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The effects of different night-time temperatures and cultivation durations on the polyphenolic contents of lettuce: Application of principal component analysis

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ABSTRACT

The present study was conducted to characterize the polyphenolic contents of lettuce leaves grown under different night-time temperatures (4, 12, and 20 °C) and cultivation durations (5, 15, and 20 days) using high performance liquid chromatography-tandem mass spectrometry

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(LC/MS/MS). The assay method was validated based on specificity, linearity, accuracy, precision, and the performance limit. The total polyphenolic contents were highest (2462.6 mg/kg) after transplantation at a night temperature of 20 °C on day 20 and lowest (1132.7 mg/kg) at the same temperature on day 5. Quantification and principal component analysis showed that the relative contents of quercetin and kaempferol were markedly higher during the early stage of cultivation (day 5) than those of day 15 and 20, and that night-time temperatures of 12 and 20 °C on day 20 were favorable for producing polyphenol-rich lettuce containing caffeic acid. In conclusion, a synergistic effect between high night-time temperatures (12 and 20 °C) and cultivation duration (20 days) produced lettuce rich in polyphenols compared to that at low temperature (4 °C).

Introduction

Lettuce (*Lactuca sativa* L.), a leafy vegetable native to the Mediterranean area, was cultivated in Egypt as early as 4500 BC [1]. It belongs to the Compositae family (Asteraceae) with a high rank both in production and economic value among vegetables grown in the Republic of Korea [2]. Lettuce is conventionally consumed in salads, and its seeds are utilized in folk medicine for treating rhinitis, asthma, cough, insomnia, and pertussis [3]. Lettuce contains multiple health-beneficial components, including polyphenols, ascorbic acid, carotenoids, and tocopherols. These compounds have protective effects against cancers, cardiovascular disorders, and other chronic diseases [4].

Polyphenols possess powerful antioxidant activities and protect animal cells from the harmful effects of reactive oxygen species (ROS), which are produced from a wide range of stressors [1]. Polyphenolic contents vary considerably among plants, depending on the type and intensity of the stressors during their growth and management [5] In this context, phenylalanine ammonia-lyase (PAL), a key plant enzyme in the biosynthesis of various polyphenols, is activated via a number of biotic and abiotic stressors, including radiation, temperature, plant hormones, wound, and disease [6–8]. Induction of this enzyme increases the production of phenolic compounds, including tannic, gallic, caffeic, chlorogenic, and cinnamic acids in lettuce grown under low temperature [5,9]. The PAL enzyme is significantly correlated with temperature in plants, and its activity increases in response to either low or high temperature [10]. Lower temperatures decrease fresh lettuce weight [11,12], whereas higher temperatures induce bolting [13]. This means that quality and productivity are not guaranteed under stressful temperatures.

Lettuce is usually cultivated under outdoor conditions with day and night-time temperatures of 17–22 °C and 3–12 °C, respectively [11]. Under controlled greenhouse conditions, the optimum night temperature is 15–20 °C, as suggested by Choi and Lee [12]. The night-time temperature has additional importance, as heating and cooling in winter and summer add an extra cost to greenhouse maintenance. However, to the best of our knowledge, there have been no reports on the role of night growth temperatures and cultivation durations on polyphenols in leaf lettuce production.

In the present study, polyphenols were determined and profiled in lettuce leaves in response to variations in growth conditions, including night-time temperatures and the duration of greenhouse cultivation using liquid chromatography-tandem mass spectrometry (LC/MS/MS) and principal component analysis (PCA). Polyphenol characterization utilizing LC/MS/MS is advantageous because it does not require extensive purification steps. LC/MS/MS is a powerful tool that provides clear and characteristic fragment patterns to identify plant polyphenols

[14]. Our results will be useful to develop cultivation guidelines for the production of health-beneficial polyphenol-rich lettuce.

Material and methods

Materials and chemicals

Lettuce (*L. sativa* L., cv Cheongchima) seeds were germinated in plug-cell trays filled with 'Tosilee' (Shinan Grow Co., Jinju, Republic of Korea) commercial media on May 10, 2011. After four leaves were opened, they were transplanted to 9 cm plastic pots and cultivated in three glass chambers (KGC-175 V, Koencon, Hanam, Republic of Korea) with a day temperature of 22 °C and night temperatures of 4, 12, and 20 °C, until harvest. The photoperiod was 12-h light/12-h dark and was provided by fluorescent lamps (approximately 450 µmol m^{-2} s^{-1}). Relative air humidity was approximately 65%. Water was supplied daily via overhead irrigation, and nutrient solution (Hoagland, pH = 5.9 ± 0.2, EC = 1.2 mS cm^{-1}) was provided every 4 days. The plant density was 36 plant/m^2 in each treatment. The plants were rearranged every 3 days to minimize position and/or edge effects in glass chamber. The leaves were washed with distilled water, lyophilized, and stored in dark glass containers at –20 °C pending analysis.

Caffeic acid, kaempferol, and quercetin were used as external standards after recrystallization in ethanol (Sigma–Aldrich Co., St. Louis, MO, USA). The purity of all standards was confirmed by HPLC to be at least 99%. All solvents and water were obtained from Duksan Pure Chemical Co., Ltd. (Ansan, Republic of Korea).

Extraction and purification

Lyophilized leaves (0.5 g) were ground into a powder and poured into 25 mL of aqueous 80% methanol. The mixture was homogenized using a Polytron blender (Brinkman Instruments, Westbury, NY, USA) for 5 min at room temperature and treated in a sonicator (100 W, 42 K*HZ*, Bransonic 3510R-DTH, Danbury, CT, USA) for 10 min. The extract was filtered through a glass filter under reduced pressure and centrifuged at 4000*g* (SCT4B centrifuge, Hitachi, Ibaraki, Japan). The supernatant was filtered through a PTFE syringe filter (Titan, 0.45 µm, SMI–Lab Hut Co., Ltd. Maisemore, UK), and the filtrate was stored at −20 °C until analysis.

LC/MS/MS

The LC/MS/MS experiment was performed according to our previously reported methodology [15] with the exception of

the column and solvent system. The column was a Cosmosil C_{18} (4.6 mm × 250 mm, 3.5 μm, Nacalai, Inc., San Diego, CA, USA), and the constituents of the solvent system were 0.1% aqueous formic acid (A) and methanol:water (6:4, v/v, B). The gradient conditions of the mobile phase were: from 0% to 10% B over 10 min, from 10% to 100% B over 50 min, and isocratic elution for 10 min. MS/MS experiments were performed using a 3200 Q TRAP LC/MS/MS system (Applied Biosystems, Forster, CA, USA) with a Turbo VTM source and a Turbo Ion Spray probe (500 °C). The mass spectrometer was operated in positive and negative ion mode. Nitrogen was used as a nebulizing as well as a drying gas. The flow rates in both cases were 45 psi. The capillary voltage was set at 5.5 kV and the source temperature was set at 500 °C. The resolutions of the first and third quadrupole were between 0.6 and 0.8 (unit resolution). Mass spectra were recorded between m/z 100 and 1000 with a step size of 0.1 amu.

Quantification

Polyphenols were quantified by chromatograms at 330 nm. Plant polyphenols can be quantified using a standard curve of compounds having the same aglycone [16]. Thus, caffeic acid (**1–6**), the quercetin derivatives (**7**, **9**, **10**), and kaempferol 3-*O*-glucuronide (**8**) were quantified using external calibration curves, which were prepared with caffeic acid, quercetin, and kaempferol, respectively.

Experimental design and statistical analysis

Experimental with three replicates per each treatment (each treatment contains three plants) were used throughout the work. PCA is a commonly used statistical tool to interpret large datasets. It reduces the number of variables in the dataset through a projection of objects onto a smaller number of new orthogonal variables, so-called PCs [17]. Extraction of the PCs is a variance-maximizing rotation of the original variable space; thus, the variance contained in the dataset is concentrated in the first PC. The following PCs progressively explain less of the variance. Two PCs are usually sufficient to explain 90% of the total variance of a given dataset. The projection of objects onto a PC is called a score. The plot of the first two object scores is called the score plot, where the objects are represented as points. It is possible to graphically identify similarities and differences between objects through the score plot. The distance between objects in a score plot indicates their degree of similarity. The PC score is the combination of the initial variables, and loading expresses how the initial variables linearly contribute to form the score. Therefore, loading is used to interpret the score, which unravels the magnitude and direction of the correlation in which the original variables contribute to the score. The loadings of the original variables can be represented as arrow lines on a score plot, which is also called a PCA biplot. Using the loadings, it is possible to determine which of the original variables are important (amount of loading is the longest distance from the origin) and whether any variables are correlated (the same or opposite direction) on a line through the origin. The PCA biplot simultaneously shows the scores and loadings and provides a graphic relationship between the samples and the variables in the data matrix. The samples are shown as points, and the variables are

exhibited as linear arrows [18]. The PCA biplot was generated using SIMCA-P 12.0.1 software (Umetrics, Umeä, Sweden).

All determinations were performed in triplicate, and data were calculated as mean ± standard deviation. Data were subjected to repeated-measures analysis of variance (SAS ver. 9.1.3; SAS Institute, Cary, NC, USA) and $P = 0.05$ was considered significant.

Results and discussion

Polyphenol separation and identification

Lyophilized samples were extracted from lettuce leaves with 80% aqueous methanol. The extracts were characterized by reversed phase-LC/MS/MS in negative ionization mode. Individual compounds were identified based upon available data in the literature. Optimized chromatographic conditions for good specificity were achieved after testing several columns and elution systems, including acetonitrile–water, methanol–water, acetonitrile–acidic aqueous solution, and methanol–acidic aqueous solution. A Cosmosil C_{18} column and a gradient elution consisting of 0.1% aqueous formic acid (A) and methanol/water (6:4) was the best for providing good chromatographic performance without peak tailing. The retention times of all polyphenols (**1–10**) were between 10 and 50 min in the chromatographic profile of the lettuce leaves recorded at 330 nm (Fig. 1). The structures and the LC/MS/MS data are shown in Fig. 2 and Table 1. The polyphenols identified in the present study have been characterized previously in other lettuce varieties [1,19,20]. Notably, the identification of the compounds in the present study with no commercially available standards could be considered "tentative".

Validation

Specificity, linearity, accuracy, precision, and the performance limit were determined according to the guidelines of the International Conference of Harmonization [21]. As shown in Fig. 1, the polyphenols were well separated without any interfering peaks, which indicates good specificity.

Linearity was determined through the determination coefficients (R^2) of the corresponding polyphenol standard calibration curves. The calibration curves were constructed from the peak area ratios as a function of concentration using a $1/x$ (x: concentration) weighted linear regression ($n = 5$). The standard concentrations spanned six points of 1, 10, 50, 100, 500, and 1000 mg/L. The R^2 was >0.9997, which indicates good linearity (Table 2).

The performance limit of the assay was represented in terms of the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were determined at signal-to-noise ratios of approximately 3 and 10, respectively. As shown in Table 2, the LOD and LOQ were 0.0375–0.1764 mg/L and 0.125–0.5882 mg/L, respectively.

Accuracy and precision were evaluated based on recovery and relative standard deviation, respectively. Recovery was calculated as $A - C/B - C$, where A is the peak area obtained for the polyphenols spiked pre-extraction, B is the peak area obtained for the polyphenols spiked post-extraction, and C is the peak area obtained for a blank extraction. The recoveries of caffeic acid, quercetin, and kaempferol at a concentration

of 10 mg/kg were ranged from 88.2% to 101.1% and those at 100 mg/kg were between 92.9% and 97.6% (Table 2). The precision of the 3 compounds was <4%. These findings demonstrate that the method exhibited good accuracy and precision.

Effect of different night-time temperatures and cultivation durations on polyphenol quantity in lettuce leaves

In general, plants exposed to temperature stress usually suffer from oxidative stress, which excites electrons in the respiratory chain reactions. Electrons in an excited state are transferred to molecular oxygen (O_2) to produce ROS [22], including singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide (O^{-2}), and hydroxyl radical ($HO^·$). These free radicals are toxic and cause oxidative damage to proteins, DNA, lipids, and membranes [23]. Plants have different defense mechanisms to reduce oxidative damage; among them the antioxidative agents scavenge ROS and act as electron and hydrogen donors. In response to temperature stress, plants activate PAL, which catalyzes the first step in phenylpropanoid metabolism [24] and triggers the biosynthesis of phenylpropanoids, including hydroxycinnamic acids, flavonoids, and other polyphenols, which act as antioxidants [23]. Therefore, the production of secondary metabolites is correlated with growth temperature in plants. Each polyphenol in lettuce leaves grown under different night temperatures was quantified (Table 3). The average total polyphenol content estimated from nine experiments was 1685.5 ± 41.7 mg/kg. Notably, total polyphenol contents increased when cultivation duration following transplantation increased. For example, the total polyphenol contents were substantially higher on day 20 after transplantation in a growth chamber with night-time temperatures of 20 and 12 °C and substantially lower on day 5 under a night-time temperature of 20 °C. The contents were not different at an early stage of cultivation (day 5), whereas they were significantly different between lettuce plants grown at different night-time temperatures at the late stage of cultivation. These findings are supported by those reported by Wang and Zheng [25], who observed that an increase in night temperature from 12 to 22 °C results in an increase in polyphenol contents in two strawberry cultivars. Higher temperatures and greater light intensity in a plastic house enhance phenolic contents and antioxidant capacity in spinach [26]. Additionally, Liu et al. [27] found that lettuce harvested at both higher temperatures and light intensities possess higher phenolic contents and antioxidant effects than that harvested under relatively lower temperature and light intensity conditions. Boo et al. [28] found that total polyphenol contents and PAL activity were higher in lettuce red cultivars subjected to 13/10 °C and 20/13 °C followed by 25/20 °C and 30/25 °C (day/night) temperature conditions. These findings suggest that activation of the antioxidative and secondary metabolism may be an integral part of plant adaptation to normal growth temperatures. However, the reason why these normal growth temperatures enhance both PAL activity and polyphenol contents is not unclear.

Among the characterized polyphenols, the average contents of caffeic acid derivatives (**1** + **2** + **4** + **5** + **6**) were the highest compared with quercetin (**7** + **9** + **10**) and kaempferol derivatives (**8**). In particular, the highest content was found for polyphenol **4** followed by polyphenol **2**. Low temperature increases the concentration of flavonoids, including rutin, quercetin, and kaempferol derivatives in some plants [29,30].
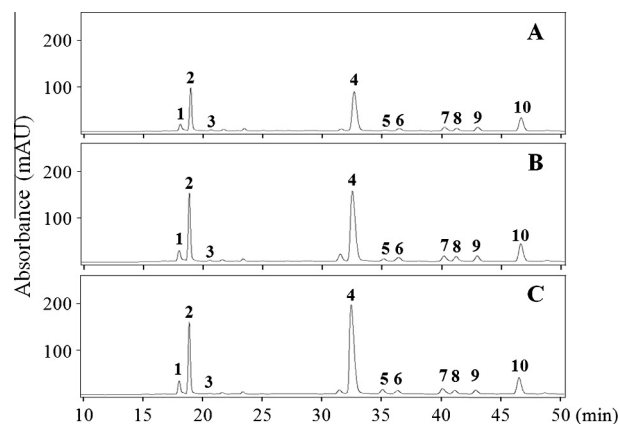

Fig. 1 High-performance liquid chromatography (HPLC) profiles (day 20 after transplantation) of lettuce leaves grown under different night-time temperatures: (A) 4 °C, (B) 12 °C, and (C) 20 °C. Peak identities: (**1**) caffeic acid, (**2**) 3-*O*-caffeoylqunic acid, (**3**) chlorogenic acid, (**4**) dicaffeoyltartaric acid, (**5**) 3,5 dicaffeoylqunic acid, (**6**) caffeoyltartaric acid, (**7**) quercetin 3-*O*-glucocide, (**8**) kaempferol 3-*O*-glucuronide, (**9**) quercetin 3-*O*-glucuronide, and (**10**) quercetin 6″-acetyl-3-*O*-glucoside.

PCA biplot

PCA was conducted to develop a clear relationship between the different conditions, including night-time growth temperatures and cultivation durations and the variation in the polyphenol levels in lettuce leaves. The results are shown
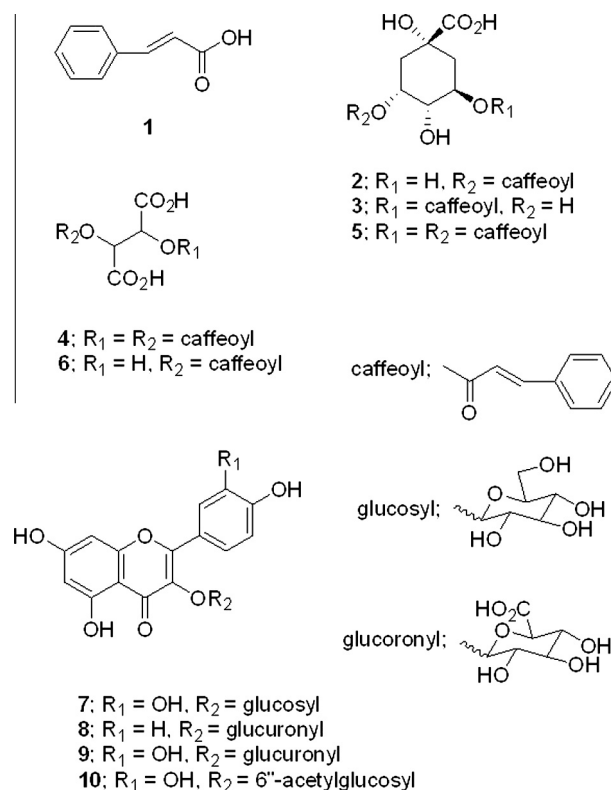

Fig. 2 Structures of the 10 polyphenols in lettuce leaves.

Table 1 Spectral data of the 10 polyphenols in lettuce leaves.

Compounds	λmax (nm)	r.t[a]	[M–H]$^-$	MS/MS
Caffeic acid (**1**)	327	17.7	179	135
3-O-Caffeoylqunic acid (**2**)	237, 326	18.3	353	191, 180, 179, 173, 135
Chlorogenic acid (**3**)	326	21.3	353	191, 179
Dicaffeoyltartaric acid (**4**)	328	33.2	473	311, 293, 179, 149, 135
3,5 dicaffeoylqunic acid (**5**)	328	35.1	515	353, 191, 179, 173, 135
Caffeoyltartaric acid (**6**)	328	37.5	311	179, 149, 135
Quercetin 3-O-glucoside (**7**)	256, 354	40.2	463	300
Kaempferol 3-O-glucuronide (**8**)	266, 348	41.9	461	285
Quercetin 3-O-glucuronide (**9**)	256, 353	43.9	477	301, 179, 151
Quercetin 6″-acetyl-3-O-glucoside (**10**)	256, 354	47.4	505	301, 300

[a] r.t: Retention time (min).

Table 2 Validation data for the external calibration standards ($n = 5$).

Standards	Calibration curve[a]	R^2	LOD (mg/L)	LOQ (mg/L)	Recovery (%) ± RSD	
					10 mg/kg	100 mg/kg
Caffeic acid	$y = 79.555x - 0.3072$	0.9998	0.0375	0.1250	88.2 ± 0.9	95.9 ± 1.3
Quercetin	$y = 27.387x - 10.001$	0.9997	0.1764	0.5882	98.8 ± 3.2	97.6 ± 3.2
Kaempferol	$y = 66.799x - 13.357$	0.9999	0.0882	0.2941	101.1 ± 3.6	92.9 ± 1.6

LOD: Limit of detection.
LOQ: Limit of quantification.
RSD: Relative standard deviation.
[a] y, Peak area of standard; x, concentration of standard (mg/L).

Table 3 Quantification (mg/kg of dry weight) of phenolic compounds in lettuce leaves grown under various temperatures and cultivation durations using liquid chromatography/tandem mass spectrometry.

Compounds	Day after transplantation								
	5			15			20		
	Night growth temperature (°C)								
	4	12	20	4	12	20	4	12	20
1	44.2^{de}	35.9 ^{e}	37.0^e	52.9^d	87.3^c	100.9^b	80.2^c	116.4^a	117.8^a
2	401.3^c	401.6^c	311.5^d	416.5^c	413.4^c	470.5^b	393.4^c	579.2^a	535.7^a
3	–	–	–	–	–	–	–	–	–
4	674.9^{ef}	636.6^f	567.8^g	754.8^{ed}	1071.0^c	1129.1^c	819.1^d	1395.4^b	1579.9^a
5	23.3^e	23.6^e	18.2^f	28.8^d	32.2^c	38.8^b	33.8^c	53.8^a	38.1^b
6	39.1^{edf}	34.3^f	43.2^d	35.6^{ef}	54.1^c	41.1^{de}	35.4^{ef}	64.1^b	86.6^a
7	2.3^e	–	–	2.6^e	3.5^c	3.2^{cd}	2.7^{ed}	4.2^b	6.1^a
8	32.8^a	33.9^a	33.0^a	21.6^c	19.5^{ed}	21.9^c	18.7^e	26.5^b	21.2^{cd}
9	46.8^b	51.2^a	47.2^{ab}	24.8^c	19.6^{ed}	24.3^c	15.7^{ef}	20.9^{cd}	13.1^f
10	73.7^c	76.9^c	74.4^c	88.9^b	79.9^c	101.1^a	65.1^d	77.6^c	64.1^d
Total polyphenol	1338.5^{cd}	1294.1^d	1132.7^e	1426.7^{cd}	1859.2^b	1853.3^b	1464.2^c	2338.1^a	2462.6^a

The compound numbers correspond to those given in Table 1.
Different letters in each row indicate a significant difference at $P = 0.05$.
– Detected but not quantified.

on the PCA biplots as illustrated in Fig. 3. The PC1 and PC2 biplots explained 68.5% and 14.5% of the total variance, respectively. Because the experiments were conducted at three different temperatures and cultivation durations, three colored sample points (blue for 4 °C, green for 12 °C, and red for 20 °C) and three different shaped points (tetragons for 5 days, triangles for 15 days, and circles for 20 days) are shown. As triplicate experiments were conducted for each cultivation condition, the plot shows three points of the same color and shape.

The direction of the arrows signifies an increase in the concentration of each polyphenol. The position on the individual arrow axis onto which each point was projected perpendicularly represents the relative concentration of the corresponding polyphenol in each sample. Three colored tetragons are projected on the rightmost of the arrow axes of kaempferol derivative **8** and quercetin derivative **9**, which indicates a relatively high concentration of these polyphenols at the early stage (5 days) of cultivation. The sample points cultivated
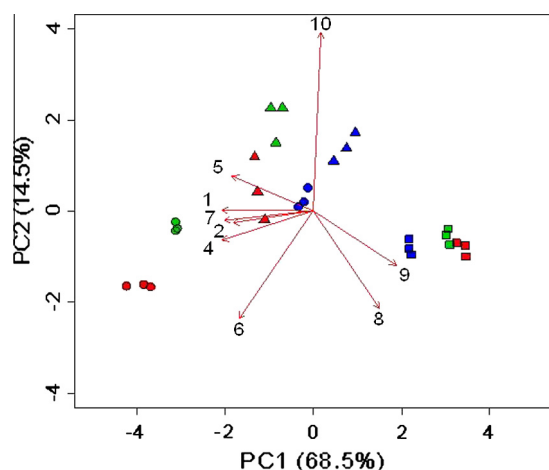
Fig. 3 Principal component analysis (PCA) biplot of nine polyphenols (except compound 3) characterized in lettuce leaves grown under different night-time temperatures and cultivation durations. The three colors represent the different night temperatures: blue, 4 °C; green, 12 °C; red, 20 °C. The three shapes represent the different cultivation durations: tetragons, 5 days; triangles, 15 days; and circles, 20 days. The compound numbers correspond to those given in Fig. 1.

under the conditions of 5 days and 20 days at 4 °C are projected around the origin of the arrow axes of the caffeic acid derivatives **1**, **2**, and **4–6,** and quercetin 3-*O*-glucoside (**7**), which indicates relatively low production of these polyphenols under these conditions. The sample points corresponding to the conditions of 20 days at 12 and 20 °C are projected on the left most of the arrow axes of the caffeic acid derivatives **1**, **2**, and **4–6** and quercetin 3-*O*-glucoside (**7**), which indicates higher production of polyphenols **1**, **2**, and **4–7**.

Conclusions

Cultivation conditions of 20 days at 12 and 20 °C were favorable for producing lettuce leaves rich in polyphenols. Profiling the variation in the levels of individual polyphenol in lettuce leaves grown under various growth conditions, including different night temperatures and durations of greenhouse cultivation, may provide cultivation guidelines for producing health-beneficial polyphenol-rich lettuce.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Acknowledgment

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