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Effects of Various High Tunnel Coverings on Color and Phenolic Compounds of Red and Green Leaf Lettuce (*Lactuca Sativa*)

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Abstract

High tunnel (HT) coverings can reduce light intensity and spectral quality, thus negatively affecting pigmentation and accumulation of several important phytochemicals in lettuce. The objectives of this study were to evaluate the effect of six HT coverings and two growing seasons (spring vs fall) on the red 'New Red Fire' and green 'Two Star' lettuce at harvest and after 5 days of storage with respect to leaf color and phenolic compound accumulation. Total phenolic content, anthocyanin accumulation, major individual phenolic acids and flavonoid compounds were measured. Chlorogenic and chicoric acid were the most prevalent phenolic compounds in both green and red lettuce. The phenolic compound accumulation in the red lettuce was significantly greater in the spring than in the fall for all measured compounds other than caffeic acid and anthocyanin. In the spring, the flavonoid accumulation was higher under the movable covering for both red and green lettuce. Red lettuce grown under clear poly (clear) and standard poly removed 2 to 3 weeks prior to initial harvest (movable) had darker leaves during both seasons (P <.001), as well as greater anthocyanin accumulation compared to the shade poly (shade) during the spring. During that season, the isoquercetin concentration of the red lettuce was 72% higher under the movable coverings compared to the shade covering (P <0.01). The effect of covering and season on phenolic compound accumulation after 5 days of storage was inconsistent. This study indicates that secondary metabolites can be manipulated due to solar light with the use of various HT coverings.

Keywords: Phytochemicals, Spectral Quality, Light Intensity, UPLC-MS

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Introduction

Phenolic compounds are available to humans through plant consumption and play a diverse role in both plants and humans. As antioxidants in humans, phenolic compounds have shown anti-inflammatory and antitumor activity in the prevention of coronary heart disease and cancer [1-3]. In plants, phenolic compounds also act as antioxidants [4,5], provide pigmentation [6-9], and have been shown to reduce electrolyte leakage in response to membrane oxidation [10].

Lettuce (*Lactuca sativa*), especially red leaf lettuce, is a good source of phenolic acids and flavonoids, including quercetin glucosides, anthocyanin conjugates, and caffeic acid derivatives [11-16]. More specifically, phenolic acids such as chicoric acid (dicaffeoyltartaric acid), chlorogenic acid (5-caffeoylquinic acid), caffeic acid, and ferulic acid have shown antidiabetic effects, prevented the formation of mutagenic compounds, and can inhibit lipid peroxidation [17-19]. Quercetin derivatives in lettuce include quercetin 3-O-(6"-O-malonyl)-glucoside, isoquercetin (quercetin-3-O-glucoside), rutin (quercetin-3-O-rutinoside), and quercetin 3-O-glucoronide, which have shown anti-inflammatory effects [20]. The flavonols, isoquercetin, and rutin, contain a catechol group on the B ring which makes them highly active antioxidants [21].

Plants exposed to various environmental stresses during production will generate reactive oxygen species (ROS). Non-enzymatic antioxidants, such as phenolic compounds, are upregulated with increased stress to scavenge ROS and prevent further oxidative stress. Thus, it has been shown that phenylalanine ammonia-lyase (PAL), a key gateway enzyme of the phenylpropanoid pathway, is typically upregulated in response to environmental and biotic stresses (light, temperature, pest damage) [22-24]. Ultraviolet light (UV-B) is known to increase PAL activity in lettuce and cucumber [25,26]. Krizek DT, et al. (1998) found that ambient UV-B increased the PAL content by 27-83% in lettuce, which resulted in increased accumulations of anthocyanins and other flavonoids [26].

HT and other forms of protected and semi-protected environment growing systems have been shown to increase the crop productivity of lettuce by minimizing environmental stresses and by providing growing seasons extension [27-30]. The HT system has been shown to alter the spectral quality, light intensity, plant growth, and antioxidant compounds through overhead polyethyelene (poly) films and shade cloth [31-36]. Stress from light intensity, as measured by photosynthetic active radiation (PAR), and spectral quality, as measured by UV radiation, has been shown to decrease under HT polyethylene (poly) film [37] and shade cloth [32, 38-40].

When grown in open field conditions with higher light intensity, both green and red lettuce cultivars accumulate higher phenolic compounds compared to those grown in HT with standard poly [33], and clear poly [41]. Several lettuce studies have shown phenolic compound accumulation inversely related to biomass accumulation [41-43]. Similarly, Li T, et al. (2017) found that the use of a 50% black shade cloth increased the fresh weight of red and green leaf lettuce, but reduced phenolic compounds compared to the open field. Studies also show that the phenolic accumulation in lettuce is especially affected 2 to 4 weeks prior to harvest [44, 45]. The concept of subjecting crops to full spectrum light prior to harvest is feasible with a movable tunnel, where the plant is established before the tunnel is moved [46]. Currently, there is a lack of published studies related to movable HTs and the impact that their use has on crop physiology and yield.

Phenolic compound accumulation may be impacted by season due to reduced UV radiation, PAR, and temperature in the fall compared to the spring [47,48]. During a study in Spain from February to May, Marin A, et al. (2015) found that temperature and radiation positively correlated with phenolic acid and flavonoid accumulation in open-field red lettuce [49]. However, Marin A, et al. (2015) found that red lettuce pigment was enhanced with wide temperature ranges, and was a deeper red when the plants experienced temperatures below 7°C.

Storage is another factor that is known to impact phenolic compound accumulation, due to continued phenolic metabolism after harvest. In addition, the physiological maturity at the time of harvest and pre-harvest conditions have shown to influence lettuce phenolic compound concentrations during storage [11,50]. Higher PAR and surface temperature pre-harvest, has shown to increase respiration rates and moisture loss throughout storage, resulting in decreased phytochemical compounds [51].

Lettuce is one of the most common crops grown in HT across the U.S. [52] and is an important source of nutrients in the American diet [53]. HTs have been increasingly utilized in leafy vegetable production across the U.S. due to higher yield and quality at harvest and throughout storage. Thus, it is critical to investigate how different HT coverings affect visual and nutrient quality in both the spring and fall season at harvest and during postharvest storage. The objectives of this study were to evaluate the effects of various types of HT coverings, growing season (spring vs fall), and storage day (0 and 5) on red and green lettuce, with respect to leaf color, TPC, anthocyanins and accumulation of individual phenolic acids and flavonoids in leaf tissue.

Materials and Methods

Experimental Design

Red leaf lettuce (RL) and green leaf lettuce (GL) trials occurred from fall 2017 to spring 2019 at the Kansas State University Olathe Horticulture Research and Extension Center (OHREC), located in Olathe, Kansas. 'New Red Fire' and 'Two Star' were used for the RL and GL trials, respectively. The trials were conducted in four, "caterpillar" HTs (39.6 m long x 3.7 m wide x 2.1 m high) that ran from east to west in a split-plot randomized complete block design. The four individual HT replications each served as a block as described by Gude KM, et al. (2022) [54].

The six main plot coverings were randomly assigned to 6.1 m long plots within each HT and an additional 2.1 m buffer area was implemented at the end of each HT as well as 1.5m at either end of each plot to minimize interplot interference (Table 1). Two parallel beds ran lengthwise in each HT (39.6 m long x 0.61 m wide). The RL and GL alternated between the parallel north and south beds within each tunnel and served as the sub-plots.

During both the spring and fall seasons, the poly over the movable covering was closed at night when outdoor temperatures fell below 0°C. Additionally, floating row covers (26 g/m) were added to all the beds at night when outdoor temperatures fell below -6°C. These steps were put in place to mitigate freezing damage to the crop, but careful steps were taken to minimize bias on light exposure.

Lettuce Trials for Nutritional Analysis

During the spring trials, lettuce was sowed into 72-cell propagation trays with potting mix (Fafard 3B; Conrad Fafard, Agawam, MA, USA) on Feb. 14, 2018, and 19, 2019 and transplanted to the HT March 19, 2018, and Apr. 1, 2019. Shade cloth was incorporated over the shade covering the same time the poly was removed from the movable covering, or three weeks prior to harvest (Apr. 13, 2018, and Apr. 15, 2019). Lettuce was harvested at commercial size, on May 3, 2018, and May 10, 2019.

During the fall trials, lettuce was sowed Sept. 7, 2017, and 19,

Table 1: Polyethylene (poly) coverings in the high tunnel system, corresponding code, ultra-violet (UV) and photosynthetic active radiation (PAR) transmission, and product descriptions.

Covering	Code	UV-A/B transmission, %	Spring/fall PAR transmission, %	Product and Manufacturer
Standard poly	Standard	16/16[a]	88/83[b]	K-50 poly; Klerk's Plastic Product Manufacturing, Inc., Richburg, SC, USA
Standard poly removed 2 to 3 wk prior to harvest	Movable[c]	100/100	100/100	K-50 poly; Klerk's Plastic Product Manufacturing, Inc., Richburg, SC, USA
Diffuse poly	Diffuse	8/7	65/76	Luminance; Visqueen Building Products, London, UK
Clear poly	Clear	61/65	79/85	6-mil Clear Plastic Sheeting; Lowes, Mooresville, NC, USA
UV-A/B Block poly	Block	24/6	77/84	Dura Film Super 4; BWI Companies, Inc., Nash, TX, USA
Standard poly + 55% black shade cloth	Shade	7/5	32/39	Sunblocker Knitted Shade; FarmTek, Dyersville, Iowa, USA

[a]Measured with the ILT5000 (International light Tech., Peabody, MA, USA) on cloudless days [54].

[b]Measured with the CID-340 (CID Bio Science, Inc., Camas, WA, USA) on cloudless days [54].

[c]Simulated a mov allowing plants to establish in a protected environment with exposure to full spectrum light before ight prior to harvest.

2018 and transplanted four weeks later (Oct. 6, 2017, and 24, 2018). The shade cloth was over the shade covering treatment the entire growing season. The poly was removed from the movable treatment two weeks prior to harvest (Oct. 27, 2017, and Nov. 26, 2018). Lettuce was harvested 4 weeks after transplanting in 2017 (Nov. 10) and 8 weeks post-transplanting in 2018 (Dec. 10). The fall lettuce season was approximately 4 weeks longer in 2018 due to field flooding that delayed planting, followed by cold winter temperatures that delayed plant growth.

Mature lettuce plants were harvested from each plot, using a lettuce knife (Harris Seeds, Rochester, NY, USA) at the soil level to remove the full plant along with any outer whorl leaves, minus the root system. Six plants, chosen at random from each treatment plot within the 4 reps, were placed in plastic bags, and transported in an air-conditioned vehicle to the postharvest physiology lab. Analysis occurred on the day of harvest, day 0, and 5 days after storage in optimum conditions (1.5°C and 90% RH) in environmental chambers (Forma Environmental Chambers; ThermoFisher Scientific Inc., Asheville, NC, USA).

Color

Color was measured from an undamaged, outermost leaf from three plants per plot with four measurements per leaf. Two measurements were taken on left and right side of the midrib, 1 to 3 cm from the tip (Ilić et al., 2017). Color measurements were made using an A5 Chroma-Meter (Minolta CR-400; Minolta Co. Ltd., Osaka, Japan). The instrument was calibrated with the Minolta calibration standard white reflector plate before sampling lettuce leaves. L*, a*, and b* readings were transformed to those of the L, a, b color space and finally to hue angle and chroma according to Setser (1984) and as recommended by McGuire (2019). Hue angle was expressed on a 360° color wheel where 0° and 360° represents red, 90° represents yellow, 180° represents green, and 270° represents blue. Chroma indicates color purity or saturation (high values are more vivid) (McGuire, 2019). Following color analysis, samples were combined by replications, lyophilized in the freeze dryer (Harvest Right, Salt Lake City, UT, USA), and ground (Waring WSG30; Conair Corporation, Torrington, CT, USA) for phenolic analysis.

Standards, Reagents, and Equipment

For TPC, Folin-Ciocalteu's phenol reagent [2,4,6-tris (2-pyridyl)-s-triazine ferrous sulphate heptahydrate (MP Biomedicals, Santa Ana, CA, USA), sodium carbonate and gallic acid [3,4,5-trihydroxybenzoic acid (Acros Organics BVBA, Geel, Belgium) were used. For phenolic compound analysis, commercial standards were all analytical grade and included caffeic acid, chicoric acid, chlorogenic acid, isoquercetin, rutin, and ferulic acid, as well as formic acid (purity >99%) purchased from Acros Organics. The stock standard solutions of individual compounds (with 1000 µg/mL concentrations) were prepared with methanol and stored at -20°C in dark bottles as described by Alarcón-Flores et al. (2013). Ammonium acetate, methanol, and ethanol were all HPLC grade (VWR, Radnor, PA, USA). Equipment used includes an analytical balance (Mettler Toledo, Columbus, OH, USA), sonicator (Ultrasonic Bath; Fisher Scientific, Hampton, NH, USA), centrifuge (Avanti J-E; Beckman Coulter, Indianapolis, IN, USA), and a 96-well spectrophotometer microplate reader (Synergy H1; BioTek Instruments, Inc. Winooski, VT, USA).

Extraction for TPC and Phenolic Compounds

Each of the six coverings had four reps at day 0 and 5. Each rep was comprised of three heads which were extracted and analyzed in a darkened room with a red safety light to avoid oxidation of the analytes, following the procedure of Vallverdú-Queralt A, et al. (2013). Lyophilized lettuce (0.2 g) was homogenized with 4 mL of ethanol/water, (80/20, v/v), vortexed (20 s), sonicated (5 min), and centrifuged (4000 rpm, 15 min, 4°C). The supernatant was transferred into a test tube and the extraction was repeated. Both supernatants were combined and evaporated to dryness under nitrogen flow (2-6 ppm) and recovered with 4 mL of 30 mM ammonium acetate in de-ionized (D.I.) water with 5 pH adjusted with formic acid (eluent B) and filtered through a 25 mm 0.22 µm filter (Supor; VWR, Radnor, PA, USA) into several 1.5 mL Eppendorf tubes (Fisher Scientific, Hampton, NH, USA) for reserve and the sample extract was stored in darkness at -70°C until TPC, anthocyanin, and individual phenolic compound assay.

Total Phenolic Content Assay

TPC was determined spectro photometrically according to the procedure of Singleton and Rossi (1965). Prior to analysis, a portion of each extracted sample was thawed, and vortexed and 100 µL of sample extract was diluted with 400 µL D.I. water. Using a 96-well microplate, 25 µL of the diluted extract was combined with 125 µL of 0.2 N Folin-Ciocalteau's reagent and 100 µL of sodium carbonate (75 g/L). The plate was incubated at 30°C for 60 min, followed by a 10 min room temperature adjustment, and absorption was read at 760 nm. TPC was expressed as milligrams gallic acid equivalents (GAE) per 100 g on a dry weight (DW) basis using a calibration curve of gallic acid.

Individual Phenolic Compound Assay

Prior to analysis, a portion of each extracted sample was thawed, vortexed and the sample extract was diluted 10x in 50:50 eluent A: eluent B (methanol + 0.1% formic acid: 30 mM ammonium acetate in D.I. water adjusted to 5 pH with formic acid) for injection. The analysis was carried out using a method adapted from Alarcón-Flores MI, et al. (2013) [56]. Samples of 3 uL were injected into the Waters Acquity UPLC System (Waters Co., Milford, MA, USA) as described in Gude KM, et al. (2020). The phenolic compounds isoquercetin, rutin, caffeic acid, chicoric acid, chlorogenic acid and ferulic acid, were quantified, related to their corresponding standard based on retention time, and confirmed by their absorption spectrum in UV. Results were expressed as mg/kg DW. For each extract, three subsamples were made.

Anthocyanin Assay

Anthocyanin absorbance was measured separately from the other flavonoids, using the microplate reader with the spectrophotometer set at 530 nm. Prior to analysis, a portion of each extracted sample was thawed, vortexed and 150 µL sample extract was pipetted in triplicate on 96-well microplates. Finally, standard curves were developed using HPLC grade cyanidin 3-glucoside (Acros Organics, Geel, Belgium) from 0.39063 to 50 µg/mL. Results were expressed as mg/kg dry weight (DW).

Statistical Analysis

Data for the two cultivars were analyzed separately and subjected to linear mixed model analysis. The fixed effects of the linear mixed model for color were season, year nested within season, covering, storage day, season x covering, season x storage day, storage day x covering, and season x storage day x covering. Random effects of the model include rep x year x season, and rep x covering x year nested within season. The values are means (±SE).

The TPC, anthocyanin, and individual phenolic data was normalized

with natural log (ln) transformation before linear mixed model analysis. The fixed effects of the model are season, year nested within season, covering, storage day, season x covering, season x storage day, storage day x covering, and season x storage day x covering. Random effects of the model include rep x year nested within season, and the rep x covering x year nested within season interactions. Within a season and storage day, the multiple comparison procedure was carried out using Tukey's method at the 0.05 significance level. Also, within a season, storage day effect for each covering was evaluated on the log scale. Back transforming the LSmean differences to the original scale corresponds to the ratio of medians. Statistical analysis was executed via Statistical Analysis Software (SAS version 9.4; Cary, NC) PROC MIXED with option DDFM=KR in the MODEL statement.

Results

Color

The Color was analyzed to examine the effects and interactions of season, HT covering, and storage day in RL and GL. The hue angle of RL differed significantly by season, covering, and storage day (*P* <.001, <.001, and <0.05), respectively; (Table 2). Similarly, the chroma values of RL differed significantly by season, covering, storage life, and interaction were observed between season and storage day (*P* <.001, <.001, <.001, and <0.05). The chroma and hue of fall RL were darker (lower chroma and hue values) compared to the spring.

During the spring RL trials, the hue angle of lettuce grown under the shade covering was higher than the ones under the movable and clear coverings (Figure 1). The fall RL hue angle was highest under the shade covering and it differed significantly from the movable, diffuse, and clear coverings at day 0, and from the clear and block at day 5. The chroma of spring and fall RL under the shade covering was higher than all other tested coverings at harvest. After 5 days of storage during the spring- the RL chroma decreased under the diffuse, clear, block, and shade coverings (*P* <0.05, <0.01, <0.05, and <0.01, respectively). During the fall, the block covering decreased significantly after 5 days of storage for hue angle and chroma (*P* <0.01 and 0.05).

The GL hue angle differed by season and covering (*P* <.001, and <0.01; Table 2). Hue angles in the spring GL were higher under the standard, block, and shade coverings, than the other tested coverings at harvest (Figure 1). Similarly, the GL chroma was affected by covering and storage day (*P* <.001, and <0.01). Chroma for the spring GL was

Table 2: Probability values[a] of effects and their interactions on chroma and hue angle of red 'New Red Fire' and green 'Two Star' leaf lettuce grown in high tunnels in Olathe, KS in the fall (2017 and 2018) and spring (2018 and 2019) at the day of harvest, day 0, and on day 5.

Parameter	Lettuce	Season (S)	Covering[b] (C)	S x C	Day (D)	S x D	C x D	S x C x D
Chroma	Red	<.001	<.001	ns	<.001	<0.05	ns	ns
	Green	ns	<.001	<0.05	<0.01	<.001	ns	<0.05
Hue (°)	Red	<.001	<.001	ns	<0.05	ns	ns	ns
	Green	<.001	<0.01	<0.05	ns	<0.01	<0.05	<0.05

[a]linear mixed model was used to test if effects and interactions had significant effect on the examined parameter (P ≤0.05).
[b]Trial was arranged in a randomized complete block design, blocking by high tunnel and year. Coverings include standard poly, standard poly with removal two weeks prior to the first harvest, diffuse poly, clear poly, UV-A/B blocking poly, and 55% shade cloth over standard poly.

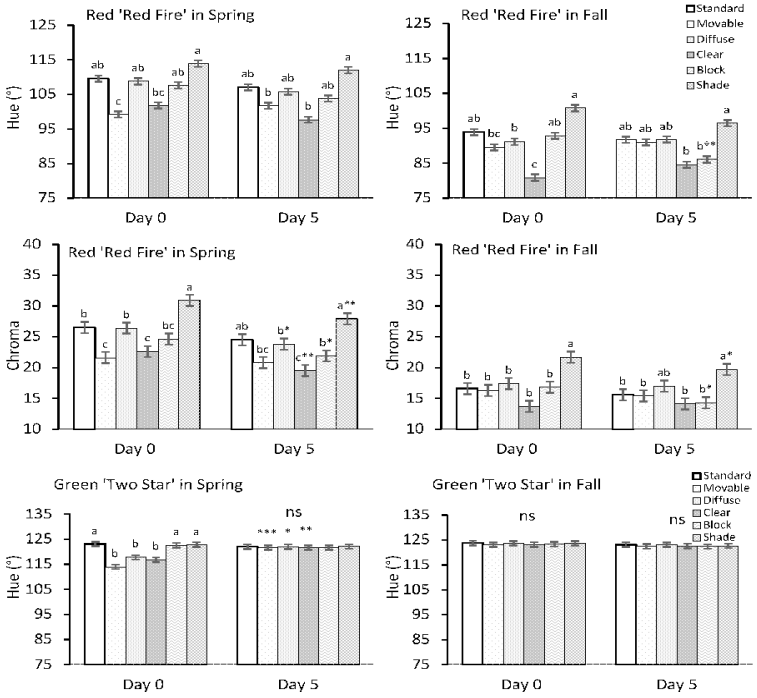

Figure 1: Effect of high tunnel covering on red 'New Red Fire' and green 'Two Star' leaf color (hue angle [$\tan^{-1}$ (b*/a*)] and chroma $(a^{*2} + b^{*2})^{0.5}$). For each day, columns with the same letter do not differ significantly between coverings at P ≤0.05, Tukey's HSD. Within each covering, *P ≤0.05, **P ≤0.01, ***P ≤0.001 denote significant differences between days. Coverings include standard poly (standard), standard poly with removal two weeks prior to the first harvest (movable), diffuse poly (diffuse), clear poly (clear), UV-A/B blocking poly (block), and 55% shade cloth over standard poly (shade).

statistically similar between the standard, block, and shade coverings, which was higher than the movable and clear coverings. By day 5, the hue angle and chroma of spring-grown GL increased under the movable, diffuse, and clear coverings.

Fixed Effects on Phenolic Compounds Accumulation of RL and GL

Individual phenolic acids were analyzed to examine the effects and interactions of season, HT covering, and storage day in RL and GL (Table 3). The effect of covering significantly altered RL accumulation of caffeic acid and chlorogenic acid (P < 0.05, and 0.05, respectively; Table 3).

Phenolic acid Compound Accumulation of RL

The TPC increased in the spring trial RL when grown under the clear covering compared to the shade covering (Table 4; P <0.05). After 5 days in storage, the TPC increased in the RL grown under the shade covering (P <0.05). No differences were detected between coverings during the fall trials for the RL. No differences were detected between coverings during the spring trials for the GL. However, the TPC increased in the fall trial GL when grown under the movable, diffuse, and clear covering compared to the shade covering (P <0.01). After 5 days in storage, the TPC increased in the GL grown under the block covering.

There was an effect of HT covering on caffeic accumulation for RL (P <0.05); however, this difference did not exist after storage when nested within season (Table 2). Chlorogenic acid and chicoric acid were found to be the dominant phenolic compounds (Table 4). Chlorogenic acid accumulation of the spring RL grown under the clear covering was 32% greater than that under the diffuse covering, although the differences were not significant. After 5 days of storage, the concentration of chlorogenic acid in RL decreased. The overall effect of covering x storage day was observed with fall chlorogenic acid, as concentration was higher under the diffuse, clear, and block coverings compared to the movable and shade coverings at day 5 (P <0.05; Table 4).

During the spring, the caffeic acid concentration under the standard, movable, block and shade covering decreased by day 5 (P <0.05, <0.01, <0.05, and <0.01, respectively; Table 4). The chlorogenic acid concentration in the spring RL that was grown under the clear and shade coverings decreased by day 5 (P <0.05, and <0.01, respectively; Table 4). During the fall, the caffeic acid concentration under standard, movable, clear, and shade covering increased by day 5 (P <0.01, <0.05, <0.05, and <0.05, respectively; Table 4). The fall RL ferulic acid concentration under the movable and clear covering increased by day 5 (P <0.05, and <.001, respectively; Table 4). Furthermore, a significant decrease in fall RL chlorogenic acid was noted for the standard and movable coverings after 5 days in storage (P <.001, and <.001, respectively). During the fall trials, the diffuse, clear, and block coverings approximately doubled their chicoric acid concentration after 5 days in storage (P <0.05, <0.05, and <0.05, respectively; Table 4).

Phenolic acid Compound Accumulation of GL

Season was found to be a prominent effect in GL with the spring season resulting in greater chlorogenic acid and chicoric acid accumulation (P <0.01, and <0.05; Table 4). The spring GL chlorogenic acid accumulation was higher under the standard and shade coverings than the diffuse covering at harvest (P <0.05; Table 4). However different from the spring lettuce, the fall GL chlorogenic acid accumulation was decreased under the shade covering relative to the others at harvest (P <.001). During the fall GL trials, the caffeic acid accumulation from the standard and clear coverings increased relative to the shade covering at harvest (P <0.01). On day 5 in the fall GL, the chicoric acid concentration was higher under the clear covering compared to the diffuse covering (P <0.05).

After 5 days in storage, the spring GL ferulic acid concentration decreased under the standard, movable, and diffuse coverings (P <0.05, <0.01, and <0.05, respectively). Similarly, the chlorogenic acid concentration decreased significantly by day 5 in the spring GL under the standard and diffuse coverings (P <0.05, and <0.05, respectively). In the spring GL, a large increase in chicoric acid was observed after 5 days under the diffuse covering (P <0.01). In the fall GL after 5 days storage, the chlorogenic acid concentration decreased under the standard, movable, and diffuse coverings (P <0.05, <0.01, <0.05) and increased under the shade covering and (P <0.01). By day 5, the chicoric acid concentration in the fall GL decreased under the movable and diffuse covering (P <0.01, <0.05), and increased under the block and shade coverings (P <0.05, and <.001).

Fixed Effects on Flavonoid Accumulation of RL and GL

Anthocyanin and the individual flavonoid compounds, isoquercetin and rutin, were analyzed to examine the effects of season, HT covering, and storage day of RL and GL (Table 5). Similar to RL phenolic acid accumulation, the spring season resulted in increased isoquercetin and rutin accumulation compared to the fall (P <.001 and <.001; Table 5).

Flavonoid Compound Accumulation of RL

During the spring RL trials, the anthocyanin concentration

Table 3: Probability values[a] of effects and their interactions on total phenolic content (TPC), and phenolic acid concentration of red 'New Red Fire' and green 'Two Star' leaf lettuce grown in high tunnels in Olathe, KS in the fall (2017 and 2018) and spring (2018 and 2019) at the day of harvest and on day 5.

Parameter	Lettuce	Season (S)	Covering (C)[b]	Storage Day (D)	(S x C)	(C x D)	(S x C x D)
TPC	Red	<0.01	ns	ns	ns	ns	ns
	Green	<0.05	ns	ns	ns	<0.05	ns
Caffeic acid	Red	ns	<0.05	ns	ns	ns	ns
	Green	ns	ns	ns	<0.05	ns	ns
Ferulic acid	Red	<.001	ns	ns	ns	ns	ns
	Green	ns	ns	ns	ns	ns	ns
Chlorogenic acid	Red	<.001	<0.05	<.001	ns	<0.05	ns
	Green	<0.01	ns	<0.05	<0.01	<0.05	<.001
Chicoric acid	Red	<.001	ns	ns	ns	ns	ns
	Green	<0.05	ns	ns	<0.05	<0.01	<.001

[a]linear mixed model was used to test if effects and interactions had a significant effect on the examined parameter (P ≤0.05).
[b]Trial was arranged in a randomized complete block design, blocking by high tunnel and year. Coverings included: standard poly, standard poly with removal two weeks prior to the first harvest, diffuse poly, clear poly, UV-A/B blocking poly, and 55% shade cloth over standard poly.

Table 4: Total phenolic content (TPC) and phenolic acid accumulation (mg/kg DW) of red 'New Red Fire' and green 'Two Star' leaf lettuce in plants grown in the fall (2017 and 2018) and spring (2018 and 2019) under six high tunnel coverings at harvest and after 5 d in storage at 1.5 °C.

Red Lettuce 'New Red Fire'											
Season	**Covering[a]**	**Total phenolic content (TPC)**		**Caffeic acid**		**Ferulic acid**		**Chlorogenic acid**		**Chicoric acid**	
	Storage Day	0	5	0	5	0	5	0	5	0	5
Spring	Standard	210.1 ab[b]	252.7	10.2	5.8*	9	4.9	555	375.9	195.8	128.6
	Movable	250.3 ab	348.7	12.7	5.8**	7.1	4	566.8	326.2	234.6	140.1
	Diffuse	270.3 ab	270.9	6.6	6.6	5.8	3.6	535.4	394.1	164.8	188.4
	Clear	410.9 a	290.8	10.3	6.2	9.2	3.3	781.7	414.6*	208.4	172.2
	Block	238.5 ab	259.5	11.1	6.1*	4.7	3.4	596.8	462.2	184.3	206.6
	Shade	199.7 b	338.5*[c]	8.2	4.1**	4	2.4	587.2	264.8**	197.6	144.4
	P-value	**<0.05**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**
Fall	Standard	266.3	197.5	6.1	10.8*	1.3	1.4	280.5	75.2 bc***	143.2	163.1
	Movable	251.1	205.8	5.7	10.2*	0.8	2.5*	184.1	66.6 c***	105.1	153.8
	Diffuse	227.9	206.1	5	8	1.6	1.8	201.6	189.5 a	91.6	179.4*
	Clear	204.3	171.7	4.4	7.9*	0.6	4.5***	142.2	180 a	78.4	154.2*
	Block	223.5	149.7	4.7	6.7	1.7	2.1	160.2	165.3 ab	97.4	183.2*
	Shade	263.3	287.3	4.6	7.9*	0.8	1.9	132.5	85.4 c	106.4	149.6
	P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**<0.05**	**ns**	**ns**
Green Lettuce 'Two Star'											
Spring	Standard	99.8	103.2	9.5	8.5	22	7*	160.1 a	58.8*	131.7	89.2
	Movable	169.3	133.8	10.6	8.4	37.2	6.8**	60 ab	53.5	116	178.3
	Diffuse	76.1	100.5	9.7	11.3	24.2	6.9*	40.3 b	110.8*	60.1	247.9**
	Clear	97.8	101.7	9.6	8.8	28.1	11.4	132.4 ab	62.3	155.3	85.5
	Block	105.6	127.7	8.7	9.2	25.9	8.8	72.7 ab	58.5	83.3	102.5
	Shade	104.9	103.7	12.8	8.3*	18.6	8	140.3 a	69.7	154.7	127.9
	P-value	**ns**	**ns**	**ns**	**ns**	**ns**	**ns**	**<0.05**	**ns**	**ns**	**ns**
Fall	Standard	70.6 ab	31.2 bc	12.8 a	9.5	4.7	15.9	106.5 a	35.8*	166.6	95.3 ab
	Movable	201.8 a	18.7 c***	11.3 ab	9.1	12.9	16.3	93.1 a	24.5**	178	56.5 ab**
	Diffuse	154.6 a	36.9 bc*	9.9 ab	8.9	9	16.4	81.4 a	28*	118.4	48.1 b*
	Clear	148.5 a	87 ab	12.1 a	12.3	8.2	20.9	48 a	55.4	82.5	168.5 a
	Block	50.0 ab	153.5 a*	8.3 ab	11.6	8.8	20.3	35.5 a	53.4	58.3	141.3 ab*
	Shade	30.0 b	34.9 bc	6.7 b	8.3	9.9	16.9	9.3 b	40.7**	11.8	116.8 ab***
	P-value	**<0.01**	**<.001**	**<0.05**	**ns**	**ns**	**ns**	**<.001**	**ns**	**ns**	**<0.05**

[a]Trial was arranged in a randomized complete block design, blocking by high tunnel and year. Coverings included standard poly, standard poly with removal two weeks prior to the first harvest, diffuse poly, clear poly, UV-A/B blocking poly, and 55% shade cloth over standard poly.

[b]Columns with same letter do not differ significantly within seasons at $P \leq 0.05$, Tukey's HSD. DW, dry weight; ns, not significant.

[c]For each covering within each compound, significant differences throughout storage are noted: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq .001$.

Table 5: Probability values[a] of effects and their interactions on flavonoid concentration of red 'New Red Fire' and green 'Two Star' leaf lettuce grown in high tunnels in Olathe, KS in the fall (2017 and 2018) and spring (2018 and 2019) at the day of harvest and on day 5.

	Lettuce	**Season (S)[c]**	**Covering (C)**	**Storage Day (D)**	**(S x C)**	**(C x D)**	**(S x C x D)**
Isoquercetin	Red	<.001	<.001	ns	ns	ns	ns
	Green	<.001	<0.01	<0.01	<.001	ns	ns
Rutin	Red	<.001	ns	ns	ns	ns	ns
	Green	<.001	ns	ns	ns	ns	ns
Anthocyanin	Red	<.001	<.001	<.001	<0.05	ns	<.001

[a]linear mixed model was used to test if effects and interactions had significant effect on the examined parameter ($P \leq 0.05$).

[b]Trial was arranged in a randomized complete block design, blocking by high tunnel and year. Covering includes the following 6 different coverings: standard poly, standard poly with removal two weeks prior to the first harvest, diffuse poly, clear poly, UV-A/B blocking poly, and 55% shade cloth over standard poly.

increased under the movable covering compared to the shade covering (P <.001; Table 6). After storage, the anthocyanin concentration in the RL grown under the clear and block coverings was greater than in the RL grown under the standard and shade coverings (P <.001; Table 6). During the fall trials, the anthocyanin concentration in the RL grown under the clear and movable coverings was greater than when grown under the shade covering (P <0.001; Table 6). Throughout storage, a significant decrease was observed from the RL lettuce grown under shade, block, and diffuse coverings.

During the spring RL trials, the movable covering increased isoquercetin accumulation by 72% compared to the shade covering (P <0.01; Table 6). Similar observations were made in the spring RL for the rutin accumulation of GL under the movable covering compared to the other tested coverings, although no significant differences were observed. In the fall RL, rutin accumulation increased under the clear, block, and shade covering compared to the standard and movable coverings (P <0.01; Table 6). The fall RL isoquercetin concentration increased under the movable covering compared to the shade covering on day 5 (P <0.05; Table 6).

After 5 days of storage in spring RL, rutin decreased significantly

Table 6: Flavonoid concentration (mg/kg DW) of red 'New Red Fire' and 'Two Star' leaf lettuce in plants grown in the fall (2017 and 2018) and spring (2018 and 2019) under six high tunnel coverings at harvest and after 5 d in storage at 1.5 °C.

Red Lettuce 'New Red Fire'								**Green Lettuce 'Two Star'**			
Season	**Covering[a]**	**Isoquercetin**		**Rutin**		**Anthocyanin**		**Isoquercetin**		**Rutin**	
	Storage Day	0	5	0	5	0	5	0	5	0	5
Spring	Standard	43.3 ab[b]	26.1	15.9	4.3***[c]	860.1 b	564.1 b*	49.0	47.4	22.7	13
	Movable	99.3 a	52	29.8	6.3***	1555.2 a	866.4 ab**	104.0	60.8	38.8	35.2
	Diffuse	36.5 ab	50.7	13.1	4**	835.9 b	802.6 ab	40.2	52.5	22.4	20
	Clear	60.4 ab	57.9	21.1	4.2***	1466.9 ab	1252.7 a	83.1	47.8	27.4	20.7
	Block	40.8 ab	58.7	17	4.2***	811.4 b	1222.5 a*	54.7	47.9	25.8	21.4
	Shade	27.7 b	21.7	11.3	3.5**	416.3 c	478.4 b	45.4	40.3	17	10.8
	P-value	**<0.01**	**ns**	**ns**	**ns**	**<.001**	**<.001**	**ns**	**ns**	**ns**	**ns**
Fall	Standard	14.1	18 ab	2.2 b	3.4	1357.5	1199.3 ab	4.7	6.3	3.3	8.9
	Movable	16.7	24.5 a	3.2 b	5.7	1883.4	1252.2 a	6.5	11.1	5	4.3
	Diffuse	12.7	19.8 ab	5.1 ab	6	1716.0	1054.5 ab*	5.3	4.9	4.5	1.3
	Clear	23.2	16.5 ab	12.2 a	3.3***	1390.0	1421.7 a	7.2	16.5	11.9	11.9
	Block	19.1	16.4 ab	10.3 a	2.7**	1588.9	953.4 ab**	8.9	21.6	21.1	4.5
	Shade	15.7	8.1 b*	10.2 a	4.2*	1267.3	672.8 b***	9.1	11.7*	11.9	4
	P-value	**ns**	**<0.05**	**<0.01**	**ns**	**ns**	**<.001**	**ns**	**ns**	**ns**	**ns**

[a]Trial was arranged in a randomized complete block design, blocking by high tunnel and year. Coverings included standard poly, standard poly with removal two weeks prior to the first harvest, diffuse poly, clear poly, UV-A/B blocking poly, and 55% shade cloth over standard poly.

[b]Columns with same letter do not differ significantly within seasons at $P \leq 0.05$, Tukey's HSD. DW, dry weight; ns, not significant.

[c]For each covering within each compound, significant differences throughout storage are noted: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq .001$.

for all coverings. The fall RL isoquercetin concentration decreased by day 5 under the shade covering (P <0.05; Table 6). While the fall RL rutin concentration decreased significantly by day 5 under the clear, block, and shade coverings.

Flavonoid Compound Accumulation of GL

In the GL, the isoquercetin and rutin accumulation increased in the spring, relative to the fall (P <.001, and <.001, respectively; Table 6). During the spring, the isoquercetin and rutin accumulation under the movable covering was 61 and 56% greater than that of shade covering, although no significant differences were noted. The shade covering increased in isoquercetin concentration by day 5 in fall GL (P <0.05; Table 6).

Discussion

The chroma and hue angle was lower in the fall RL compared to the spring, resulting in darker leaves in the fall. Previous studies found that darker RL was observed in cooler temperatures compared to warmer ones [49,50]. The fall canopy temperatures were 6 to 18°C, while the spring was 11 to 26°C [54], which may have contributed to the leaf darkening.

Hue angle and chroma in the spring RL were lowest under the movable and clear coverings. According to the hue angles, the shade covering presented the greenest leaf coloring, while the clear and movable presented the reddest. Springtime PAR transmission under the movable covering was 100% with no light obstruction and lowest under the shade with only 32% PAR transmission. Although the standard covering had comparable PAR transmission to the clear, the clear covering transmitted more UV-A and B. This suggests that the 100% UV radiation with the movable covering and ~60% under the clear covering may have contributed to the dark red pigmentation during the spring [54,56]. Our findings of covering differences in lettuce color induction by UV-radiation and light intensity are in agreement with past reports [8,15, and 49]. Shioshita R, et al. (2006) found that the effect of UV radiation is more determinate of darker RL pigmentation compared to high light intensity (PAR). During the fall, it was the RL under the clear covering that had the darkest red (lowest hue angle and chroma) on the day of harvest. In the fall, the low temperatures often required the replacement of the standard poly over the movable covering (section Experimental Design), which may have reduced the movable covering effect of unobstructed light prior to harvest. To our knowledge, this is the first study to report the effect of a movable covering on lettuce color development at harvest and after 5 days of storage.

For the spring GL at the day of harvest, chroma and hue angle increased under the shade, standard, and block coverings relative to the other tested coverings. Similarly, Ilić Z, et al. (2017) found that a 50% black shade net increased the chroma of green lettuce at harvest compared to the open field control [57]. However, this effect was not detected after 5 days in storage.

The individual phenolic compounds reported here included chicoric acid, chlorogenic acid and quercetin glycosides, which were previously identified as the prominent phenolic compounds in lettuce [32,33, and 40]. Our study focused on the effects of season and HT covering total and individual phenolics at harvest and after 5 days in storage. Effects of covering on phenolic compound accumulation varied with season. The season was found to be the prominent factor with the spring lettuce accumulating the greater total and individual phenolic acid accumulation in RL. The individual phenolic acids, ferulic acid, chlorogenic acid, and chicoric acid of the RL were significantly less during the fall. Similarly, with the GL, the total phenolic content and individual phenolic acid accumulations of chlorogenic acid and chicoric acid were greater in the spring than in the fall. Typically, the fall seasons have lower levels of UV-radiation [47], PAR, and temperature [54], which may have contributed to the decrease in secondary metabolites due to altered phenolic metabolism. In agreement with our work, other studies have shown RL phenolic compound accumulation positively correlates with solar radiation and temperature; although, it may vary by cultivar [15,49].

Previous studies have shown that solar UV-radiation resulted in increased phenolic compound accumulation in HT lettuce. During the spring, the total phenolic content in the RL grown under the clear covering (with high UV transmission) was the highest of the studied coverings and exceeded that of the shade covering. It has been suggested that the dark red color is positively influenced by total phenolic accumulation [15], which was found true in spring RL. Similarly, the RL phenolic acid accumulation of caffeic acid, ferulic acid, chlorogenic acid, and chicoric acid was highest under the movable or clear covering at harvest, but not by a significant amount. In HT, García-Macías P, et al. (2007) found that individual flavonoid and phenolic acid accumulation of lettuce under UV-transparent poly were greater than those grown under UV-reduce or UV-block poly coverings [12]. To our knowledge, this is the first report to investigate the utility of a movable covering with phenolic compound accumulation in lettuce. However, only a few studies have included pre-harvest light-altering coverings in their trial design [43,44, and 57]. A study with accidental poly removal from HTs due to weather events found that RL with 4 weeks of full solar exposure prior to harvest, resulted in the same antioxidant accumulation as the open field.

There was no effect of storage on total phenolic content in spring or fall RL trials, as some past studies suggest. Caffeic acid and chlorogenic acid decreased in spring RL under some coverings after 5 days of storage, but it was inconsistent. In the fall RL, chlorogenic acid decreased under some coverings by day 5, while caffeic acid, ferulic acid, and chicoric acid increased under some coverings by day 5. The decrease in chlorogenic acid has been well documented in tomatoes [58,59], and lettuce [60,61]. It is suggested that the rise in levels of caffeic acid derivatives may happen at the expense of chlorogenic acid, due to chlorogenic acid's high redox potential [58]. Another theory is that chlorogenic acid is a good polyphenol oxidase substrate [60].

Green leaf lettuce had a lower amount of phenolic compounds compared to the RL. It has been reported that the GL lettuce allocates less carbon to the biosynthesis of phenolics and more to biomass growth [25], which may explain the lower amounts in secondary metabolites. The total phenolic content in the fall grown GL grown under the movable, diffuse, and clear coverings exceeded that of the shade covering. The individual phenolics show similar results during the fall with increased chlorogenic acid accumulation under all coverings besides shade and increased chicoric acid accumulation (by 93%) under the movable covering compared to the shade covering, although the two were not significantly different. Furthermore, the fall GL lettuce had higher caffeic acid accumulation under the standard and clear covering relative to the black shade. In agreement, a previous study showed that total phenol accumulation in green leaf lettuce decreased under a 55% black shade relative to a pearl shade cloth [57].

It has been suggested that higher PAR and surface temperature pre-harvest, may result in phytochemical loss throughout storage due to increased plant respiration [20]. However, the effect of covering throughout storage was inconsistent for GL, as seen with RL. Previous studies have suggested that phenolic acid and total phenolic concentration remain stable or increase after storage of green lettuce [57,60-65], but we did not find this to be true.

Cooler temperatures (10 to 25°C) and direct solar UV radiation have been shown to positively influence flavonoid accumulation [15]. The spring solar radiation was increased compared to the fall, which may explain the increase in isoquercetin and rutin accumulation in the spring compared to the fall for both RL and GL. The exception to the other flavonoids was the increase in anthocyanin accumulation during the fall trials. Flavonoid increases have been shown to increase in cool-cultivated environments [66]. Although the spring trials in the present study were warmer than during the fall (11 to 26°C compared to 6 to 18°C, respectively), the spring temperatures were still cooler than the studies mentioned above. Becker et al., 2014a measured the change in several phenolic compounds due to warm and cool environments. They found that of the measured phenolics in mature lettuce, only the anthocyanin cyanidin-3-O-(6″-O-malonyl)-glucoside responded to changes in the studied temperatures and increased in the cooler climate (7 to 12°C). They suggest that the different ROS typically scavenged by quercetin and cyanidin activate different stress response genes, which may help to explain the differential regulation of the flavonoids due to temperature.

The effect of high UV transmission on flavonoid content was seen with the increase in isoquercetin under the movable covering compared to the shade covering in the RL during spring and fall trials. Although not significantly different, the isoquercetin and rutin accumulation of the spring GL increased under the movable covering at harvest by 61% and 56%, respectively, compared to the lettuce growing under shade. Past studies have shown similar results with red and green cultivars under 50 to 55% black shade [57]. Similar to the movable covering in the present study, Becker et al. (2013) tested the effect of switching RL from shade to no shade and vice versa on quercetin derivative accumulation by harvest. They found that the RL grown under shade and then moved to no shade 2 weeks before harvest had the same accumulation of isoquercetin and other quercetin derivatives as the lettuce grown under no shade throughout the full trial. In the present study, the effect of storage was mostly inconsistent and altered by season. A general decrease of isoquercetin was observed throughout the storage of spring GL; however, a general increase of isoquercetin was observed throughout the storage of fall GL.

Both covering and season factors had an equally high effect on anthocyanin accumulation. The movable covering resulted in greater anthocyanin accumulation in RL compared to the other tested coverings (besides clear) in the spring trials. In general, our study demonstrates that environmental conditions, especially UV radiation and temperature, alter the biosynthesis of phenolic compounds in lettuce.

Conclusion

The experimental design enabled us to assess the effects of high tunnel covering and environmental conditions by season on leaf color and the accumulation of total phenolics, anthocyanins, and individual phenolic acid and flavonoid compounds of RL and GL. Increased reddening and anthocyanin accumulation of the RL along with the accumulation of total phenolics and select flavonoids and phenolic acids of both GL and RL was observed under the movable and clear coverings with UV-transmitting properties compared to the other studied coverings. The environmental temperature that varied by season was a major factor affecting all measured parameters. In the spring with warmer temperatures, there was an increase in total phenolic content and individual phenolic acids and flavonoids (mostly chlorogenic acid, chicoric acid, isoquercetin, and rutin) for both RL and GL. While in the fall, there was an increase in anthocyanin and leaf redness from the RL. The biosynthesis of phenolic compounds in the spring was enhanced by growing the crop under the clear and movable coverings that allowed a higher exposure to UV radiation. This study provides scope to consider clear poly or movable coverings in the production of 'New Red Fire' RL and 'Two Star' GL lettuce, to enhance phenolic compounds. High tunnel coverings that alter the light intensity and UV, can potentially add value to protected salad crops, such as RL and

GL. Since phenolic compounds are known to play an important role as antioxidants in human nutrition, the UV transmission properties of these coverings may be important from a nutritional significance. Future studies may help to further understand the production method and implementation of movable tunnels.

Author Roles

- Kelly Gude: Data curation, conducted the experimental investigation, development of phenolic assay, wrote the original and final draft.
- C.B. Rajashekar: Funding acquisition, review, and editing of drafts.
- Weiqun Wang: review and editing of drafts.
- Kanwal Ayub and Qing Kang: Statistical consultant and model design.
- Cary Rivard: Project administration, the conceptualization of field trials, review of a final draft.
- Eleni Pliakoni: Supervision of postharvest trials, the conceptualization of postharvest trials, review, and editing of drafts

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