Synergistic Effects of a Night Temperature Shift and Methyl Jasmonate on the Production of Anthocyanin in Red Leaf Lettuce

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Abstract

The production of a secondary metabolite such as anthocyanin is coordinately regulated by plant intrinsic factors and influenced by multiple environmental factors. In red leaf lettuce, the red pigment component anthocyanin is important for the commercial value of the crop, but its synchronous regulation by multiple factors is not well understood. Here, we examined the synergistic effects of a night temperature shift and methyl jasmonate (MJ) on the production of anthocyanin in red leaf lettuce. Low or high night temperature treatment for 3 days just before harvesting induced the production of anthocyanin without affecting plant biomass. Temperature-dependent activation of anthocyanin accumulation was accelerated by treating with MJ. Night temperature shifts and MJ triggered oxidative stresses in leaves, as indicated by hydrogen peroxide accumulation and lipid peroxidation. Interestingly, these oxidative stresses were more evident in leaves simultaneously treated with both a high night temperature and MJ. The activity of the superoxide dismutase (SOD) was increased alongside the elevation of oxidative stress. Taken together, these results indicate that the combined treatment of a night temperature shift with MJ may accelerate anthocyanin production by increasing the levels of oxidative stress to the leaves of red leaf lettuce.

1. Introduction

Plants can activate secondary metabolic pathways in response to a variety of environmental stresses, such as light, temperature, salinity, drought, and pathogens

[1] [2]. Indeed, a high or low temperature will often increase the production of secondary metabolites [3] [4] [5]. High temperature stress, for example, induced leaf senescence and increased the secondary metabolite concentrations in the root of the herb *Panax quinquefolius* [6]. Spinach plants grown at a low temperature increased their production of sugar and ascorbic acid in leaves compared with those grown under ambient conditions [7]. In strawberry plants, the amount of ascorbic acid in the fruits increased when these plants were exposed to low temperature [8], whereas anthocyanin decreased at a high temperature [9]. Thus, controlling the cultivation temperature may be an effective strategy for producing value-added crops that contain high amounts of useful secondary metabolites for humans.

Methyl jasmonate (MJ) is one of the organic volatile compounds involved in various developmental processes, such as those of seed germination, root elongation, flowering, fruit ripening, and aging [10] [11]. In addition, when a plant undergoes several stresses such as necrotrophic bacteria, insect attacks, and wounding, it releases MJ to initiate the local and systemic responses, which includes secondary metabolite production, to defend itself and neighboring plants [11]. Utilizing this property of MJ, the application of exogenous MJ treatment has been studied for enhancing the amount of secondary metabolites in various crop plants [12] [13] [14]. In sweet basil, the total phenolic content and two valuable phenolic compounds, rosmarinic acid and caffeic acid, are induced by an MJ treatment [12]. Anthocyanins, a phenolic compound better known as plant red pigments, have also been induced by MJ in *Arabidopsis* leaves, accompanied by the expression of a series of genes involved in the biosynthesis of anthocyanin [15].

In recent years, lettuces have been cultivated in plant factories, and there is more demand for technology to control these cultivation environments to increase the crop value to offset the high cultivation costs. Antioxidants, such as anthocyanins, are considered one of the functional ingredients that can increase the added value of crops [16]. To date, various environmental stresses have been shown that enhance the production of anthocyanin in plants [17]. In lettuces, it is reported that lowering the growth temperature during the night promotes the production of anthocyanin [18] [19] [20]. In these studies, the temperature shift is considered to impose only a mild stress to the plants since the growth temperature conditions are set within the range of 10°C to 30°C. Lettuce anthocyanins are also induced by MJ treatment, as seen in other plants and for other metabolites [21] [22]. The MJ-induced anthocyanin accumulations are further enhanced by exposing plants to high light conditions in several plants [21] [23] [24]. Similarly, low temperature-induced anthocyanin production is dependent on the intensity of light in red leaf lettuce [20]. Although the light intensity is closely linked to the anthocyanin production triggered by temperature and MJ, the relationship between MJ and temperature in lettuce anthocyanin production is largely unknown. In the present study, we investigated the influence of high or low night temperature in combination with an MJ treatment on the anthocyanin

production in red leaf lettuce. We also examined the oxidative stress parameters in these plants as exposed to different night temperatures and to MJ for a better understanding of the oxidative stress in the lettuce anthocyanin production.

2. Materials and Methods

2.1. Plant Growth Conditions and the MJ Treatment

Red leaf lettuce seeds (*Lactuca sativa* L. cv. Red Wave; Sakata Seed Co., Japan) were pre-germinated for 1 day at 20°C under 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux (PPF) for 12 h by using fluorescent lamps (FL40SBR-A; NEC Co., Japan). The germinated seeds were sown in sponge cubes of $2 \times 2 \times 2$ cm where they were grown under the same prior conditions. The nutrient solution was based on a half-strength culture solution of the Otsuka House A-recipe (Otsuka Chemical Co., Japan). Plants were grown under a 12-h day/night cycle throughout the experiments. After 14 days from the date of sowing, the night temperature treatment was initiated by transferring plants to different temperature conditions at nighttime. The temperature of the day period was set at 20°C in all plants. MJ (5 mM) was sprayed onto the whole leaves for the last 1 h of night temperature treatment and all the plants were kept in the dark for 1 h at 20°C. The control plants were sprayed with distilled water. These treatments were repeated for 3 days. After 17 days from the date of sowing, and just after exposure to light for 12 h, the plants were harvested, and the growth and metabolite analyses were conducted. All experiments were repeated at least four times—all obtained similar results.

2.2. Measurement of the Anthocyanin Content

The anthocyanin content was spectrophotometrically measured as described elsewhere [25]. The second true leaves (50 mg) were homogenized with 1 mL methanol containing 1% hydrochloric acid. After storage at 4°C overnight, each sample was centrifuged at 10,000 g for 5 min at room temperature. The absorbance of the supernatant was measured at 533 nm, and a standard curve was prepared by using cyanidin-3-glucoside.

2.3. Measurement of the Total Phenol Content

The total phenol content was measured using the modified Folin-Ciocalteu method, as described previously [25]. The second true leaves (50 mg) were homogenized with 500 μL of 90% methanol. After storage at 4°C overnight, each sample was centrifuged at 10,000 g for 5 min at room temperature. The supernatant (50 μL) was diluted with distilled water to 650 μL, and a 50 μL of phenol reagent was mixed with it. After adding 300 μL of 5% sodium carbonate the mixture was incubated at 25°C for 30 min. The absorbance of the supernatant was measured at 765 nm, and a standard curve was prepared by using gallic acid.

2.4. Measurement of the Hydrogen Peroxide Content

The hydrogen peroxide (H_2O_2) content was spectrophotometrically measured as

described previously [26] but with slight modifications. The second true leaves (100 mg) were homogenized in 600 μL of 1% trichloroacetic acid (TCA). Then each sample was centrifuged at 10,000 g for 10 min. The supernatant (250 μL) was added to 250 μL of a 10 mM K-phosphate buffer (pH 7.0) and 500 μL of 1 M KI. The absorbance of the mixture was measured at 390 nm, and a standard curve was prepared by using H_2O_2 in the range from 25 - 500 μmol mL^{-1}.

2.5. Measurement of the Thiobarbituric Acid Reaction Content

The level of malondialdehyde—MDA, a lipid peroxidation product—was determined via the thiobarbituric acid reaction (TBAR) method [25] [27]. The second true leaves (100 mg) were homogenized in 0.5 mL of a 0.1% TCA solution. After centrifuging at 10000 g for 5 min, 0.2 mL of the supernatant was added to 0.8 mL of 0.5% thiobarbituric acid in 20% TCA. The mixture was incubated at 95°C for 30 min; the reaction was stopped by cooling on ice. After centrifuging at 10,000 g for 5 min at room temperature, the absorbance of the supernatant was measured at 532 nm and the nonspecific absorbance at 600 nm was subtracted. The concentration of MDA was quantified by using a molar extinction coefficient of 155 mM^{-1}·cm^{-1}.

2.6. Measurement of Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was spectrophotometrically measured as described previously [28], but with slight modifications. The second true leaves (100 mg) were homogenized in 0.5 mL of a 50 mM phosphate buffer (pH 7.0, 0.1 mM EDTA). The sample was centrifuged at 10,000 g for 10 min. The supernatant (2.5 μL) was added to 1 mL of a SOD reaction mixture (50 mM phosphate buffer [pH 7.7], 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium [NBT], 2 μM riboflavin). The reaction mixture was illuminated for 15 min at a light intensity of 300 μmol·m^{-2}·s^{-1}, and the absorbance was then monitored at 550 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the NBT reduction by 50%. The total soluble proteins were determined spectrophotometrically by using the reagent Protein Assay Rapid Kit (Wako Ltd., Japan) and by applying the Bradford method in which bovine serum albumin was used as the standard.

3. Results

3.1. Effects of Night Temperature on the Leaf Biomass in Red Leaf Lettuce

Three-day treatments of five different night temperatures (5°C, 10°C, 20°C, 30°C, and 37°C) did not significantly influence the leaf biomass of red leaf lettuce (Figure 1, Figure 2(A) and Figure 2(B)). By contrast, the water content of the leaves was significantly decreased by subjecting the lettuce plants to 5°C and 10°C low night temperatures as compared with the non-shifted control temperature (20°C), while the high night temperature shifts did not significantly influence the leaf water content (Figure 2(C)). Water contents of plants grown at
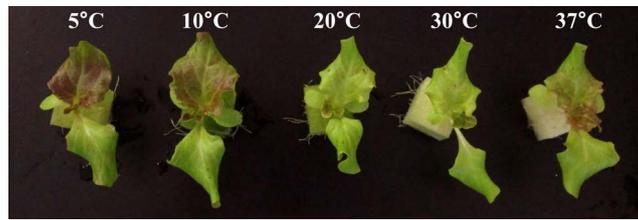
Figure 1. Effect of night temperature on the growth of red leaf lettuce plants.
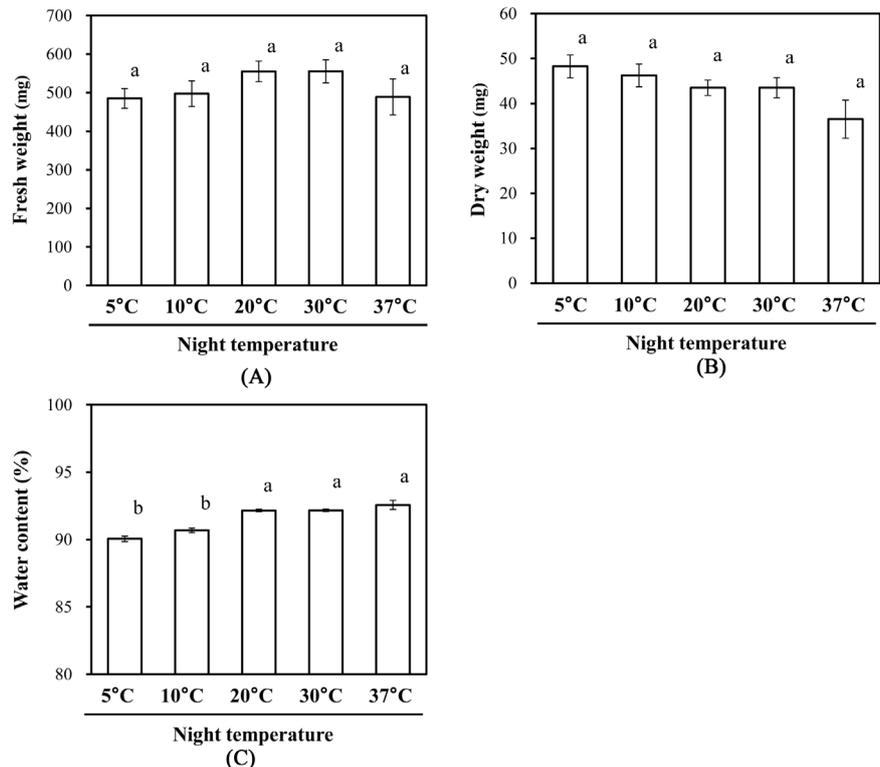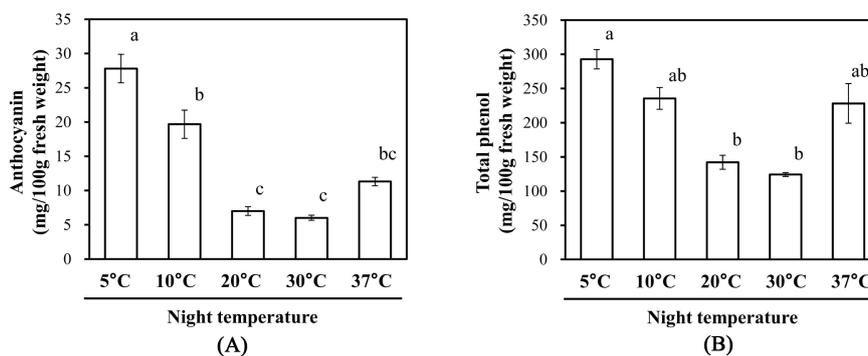

Figure 2. Effect of night temperature on the fresh weight (A); dry weight (B); and water content (C) of red leaf lettuce leaves. Vertical bars represent the means ± SEs ($n = 4$). Different letters indicate significant differences as determined by the Tukey's multiple comparison test ($p < 0.05$).

night temperature of 5˚C, 20˚C, and 37˚C were 90.1%, 92.2%, and 92.6%, respectively.

3.2. Effect of Night Temperature on the Anthocyanin and Total Phenol Content in Red Leaf Lettuce

Red pigmentation of the leaves was enhanced in those plants treated with low (5˚C and 10˚C) or high (37˚C) night temperatures, especially in second true leaves (Figure 1). In agreement with this result, the production of anthocyanin in second true leaves was significantly increased by the treatment of low night temperatures as compared with the control plants grown at 20˚C (Figure 3(A)). In plants grown at 5˚C and 10˚C, their total leaf anthocyanin content increased about 4.0- and 2.8-fold, respectively, when compared with the control plants. The plants grown at a 37˚C night temperature also displayed a 1.6-fold increase

Figure 3. Effect of night temperature on the anthocyanin (A) and total phenol (B) contents of red leaf lettuce leaves. Vertical bars represent the means ± SEs ($n = 4$). Different letters indicate significant differences as determined by the Tukey's multiple comparison test ($p < 0.05$).

in their amount of anthocyanin, though this difference was not significant (Figure 3(A)). Consistent with these results, the total phenol content of leaves exposed to either low or high temperatures at night was comparably increased as well (Figure 3(B)). The exposure of plants to temperatures of 5°C, 10°C, and 37°C at night caused about 2.1-, 1.7-, and 1.6-fold increase in their total phenol content, respectively, as compared with the control plants. However, no apparent differences in the anthocyanin and total phenol contents were observed between 20°C (control) and the 30°C treated plants (Figure 3).

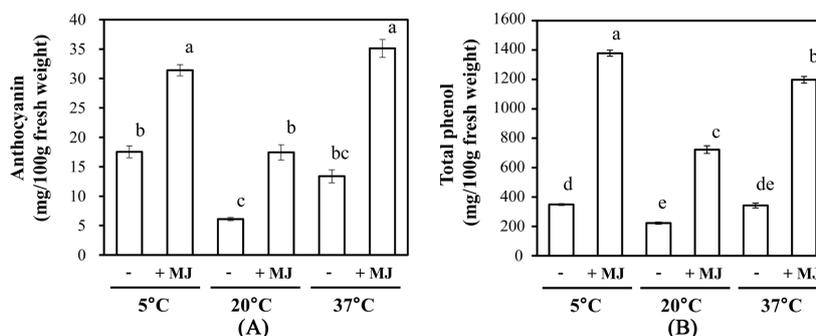### 3.3. Synergistic Effects of Night Temperature and MJ on the Leaf Biomass in Red Leaf Lettuce

Next, we tested the combined effects of either a low (5°C) or high (37°C) night temperature with MJ on the leaf biomass and metabolites in red leaf lettuce. After the 3-day treatment, MJ tended to reduce the leaf biomass regardless of the night temperature (Figure 4(A) and Figure 4(B)). The MJ-induced biomass reduction was greater in plants exposed to the low night temperatures as compared with plants exposed to the high night temperature. Similarly, those plants exposed to MJ showed a tendency of decreased leaf water content irrespective of the night temperature treatment (Figure 4(C)).

3.4. Synergistic Effects of Night Temperature and MJ on the Anthocyanin and Total Phenol Contents in Red Leaf Lettuce

The application of MJ increased the anthocyanin content in leaves regardless of the night temperature (Figure 5(A)). The effect of enhanced anthocyanin content by MJ was higher in those plants that were exposed to the high night temperature as compared with those that were co-treated with the low night temperature. The amounts of anthocyanin in leaves that were exposed to 5°C, 20°C, and 37°C night temperatures and co-treated with MJ respectively were approximately 1.8-, 2.9-, and 2.7-fold higher than that in corresponding leaves untreated with MJ. Consistent with these results, the production of total phenol was up regulated by the MJ treatment at all tested temperatures (Figure 5(B)).
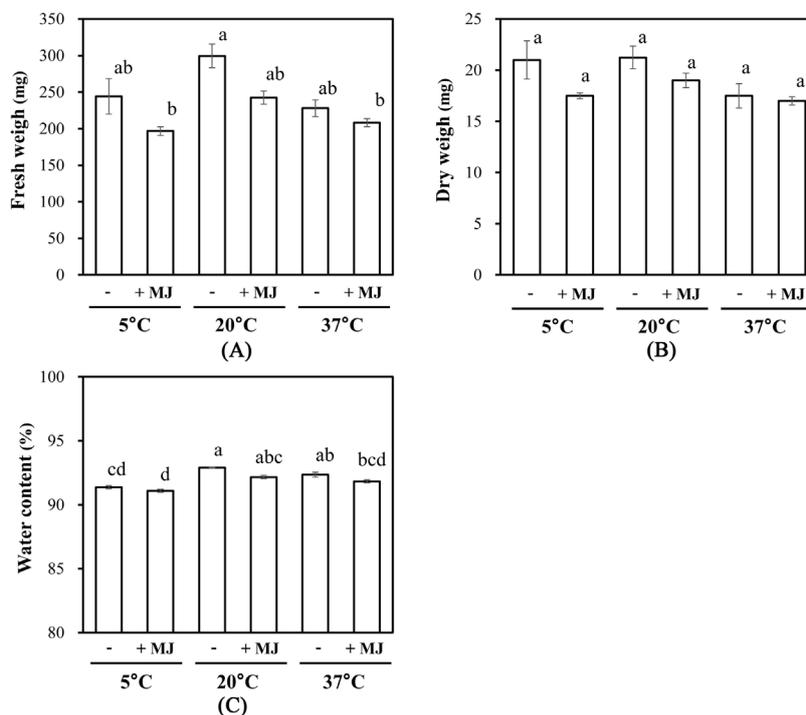
Figure 4. Effects of night temperature and MJ on the fresh weight (A), dry weight (B), and water content (C) of red leaf lettuce leaves. Vertical bars represent the means ± SEs ($n = 4$). Different letters indicate significant differences as determined by the Tukey's multiple comparison test ($p < 0.05$).

Figure 5. Effects of night temperature and MJ on the anthocyanin (A) and total phenol (B) contents of red leaf lettuce leaves. Vertical bars represent the means ± SEs ($n = 4$). Different letters indicate significant differences as determined by the Tukey's multiple comparison test ($p < 0.05$).

The total phenol contents of leaves that were exposed to 5°C, 20°C, and 37°C night temperatures and co-treated with MJ respectively were about 4.0-, 3.3-, and 3.5-fold higher than that in corresponding leaves untreated with MJ.

3.5. Synergistic Effects of Night Temperature and MJ on the Oxidative Properties in Red Leaf Lettuce

Anthocyanin accumulation is triggered by several oxidative stresses in plants [29] [30]. Therefore, we investigated the leaf H_2O_2 content in the experimental plants treated with night temperatures in combination with MJ. Low or high

night temperatures triggered the production of H_2O_2 in the absence of MJ (Figure 6(A)). The MJ treatment up regulated the production of H_2O_2 at any of the night temperatures tested. The level of H_2O_2 was significantly higher in those plants exposed to a high night temperature in combination with MJ as compared with those exposed to a low night temperature in combination with MJ. The amounts of H_2O_2 in leaves that were exposed to 5˚C, 20˚C, and 37˚C night temperature and co-treated with MJ respectively were about 1.4-, 1.5-, and 2.0-fold higher than that in corresponding leaves untreated with MJ.

To monitor the marker for oxidative stress status we measured a lipid peroxidation products, MDA using the TBAR method. Consistent with the above changes found in the amount of leaf H_2O_2, MDA was induced by either low or high night temperatures, and it was accelerated by the MJ treatment (Figure 6(B)). The amount of MDA in leaves that were exposed to 5˚C, 20˚C, and 37˚C night temperatures and co-treated with MJ respectively were approximately 1.3-, 1.2-, and 2.0-fold greater than that in corresponding leaves without MJ.
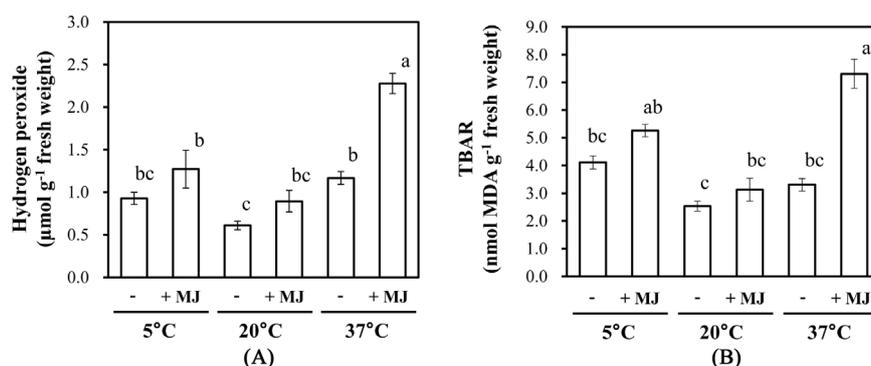
3.6. Synergistic Effects of Night Temperature and MJ on the Activity of SOD in Red Leaf Lettuce

Plants are able to mitigate oxidative stresses by activating antioxidative enzymes, such as SOD [31]. Hence, we also monitored the SOD activity of leaves in plants that were exposed to different night temperatures in combination with MJ. The SOD activity tended to increase at either a low or high night temperature treatment in the absence of MJ (Figure 7). Co-treatment with MJ up regulated the SOD activity, irrespective of any night temperature.

4. Discussion

4.1. Effect of Low Night Temperature on the Production of Anthocyanin

Low temperature induces the production of plant secondary metabolites, including anthocyanin, in various plants [17]. Similar to this view, our present re-

Figure 6. Effects of night temperature and MJ on the H_2O_2 content (A) and lipid peroxidation (B) of red leaf lettuce leaves. Vertical bars represent the means ± SEs (n = 4). Different letters indicate significant differences as determined by the Tukey's multiple comparison test ($p < 0.05$).
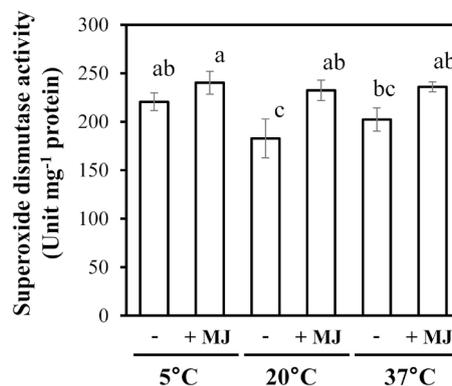
Figure 7. Effects of night temperature and MJ on the activity of SOD in red leaf lettuce leaves. Vertical bars represent the means ± SEs ($n = 4$). Different letters indicate significant differences as determined by the Tukey's multiple comparison test ($p < 0.05$).

sults show that the exposure of red leaf lettuce plants to a low night temperature triggers anthocyanin accumulation in their leaves. When stressed by low temperatures, plants can activate the expression of a variety of genes, some of which are involved in the biosynthesis of anthocyanin, such as *PAL*, *CHS,* and *CHI* in many plant species [32] [33] [34] [35] [36]. In lettuce, these anthocyanin biosynthesis genes can be transcriptionally regulated by the *Arabidopsis* MYB transcription factor AtMYB60 [37]. Given that MYB transcriptional factors are common regulators of the anthocyanin synthesis pathway in many plant species [38], the modulation of the temperature-responsive transcriptional factors that orchestrate the expression of the involved anthocyanin biosynthesis genes may offer a strategy for increasing the content of this valuable metabolite in lettuce crops. In this study, the plants underwent the temperature stresses during the night period only, which did not significantly affect their leaf biomass. This suggests that the method of a periodical temperature stress treatment may allow for the efficient production of valuable metabolites.

4.2. Effect of High Night Temperature on the Production of Anthocyanin

High temperature also modulates the production of anthocyanin, a process that is well studied, especially in fruit vegetables [9] [39] [40]. In apples and grapes, the colors of their fruits decrease under high temperatures due to the downregulation of anthocyanin accumulation [39] [40]. In accord with this, increasing the growing temperature decreases the expression of several genes encoding the transcription factors that regulate the anthocyanin synthetic genes [39] [41]. In prior studies, anthocyanin production in lettuce decreased when the growing temperatures were increased [18] [20] [42]. In contrast to that finding, here we found that a high night temperature enhances the anthocyanin content in red leaf lettuce leaves. Because the high temperature treatment in those previous studies was set 25°C - 30°C lower than that used in our present study (37°C), those temperatures might be insufficient to activate the heat stress signaling associated with the anthocyanin synthesis. It is also possible that the modest high

temperature stimuli used in previous studies may have caused alternations of the growth and metabolites as a result of a plant acclimation response to the modest temperature shift. Indeed, when 1-month-old sugarcane sprouts were exposed to more severe condition of heat stress (40˚C), within 24 h this treatment had triggered anthocyanin accumulation in them [43]. Given that exposure to 35˚C increased the phenolic compounds in tomato though this same treatment did not do so in watermelon plants [44], the particular temperature required for the induction of heat stress responses is likely species- and cultivar-dependent.

4.3. Synergistic Effects of Night Temperature and MJ on the Production of Anthocyanin and the Oxidative Stress Response

Previously, we demonstrated that MJ induced the anthocyanin production in red leaf lettuce in a light-dependent manner [21]. Here, we show that MJ accelerated the accumulation of anthocyanin in leaves triggered by the treatment with low or high night temperatures. Jasmonic acid (JA) is produced in response to biotic and abiotic stresses, and it acts as a signal molecule in the synthesis of flavonoids, such as anthocyanin [10] [45]. Alone, both JA and MJ can induce anthocyanin production in *Arabidopsis* by activating the expression of the anthocyanin biosynthesis genes *PAL*, *CHS*, *DFR*, *LDOX*, and *UF3GT* [15]. In guava fruits, an MJ treatment in combination with a 5°C-low temperature treatment increased PAL activity, which in turn led to the accumulation of total phenols [46]. Given that a low temperature stress is known to activate the anthocyanin biosynthetic genes in several plants [32] [33] [34] [35] [36], and that the low temperature-induced expressions of these genes were dependent on light [47], the augmentation of a low night temperature-induced anthocyanin accumulation by MJ in the red leaf lettuce plants may be caused by the cumulative upregulation of gene expression related to anthocyanin synthesis.

Chilling and heat stresses can trigger oxidative stress responses, including the production of reactive oxygen species which could play a key role in mediating important signal transduction responses involved in plant tolerance to changing temperatures [48]. Similar to this, in the present study, either a high or low night temperature treatment was able to induce H_2O_2 production and lipid peroxidation in the red leaf lettuce plants. These oxidative stress parameters were also upregulated by the MJ treatment regardless of the night temperature condition, and they were closely related to the production of anthocyanin. Anthocyanins are involved in several stress responses, namely as antioxidants that alleviate the oxidative damage in plants [49] [50] [51] [52] [53]. Considering that JA is also a mediator of oxidative stress responses and is involved in plant adaptation to mitigate several stresses [54], the increased production of anthocyanin in our experiments is among the antioxidative responses that resulted from the heat, chilling, and JA-mediated stress responses. The augmentation effects of MJ on anthocyanin synthesis, H_2O_2 accumulation, and lipid peroxidation, were more evident in those lettuce plants exposed to high night temperature as compared with those exposed to the low or control (20˚C) night temperatures. Interestingly, in

rice plants, the endogenous JA content is markedly increased under cold stress but it is reduced by heat stress [55]. In soybean plants, ethylene synthesis is activated under high temperature stress, which triggers premature leaf senescence by increasing the superoxide radical and H_2O_2 content, as well as membrane damage [56] [57]. Given that the color pigmentation of apple skin is enhanced by both MJ and ethylene, and enhanced by their combined treatment [58], the augmentation effect of the oxidative responses triggered by the high night temperature in combination with the MJ treatment may be driven by the dual induction of ethylene-dependent and JA-dependent signaling for anthocyanin synthesis.

In the present study, the low night temperature and the MJ treatment significantly decreased the leaf water content in red leaf lettuce, thus indicating a modest drought stress to leaves. Anthocyanin is produced in response to drought stress and it is involved in tolerance to this stress [17] [59] [60]. In *Arabidopsis*, the oxidative and drought tolerances are enhanced by an overaccumulation of antioxidant flavonoids, including anthocyanins [59]. Given that anthocyanin production is negatively related with the leaf water content in the present study, the low night temperature and MJ treatments may induce anthocyanin production in red leaf lettuce in part via the activation of drought stress signaling. Thus, the regulation of plant water status by manipulating the hydroponic cultivation system might be a useful way to further modulate the production of anthocyanin in crops of red leaf lettuce.

4.4. Conclusion

Our experimental results indicate that the combined treatment of multiple stimuli, such as the night temperature shift and the MJ treatment, could be a useful strategy to modulate plant secondary metabolite production in red leaf lettuce. Even if a single stress treatment is effective at increasing the content of valuable plant metabolites, this effect seems to be less than that arising from multiple stimuli treatments. Given that a single stimulus is required for a relatively long-term period to accumulate anthocyanin in plants, this may eventually affect the plant biomass of lettuce. Therefore, providing multiple short-term stimuli just before harvesting potentially offers a simple and effective way for many plants of a crop to gain more valuable metabolites while minimizing the stimuli's influence on plant biomass.

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