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Changes in Agronomic, Antioxidant Compounds, and Morphology Parameters of Green and Red Lettuces (*Lactuca sativa* L.) by Successive Harvests and UV-B Supplementation

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Abstract: The growing demand for lettuce has prompted the need for higher quality standards. Consequently, researchers have focused their efforts on identifying cultural management strategies that can enhance the synthesis of antioxidant compounds, leading to improved functional properties of lettuce. In this regard, two experiments were conducted on hydroponically grown Lollo Bionda 'Levistro' and Lollo Rosso 'Carmoli' lettuces, known, respectively, for their green and red crispy leaves. The first experiment assessed the effects of harvest time and cutting on fresh weight (FW), dry weight (DW), total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and antioxidant capacity (AC). The second experiment evaluated the response of FW, DW, TPC, TFC, TAC, AC, proline content, and morphological cell changes to UV-B supplementation in greenhouse conditions as well as the impact of successive harvests on the same plant. UV-B radiation and cutting led to a reduction in FW, but they also showed an increase in DW. Furthermore, UV-B radiation, cutting, and plant growth stage had significant effects on TPC, TFC, and AC in both cultivars. Applying 10.5 kJ m^{-2} of UV-B radiation or performing different harvests resulted in increased TFC in 'Levistro', exhibiting a remarkable 91% increase at the third harvest compared to the control group (0 kJ m^{-2} at the first harvest). UV-B radiation also induced changes in anatomical cell distribution in both cultivars, leading to a 37% increase in intracellular space in 'Levistro' and a reduction of up to 8.2% in 'Carmoli'. Lastly, at a later stage of plant development (9-10th true leaves), 'Carmoli' demonstrated a 51% increase in TPC, 95% in TFC, and 65% in TAC, highlighting its potential as an intriguing strategy to obtain lettuce varieties with higher antioxidant properties. These findings underscore the significance of implementing cultural management techniques to enhance the antioxidant composition of lettuce.

Keywords: UV-B radiation; cutting; leafy vegetables; plant growth stage; phenolic compounds

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1. Introduction

The consumption of assorted fresh fruit and vegetable diets has demonstrated a notable inverse association with the incidence of noncommunicable chronic diseases, such as obesity, diabetes, cancer, hypertension, and other related conditions. These beneficial effects have been primarily attributed to the presence of secondary plant metabolites, notably polyphenols, which are known for their antioxidant properties [1–3]. Consequently, there is a surging demand for fresh products, such as leafy salads, driven by the recognition of their benefits beyond basic nutrition. Remarkably, approximately 35% of consumers exhibit a willingness to pay a premium for these high-quality food items that offer enhanced

advantages beyond fundamental nutritional content [4,5]. Given the growing interest in vegetables with heightened antioxidant compounds and the escalating consumer demand for assortment featuring types, shapes, and colors in salads, lettuce varieties and cultivars have emerged as a particularly prominent option [6,7]. Lettuce (*Lactuca sativa* L.) has many healthy properties, attributed to different secondary metabolites such as fiber, minerals, carotenoids, vitamins K and B complex, vitamins with antioxidant properties (E and C), and especially polyphenols [1]. Among the polyphenolic compounds, flavanols and anthocyanins, classified as flavonoids, have been recognized for exhibiting superior antioxidant activity compared to vitamin C. This notable disparity has underscored the heightened interest in exploring and harnessing the potential benefits of these specific polyphenolic compounds [8]. The phenolic compound content is influenced by both internal and external factors. Even a slight alteration in the environmental conditions can give rise to substantial variations in the concentration of antioxidant compounds [9–11]. In this context, light is a pivotal environmental factor governing plant growth and development, serving as the primary energy source for photosynthesis and playing a vital role in triggering diverse physiological responses in plants [12,13].

UV radiation, categorized into three distinct types based on its electromagnetic wavelength spectrum, namely UV-C (100–280 nm), UV-B (280–320 nm), and UV-A (320–400 nm), significantly affects various aspects of plant physiology. It serves as an inducer of diverse plant responses, impacting not only growth, development, and reproduction but also physiological, biochemical, and even genetic aspects of plant functioning [14]. Despite UV-B radiation accounting for less than 0.5% of the total solar energy, its photons possess the highest energy within the electromagnetic spectrum that reaches the Earth's surface. Therefore, even a small increase in UV-B radiation levels can exert substantial impacts on critical processes spanning various levels of biological organization, ranging from individual organisms to entire ecosystems [14]. UV-B radiation causes plenty of responses in the plant and impacts the levels of a broad range of metabolites, including phenolic, terpenoid, and alkaloid compounds [15,16]. Specifically, flavonoid, and phenolic acid biosynthesis is strongly influenced by light quality and specially by UV-B radiation [9,17–19]. However, exposure to high UV-B doses can interrupt the plant's metabolic process and cause leaf damage. Elevated levels of UV-B radiation can have deleterious effects on the photosynthetic machinery, particularly on Photosystem II (PSII), resulting in alterations in photosynthetic rates, photoinhibition, and the activation of photoprotective mechanisms [10,18,20,21]. Energy-rich UV-B photons can increase the overproduction of free radicals, thereby leading to the development of oxidative stress within plant cells. Nevertheless, emerging evidence from experimental studies suggests that UV-B radiation functions more as a regulatory and acclimatizing factor rather than as a limiting environmental stressor [15]. In this context, UV-B radiation can efficiently stimulate the synthesis of secondary metabolites such as flavonoids, phenolic compounds, and other antioxidants, which contribute to plant defense mechanisms against UV-induced oxidative stress [15,22].

Since plastic greenhouse films block UV-B wavelengths, there are lamps to supply this type of radiation and promote antioxidant compounds biosynthesis in controlled conditions [16]. Consequently, UV-B radiation has been employed in cultivation protocols as a strategic approach to promote the proper development of oil glands in *Ocimum basilicum* L. (Sweet Basil) [23] and get better results in biosynthesis and accumulation of phenolic compounds in particular flavonoids, phenolic acids, and other antioxidants [14,16,24,25]. Therefore, understanding the complex interactions between plants and UV-B radiation is crucial to understanding the adaptive strategies and responses that plants employ to cope with varying levels of UV exposure and produce healthy and antioxidant-enriched vegetables. UV-B radiation can also trigger responses of photoprotection and decreased leaf area and yield [26,27] and generate morphological changes in plant cells [28,29], resulting in the reduction of the leaf area and modifying the stomatal frequency [30,31]. To address this approach, both an evaluation of cell and stomatal density and a quantification evaluation of the intercellular space in the leaf tissue were carried out.

On the other hand, physical damage, such as cutting leaves during culture, could also generate an enhanced production of phenolic compounds by enhancing phenylalanine ammonia-lyase activity [32]. In plant cells, one of the first responses to wounding is an oxidative burst of reactive oxygen species (ROS) as a protective anti-stress mechanism, a response associated with a metabolic cost [33]. Phenolic compounds stand out as one of the most prevalent and ubiquitous groups of defensive secondary metabolites. Therefore, different stress sources, such as microorganism attacks or competing plants, increase phenolic compounds acting as a defensive mechanism [33,34].

In this context, and because environmental, cultural, and management practices have been used to enhance the lettuce quality and its phytochemical contents and health-promoting attributes [6], the purpose of the study was to evaluate the effect of successive cuttings and UV-B supplementation on phenolic compound accumulation, agronomic growth parameters, and leaf anatomy of green and red hydroponic lettuces.

2. Materials and Methods

2.1. Plant Material, Growth Conditions, and Experimental Design

The experiments were performed in the Postharvest Study Center (CEPOC) in the Faculty of Agricultural Sciences at the University of Chile in La Pintana, Santiago, Chile.

Lettuce plants of Lollo Bionda cv. 'Levistro', with crispy green leaves, and Lollo Rosso cv. 'Carmoli', with crispy red leaves, were grown in a floating root hydroponic system under a plastic chapel greenhouse (8 m wide, 33 m long, and 5.8 m high at the peak). The greenhouse structure was wrapped with a polyethylene film 200 µm thick, providing 90% and 20% transmission and diffusion of global light, respectively.

Lettuce seeds, obtained from Rijk Zwaan, were germinated on pre-hydrated granulated rock wool and expanded perlite A6 using a dark germination chamber (T: 22 °C; and RH: 80% for 24 h) following the recommendation by [35]. After root emission, the trays were transferred to the greenhouse and irrigated in accordance with the recommendations by [35]. The nutrient solution proposed by [36] for leafy vegetables was used during culture. The pH of the nutrient solution was kept between 5.5 and 5.8 to maximize nutrient absorption from the crop using nitric acid solution. Seedlings at the true four-leaf stage were transplanted to a recirculating floating root hydroponic system and established in floating rafts made of high-density expanded polystyrene (25 kg m^{-3}) of 0.5·1.5·0.025 m with a density of 50 plants m^{-2} [35]. Two days after the transplant, the complete nutrient solution was added. The plant culture was carried out in autumn. The minimum and maximum temperatures of the air in the greenhouse and nutrient solution temperature (°C) during culture are in Figure 1.

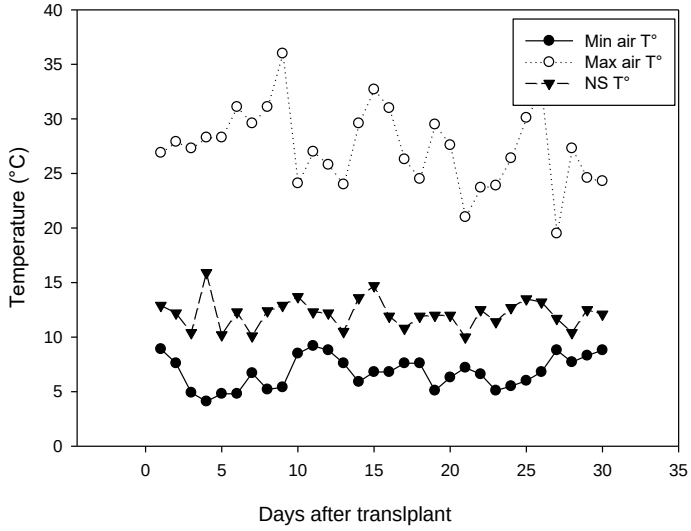

Figure 1. Means of maximum and minimum air temperatures in the greenhouse and nutrient solution (NS) temperature during culture.

Two continuous experiments were performed to determine the effect of the cutting, harvest time, and the use of UV-B radiation as a culture practice on growth parameters and phenolic compound concentration in green and red lettuces. The aim of the first experiment was to isolate the effect of the cutting and harvest time on the aforementioned parameters. The aim of the second experiment was to evaluate the effect of UV-B supplementation in combination with different harvest times on the same parameters. In this second experiment, as additional evaluation, the proline content was considered a stress indicator, and leaf anatomical changes were also evaluated.

2.1.1. Experiment 1: Effect of the Cutting by Harvest on Green and Red Lettuces

This experiment was performed on two types of lettuce: Lollo Bionda cultivars 'Levistro' characterized by crispy green leaves, and Lollo Rosso cv. 'Carmoli' with crispy red leaves. The lettuces were arranged on three blocks of divided plots designed with two factors. The first factor was the harvest times (1st, 2nd, and 3rd), and the second was the previous cut (w) or without cutting (wo). Data obtained from the first harvest, which did not undergo any prior cutting, were presented solely as reference values for comparison or as a starting point in the experiment.

2.1.2. Experiment 2: Effect of UV-B Radiation and Successive Harvests on Green and Red Lettuces

UV-B radiation treatments were applied using 2 UV-B Broadband TL lamps (Philips, Holland, The Netherlands). The lamps were set in a transparent acrylic box 50 cm above the culture. The different accumulated UV-B radiation doses—0 kJ m^{-2} (control), 5.2 kJ m^{-2}, and 10.5 kJ m^{-2}—took lamp intensity and time of exposition into consideration. To achieve the mentioned doses, the lamps were turned on for 0, 30, and 60 min, once a day, for 10 consecutive days before harvest. The intensity of UV-B radiation emitted by the lamps was measured by a photometer and radiometer (Solar Light, model PMA 2200, Glenside, PA, USA) using a PMA2101 UV-B erythema sensor (Solar Light, Glenside, PA, USA). The plant only received the UV-B radiation applied by the lamps because the greenhouse cover plastic did not allow the UV-B radiation to penetrate. The application of UV-B treatments began at 10 am, and because the acrylic box allowed the passage of the photosynthetic active radiation (PAR) and UV-A, the lettuce plants received the UV-B radiation in the presence of other light wavelengths. This experiment was also performed for two cultivars ('Levistro' and 'Carmoli') and arranged on three blocks of divided plots with a two-factor design (Figure 2). In this case, the first factor was harvesting times (1st, 2nd, 3rd), and the second was UV-B radiation dose (0, 5.2, 10.5 kJ m^{-2}). For this experiment, several evaluations were carried out, including the measurement of growth parameters and phenolic compounds. The evaluation of proline concentration as an indicator of stress and the use of microscopic analysis to evaluate stomatal and cell densities, as well as intracellular spaces in leaf tissue after UV-B application, were considered.

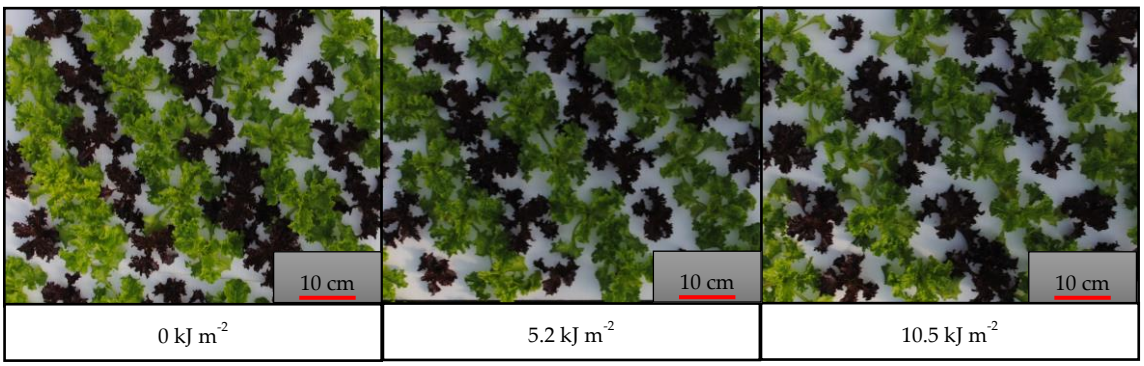

Figure 2. Lettuce types Lollo Bionda 'Levistro' cv. (green) and Lollo Rosso 'Carmoli' cv. (red), growing in a recirculating floating root hydroponic system. The image was captured before the initial harvest, after 10 days of UV-B treatment. Red bars represent 10 cm.

2.2. Plant Growth Analytical Parameters

For the first experiment, the plants were divided into two groups labeled with (w) and without (wo) previous cutting. For the first group, fully expanded leaves of the same plants were cut at day 10 (5th + 6th leaves), 20 (7th + 8th leaves), and 30 (9th + 10th leaves), and labeled first (1st), second (2nd), and third (3rd) harvest. For the second group, fully expanded leaves of the same phenological stage coming from different w and wo plants were sampled.

In the second experiment, after the application of UV-B radiation (0, 5.2, and 10.5 kJ m^{-2}), fully expanded leaves of the same plants were cut at day 10 (5th + 6th leaves), 20 (7th + 8th leaves), and 30 (9th + 10th leaves), and categorized as first (1st), second (2nd), and third (3rd) harvest, respectively.

For fresh (FW) and dry weights (DW) measurements, the recommendations by [35] were followed. Briefly, for each harvest, five biological replicates per repetition (n = 15 per treatment) were randomly selected for FW. Then, for DW, the samples were dried in a forced air oven until constant mass. FW and DW were expressed in grams (g). The percentages of DW were estimated by the DW:FW ratio.

2.3. Color Parameters

Color parameters were evaluated as a quality characteristic of the leaves. For this measurement, the recommendation made by [35] was followed. For each harvest, two measurements were taken for each one of the nine biological replicates per treatment repetition (n = 27 plants per treatment). Data were analyzed with the color data software SpectraMagic NX and expressed as luminosity (L), chroma (C*), and hue angle (Hue) [37].

2.4. Antioxidant Extraction

The soluble antioxidants were extracted using the method proposed by [11] and following the recommendations by [35]. Fresh lettuce leaves were collected, frozen at −80 °C, lyophilized, and crushed until powder. An exact amount of powder was mixed with 10 mL of methanol: Water (MeOH: H_2O) solution in a 70:30 proportion. Each mixture was stirred in a vortex and put in an ultrasonic bath for complete extraction. Subsequently, the samples were centrifuged (4180× g for 10 min at 4 °C), and the supernatant was passed through a PVDF membrane filter (0.45 µm) using a sterile syringe. The filtrate was carefully preserved in amber tubes at −20 °C until total phenolic, flavonoid, anthocyanin compounds, and antioxidant activity analysis. For each harvest, three hydro-methanolic extracts per repetition were taken (n = 9 per treatment). Each extract came from a sample of five biological replicates.

2.5. Total Phenolic Content (TPC)

TPC was quantified employing the methodology introduced by [38] and following the recommendations by [35]. First, 10% Folin–Ciocalteu reagent was mixed with 100 µL of hydro-methanolic extract. 0.7 mol L^{-1} Na_2CO_3 solution was added to complete the reaction and left for 2 h. The absorbance was read at 765 nm using a multi-plate spectrophotometer. A control without samples was prepared and used as a baseline correction. TPC was estimated using a gallic acid calibration curve and expressed as mg of gallic acid equivalent (GAE) per 100 g^{-1} of FW and calculated as means of 9 data points (3 samples per treatment repetition).

2.6. Total Flavonoid Content (TFC)

TFC were measured following the method proposed by [39] and following the recommendation by [35]. First, 100 µL of hydro-methanolic extract was mixed with 100 µL of 5% $NaNO_2$ solution and reacted for 5 min in darkness. Then, 100 µL of 10% $AlCl_3$ solution was added and homogenized. To complete the reaction, 670 µL of 1 mol L^{-1} NaOH was added. The absorbance reading was at 510 nm using a multi-plate spectrophotometer. TFC was estimated using a rutin calibration curve (Merck KGaA, Darmstadt, Germany) and

expressed as mg rutin equivalents (Rut Eq) in 100 g^{-1} of FW and calculated as means of 9 data points (3 samples per treatment repetition). A control without a sample was prepared and used as a baseline correction.

2.7. Total Anthocyanin Content (TAC)

TAC was determined using a differential pH method following the recommendations by [40] recommendations. This method consisted of two buffer systems: The first buffer was a 0.025 mol L^{-1} potassium chloride buffer (pH 1.0), and the second was 0.4 mol L^{-1} sodium acetate buffer (pH 4.5). For the reaction, a 100 µL aliquot of the MeOH: H_2O extract was mixed separately with 900 µL of each buffer. The absorbance of each reaction was read against a blank without sample at 520 and 700 nm using a multi-plate spectrophotometer (Asys UVM 340, Biochrom, Cambridge, UK). Final absorbance was calculated using the following expression:

$$A = (A_{520}\ \text{nm} - A_{700}\ \text{nm})\ pH_{\ 1.0} - (A_{520}\ \text{nm} - A_{700}\ \text{nm})\ pH_{\ 4.5} \quad (1)$$

Once final absorbance was obtained, total anthocyanin was reported as Cyanidin-3 glucoside equivalents (mg L^{-1}) using the following equation:

$$\textit{Total anthocyanin}\ (\text{mg L}^{-1}) = A \times MW \times DF \times 1000/\varepsilon \times l \quad (2)$$

where A is absorbance, MW is the molecular weight of cyanidin-3-glucoside (449.2 g mol^{-1}), DF is the dilution factor, ε is the molar absorptivity of Cyanidin-3-glucoside coefficient (26,900 L mol^{-1} cm^{-1}), and l is the bucket width (cm). The total anthocyanin concentration was finally expressed as Cyanidin-3-glucoside equivalents (Cyn3gluc Eq) in mg 100 g^{-1} FW [35].

2.8. Antioxidant Capacity (AC)

As measurements of AC, ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were conducted.

The FRAP assay was performed according to the method described by [41] with some modifications. For the FRAP reagent, 0.3 mol L^{-1} acetate buffer (pH 3.6), 0.01 mol L^{-1} 2,4,6-Tripyridyl-s-triazine (TPTZ) in 0.04 mL L^{-1} HCl and 0.02 mol L^{-1} $FeCl_3 \cdot 6H_2O$ were mixed at a 10:1:1 ratio and heated at 37 °C for 10 min. On the other hand, dilutions of the original hydro-methanolic extract were obtained using a dilution factor of 4 and 8 for 'Levistro' and 'Carmoli', respectively. An aliquot of the dilution was mixed with a previously heated FRAP reagent. The reaction absorbance was measured at 593 nm using a multi-plate spectrophotometer every 30 min until reading stabilization (2 h). A control without samples was prepared and used as a baseline correction. The equivalent antioxidant capacity was calculated using a Trolox (Merck KGaA, Darmstadt, Germany) calibration curve as described in [42]. The results were expressed as mg of Trolox equivalent (Trolox eq) in 100 g^{-1} of FW and calculated as means of 9 data points (3 samples per treatment repetition).

For the free radical scavenging activity assay, a 2.2-diphenyl-1-picrylhydrazyl (DPPH) reagent was used, according to [43]. The same dilution samples mentioned for the FRAP assay were used for this measurement. For each reaction, an aliquot of the diluted extract was mixed with 1 mL of $0.2 \cdot 10^{-3}$ mol L^{-1} DPPH solution. The absorbance of the reaction was read at 517 nm using a multi-plate spectrophotometer every 30 min until reading stabilization (2 h). A control without a sample was prepared and used as a baseline correction. The equivalent antioxidant capacity was calculated using a Trolox (Merck KGaA, Darmstadt, Germany) calibration curve, expressed as mg of Trolox equivalent (Trolox eq) in mg 100 g^{-1} of FW and calculated as means of 9 data points (3 samples per treatment repetition).

2.9. Total Proline Content (TPrC)

TPrC was determined according to the protocol proposed by [44]. For this, 0.2 g of lyophilized lettuce powder was suspended in 10 mL of 3% sulfosalicylic acid solution and put in an ultrasound bath for 10 min. The previous mixtures were centrifuged at 6000× *g* (Z 326 K) for 10 min at 4 °C to separate solid residue. Once the samples were centrifuged, 1 mL of the supernatant was placed in a glass tube, and 1 mL of acid-ninhydrin solution and 1 mL of glacial acetic acid were added. For the acid-ninhydrin solution, 1.25 g ninhydrin, 30 mL of glacial acetic acid, and 20 mL of 6 M phosphoric acid were mixed. The reaction mix (sample, acid-ninhydrin, and acetic acid) was put in a thermo-regulated bath for 1 h at 100 °C. Once the time had elapsed, the reaction was stopped using an ice bath for 2 min. When the mixture was cold, 2 mL of toluene was added and stirred for 20 s in a vortex. The solution was maintained at room temperature for 30 min until the two phases had clearly separated. The absorbance of the toluene phase (upper phase) was measured at 520 nm using a multi-plate spectrophotometer (Asys UVM 340, Biochrom, Cambridge, UK). A control without a sample was prepared and used as a baseline correction. Proline content was determined using a D-proline standard curve (Merck KGaA, Darmstadt, Germany). The results were expressed as μg proline 100 g^{-1} FW and calculated as means of 9 data points (3 samples per treatment repetition). For each harvest, three extracts per repetition were taken (n = 9 per treatment). Each extract came from a sample of five biological repetitions.

2.10. Microscopic Cell Analysis

For the third harvest, microscopic leaf anatomical changes evaluation was made. Two circular sample sets of fresh leaves were cut from each of 5 biological replicates per replicate (15 samples per treatment) using a 1 cm diameter punch. One set of samples was used for the assessment of the stomatal and cellular densities, while another set of samples was fixed in a formalin-acetic-alcohol (FAA) solution (10% formaldehyde, 5% acetic acid, 50% ethanol, v/v in water) and used to observe the histological cell distribution in the leaf and determine the intercellular space.

2.10.1. Stomatal and Cellular Densities

Using fresh leaf tissue from the previous circular samples set and following the recommendation of [45], stomatal quantification was performed on the abaxial epidermis at the midsection of the leaf lamina, precisely positioned between the midrib and the leaf margin. A 5·5 mm leaf tissue was placed on a glass slide and gently scraped to facilitate optimal fixation of the neutral red staining. The samples were observed under a trinocular brightfield microscope (BA310, Motic, Hong Kong, China), photographed with a digital camera (Moticam 5.0 MP), and counted using the ImageJ software developed by NIH [46] following the recommendation by [35]. Stomatal density was expressed as stomata mm^{-2} and cellular density as cell mm^{-2}. Finally, the stomatal index (SI) was determined by employing the subsequent formula [45]. Values are expressed as means of five biological replicates per repetition. The analysis of 7 images contributed to the data obtained from each of the biological replicates.

$$\text{Stomatal Index (SI)} = (\text{Stomatal density}/(\text{Stomatal density} + \text{epidermal cell density})) \times 100 \quad (3)$$

2.10.2. Intercellular Space

FAA fixed leaf tissue from five biological replicates (15 plants per treatment) was processed as detailed in [35]. The samples underwent a washing process with a series of ethanol dilutions [47] and resin included using the JB-4 Embedding Kit (Sigma, Aldrich, Darmstadt, Germany). Using a manual microtome, 10 μm thick samples were cut and stained with periodic acid–Schiff (PAS) and aniline blue following the method by [48]. Briefly, the glass slides were immersed in 1% periodic acid for 10 min, washed, and stained with Schiff's reagent for 15 min. Later, the slides stayed for 2 min in 2% sodium sulfate

to eliminate possible interferences and improve the visibility of the stained structures. Finally, aniline blue-black was used as the counterstain. The samples were observed under a trinocular brightfield microscope with a 100X zoom lens. The images were taken with a digital camera and processed using the ImageJ software [46] to obtain total and intracellular areas [35]. The results were expressed as a percentage of intracellular space of the total area. The analysis of 10 images contributed to the data obtained from each of the biological replicates.

2.11. Statistical Analysis

The statistical analysis was conducted using InfoStat, a statistics software developed by Córdoba National University, Argentina [49]. The collected data underwent a multifactor analysis of variance (ANOVA). Statistically significant differences between means were determined at a significance level of $p < 0.05$, employing Fisher's least significant difference (LSD) test for pairwise comparison.

3. Results and Discussion

3.1. Effect of Successive Harvests on Green and Red Lettuces (Experiment 1)

3.1.1. Plant Growth Analytical Parameters

A significant effect was not on the 'Carmoli' FW harvests. At the third harvest, the value was 18.98 g, significantly higher than the 14.60 g reached in the second harvest. However, for 'Levistro', no differences were found among harvest times. There was also a significant effect of cutting for FW in 'Levistro'. Higher values in plants without previous cuts (wo = 22.66 g) compared to cut plants (w = 16.58 g) were found (Table 1). Conversely, there was a significant interaction between harvest time and cutting for 'Carmoli' FW and DW% (Table 1). The highest 'Carmoli' FW value was observed on the 3rd harvest without cutting (wo = 25.53 g). Moreover, an increase in DW% was observed at the same harvest with cut, reaching a value of 7.84 %, compared to 2nd and 3rd harvest without cutting, which had lower values. This rise in DW% may be due to the increase in lignin synthesis and other compounds triggered in response to the stress caused by cutting [33].

Table 1. Effect of three different harvest times and cutting on fresh weight (g) and dry weight content (%) for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	FW (g)		DW (%)	
		'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
Harvest time (1)	1st	(7.82)	(6.72)	(9.04)	(8.54)
	2nd	18.93	14.6	6.80 ^{b}	7.11
	3rd	20.31	18.98	8.64 ^{a}	7.12
Cutting (2)	With (w)	16.58 ^{b}	12.15	7.93	7.53
	Without (wo)	22.66 ^{a}	21.43	7.50	6.70
1·2	2nd·w	16.55	11.87 ^{c}	7.06	7.23 ^{b}
	3rd·w	16.61	12.42 ^{c}	8.80	7.84 ^{a}
	2nd·wo	21.31	17.32 ^{b}	6.54	6.99 ^{b}
	3rd·wo	24.00	25.53 ^{a}	8.47	6.41 ^{c}
p-value 1		ns	**	***	ns
p-value 2		***	***	ns	***
p-value 1·2		ns	**	ns	**

Harvest time: First harvest (1st) data presented in parentheses ( ), were incorporated as an initial reference value but not used for statistical analysis. As it was the first harvest, there were no plants that had been previously cut. For statistical analysis, the second (2nd) and third (3rd) harvests were used because data from with (w) and without (wo) cutting were obtained from both. FW: Fresh weight; DW: Dry weight. The reported values represent the means derived from 10 data points for harvest time and 15 data for cutting, while for the interaction (1·2), the means were calculated from 5 measurements. ^{a,b,c} Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, **: < 0.01, ***: < 0.001.

3.1.2. Color Parameters

The first experiment showed significant differences in color parameters in 'Levistro' at different harvest times. Luminosity (48.27 to 52.54) and hue angle (105.28 to 108.24°) significantly increased, whereas the chroma decreased (49.07 to 46.73) from the 2nd to 3rd harvest (Table 2). This 3-point increase in hue angle indicates a slight movement toward greener hues and away from yellow, and the highest chroma value reached in the second harvest indicated a more defined green color [37]. For 'Carmoli', there was a significant interaction between harvest times and cutting in luminosity. Similarly, significant changes were observed in the chroma by cutting (w: 16.73 and wo: 22.09), whereas the hue angle did not respond to harvest time nor cutting (Table 2). These results agree with [9] where it is commented that the differences in the color parameters are sensitive to the environmental conditions to which the plants are exposed.

Table 2. Effect of three different harvest times and cutting for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces on the luminosity, chroma, and hue angle color parameters.

Factor	Level	Luminosity		Chroma		Hue	
		'Levistro'	'Carmoli'	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
Harvest time (1)	1st	(46.47)	(4.80)	(51.11)	(17.49)	(106.23)	(22.54)
	2nd	48.27 ^{b}	6.32	49.07 ^{a}	18.90	105.28 ^{b}	25.14
	3rd	52.54 ^{a}	11.21	46.73 ^{b}	19.92	108.24 ^{a}	42.03
Cutting (2)	With (w)	48.54	12.25	49.05	16.73 ^{b}	106.76	33.61
	Without (wo)	49.65	5.29	48.90	22.09 ^{a}	106.40	26.19
1·2	2nd·w	47.62	6.05 ^{b}	47.85	16.42	105.06	22.79
	3rd·w	51.53	4.52 ^{b}	47.73	17.03	107.92	33.24
	2nd·wo	48.93	6.60 ^{b}	50.29	21.38	105.51	27.49
	3rd·wo	53.55	17.09 ^{a}	45.74	22.81	108.55	50.82
p-value 1		***	***	***	ns	***	ns
p-value 2		ns	***	ns	***	ns	ns
p-value 1·2		ns	***	ns	ns	ns	ns

Harvest times: First harvest (1st) was incorporated as an initial reference value but not used for the statistical analysis because no cutting plants were analyzed. For the statistical analysis, the second (2nd) and third (3rd) harvests were used because data from with (w) and without (wo) cutting were obtained from both. The reported values represent the means derived from 40 data points for harvest time and 60 data points for cutting, while for the interaction (1·2), the means were calculated from 20 measurements. ^{a,b} Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, ***: < 0.001.

3.1.3. Total Phenolic, Flavonoid, Anthocyanin Content, and Antioxidant Capacity

According to this experiment, TPC and TFC showed significant differences caused by the harvest time in 'Levistro'. For this cultivar, there was an increase of 373.71 mg GAE 100 g^{-1} FW on TPC, between the 2nd and 3rd harvests, representing an increase of 29%. Conversely, there was an increase from 737.69 to 1029.72 mg Rut eq 100 g^{-1} FW for TFC, showing an increase of about 40% (Table 3). On the other hand, a significant interaction between harvest time and cutting for TPC and TFC was found in 'Carmoli'. In both parameters, the highest values were recorded for the 3rd harvest with the previous cut (3rd x w), registering an increase of 46 and 62% for TPC and TFC, respectively, compared to the same harvest time without cutting (Table 3). In the same way, a higher TAC was found in 'Carmoli' plants with the previous cut, increasing almost 30% compared to plants without cutting (Table 3). Cutting some leaves of plants during cultivation could generate a response similar to herbivory, generating a greater production of phenolic compounds by increasing the activity of phenylalanine ammonium lyase (PAL) in the injured tissue [32]. Moreover, according to [33,34], phenols constitute one of the most common and widespread groups of defensive compounds, which could explain the differences registered as a result of cutting. Similarly, [50] demonstrated that there was an effect of the growth stage on

the concentration and composition of the phenolic fraction of sweet marjoram, and [51] reported that the nutritional value of lettuce depended to a great extent on the growth stage, which responds to the differences found as a result of harvest time. Furthermore, the evidently higher amounts of total phenols, flavonoids, and anthocyanins detected for the 'Carmoli' cultivar over the 'Levistro' cultivar confirmed once again the differences between red and green cultivars in the amount of antioxidant compounds [52,53].

Table 3. Effect of harvest and cutting on total phenolic (mg GAE 100 g^{-1} FW), flavonoid (mg Rut eq 100 g^{-1} FW), anthocyanin contents (mg Cyn3gluc eq 100 g^{-1} FW), and antioxidant capacity (AC: mg Trolox eq 100 g^{-1} FW) for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	Phenolics (TPC, mg GAE 100g^{-1} FW)		Flavonoids (TFC, mg Rut eq 100g^{-1} FW)		Anthocyanins (TAC, mg Cyn3gluc eq 100g^{-1} FW)	FRAP (mg Trolox eq 100 g^{-1} FW)		DPPH (mg Trolox eq 100 g^{-1} FW)	
		'Levistro'	'Carmoli'	'Levistro'	'Carmoli'	'Carmoli'	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
Harvest time (1)	1st	(1811.16)	(2944.71)	(953.04)	(1464.47)	(5.81)	(469.64)	(787.49)	(163.05)	(321.33)
	2nd	1280.44 ^{b}	2706.75	737.69 ^{b}	1398.22	5.41	334.97	752.39	131.21 ^{b}	269.36
	3rd	1654.15 ^{a}	2774.52	1029.72 ^{a}	1336.71	6.06	414.58	801.63	159.95 ^{a}	271.91
Cutting (2)	With (w)	1532.54	3057.98	928.39	1561.53	6.44 ^{a}	389.40	797.52	146.53	278.66
	Without (wo)	1402.06	2423.30	839.02	1173.39	5.04 ^{b}	360.14	756.50	144.62	262.61
1·2	2nd·w	1362.19	2821.10 ^{b}	797.06	1471.56 ^{b}	5.89	326.93 ^{b}	732.91 ^{b}	132.51	266.74 ^{c}
	3rd·w	1702.89	3294.86 ^{a}	1059.71	1651.51 ^{a}	7.00	451.88 ^{a}	862.13 ^{a}	160.55	290.59 ^{a}
	2nd·wo	1198.70	2592.41 ^{b}	678.32	1324.87 ^{c}	4.94	343.01 ^{b}	771.87 ^{ab}	129.90	271.99 ^{b}
	3rd·wo	1605.42	2254.19 ^{c}	999.72	1021.90 ^{d}	5.13	377.28 ^{b}	741.13 ^{b}	159.34	253.23 ^{d}
p-value 1		**	ns	***	ns	ns	***	ns	***	***
p-value 2		ns	***	ns	***	*	ns	ns	ns	ns
p-value 1·2		ns	***	ns	***	ns	*	*	ns	***

Harvest time: The first (1st) was incorporated as the initial reference value, second (2nd), and third (3rd). TPC: Total phenolic content; TFC: total flavonoid content; TAC: Total anthocyanin content; AC: Antioxidant capacity; FRAP: Ferric reducing antioxidant power; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: Gallic acid equivalent; Rut eq: Rutin equivalents; Cyn3gluc Eq: Cyanidin-3-glucoside equivalents; Trolox eq: Trolox equivalent; FW: Fresh weight. The reported values represent the means derived from 6 data points for harvest time and 9 for cutting, while for the interaction (1·2), the means were calculated from 3 measurements. ^{a,b,c,d} Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, *: < 0.05, **: < 0.01, ***: < 0.001.

Regarding the antioxidant capacity (CA) 'Levistro' cultivar had a lower CA than the 'Carmoli' cultivar, following a typical behavior of green and red lettuces [9,42]. AC (by DPPH) showed the same behavior as TPC and TFC for 'Levistro', presenting differences caused by subsequent harvests. On the 3rd harvest, an increase of about 22% compared with the 2nd harvest was found (Table 3). An expected behavior could be due to the antioxidant activity of compounds, such as phenols and flavonoids [54]. In this green cultivar, AC (by FRAP) had a significant interaction between harvest time and cutting. The highest value was observed at 3rd harvest with cutting (451.88 mg Trolox eq 100 g^{-1} FW), reaching an increase of 38% compared to the lower value, registered in the 2nd harvest with cutting (326.93 mg Trolox eq 100 g^{-1} FW), and it was higher than the values reported by [42] for green lettuces. For 'Carmolí', ACs measured by FRAP and DPPH showed significant interactions between harvest time and cutting. The highest value for DPPH was reached at the 3rd harvest with cutting (290.59 mg Trolox eq 100 g^{-1} FW). Despite showing a trend similar to TPC and TFC, the increase was about 15% compared with the lowest value, registered at the 3rd harvests without cutting (253.23 mg Trolox eq 100 g^{-1} FW). Similarly, the highest value obtained by FRAP (862.13 mg Trolox eq 100 g^{-1} FW) was also recorded at the 3rd harvest with cutting (Table 3) a value that corresponds to the range reported by [42] for Lollo Rosso lettuces. These results confirm the antioxidant response to cutting and the phenological age described by [32,51]

3.2. Effect of UV-B Radiation and Harvest Times on Green and Red Lettuces (Experiment 2)

3.2.1. Plant Growth Analytical Parameters

The application of UV-B radiation during culture and at different harvest times independently affected both FW and DW% for 'Levistro'. However, only different harvest times affected these same parameters in 'Carmolí' (Table 4). At successive harvests, FW and DW% increased in both cultivars. In fact, an increase from 4.64 to 15.52 g, which represents a 230% increase, was observed between the first and third harvest for 'Levistro' FW (Table 4). Meanwhile, 'Carmoli' showed an increase from 3.34 to 4.66 g (41%) between the same harvest times. In the case of DW%, for 'Levistro' and 'Carmoli', an increase from 6.55 to 6.72% and 5.80 to 7.46%, respectively, were observed between the first and third harvests. These significant differences in FW and DW% among plant stages by harvest time and UV-B were consistent with [27]. This author revealed that 2 and 4 kJ m^{-2} day^{-1} UV-B significantly affected the early growth stage of *Ocimum basilicum*, reaching differences of 2.7 g in FW and 0.24 g in DW, for the 3 to 4 leaf pair stage and lower differences of 1.4 g in FW and 0.15 g in DW for the flowering stage.

Table 4. Effect UV-B radiation and three different harvest times on fresh weight (g) and dry weight content (%) for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	FW (g)		DW (%)	
		'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
Harvest time (1)	1st	4.64 ^{c}	3.34 ^{b}	6.55 ^{a}	5.80 ^{b}
	2nd	9.55 ^{b}	4.84 ^{a}	6.01 ^{b}	5.83 ^{b}
	3rd	15.52 ^{a}	4.66 ^{a}	6.72 ^{a}	7.46 ^{a}
UV-B Doses (2)	0 kJ m^{-2}	10.93 ^{a}	4.09	6.30 ^{b}	6.30
	5.2 kJ m^{-2}	10.12 ^{b}	4.33	6.36 ^{b}	6.38
	10.5 kJ m^{-2}	8.66 ^{c}	4.42	6.62 ^{a}	6.41
p-value 1		***	***	***	***
p-value 2		***	ns	**	ns
p-value 1·2		ns	ns	ns	ns

UV-B doses: 0 kJ m^{-2} (control); 5.2 kJ m^{-2} and 10.5 kJ m^{-2}; harvest times: First (1st), second (2nd), and third (3rd). FW: Fresh weight; DW: Dry weight. The reported values represent the means derived from 27 data points for harvest time and UV-B doses. ^{a,b,c} Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, **: < 0.01, ***: < 0.001.

On the other hand, for 'Levistro' FW UV-B treatment decreased by 20% between the highest UV-B treatment of 10.5 kJ m^{-2} and the control (0 kJ m^{-2}) (Table 4). By contrast, an increase in DW% exhibited a consistent response with the accumulation of dry matter against abiotic stress. For 'Carmoli' under UV-B treatment, neither FW nor DW% showed significant changes between doses, with the values varying from 4.09 to 4.42 g and 6.30 to 6.41% for FW and DW%, respectively (Table 4). These results differed from those obtained by [26], who reported that Lollo Rosso lettuces strongly responded to reducing vegetative growth at high UV levels. These authors reported 21 and 27 leaf numbers when lettuces were grown under 81% and 0% UV 280–400 nm transmission, respectively. They also found that under a complete UV blocking film (0% UV 280–400 nm transmission), Lollo Rosso reached between 40 and 122% more total dry weight than plants under the UV transparent treatment (81% UV 280–400 nm transmission).

3.2.2. Color Parameters

Several researchers have observed a positive correlation between alterations in color and phenolic concentrations in both green and red lettuce cultivars grown under diverse environmental conditions. Therefore, color parameter measurements must be considered [9,55,56]. UV-B radiation had a significant decrease in luminosity in 'Levistro', showing that treated leaves by 5.2 or 10.5 kJ m^{-2} UV-B were darker than the control (Table 5). Moreover, there was a significant effect of harvest time on the chroma and its behavior

depending on the cultivar. 'Levistro' reached the highest value at the second harvest (55.82), reaching the most intense color, while the lowest value was registered at the first harvest (Table 5). By contrast, 'Carmoli' showed the lowest values in the second harvest (5.06), exhibiting the least intense color. There was also a harvesting effect on the hue angle for 'Levistro', where the highest values were found at the first harvest, reaching 105.74°. However, there was no significant effect of UV-B or harvest time on the hue angle for 'Carmoli' (Table 5). As expected, 'Levistro' presented hue angle values belonging to green and 'Carmoli' values closer to red [37] in accordance with these types of lettuces. Finally, [29] described coloration changes in plants under excessive UV-B radiation, beginning from bronze or brown spots to chlorosis, necrosis, and desiccation of the leaves. In this sense, a few bronze spots were observed on Lollo Bionda lettuce, and green blemishes were observed on Lollo Rosso lettuce caused by UV-B at the highest tested UV-B radiation doses (10.5 kJ m^{-2}).

Table 5. Effect of UV-B radiation and three different harvest times on color parameters (luminosity, chroma, and hue angle) for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	Luminosity		Chroma		Hue	
		'Levistro'	'Carmoli'	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
Harvest time (1)	1st	40.74	1.78 ^{a}	50.03 ^{c}	9.51 ^{a}	105.74 ^{a}	16.27
	2nd	42.15	0.94 ^{b}	55.82 ^{a}	5.06 ^{b}	104.54 ^{b}	15.59
	3rd	40.43	1.89 ^{b}	52.87 ^{b}	8.78 ^{a}	104.75 ^{b}	15.22
UV-B Doses (2)	0 kJ m^{-2}	42.75 ^{a}	1.61	52.69	8.63	105.10	15.96
	5.2 kJ m^{-2}	40.55 ^{b}	1.74	53.82	7.83	105.08	15.87
	10.5 kJ m^{-2}	40.02 ^{b}	1.28	52.20	6.89	104.87	15.26
p-value 1		ns	**	***	***	***	ns
p-value 2		**	ns	ns	ns	ns	ns
p-value 1·2		ns	ns	ns	ns	ns	ns

UV-B doses: 0 kJ m^{-2} (control); 5.2 kJ m^{-2} and 10.5 kJ m^{-2}; harvest times: First (1st), second (2nd), and third (3rd). Values are means of 54 data points per harvest time and UV-B doses. ^{a,b,c} Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, **: < 0.01, ***: < 0.001.

In regions near the equator, the daily UV-B radiation levels typically range between 0 and 12 kJm^{-2} [57]. Although, in these study, lower doses were used (1.05 kJ m^{-2} d^{-1}), certain studies conducted in growth chambers and greenhouses, characterized by low PAR, low UV-A, and high UV-B conditions, have shown an increased vulnerability to UV-B damage [57]. These observations can explain the spots on some leaves and can be associated with the specific season in which the experiments were carried out (autumn), highlighting the influence of seasonal variations on plant responses to UV-B radiation.

3.2.3. Total Phenolic, Flavonoid, and Anthocyanin Contents, and Antioxidant Capacity

For this experiment, the harvest time showed a significant effect on all antioxidant compound measurements for both cultivars. In the case of TPC, 'Levistro' and 'Carmoli' increased by 31% (from 207.04 to 271.91 mg GAE 100 g^{-1} FW) and 51% (from 430.85 to 650.73 mg GAE 100 g^{-1} FW) between the 1st and 3rd harvest (Table 6). These TPC values for 'Levistro' were higher than those between 18 and 125 mg 100 g^{-1} FW found by [42], and higher than those measured by [54] between 30 and 140 mg GAE 100 g^{-1} FW for green lettuces. For 'Carmoli', TPC values were like those reported by [42] for Lollo Rosso lettuce, where they determined a range between 259 and 571 mg 100 g^{-1} FW. There was no significant effect of UV-B on TPC, nor green or red cultivars (Table 6).

Table 6. Effect of UV-B radiation and three different harvest times on total phenolic (mg GAE 100 g^{-1} FW), flavonoid (mg Rut eq 100g^{-1} FW), anthocyanin contents (mg Cyn3gluc eq 100 g^{-1} FW), and antioxidant capacity (AC: mg Trolox eq 100 g^{-1} FW) for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	Phenolics (TPC, mg GAE 100 g^{-1} FW)		Flavonoids (TFC, mg Rut eq 100 g^{-1} FW)		Anthocyanins (TAC, mg Cyn3gluc eq 100 g^{-1} FW)	FRAP (mg Trolox eq 100 g^{-1} FW)		DPPH (mg Trolox eq 100 g^{-1} FW)	
		'Levistro'	'Carmoli'	'Levistro'	'Carmoli'	'Carmoli'	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
Harvest time (1)	1st	207.04 ^{b}	430.85 ^{c}	692.18	1130.18 ^{c}	5.22 ^{c}	273.63 ^{b}	891.91 ^{b}	239.05 ^{b}	289.25 ^{c}
	2nd	222.70 ^{b}	505.72 ^{b}	813.02	1595.79 ^{b}	7.43 ^{b}	305.09 ^{b}	782.91 ^{b}	267.59 ^{b}	334.77 ^{b}
	3rd	271.91 ^{a}	650.73 ^{a}	1069.67	2214.71 ^{a}	8.61 ^{a}	398.84 ^{a}	1075.08 ^{a}	333.70 ^{a}	419.71 ^{a}
UV-B Doses (2)	0 kJ m^{-2}	217.99	500.69	776.92	1522.25 ^{b}	6.57 ^{b}	310.88	893.34	256.35	318.34 ^{b}
	5.2 kJ m^{-2}	247.40	537.35	906.07	1681.16 ^{a}	7.63 ^{a}	342.36	929.15	300.07	351.81 ^{a}
	10.5 kJ m^{-2}	236.26	549.26	891.89	1737.27 ^{a}	7.06 ^{ab}	324.31	927.41	283.91	373.58 ^{a}
1·2	1st·0	198.16	407.10	657.92 ^{de}	993.95	5.02	261.97	873.00	223.98	257.33
	2nd·0	219.44	484.58	792.31 ^{cde}	1560.72	6.85	316.16	757.57	267.26	314.37
	3rd·0	236.37	610.38	880.54 ^{bc}	2012.09	7.84	354.52	1049.44	277.82	383.31
	1st·5.2	232.81	430.78	805.61 ^{cde}	1142.24	5.37	314.62	904.55	270.52	284.99
	2nd·5.2	228.13	515.23	841.10 ^{cd}	1615.40	8.01	315.40	805.32	279.16	339.48
	3rd·5.2	281.25	666.04	1071.48 ^{ab}	2285.83	9.51	397.06	1077.59	350.54	430.97
	1st·10.5	190.15	454.68	613.02 ^{e}	1254.33	5.29	244.30	898.20	222.64	325.43
	2nd·10.5	220.52	517.33	805.65 ^{cde}	1611.25	7.43	283.71	785.83	256.35	350.47
	3rd·10.5	298.10	675.77	1257.0 ^{a}	2346.21	8.46	444.93	1098.20	372.74	444.85
p-value 1		***	***	***	***	***	***	**	***	***
p-value 2		ns	ns	ns	*	*	ns	ns	ns	**
p-value 1·2		ns	ns	*	ns	ns	ns	ns	ns	ns

UV-B doses: 0 kJ m^{-2} (control); 5.2 kJ m^{-2} and 10.5 kJ m^{-2}; harvest time: First (1st), second (2nd), and third (3rd). TPC: Total phenolic content; TFC: Total flavonoid content; TAC: Total anthocyanin content; AC: Antioxidant capacity; FRAP: Ferric reducing antioxidant power; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: Gallic acid equivalent; Rut eq: Rutin equivalents; Cyn3gluc Eq: Cyanidin-3-glucoside equivalents; Trolox eq: Trolox equivalent; FW: Fresh weight. The reported values represent the means derived from 27 data points for harvest time and UV-B doses, while for the interaction (1·2), the means were calculated from 9 measurements. ^{a,b,c,d,e} Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, *: < 0.05, **: < 0.01, *** < 0.001.

The synthesis and accumulation of phenolic compounds, especially flavonoids, and anthocyanins yield commonly observed responses in plants when exposed to UV-B radiation by increasing the enzimatic activity of the phenylpropanoid pathway [15,17,19,58], particularly in red lettuces [26,56]. In this context, for total flavonoid content (TFC) on 'Levistro', a noteworthy interaction between harvest times and UV-B radiation factors was found. The peak concentration of flavonoids was identified during the third harvest in the plants treated with UV-B radiation with values between 1071.48 and 1257.0 mg Rut eq 100 g^{-1} FW (Table 6). This represents an increase of 91% when 10.5 kJ m^{-2} UV-B treatment at the 3rd harvest was compared with the control (0 kJ m^{-2}) at the 1st harvest. Moreover, these flavonoid concentrations were in accordance with those found by [54] for green lettuces, who reported between 900 and 3300 mg QE 100 g^{-1} FW.

The TFC and TAC in red lettuce showed an independent increased response by following harvest or higher UV-B doses. The highest TFC values were in plants at the 3rd harvest, with 2214.71 mg Rut eq 100 g^{-1} FW or when UV-B dose from 5.2 kJ m^{-2} (1681.16 mg Rut eq 100 g^{-1} FW) to 10.5 kJ m^{-2} (1737.27 mg Rut eq 100 g^{-1} FW) were used (Table 6). In this context, 'Carmoli' showed an increase of 95% in total flavonoids between the 1st and 3rd harvest and only between 10.4 and 14.0% when 5.2 and 10.5 kJ m^{-2} were compared with the control (0 kJ m^{-2}) confirming the results presented by [19,26] who show that UV can induce the synthesis of anthocyanins and other flavonoids. Nevertheless, even the lowest recorded value of flavonoids in this study was higher than the values reported by [7] for Lollo Rosso lettuce. These authors found between 4 and 280 mg quercetin 3-glucoside 100 g^{-1} FW for this type of lettuce, showing that 'Carmoli' was a richer flavonoid cultivar. Moreover, total anthocyanin content (TAC) increased from 5.22 to 8.61 mg Cyn3gluc eq 100 g^{-1} FW, by 65% between 1st and 3rd harvest, showing an important response against harvest time. As mentioned above, growth stage has an effect

on the concentration and composition of the phenolic fraction of sweet marjoram [50], and the nutritional value of lettuce was strongly dependent on the growth stage [51], as observed in this trial.

Both flavonoid and anthocyanin biosynthesis were involved in UV-B acclimation [15,19,24] also [26] described an important accumulation of anthocyanins promoted by UV-B wavelength, showing that TAC of plants grown under UV-blocking film was eight times lower compared to plants grown under a UV-transparent film. However, in this experiment, there was an increase in TAC of only 16% between the control (0 kJ m^{-2}) and 5.2 kJ m^{-2} UV-B, which achieved 7.63 mg Cyn3gluc eq 100 g^{-1} FW, the highest anthocyanin concentration found (Table 6). Although this increase does not seem to be so relevant, we must consider that the response to UVB radiation is affected by the intensity of PAR and UV-A wavelengths [57]. Despite this, the TAC values were consistent with the range collected by [7] for Lollo Rosso lettuces, which showed values between 2 and 100 mg cyanidin 100 g^{-1} FW.

Antioxidant capacity (AC) measured by FRAP had a behavior similar to TPC, showing differences between harvest times (Table 6). In both cultivars, the values increased from the first to the third harvest. The increase was about 125 mg Trolox eq 100 g^{-1} FW for 'Levistro' and about 183 mg Trolox eq 100 g^{-1} FW for 'Carmoli' (Table 5), which represents an increase in AC by FRAP of 45 and 20%, respectively. In the same vein, harvest time influenced AC (by DPPH) in the 'Levistro' and 'Carmoli' cultivars. Harvests from the first to the third enhanced AC by DPPH in 94.65 and 130.46 mg Trolox eq 100 g^{-1} FW, yielding an increase of 39 and 44% for 'Levistro' and 'Carmoli', respectively (Table 6). In the case of UV-B radiation, AC (by DPPH) was only affected for 'Carmoli', increasing 55.24 mg Trolox eq 100 g^{-1} FW from 0 to 10.5 kJ m^{-2} (17%) and showing behavior similar to total flavonoid and anthocyanin content. This result could explain the strong relationship between total anthocyanins and the antioxidant capacity of 'Carmoli'. According to [59], cyanidin 3-malonylglucoside is an anthocyanin present in red lettuce with a high antioxidant capacity, representing about 15% of the total antioxidant activity in this type of lettuce.

3.2.4. Total Proline Content

Proline is an amino acid that acts as an organic osmoprotectant, metal chelator, inhibitor of lipid membrane peroxidation, and ROS scavenger [31]. It is accumulated when the plant needs osmotic adjustment and plays an important role in stress conditions [60–62]. Ref. [60] described that osmolyte accumulation as proline was also involved in the response to UV-B radiation. According to [31], in lettuce, the addition of exogenous proline affects the accumulation of endogenous hormones and improves adaptation to UV-B stress. However, for both green and red cultivars, proline concentration was not significantly increased by UV-B. However, a significant reduction in proline accumulation due to harvest time was found (Table 7). For 'Levistro', a significant decrease was observed at the third harvest (16.02 µg 100 g^{-1} FW) compared to the first one (52.65 µg 100 g^{-1} FW) (Table 7). Similarly, 'Carmoli' also had a significant decline from the first to the third harvest, from 14.82 to 7.04 µg 100 g^{-1} FW. In line with this finding, [63] reported that the concentration of proline in peas was higher in younger and undeveloped leaves compared to older and well-developed leaves. The researchers emphasized that the maximum proline content in the youngest leaves corresponded to the highest transcript levels of two genes, PsP5CS1 and PsP5CS2, which encode enzymes involved in the synthesis of D1-pyrroline-5-carboxylate, a precursor of proline. By contrast, the mRNA expression of PsPDH1, a gene responsible for encoding proline dehydrogenase, which participates in proline degradation, was lower in younger than older leaf stages, showing contrasting patterns. Therefore, this gene expression could explain the highest amount of proline found at the 1st harvest in green and red lettuces.

Table 7. Impact of UV-B radiation and three distinct harvest times on the proline content (μg·100 g^{-1} FW) for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	Proline (μg·100 g^{-1} FW)	
		'Levistro'	'Carmoli'
Harvest time (1)	1st	52.65 [a]	14.82 [a]
	2nd	13.46 [b]	7.56 [b]
	3rd	16.02 [b]	7.04 [b]
UV-B Doses (2)	0 kJ·m^{-2}	26.24	9.95
	5.2 kJ·m^{-2}	28.86	10.48
	10.5 kJ·m^{-2}	27.02	9.00
p-value 1		***	***
p-value 2		ns	ns
p-value 1·2		ns	ns

UV-B doses: 0 kJ m^{-2} (control); 5.2 kJ m^{-2} and 10.5 kJ m^{-2}; harvest time: First (1st), second (2nd), and third (3rd). FW: Fresh weight. The reported values represent the means derived from 27 data points for harvest time and UV-B doses. [a,b] Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, ***: < 0.001.

3.2.5. Stomatal, Cellular Density, and Intercellular Space

UV-B radiation can modify plant morphology. The observed effects included an increase in leaf thickness, stimulation of axillary branching, and a decrease in hypocotyl length leaf area and stomatal frequencies [30,31]. In this context, stomatal density on 'Levistro' showed a significant interaction between harvest time and UV-B treatment, indicating their combined influence on the outcome and reaching the highest values of 67.1 stomata mm^{-2} at 3rd harvest with 5.2 and 10.5 kJ m^{-2} (Table 8). Conversely, for 'Carmoli', there was only an effect by UV-B radiation, which increased by 22% on stomatal density at 5.2 compared to 0 kJ m^{-2}. UV-B radiation also generated changes in cell size and shape by modifying the cytoskeleton [28]. In this context, both factors, harvest time and UV-B, had a significant effect on the cell density of green and red lettuces (Table 8). 'Levistro' registered an increase from 432.7 to 682.2 cell m^{-2} between the first and third harvests representing a 57%increase. On the other hand, 'Carmoli' recorded the highest value at the second harvest, reaching 923 cells m^{-2}. Moreover, 'Levistro' and 'Carmoli', reached the highest cell counts at 10.5 kJ m^{-2} of 636.6 and 916.8 cells mm^{-2}, respectively (Table 8). Finally, the stomatal index for 'Levistro' significantly decreased after the 1st harvest, but UV-B radiation had no significant impact on this parameter (Table 8). Meanwhile, in 'Carmoli', the stomatal index responded independently to harvest time and UV-B and reached the lowest values at 2nd harvest and 10.5 kJ m^{-2} (Table 8), showing a cultivar-dependent response.

At the end of the experiment and after the UV-B application, leaves showed no evidence of visual histological changes. The intercellular space (is) palisade (pp) and spongy (sp) parenchymal tissue maintained apparently normal in 'Levistro' (Figure 3) but not in 'Carmoli' (Figure 4). After the image analysis, it was shown that percentages of intercellular space of leaf tissue of green and red lettuce were affected by the UV-B radiation studied (Table 9). In fact, among cultivars, the response to UV-B was the opposite, and 'Levistro' registered the highest intercellular percentage (37.07%) at 10.5 kJ m^{-2}, while 'Carmoli' had the lowest percentage (8.25%) at the same dose (Table 9). Therefore, these results indicate that the different cultivars, particularly green and red leaves, had contrasting cell distribution responses as a strategy to protect themselves against UV-B radiation.

Table 8. Impact of UV-B radiation and three distinct harvest times on stomatal density, cellular density, and stomatal index for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	Stomatal Density Stomata mm^{-2}		Cellular Density Cell mm^{-2}		Stomatal Index	
		'Levistro'	'Carmoli'	'Levistro'	'Carmoli'	'Levistro'	'Carmoli'
Harvest time (1)	1st	44.8	58.0	432.7 ^{c}	715.4 ^{b}	9.4 ^{a}	8.0 ^{a}
	2nd	45.6	51.4	546.3 ^{b}	923.4 ^{a}	7.8 ^{b}	5.3 ^{b}
	3rd	59.7	54.7	682.2 ^{a}	719.5 ^{b}	8.1 ^{b}	7.2 ^{a}
UV-B Doses (2)	0 kJ m^{-2}	43.9	51.4 ^{b}	527.2 ^{b}	695.5 ^{b}	7.8	7.1 ^{a}
	5.2 kJ m^{-2}	50.6	63.0 ^{a}	497.4 ^{b}	746.0 ^{b}	9.2	8.0 ^{a}
	10.5 kJ m^{-2}	55.5	49.7 ^{b}	636.6 ^{a}	916.8 ^{a}	8.2	5.4 ^{b}
1·2	1st·0	44.8 ^{bc}	59.7	455.1	567.0	8.9	9.5
	2nd·0	42.3 ^{bc}	44.8	489.9	850.5	7.9	4.9
	3rd·0	44.8 ^{bc}	49.7	636.6	669.0	6.6	6.9
	1st·5.2	47.2 ^{bc}	59.7	397.9	666.5	10.6	8.4
	2nd·5.2	37.3 ^{c}	64.7	457.6	937.5	7.6	6.6
	3rd·5.2	67.1 ^{a}	64.7	636.6	634.1	9.3	9.0
	1st·10.5	42.3 ^{bc}	54.7	445.1	912.7	8.7	6.2
	2nd·10.5	57.2 ^{ab}	44.8	691.3	982.3	7.9	4.5
	3rd·10.5	67.1 ^{a}	49.8	773.4	855.4	8.2	5.6
p-value 1		**	ns	***	***	*	***
p-value 2		ns	*	***	***	ns	***
p-value 1·2		*	ns	ns	ns	ns	ns

UV-B doses: 0 kJ m^{-2} (control); 5.2 kJ m^{-2} and 10.5 kJ m^{-2}; harvest time: First (1st), second (2nd), and third (3rd). The reported values represent the means derived from 15 data points for harvest time and UV-B doses, while for the interaction (1·2), the means were calculated using 5 biological replicates. The analysis of 7 images contributed to the data obtained from each of the biological replicates. ^{a,b,c} Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, *: < 0.05, **: < 0.01, *** < 0.001.

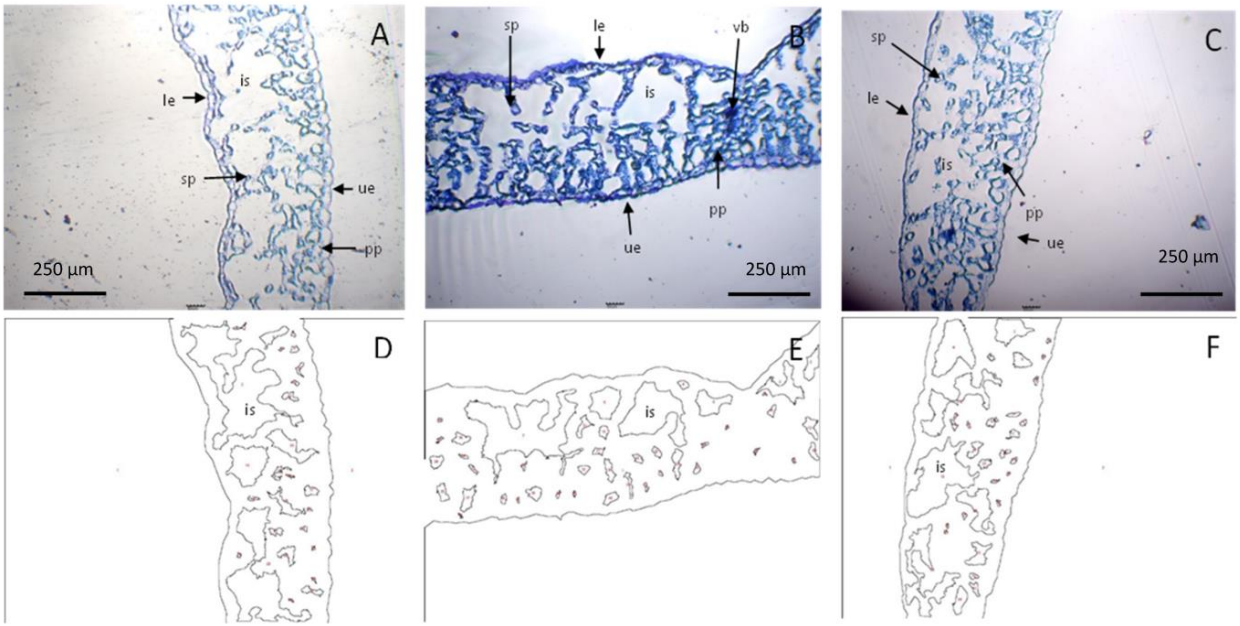

Figure 3. (**A**–**C**) Transverse sections of leaf tissue were captured using an optical microscope at 100X magnification, showcasing 'Levistro' cultivar under 0 kJ m^{-2} (**A**), 5.2 kJ m^{-2} (**B**) and 10.5 kJ m^{-2} (**C**) UV-B growth conditions. (**D**–**F**) correspond to ImageJ processed images for A, B and C. ue: Upper epidermis; le: Lower epidermis; pp: Palisade parenchyma; sp: Spongy parenchyma, is: Intercellular spaces; vb: Vascular bundle.

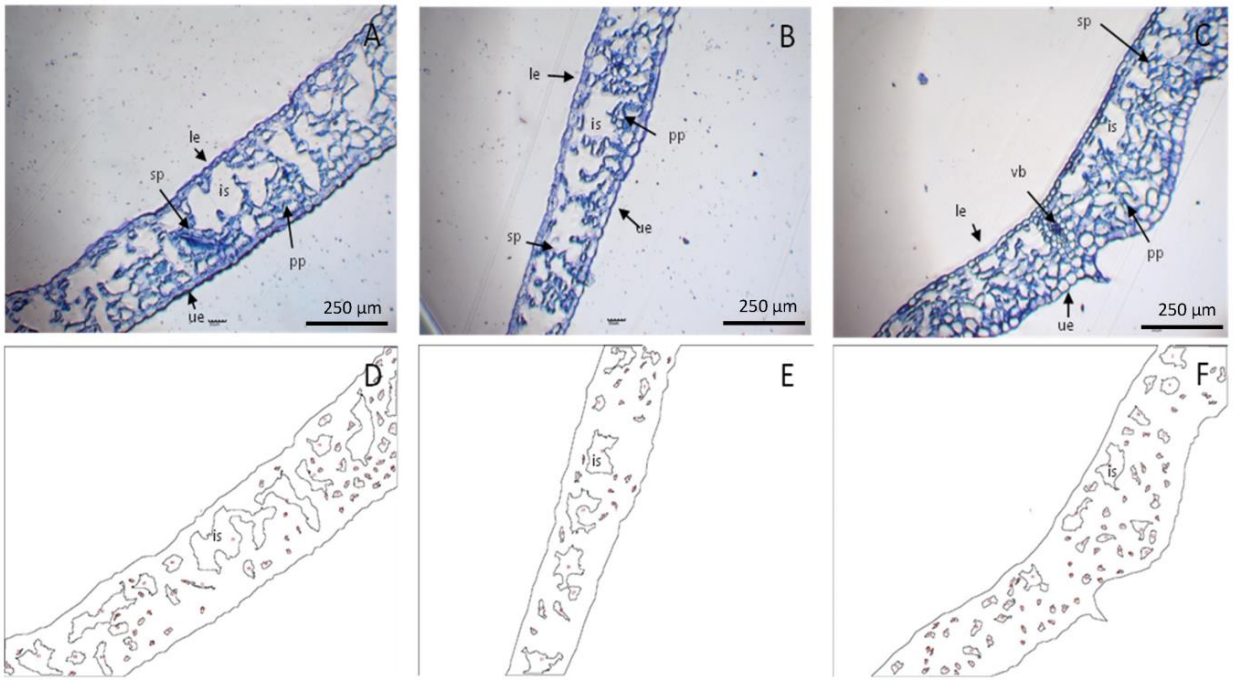

Figure 4. (**A**–**C**) Transverse sections of leaf tissue were captured using an optical microscope at 100X magnification, showcasing 'Carmoli' cultivar under 0 kJ m^{-2} (**A**), 5.2 kJ m^{-2} (**B**), and 10.5 kJ m^{-2} (**C**) UV-B growth conditions. (**D**–**F**) correspond to ImageJ processed images for A, B and C. ue: Upper epidermis; le: Lower epidermis; pp: Palisade parenchyma; sp: Spongy parenchyma, is: Intercellular spaces; vb: Vascular bundle.

Table 9. Effect of UV-B radiation at the third harvest time on intercellular space (%) for 'Levistro' Lollo Bionda and 'Carmoli' Lollo Rosso lettuces.

Factor	Level	Intercellular Space (% Total Area)	
		'Levistro'	'Carmoli'
UV-B Doses	0 kJ m^{-2}	24.02 ^{b}	17.17 ^{a}
	5.2 kJ m^{-2}	19.57 ^{b}	16.17 ^{a}
	10.5 kJ m^{-2}	37.07 ^{a}	8.25 ^{b}
p-value		***	

UV-B doses: 0 kJ m^{-2} (control); 5.2 and 10.5 kJ m^{-2}. The reported values represent the means derived from 150 processing image data points. Different letters represent significant differences as determined by Fisher's least significant difference (LSD) test at a significance level of $p < 0.05$. ns: Not significant, ***: < 0.001.

4. Conclusions

Cultural practices, such as cutting made by successive harvests and UV-B supplementation, have been found to have a significant impact on the fresh and dried weights of lettuce leaves, specifically the 'Levistro' and 'Carmoli' varieties. These practices also play a crucial role in stimulating the synthesis of TPC, TFC, and AC, resulting in their enhanced accumulation in both lettuce cultivars. TFC in 'Levistro' increased considerably in response to the use of a combined strategy of UV-B radiation and cutting made by successive harvests, while for 'Carmoli', each strategy by itself was able to increase the concentration of antioxidant compounds. By implementing these strategies, it becomes possible to cultivate specific lettuce varieties with elevated concentrations of phenolic compounds, offering a promising avenue for obtaining antioxidant-rich vegetable products. Moreover, depending on the stage of leaf development, notable variations in the accumulation of TPC, TFC, and TAC in the 'Carmoli' cultivar were observed, further highlighting the great potential of

this approach to achieve optimal functional product content. Moreover, it is noteworthy that different lettuce cultivars, such as the Lollo Bionda and Lollo Rosso varieties, display diverse anatomical cell distribution strategies in response to UV-B stress. Specifically, the 'Levistro' cultivar demonstrates an increase in intercellular space, whereas the 'Carmoli' cultivar exhibits a reduction in intercellular space. To cultivate vegetables with improved health-promoting characteristics, the implementation of precise, controlled agricultural strategies is essential. It is crucial to consider the potential variability in response among different cultivars to the same stimulus, particularly when employing different color varieties. This recognition of variability allows for tailored cultivation approaches that optimize the desired traits and outcomes in the specific cultivar being cultivated.

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