growth chamber. Each tank was covered with a floating 4-cm-thick extruded polystyrene foam sheet. The foam sheets had 4-cm diameter holes drilled in them to accommodate plastic net baskets that held rockwool starter cubes/seedlings in contact with the nutrient solution. DI water

supplemented with water-soluble fertilizer providing ($mg \cdot L^{-1}$): 150 N, 22 P, 166 K, 87 Ca, 25 Mg, 1.9 Fe, 0.62 Mn, 0.44 Zn, 0.25 B, 0.25 Cu, and 0.01 Mo (Jack's 12N–4P–16K; JR Peters, Inc.), and 0.28 g magnesium sulfate (Pennington Epsom salt; Madison, GA). The pH and EC were measured and adjusted daily to maintain an EC of $1.7 \pm 0.05$ $mS \cdot cm^{-1}$ by adding DI water or concentrated nutrient solution as needed, while the pH was adjusted to $5.6 \pm 0.05$ using potassium bicarbonate or sulfuric acid. A dissolved oxygen concentration of $10.0 \pm 0.03$ $mg \cdot L^{-1}$ was maintained using air pumps (Active Aqua 70 $L \cdot min^{-1}$ commercial air pump; Hydrofarm) connected to air stones (Active Aqua air stone round 10.2 cm × 2.5 cm; Hydrofarm).

*Growth chamber environmental conditions*

The air day/night temperature (17 /7 h) was 28/21 °C (MDT 26 °C), measured every 5 seconds by a resistance temperature detector (Platinum RTD RBBJL-GW05A-00-M 36B; SensorTec, Inc., Fort Wayne, IN) and logged by a C6 controller (Environmental Growth Chambers, Chagrin Falls, OH). A photosynthetic photon flux density (PPFD)of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was provided for 17 $h \cdot d^{-1}$ by LED fixtures (GreenPower LED production module 3.0; Phillips) achieving a DLI of 18.4 $mol \cdot m^{-2} \cdot d^{-1}$ with the LED fixtures mounted 10-12 cm above the crop canopy. Water and leaf temperature, and PPFD were monitored using a thermistor (ST-100; Apogee Instruments, Logan, UT), infrared thermocouple (OS36-01-T-80F; Omega Engineering, INC. Norwalk, CT), and quantum sensor (LI-190R; LI-COR Biosciences, Lincoln, NE), respectively, every 15 seconds and means were logged each hour by a CR-1000 datalogger (Campbell Scientific, Logan, UT). $CO_2$ concentration was maintained via compressed $CO_2$ injection at 800 $\mu mol \cdot mol^{-1}$ during the day, monitored with a $CO_2$ sensor (GM86P; Vaisala, Helsinki, Finland), and logged by a C6 Controller (Environmental Growth Chambers) every 5 seconds. A VPD of 1.1 kPa was maintained by having a day and night relative humidity of

70/55%. Overhead fans were utilized to maintain an average air velocity of 0.7 $m^{-3} \cdot s^{-1}$ at plant height.

*End-of-production temperature and radiation treatments*

EOP treatment for 'Thurinus' was initiated 24 d after sowing and continued for 6 d. 'Rouxai' and 'Barlach' were initiated 30 d after sowing and continued for 6 or 8 d, respectively. The EOP radiation intensity in each growth chamber was reduced to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to provide a B:R light ratio (%)of 75:25 for 16-$h \cdot d^{-1}$. The air temperature within each chamber was reduced to 20, 14, or 8 °C.

*Growth data collection and analysis*

Using a tristimulus colorimeter (Chroma Meter CR-400; Konica Minolta Sensing, Inc., Chiyoda, Tokyo), foliage coloration measurements were taken for 'Thurinus' on days 24, 26, 28, and 30, 'Rouxai' on days 30, 32, 34, and 36, and 'Barlach' on days 30, 32, 34, 36, and 38 after sowing. The relative chlorophyll concentration (RCC) of the most recent fully expanded leaf from each sample plant was estimated using a SPAD chlorophyll meter (MC-100 Chlorophyll Meter; Apogee Instruments, Logan, UT). A second leaf was then dark acclimated for >15 minutes using the manufacturer-supplied clips and exposed to 3,500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of R light (peak wavelength 650 nm) to achieve saturation of photosystem II and the fluorescence was then measured, averaged, and reported as $F_v/F_m$ by a portable chlorophyll fluorescence (CF) meter (Handy Plant Efficiency Analyzer; Hanstech Instruments Ltd. Norfolk, U.K.).

Harvest occurred on day 30 for 'Thurinus', day 36 for 'Rouxaï', and day 38 for 'Barlach'. Shoot fresh mass (g), length and width (cm) of the sixth most recent fully expanded leaf, and leaf number (when >5 cm) was taken on plants of each cultivar and treatment. Growth index (GI) was calculated (GI = {plant height + [(diameter 1 + diameter 2)/2]}/2) (Krug et al.,

2010) after measuring plant height from the root to the apical meristem, and plant width at the widest point and perpendicular from the widest point. After recording the shoot fresh mass, leaves were removed at random and weighed to collect 40 g of leaves. The leaves were then placed into a polyethylene sampling bag and submerged in liquid N to flash freeze. Frozen samples were stored in a –80 ºC freezer until they were ready to be freeze dried (SP VirTis Genesis Freeze Drier; ATS Life Sciences, Warminster, PA). The remaining plant material from each sample was dried at 75 ºC for a minimum of 3 days using a forced-air drier. Freeze dried and oven dried samples were weighed independently, added together, and recorded as the shoot dry mass.

*Mineral nutrient analysis*

Air dried samples were homogenized individually using a mortar and pestle before being sent to the USDA-ARS for tissue elemental analysis. Foliar nitrogen (N) was determined by measuring 2.5 mg of dry tissue into tin capsules (Costech Analytical, Valencia, CA) and using a CHN analyzer to conduct analysis (vario MICRO cube; Elementar, Hanau, Germany). Additional elements were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES). For these macronutrients and micronutrients, 0.25 g of dry tissue was loaded into a Teflon vessel and 5 mL of nitric acid was added. A programmable microwave (MARS 6; CEM Corp., Matthews, NC) was used to increase the temperature to 200 °C over 15 min., maintain 200 °C for 15 min., and then cooled to room temperature. After reaching room temperature, 1.5 mL of hydrogen peroxide was added, solutions were reheated to 200 °C and maintained for 5 min. After samples cooled to room temperature, 12 mL of 18 MΩ water was added, and each solution was filtered (Whatman #2). Then a 1.3 mL aliquot of solution was diluted with 8.7 mL 18 MΩ water and analyzed using ICP-OES (Brewer et al., 2023)

*Anthocyanin isolation and quantification*

Before being sent to the University of Tennessee for extraction and analysis, tissue samples were homogenized with a ceramic mortar and pestle containing liquid N. Total anthocyanin extraction and analysis was conducted according to a method derived from Islam et al (2019). Extractions were performed under R light (peak wavelength: 654 nm). One hundred mg of each sample was weighed and placed into a 15 mL polypropylene centrifuge tube, then saturated with 5 mL of 95% ethanol/1.5 N HCl (85:15, v:v). An orbital shaker was used to maintain samples at 200 rpm for 15 min., afterwards samples were stored in the dark at 4 °C for 24 h. Sample contents were then filtered into a 25 mL Erlenmeyer flask and a 200-µL aliquot of extractant was pipetted into a 96-well assay plate. Samples were analyzed using a Biotek PowerWave XS Microplate Reader (Agilent Technologies, Santa Clara CA). Optical density was measured at 530 nm and total anthocyanin concentrations were calculated based on the calibration curve of cyanidin-3-o-glucoside chloride (MilliporeSigma, Burlington MA).

*Carotenoid Isolation and Quantification*

Before being sent to the University of Tennessee for extraction and analysis, tissue samples were homogenized with a ceramic mortar and pestle containing liquid N. Carotenoids α-carotene, β carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin were extracted and analyzed according to the method described by Darby et al. (2023) and derived from Kopsell et al. (2012). Under R light, chilled samples were extracted using purified water and tetrahydrofuran. An internal carotenoid standard was used to quantify sample loss during homogenization. Samples were homogenized then a second extraction using tetrahydrofuran was conducted, and samples were homogenized again. The solution was then placed in a centrifuge, and the eluent was dried using a stream evaporator. Acetone was added, then samples were

filtered, and an aliquot was stored for identification and quantification on a 1200 Agilent Series HPLC unit equipped with a diode array detector. Lastly a reverse phase C30 column was used with a mobile phase composed of methyl tert-butyl ether, methanol, and triethylamine.

**Results**

*Shoot fresh and dry mass*

The SFM and SDM of each cultivar was negatively influenced by EOP cooling (Table III-3) For instance, as the MDT was reduced from 26 (control) to 20 ℃, the SFM and SDM of 'Thurinus' decreased by 35% (34.8 g) and 34% (1.1g), respectively. Further reducing the MDT to 14 ℃, resulted in a 27% (46.0 g) and 17% (0.8 g), 25% (37.6 g) and 20% (0.9 g), and 51% (50.6 g) and 52% (1.8 g) reduction of SFM and SDM for 'Barlach', 'Rouxai', and 'Thurinus', respectively. At 8 ℃ EOP cooling, the SFM of Barlach was 45% (75.1 g) lower than the control; however, the SFM and SDM of 'Rouxai' and 'Thurinus' did not further decrease beyond that of plants at 14 ℃.

*Plant morphology*

The leaf unfolding rate and GI of all cultivars was negatively influenced by EOP cooling treatments (Table III-3). 'Barlach', 'Rouxai', and 'Thurinus' unfolded 21, 4, and 5 fewer leaves as the MDT was reduced from 26 to 20 ℃, respectively. Furthermore, at an EOP temperature of 14 or 8 ℃, 'Rouxai' and 'Thurinus' unfolded 3 to 4 fewer leaves, respectively, than at 20 ℃. Compared to uncooled plants, the GI of 'Barlach', 'Rouxai', and 'Thurinus' was reduced by 14%, 6%, and 26%, respectively, at an EOP temperature of 20 ℃. At the lower EOP temperature of 14 or 8 ℃, the GI of 'Barlach', 'Rouxai', and 'Thurinus' was further reduced by 20%, 14%, and 32%, respectively, compared to the control.

*Chlorophyll fluorescence and pigmentation*

CF of ‘Barlach’ and ‘Rouxai was greatest at 20 °C with CF values of 0.8509 and 0.8512; however, in ‘Thurinus the greatest CF value of 0.8373 was recorded in plants not receiving a cooling treatment. At 8 °C ‘Barlach’, ‘Rouxai’ and ‘Thurinus had reduced CF values of 0.8095, 0.8213 and 0.8056, respectively (Table III-3).

EOP cooling treatments and duration influenced the foliage lightness (L*), greenness and redness (Chromametric a* value), and blueness and yellowness (Chromametric b* value) of each cultivar (Fig. III-1). On day 2, L* and b* of ‘Barlach’ exposed to 8 and 14 °C had decreased by 12 and 14% and 26 and 32%, respectively, indicating a darker and bluer color compared to the pretreatment measurement. By day 6, a* of ‘Barlach’ exposed to the 8, 14, and 20 °C treatment had increased (becoming more red than green) from −4.27, −4.52, and −4.18 to −1.66, −1.99, and −3.32, respectively, while control plants decreased from −5.06 to −6.97 (Fig. III−1). On day 8, L* of plants under 8, 14, and 20 °C were 30, 28, and 22% lower than the control (Fig. III-1).

On day 2, L* of ‘Rouxai’ exposed to EOP cooling temperatures of 8 and 14 °C had decreased by 10 and 8% and a* had increased from −2.22 to −0.57 and −1.7 to 0.06, respectively, compared to uncooled plants. By day 4, b* of ‘Rouxai’ at 8 and 14 °C was 25 and 38% lower, respectively, then plants under the control (Fig. III-2). On day 6, L* of plants at 8 and 14 °C were 21 and 11% lower, respectively, than the control (Fig. III-2).

From day 0 to 6, L* of untreated ‘Thurinus’ increased 13% from 27.1 to 31. In contrast, from day 0 to 2, L* of ‘Thurinus’ exposed to 8, 14, and 20 °C decreased by 10, 22, and 15%, respectively (Fig. III-3). By day 6, L* of plants cooled at 8 and 14 °C had decreased or were unchanged, while at 20 °C, L* increased from days 2 to 6. The greatest increase in a* occurred from day 0 to 2 for cooled plants, increasing from −0.36, −0.99, and −0.78 to 1.08, 1.22, and 1.14, respectively, for plants at EOP treatments of 8, 14, and 20 °C, while the control decreased

from −0.24 to −1.39 (Fig. III-3). Beyond day 2, a* of plants at 8 and 14 °C generally remained constant. On day 2, b* of the 8, 14, and 20 °C treatments decreased from 3.21, 4.38, and 4.18 to 0.52, −0.74, and 1.03, respectively, while b* of the control plants increased by from 3.11 to 4.57 (Fig. III-3). Beyond day 2, only plants at 8 °C continued to show a decrease in b*.

*Mineral nutrient concentration*

EOP cooling influenced macro- and micro-nutrient concentration of cultivars differently (Fig. III-4). Compared to uncooled plants, 'Barlach' in the 20 °C EOP treatment contained 21, 9, and 12% greater foliar tissue concentrations of Ca, B, and Mo respectively (Fig. III-4; Fig. III-5). After 8 d at 14 °C, the concentrations of N, K, Mg, Fe, Mn, and Zn of 'Barlach' increased by 14, 9, 23, 27, 20, and 21%, respectively (Fig. III-4; Fig. III-5). Similarly, plants at 8 °C contained a 15, 18, and 20% greater concentration of S, Cu, and Zn, respectively, compared to the control. However, those same plants contained 5, 12, and 7% less N, P, and B, respectively, than those plants not placed under EOP treatments (Fig. III-4; Fig. III-5).

At 20 °C, 'Rouxai' had 7, 24, 21, 13, and 26% greater concentrations of P, K, Ca, B, and Mo, respectively, compared to the control (Fig. III-4; Fig. III-5). When 'Rouxai' was placed at the 14 °C EOP treatment, the concentration of N, Mg, S, Mn, and Zn increased by 3, 26, 15, 21, and 13% respectively (Fig. III-4; Fig. III-5). Conversely, at 8 °C, plants possessed 11% less N (Fig. III-4; Fig. III-5).

At an EOP temperature of 20 °C, 'Thurinus' contained 8, 22, and 20% greater P, B, and Mo, respectively, than uncooled plants. However, they contained 11% less Zn (Fig. III-4; Fig. III-5). At 14 °C, 'Thurinus' had 12, 11, and 40% greater concentrations of Mg, S, and Cu respectively. However, plants had 12 and 27% less K and Ca than the control (Fig. III-4; Fig.III-

5). Lastly, at 8 °C, plant tissue concentration of N, P, K, Ca, Mn, Mo, and Zn was 17, 18, 26, 39, 21, 31, and 19% lower than the control, respectively (Fig. III-4; Fig.III-5).

*Anthocyanin and carotenoids*

Anthocyanin concentration of each cultivar increased when they were placed in EOP cooling treatments of 8, 14, or 20 °C (Fig. III-6). 'Barlach' had the greatest anthocyanin concentration at an EOP temperature of 8 °C, which was 69% greater than the control. At 14 °C, 'Barlach' had less anthocyanins than at 8 °C, but still possessed 62% greater anthocyanin concentration than uncooled plants (Fig. III-6). 'Rouxai' displayed 32, 53, and 52% greater anthocyanin concentration at 20, 14, and 8 °C, respectively, than plants in the control. Similarly, 'Thurinus' exposed to 8 or 14 °C displayed a 59% greater anthocyanin concentration than plants not receiving an EOP treatment.

EOP cooling altered carotenoid composition in each cultivar (Table III-4). 'Barlach' exposed to 14 °C had 59% greater violaxanthin, 38% lutein, and 37% total carotenoid concentration compared to the control (Table III-4). All EOP cooling treatments correlated with reduced neoxanthin concentration in 'Rouxai', ranging from a 36% decrease at 20 °C to a 74% decrease at 8 °C. However, there was no difference in total carotenoid concentration (Table III-4). Exposing 'Thurinus' to 20 °C resulted in a 29% increase of both violaxanthin and lutein, and 15% total carotenoid concentration. However, exposure to 8 °C reduced neoxanthin concentration by 62% (Table III-4).

**Discussion**

As expected, SFM and SDM decreased with a 6-to-8-day exposure to suboptimal temperatures (Tarr et al., 2023; Marsh and Albright, 1991). The greatest SFM and SDM of 'Barlach' and 'Thurinus' was achieved at an MDT of 26 °C; however, 'Rouxai' had the greatest

SFM and SDM at an EOP temperature of 20 °C. This is in agreement with the results of Tarr et al. (2023) that indicated biomass accumulation was greatest at an MDT of 23 °C and thus lower than that of 'Barlach' and 'Thurinus'. The lowest SFM and SDM of each cultivar was recorded after 6 to 8 days at 8 °C.

EOP cooling resulted in morphological changes of each cultivar. As EOP temperature decreased, leaf number, growth index, and chlorophyll fluorescence of each cultivar decreased, while total chlorophyll content increased (Table III-3). A reduction in both the leaf unfolding rate and total leaf area or growth index in response to MDT is consistent with the understanding that development is primarily dependent on temperature (Hatfield and Prueger, 2015; Tarr et al., 2023; Kong et al., 2023). CF analysis is a rapid and non-destructive technique used to assess stress in many crops (Gorbe and Calatayud, 2012). In the current study, CF of 'Barlach' was greatest (i.e., the lowest stress) when grown at 20 °C for 8 days or for 38 days at 26 °C or 36 days at 26 °C for 'Rouxai'. All cultivars possessed the lowest CF at 8 °C, indicating a stress response. This is consistent with findings by Chen et al., (2021) who reported that romaine lettuce grown at an MDT of 17.5 °C and PPFD of 200 µmol $m^{-2}$ $s^{-1}$ had lower CF than plants grown at the same light intensity and an MDT of 21.5 °C.

Typically, cold stress results in reduced chlorophyll concentrations, however, in the present study, total chlorophyll content (TCC) was higher in plants exposed to EOP cooling. 'Rouxai' had the greatest TCC after 6 days at 8 °C, despite a decrease in CF indicating a stress response (Zhou et al., 2020). 'Barlach' and 'Thurinus' had the greatest TCC when exposed to 8 and 6 days of EOP cooling at 14 °C. This aligns with past research by Gazula et al. (2005) which compared chlorophyll content of 3 lettuce cultivars when grown at day/ night temperatures of 30/30, 30/20, and 20/20 °C and found that chlorophyll content was greatest at 20/20 °C.

Color is a key attribute in consumer perception of quality, food preference, and acceptability, with red coloration described as a major marketable attribute responsible for the commercial value of pigmented leaf lettuce (Marin et al., 2015). Past research has indicated that red leaf lettuce grown at an MDT of 26 °C or higher was a lighter, greener color as opposed to the desired darker red foliage d when produced at temperatures of <20 °C (Tarr et al., 2023; Marin et al., 2015). We observed a similar appearance in each cultivar produced at 26 °C (without cooling). In plants exposed to cooling, by day 2, we observed lower L* at 8 and 14 °C for 'Barlach' and 'Rouxai' while L* of 'Thurinus' was lower at 8, 14, and 20 °C. Additionally, a* of 'Rouxai' exposed to 8 or 14 °C increased and a* of 'Thurinus' under any treatment increased. Lastly, b* of 'Barlach' decreased at 8 and 14 °C and b* of 'Thurinus' increased under each treatment. On day 4, b* of 'Rouxai' had increased at temperatures of 8 and 14 °C. By day 6, a* of 'Barlach' had increased when exposed to any EOP treatment (Fig. III-1, Fig. III-2, Fig. III-3). Collectively, these changes indicate that EOP cooling altered foliage coloration as it became darker and more red/purple within 48 hours.

EOP cooling resulted in an increased macro- and micro-nutrient concentration in 'Barlach'; it contained the highest concentration of N, K, Ca, Mg, S, B, Cu, Mn, Mo, and Zn when exposed to an EOP cooling temperature of 20 °C and/or 14 °C (Fig. III-4). 'Rouxai' achieved the highest concentration of K, Ca, B, Mn, and Mo when exposed to EOP cooling at 20 °C and had the highest concentration of N, Mg, S, Cu, and Zn when exposed to 14 °C (Fig. III-5). This is similar to findings by Kong et al. (2023) which found that lettuce grown at 21 °C had 18, 20, 9, 24, 13, 21, 19, and 34% greater concentration of N, P, K, Mg, Fe, B, Cu, Mn and Zn than plants at 30 °C. It is likely that the P, Ca, Fe, and Mo may be more heavily influenced by other environmental or cultivar differences. Furthermore, research on the effect of preharvest

environmental parameters has shown that both Ca and Mg of lettuce increase with minor reductions in temperature (Weerakkody, 1994).

'Thurinus' displayed the greatest concentration of N, P, K, Ca, Mg, S, Mo, and Zn when it was not cooled. We observed that 'Thurinus' typically undergoes a period of rapid biomass accumulation during the final week of production; however, when exposed to EOP cooling, this did not occur. It is possible that cooling at a critical point of development delayed root development, inhibiting overall growth and hindering nutrient uptake (Cutforth et al., 1986).

Cold temperatures can limit the activity of enzymes such as Rubisco as well as decrease efficiency of the electron transport chain, reducing the photosynthetic rate. In response to high-light environments, plants develop increased anthocyanin contents to filter excessive light energy (Lev-Yadun and Gould, 2009). The function of anthocyanins in both scenarios is to protect the photosystem from damage from excessive photons. Despite reducing the radiation intensity from 300 to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, we observed a negative correlation between anthocyanin concentration and temperature, similar to results by Lovdal et al. (2010) in which lowering the MDT from 30 and 24 to 18 and 12 ℃ resulted in increased anthocyanin concentration. Each cultivar in the current study accumulated the greatest anthocyanin concentration at 8 or 14 ℃ and the lowest concentration at 26 ℃ (control). This indicates that temperature had a greater influence on anthocyanin accumulation than light intensity.

Carotenoids are the most common tetraterpene pigments in nature and responsible for the yellow, orange, and red coloration in plants (Maoka, 2020). The most common carotenoids in lettuce are violaxanthin, lutein and β-carotene (Kim et al., 2018). EOP cooling increased carotenoid concentration in both 'Rouxai' and 'Thurinus', however, it had no effect on carotenoid concentration of 'Barlach'. We observed the highest concentration of violaxanthin,

neoxanthin, lutein, α- carotene, and β-carotene in both cultivars at 14 °C and the lowest under the control treatment. Similar to anthocyanins, carotenoids also accumulate when a plant is faced with stress and elevated carotenoid concentration in our study is likely due to the decreased photosynthetic capability of plants under cooling treatments (Couso et al., 2011).

In conclusion, despite reduced light intensity EOP cooling was able to improve or increase coloration, carotenoid, and anthocyanin concentration of red leaf lettuce. Reducing the MDT to 8 or 14 °C for 'Barlach', 'Rouxai', and 'Thurinus' for 2-3 days at the EOP may increase foliage coloration without sacrificing fresh mass and leaf number.

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## APPENDIX B: SECTION III FIGURES AND TABLES

Table III-1. Mean (± SD) day and night air, canopy, and water temperatures; carbon dioxide ($CO_2$) concentrations; total photon flux density (PPFD); and vapor pressure deficit (VPD) during 30 days of indoor deep-flow hydroponic production for red leaf lettuce (*Lactuca sativa*) 'Barlach', 'Rouxaï RZ', and 'Thurinus'.

| Replication | Temperature (°C) | | | | $CO_2$ | PPFD | VPD |
|---|---|---|---|---|---|---|---|
| | Air day | Air night | Canopy | Water | ($\mu mol \cdot mol^{-1}$) | ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | (kPa) |
| 1 | 27.8 ± 0.1 | 22.0 ± 0.3 | 28.1 ± 2.8 | 25.9 ± 2.5 | 794.3 ± 20.2 | 299.4 ± 5.4 | 1.02 ± 0.12 |
| | 27.9 ± 0.3 | 21.9 ± 0.5 | 28.4 ± 3.2 | 25.3 ± 1.2 | 802.9 ± 28.4 | 302.7 ± 3.5 | 1.04 ± 0.07 |
| | 27.7 ± 0.1 | 21.7 ± 0.4 | 25.9 ± 3.2 | 25.8 ± 3.2 | 803.1 ± 32.0 | 299.5 ± 8.7 | 1.03 ± 0.11 |
| 2 | 27.9 ± 0.1 | 21.9 ± 0.4 | 28.4 ± 3.5 | 25.6 ± 2.7 | 799.0 ± 34.0 | 301.6 ± 11.5 | 1.06 ± 0.12 |
| | 27.6 ± 0.2 | 21.9 ± 0.3 | 26.6 ± 3.6 | 26.0 ± 3.6 | 806.8 ± 19.0 | 293.8 ± 5.8 | 1.11 ± 0.18 |
| | 27.9 ± 0.2 | 21.9 ± 0.3 | 29.1 ± 3.1 | 25.3 ± 1.7 | 802.9 ± 40.4 | 299.6 ± 5.0 | 1.09 ± 0.03 |

Table III-2. Mean (± SD) end-of-production (EOP) air, canopy, and water temperatures; carbon dioxide ($CO_2$) concentration; photosynthetic photon flux density (PPFD); and vapor-pressure deficit (VPD) of indoor deep flow hydroponic production for red leaf lettuce (*Lactuca sativa*) 'Barlach', 'Rouxaï RZ', and 'Thurinus', respectively.

| Replication | EOP | Temperature (°C) | | | $CO_2$ | PPFD | VPD |
|---|---|---|---|---|---|---|---|
| | Temperature (°C) | Air | Canopy | Water | ($\mu mol \cdot mol^{-1}$) | ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | (kPa) |
| 1 | 8 | 8.2 ± 1.1 | 12.6 ± 3.4 | 12.6 ± 3.4 | 9.5 ± 3.6 | 794.3 ± 20.2 | 154.2 ± 15.4 |
| | 14 | 14.3 ± 1.3 | 17.1 ± 1.9 | 17.1 ± 1.9 | 14.8 ± 3.8 | 802.9 ± 28.4 | 154.8 ± 6.2 |
| | 20 | 20.0 ± 0.2 | 19.8 ± 0.2 | 19.8 ± 0.2 | 19.8 ± 0.2 | 803.1 ± 32.0 | 155.4 ± 9.3 |
| 2 | 8 | 9.4 ± 4.5 | 12.1 ± 1.0 | 12.1 ± 1.0 | 8.9 ± 2.1 | 802.9 ± 40.4 | 156.3 ± 3.0 |
| | 14 | 14.7 ± 2.9 | 14.5 ± 0.3 | 14.5 ± 0.3 | 13.7 ± 0.3 | 806.8 ± 19.0 | 150.9 ± 11.5 |
| | 20 | 20.7 ± 2.3 | 20.7 ± 0.6 | 20.7 ± 0.6 | 18.8 ± 0.6 | 779.0 ± 34.0 | 155.0 ± 4.5 |

Table III-3. Influence of end-of-production (EOP) cooling temperatures (8, 14, and 20 °C) for 6 or 8 d or no EOP cooling (control) on leaf number (no.); shoot fresh and dry mass; growth index; chlorophyll fluorescence ($F_v/F_m$); and total chlorophyll content of red leaf lettuce cultivars (*Lactuca sativa*) 'Barlach', 'Rouxai', and 'Thurinus'. Data represent the mean of two replications and cultivars with 13 samples. Different letters within columns signify significantly different means according to Tukey's honestly significant difference (HSD) test ($P < 0.05$).

| Treatment °C | Leaf (no.) | Fresh mass (g) | Dry mass (g) | Growth index | $F_v/F_m$ | Total chlorophyll µg/mL |
|---|---|---|---|---|---|---|
| | | | **'Barlach'** | | | |
| Control | 56.7 a | 168.7 a | 4.8 a | 19.8 a | 0.8468 a | 5.0 c |
| 8 | 22.2 b | 93.6 c | 3.7 b | 14.7 d | 0.8095 c | 16.8 b |
| 14 | 29.2 b | 122.7 b | 4.0 b | 16.0 c | 0.8356 b | 26.6 a |
| 20 | 36.0 b | 167.8 a | 4.5 ab | 17.0 b | 0.8509 a | 12.2 b |
| | | | **'Rouxai'** | | | |
| Control | 26.3 a | 141.1 a | 4.0 a | 20.5 a | 0.8429 a | 4.9 b |
| 8 | 17.5 c | 97.3 b | 2.9 b | 17.3 c | 0.8213 b | 15.1 a |
| 14 | 19.7 c | 115.3 b | 3.5 ab | 17.6 c | 0.8491 a | 13.3 a |
| 20 | 22.9 b | 152.9 a | 4.4 a | 19.4 b | 0.8512 a | 14.4 a |
| | | | **'Thurinus'** | | | |
| Control | 21.3 a | 99.9 a | 3.2 a | 25.1 a | 0.8373 a | 8.9 c |
| 8 | 12.1 d | 31.6 c | 1.3 b | 15.8 c | 0.8056 c | 14.6 b |
| 14 | 14.3 c | 49.3 c | 1.4 b | 17.1 bc | 0.8249 b | 21.4 a |
| 20 | 16.7 b | 65.1 b | 2.1 b | 18.6 b | 0.8279 b | 21.0 ab |

Table III-4. Influence of end-of-production (EOP) cooling temperature (8, 14, and 20 °C) for 6 or 8 d or no EOP cooling (control) on violaxanthin; neoxanthin; lutein; alpha carotene; beta carotene; and total carotenoid content (μg/mL) of red leaf lettuce cultivars (*Lactuca sativa*) 'Barlach', 'Rouxai', and 'Thurinus'. Means followed by different letters within each cultivar are significantly different based on Tukey's honestly significant difference test (α = 0.05).

| Treatment °C | Violaxanthin | Neoxanthin | Lutein | α carotene | β carotene | Total carotenoids |
|---|---|---|---|---|---|---|
| | | | (μg/mL) | | | |
| | | | **'Barlach'** | | | |
| Control | 0.6 a | 0.4 a | 0.8 a | 0.01 a | 1.5 a | 3.3 a |
| 8 | 0.7 a | 0.1 b | 0.8 a | 0.02 a | 1.3 a | 3.0 a |
| 14 | 0.6 a | 0.1 b | 0.7 a | 0.01 a | 1.2 a | 2.9 a |
| 20 | 0.7 a | 0.3 ab | 0.8 a | 0.02 a | 1.3 a | 3.1 a |
| | | | **'Rouxai'** | | | |
| Control | 0.4 c | 0.4 b | 0.7 b | 0.01 a | 1.2 a | 3.0 b |
| 8 | 0.7 b | 0.5 b | 0.9 ab | 0.01 a | 1.6 a | 3.6 ab |
| 14 | 1.0 a | 1.1 a | 1.2 a | 0.04 a | 2.0 a | 4.9 a |
| 20 | 0.6 b | 0.5 b | 0.8 b | 0.01 a | 1.2 a | 3.0 b |
| | | | **'Thurinus'** | | | |
| Control | 0.8 b | 0.5 ab | 0.9 b | 0.02 a | 1.9 b | 4.1 c |
| 8 | 0.9 b | 0.3 b | 1.0 ab | 0.01 a | 2.1 ab | 4.4 bc |
| 14 | 1.1 a | 0.7 a | 1.2 a | 0.02 a | 2.5 a | 6.0 a |
| 20 | 1.1 a | 0.6 ab | 1.3 a | 0.02 a | 2.3 a | 5.5 ab |

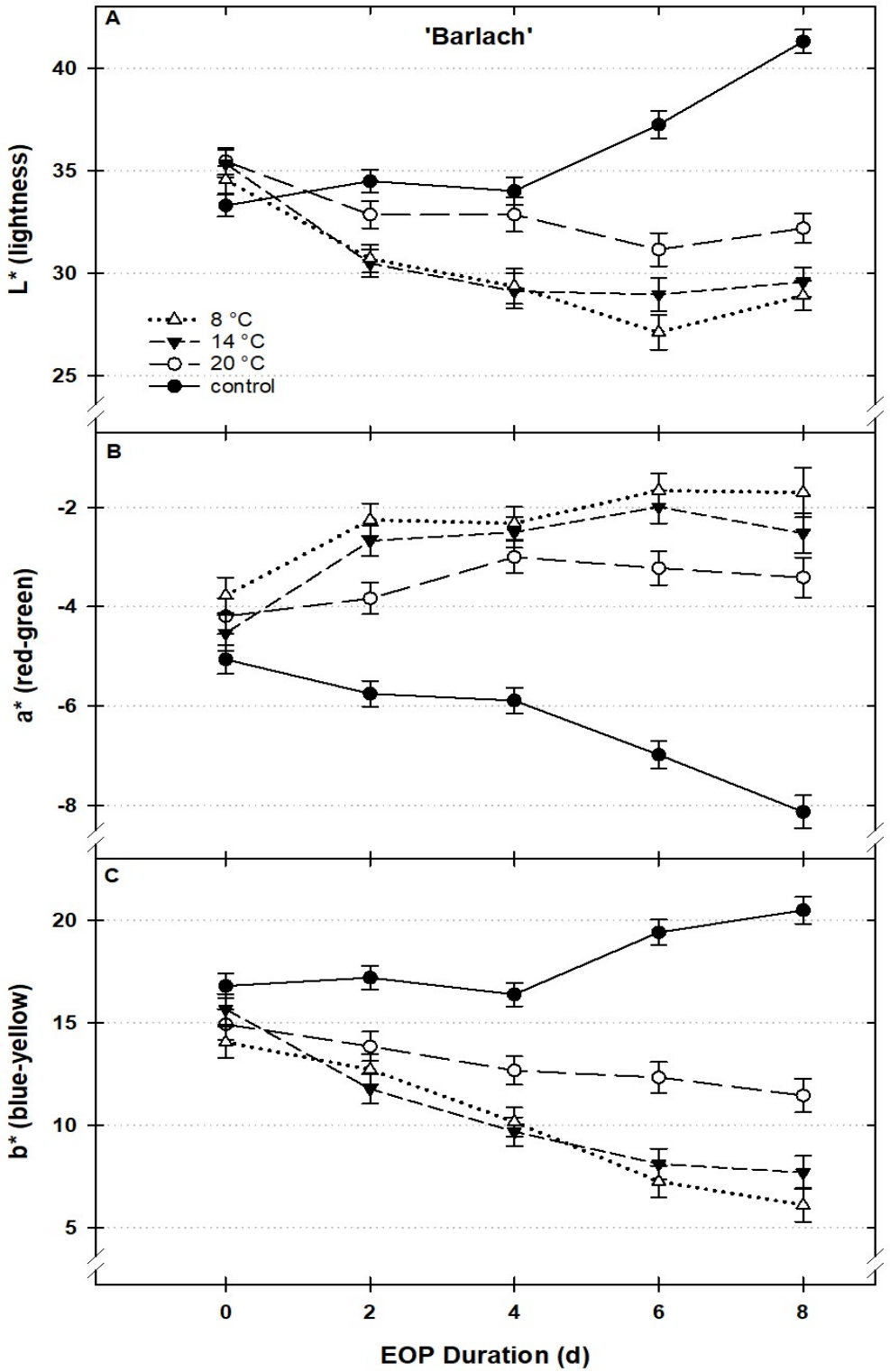


Figure III-1. Effect of end-of-production (EOP) cooling temperature (8, 14, and 20 °C) or no EOP cooling (control) by day on lightness (L*), a*, and b* of red leaf lettuce (*Lactuca sativa*) 'Barlach'. Error bars indicate the standard error of the mean.

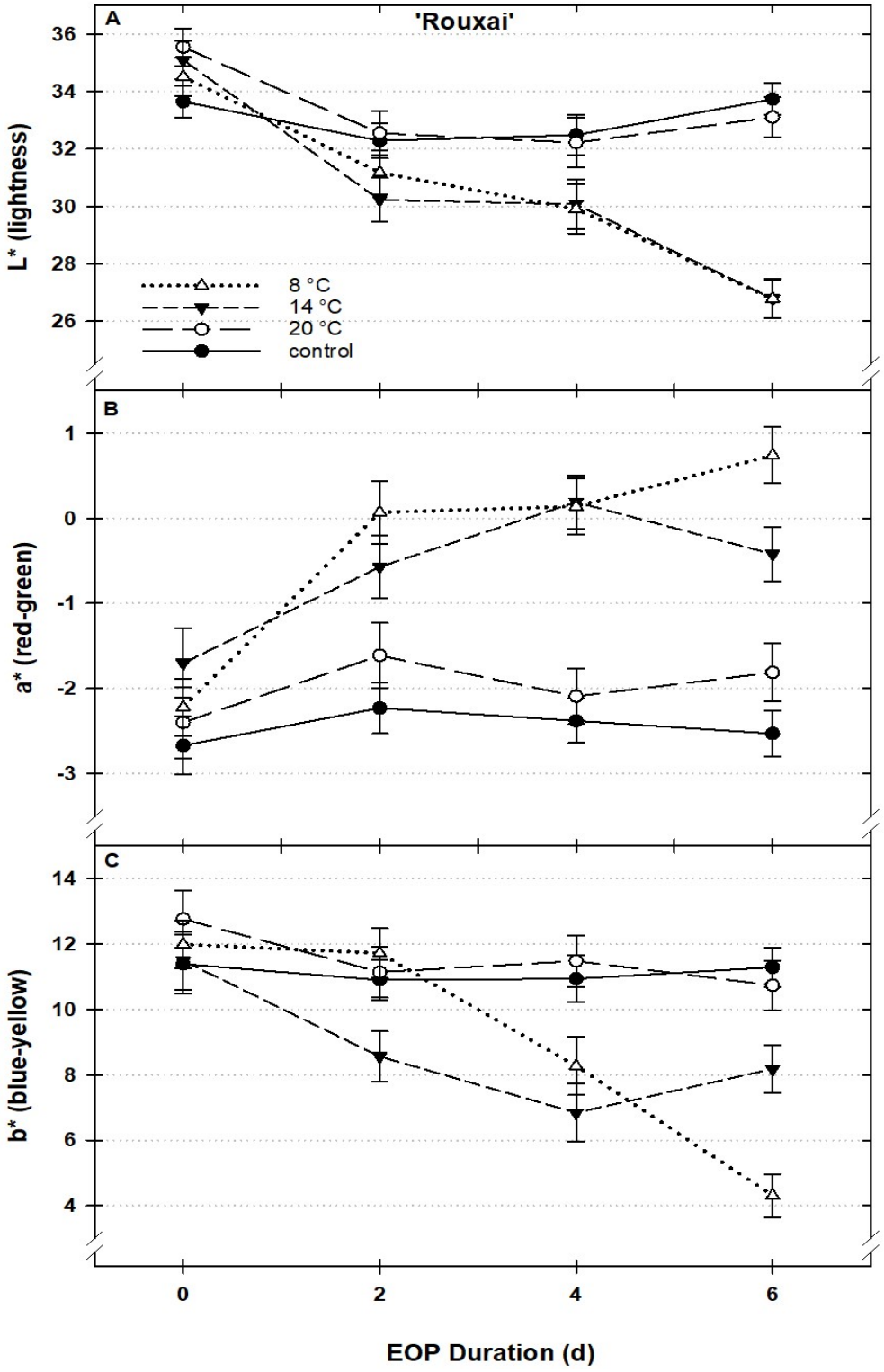


Figure III-2. Effect of end-of-production (EOP) cooling temperature (8, 14, and 20 °C) or no EOP cooling (control) by day on lightness (L*), a*, and b* of red leaf lettuce (*Lactuca sativa*) 'Rouxai'. Error bars indicate the standard error of the mean.

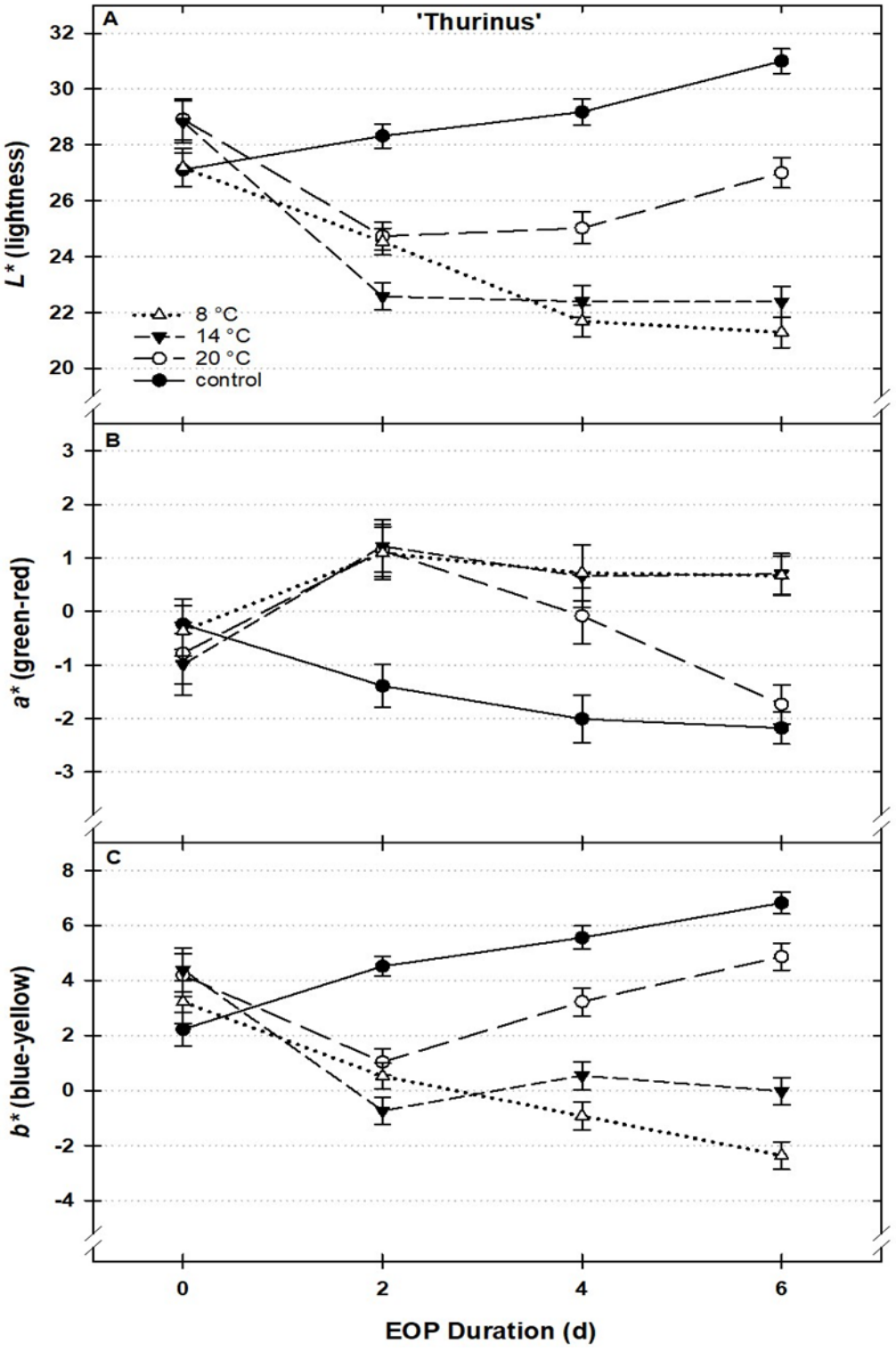


Figure III-3. Effect of end-of-production (EOP) cooling temperature (8, 14, and 20 °C) or no EOP cooling (control) by day on lightness (L*), a*, and b* of red leaf lettuce (*Lactuca sativa*)'Thurinus'. Error bars indicate the standard error of the mean.

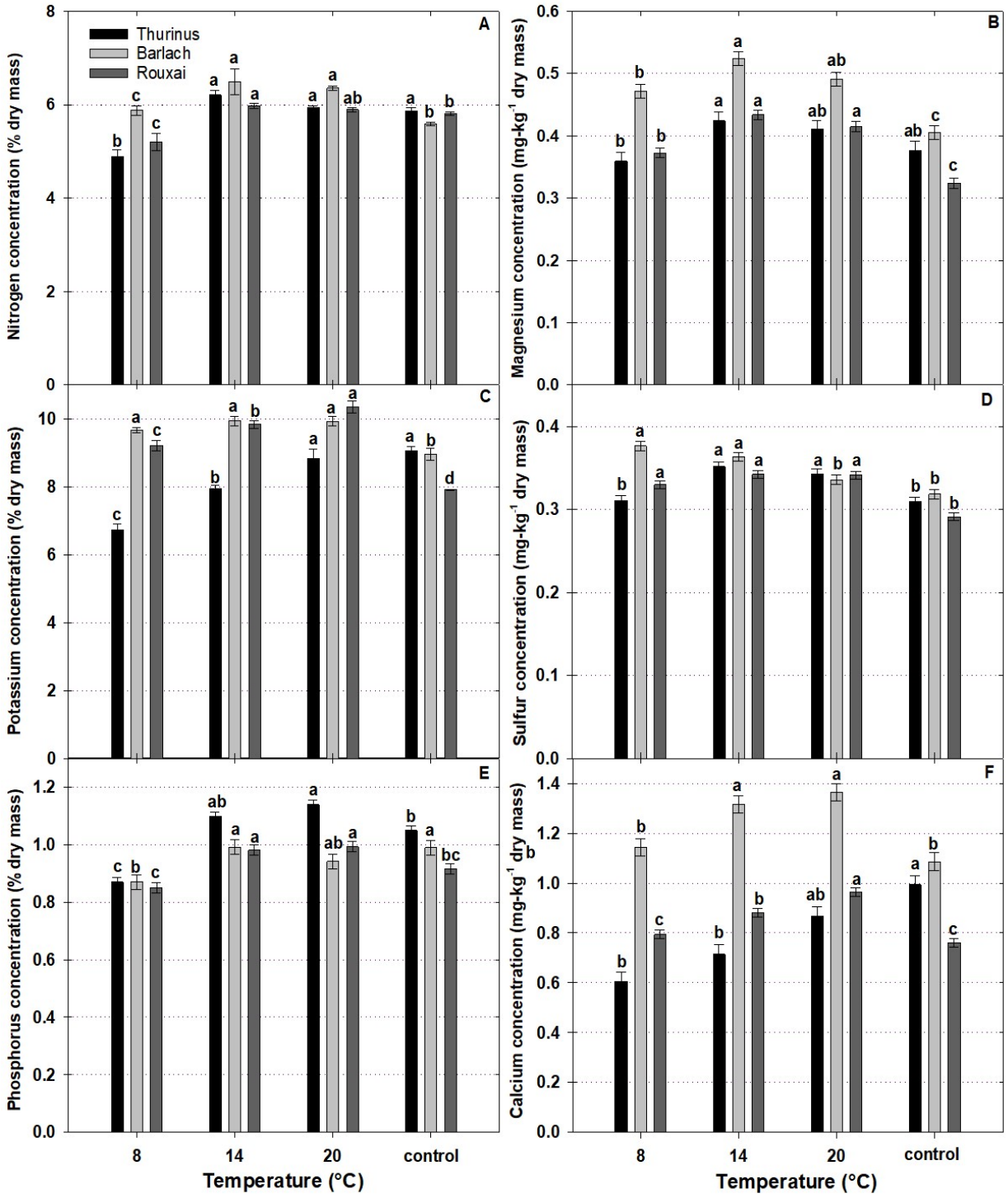


Figure III-4. Concentrations of macronutrients (nitrogen, potassium, phosphorus, calcium, magnesium, and sulfur) of red leaf lettuce (*lactuca* sativa) under end-of-production (EOP) cooling temperature (8, 14, and 20 °C) treatments or no EOP cooling (control). Means followed by different letters within each cultivar are significantly different based on Tukey's honestly significant difference test ($\alpha = 0.05$). Error bars indicate the standard error of the mean.

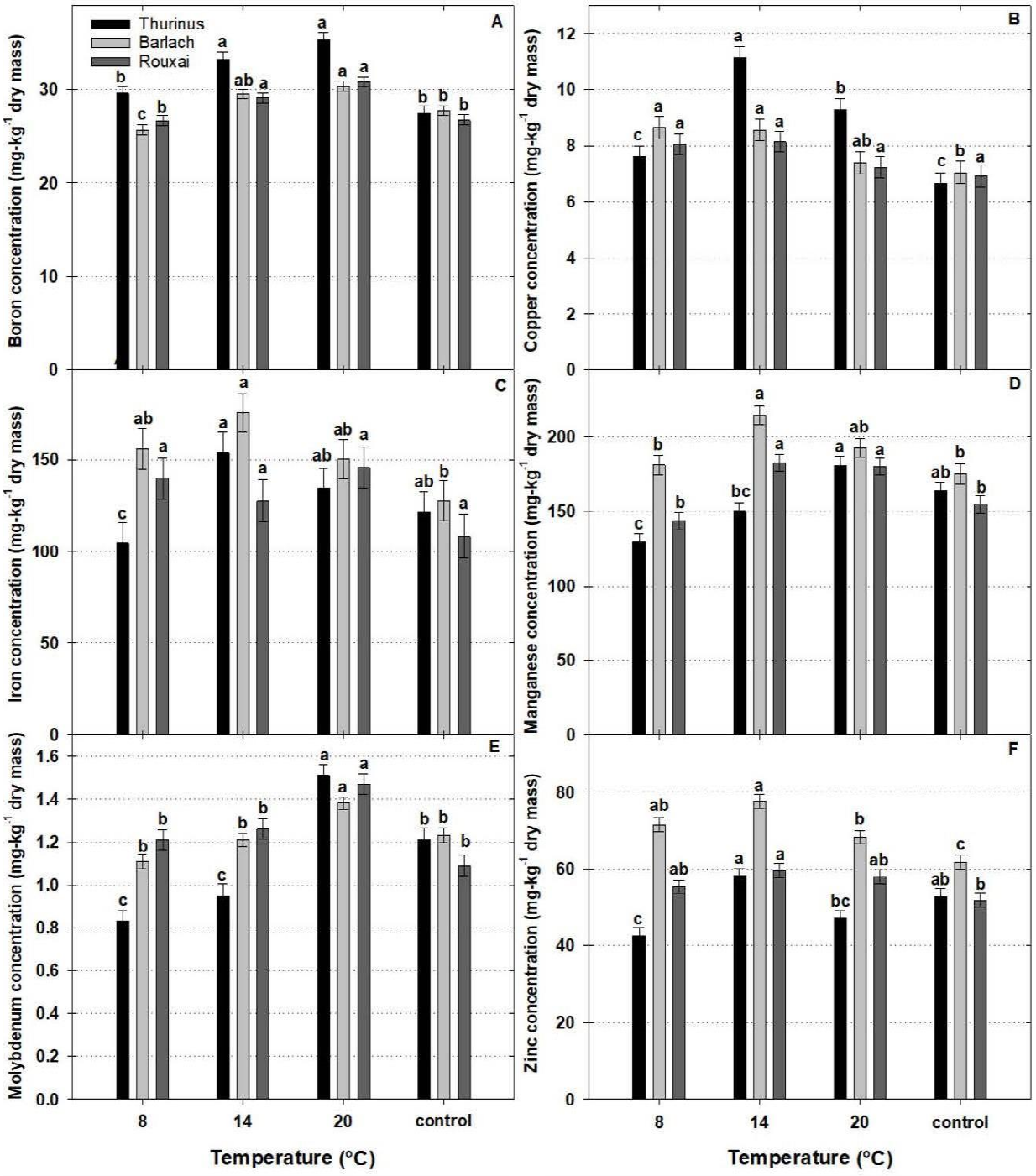


Figure III-5. Concentrations of micronutrients (iron, manganese, zinc, boron, copper, and molybdenum) in leaf tissues of 'Barlach', 'Rouxai', and 'Thurinus' red leaf lettuce (*Lactuca sativa*) under end-of-production (EOP) cooling temperature (8, 14, and 20 °C) treatments or no EOP cooling (control). Means followed by different letters within each cultivar are significantly different based on Tukey's honestly significant difference test ($\alpha = 0.05$). Error bars indicate the standard error of the mean.

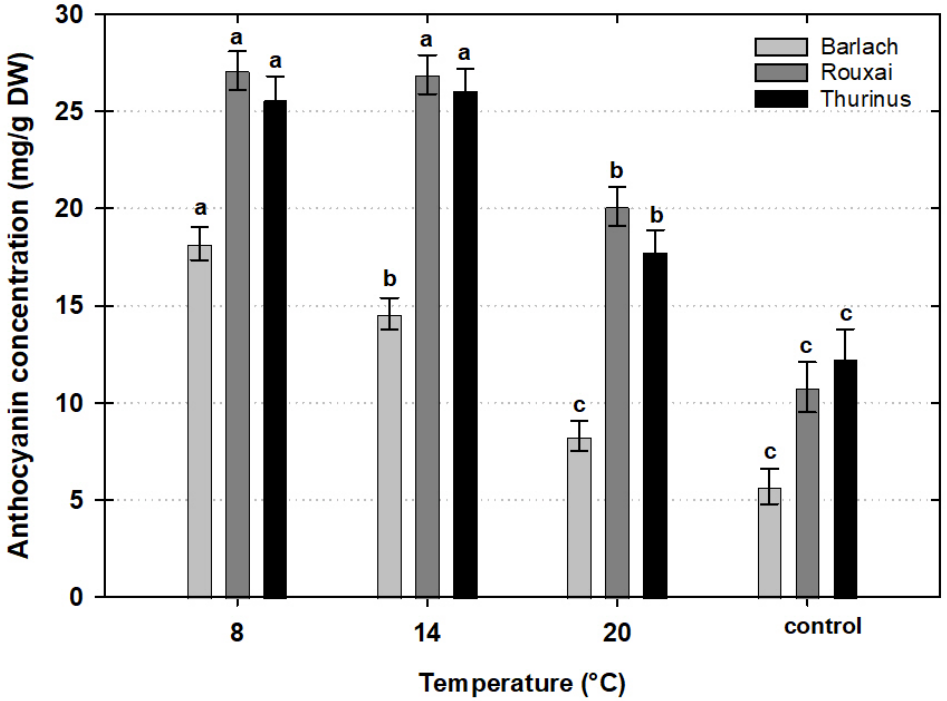


Figure III-6. Influence of EOP temperature (8, 14, and 20 °C) or no EOP cooling (control) on anthocyanin concentration of red leaf lettuce cultivars (*Lactuca sativa*) 'Barlach', 'Rouxai', and 'Thurinus'. Means followed by different letters within each cultivar are significantly different based on Tukey's honestly significant difference test ($\alpha = 0.05$). Error bars indicate the standard error of the mean.

# SECTION IV

# INFLUENCE OF END-OF-PRODUCTION COOLING ON FOLIAGE COLOR, MORPHOLOGY, AND YIELD OF MICROGREENS

Influence of Indoor End-of-Production Cooling on Foliage Color, Morphology, and Yield of Microgreens.

Devin S. Brewer and Roberto G. Lopez*

Department of Horticulture, 1066 Bogue Street, Michigan State University, East Lansing, MI 48824-1325, USA

***** Corresponding author. Tel.: +1 517-353-0342. E-mail address: rglopez@msu.edu (R.G. Lopez).

**Abstract**

Microgreens are a relatively new specialty crop, popularized by their assortment of colors and significantly higher concentrations of vitamins, minerals, and phenolic compounds than their mature counterparts. In controlled environments, parameters such as temperature can be manipulated and used to illicit stress responses, promoting the accumulation of phenolic compounds including anthocyanin and carotenoids, subsequently improving color and nutrition. Our objectives were to 1) quantify how end-of-production (EOP) cooling influences foliage color, growth, development, quality, and yield of microgreens. Beet 'Bulls Blood' (*Beta vulgaris*), mustard 'Miz America' (*Brassica juncea*), pac choi 'Red Pac' (*Brasica rapa var chinensis*), cabbage 'Red Jewel' (*Brassica oleracea*), and daikon radish 'Shunkyo' (*Raphanus sativus*) seeds were sown in a growth chamber with a mean daily temperature (MDT) set point of 20 °C and covered for 3 d. After the germination period, the air day/night temperature (18 /6 h) was set to 21/17 °C (MDT 20 °C) along with a constant relative humidity (RH) of 80%. A daily light integral (DLI) of 9.7 $mol \cdot m^{-2} \cdot d^{-1}$ was achieved with sole-source light-emitting diode fixtures that provided a photosynthetic photon flux density (PPFD) of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 18 $h \cdot d^{-1}$. During the last 3 days of production, microgreens were either left in the same conditions or transferred to growth chambers with a constant MDT of either 8 or 14 °C with all other parameters remaining unchanged. EOP cooling altered the shoot fresh mass (SFM) of 'Bulls Blood', 'Red Pak', and 'Miz America' and percentage shoot dry mass (SDM) of 'Bulls Blood', 'Miz America', and 'Red Jewel'. SFM of 'Bulls Blood', 'Red Pak', and 'Miz America' decreased 25, 19, and 22% after the 8 °C EOP treatment. Percentage SDM of SFM of 'Bulls Blood' increased by 20% after placed at 8 °C, while 'Miz America' and 'Red Jewel' increased by 21 and 10%, respectively, when exposed to 14 °C. On day 3, a* of 'Miz America' placed at 8

°C had increased 29 %, indicating a shift of leaf color from green to red, while a* of untreated plants decreased by 31%. Furthermore, placing 'Red Pak' and 'Bulls Blood' at 14 °C for 3 d resulted in b* decreasing from 2.84 to -0.77 and 2.24 to -1.3, respectively, indicating a subtle shift from yellow towards blue. Exposing 'Bulls Blood', 'Shunkyo', and 'Miz America' to 8 °C resulted in an additional 2, 2.5, and 2 days of shelf-life, respectively. Based on these results we recommend exposing microgreens to 3 d of EOP cooling at 8 or 14 °C to improve coloration and/or shelf life.

Keywords: anthocyanin, controlled-environment agriculture, microgreens, coloration, vertical farming, end-of-production

**Introduction**

Microgreens are seedlings that are harvested within 20 days of emergence and after the first true leaves have unfolded (Xiao et al., 2014). These young greens are used to enhance the color, texture, or flavor of meals (Zhang et al., 2021). They are a relatively new specialty crop category that is referred to as "an exotic genre of edible greens or sprouts" (Mir et al., 2017). This label is appropriate as microgreens are not a species of plant, but a term for immature vegetable greens of many species (Zhang et al., 2021) Microgreens have risen in popularity partly due to their status as a functional food, as they contain significantly higher concentrations of health promoting or disease preventing properties including vitamins, minerals, and phenolic compounds than their mature counterparts (Xiao et al., 2015; Lenzi et al., 2019; Zhang et al., 2021). However, microgreens suffer from a short post-harvest shelf-life due to their relatively high respiration rate (Chandra et al., 2012). Therefore, they must be kept between 1 to 5 °C after harvest, along with high humidity to mitigate respiration and achieve a shelf-life of up to 14 days

(Kou et al., 2013). These conditions exacerbate issues with microbial growth and subsequent decay (Zagory and Kader, 1988).

The striking foliage color of microgreens is associated with the accumulation of phenolic compounds such as anthocyanins and carotenoids (Li and Kubota, 2009; Samuoliené et al., 2012; Goins et al., 1998). Anthocyanins are phenolic compounds of particular interest due to their health promoting effects such as increased antioxidant activity, anti-cancer effects, and anti-neurodegeneration effects (Blesso, 2019; Wallace et al., 2016; Zhu, 2018). He et al. (2020) found that anthocyanin accumulation is strongly induced by low temperatures by comparing anthocyanin content of purple head Chinese cabbage seedlings (*Brassica rapa* L. ssp. *pekinensis*) grown at temperatures of 12 or 25 °C. This supports findings that anthocyanin content is negatively correlated with high temperatures and that, generally, the accumulation of anthocyanins occurs at lower temperatures (Lovdal et al., 2010). Likewise, carotenoids are a pigment that contributes to the vibrant yellow, orange, and red foliage and flower color of many plants (Edge et al., 1997). Furthermore, carotenoids also possess health-promoting effects such as reducing the risk of age-related macular degeneration and cataracts, as well as decreasing the risk of certain types of cancer (Kaulmann and Bohn, 2014). Since carotenoids work as an antioxidant reducing oxidative stress, it is possible that exposure to low temperature has the potential to increase accumulation (Oh et al., 2009).

End-of-production (EOP) treatments are a strategy made possible due to the advancement of CEA technology. EOP treatment refers to altering one or more environmental parameters such as light intensity, light quality, or temperature during the last days of the production cycle in order to illicit a response that enhances targeted characteristics such as coloration, nutrition, and phenolics accumulation (Gómez and Jiménez, 2020). Enhancing these characteristics is valuable

as consumer demand for nutrient and vitamin rich, as well as preference for more colorful vegetables, is well documented (Llorach et al., 2008).

Currently there is a lack of research on the effectiveness of EOP temperature cooling to increase anthocyanin, carotenoid, and mineral nutrient content of microgreens and the extent to which EOP cooling treatments affect production costs for growers. Therefore, the objectives of this study were 1) to identify the effect of EOP temperature treatment on anthocyanin and carotenoid concentrations, color, shelf-life, plant quality, and yield of microgreens. Our hypothesis was that EOP temperature treatments of 8 and 14 °C would improve shelf-life, coloration, and overall plant quality.

**Materials and Methods**

*Plant material and propagation conditions*

Twenty, 5, 5, 19 and 18 g of beet 'Bulls Blood' (*Beta vulgaris*), mustard 'Miz America' (*Brassica juncea*), pac choi 'Red Pac' (*Brasica rapa* var *chinensis*), cabbage 'Red Jewel' (*Brassica oleracea*), and daikon radish 'Shunkyo' (*Raphanus sativus*) seeds, respectively, were sown from 13 to 18 Aug. 2023 and 31 Aug. to 05 Sept. 2023. They were sown evenly onto rockwool pads (50.8 cm × 24.7 cm × 0.89 cm) in plastic trays (52 cm × 26 cm × 6 cm) that had been presoaked in deionized (DI) water with a pH of 4.4 to 4.5 adjusted using diluted (1:31) 95 to 98% sulfuric acid (J.Y. Baker, Inc.; Phillipsburg, NJ). Six trays of each species were then divided evenly among three walk-in growth chambers (Hotpack environmental room UWP 2614-3; SP Scientific). Seedlings were sub-irrigated with deionized water supplemented with water-soluble fertilizer providing (in $mg \cdot L^{-1}$): 125 N, 18 P, 138 K, 73 Ca, 47 Mg, 1.56 Fe, 0.52 Mn, 0.36 Zn, 0.21 B, 0.21 Cu, 35 S, and 0.01 Mo (12N–4P–16K RO Hydro FeED; JR Peters, Inc., Allentown, PA). The pH and electrical conductivity of the solution was measured using a

pH/EC probe and adjusted to 5.6 and 1.6 $mS \cdot cm^{-1}$, respectively, (HI 991301 pH/TDS/Temperature Monitor; Hanna Instruments, Smithfield, RI). Seedlings were then germinated under darkness for 3 d with environmental conditions consisting of 20 °C, 80% relative humidity (RH), and atmospheric carbon dioxide ($CO_2$) concentration.

*Growth chamber environmental conditions*

After the germination period, the air day/night temperature (18 /6 h) and mean daily temperature (MDT) were set to 21/17 °C (MDT 20 °C) along with a constant RH of 80%. These parameters were measured every 5 s by a resistance temperature detector (Platinum RTD RBBJL-GW05A-00-M 36B; SensorTec, Inc., Fort Wayne, IN) and logged by a C6 controller (Environmental Growth Chambers, Chagrin Falls, OH). A daily light integral (DLI) of 9.7 $mol \cdot m^{-2} \cdot d^{-1}$ was achieved with sole-source light-emitting diode (LED; GreenPower LED production module 3.0; Phillips, Amsterdam, Netherlands) fixtures mounted 24 cm above the crop canopy that provided a PPFD of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 18 $h \cdot d^{-1}$. Leaf temperature and the *TPFD* were monitored using an infrared thermocouple (OS36-01-T-80F; Omega Engineering, INC. Norwalk, CT), and quantum sensor (LI-190R; LI-COR Biosciences, Lincoln, NE), respectively, every 15 seconds and means logged each hour by a CR-1000 datalogger (Campbell Scientific, Logan, UT). The $CO_2$ concentration was monitored with a $CO_2$ sensor (GM86P; Vaisala, Helsinki, Finland), and logged by a C6 Controller (Environmental Growth Chambers) every 5 seconds. On day 12, 9, 9, 9, and 7, two trays of beet, mustard, pac choi, cabbage and daikon radish, respectively, were placed in growth chambers with the same environmental parameters but mean daily air temperature setpoints of 8, 14, or 20 °C for 3 d of for EOP cooling.

*Growth data collection and analysis*

A colorimeter (Chroma Meter CR-400; Konica Minolta Sensing, Inc., Chiyoda, Tokyo) was used to take 10 randomly selected readings of the most recent fully expand leaf from each tray prior to EOP cooling as well as each day of the EOP treatment. Relative chlorophyll concentration (RCC) of the most recent fully expanded leaf from 10 randomly selected seedlings in each tray was estimated using a SPAD chlorophyll meter (MC-100 Chlorophyll Meter; Apogee Instruments, Logan, UT). Each species was harvested on 28 Aug. 2023 and 15 Sept. 2023. Ten samples, consisting of 10 randomly selected seedlings per species and treatment, were used to determine the shoot fresh mass (SFM), hypocotyl length (cm), and leaf area ($cm^2$), measured using a leaf area meter (LI-300; LI-COR Biosciences). After recording the SFM of each sample, they were placed in a drying oven at 75 °C for a minimum of 3 d before the shoot dry mass (DM) was recorded. SFM and SDM were then used to calculate the percentage SDM (SDM/SFM × 100).

*Post-harvest shelf-life*

The remaining plant material of each species and treatment was harvested and distributed into respective transparent 4 oz. clamshells (Genpak, Charlotte NC) to be stored in a refrigerator with a setpoint of 4 °C. Each clamshell was removed and scored daily according to the overall visual quality (OVQ) (Kader et al., 1973) guidelines by two lab members and scores were averaged, this process was repeated for 10 days.

**Results**

*Shoot fresh and dry mass*

EOP cooling decreased the SFM of ‘Bulls Blood’, ‘Red Pak’, and ‘Miz America’ and percent SDM of ‘Bulls Blood’ and ‘Miz America’ and ‘Red Jewel’. For example, SFM of ‘Bulls

Blood' decreased by 15 (0.15 g) and 25% (0.25 g) respectively, after the 14 and 8 °C EOP treatments. Percentage dry mass of 'Bulls Blood' increased by 28 and 20% at 8 and 14 °C compared to 20 °C. The SFM of 'Red Pak' was 19% (0.25 g) lower at 8 °C compared to noncooled plants. Compared to the control, the SFM of 'Miz America' was 23 (0.33 g) and 22% (0.32 g) lower at 8 and 14 °C, respectively (Table IV-3). Additionally, percentage SDM of 'Miz America' increased by 21 and 19% at 14 and 8 °C. Lastly, there were no significant changes in SFM or SDM for 'Red Jewel' or 'Shunkyo'. Across all the microgreen species, SDM was similar among treatments (Table IV-3).

*Morphology*

Hypocotyl length of 'Bulls Blood', 'Miz America', 'Red Jewel', and 'Shunkyo' was reduced under EOP cooling. Hypocotyl length of 'Miz America' was 25% (1.6 cm) shorter at 8 °C compared to seedlings at 20 °C. Likewise, hypocotyl length of 'Red Jewel' was greatest at 20 °C and was 29 (1.37 cm) and 17% (0.82 cm) greater than plants harvested at 8 and 14 °C, respectively. 'Shunkyo' hypocotyl length at 20 °C was 17 (1.11 cm) and was 15% (0.96 cm) greater than plants exposed to EOP temperatures of 8 and 14 °C. Similarly, hypocotyl length of 'Bulls Blood' was 16 % (0.52 cm) shorter at 8 °C compared to plants at 20 °C (Table IV-3).

Leaf area of 'Bulls Blood' and 'Miz America' generally decreased with temperature. Leaf area of 'Bulls Blood' at 14 °C was 34% (0.31 $cm^2$) greater compared to plants at 8 °C. However, leaf area of 'Miz America' was 33% (0.7 $cm^2$) larger at 20 °C compared to 8 °C. Leaf area of 'Red Pac', 'Red Jewel', and 'Shunkyo' was not altered by any EOP treatment (Table IV-3).

*Pigmentation*

Coloration was enhanced by EOP cooling in species with the genetic disposition for red and purple color; however, color was not altered in plants lacking that genetic disposition. On day 3, a* of 'Miz America' placed at 8 °C had increased 29%, indicating a shift from green to red, while a* of plants at 20 °C was 31% lower. Placing 'Red Pak' and 'Bulls Blood' at 14 °C for 3 d resulted in b* decreasing from 2.84 to -0.77 and 2.24 to -1.3, respectively, indicating a color shift from yellow to blue (Fig.IV-3). At 20 °C b* of 'Red Pak' and 'Bulls Blood' increased from 2.17 to 2.84 and 2.59 to 3.36, respectively.

*Post harvest*

EOP cooling extended the post-harvest shelf-life of 'Bulls Blood', 'Shunkyo', and 'Miz America'. 'Bulls Blood' exposed to 8 °C maintained an acceptable average OVQ rating (5 or greater) for 9 days compared to 7 days for plants that were not exposed to EOP cooling. Regardless of EOP cooling temperature, the shelf-life of 'Daikon Radish was acceptable for 8.5 days compared to 6 days for plants that did not undergo EOP cooling. 'Miz America' that was not exposed to EOP cooling was acceptable for 8 days, while those that received either EOP treatment were acceptable for 10 days (Table IV-3).

**Discussion**

It is well documented that SFM decreases when plants are exposed to suboptimal temperatures, as was the case with 'Bulls Blood', 'Miz America', and 'Red Pac' (Tarr et al., 2023; Marsh and Albright, 1991). The greatest SFM of each of these species occurred when the MDT remained at 20 °C; however, SFM of 'Red Jewel' and 'Shunkyo' was not affected by EOP cooling. This may indicate that the optimal temperature of 'Red Jewel' and 'Shunkyo' is lower, or that EOP duration was not long enough to affect the SFM. SDM of each species was not

altered by EOP cooling, however the percent SDM of 'Bulls Blood' increased by 29% at 8 °C, while the percent SDM of 'Miz America' increased by 21% at 14 °C, compared to plants that remained at 20 °C. Interestingly, 'Red Jewel' displayed a 10% increase in percent SDM at 14 °C compared to plants exposed to EOP temperatures of either 8 or 20. Increased percent SDM at cool temperatures has been documented in the past (Steer, 1982; Gent, 2016).

Temperature primarily influences the rate of biochemical reactions within a plant and the time required for leaves, stems, roots, and flowers to develop (Erwin and Heins, 1995). Hypocotyl length is an important growth attribute for commercial microgreen producers as elongated hypocotyls (e.g., ≥5 cm) facilitate mechanical harvesting (Baumgardt et al., 2020). The role of temperature in hypocotyl development was apparent as EOP cooling linearly reduced hypocotyl length of 'Bulls Blood', 'Shunkyo', 'Miz America', and 'Red Jewel' as temperature decreased. Research by Gray et al. (1998) notes that Arabidopsis (*Arabidopsis thaliana*) seedlings exhibited dramatic hypocotyl elongation when grown at 29 °C compared to 20 °C. They postulated that higher temperatures increased auxin levels which in turn promoted cell elongation. In addition to the reduction of hypocotyl length, leaf area of 'Bulls Blood' and 'Red Pac' were also reduced at 8 °C. However, 'Bulls Blood' possessed the greatest leaf area when placed at 14 °C, suggesting that it may have a lower optimal temperature than the other species examined. This is congruent with past research that has indicated that leaf area of lettuce (*Lactuca sativa*) increased when grown at temperatures of 20 °C or higher compared to 10 °C (Gent, 2016).

The popularity of microgreens is due in part to their vivid colors, and among quality attributes, appearance is partly responsible for customers' initial attraction, as well as likelihood of first-time purchase (Xiao et al., 2015; Kyriacou et al., 2016). Therefore, color is an important

quality parameter when producing microgreens. In the current study, EOP cooling increased the a* of 'Miz America' over the course of 3d, with greater increases at 8 °C than at 14 °C. This increase in a* signals a shift away from green towards red leaf color. Furthermore, EOP cooling resulted in b* of 'Bulls Blood' and 'Red Pac' decreasing, with the lowest b* reading occurring at 14 °C. This reduction in b* indicates a shift from yellow to blue leaf color. Red and purple leaf and flower color is typically associated with anthocyanin accumulation, while orange and yellow is associated with carotenoid accumulation (Li and Kubota, 2009; Samuoliené et al., 2012; Edge et al., 1997). Previous research suggests that anthocyanin content is negatively correlated with high temperatures and generally, sub-optimal or large fluctuations in temperature stimulate their accumulation (Lovdal et al., 2010). Conversely, carotenoid accumulation generally increases with rising temperature and heat stress or wounding decreases its accumulation (Juneja et al., 2013).

A key challenge for microgreen producers is rapid senescence, which translates into a short shelf-life of 3-5 days at room temperature (Chandra et al., 2012). This rapid deterioration in post-harvest quality is due to several factors including high respiration rates, high surface area to volume ratio, delicate leaves prone to wilt, and tissue damage (Berba and Uchanski, 2012; Kou et al., 2013). Storage of radish (*Raphanus sativus*) microgreens between 1 to 5 °C has been reported to extend the shelf-life to 7 to 10 days (Xiao et al., 2014). Furthermore, Kou et al. (2014) discovered that applying 10 mM of calcium chloride prior to harvest delayed decline of overall quality and extended shelf-life of broccoli (*Brassica oleracea* L. var. *italica*) microgreens stored at 5 °C from 7 to 10 days to 14 to 21 days. In the current study, EOP cooling enhanced shelf-life of 'Bulls Blood', 'Shunkyo', and 'Miz America' by 2, 2.5, and 2 days, respectively.

In conclusion, EOP cooling reduced SFM, increased percentage SDM, reduced hypocotyl length, improved coloration of species exhibiting red/purple foliage, and improved shelf-life. We recommend exposing microgreens to 3 d of EOP cooling at 8 or 14 °C if improved coloration and/or shelf life is desired, but foregoing EOP cooling if the primary target is yield.

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## APPENDIX C: SECTION IV FIGURES AND TABLES

Table IV-1. Mean (± SD) day and night air and canopy temperatures; carbon dioxide ($CO_2$) concentrations; total photon flux density (TPFD); and vapor-pressure deficit (VPD) during 12 days of indoor sub-irrigated hydroponic production of beet 'Bulls Blood' (*Beta vulgaris*), mustard 'Miz America' (*Brassica juncea*), pac choi 'Red Pac' (*Brasica rapa var chinensis*), cabbage 'Red Jewel' (*Brassica oleracea*), and daikon radish 'Shunkyo' (*Raphanus sativus*) microgreens.

| | Temperature (°C) | | | $CO_2$ | *TPFD* | VPD |
|---|---|---|---|---|---|---|
| Replication | Air day | Air night | Canopy | ($\mu mol \cdot mol^{-1}$) | ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | (kPa) |
| 1 | 20.9 ± 0.2 | 17.0 ± 0.1 | 21.4 ± 1.9 | 426.3 ± 18.6 | 148.9 ± 5.1 | 0.50 ± 0.14 |
| | 21.0 ± 0.4 | 17.2 ± 0.2 | 21.4 ± 1.8 | 428.7 ± 24.8 | 152.1 ± 4.3 | 0.51 ± 0.10 |
| | 21.3 ± 0.3 | 17.4 ± 0.4 | 21.6 ± 2.4 | 432.0 ± 21.5 | 149.9 ± 5.6 | 0.53 ± 0.08 |
| 2 | 21.2 ± 0.3 | 17.2 ± 0.2 | 21.3 ± 1.7 | 431.1 ± 24.0 | 153.4 ± 6.7 | 0.55 ± 0.13 |
| | 21.0 ± 0.1 | 16.9 ± 0.3 | 21.5 ± 2.3 | 421.9 ± 18.1 | 154.3 ± 6.2 | 0.52 ± 0.15 |
| | 21.4 ± 0.4 | 17.5 ± 0.5 | 21.7 ± 2.6 | 436.7 ± 25.4 | 155.0 ± 6.9 | 0.60 ± 0.09 |

Table IV-2. Mean (± SD) end-of-production (EOP) air and canopy temperatures; carbon dioxide ($CO_2$) concentration; photosynthetic photon flux density (PPFD); and vapor pressure deficit (VPD) of indoor sub-irrigated hydroponic production beet 'Bulls Blood' (*Beta vulgaris*), mustard 'Miz America' (*Brassica juncea*), pac choi 'Red Pac' (*Brasica rapa var chinensis*), cabbage 'Red Jewel' (*Brassica oleracea*), and daikon radish 'Shunkyo' (*Raphanus sativus*) microgreens.

| Replication | EOP temperature (°C) | Actual temperature (°C) | | $CO_2$ ($\mu mol \cdot mol^{-1}$) | *PPFD* ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | VPD (kPa) |
|---|---|---|---|---|---|---|
| | | Air | Canopy | | | |
| 1 | 8 | 8.3 ± 1.2 | 21.4 ± 1.9 | 426.3 ± 28.6 | 148.9 ± 5.1 | 0.27 ± 0.11 |
| | 14 | 14.4 ± 0.7 | 21.4 ± 1.8 | 428.7 ± 24.8 | 152.1 ± 4.3 | 0.38 ± 0.08 |
| | 20 | 20.5 ± 0.6 | 21.6 ± 2.4 | 432.0 ± 21.5 | 149.9 ± 5.6 | 0.60 ± 0.14 |
| 2 | 8 | 8.1 ± 0.3 | 21.3 ± 1.7 | 431.1 ± 23.0 | 153.4 ± 6.7 | 0.26 ± 0.13 |
| | 14 | 14.0 ± 0.3 | 21.5 ± 2.3 | 421.9 ± 28.1 | 154.3 ± 6.2 | 0.40 ± 0.13 |
| | 20 | 20.7 ± 1.1 | 21.7 ± 2.6 | 436.7 ± 25.4 | 155.0 ± 6.9 | 0.62 ± 0.16 |

Table IV-3. Influence of end-of-production (EOP) cooling temperatures (8 and 14 °C) or no EOP cooling (20°C) on leaf area (cm2); hypocotyl length (cm); shoot fresh and dry mass; and percentage dry mass of beet 'Bulls Blood' (Beta vulgaris), mustard 'Miz America' (Brassica juncea), pac choi 'Red Pac' (Brasica rapa var chinensis), cabbage 'Red Jewel' (Brassica oleracea), and daikon radish 'Shunkyo' (Raphanus sativus) microgreens. Data represents the mean of two replications with 10 samples per species. Different letters within columns signify significantly different means according to Tukey's honestly significant difference (HSD) test ($P < 0.05$).

| Treatment (°C) | Leaf area ($cm^2$) | Hypocotyl length (cm) | Fresh mass (g) | Dry mass (g) | Percentage dry mass | Shelf life (d) |
|---|---|---|---|---|---|---|
| **'Bulls Blood'** | | | | | | |
| 8 | 0.84 b | 2.66 b | 0.77 b | 0.13 a | 13.9 a | 9 a |
| 14 | 1.21 a | 3.09 a | 0.87 b | 0.10 a | 12.4 ab | 7 b |
| 20 | 1.15 ab | 3.18 a | 1.02 a | 0.09 a | 9.9 b | 7 b |
| **'Miz America'** | | | | | | |
| 8 | 1.44 b | 4.97 b | 1.14 b | 0.07 a | 7.7 a | 10 a |
| 14 | 1.86 a | 6.23 a | 1.15 b | 0.07 a | 7.8 a | 10 a |
| 20 | 2.14 a | 6.59 a | 1.47 a | 0.08 a | 6.2 b | 8 b |
| **'Red Pac'** | | | | | | |
| 8 | 1.98 a | 3.23 a | 1.04 b | 0.07 a | 10.4 a | 10 a |
| 14 | 2.32 a | 3.12 a | 1.19 ab | 0.08 a | 9.6 a | 9.5 a |
| 20 | 2.22 a | 3.40 a | 1.29 a | 0.07 a | 8.9 a | 10 a |
| **'Red Jewel'** | | | | | | |
| 8 | 3.40 a | 3.37 c | 2.16 a | 0.13 a | 7.17 b | 8 a |
| 14 | 3.85 a | 3.92 b | 2.05 a | 0.13 a | 7.99 a | 8 a |
| 20 | 3.76 a | 4.74 a | 2.18 a | 0.12 a | 7.19 b | 7.5 a |
| **'Shunkyo'** | | | | | | |
| 8 | 4.92 a | 5.31 b | 2.88 a | 0.18 a | 9.3 a | 8.5 a |
| 14 | 4.22 a | 5.46 b | 3.13 a | 0.19 a | 8.9 a | 8.5 a |
| 20 | 4.51 a | 6.42 a | 3.34 a | 0.18 a | 8.0 a | 6 b |

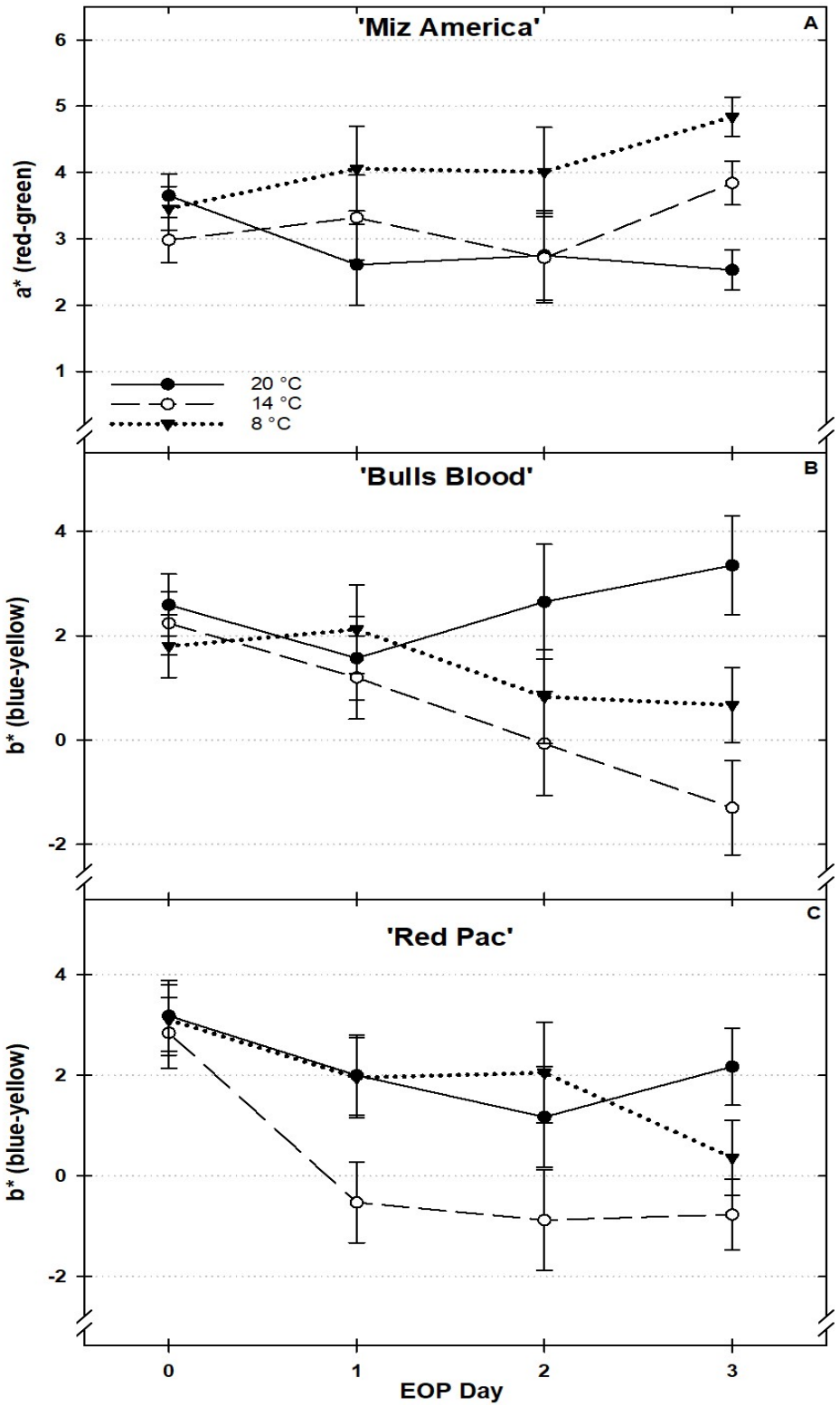


Figure IV-1. Effect of end-of-production (EOP) cooling temperature (8 and 14 °C) or no EOP cooling (20 °C) by day on a* of mustard 'Miz America' (*Brassica juncea*), and b* of beet 'Bulls Blood' (*Beta vulgaris*) and pac choi 'Red Pac' (*Brasica rapa var chinensis*) microgreens. Error bars indicate the standard error of the mean.