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Changes in the Flavonoid and Phenolic Acid Contents and Antioxidant Activity of Red Leaf Lettuce (Lollo Rosso) Due to Cultivation under Plastic Films Varying in Ultraviolet Transparency

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Red leaf lettuce (Lollo Rosso) was grown under three types of plastic films that varied in transparency to UV radiation (designated as UV block, UV low, and UV window). Flavonoid composition was determined by high-performance liquid chromatography (HPLC), total phenolics by the Folin−Ciocalteu assay, and antioxidant capacity by the oxygen radical absorbance capacity (ORAC) assay. Exposure to increased levels of UV radiation during cultivation caused the leaves to redden and increased concentrations of total phenols and the main flavonoids, quercetin and cyanidin glycosides, as well as luteolin conjugates and phenolic acids. The total phenol content increased from 1.6 mg of gallic acid equivalents (GAE)/g of fresh weight (FW) for lettuce grown under UV block film to 2.9 and 3.5 mg of GAE/g of FW for lettuce grown under the UV low and UV window films. The antioxidant activity was also higher in lettuce exposed to higher levels of UV radiation with ORAC values of 25.4 and 55.1 μmol of Trolox equivalents/g of FW for lettuce grown under the UV block and UV window films, respectively. The content of phenolic acids, quantified as caffeic acid, was also different, ranging from 6.2 to 11.1 μmol/g of FW for lettuce cultivated under the lowest and highest UV exposure plastic films, respectively. Higher concentrations of the flavonoid glycosides were observed with increased exposure to UV radiation, as demonstrated by the concentrations of aglycones after hydrolysis, which were cyanidin (ranging from 165 to 793 μg/g), quercetin (ranging from 196 to 880 μg/g), and luteolin (ranging from 19 to 152 μg/g). The results demonstrate the potential of the use of UV-transparent plastic as a means of increasing beneficial flavonoid content of red leaf lettuce when the crop is grown in polytunnels.

KEYWORDS: Anthocyanins; flavonoids; lettuce; luteolin; ultraviolet

INTRODUCTION

Epidemiological and intervention studies have provided evidence of beneficial health effects of dietary fruits and vegetables, and the beneficial effects have been attributed at least in part to secondary metabolites, including flavonoids and hydroxycinnamic acids (*1*). Effects of selected flavonoids in reducing the risk of various diseases including cardiovascular disease, cancer, atherosclerosis, and other age-related diseases have been demonstrated (*2, 3*), and it is thought that these phytochemicals may provide health benefits as antioxidants or by other mechanisms including effects on gene expression or cell signaling (*4, 5*). Therefore, increasing the levels of phenolic compounds in food plants has potential for improving the health of the population.

The accumulation of secondary products in plants depends on both external and internal factors. Intervarietal genetic variation is in general more important than environmental influences (compare refs *6*, *7*, and *8*), but agronomic factors can also influence the phytochemical content of plants (*9*). In this context, variation in light exposure has been the focus of increased attention. The salad crop *Gynura bicolor* had lower levels of flavonoids when grown in a glasshouse, protected against UV-B light, compared to open field conditions (*10*). In

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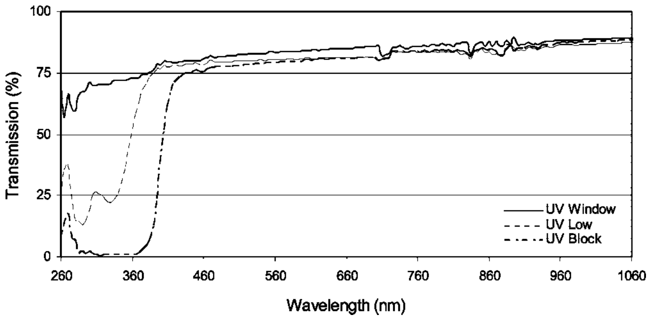

Figure 1. Spectral properties of filters.

lettuce, whenever the effect of light on the phenolic content has been investigated (*6–9*, *11*, *12*), leaves exposed to higher light levels have been found to have higher concentrations of flavonoids.

Lettuce is a widely available crop; production worldwide increased by 17% between 2000 and 2004 (*13*). In temperate countries, lettuce is increasingly being grown in polythene tunnels to extend the seasonal availability and enhance crop quality. The present study describes the effect of cultivation of lettuce under three types of plastic, varying in UV transparency, on the concentration of the main phenolic components and the antioxidant activity. The standard plastic film currently used by soft fruit growers in Europe filters UV light, and similar films are increasingly being used by lettuce growers. The effects of replacing this film with a UV blocking (UV block) or UV transparent film (UV window) were studied. This paper represents a rigorous and detailed study of the effect of UV radiation on several classes of phenolic compounds in lettuce (Lollo Rosso type, cv. Revolution), and it is the first report of the effects of exposure to different levels of UV radiation on the antioxidant activity of extracts from lettuce.

MATERIALS AND METHODS

Chemicals. 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), Folin−Ciocalteu reagent, Trolox, quercetin, gallic acid, disodium fluorescein, luteolin, chlorogenic acid, and caffeic acid were purchased from Sigma-Aldrich Co. Ltd. (Poole, U.K.). Dipotassium hydrogen orthophosphate and dihydrogen orthophosphate dihydrate were purchased from Merck (Nottingham, U.K.), and cyanidin was purchased from Polyphenols Laboratories (Sandnes, Norway).

Samples and Radiation Exposure. Lollo Rosso type red leaf lettuce (cv. Revolution) was grown at Shinfield Field Station, University of Reading, under plastic films with three different degrees of UV transparency. The properties of the films when applied in autumn 2005 were (1) UV block film, which blocked UV up to 380 nm (all UV-B and most UV-A); (2) the standard UV low film, with low transmission of UV light up to 330 nm and lower transmission than UV window from 330 to 380 nm; and (3) UV window, which transmitted the full UV range (UV-A and UV-B) as seen in **Figure 1**. All films contained the infrared-reducing and light-diffusing components of Luminance THB (British Polythene Industries PLC, Greenock, U.K.).

The plastic tunnels formed nine individual replicates of 25 × 6.5 m, resulting in three independent replicated groups for each UV treatment. Lettuce seeds were sown in April 2006. Seed was germinated and grown for 21 days in a greenhouse before transplantation to peat bags in the tunnels. Plants were grown for a further 31 days to maturity. The plants were watered and fed using an automatic irrigation system.

From each replicate, three heads of lettuce were harvested for chemical analysis and seven heads for analysis of yield, and they were extracted immediately.

Extraction. Phenolic compounds were extracted on the day of harvest. Each whole head of lettuce was cooled to 6 °C, washed, and ground for 5 min in a food processor, and 1 g was extracted with 20 mL of acidified methanol (1% HCl). The mixture was left for 20 h at 6 °C in the dark. Afterward, the extracts were vacuum filtered through Whatman no. 1 (11 μm) and kept at −20 °C until analysis. Alternatively, the portion of the leaves assessed as reddest by eye was separated from the green part and extracted in the same way.

Determination of Total Phenolic Content. The total phenolic content was determined according to the Folin−Ciocalteu method (*14*). Diluted samples (200 μL) were added to deionized water (1.8 mL) followed by Folin−Ciocalteu reagent (200 μL). After 3 min, sodium carbonate (400 μL, 35% w/v) was added, followed by deionized water (1.4 mL). Samples were left in the dark for 1 h before absorbance was measured at 725 nm using a Perkin-Elmer Lambda bio 20 UV–vis spectrometer. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh weight (FW).

Total Antioxidant Activity. Antioxidant activity was determined by using the oxygen radical absorbance capacity (ORAC) method based on that of Ou et al. (*15*) performed on a GENios TECAN 96-well plate reader. All reagents and extracts were diluted in 75 mM potassium phosphate buffer (pH 7.4) for analysis. Extract, diluted as required (25 μL), was taken into a well followed by 150 μL of disodium fluorescein solution (96 nM, 37 °C) and 75 μL of AAPH solution (153 mM). Readings were taken every 2 min for 1 h at 37 °C. Results were expressed as micromoles of Trolox equivalent (TE) per gram of fresh weight.

Flavonoids and Phenolic Acids. Flavonoids and phenolic acids were determined by high-performance liquid chromatography (HPLC) using a HP 1050 chromatograph equipped with a diode array detector. Hydrochloric acid was added to the extract (2.5 M final concentration), and extracts were heated for 1 h at 90 °C prior to analysis. The extracts were removed from the water bath, allowed to cool in ice, and filtered through a 0.22 μm syringe filter. HPLC analysis was performed with a Prodigy ODS3 column (250 × 4.6 mm, 5 μm particle size) from Phenomenex (Macclesfield, U.K.) equipped with a 5 μm ODS guard column (4.0 × 4.6 mm). The solvent flow rate was 1 mL/min, and the column was allowed to equilibrate for 15 min between injections (50 μL). Flavonoids and caffeic acid were identified by retention time, cochromatography with standards, and UV spectra. External standard calibrations were used for the quantification of the respective hydrolyzed product in the chromatograms. The results were expressed as micrograms per gram of fresh weight. Quercetin and luteolin were quantified at 360 nm. Caffeic acid derivatives and cyanidin were quantified at 320 and 520 nm, respectively. The mobile phase for separation consisted of (A) 1% formic acid and (B) methanol, using a gradient starting with 25% B for 10 min, increasing to 27% B at 12 min, 31% B at 20 min, 31% B at 30 min, 38% B at 40 min, 47% B at 44 min, 60% B at 46 min, and 65% at 54 min and then reverting to 10% at 60 min.

Statistical Analysis. The program used for statistical analysis was SPSS. Differences among the means were compared between treatments using one-way analysis of variance. Differences at $P < 0.05$ were considered to be significant. Data in tables are quoted as mean ± standard error.

RESULTS

Yield. The yield of lettuce decreased with exposure to UV radiation. Lettuce grown under UV block had an average fresh weight of 315.1 g, whereas lettuce grown under the UV low and UV window films had average decreases in weight of 34 and 42%, respectively. In the same way, mean leaf number decreased with exposure to UV radiation by 10 and 13% from that found in samples grown under UV block (**Figure 2**).

Phenol Content. The phenol content in the extracts was significantly higher with increased exposure to UV radiation on both fresh weight and dry weight bases (**Table 1**). Lettuce grown under the UV block film contained 55% of the phenol content of samples grown under the UV low plastic, when calculated on a fresh weight basis (63% on a dry weight basis), whereas lettuce grown under the UV window film contained 21% more than that grown under the UV low film on a fresh weight basis (12% on a dry weight basis) (**Table 1**). The range of total phenol content for samples of leaves separated by color

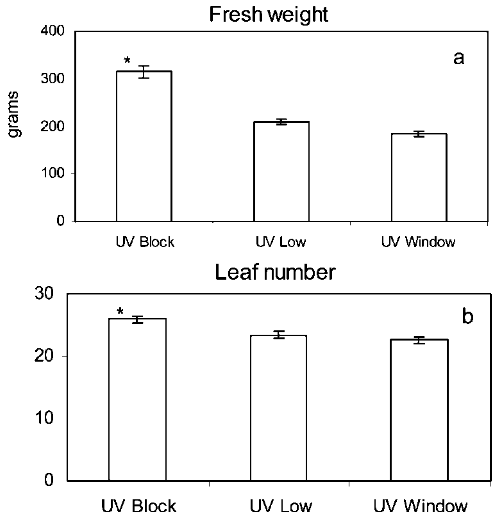

Figure 2. Mean values for fresh weight (**a**) and leaf number (**b**) of Lollo Rosso lettuce grown under different UV-transmitting films.

was 0.7–1.2 mg of GAE/g of FW for the green leaves and 3.8–6.5 mg of GAE/g of FW for the red leaves in the current study.

Flavonoids. Quercetin and cyanidin glycosides were the main flavonoid components in the lettuce. After hydrolysis of the extracts, the amount of quercetin found in Lollo Rosso represented 44.5% of the flavonoid content, whereas the amount of cyanidin was, on average, 47.7% of the flavonoid content (**Table 2**). The concentration of flavonoids in lettuce was highly sensitive to the transmission of UV light. The mean flavonoid concentration (**Table 2**) found in lettuce grown under the UV low film was 1347 μg/g (expressed as the aglycon concentration). Lettuce grown under the UV block film had 3.5 times lower flavonoid concentration than that grown under UV low conditions (381 μg/g of FW), and lettuce grown under UV window conditions contained flavonoids at a concentration of 1825 μg/g of FW, which was 1.3 times that found in samples grown under the UV low film.

Cyanidin. After acid hydrolysis, the extracts showed a single peak in the HPLC chromatogram at 520 nm, which corresponded to cyanidin. Lettuce grown under the UV block film had the lowest concentration of cyanidin glycosides, yielding cyanidin at a concentration of 165 μg/g of FW, which was 25% of that found in lettuce grown under the UV low film. Samples grown under the UV window film had a higher cyanidin glycoside content, which was 50% higher compared with UV low. A high correlation was found between cyanidin content after hydrolysis and antioxidant activity ($r = 0.723$; $P < 0.01$).

Red coloration was found mainly in the outer leaves and toward the extremities of the inner leaves, where the leaves were exposed to most light. **Table 3** shows the effects of the three different UV treatments on the coloration of the lettuces, where the red color, due to the anthocyanins present, is clearly more apparent in the plants grown under the film allowing a higher UV exposure. The concentration of cyanidin glycosides in the red leaves was more than 10 times higher than in the green leaves, with cyanidin present at 992 μg/g of FW after hydrolysis.

Quercetin. The level of quercetin glycosides in the lettuce was also sensitive to exposure to UV radiation during cultivation. Lettuce grown under the UV low film contained quercetin glycosides, which yielded an average quercetin concentration of 586 μg/g of FW. A decrease of 77% in quercetin glycosides was found in lettuce grown under the UV block film. Lettuce grown under the UV window film contained a 50% higher quercetin glycoside concentration compared with lettuce grown under the UV low film. These concentrations of quercetin glycosides are based on the quantification of flavonoids from the whole head of the lettuce.

Luteolin. As for quercetin and cyanidin glycosides, concentrations of luteolin conjugates increased significantly with exposure to higher levels of UV radiation during cultivation. Lettuce grown under the UV block film yielded 19 μg of luteolin/g of FW on hydrolysis, which was 17% of the concentration in lettuce grown under the UV low film, whereas lettuce grown under the UV window film contained the highest concentration of luteolin conjugates, which was 35% higher than that of the lettuce grown under the UV low film.

Phenolic Acids. Prior to hydrolysis, four components were identified as cinnamic acid derivatives from their identical UV spectra in the HPLC chromatogram. Caffeic acid, which was identified by comparison of the retention time and spectrum with those of a standard, was formed from all of the main phenolic acids by the acid hydrolysis step in the analytical procedure. The concentration of caffeic acid derivatives also increased with increased exposure to UV radiation preharvest (**Table 2**). There was no evidence for isomerization of trans to cis isomers at any of the UV exposure levels.

Antioxidant Activity. The antioxidant activity of extracts from the lettuce assessed by the ORAC assay increased with increasing total phenol content, and hence it increased with exposure to UV radiation during cultivation (**Table 1**). The values for the ORAC assay for lettuce grown under UV block and UV window films were 52 and 113%, respectively, of the values for the lettuce grown under the UV low film. A highly significant correlation ($r = 0.936$; $P < 0.01$) was found between the total phenol content and the antioxidant activity assessed by the ORAC assay. Lettuce plants grown under the UV window and the UV low film had the highest antioxidant capacities.

The antioxidant activity of extracts from the red leaves of lettuce was in the range of 100–144 μmol of TE/g of FW. This compared with the range of 29–42 μmol of TE/g of FW for the green leaves.

DISCUSSION

The inhibitory effect of UV radiation on fresh weight of lettuce (**Figure 2a**) confirms the findings of Krizek et al. (*12*) and Diaz et al. (*16*). In the same way, leaf number was affected by exposure to UV radiation (**Figure 2b**). Krizek et al. (*12*) suggested that the growth reduction in a UV environment could be due to damage to the photosynthetic apparatus. However, recent work suggests this is not the case (*17*). Other possibilities are that the biosynthesis of anthocyanins, which absorb in the photosynthetic part of the spectrum, reduces the photosynthetic capability of the leaves or that the increased production of secondary compounds acts in direct competition for assimilated carbon to the plants' growth process.

Increasing exposure of lettuce to UV radiation during cultivation allowed the cyanidin glycoside concentration of the whole lettuce head to increase by 381%, with cyanidin concentrations after hydrolysis increasing from 165 to 793 μg/g of FW. The finding that cyanidin glycosides were the main anthocyanins is consistent with previous reports for Lollo Rosso (*18–20*). The differences induced by exposure to UV light were most clearly seen in the color of the leaves, due to the anthocyanin content, with cyanidin concentrations in the red leaves after hydrolysis being up to 992 μg/g of FW compared with up to 75 μg/g of FW in the green leaves (**Table 3**). This

Table 1. Phenol Content and Antioxidant Activity (ORAC)

treatment	total phenol content		antioxidant activity (ORAC)	
	mg of GAE/g of FW	mg of GAE/g of DW	μmol of TE/g of FW	μmol of TE/g of DW
UV block	1.6 ± 0.1 a	31.3 ± 1.4 a	25.4 ± 0.5 a	480.3 ± 6.1 a
UV low	2.9 ± 0.2 b	49.8 ± 2.0 b	48.7 ± 3.7 b	795.3 ± 34.6 b
UV window	3.5 ± 0.1 c	55.6 ± 1.0 c	55.1 ± 2.2 c	850.5 ± 19.9 c

Table 2. Flavonoid and Phenolic Acid Contents in Lettuce

treatment	cyanidin		quercetin		luteolin		flavonoid content		caffeic acid	
	μg of Cy/g of FW	mg of Cy/g of DW	μg of Que/g of FW	mg of Que/g of DW	μg of Lut/g of FW	mg of Lut/g of DW	μg/g of FW	mg/g of DW	μmol of CA/g of FW	μmol of CA/g of DW
UV block	165 ± 16 a	3.11 ± 0.2 a	196 ± 16 a	3.7 ± 0.3 a	19 ± 2 a	0.37 ± 0.04 a	381 ± 41 a	7.2 ± 0.2 a	6.2 ± 0.3 a	117.3 ± 7.4 a
UV low	649 ± 70 b	10.6 ± 0.7 b	586 ± 53 b	10.6 ± 0.7 b	113 ± 10 b	1.84 ± 0.1 b	1412 ± 149 b	23.0 ± 0.8 b	9.7 ± 0.3 b	159.2 ± 5.6 b
UV window	793 ± 21 c	12.2 ± 0.2 c	880 ± 35 c	13.2 ± 0.5 c	152 ± 7 c	2.35 ± 0.09 c	1803 ± 69 c	27.8 ± 0.35 c	11.1 ± 0.2 c	171.6 ± 1.9 c

Table 3. Total Phenols, Anthocyanins, and Antioxidant Activity of Green and Red Leaves of Lollo Rosso Lettuce

lettuce sample	total phenols, GAE (mg/g of FW)	anthocyanin concn (μg of Cy3glc/g of FW)	ORAC value (μmol of TE/g of FW)
green, UV block	0.7 ± 0.04 a	25 ± 3 a	29 ± 2.2 a
green, UV low	0.7 ± 0.05 a	24 ± 1.6 a	31 ± 3.6 a
green, UV window	1.2 ± 0.1 b	75 ± 1.5 b	42 ± 2.7 b
red, UV block	3.7 ± 0.1 c	375 ± 9 c	100 ± 3.2 c
red, UV low	6.2 ± 0.1 d	970 ± 10 d	135 ± 3.6 d
red, UV window	6.5 ± 0.1 d	992 ± 26 d	144 ± 4.3 e

gives some indication of the potential for increase in anthocyanin biosynthesis if more leaves are exposed to the light. Our results are consistent with the finding of Kleinhenz et al. (*7*) that shading, which reduced ambient light intensity by 50%, decreased the concentration of anthocyanins by 48–59% in four varieties of red leaf lettuce. Increasing UV radiation in lettuce has shown effects on the fresh weight of the heads and on the concentration of phenolic acids, flavonols, flavones, and total flavonoids per gram of fresh weight. Even taking into account the reduction in weight of lettuce grown under higher UV conditions, a high increase in the total mass of cyanidin glycosides per head of lettuce was observed depending on UV treatment. Lettuce grown under UV block film yielded 52 mg of cyanidin per head of lettuce on hydrolysis. This value was 262% higher for lettuce grown under UV low film, which was close to the value for lettuce grown under the UV window film, which had an increase of 282%.

The phenol concentration of red leaf lettuce was reported by Hassimoto et al. as 1.7 mg/g of FW (*21*), which is similar to that of lettuce grown under UV block film (1.6 mg/g of FW) in the present study. The phenolic acid content of lettuce (cv. Audran) was also reported to increase by an average of 50% when grown outdoors compared with glasshouse-grown plants, thus supporting the conclusion that UV-B exposure raises phenolic acid levels (*6*). Overall, we have found that increased exposure of lettuce to UV radiation caused increases in total phenol content, glycosides of cyanidin, quercetin, and luteolin, and phenolic acids. Previously Ferreres et al. (*19*) determined the concentration of phenolic acids in Lollo Rosso (quantified as chlorogenic acid) and found that phenolic acid levels were higher in the red tissue (1696 μg/g of FW) compared with the green tissue (570 μg/g of FW) and white tissue (213 μg/g of FW), in agreement with the results of our study.

The flavonoid content of extracts from the lettuce after hydrolysis increased from 381 μg/g of FW (UV block) to 1825 μg/g of FW (UV window). The latter levels are high compared with flavonoid concentrations reported previously in Lollo Rosso, for example, 1384 μg/g in red tissue, 244 μg/g in green tissue, and 43 μg/g in white tissue (*19*), and 207 μg/g was reported by Du Pont et al. (*20*). The importance of flavonoids and phenolic acids in photoprotection of plants is well recognized (*22*).

Turning to individual flavonoid components, the quercetin glycoside levels present in the lettuce grown under the UV window plastic are similar to levels reported previously by Crozier et al. (*8*) for Lollo Rosso. There are few reports of luteolin conjugates in lettuce. Neither Crozier et al. (*8*) nor Du Pont et al. (*20*) reported any luteolin in lettuce, although Hohl et al. (*11*) reported luteolin conjugates in the outer leaves of lettuce cv. Newton.

The flavonoid content and antioxidant capacity of fruits and vegetables are important for their nutritional quality. The antioxidant activity of extracts from the red leaves of lettuce was in the range of 100–145 μmol of TE/g. Strawberries had the highest ORAC values among the fruits studied by Wang et al. (*23*), so samples of Elsanta and Everest strawberries were analyzed for antioxidant capacity, and the values were found to be in the range of 60.1–64.9 and 38.8−44.3 μmol of TE/g. It is therefore clear that extracts from the red leaves were more active as antioxidants than extracts from these samples of strawberries, when expressed on a fresh weight basis. However, the data for the whole lettuce (**Table 1**) showed that growing lettuce under the UV window film gives a product with comparable antioxidant activity to the strawberries at 55.1 μmol of TE/g. Soft fruit is commonly regarded as a good source of phenolic antioxidants (*24*), but it is clear from this study that extracts from Lollo Rosso lettuce are of comparable antioxidant activity. Many components in the lettuce contribute to the high antioxidant activity, but Caldwell (*18*) found that cyanidin and quercetin derivatives contributed the highest antioxidant activity to red leaf lettuce. This is consistent with the high concentrations and high radical scavenging activity of these components.

In conclusion, biosynthesis of flavonoids, and consequently the antioxidant activity of extracts from red leaf lettuce, was enhanced dramatically by growing the crop under plastic films that allowed a higher exposure to UV radiation. UV window plastics are potentially useful in growing lettuce with increased phytochemical content and thus adding value to protected salad crops such as red lettuce. This provides scope to consider Lollo Rosso as a potential source of flavonoids, essentially quercetin and cyanidin glycosides, with consequent health benefits (*25*, *26*). However, UV-transparent films do have the disadvantage that the fresh weight of the lettuces is reduced.

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