



Positive effects of temperature and growth conditions on enzymatic and antioxidant status in lettuce plants

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ABSTRACT

The contents of two bioactive compounds (polyphenols and flavonoids) and their antioxidant and enzyme activities were determined in the leaves of six lettuce (*Lactuca sativa* L.) cultivars subjected to 4 different day/night temperatures for 6 weeks.

The total polyphenol and anthocyanin contents and the corresponding antioxidant activities were the highest at 13/10 °C and 20/13 °C, followed by 25/20 °C and 30/25 °C. The enzymatic activities of polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) were also the highest at low day/night temperatures, but the peroxidase (POD) activity was decreased at low day/night temperatures and increased at high day/night temperatures.

The most significant positive correlation existed between anthocyanin content and PPO activity, total polyphenols and their antioxidant activities. The results showed that at relatively low temperatures, lettuce plants have a high antioxidant and enzymatic status. These results provide additional information for the lettuce growers.

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1. Introduction

Lettuce is an annual herbaceous plant of the Compositae family, which is one of the largest and the most diverse families of flowering plants. The pigments of red lettuce are attributed to the accumulation of anthocyanins, which are water-soluble and are derived from flavonoids via the phenylpropanoid pathway. The phytochemicals in lettuce are mostly secondary metabolites that are synthesized during the normal growth of plants or in response to a number of environmental conditions [1,2]. The photoinduction, signal transduction, gene expression and biosynthetic pathway of anthocyanins have been studied [3]. The phytochemicals in lettuce, such as anthocyanins and polyphenols, have a positive effect on the prevention of cardiovascular disease [4]. The accumulation of anthocyanins in fruits [5] and lettuce [6] is higher at low temperatures. Various antibrowning agents had been evaluated for their

influence on phenolic compounds in lettuce. Oxalic and ascorbic acids were more effective in preserving the phenolic compounds in comparison with cysteine and citric acid [7]. It was shown that polyphenol oxidase (PPO, o-diphenol: oxidoreductase, EC 1.10.3.1) catalyzes the oxidation of phenol to quinone. Quinone can covalently modify and crosslink various cellular nucleophiles, undergo melanin-forming autoxidation reactions, or participate in other redox reactions [8]. PPO is found in most higher plants and is responsible for the enzymatic browning of raw fruits and vegetables. Such reactions are generally considered undesirable in food preservation and processing because of the unpleasant appearance and the concomitant development of a substandard flavor [7]. During the growth of lettuce, changes also occur in peroxidase (POD, EC 1.11.1.7). PPO is an important reagent for clinical diagnosis and micro-analytical immunoassays because of its high sensitivity [9]. Many fruits and vegetables contain POD in amounts that contribute to browning-like reactions [10,11]. Although PPO primarily degrades phenolics, they can also be degraded by POD [12,13]. The activities of both enzymes are increased in response to biotic and abiotic stresses [14]. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is a key plant enzyme in the biosynthesis of phenolic compounds.

PAL catalyzes the conversion of phenylalanine to cinnamic acid. *Trans*-cinnamic acid, a product of the PAL reaction, may act as

Abbreviations: PPO, polyphenol oxidase; POD, peroxidase; PAL, phenylalanine ammonia-lyase; ANT, anthocyanins; POL, polyphenols; TE, trolox equivalent; GAE, gallic acid equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

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a modulator of PAL turnover. Treatments that prevent the accumulation of endogenous cinnamic acid *in vivo* may result in the superinduction of PAL [15–20]. In contrast, conditions that inhibit cinnamic acid metabolism correspondingly inhibit PAL activity [21]. PAL is induced by a variety of stimuli, including radiation, temperature, plant hormones, wounding, and disease. This induction is often characterized by the concurrent *in vivo* development of a PAL inactivation system. The association of anthocyanin synthesis with PPO and POD activities is not well documented in literature. However, anthocyanin synthesis in several plant tissues is known to be associated with increased PAL activity. PAL activity is more closely related with the synthesis of simple phenol than with that of anthocyanin [15]. The induction of PAL was also associated with the increased synthesis of phenolic compounds, such as tannic, gallic, caffeic, chlorogenic, and cinnamic acids in the chickpea [19]. PPO and PAL activities show a significant correlation with the storage or ripening temperatures in most plants [22]. The increase in PAL and PPO activities occurred more rapidly at low temperatures. Differential display was used to characterize gene expression in banana fruit in response to low temperature stress. Banana fruits were kept at 10 °C for 8 h. Differentially expressed fragments in the pulp and peel were obtained [22]. Other fruits had similar relationships with the enzymes [23,24]. In another investigation, the activity of PPO in the leaves of silver birch (*Betula pendula* Roth) seedlings was low but increased with elevated temperatures [24]. Therefore, it was interesting to investigate the effect of temperature, especially various combinations of day/night temperatures, on anthocyanin and polyphenol contents, their antioxidant abilities and the enzymatic activities of PPO, POD and PAL in six lettuce cultivars. Because no previous studies have been conducted on the properties of enzymes in lettuce, the aim of this study was to determine the temperature dependence of anthocyanins and polyphenols and to characterize their antioxidant and enzymatic activities.

2. Materials and methods

2.1. Plant samples

The seeds were purchased from a seed company (“Seoul Seed”, Seoul, Korea). The experiment was performed in three replicates and consisted of a randomized complete block design.

The seeds of six red-colored lettuce cultivars, namely, ‘Hongyil’, ‘Red Fire’, ‘Jinjuck’, ‘Dazzler’, ‘Fire’, and ‘Seoul Red’, were planted into plastic pots (10 cm dia. × 15 cm high) and were cultured for 35 days in a greenhouse maintained at a temperature of 22/20 °C (day/night). A high organic matter-potting medium (Hanter 21, Seoul, Korea) that contained 30% sphagnum peat moss, 50% vermiculite, 18% zeolite, and 2% sand (v/v) per 200 cm³-pot was used for lettuce cultivation. The seedlings were developed from four to five true leaves. The plants were transferred into a temperature-controlled chamber at 13/10 °C, 20/13 °C, 25/20 °C, and 30/25 °C (day/night) under 150 μmol m⁻² s⁻¹ of photosynthetically active radiation (PAR). The low temperature regime at 13/20 °C was added to observe the physiological responses against an abnormal temperature combination. The photoperiod and duration of the day temperature was 12 h.

The plants were sampled after being subjected to the treatment conditions for 6 weeks, which ensured that the leaf tissue had been acclimated to the experimental conditions. The outer leaves of at least three replicate plants were sampled in the middle of the photoperiod and analyzed.

2.2. Determination of anthocyanin content

The anthocyanins were extracted from the leaves, which were soaked in methanol containing 1% HCl, in the dark overnight at 6 °C.

The crude pigment extract was filtered through Whatman No. 42 filter paper, evaporated and kept at –20 °C prior to the analysis. The samples were hydrolyzed with 15% HCl, boiled for 1 h prior to analysis, cooled on ice, and filtered through a 0.22 μm-syringe filter [1]. The anthocyanin composition and the concentration of the purified aglycone were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC Waters 2996 Alliance System, Milford, MA, USA). An acetic acid:HCl:water (30:3:10) solvent was used, and the separation was performed on TLC plates (DC – Plastikfolien Cellulose, 20 × 20 cm, 0.1 mm thickness; Merck). The extract for the HPLC analysis was injected into a μBondapak C18 column, 3.9 mm × 300 mm (Waters, Dublin, Ireland) with a solvent water:methanol:acetic acid (58:40:2) mixture at a flow rate of 0.7 ml/min. The detection was obtained at 530 nm [25].

2.3. PPO activity assay

Fresh plant material was ground in a mortar with 0.1 M buffer (Na₂HPO₄/KH₂PO₄, pH 7.0) containing 1.5% PVP (solid polyvinylpyrrolidone). Subsequently, the homogenate was filtered and centrifuged at 15,000 × g for 15 min [26]. The supernatant was used for the PPO assays. All procedures were carried out at 4 °C. The assay mixture contained 100 mM buffer (Na₂HPO₄/KH₂PO₄, pH 7.0) and 0.58% Triton X-100 in the enzyme extracts. The reaction was initiated by the addition of 30 μM of caffeic acid. The PPO activity was measured spectrophotometrically at 370 nm and 30 °C by the disappearance of caffeic acid. The activity was expressed as μmol mg⁻¹ protein min⁻¹.

2.4. POD activity assay

Fresh material was ground with 50 mM Tris–acetate buffer, pH 7.5, containing 2 mM EDTA and 0.5% PVP [27]. The homogenate was filtered and centrifuged at 40,000 × g for 10 min [27]. The reaction mixture of 3 ml contained 100 μM Tris–acetate buffer, pH 5.0, 1 μM EDTA, 0.1 μM guaiacol and 0.003 μM H₂O₂. All procedures were conducted at 4 °C. The POD activity in the supernatant was determined by the absorbance change at 485 nm due to guaiacol oxidation. The activity was expressed as μmol mg⁻¹ protein min⁻¹.

2.5. PAL activity assay

The enzyme PAL was extracted, purified and assayed according to the method described by Nagarathna et al. [28], where 1 g of the leaf tissue was macerated to a fine paste in a mortar with 1 g of acid-purified sand, 2 ml of 3 mM β-mercaptoethanol and 10 ml of 25 mM sodium borate buffer (pH 8.8). The homogenate was centrifuged at 20,000 × g for 20 min at 4 °C. The reaction mixture of 3 ml contained 0.5 ml of enzyme extract, 2.5 ml of 10 mM L-phenylalanine and 2.5 ml of 25 mM sodium borate buffer (pH 8.8). After incubating for 2 h at 40 °C, the activity was stopped by the addition of 0.1 ml 5 N HCl. The enzymatic activity in the supernatant was measured spectrophotometrically at 290 nm by the production of *trans*-cinnamic acid from L-phenylalanine. The absorbance was read against the same volume of the reaction mixture without L-phenylalanine. The enzymatic activity was expressed as mmol of *trans*-cinnamic acid produced h⁻¹ g⁻¹ FW.

2.6. Polyphenol content and antioxidant activity of lettuce plant

The leaves from lettuce plants were freeze-dried, and the moisture losses were recorded. The freeze-dried lettuce samples were blended into powder for solvent extraction.

2.6.1. Polyphenol extraction

Freeze-dried lettuces (0.25 g) were weighed into 30 ml screw-capped glass tubes, and 20 ml of methanol:water:acetic acid (85:15:0.5, v/v) was added as extraction solvent [29]. The extraction tubes were vortexed for 30 s, sonicated for 5 min, incubated at room temperature for 20 min and vortexed again for another 30 s. The tubes were centrifuged at 3000 rpm for 10 min. The supernatants were decanted and stored at -20°C until further analysis.

2.6.2. Folin-Ciocalteu assay

The total phenolic content was determined by the Folin-Ciocalteu assay [30]. Lettuce extracts were mixed with diluted Folin-Ciocalteu reagent and 15% sodium carbonate. The absorbance at 765 nm was measured spectrophotometrically (Hewlett-Packard, model 8452A, Rockville, USA) after 30 min of incubation at room temperature. Gallic acid was used to generate a standard curve. The results for the total phenolic content of the lettuces were expressed as milligram of gallic acid equivalent per gram of freeze-dried leaf samples (mg GAE/g).

2.6.3. DPPH assay

The DPPH scavenging activities of freeze-dried lettuce leaves were measured using a published method [31,32]. Twenty grams of DPPH was dissolved into 100 ml of methanol to make a DPPH stock solution. The DPPH working solution was freshly prepared by mixing 3.5 ml of DPPH stock solution and 6.5 ml of methanol. The lettuce leaf extracts (50 μl) were added to 950 μl of DPPH working solution and were incubated in darkness for 60 min. Trolox solutions from 100 to 1000 μM were added to the DPPH working solution as standards.

The absorbance at 515 nm was measured on a spectrophotometer (Hewlett-Packard, model 8452A, Rockville, USA). The initial absorbance of the DPPH working solution was between 0.9 and 1.0. The results of the DPPH scavenging activity of the lettuce leaves were expressed as μmol Trolox equivalent per gram of freeze-dried leaf samples ($\mu\text{mol TE/g}$).

2.7. Data analysis

The statistical analysis was performed using the procedures of the Statistical Analysis System [33]. The means were separated by the least significant difference (LSD) using the *F*-test. Lastly, a correlation analysis was adopted, with the correlation coefficient methods, among the anthocyanin levels and the activities of PPO, POD, and PAL at different day/night temperatures.

3. Results and discussion

3.1. Anthocyanin, polyphenol contents and antioxidant activity

Table 1 shows the temperature-dependent changes in the anthocyanin contents among the averaged data of 6 cultivars. The total anthocyanin contents were the highest at $13/10^{\circ}\text{C}$ and fol-

lowed by $20/13^{\circ}\text{C}$, $25/20^{\circ}\text{C}$ and $30/25^{\circ}\text{C}$. However, no significant difference in the total anthocyanin contents between $13/10^{\circ}\text{C}$ and $20/13^{\circ}\text{C}$ was observed, showing a higher anthocyanin content at lower temperatures. The total anthocyanins content in all the cultivars was significantly higher at low temperatures ($p < 0.05$). The anthocyanin content was more stimulated when the low temperatures occurred during the photoperiod than during the dark period. The cultivars differed in anthocyanin content, specifically, 'Hongyi' at $13/10^{\circ}\text{C}$ and $20/13^{\circ}\text{C}$ had the highest content and 'Fire' had the lowest [34]; the relative differences among the cultivars were less pronounced at low temperatures (data are not shown). These results show that the temperature effects on the change of anthocyanin content were higher than the cultivar effects; therefore, we use the averaged data of all 6 cultivars. Our data are consistent with reports that strawberry cells produce the highest anthocyanin content at 20°C . The high temperatures have a negative influence on the amount of anthocyanins [5]. It was reported that the exposure of lettuce to light caused a decrease in flavonol content and a significant increase in anthocyanin content [6]. Our data are consistent with other reports [35,36], which consider a variety of plants, including fruits and vegetables. Intense sunlight caused excessive sunburn in exposed berries and reduced the anthocyanin accumulation. The associated high temperature also inhibits the color development [35,36]. In other studies involving fruit-zone leaf removal, the increased sunlight exposure caused sunburn damage and reduced anthocyanin accumulation. Thus, for the maximum production of anthocyanins in grape berries, moderate sunlight exposure is necessary, but the extent varies among different cultivars. Generally, low temperatures, such as 25°C , are optimal for anthocyanin biosynthesis. High temperatures, such as 35°C , are associated with anthocyanin degradation and the inhibition of anthocyanin accumulation. Our results can also be partly compared with other research [37] involving two C4 plants, *Miscanthus x giganteus* and *Cyperus longus* L., grown at suboptimal growth temperatures. The relationships between the quantum efficiencies of photosynthetic electron transport through photosystem II (PSII) (PSII operating efficiency; F_q'/F_m') and CO_2 assimilation (phiCO_2) in leaves were examined. When *M. x giganteus* was grown at 10°C , the ratio of the PSII operating efficiency to the phiCO_2 increased relative to that found in leaves grown at 14 and 25°C . Similar increases in the F_q'/F_m' : phiCO_2 occurred in the leaves of two *C. longus* ecotypes when the plants were grown at 17°C compared with 25°C . These elevations of F_q'/F_m' : phiCO_2 at low growth temperatures were not attributable to the development of anthocyanins, as has previously been suggested for maize [38].

In our reported results, at $13/10^{\circ}\text{C}$, the content of anthocyanins was the highest. However, the temperature is not applicable to a real culture environment. Thus, $20/13^{\circ}\text{C}$ is a more reasonable environment for lettuce culture than $13/10^{\circ}\text{C}$. However, this temperature setting was added as a scientific approach and could suggest that storage at low temperatures after a harvest could induce higher anthocyanin accumulation. These results can be supported by other reports [37], where further protection of PSII was effected by a 20-fold increase in zeaxanthin content in the dark-adapted leaves of *M. x giganteus* grown at 10°C . This observation was associated with much higher levels of non-photochemical quenching of excitation energy compared with that observed in leaves grown at 14 and 25°C . These differences may explain the long growing season and the remarkable productivity of this C4 plant in cool climates in comparison with other C4 species, such as *C. longus*, which occur naturally in such climates. A similar relationship involving temperature was observed for the amount of polyphenols and their antioxidant activities, which was consistent with other reports [29].

Table 1

Effects of day/night combined temperatures on anthocyanins, polyphenols and antioxidant activities of lettuce plants.

Average	Day/night temperature				LSD (0.05)
	13/10 $^{\circ}\text{C}$	20/13 $^{\circ}\text{C}$	25/20 $^{\circ}\text{C}$	30/25 $^{\circ}\text{C}$	
ANT	69.41 a	61.90 a	33.20 b	4.42 c	22.21
POL	29.14 a	28.45 a	16.74 b	2.48 c	4.70
DPPH	60.18 a	59.87 a	36.96 b	5.87 c	3.18

ANT, anthocyanin content, $\mu\text{g/g}$ FW; POL, polyphenols, mg GAE/g DW; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DPPH, $\mu\text{M TE/g DW}$; Data are means for three replications.

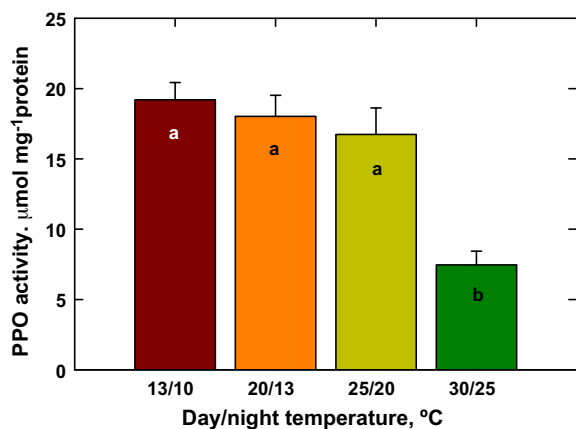


Fig. 1. Effects of day/night combined temperatures on the PPO activity of 6 lettuce cultivars (averaged data). The PPO activity was determined 6 weeks after temperature treatment. The various lowercase letters within the columns indicates significant differences among the treatments at different temperatures ($p < 0.05$). The bars represent the standard error (SE).

3.2. PPO activity

The average PPO activity of cultivars was highest at 20/13 °C ($18.6 \mu\text{mol mg}^{-1} \text{protein min}^{-1}$) and reduced at 30/25 °C ($7.5 \mu\text{mol mg}^{-1} \text{protein min}^{-1}$) (Fig. 1). The cultivar rankings were not consistent among day/night temperatures, and no significant difference among cultivars at each temperature regime was observed. At 20/13 °C, “Dazzler” had the highest PPO activity followed by ‘Fire’ and ‘Junjuck’ whereas at 30/25 °C, the PPO activities for ‘Dazzler’ and ‘Seoul red’ were relatively lower than the others (data not shown). The oxidation of phenolic substrates by PPO is a major cause of the brown discoloration of many fruits and vegetables [39].

3.3. POD activity

Both PPO and POD have been considered defensive mechanisms for plants against stress [11]. The POD activity in lettuce, in contrast with that of PPO, was the lowest at 20/13 °C and was increased at higher temperatures (Fig. 2). At 25/20 °C, specifically, ‘Junjuck’ had the highest POD activity, and ‘Fire’ had the lowest POD activity (data are not shown). However, the POD activities

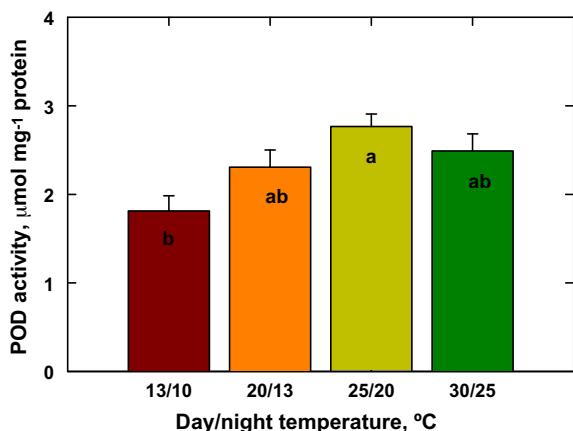


Fig. 2. Effects of day/night combined temperatures on POD of 6 lettuce cultivars of (averaged data). The POD activity was determined 6 weeks after temperature treatment. The various lowercase letters within the columns indicates significant differences among the treatments at different temperatures ($p < 0.05$). The bars represent SE.

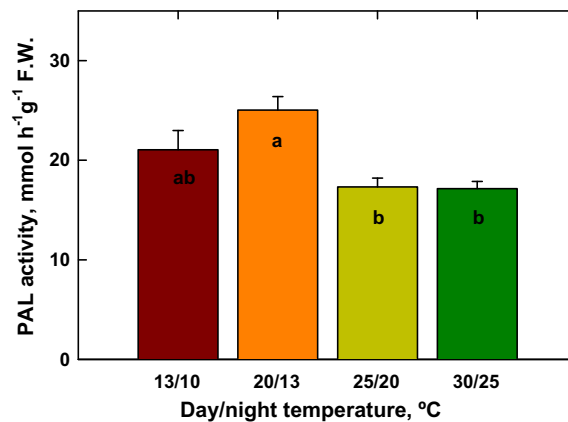


Fig. 3. Effects of day/night combined temperatures on PAL of 6 lettuce cultivars of (averaged data). The PAL activity was determined 6 weeks after temperature treatment. The various lowercase letters within the columns indicates significant differences among the treatments at different temperatures ($p < 0.05$). The bars represent SE.

of lettuce responded differently, depending on temperature, but not on the cultivar. The POD activity was highest at 25/20 °C while it was the lowest at 20/13 °C. Such results demonstrate a potential relationship between POD activity and physiological disorders in vegetables [11], where the POD activities often increase with the plant development and senescence [3]. Our results are not consistent with another report [40] where the peel browning of harvested litchi fruit was largely attributed to the rapid degradation of red anthocyanin pigments. This process was associated with the enzymatic oxidation of phenolics by PPO and POD. However, the direct oxidation of anthocyanins does not occur by these enzymes in litchi pericarp. Thus, litchi enzymatic browning might involve an anthocyanase–anthocyanin–phenolic–PPO reaction [40]. Future studies should be conducted to determine the mechanism of browning reactions in lettuce.

3.4. PAL activity

The PAL activity at 13/10 °C and 20/13 °C was significantly higher than that at higher day/night temperatures (Fig. 3). The PAL activity was higher at 20/13 °C than at 25/20 °C or at 30/25 °C ($p < 0.05$). This result indicates that the low temperatures during the photoperiod enhanced PAL activity. The PAL activity in lettuce *in vitro* was the highest at lower temperatures. However, the activity declined at higher temperatures, suggesting an inactivation of the enzyme. The relationship between the anthocyanin content (Table 1) and the temperature regime was similar to the changes in PAL activities. However, the range in PAL activities among cultivars showed no genetic differences. This result indicates that PAL activities were more affected by temperatures than by cultivars [34]. Our data are consistent with other reports that the increase in PAL activity is associated with increased anthocyanin content in several plant tissues [15,19]. The PAL activity in plant tissues is increased by factors such as temperature, light, and plant growth regulators. The increased PAL activity in lettuce facilitates a high rate of anthocyanin content. The relationships between PAL activity and anthocyanin accumulation in the apple varied depending on the development stage of the fruit [15]. The increase in PAL and PPO activities occurred more rapidly at 6 °C than at 10 °C in the banana, showing highly significant correlations between chilling injury (browning) and the activities of PPO and PAL. The banana fruits were kept at 10 °C for 8 h, and 60 differential expressed fragments in the pulp and peel were obtained [22]. In another investigation, the activity of PPO in the leaves of sil-

Table 2

Correlation between anthocyanin content, polyphenols, DPPH, PPO, POD and PAL activities and polyphenols and DPPH of lettuce plants.

	ANT	POL	DPPH	PPO	POD	PAL
ANT	1.0000	0.9883	0.9792	0.8648	0.4700	0.6127
POL		1.0000	0.9986	0.9059	0.3649	0.6315
DPPH			1.0000	0.9215	0.3293	0.6217
PPO				1.0000	0.1768	0.3659
POD					1.0000	0.2606
PAL						1.0000

ANT, anthocyanin content, $\mu\text{g/g}$ FW; POL, polyphenols, mg GAE/g DW; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DPPH, $\mu\text{M TE/g}$ DW; PPO, polyphenol oxidase; POD, peroxidase; PAL, phenylalanine ammonia-lyase.

ver birch (*Betula pendula* Roth) seedlings was low but increased with elevated temperature [24]. A close correlation between the increase in PAL activity and the anthocyanin content in strawberry fruits and grape berries during various ripening stages was investigated. It was proposed that the increased PAL activity is required for a high rate of anthocyanin synthesis [41,42]. This relationship suggests that the phenylpropanoid pathway is synchronized with fruit ripening, as observed in other plant systems [43], and that the activity of the enzyme is involved in the synthesis of flavonoids in leaves, including anthocyanins and polyphenols. The obtained correlation between the polyphenols and the antioxidant activity corresponds with our previous investigations of the Korean lotus [32]. Other research reports [44] show that PAL inhibition by 2-aminoindan-2-phosphonic acid rendered these plants more sensitive to chilling and heat shock treatments. These results suggest that the activation of secondary and antioxidative metabolism is an integral part of plant adaptation to normal growing conditions in lettuce plants. In our research, the polyphenols in different varieties do not differ significantly. In another report [45], in which the most widespread lettuce cultivars ('Montego', 'Great Lakes' and 'Salad Bowl. Contrary') were studied, it was found that the concentration of polyphenols differs among the three varieties and that it was highest in 'Salad Bowl'.

The correlations between the anthocyanin level and the PPO, POD, or PAL activities in lettuces influenced by day/night temperatures were analyzed. The most significant positive correlation (Table 2) existed between anthocyanin content and PPO activity ($r^2=0.8648$), followed by a correlation with PAL activity ($r^2=0.6127$). The polyphenols (POL) were highly correlated with DPPH and the enzymes: POL and DPPH ($r^2=0.9986$); POL and PPO ($r^2=0.9059$), POL and PAL ($r^2=0.6315$), DPPH and PPO ($r^2=0.9215$), and DPPH and PAL ($r^2=0.6117$). However, there was a low correlation coefficient between anthocyanin content and POD activity. This result showed that the low temperature induced an increased level of anthocyanins and polyphenols and PPO and PAL activities, showing more polyphenol and anthocyanin production and the enzyme activities at low temperatures. Growth conditions are one of the most important factors of the nutritional value of this plant [46]. Although the cost of red lettuce is higher than that of green lettuce, it is becoming popular among consumers. This popularity is probably due to its red color and its association with increased health, as in the case of red fruits and berries [47]. The results show that at relatively low temperatures, lettuce plants have a high antioxidant and enzymatic status, and this result provides additional information for lettuce growers.

In conclusion, this study shows that low temperatures lead to an increase of anthocyanin and polyphenol production as well as an increase in antioxidant, PPO and PAL activities in lettuce plants. The cultivar responses of lettuce plants were not consistent for the anthocyanin and polyphenol contents and the activities of PPO, POD and PAL. PPO and PAL activities were high at low temperatures of 20/13 °C, and their activities were low at higher temperatures. Specifically, the anthocyanin content in lettuce was highly corre-

lated with PPO and PAL activities. Day/night temperatures affect anthocyanin content and the activities of PPO, POD and PAL in lettuce leaves.

References

- [1] M. Ordidge, P. Garcia-Macias, N.H. Battey, M.H. Gordon, H. Hadley, P. John, J.A. Lovegrove, E. Vysini, A. Wagstaffe, Phenolic contents of lettuce, strawberry, raspberry, and blueberry crops cultivated under plastic films varying in ultraviolet transparency, *Food Chem.* 119 (2010) 224–227.
- [2] R. Llorach, A. Martínez-Sánchez, F.A. Tomás-Barberán, M.I. Gil, F. Ferreres, Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole, *Food Chem.* 108 (2008) 1028–1038.
- [3] A. Grover, S.K. Sinha, Senescence of detached leaves in pigeon pea and chick pea: regulation by developing pods, *Physiol. Plant.* 65 (1985) 503–507.
- [4] H. Lee, C. Aedin, A review of the health care potential of bioactive compounds, *J. Sci. Food Agric.* 86 (2006) 1805–1813.
- [5] W. Zhang, M. Seki, S. Furusaki, Effect of temperature and its shift on growth and anthocyanin production in suspension cultures of strawberry cells, *Plant Sci.* 127 (1997) 207–214.
- [6] A. Romani, P. Pinelli, C. Galardi, G. Sani, A. Cimato, D. Heimler, Polyphenols in greenhouse and open-air-grown lettuce, *Food Chem.* 79 (2002) 337–342.
- [7] A. Altunkaya, V. Gökmen, Effect of various anti-browning agents on phenolic compounds profile of fresh lettuce (*L. sativa*), *Food Chem.* 117 (2009) 122–126.
- [8] A. Sanches-Ferrer, J. Villaba, G. Garcia Carmona, Partial purification of a thylakoid-bound enzyme using temperature-induced phase partitioning, *Anal. Biochem.* 72 (1990) 248–254.
- [9] H. Siers, Oxidative stress: from basic research to clinical application, *Am. J. Med.* 91 (1991) 31–38.
- [10] A.M. Mayer, Polyphenol oxidases in plants. Recent progress, *Phytochem.* 26 (1987) 11–20.
- [11] L. Vamos-Vigyazo, Polyphenoloxidase and peroxidase in fruits and vegetables, *CRC Crit. Rev. Food Sci. Nutr.* 15 (1981) 49–126.
- [12] I. Söderhäll, Properties of carrot polyphenoloxidase, *Phytochemistry* 39 (1995) 33–38.
- [13] P. Thyapong, M.D. Hunt, J.C. Steffens, Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase, *Phytochemistry* 40 (1995) 673–676.
- [14] S.S. Kwak, S.K. Kim, I.H. Park, J.R. Liu, Enhancement of peroxidase activity by stressed-related chemicals in sweet potato, *Phytochemistry* 43 (1996) 565–568.
- [15] Z. Ju, Y. Yuan, C. Liu, H. Dai, S. Zhan, Study on characteristics of phenolics synthesis in apple peel, *J. Laiyang Agric. Coll.* 9 (1992) 226–230.
- [16] J.E. Lancaster, J.E. Grant, C.E. Lister, Skin color in apples-Influence of copigmentation and plastid pigmentation shade and darkness of red color in five genotypes, *J. Am. Soc. Hortic. Sci.* 119 (1994) 63–69.
- [17] K.B. MacRae, P.D. Lidster, A.C. De Marco, A.J. Dick, Comparison of the polyphenol profiles of apple fruit cultivars by correspondence analysis, *J. Sci. Food Agric.* 50 (1990) 329–342.
- [18] W. Oleszek, C.Y. Lee, A.W. Jaworski, K.R. Price, Identification of some phenolic compounds in apples, *J. Agric. Food Chem.* 36 (1988) 430–432.
- [19] S.A. Basha, B.K. Sarma, D.P. Singh, K. Annapurna, U.P. Singh, Differential methods of inoculation of plant growth-promoting rhizobacteria induce synthesis of phenylalanine ammonia-lyase and phenolic compounds differentially in chickpea, *Folia Microbiol. (Praha)* 51 (2006) 463–468.
- [20] C. Gerrish, M.P. Robbins, R.A. Dixon, Trans-cinnamic acid as a modulator of chalcone isomerase in bean cell suspension cultures, *Plant Sci.* 38 (1985) 23–27.
- [21] N. Fujita, E. Tanaka, M. Murata, Cinnamaldehyde inhibits phenylalanine ammonia-lyase and enzymatic browning of cut lettuce, *Biosci. Biotechnol. Biochem.* 70 (2006) 672–676.
- [22] J.H. Caamal-Velázquez, B.H. Chi-Manzanero, J.J. Canche-Yam, E. Castaño, L.C. Rodríguez-Zapata, Low temperature induce differential expression genes in banana fruits, *Sci. Hortic.* 114 (2007) 83–89.
- [23] P. Yingsanga, V. Srilaong, S. Kanlayanarat, S. Noichinda, W.B. McGlasson, Relationship between browning and related enzymes (PAL, PPO and POD) in rambutan fruit (*Nephelium lappaceum* Linn.) cvs. Rongrien and See-chompoo, *Postharvest Biol. Technol.* 50 (2008) 164–168.

- [24] T. Tegelberg, R. Julkunen-Tiitto, M. Vartiainen, R. Paunonen, M. Rousi, S. Kellomaki, Exposures to elevated CO₂, elevated temperature and enhanced UV-B radiation modify activities of polyphenol oxidase and guaiacol peroxidase and concentrations of chlorophylls, polyamines and soluble proteins in the leaves of *Betula pendula* seedlings, *Environ. Exp. Bot.* 62 (2008) 308–315.
- [25] T. Mori, T.M. Sakurai, Production of anthocyanin from strawberry cell suspension cultures; effects of sugar and nitrogen, *J. Food Sci.* 59 (1994) 588–593.
- [26] V. Kahn, Polyphenol oxidase isoenzymes in avocado, *Phytochem.* 15 (1976) 267–272.
- [27] J.M. Ruiz, G. Bretones, M. Baghour, L. Ragala, A. Belakbir, L. Romero, Relationship between boron and phenolic metabolism in tobacco leaves, *Phytochemistry* 48 (1998) 269–272.
- [28] K.C. Nagarathna, A.S. Sudheer, H.S. Shetty, Phenylalanine in pearl mollet seedlings and its relation to down mildew diseases resistance, *J. Exp. Bot.* 44 (1993) 1291–1296.
- [29] Z. Li, X. Zhao, A.K. Sandhu, L. Gu, Effects of exogenous abscisic acid on yield, antioxidant capacities, and phytochemical contents of greenhouse grown lettuces, *J. Agric. Food Chem.* 58 (2010) 6503–6509.
- [30] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, *Methods Enzymol.* 299 (1999) 152–178.
- [31] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, *LWT, Food Sci. Technol.* 28 (1995) 25–30.
- [32] Y.-S. Park, K. Towantakavanit, T. Kowalska, S.-T. Jung, K.-S. Ham, B.-G. Heo, J.-Y. Cho, J.-G. Yun, H.-J. Kim, S. Gorinstein, Bioactive compounds and antioxidant and antiproliferative activities of Korean white lotus cultivars, *J. Med. Food* 12 (2009) 1057–1064.
- [33] SAS (Statistical Analysis Systems) Institute, SAS/STAT user's guide. Version 7. Electronic Version, Cary, NC, USA, 2000.
- [34] S.-U. Chon, H.-O. Boo, B.-G. Heo, S. Gorinstein, Anthocyanin content and the activities of polyphenol oxidase, peroxidase, and phenylalanine ammonia-lyase in lettuce cultivars, *Int. J. Food Sci. Nutr.* (2011), doi:10.3109/096374486.2011.595704.
- [35] F. He, L. Mu, G.-L. Yan, N.-N. Liang, Q.-H. Pan, J. Wang, M.J. Reeves, C.-Q. Duan, Biosynthesis of anthocyanins and their regulation in colored grapes, *Molecules* 15 (2010) 9057–9091.
- [36] S.T. Jeong, N. Goto-Yamamoto, S. Kobayashi, M. Esaka, Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins, *Plant Sci.* 167 (2004) 247–252.
- [37] P.K. Farage, D. Blowers, S.P. Long, N.R. Baker, Low growth temperatures modify the efficiency of light use by photosystem II for CO₂ assimilation in leaves of two chilling-tolerant C4 species, *Cyperus longus* L. and *Miscanthus x giganteus*, *Plant Cell Environ.* 29 (2006) 720–728.
- [38] M.J. Fryer, J.R. Andrews, K. Oxborough, D.A. Blowers, N.R. Baker, Relationship between CO₂ assimilation, photosynthetic electron transport and active oxygen metabolism in leaves of maize in the field during periods of low temperature, *Plant Physiol.* 116 (1998) 571–580.
- [39] K.C. Vaughn, A.R. Lax, S.O. Duke, Polyphenol oxidase: the chloroplast oxidase with no established function, *Physiol. Plant.* 72 (1988) 659–665.
- [40] Y. Jiang, X. Duan, D. Joyce, Z. Zhang, J. Li, Advances in understanding of enzymatic browning in harvested litchi fruit, *Food Chem.* 88 (2004) 443–446.
- [41] N.K. Given, M.A. Venis, D. Grierson, Purification and properties of phenylalanine ammonia-lyase from strawberry fruit and its synthesis during ripening, *J. Plant Physiol.* 133 (1988) 31–37.
- [42] I. Kataoka, Y. Kubo, A. Sugiura, T. Tomana, Changes in L-phenylalanine ammonia-lyase activity and anthocyanin synthesis during berry ripening of three grape cultivars, *J. Jpn. Soc. Hortic. Sci.* 52 (1983) 273–279.
- [43] D.D. Templeton, C.J. Lamb, Elicitors and defense gene activation, *Plant Cell Environ.* 11 (1988) 395–401.
- [44] M.-M. Oha, H.N. Trick, C.B. Rajashekar, Secondary metabolism and antioxidants are involved in environmental adaptation and stress tolerance in lettuce, *J. Plant Physiol.* 166 (2009) 180–191.
- [45] R. Pernice, D. Scuderi, A. Napolitano, V. Fogliano, C. Leonardi, Polyphenol composition and qualitative characteristics of fresh-cut lettuce in relation to cultivar, mulching, and storage, *J. Hortic. Sci. Biotechnol.* 82 (2007) 420–427.
- [46] C.N. Nicolle, E. Cardinault, G.L. Jaffrelo, E. Rock, Health effect of vegetable-based diet, lettuce consumption improves cholesterol metabolism and antioxidant status in the rat, *Clin. Nutr.* 23 (2004) 605–614.
- [47] P. Arancibia-Avila, F. Toledo, E. Werner, M. Suhaj, H. Leontowicz, M. Leontowicz, A.L. Martinez-Ayala, P. Paško, S. Gorinstein, Partial characterization of a new kind of Chilean Murtilla-like berries, *Food Res. Intern.* 44 (2011) 2054–2062.