Effects of nutrient solution temperature on the concentration of major bioactive compounds in red perilla
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Abstract
Red perilla (Perilla frutescens) is used as food, stomachic and antitussive crude drugs, and anti-allergy supplements. The major bioactive compounds of red perilla are perillaldehyde (PA), rosmarinic acid (RA), luteolin (LU), and anthocyanin (ANT). In this study, we investigated the effects of 6 days low (10, 12, and 15°C) and control (20°C) nutrient solution temperature (NST) treatments on plant growth and major bioactive compound concentrations in red perilla leaves at the node position. No significant difference was detected in the dry weight of leaves of the main shoot, which is the main part harvested, among the treatments. However, leaf water content (%) tended to decrease with a decrease in NST, especially in plants grown at 10°C NST, which exhibited values significantly lower than those of plants grown at 15°C and 20°C NSTs. PA and ANT concentrations in the 3rd to 5th nodes did not differ among treatments. Conversely, RA concentration increased with a decrease in NST, irrespective of the node position. LU concentration at 10°C NST was the highest in all treatments, irrespective of the node position. This suggested that the production of bioactive compounds under the low NSTs differed depending on leaf maturity stage and compound. Additionally, the contents of RA and LU in the whole plant increased because there was no reduction in growth of the harvested part under the low NSTs. Therefore, 6 days exposure to root-zone temperatures at 10°C appears to be an effective method to increase both RA and LU concentrations and their contents in the whole plant of red perilla for its use as a crude drug or medicinal material.

Key words: Anthocyanin, Environmental stress, Luteolin, Perillaldehyde, Rosmarinic acid

1. Introduction
Red perilla (Perilla frutescens) is an annual plant that belongs to the Lamiaceae family and is used as a food and a food coloring agent. It is also used as an ingredient for over-the-counter drugs (non-prescription drugs), Kampo medicines, and supplements. Dried red perilla leaves are used as a crude drug and are listed as ‘Soyou’ in the 17th Japanese Pharmacopoeia for the treatment of stomach ailments, anxiety neurosis, asthma, and bronchitis. The domestic consumption of ‘Soyou’ has increased in recent years (Japan Kampo Medicines Manufacturers Association, 2017).

The major compounds in red perilla are perillaldehyde (PA) and anthocyanin (ANT). It is stipulated by the 17th Japanese Pharmacopoeia (The Ministry of Health, Labor and Welfare, Japan, 2016) that PA must constitute more than 0.08% of the dry matter of ‘Soyou’. PA is an odorant, and has neuropharmacological effects (Sugaya et al., 1981), vasodilative effects (Takagi et al., 2005), antidepressant-like effects (Ito et al., 2008), and bactericidal action (Oda et al., 1982a; 1982b). ANT is a red pigment, and an antioxidant. In recent years, red perilla has been used in anti-allergy supplements; compounds with anti-allergy effects in red perilla leaves include rosmarinic acid (RA) (Sanbongi et al., 2003; 2004) and luteolin (LU) (Inoue et al., 2002), which also have anti-inflammatory and anti-oxidative effects. Therefore, we defined PA, RA, LU, and ANT as the major bioactive compounds in red perilla in this study.

Generally, the constitution and concentration of bioactive compounds in medicinal plants, including red perilla, are largely influenced by factors such as the growth environment, genetic background, harvest time, and methods of processing and storage of medicinal plants (Yoshimatsu, 2012). However, most commercial red perilla plants are currently cultivated outdoors in Japan. In addition, the amount of bioactive compounds in red perilla decreases during both post-harvest drying and storage; therefore, the production of red perilla leaves with stable and high concentrations of bioactive compounds at harvest is needed. Hikosaka et al. (2017) reported that greenhouse cultivation is an effective method for the steady production of medicinal plants because environmental conditions can be controlled to be suitable for plant growth and quality; furthermore, safer medicinal plants are produced with the use of fewer agrochemicals. Moreover, there is a possibility that the bioactive compound concentration in medicinal plants increases with environmental control in a greenhouse.

Applying environmental stresses during greenhouse cultivation is one method of environmental control for increasing the concentrations and contents of bioactive compounds in plants. Previous studies have reported that the concentrations and/or contents of bioactive compounds in many kinds of herbs and medicinal plants were increased by low air temperature (Miura and Iwata, 1983; Christie et al., 1994; Leyva et al., 1995), low root-zone temperature (Voipio and Autio, 1994; Sakamoto and
2.2 Treatments

Red perilla has two leaves per node. Thus, leaf positions are described as node numbers in this study. Moreover, node numbers were counted beginning at the base of the main stem. NST treatments started 42 days after sowing, when the leaves in the 5th node had appeared; the 5th node leaves appeared 0–3 days after the start of foliation. Red perilla leaves developed to the fully expanded stage approximately 12–15 days after the beginning of foliation. At the start of treatment, the 3rd and 4th node leaves appeared 12–15 days and 6–9 days after the beginning of foliation, respectively. The leaves at the 3rd, 4th, 5th, and 6th nodes were used for analysis of bioactive compounds, and foliation of the 6th node started during the NST treatment. The NSTs were set at 10, 12, and 15°C (low NSTs), and 20°C (control NST), and exposure was continued for 6 days. The 20°C NST was closer to the NST under air temperature at 25/20°C (light/dark period), and served as the control. Eight plants were transplanted to containers with the nutrient solution previously brought to the treatment temperatures. The volume of nutrient solution in a container was 16 L during the treatment period.

A cooler (TRL-107NHF, Thomas Kagaku Co., Ltd., Tokyo, Japan) and thermal controller (TC-107, Thomas Kagaku Co., Ltd., Tokyo, Japan) were used for the control of NST in this study. In addition, an underwater pump (Compact 300, EHEIM GmbH & Co., KG. Esslingen, Germany) was used to keep the NST uniform, and air pumps and air stones were used for aeration.

2.3 Growth analyses

Measurements of plant growth were taken 6 days after the start of treatment. 6 plants were randomly selected in each treatment. Fresh and dry weights of each plant part and leaf area were measured. In this study, because the leaves and stems of lateral shoots were small, their leaves and stems were not divided for measurement. One leaf at the 3rd or one of the higher nodes was measured for analysis of the main bioactive compounds, and the other leaf at each node was used for the measurement of fresh and dry weights. The water content at each node was calculated by dividing dry weight by fresh weight and multiplying by 100 (% dry matter ratio), and subtracting that value from 100.

2.4 PA, RA and LU analyses

Extraction of PA, RA, and LU was in accordance with the extraction method of perillaldehyde from red perilla in the 17th Japanese Pharmacopoeia. The extraction method was changed from static extraction to ultrasonic extraction, and the number of extractions was changed from 3 to 2 in this study. We modified the analytical method for several polyphenols in mint, as described by Krzyzanowska et al. (2011).

The leaves at the 4th, 5th, and 6th nodes were used for the analysis of the main bioactive compounds. First, 1.0 mL methanol was added to each sample (0.05–0.10 g), which were obtained from a ground whole leaf at each node, in a 2.0 mL microtube. The samples were sonicated in an ultrasonic bath (ASU-2; output: 40 W; AS ONE Corp., Osaka, Japan) for 30 min. The sonicated samples were centrifuged (10 min, 4°C, 20,000 × g) with an MX-305 centrifuge (TOMY SEIKO Co., Ltd., Tokyo, Japan) and the supernatants were collected. Then, 0.5 mL
methanol was added to the residue, and the samples were sonicated (40 W, 30 min) and centrifuged (10 min, 4°C, 20,000 × g) again. Then, the combined supernatant was filtered through a disposable syringe filter units (13HP020AN; pore size: 0.20 μm; Advantec Toyo Kaisha, Ltd., Tokyo, Japan), and the filtered supernatant was used for ultra-high-performance liquid chromatography (UHPLC) analysis.

A UHPLC (Nexera, Shimadzu Corporation, Kyoto, Japan) was used for PA, RA, and LU analysis. A diode array detector (SPD-M20A, Shimadzu Corporation, Kyoto, Japan) was used for detecting the UV absorption spectrum in this analysis. The analytical column was the UPLC BEH C18 column (ϕ1.7 μm, 2.1 × 50 mm, Waters Corporation, Massachusetts, USA). The column was thermostatically controlled at 50°C and the flow rate was constant at 0.4 mL min⁻¹. A gradient elution program was conducted for chromatographic separation with mobile phase A (water containing 0.5% formic acid) and mobile phase B [water-acetonitrile (60:40) containing 0.5% formic acid] as follows: 0 min (80% A); 5.1 min (50% A); 6.0 min (0% A); 9.0 min (0% A); 10.0 min (80% A); and ending at 10.0 min. The injection volume of the sample was 1.0 μL. Detection of PA, RA, and LU was performed at 230 nm, 327 nm, and 347 nm, respectively. The retention times of PA, RA, and LU were 8.0 min, 5.7 min, and 6.2 min, respectively.

Quantitation was conducted with the absolute calibration curve method, and the standard curve was made with a diluted solution of perillaldehyde standard reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan), rosmarinic acid standard reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and luteolin standard reagent (Sigma-Aldrich Japan, Co. LLC., Tokyo, Japan) with methanol.

2.5 ANT analysis

The analytical method for ANT was in accordance with Gong et al. (1997), who also measured the concentration of ANT in red perilla. First, 1.5 mL of 1% HCl–methanol was added to each sample (0.02–0.05 g), which were obtained from a ground whole leaf at each node, in 2.0 mL microtubes. The samples were refrigerated (4°C) for 48 h. Thereafter, the sample was mixed and incubated for 30 min, and the supernatant was diluted 10-fold with 1% HCl–methanol. Thereafter, the absorption of the sample at 510 nm was measured by a spectrophotometer (V-550, JASCO Corp., Tokyo, Japan). Quantitation was conducted with an absolute calibration curve method, and the concentration of ANT was calculated as an equivalent of cyanidin 3-glucoside (C3G). The standard curve was made with a diluted solution of cyanidin 3-glucoside chloride (Funakoshi Ltd., Tokyo, Japan) standard reagent.

2.6 Calculation and statistical analysis

Several studies have shown that water contents in Zea mays (Aroca et al., 2001) and Cucumis sativus (Yan et al., 2013) decreased with a decrease in root-zone temperature. In the present study, to elucidate the effects of NST on the biosynthesis and accumulation of bioactive compounds in red perilla, the concentrations of these compounds were determined on a dry weight basis rather than a fresh weight basis. Total contents of leaves (or whole plant) were the sum of the content of the 3rd to 5th nodes.

In addition, the PA content was determined on the basis of leaf area because PA is biosynthesized and accumulated in the trichomes on the leaf surface. PA content was calculated by division of the total PA content in a sample by the leaf area. The leaf area of the analyzed sample was calculated by multiplying the dry weight of analyzed sample by the specific leaf area of other leaves.

All data were presented as average values. The averages among the treatments were compared using Tukey-Kramer’s method, and the levels of significance were set at F < 0.05 and 0.01. All data were analyzed using IBM SPSS Statistics 24.0 for Windows (IBM Corp., New York, USA).

3. Results

3.1 Growth

The average measured NSTs on average for 6 days for the 10, 12, 15, and 20°C treatments were 10.0, 12.4, 15.2, and 20.2°C, respectively. The number of nodes in the main shoot, fresh and dry weights, and leaf areas increased from the start of the treatments, but no significant difference was observed for these parameters among the treatments (Tables 1 and 2). The water content of above ground part of plant tended to decrease with a decrease in NST, especially those of plants grown at 10°C NST, which were significantly lower than those of plants grown at 15 and 20°C NSTs (Table 2). Additionally, droopy leaves were observed approximately 30 min after transplanting at 12°C NST or less, and they gradually recovered after 90–120 min (data not shown, Fig. S1).

| Table 1. Effects of nutrient solution temperature (NST) on the number of nodes and fresh red perilla plants. |
|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| The number of nodes                                           | Fresh weight (g)                                             | Leaf area of leaves in main shoot (cm²)                        |
|                                                              | Leaves            | Main shoot       | Lateral shoot |                                                  |
| Before the start of treatment                                 | 3.56±0.26         | 1.35±0.07        | -             | 217±9.9                                           |
| NST (°C)                                                      | 10                | 6                | 16.6±0.15     | 0.75±0.15                                         |
|                                                              | 5.21±0.31         | 1.85±0.18        | 1.37±0.31     | 276±14.6                                          |
|                                                              | 5.65±0.30         | n.s.             | 1.99±0.23     | 323±14.1                                          |
|                                                              | 5.69±0.55         | 1.76±0.21        | 1.39±0.36     | 309±24.1                                          |

The plants were harvested at 6 days after the treatments began (n = 6). n.s. indicates no significant difference by Tukey-Kramer’s test.
3.2 Main bioactive compounds

Concentrations on a dry weight basis of PA at all node positions and all treatments were higher than the 0.08% prescribed by the 17th Japanese Pharmacopoeia as a minimum concentration (Fig. 1 (A)). In all treatments, PA concentrations were the highest at the 6th node, which was the highest (youngest) node on the main shoot at the end of treatments (Fig. 1 (A) and Fig. 2). Although PA concentrations in the 6th node at 12°C NST or less tended to be higher than that at 20°C NST (control NST), PA concentrations (Fig. 1 (A)) in the 3rd, 4th (fully expanded), and 5th (before fully expanded) nodes did not differ among treatments. RA concentrations in all treatments were the lowest at the 6th node among the nodes on the main shoot, and those in lower leaf position were higher than those in higher position (Fig. 1 (B)). RA concentration at all nodes increased with a decrease in NST.

LU concentrations in all treatments tended to be higher with an increased node position on the main shoot, especially at 10 and 12°C NSTs (Fig. 1 (C)). LU concentrations in all nodes at 10°C NST were higher than at other NSTs (Fig. 1 (C)). ANT concentrations under 20°C NST did not differ among the nodes (Fig. 1 (D)). ANT concentrations under 15°C or less NST were the highest at the 6th node, and those were the same level.

### Table 2. Effects of nutrient solution temperature (NST) on dry weights and water content of red perilla plants.

| NST (°C) | Dry weight (g) | Water content (%) of leaves in main shoot |
|---------|----------|-----------------------------------------|
|         | Above part | Root | Total |
|         | Main shoot | Lateral shoots |          |
| Before the start of treatment | 0.44±0.05 | 0.10±0.01 | - | 0.07±0.02 | 0.61±0.07 | 87.9 |
| 10      | 0.81±0.06 | 0.18±0.02 | 0.12±0.02 | 0.17±0.02 | 1.28±0.11 | 82.8 b |
| 12      | 0.79±0.05 | 0.19±0.05 | 0.19±0.05 | 0.21±0.03 | 1.37±0.13 | 84.7 ab |
| 15      | 0.79±0.04 | 0.19±0.04 | 0.17±0.04 | n.s. | 0.27±0.04 | 1.42±0.12 | n.s. | 85.9 a |
| 20      | 0.84±0.08 | 0.18±0.05 | 0.20±0.06 | 0.29±0.05 | 1.51±0.20 | 85.2 a |

The plants were harvested at 6 days after the treatment began ($n = 6$). Different letters indicate significant differences among treatments at $P < 0.05$, and n.s. indicates no significant difference by Tukey-Kramer’s test.

![Fig. 1.](image-url) Effects of nutrient solution temperature (NST) on the concentrations on dry weight basis of bioactive compounds (A; perillaldehyde (PA), B; rosmarinic acid (RA), C; luteolin (LU), D; anthocyanin (ANT)). The plants were harvested at 6 days after the treatment began. The concentration of ANT was calculated as an equivalent of cyanidin 3-glucoside (C3G). Vertical bars indicate SE ($n = 6$). Different letters indicate significant differences among treatments at $P < 0.05$, and n.s. indicates no significant difference by Tukey-Kramer’s test.
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at 5th or lower node. Although the ANT concentrations in the 3rd, 4th, and 5th nodes did not differ among the treatments, the ANT concentration in the 6th node at 15°C or less NSTs tended to be higher than that at the control NST (Fig. 1 (D)).

In all treatments, the dry weights of leaves on the main shoot at the 3rd, 4th, and 5th nodes were bigger than that at the 1st, 2nd, and 6th nodes, and represented more than 85% of the total dry weight of leaves on the main shoot (Fig. 2). The leaves at the 1st and 2nd nodes were smaller than those at the 3rd to 5th nodes, in spite of them being fully expanded, and the contents and concentrations of bioactive compounds in leaves at the 1st and 2nd nodes were lower than in those at the 3rd to 6th nodes. Therefore, total contents of bioactive compounds in the whole plant were calculated by the sum of the contents at the 3rd to 6th nodes. The results indicated that PA and ANT content did not differ among treatments (Fig. 3 (A) and (D)). RA contents increased with a decrease in NST (Fig. 3 (B)), and LU content at 10°C NST (Fig. 3 (C)) was significantly higher than that at other NSTs.

4. Discussion

4.1 Growth

There was no significant difference among treatments in the dry weight of above ground part of the plants in this study. These results corresponded to studies that investigated the effect of low root-zone temperature at 15°C on Zea mays (Beauchamp and Lathwell, 1967) and 10°C on Brassica napus (Cumbus and Nye, 1982). On the other hand, some studies suggest that low air and/or root-zone temperature inhibit plant growth; for example, 7

Fig. 2. Effects of nutrient solution temperature (NST) on dry weight of leaves on the main shoot. The plants were harvested at 6 days after the treatment began. Vertical bars indicate SE (n = 6). n.s. indicates no significant difference by Tukey-Kramer’s test.

Fig. 3. Effects of nutrient solution temperature (NST) on the contents of bioactive compounds (A; perillaldehyde (PA), B; rosmarinic acid (RA), C; luteolin (LU), D; anthocyanin (ANT)). The plants were harvested at 6 days after the treatment began. The concentration of ANT was calculated as an equivalent of cyanidin 3-glucoside (C3G). Vertical bars indicate SE (n = 6). Different letters indicate significant differences among treatments at same node at P < 0.05 by Tukey-Kramer’s test.
days of 10°C NST and 14 days of 5°C NST treatment inhibited the growth of lettuce (Sakamoto and Suzuki, 2015) and spinach (Chadirin et al., 2011) compared to the non-controlled NST, respectively. Therefore, it is considered that low NST treatment inhibits the plant growth depending on the crop, the stage of the plant, and the duration and temperature of the NST treatment. It was indicated that 6 days low NSTs (10–15°C) did not decrease the growth of red perilla plants in the NST treatment condition and plant growth stage of this study. It was considered that we need to investigate the effects of shorter or longer low NST treatments in further experiments.

In this study, it appeared that the sudden decrease in NST at 12°C or less caused Wilted leaves at the start of treatment. Previous studies have shown that water absorption rate in cucumbers (Tachibana, 1987) and rice (Takehima, 1964; Nagasuga et al., 2011) under lower root-zone temperature was lower than those under higher root-zone temperatures (within 12–30°C). Therefore, it is possible that the plants in the present study also experienced water stress, which was caused by water-uptake inhibition from root in that period. Plants close their stomata to inhibit evaporation and water deficiency when extractable water from the root-zone decreases (Jones, 1992). In this study, it was considered that stomata were closed by the inhibition of water absorption through the roots under low NST, although there was sufficient water in the root-zone.

Because the droopy leaves were recovered within 90–120 min in the present study, the accurate water deficit within the plants seemed to be gradually alleviated by a low evaporation rate. However, water content at 6 days after the start of treatment tended to decrease with a decrease in NST. Therefore, it is suggested that the water stress condition of 12°C or less continued for 6 days, although the appearance and dry weight of the plants did not differ among treatments. Therefore, it is possible that more than 6 days of low NST treatment could decrease the dry weight of red perilla.

### 4.2 Bioactive compounds

**Variation of concentrations of bioactive compounds among the node positions**

In all treatments, concentrations of PA and LU were higher in younger leaves, although RA concentration was higher in older leaves. In addition, ANT concentration in the control NST was not varied among the node positions. These results indicate that the variation trend of the bioactive compound concentrations among the node position differed depending on compounds.

PA is biosynthesized and accumulated in trichomes on the abaxial leaf surface (Yoshida et al., 1968). Previous studies have shown that trichome density on the leaf area decreased with an increase in leaf age (Yoshida et al., 1969), and PA concentration of perilla leaves is high when trichome density on the leaf is high (data not shown, Fig. S2); therefore, PA concentration was estimated to be highest in the 6th node leaves, which were the youngest at the end of the treatment. Additionally, possible reasons for this result are that the increase rate of dry weight exceeds the rate of PA accumulation in trichomes and/or the decomposition rate of PA exceeds the PA biosynthesis rate, i.e., volatilization from trichomes, and the aging and bursting of trichomes (dissipation of PA accumulation sites).

Although LU accumulation sites are not trichomes, the exceeded rate of dry weight accumulation beyond LU and/or the degradation and decreased biosynthesis of LU were also considered as the reasons for the decrease in LU concentration with leaf foliation.

In contrast, the RA concentration increased with leaf foliation. Possible reasons for this result are that the accumulation rate of RA exceeds the rate of dry weight increase and/or the biosynthesis rate of RA exceeds the RA decomposition rate.

ANT concentration was estimated to be balanced between the biosynthesis rate and decomposition rate throughout leaf foliation.

**Effects of NST**

RA and LU concentrations on a dry weight basis under 10°C NST were higher than those under the control NST at all nodes, while PA and ANT concentrations under low NSTs tended to be higher than those under the control NST at the 6th node only. Therefore, it was suggested that the responses of bioactive compound concentration to low NST treatment differed among the bioactive compounds.

Generally, reactive oxygen species (ROS) generation are causally increased under water stress conditions (Ksouri et al., 2007). It is known that increases in biosynthesis and accumulation of bioactive compounds with antioxidative effects under these environmental stresses are induced by an increase in the generation of ROS (Blokhina et al., 2003). Voipio and Autio (1994) reported that ANT concentration in lettuce leaves under 13–14°C NST was higher than that under non-controlled NST (20–21°C NST). Gazula et al. (2005) reported that the ANT concentration in lettuce leaves under lower air temperatures was higher than that under higher air temperatures (within 20–30°C). Sakamoto and Suzuki (2015) reported that the concentrations of ANT and total phenol, and antioxidant capacity in red leaf lettuce increased at 10°C NST compared to that at 15°C or higher NST in hydroponics. In addition, the gene expression of phenylalanine ammonia-lyase (PAL), the rate-limiting enzyme in the biosynthesis pathway of phenylpropanoids and flavonoids, was often promoted by low air temperature; this was 4°C in the case of Arabidopsis (Leyva et al., 1995) and Zea mays (Christie et al., 1994). Because RA is a phenylpropanoid, and LU and ANT are flavonoids, the biosynthesis of these three compounds might also increase at low NSTs.

On the other hand, PA and ANT concentrations did not increase under low NSTs at the 3rd to 5th node. Lu et al. (2017) reported that high light intensity (PPFD 300 μmol m⁻² s⁻¹) and low EC in nutrient solution (EC 1.0 dS m⁻¹) increased the RA concentration in red perilla, while the concentrations of PA and ANT were not affected by light intensity and EC. In our study, the concentrations of PA, ANT, and RA in leaves at the 3rd to 5th nodes showed similar tendencies as the results of Lu et al. (2017). According to this, and the results of the present study, it was suggested that biosynthesis and accumulation of PA and ANT were less susceptible to changes in environmental conditions, while biosynthesis and accumulation of RA were susceptible to these changes.

In this study, RA and LU contents in whole plants increased.
twofold at 10°C NST compared to the control NST because the dry weights of leaves at 10°C and 20°C NSTs were the same, and the concentrations of RA and LU at 10°C NST were about twofold higher than those at 20°C NST in all nodes. In addition, PA and ANT concentrations did not decrease under 10°C NST. Thus, it is possible that 6 days exposure to a low NST treatment (10°C NST) is an effective method to increase the concentrations and yields of RA and LU. Further studies are needed to elucidate the effects of air temperature during low NST treatment on the growth and bioactive compound concentrations of red perilla, to enable the efficient production of medicinal red perilla in greenhouses.

**Supplemental information**

Supplemental information for this paper is available at http://doi.org/10.2480/agrmet-D-17-00037

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