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The Effects of Root Temperature on Growth, Physiology, and Accumulation of Bioactive Compounds of *Agastache rugosa*

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Abstract: Plants respond to root temperature stresses by producing antioxidants as a defense mechanism. Since a number of these are phytochemicals with enhancing effects on human health, we examined the effects of 4 root-zone temperature (RZT) treatments (10, 20, 28, and 36 °C) on plant growth and the main bioactive compound concentrations in each organ of *Agastache rugosa* plants. We aimed to determine the optimal RZT treatment to increase bioactive compound concentrations with no deleterious effects on plant growth. Four-week-old seedlings were grown in a plant factory for 32 days. Nine plant growth parameters, namely, shoot and root fresh weights, stem and root lengths, leaf length and leaf width, leaf area, and shoot and root dry weights were significantly decreased at 10 and 36 °C compared with other treatments. A similar pattern was observed for the chlorophyll content and leaf gas exchange parameters. Of all the RZT treatments, RZT at 28 °C produced the significantly greatest accumulation of two major bioactive compounds, namely, rosmarinic acid (RA) and tilianin contents per the *A. rugosa* plant, and had no adverse effects on the overall growth of *A. rugosa*. This supports the use of 28 °C RZT to successfully improve the bioactive compounds with no adverse influence on plant growth or yield.

Keywords: acacetin; chlorophyll content; plant growth; rosmarinic acid; tilianin

1. Introduction

Agastache rugosa (Lamiaceae family), a perennial herb that is widely distributed in China, Korea, and Japan [1,2], contains some important phytochemicals such as rosmarinic acid (RA), chlorogenic, caffeic, and ferulic, as well as flavone glycosides, such as tilianin, acacetin, sesquiterpenes, diterpenes, and triterpene [1–3]. Biological analysis of *A. rugosa* has revealed its antiallergic, antimicrobial, antitumor, anticancer, antiviral, antidepression, antioxidant activities, and antiasthmatic efficacy accumulation of tilianin and rosmarinic acid and expression of phenylpropanoid biosynthetic genes in *Agastache rugosa* [1,2]. Although it is used as a functional food worldwide, its organized production is limited in some regions despite its many uses and benefits [4].

A plant factory system is useful for medicinal plant cultivation because of the managed and optimized environmental conditions such as light intensity, air and root-zone temperature (RZT), humidity, CO_2 concentration, and a nutrient solution [5–7]. Controlling environmental conditions significantly affected the plant's synthetic pathways, plant metabolic, and secondary metabolites content [7,8]. Besides air temperature, root-zone temperature (RZT) is an important factor for plant

growth and development, nutrient uptake, and accumulation and biosynthesis process of bioactive compounds [9,10]. For example, 10 °C RZT decreased plant growth but increased RA and luteolin concentrations and the contents of red perilla (*Perilla frutescens*, Labiatae) due to water stress [10]. Optimal RZT for nutrient uptake and plant growth of snapdragons (*Antirrhinum majus* L. "Peoria") was 22 °C [11]. Root zone temperature at 26 °C significantly increased shoot growth; however, lower RZT (10 °C) significantly decreased shoot growth and increased soluble sugars in both roots and leaves of cucumber (*Cucumis sativus* L.) at 10 °C RZT in the hydroponic system after four weeks of RZT treatment [12].

Mineral nutrient absorption and plant biomass were increased at higher RZT. In addition, the uptake of total N, P, and K of cucumber (*Cucumis sativus* L.) was decreased at low RZT (10 °C) compared with other treatments [13]. Phenols, anthocyanin, nitrate, antioxidant enzymes, and sugar concentrations at low RZT at 10 °C were increased in leaves of lettuce (*Lactuca sativa* L.) compared with higher RZT (20, 25, and 30 °C) [9]. Plant growth and water content were decreased at high RZT (33 °C); however, soluble-solid content and total phenolic compounds in carrots (*Daucus carota* L.) were increased at high RZT treatment compared with other treatments (20, 25, and 29 °C), because high RZT caused drought stress [14]. Plant fresh and dry weights of muskmelons (*Cucumis melo* L. "Gold Star") were the highest and the lowest at 25 °C RZT and 35 °C RZT, respectively [15]. Biomass accumulation, antioxidant power, vitamin C, and sugar content of lettuce (*Lactuca sativa* L.) were affected by root-zone heating [16]. The concentration of N and P and total mineral accumulation K, NO_3^-, Ca, Fe, Cu, Mn, Mg, and Zn in plants at 20 °C RZT were higher than other treatments [17].

These reports indicated that RZT plays a significant role on the nutritional quality and phytochemical concentrations in plants. Several studies have examined the effects of RZT on the accumulation of secondary metabolites in plants, but no information exists on *A. rugosa* [7,10]. Therefore, the objectives of this study were to clarify the influences of RZT on the growth, chlorophyll content, leaf gas exchange parameters, and accumulation of major bioactive compounds in *A. rugosa* plants grown in a closed plant production system and to determine the optimal RZT treatment to increase bioactive compounds with no adverse influence on plant growth.

2. Materials and Methods

2.1. Plant Materials and Seedling Conditions

Agastache rugosa seeds were sown in a rockwool cube (CP-20, UR rockwool, Suwon, Korea) and laid in the dark for one day. Afterward, the seed tray was moved to a cultivation room with the relative humidity and air temperature set to 60%–80% and 22/18 °C (light/dark periods), respectively. The photosynthetic photon flux density (PPFD) provided by fluorescent lamps (TL5 14W/865 Philips, Amsterdam, Netherlands) was set to 180 ± 10 μmol·m^{-2}·s^{-1}. The *A. rugosa* seedlings were irrigated daily with tap water. Two true leaves appeared from two weeks after sowing. Hoagland nutrient solution was used to irrigate for seedlings. Electrical conductivity (EC) and pH values were adjusted to 1.2 dS·m^{-1} and 6.0 [18].

2.2. Root Zone Temperature Experiment and Growth Conditions

Four weeks after sowing, *A. rugosa* seedlings with the same uniformity were transplanted into a deep-flow technique system (0.7 m (W) × 1.2 m (L) × 0.11 m (H)) in a plant factory (Figure 1) and subjected to PPFD levels of 200 μmol·m^{-2}·s^{-1} with a photoperiod of 14 h per day supplied by light-emitting diode (LED) light (TL5 14W/865 Philips, Amsterdam, Netherlands) at a red:green:blue ratio of 60%:10%:30% for 32 days after transplant. The relative humidity, air temperature, and CO_2 concentration were set at 70%, 20 °C, and 1000 μmol·mol^{-1}, respectively. Root zone temperature for the initial one week was set at 20 °C in order the plants to avoid low or high RZT transplant shock. From 8 days after transplanting, plants were treated 4 RZT treatments at 10, 20, 28, and 36 °C and were imposed for 25 days. A thermal controller (CN-25-1, JS Electrical Contractors Co., Ltd., Seoul, Korea)

and compact handy cooler (102TCN, Thomas Kagaku Co., Ltd., Tokyo, Japan) were used to control the nutrient solution temperature. Hoagland nutrient solution around 6.0 pH and 2.0 dS·m^{-1} EC was supplied from transplant to harvest. The EC and pH values of the nutrient solution were determined every two days by a portable conductivity meter and pH tester combo (HI98129, Hanna instrument Co., Ltd., Woonsocket, RI, USA).

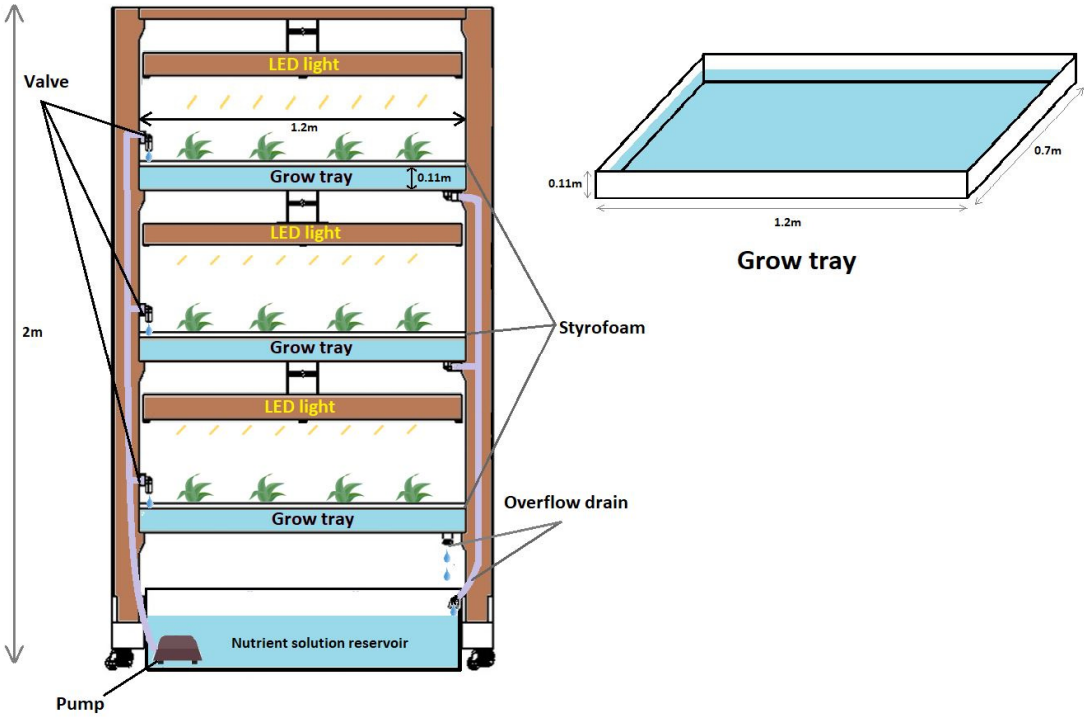

Figure 1. A deep flow technique (DFT) system in a plant factory. The plants were transplanted on a Styrofoam board, which was placed on the surface of the nutrient solution.

2.3. Measurements of Plant Growth Parameters

Nine growth parameters were determined at 32 days after transplant with eight plants per each replication (n = 8). The number of leaves both wider and longer than 1cm was counted, and a leaf area meter (Li-3100, LiCor, Lincoln, NE, USA) was used to determine the leaf area. Leaf length, leaf width, root, and stem lengths were determined by a measuring tape (STHT-36127, Stanley Electric Co., Ltd., Tianjin, China). An micro weighing scale (CAS MW-II, CAS Co., Ltd., East Rutherford, NJ, USA) was used to determine the root fresh weight and shoot fresh weight after washing roots and eliminating water from the root surface with paper. Samples were then dried in a drying oven (HB-502M, Hanback Sci, Suwon, Korea) for seven days at 70 °C, and an electronic scale was used to determine the root dry weight and shoot dry weight.

2.4. Measurements Chlorophyll fluorescence (F_v/F_m) and Relative Chlorophyll Values

The relative chlorophyll value was measured at 32 days after transplant using a portable chlorophyll meter (SPAD 502, Minolta Camera Co., Ltd., Tokyo, Japan). A portable fluorometer (Fluorpen Pen FP 100, Photon System Instruments Ltd, Drasov, Czech Republic) was used to determine the ratio of variable to maximum fluorescence (F_v/F_m) at 32 days after transplant. Chlorophyll fluorescence (Fv/Fm) and relative chlorophyll values were measured on 3 and 8 plants (n = 3 and n = 8) in each replication. Relative chlorophyll and Fv/Fm values were recorded on the third fully expanded leaf from the plant apex.

2.5. Leaf Gas Exchange Measurement

The leaf gas exchange parameters consisting of the net photosynthetic rate (P_n), intercellular CO_2 concentration (C_i), transpiration rate (T_r), and stomatal conductance (g_s) of *A. rugosa* were measured at 32 days after transplant by using a LICOR 6400 portable photosynthesis system (IRGA, Licor. Inc., Nebraska, NE, USA). The machine had been calibrated at the ZERO IRGA mode. The optimum conditions in the transparent leaf chamber were installed with a leaf temperature of 25 °C, 1500 μmol·m^{-2}·s^{-1} PPFD, 400 μmol·mol^{-1} ambient CO_2 concentration, 60% relative humidity, and 500 cm^3·s^{-1} air flow rate. The measurement process was automatically executed on the third fully expanded leaf from the plant apex. "Photosyn Assistant" software (Version 3, Lincoln Inc., Columbus, OH, USA) was used to analyze the data, and the LI-6400 system was used to save the data. Five plants were used for measurement per each replication (n = 5).

2.6. Analysis of Rosmarinic Acid (RA), Tilianin, and Acacetin Concentrations and Contents

The leaves, roots, flowers, and stems of *A. rugosa* at 32 days after transplant from each replication were divided and promptly put in liquid nitrogen, stored at −70 °C in a deep freezer, and later dried at −50 °C in a dry freezer (TFD5503, IL Shinbiobase Co. Ltd., Gyeonggi-do, Korea) for 4 days. Bioactive compounds were analyzed from three plants (n = 3) in each replication. Plant organs were milled to a fine powder in a blender, and the powder was passed through mesh sieves. The dried powder (0.1 g) of each plant organ was blended with 80% (v/v) MeOH (1.5 mL) and then sonicated for 60 min. The solution was centrifuged at 12,000× *g* for 10 min using a microcentrifuge (R17 Plus, Hanil Scientific Co., Ltd., Gimpo, Korea) at 4 °C. The rosmarinic acid (RA), tilianin, and acacetin concentrations were determined by a HPLC system (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) [3,18]. The supernatant was filtered through a filter (0.45 μm), and then a high-performance liquid chromatography (HPLC) analysis was conducted with a C18 column (250 mm × 4.6 mm, 5 μm; RS tech, Daejeon, Korea) at 30 °C. Standard compounds of pure RA, tilianin, acacetin, HPLC grade acetic acid, methanol, the pure compounds, analytical grade solvents, and reagents from Sigma-Aldrich, Co., Ltd., (Seoul, Korea) were used in this experiment. Methanol 100% (solvent B) and 0.20% (v/v) acetic acid (solvent A) were used as the solvent systems. The gradient profile was run as follows: 0–15 min: 45% B; 15–20 min: 45%–55% B; 20–45 min: 55%–80% B; 45–50 min: 80% B; 50–52 min: 80%–45% B; and 52–60 min: 45% B (total of 60 minutes). The injected volume was 20 μL, the detection wavelength was 275 nm, and the flow rate was kept at 1.0 mL·min^{-1}. The mean values of each plant organ (leaf, root, stem, and flower) were calculated from three independent replicates. The tilianin, RA, and acacetin concentrations (mg/each plant organ dry weight (DW)) were determined. Whole plant concentration (mg·g^{-1} plant DW) means the total tilianin, RA, and acacetin concentrations of all plant organs. The tilianin, RA, and acacetin contents in flowers, leaves, roots, and stems (mg/plant organs DW) are the tilianin, RA, and acacetin concentrations in plant organs (mg·g^{-1} DW) multiplied by the plant organs' DW (g). The whole plant's RA, tilianin, and acacetin contents (mg/plant DW) are the RA, tilianin, and acacetin concentrations (mg·g^{-1} plant DW) multiplied by the whole plant DW (g).

2.7. Statistical Analysis

This experiment consisted of two repetitions in one experiment, and a repetition consisted of a floating bed. The data was pooled and then analyzed. Two replications were performed in the RZT experiment by completely randomized designs. One-way ANOVA was performed by the SPSS 20.0 soft program (SPSS 20, SPSS Inc., Chicago, IL, USA). To check for significant differences among the means of all treatments, Tukey's multiple range test was used. All graphs were built by using SigmaPlot 10 (Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

3.1. Plant Growth Parameters

The following plant growth parameters of *A. rugosa* grown differed significantly at different root zone temperatures (RZTs): leaf size, leaf area and number, root and stem lengths, and the fresh and dry weights of shoots and roots (Figure 2 and Table 1). Table 1 shows that all plant growth parameters of *A. rugosa* were significantly decreased at cold and heat root stress (10 and 36 °C). Especially, the reductions at 36 °C were greater than at 28 °C as follows: 56.97% in the number of leaves, 49.71% in leaf area, 46.91% in shoot fresh weight (SFW), 10.66% in root fresh weight (RFW), 54.54% in shoot dry weight (SDW), and 21.14% in root dry weight (RDW). Some other studies confirmed that low RZT can decrease shoot and root growth and is mostly ascribed to water stress [19,20]. On the other hand, some studies reported that RZT restricted shoot and/or root growth. For example, plant growth of red perilla (*Perilla frutescens*, Labiatae) was decreased at a low nutrient solution temperature (10 °C) compared with 15 and 20 °C [10]. The shoot size of lettuce (*Lactuca sativa* L.) was decreased at low RZT (10 °C) at seven days after treatment compared with other treatments (20, 25, and 30 °C) [9]. Total fresh weights of cucumber (*Cucumis sativus* L.) at lower RZT (12 °C) were significantly lower than higher RZT (20 °C), because plant growth was restricted by membrane lipid peroxidation, water stress, and root cell viability [21]. Moreover, low root zone and air temperatures can affect the imbalance between growth inhibitors and promoters in plants such as abscisic acid, cytokinin, and gibberellins, which are mainly synthesized in the root apical meristems [22,23]. Therefore, *A. rugosa* plants at low RZT (10 °C) may also have suffered water stress, because water uptake was inhibited from root to shoot in the treatment period.

High RZT is a factor that affects plant growth. In our results, the plant growth parameters were markedly and significantly decreased at the 36 °C treatment compared to the other treatments. Since plant roots are more sensitive to heat stress than the above-ground parts, the high RZT damages the roots, which can restrict the stem length and diameter [24]. The RZT at 36 °C treatment caused the shortest root length (3.8 cm) (Table 1). This demonstration of heat stress-induced decrement of root growth agrees with observations by Odhiamboa et al. (2018). For example, high RZT leads to a reduction of plant growth and fruit yield of tomatoes because high RZT can lead to water or nutrient stress [25]. High RZT can affect the plant growth, morphology, and physiology of some plants, such as peaches (*Prunus persica* L.) [26] and cucumbers (*Cucumis sativus* L.) [27]. Additionally, high RZT stress reduced the leaf, stem, and fresh and dry weights of coriander (*Coriandrum sativum* L.), because water deficits occurred in the plants [7]. Water uptake of the root was affected by both the root volume and the water permeability of the root surface [28]. High RZT limited root growth, such as root length and weight. Plant water content relies on the balance between root water absorption and transpiration from the shoot. High RZT affects this relationship by decreasing plant water uptake and increasing transpiration [7]. Therefore, high RZT caused water a deficit in the plants. Therefore, the high and low RZTs (36 and 10 °C) in our study reduced the plant growth parameters of *A. rugosa*, because they can lead to water stress, ion imbalance, and growth inhibitors and promoters imbalance in the plant. In contrast, 28 °C treatment showed the longest root length (Table 1). Likewise, the longest root lengths of pepper plants were observed at 25 °C RZT [29].

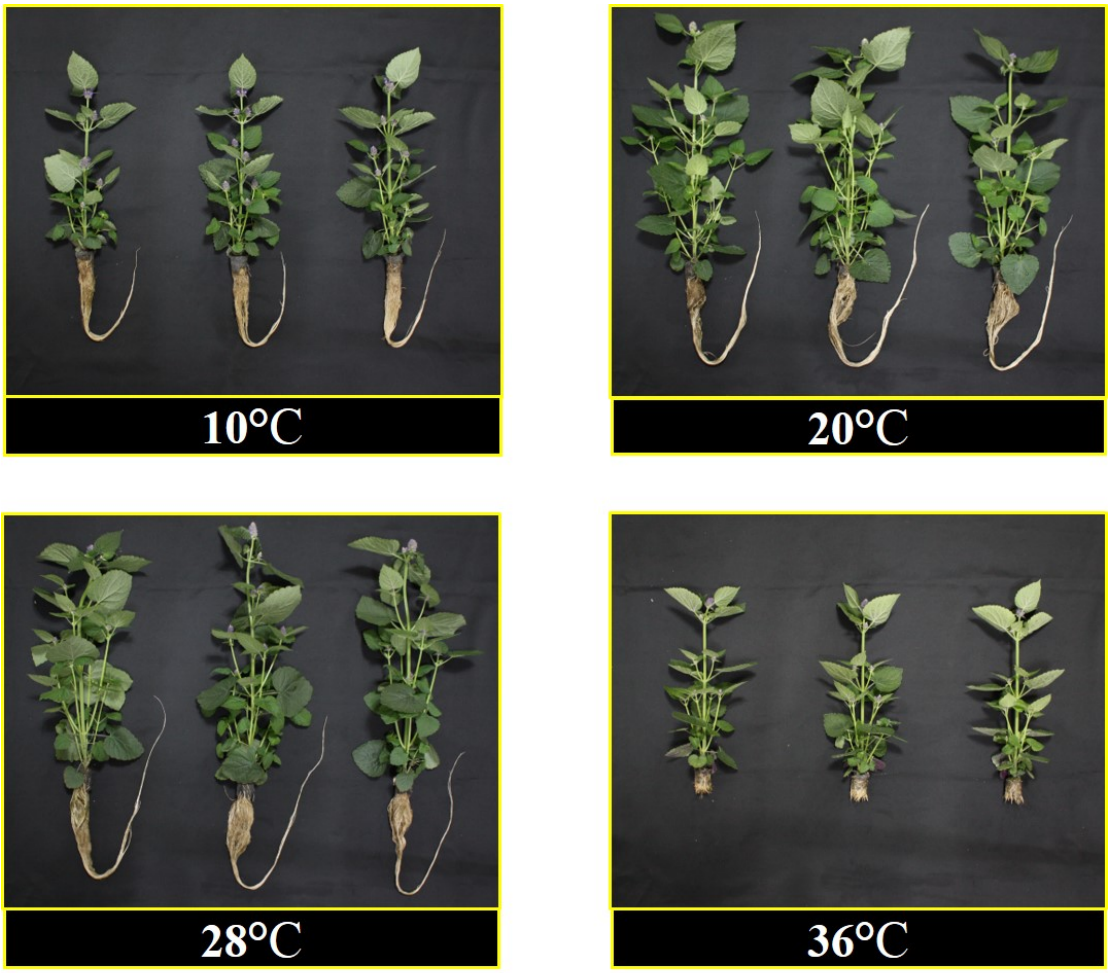

Figure 2. Images of *A. rugosa* plants grown at four different root zone temperature (RZT) treatments (10, 20, 28, and 36 °C) at 32 days after transplant.

Table 1. Plant growth parameters of *A. rugosa* grown at four different root zone temperature (RZT) treatments (10, 20, 28, and 36 °C) at 32 days after transplant.

RZT [z] (°C)	Leaf Length (cm)	Leaf Width (cm)	Number of Leaves (leaves)	Leaf Area (cm^2)	Stem Length (cm)	SFW (g)	RFW (g)	Root Length (cm)	SDW (g)	RDW (g)
10	7.92 b [y]	7.29 b	51.12 b	563.71 b	34.12 b	16.56 b	9.43 b	29.56 b	1.91 b	0.54 b
20	8.55 a	8.39 a	75.50 a	819.20 a	40.23 a	24.49 a	14.31 a	49.99 a	2.98 a	0.77 a
28	8.38 a	8.17 a	77.00 a	810.25 a	40.61 a	25.43 a	13.98 a	51.15 a	2.93 a	0.79 a
36	6.57 c	6.02 c	43.87 b	402.77 c	23.29 c	11.93 c	1.49 c	3.80 c	1.60 c	0.17 c
Significance [x]	***	***	***	***	***	***	***	***	***	***

[z] Root zone temperature treatments. [y] Mean separation within columns by Tukey's multiple range test. [x] Significant at *** $p \leq 0.001$. Data are the means ± SE (n = 8). Different letters (a–c) indicate significant differences among RZT treatments at the level of 5%, according to Tukey's test. SFW: shoot fresh weight, SDW: shoot dry weight, RFW: root fresh weight, and RDW: root dry weight.

3.2. Chlorophyll Fluorescence (F_v /F_m) and Relative Chlorophyll Values

The RZT at 20 °C and 28 °C generated significantly higher relative chlorophyll value and Fv/Fm ratios than those of the 10 °C and 36 °C treatments. There was no significant difference in the relative chlorophyll value and Fv/Fm ratios between the 20 and 28 °C treatments and between the 10 and 36 °C treatments, respectively (Figure 3). Cold and heat root stress (10 and 36 °C) reduced the chlorophyll content, whereas moderate RZT (20 and 28 °C) enhanced it. These results agree with findings by Odhiamboa et al. (2018), who reported that cold and heat stress reduced chlorophyll contents. Similarly, RZT at 10 °C decreased the chlorophyll (chl a, chl b, and total chl) contents in red romaine lettuce (*Lactuca sativa* L.) compared with that at 15 °C [30], because the optimal RZT positively affected the uptake of essential nutrients such as nitrogen, which is essential for the synthesis of chlorophyll [13].

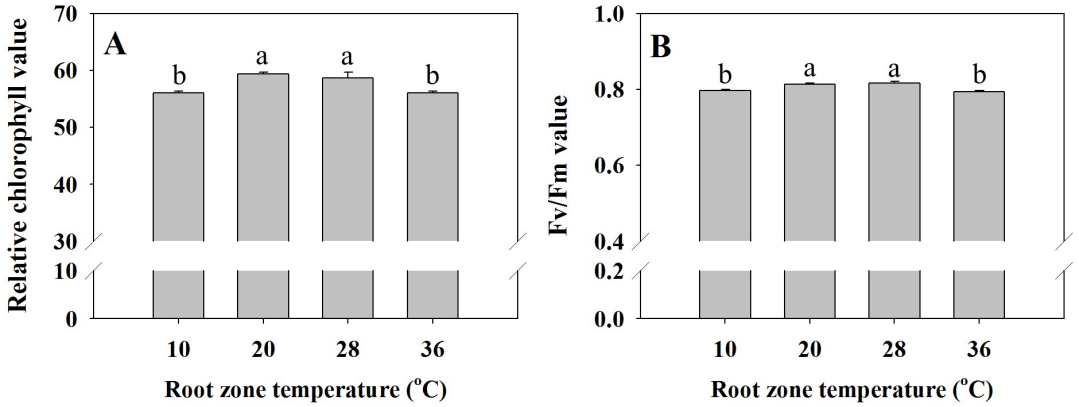

Figure 3. Relative chlorophyll value (**A**) and the ratio of variable to maximum fluorescence (F_v /F_m) (**B**) of *A. rugosa* at four different root zone temperature (RZT) treatments (10, 20, 28, and 36 °C) at 32 days after transplant. Data are shown as the means ± standard error (SE) (relative chlorophyll value (n = 8) and Fv/Fm value (n = 3)). Different letters (a and b) show significant differences among the RZT treatments (Tukey's test, $p < 0.05$).

3.3. Leaf Gas Exchange Parameters

Figure 4 shows that the net photosynthesis rate (P_n), stomatal conductance (g_s), and transpiration rate (T_r) at low and high RZTs (10 and 36 °C) were significantly lower than those at 20 and 28 °C RZT treatments, respectively. However, the intercellular CO_2 concentration (C_i) was significantly lower at the highest RZT treatment (36 °C) compared to the rest of the treatments. Since low RZT decreases water uptake, the resulting water stress causes stomata closure, which sustains positive turgor pressure in the plant; therefore, this reduces the CO_2 absorption, photosynthetic rate, and carbon production [31]. Similarly, cold stress significantly reduced the photosynthesis rate in *Stevia rebaudina* [32]. Cold stress often reduces the photosynthesis rate, but the effect is species-dependent. For example, the stomatal conductance and photosynthesis rate of the tomato (*Solanum lycopersicum* L.) plant were not restricted by low RZT at 12 °C [33].

Photosynthesis is extremely sensitive to higher than optimum temperatures and is often limited before other cell functions are affected [34]. Our study showed that RZT at 36 °C reduced the photosynthesis rate by 64% compared with that at 28 °C. Likewise, leaf water status, intercellular CO_2 concentration, leaf stomatal conductance, and photosynthesis rate were reduced by high temperatures [34]. The high RZT was shown to reduce the water uptake in roots, which caused stomatal closure. CO_2 absorption was negatively influenced by stomatal closure [35]. Stomata was closed when hydraulic conductance in the shoot was decreased by a xylem embolism [36]. High RZT reduced the photosynthesis rate because of inadequate carbon acquisition by the stomatal closure [37]. Therefore, the photosynthesis rate was reduced by high RZT.

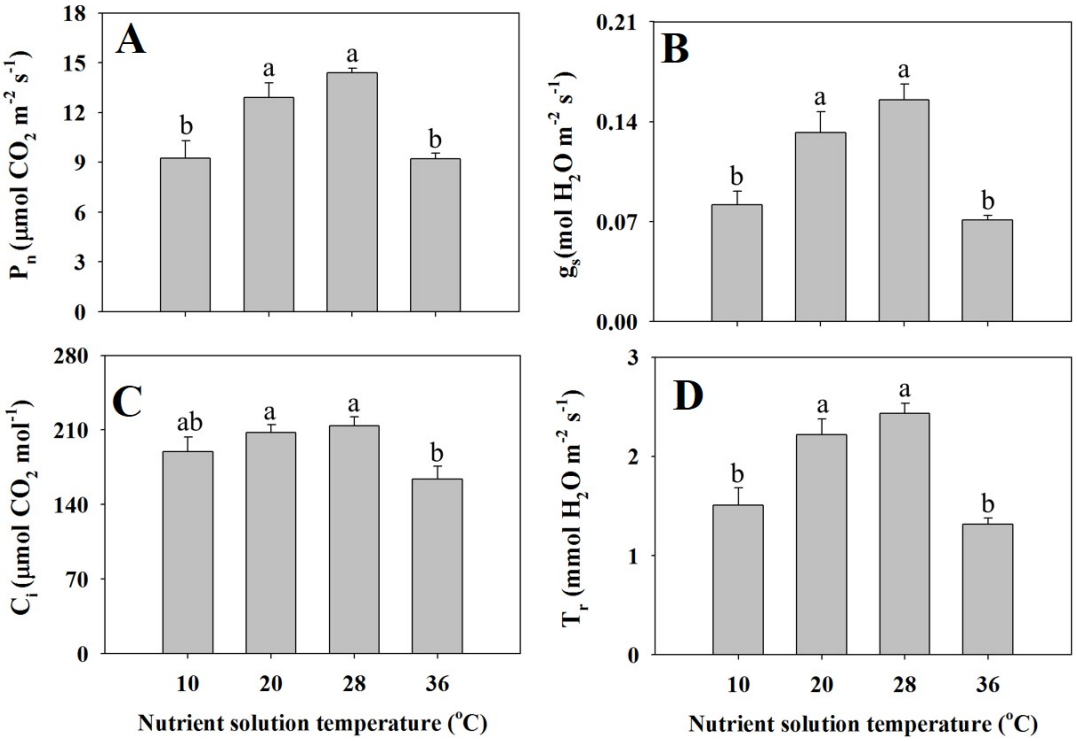

Figure 4. Net photosynthetic rate (Pn) (**A**), stomatal conductance (gs) (**B**), intercellular CO_2 concentration (Ci) (**C**), and transpiration rate (Tr) (**D**) of *A. rugosa* at four different root zone temperature (RZT) treatments (10, 20, 28, and 36 °C) at 32 days after transplant. Data are shown as the means ± standard error (SE) (n = 5). Different letters (a and b) show significant differences among the RZT treatments (Tukey's test, $p < 0.05$).

3.4. Rosmarinic Acid (RA), Tilianin, and Acacetin Concentrations and Contents

The flowers, leaves, stems, and roots were separated to analyze the tilianin, RA, and acacetin concentrations of *A. rugosa* at 32 days after transplant (Table 2). The highest RA concentrations in leaves and stems were observed at 36 °C. The lowest RA concentration in flowers was observed at 28 °C RZT; however, the lowest RA concentration in roots was observed at 20 °C. These results revealed an increasing trend in RA concentration of *A. rugosa* organs at low or high root zone temperatures (10 or 36 °C) (Table 2). Furthermore, the tilianin and acacetin concentrations were lower than the RA concentration in the whole plant, as well as in the organ parts of the *A. rugosa* plant (Table 2 and Figure 5). Similarly, Tuan et al. (2012) reported that the RA concentration was the highest compared with tilianin and acacetin concentrations of *A. rugosa* plant grown under farm conditions. Moreover, RA was the main phenolic compound that accumulated in the root of *A. rugosa* [38]. This also agrees with the result of this study that the RA concentration of *A. rugosa* was mostly concentrated in roots compared with other plant organs (Table 2). Moreover, the results indicated that the highest RA content was observed in leaves at 36 °C, in flowers at 10 °C, in stems at 20 °C, and in roots at 28 °C, since it is based on the DW of plant organs (Table 3). Significantly higher RA concentrations in the whole plant were observed at low or high root zone temperatures (10 and 36 °C) (Figure 5A). Similarly, the nutrient solution at 10 °C was beneficial for raising both RA and luteolin concentrations and the contents of the whole plant in red perilla (*Perilla frutescens*, Labiatae) [10]. Bioactive compounds in the tomatoes (*Solanum lycopersicum* L.) were increased under cold stress at 10 °C [39]. Low root temperature at 10 °C caused water stress, which increased the sugar concentrations in the tomatoes [39]. Furthermore, the high RZT treatment at 33 °C increased the total phenolic compound content in the carrots (*Daucus carota* L.) [14]. High RZT at 35 °C significantly increased the soluble protein content, proline, and antioxidant enzymes in the cucumbers (*Cucumis sativus* L.) [27]. However, the highest

RA content in the plants was observed at 28 °C RZT, because the whole plant's RA content is the RA concentration multiplied by whole plant DW (Figure 5B).

The tilianin concentration in the leaves and flowers was significantly higher at 28 and 36 °C RZT compared with the other treatments. However, the tilianin concentration in the stems was higher at 20 and 36 °C RZT than that in the other treatments. The tilianin concentration in the roots was maximized at 36 °C RZT (Table 2). The results showed that the tilianin content in leaves and flowers was the highest at 28 °C RZT. The tilianin content was the highest in stems at 20 °C and the lowest in roots at 10 °C RZT (Table 3). The tilianin concentration in the whole plant increased significantly with increasing RZT up to a maximum at 28 °C, because the whole plant's tilianin content is the tilianin concentration multiplied by whole plant DW (Figure 5C,D).

The highest acacetin concentration in the flowers was observed at 10 °C RZT. The lowest acacetin concentration in the stems was observed at 36 °C RZT. The acacetin concentration did not appear in the leaves and roots of *A. rugosa* at different RZTs (Table 2). These results corresponded with the results of Tuan et al. (2012), who reported that the acacetin concentration did not appear in the roots, and the small amount of acacetin concentration appeared in the leaves of *A. rugosa* plants grown under farm conditions. The highest acacetin content was obtained at 10 °C in the flowers and 20 °C in the stems (Table 3). The acacetin concentration in the whole plant was significantly higher at 10 °C and significantly lower at 28 °C; however, the significantly higher acacetin content was observed at 10 and 20 °C (Figure 5E,F). These results were attributed to the RZT stress-induced increase of water stress, which increased the bioactive compounds in the plant [40].

The highest RA, tilianin, and acacetin concentrations in *A. rugosa* were mostly found at low and high RZTs (10 and 36 °C) (Figure 5), because reactive oxygen species (ROS) such as $^1O_2$, O_2^-, H_2O_2, and OH can increase in the cells at low or high RZT. Reactive oxygen species are accumulated in plants and required for cellular signaling. However, under low or high root zone temperature conditions, the antioxidant defense was produced to prevent the detrimental oxidative damage of ROS, which can break the ordinary metabolism of plants through the oxidation process of membrane proteins, lipids, and nucleic acids. Therefore, this process accumulates the secondary metabolisms and limits the plant growth [10,14,27].

These results indicated that RZT at 28 °C significantly increased the RA and tilianin contents per plant and that 20 °C increased the acacetin content in *A. rugosa* without decreasing the plant growth (Figure 5B,D,F). The lowest RZT treatment (10 °C) restricted plant growth, chlorophyll content, leaf gas exchange parameters, and tilianin concentration, probably due to water inadequacy and ion imbalance uptake, while the highest RZT treatment (36 °C) limited chlorophyll content, leaf gas exchange parameters, acacetin concentration, and plant growth, probably due to drought stress and ion imbalance uptake. Medium RZT treatment (20 °C) restricted RA and tilianin contents. Root zone temperature stresses affected membrane permeability and fluidity, water viscosity, and root growth. Water uptake of the root was affected by the root volume and the water permeability [36]. Moreover, water viscosity was increased under low root temperatures [41]. Therefore, water contents in plants can be reduced under low and high root zone temperatures.

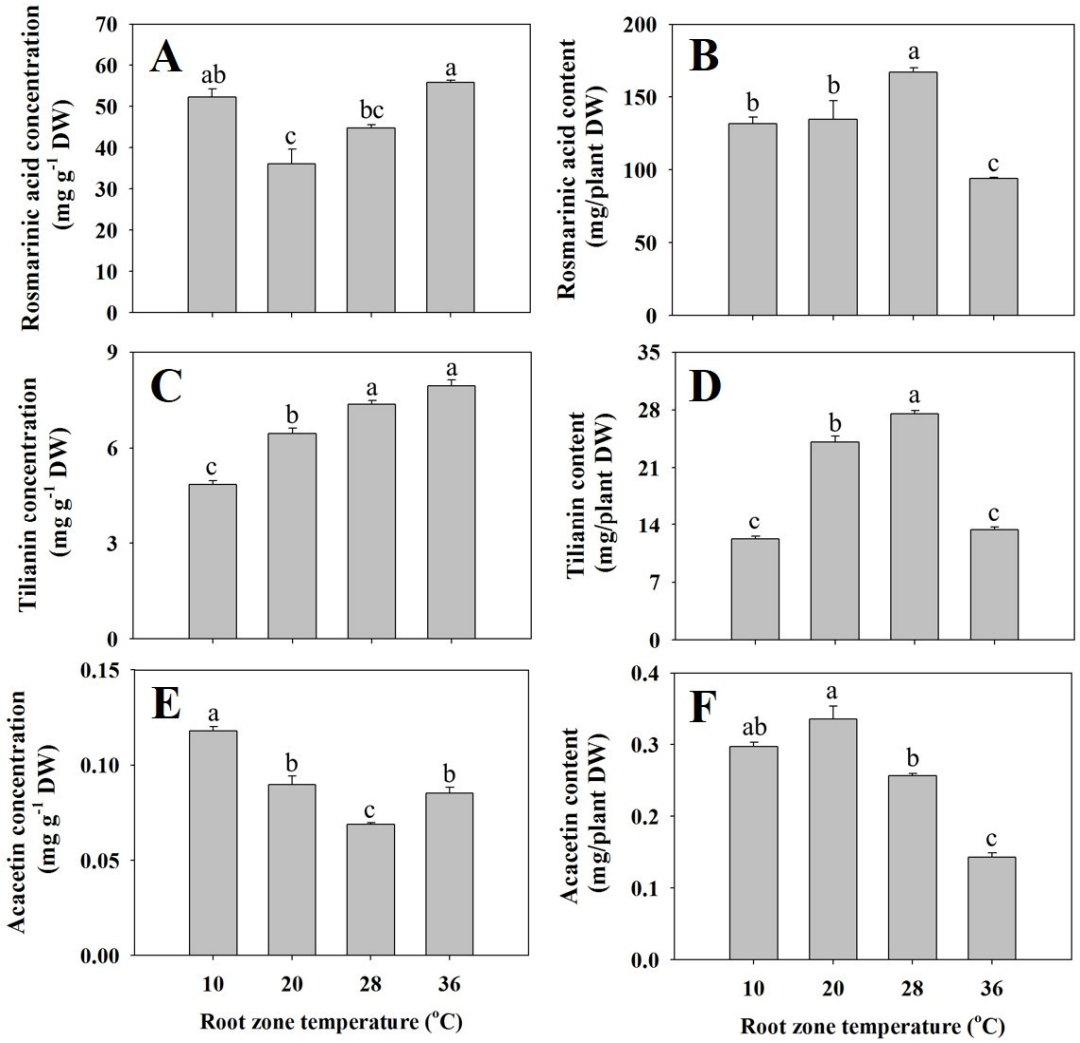

Figure 5. Rosmarinic acid (RA) (**A** and **B**), tilianin (**C** and **D**), and acacetin (**E** and **F**) concentrations and contents in the whole plant of *A. rugosa* at four different root zone temperature (RZT) treatments (10, 20, 28, and 36 °C) at 32 days after transplant. Data are shown as the means ± standard error (SE) (n = 3). Different letters (a–c) show significant differences among the RZT treatments (Tukey's test, $p < 0.05$). DW: dry weight.

Table 2. Rosmarinic acid (RA), tilianin, and acacetin concentrations (mg·g^{-1} DW) in leaves, flowers, stems, and roots of *A. rugosa* grown at four different root zone temperature (RZT) treatments (10, 20, 28, and 36 °C) at 32 days after transplant.

RZT [z] (°C)	RA Concentration in Plant Organs (mg·g^{-1} DW)				Tilianin Concentration in Plant Organs (mg·g^{-1} DW)				Acacetin Concentration in Plant Organs (mg·g^{-1} DW)			
	Leaves	Flowers	Stems	Roots	Leaves	Flowers	Stems	Roots	Leaves	Flowers	Stems	Roots
10	5.122 c[y]	5.948 a	3.357 b	37.915 a	1.078 c	2.606 c	1.149 b	0.033 b	ND	0.086 a	0.032 a	ND
20	5.496 bc	5.890 a	3.585 b	21.083 b	1.387 b	3.485 b	1.549 a	0.027 b	ND	0.057 b	0.032 a	ND
28	5.781 b	5.065 b	3.316 b	30.587 a	1.858 a	4.302 a	1.183 b	0.028 b	ND	0.036 c	0.032 a	ND
36	14.380 a	6.485 a	4.195 a	30.819 a	1.672 a	4.309 a	1.799 a	0.643 a	ND	0.057 b	0.028 b	ND
Significance [x]	***	***	***	**	***	***	***	***	ND	***	**	ND

[z] Root zone temperature treatments. [y] Mean separation within columns by Tukey's multiple range test. ** and ***[x] Significant at $p \leq 0.01$ and $p \leq 0.001$, respectively. Data are the mean ± SE (n = 3). Different letters (a–c) indicate significant differences among RZT treatments at the level of 5%, according to Tukey's test. ND: not detected. DW: dry weight.

Table 3. Rosmarinic acid (RA), tilianin, and acacetin contents (mg/plant organs DW) in leaves, flowers, stems, and roots of *A. rugosa* grown at four different root zone temperature (RZT) treatments (10, 20, 28, and 36 °C) at 32 days after transplant.

RZT [z] (°C)	RA Content in Plant Organs (mg/Plant Organs DW)				Tilianin Content in Plant Organs (mg/Plant Organs DW)				Acacetin Content in Plant Organs (mg/Plant Organs DW)			
	Leaves	Flowers	Stems	Roots	Leaves	Flowers	Stems	Roots	Leaves	Flowers	Stems	Roots
10	5.719 c[y]	2.259 a	1.544 c	21.240 ab	1.203 c	0.990 b	0.528 c	0.018 b	ND	0.032 a	0.015 c	ND
20	10.497 b	1.649 c	2.761 a	16.498 b	2.649 b	0.976 b	1.193 a	0.021 ab	ND	0.016 b	0.025 a	ND
28	10.773 b	1.874 b	2.409 b	23.658 a	3.463 a	1.593 a	0.860 b	0.022 ab	ND	0.013 bc	0.023 b	ND
36	13.373 a	1.360 d	1.594 c	5.029 c	1.555 c	0.903 b	0.683 bc	0.026 a	ND	0.012 c	0.011 d	ND
Significance [x]	***	***	***	***	***	***	***	***	***	***	***	***

[z] Root zone temperature treatments. [y] Mean separation within columns by Tukey's multiple range test. [x] Significant at *** $p \leq 0.001$. Data are the mean ± SE (n = 3). Different letters (a–d) indicate significant differences among RZT treatments at the level of 5%, according to Tukey's test. ND: not detected. DW: dry weight.

4. Conclusions

The RZT treatment at 28 °C can affect concentrations of bioactive compounds such as RA and tilianin without reducing plant growth. Our results confirmed that RZT control effectively enhances bioactive compounds in *A. rugosa*. Furthermore, the RA, tilianin, and acacetin concentrations in each part of the plant (flowers, roots, stems, and leaves) were increased, which will help users to optimize each organ's quality according to their requirements. Further studies are needed to clarify the effects of different air temperatures during low or high RZT treatments on the plant growth and bioactive compound concentrations of *A. rugosa* to enhance its effective production.

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