

Anthocyanin content and the activities of polyphenol oxidase, peroxidase and phenylalanine ammonia-lyase in lettuce cultivars

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Abstract

Anthocyanin content and the activities of polyphenol oxidase (PPO), peroxidase (POD) and phenylalanine ammonia-lyase (PAL) and their relationships were determined in the leaves of six lettuce (*Lactuca sativa* L.) cultivars, exposed for 6 weeks to alternating three different day/night temperatures. Anthocyanin content was found to be highest at 20/13°C, followed by 25/20°C and 30/25°C, showing accumulation of anthocyanin at low temperatures. Activities of PPO and PAL were also found to be highest at low day/night temperatures, whereas the POD activity was decreased at low day/night temperatures. The most significant positive correlation existed between anthocyanin content and PPO activity ($r^2 = 0.71$). The results suggest that various day/night temperature regimes affect anthocyanin content and the activities of PPO, POD and PAL in lettuce.

Keywords: lettuce, anthocyanin content, PPO, POD, PAL

Introduction

Anthocyanins are water-soluble pigments, which derive from flavonoids via the shikimic acid pathway. Anthocyanins can be transient, appearing only in juvenile or senescing tissues, or permanent. Likewise, they may be environmentally transient, appearing and disappearing with changes in photoperiod, temperature or other signals. Anthocyanins, as secondary metabolites, play an important role in plant-derived food quality, affecting characteristics such as appearance, flavour and health-promoting properties. Anthocyanin content may be influenced by several factors including temperature (Zhang et al. 1997), UV radiation (Wellmann et al. 1976), light intensity (Zhong et al. 1991; Kobayashi et al. 1993), sugar content (Mori and Sakurai 1994; Nakajima et al. 1989) and osmotic stress (Do and Cormier 1991). It was reported that the maximum anthocyanin production occurred at 20°C (Zhang et al. 1997).

The data of the relationship between anthocyanin synthesis and polyphenol oxidase (PPO) or peroxidase (POD) activities were limited. However, anthocyanin synthesis in several plant tissues is known to be associated with increased phenylalanine ammonia-lyase (PAL) activity (Ju et al. 1995). It is generally accepted that PPO and PAL activities and the storage or ripening temperatures in most investigations of food, fruits and vegetables showed a significant correlation (Nguyen et al. 2003; Xu 2005). Therefore, in this investigation, various combinations of day/night temperatures on anthocyanin content and PPO, POD and PAL activities in six lettuce cultivars were examined. As far as we know, no studies of the properties of enzymes in lettuce, describing the temperature dependence of anthocyanin content and activities of PPO, POD and PAL, were published.

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Materials and methods

Plant samples

The seeds were purchased from a seed company ('Seoul Seed', Seoul, Korea). The experimental design was a randomized complete block with three replicates. Seeds of six red-coloured lettuce cultivars, namely 'Hongyil', 'Red Fire', 'Jinjuck', 'Dazzler', 'Fire' and 'Seoul Red', were planted into plastic pots (10 cm diameter × 15 cm high) and cultured in a greenhouse that maintained the temperature at 22/20°C (day/night) for 35 days. Leaves were sampled after being subjected to the treatment conditions for 6 weeks, ensuring that the leaf tissue had acclimated to the treatment conditions.

Determination of anthocyanin content

Anthocyanins were extracted from the leaves that had been soaked overnight in methanol containing 1% HCl. The crude pigment extract was filtered through Whatman 42 filter paper, evaporated and hydrolysed with 15% HCl. Anthocyanin concentration of the purified aglycone was analysed using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC Waters 2996 Alliance System (Milford, MA, USA)). The TLC solvent for analysis was acetic acid–HCl–water (30:3:10), and the TLC plate was DC-Plastikfolien Cellulose, 20 × 20 cm, 0.1 mm in thickness. For HPLC analysis, the extract was injected into a μ Bondapak C₁₈ column, 3.9 × 300 mm (Waters, Dublin, Eire, Ireland). The solvent was water–methanol–acetic acid (58:40:2), flow rate was 0.7 ml/min and the detector operated at 530 nm (Mori and Sakurai 1994; Jakobek et al. 2011).

PPO, POD and PAL activity assays

PPO activity ($\mu\text{mol mg}^{-1}$ protein min^{-1}) was carried out in fresh plant material, which was grounded in a mortar with 0.1 M buffer ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7.0, containing 1.5% solid polyvinylpyrrolidone (PVP). The homogenate was filtered and centrifuged at 15,000 *g* for 15 min (Kahn 1976). PPO activity was measured at 30°C spectrophotometrically at 370 nm by the disappearance of caffeic acid.

POD activity assay was done according to Ruiz et al. (1998). Fresh material was grounded with 50 mM Tris–acetate buffer, pH 7.5, containing 2 mM EDTA and 0.5% PVP. The homogenate was filtered and centrifuged at 40,000 *g* for 10 min. All procedures were carried out at 4°C. The reaction mixture of 3 ml consisted of 100 μM Tris–acetate buffer, pH 5.0, 1 μM EDTA, 0.1 μM guaiacol and 0.003 μM H_2O_2 . POD activity ($\mu\text{mol mg}^{-1}$ protein min^{-1}) in the supernatant was determined following the absorbance changes at 485 nm due to guaiacol oxidation.

The enzyme PAL was extracted, purified and assayed according to the method of Nagarathna

et al. (1993). The reaction mixture of 3 ml contained 0.5 ml enzyme extract, 2.5 ml of 10 mM L-phenylalanine and 2.5 ml of 25 mM sodium borate buffer (pH 8.8). After incubation for 2 h at 40°C, the activity was stopped using 0.1 ml 5N HCl. The enzyme activity in the supernatant was measured spectrophotometrically as a product of *trans*-cinnamic acid from L-phenylalanine. Absorbance at 290 nm was read against the same volume of reaction mixture without L-phenylalanine. Enzyme activity was expressed as millimoles of *trans*-cinnamic acid produced per hour per gram of FW.

Statistical analysis

Statistical Analysis System (SAS Institute, 2000) was used. The means were separated by least significant difference (LSD) *F*-test. Correlation analysis was adopted with the correlation coefficient methods, among anthocyanin level and the activities of PPO, POD and PAL at different day/night temperatures.

Results and discussion

Anthocyanin content

Anthocyanin content was high at day/night temperatures of 20/13°C and progressively decreased as day/night temperatures were increasing (Table I). Anthocyanin content of all cultivars at 20/13°C was higher than at 25/20°C or at 30/25°C ($p < 0.05$). The significantly highest anthocyanin content was determined in 'Hongyil' cultivar at all investigated temperatures in comparison with other five cultivars. This observation is consistent with data of Zhang et al. (1997), who reported that low temperature induced an increased level of anthocyanin content in suspension cultures of strawberry cells.

PPO activity

PPO activity was found to be highest at 20/13°C (18.6 $\mu\text{mol mg}^{-1}$ protein min^{-1}), and was decreased

Table I. Effects of day/night combined temperatures on anthocyanin content of lettuce cultivars.

Lettuce cultivar	Anthocyanin content (mg kg ⁻¹ FW)			LSD (0.05)
	20/13°C	25/20°C	30/25°C	
Hongyil	70.5 ± 4.2	42.2 ± 2.2	6.3 ± 0.6	13.43
Red fire	59.3 ± 2.6	39.0 ± 1.8	5.1 ± 0.3	8.92
Jinjuck	59.8 ± 2.8	31.1 ± 1.4	3.8 ± 0.2	8.81
Dazzler	66.7 ± 3.6	33.1 ± 1.5	5.2 ± 0.4	11.01
Fire	57.1 ± 2.0	28.7 ± 1.2	2.9 ± 0.1	6.56
Seoul red	58.1 ± 1.6	25.3 ± 1.0	3.1 ± 0.1	5.31
LSD (0.05)	10.35	5.52	1.17	

Note: Data are means for three replications.

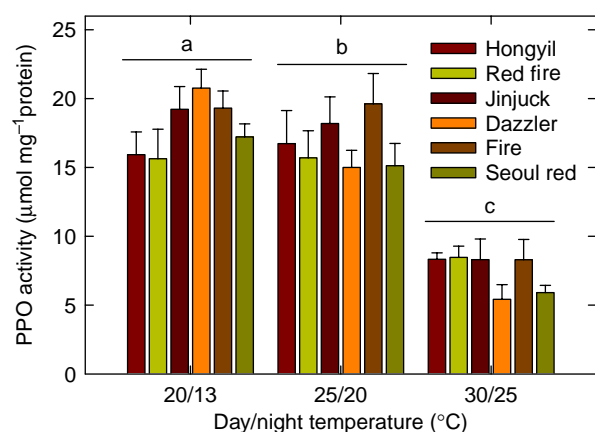


Figure 1. Effects of day/night combined temperatures on PPO activity of lettuce cultivars. PPO activity was determined 6 weeks after temperature treatment. Different small letters within the columns mean significant differences among plant species ($p < 0.05$). Bars represent SE ($n = 3$). No difference in activity of PPO among cultivars was observed.

at 30/25°C ($7.5 \mu\text{mol mg}^{-1} \text{protein min}^{-1}$) (Figure 1). Oxidation of phenolic substrates by PPO is a major cause of the brown discoloration of many fruits and vegetables (Walker and Ferrar 1998). The obtained results show that temperature effects influenced the anthocyanin content greater than that in the cultivar effects. Solecka et al. (1999) reported that leaves of oilseed rape grown in cold (2°C) for 3 weeks accumulated the amount of anthocyanins as much as twice at 20°C . This may indicate that enzymes of the anthocyanin pathway in plants act effectively after adjusting their metabolism to low-temperature conditions (Kacperska 1989). Nguyen et al. (2003) stored bananas at 6 and 10°C and found that the PPO activity of cultivar 'Kluai Khai' was considerably higher than that in cultivar 'Kluai Hom Thong'. The increase in PPO activity occurred more rapidly at 6°C than at 10°C in both cultivars.

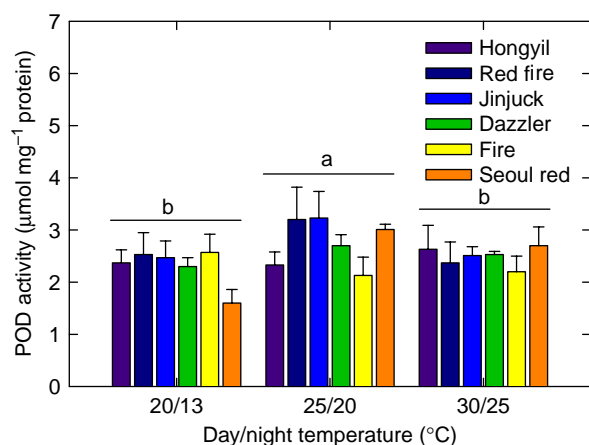


Figure 2. Effects of day/night combined temperatures on POD of lettuce cultivars. POD activity was determined 6 weeks after temperature treatment. Different small letters within the columns mean significant differences among plant species ($p < 0.05$). Bars represent SE ($n = 3$). No difference in activity of POD among cultivars was observed.

POD activity

POD activity in lettuce, in comparison with that of PPO, was the lowest at 20/13°C and was increased at higher temperatures (Figure 2). At 25/20°C, 'Junjuck' had the highest POD activity and cultivar 'Fire' had the lowest. This observation corroborates with other results that some relationship exists between POD activity and physiological disorders in vegetables (Robinson 1991). Activities of POD often increase with plant development and senescence (Grover and Sinha 1985).

PAL activity

At 20/13°C, PAL activity was significantly higher than at 25/20°C and 30/25°C ($p < 0.05$) (Figure 3). This indicates that low temperatures during the photo-period enhanced PAL activity more than high temperatures. However, the PAL activities among cultivars showed no genetic differences. This indicates that PAL activities were more affected by temperatures than by cultivar. Other authors reported similar results in studies of wampee fruit (Zhang and Li 2008). They investigated the effects of different storage temperatures on PAL, POD and PPO activities of wampee fruit and found that the best storage temperature was $2-4^\circ\text{C}$. The correlations between anthocyanin level and PPO, POD and PAL activities of lettuce cultivars influenced by day/night temperatures were calculated. PAL is induced by a variety of stimuli, including radiation, temperature, plant hormones, wounding and disease. Nguyen et al. (2003) reported that the increase in PAL and PPO activities occurred more rapidly at 6°C than at 10°C in banana, showing highly significant correlations between low temperature (browning) and the activities of PPO and PAL. The most significant positive correlation existed between

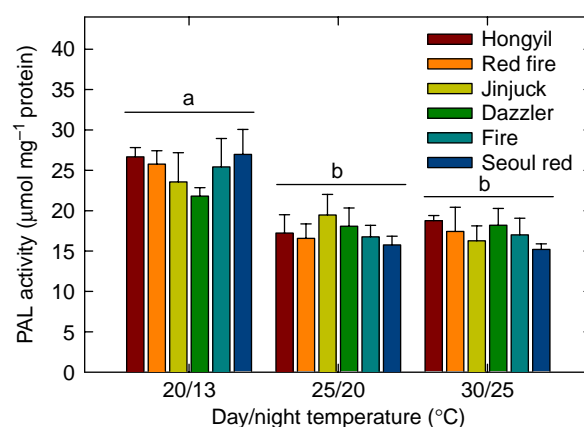


Figure 3. Effects of day/night combined temperatures on phenylalanine ammonia-lyase (PAL) activity of lettuce cultivars. PAL activity was determined 6 weeks after temperature treatment. Different small letters within the columns mean significant differences among plant species ($p < 0.05$). Bars represent SE ($n = 3$).

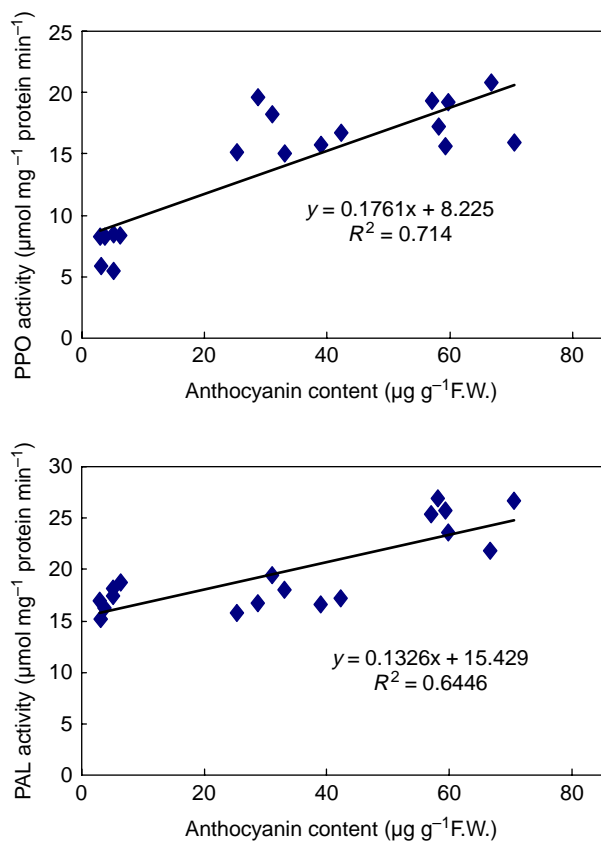


Figure 4. Correlations between anthocyanin level and PPO activity (top) and between anthocyanin level and PAL activity (bottom) of lettuce cultivars influenced by day/night temperatures.

anthocyanin content and PPO activity ($r^2 = 0.71$), and followed by the correlation between anthocyanin synthesis and PAL activity ($r^2 = 0.64$) (Figure 4).

Conclusion

- i) This study shows that low temperature leads to an increase in synthesis of anthocyanin and the activities of PPO and PAL in lettuce plants.
- ii) Cultivar differences of lettuce plants have limited effect on anthocyanin content and the activities of PPO, POD and PAL. The activities of PPO and PAL were higher at low temperatures of 20/13°C and lower at high temperatures.
- iii) Anthocyanin content in lettuce was highly correlated with PPO and PAL activities.
- iv) Day/night temperatures affect anthocyanin content and the activities of PPO, POD and PAL in lettuce leaves in laboratory experimental conditions.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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