CONTROL OF UV RADIATION AND ROOT-ZONE TEMPERATURE FOR PRODUCTION OF HIGH-QUALITY BABY LEAF *AMARANTHUS TRICOLOR* L.

AUGUST 2022

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(千葉大学審査学位論文)

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ABSTRACT

Baby-leaf vegetables are a trading name for leafy vegetables sold as leaves with petioles at the seedling stage. Amaranth (*Amaranthus tricolor* L.) is a nutritious baby-leaf vegetable containing many bioactive compounds. Although baby-leaf amaranth product has been established in the market, short-term application of abiotic stress for enhancing a quality especially bioactive compounds, without causing an abnormal appearance has not been revealed yet. The preliminary study was conducted for establishing a quality index, outline for forthcoming studies (Chapter 2). The quality index included 4−5 cm leaf lengths with no abnormal appearance, such as discoloration, yellowing, or uneven surface, and with adequate colorimetric parameters and bioactive compounds. While the seedlings grown in high PPFD (300 $\mu m^{-2}s^{-1}$) had high bioactive compounds, their leaves were rigid and unsuitable for consumption, whereas the leaf grown in the low PPFD (200 $\mu m^{-2}\ s^{-1}$) had low bioactive compounds, but their leaves were smooth and soft. The cultivation under low PPFD followed by the application of abiotic stress for enhancing bioactive substances could be a feasible option for manufacturing. Two abiotic stress treatments were demonstrated: UV-B irradiation (Chapter 3) and cooling root-zone temperature (RZT; Chapter 4). It was concluded that UV-B irradiation at 6 W m^{-2} with cumulative energy of 150 kJ m^{-2} and a 24 h post-irradiation recovery period could be an appropriate treatment to increase bioactive compounds in baby-leaf amaranth without causing the appearance abnormalities. For RZT treatment, the combination of RZT treatments at 5 and 10ºC for one day prior or followed by 20ºC for two days had varied effects on the growth and quality of amaranth leaves. After one day of RZT at 5ºC followed by two days of RZT at 20ºC, the highest concentration of bioactive compounds, nutrients, and antioxidant capacity was demonstrated in the leaves without impairing growth. Both treatments, two days of UV-B treatment and three days of cooling RZT treatment showed a significant feature in these studies, producing high-quality green and red baby-leaves, respectively. This study's findings could be used for the advancement of the baby-leaf industry.

CHAPTER 1
Introduction

1.1 Background

1.1.1. Functional foods

Consumption of foods that provide health advantages, known as functional food (FF), has recently gained popularity. The FF contains bioactive compounds known or unknown, potentially reducing the risk of chronic diseases such as cancer, atherosclerosis, diabetes, Alzheimer's, and Parkinson's disease (Rudkowska, 2009). In addition, FF improves human well-being in ways beyond the aspect of nutrients themselves, all while providing no or significantly less toxicity (Eussen et al., 2011). There are various FFs available in the market; however, among the FFs, plant-based FF is regarded as a more accessible daily because of its lower cost and versatile consumption style (Bleiel, 2010).

It is well established that plant-based FF can prevent lifestyle diseases due to the presence of bioactive compounds. The consumption of bioactive compound cocktails from plant-based FF has been shown in certain studies to have greater health benefits in preventing lifestyle diseases than consuming a single bioactive compound (Kapinova et al., 2017). There are various plant-based FFs available in the market; in brief, cereal, fruit, and vegetable, edible seeds, sprouts, microgreens, and baby-leaf or are all examples of FFs (Khan et al., 2013).

1.1.2. Baby-leaf amaranth

Commercially, baby-leaf vegetables, also known as baby greens, are a term for leafy vegetables sold as leaves with a petiole at the seedling stage. Seedling plants often contain more bioactive compounds, nutrients, and minerals than their mature counterpart (Paradiso et al., 2018); consequently, baby-leaf is one of the FFs. The typical quality index of baby-leaf vegetables is leaf length, leaf area, color, texture, taste, and specific bioactive compounds such as ascorbic acid, anthocyanin, and β-carotene. (Takahama et al., 2019).

A wide variety of baby-leaf families, including *Amaranthaceae*, *Asteraceae*, and *Brassicaceae*, are available on the market presently (Fig. 1-1). For each baby-leaf family, each variety's appearance, taste, and bioactive compounds are selected according to their intended use and their versatility to be eaten in various ways (Fig. 1-2).

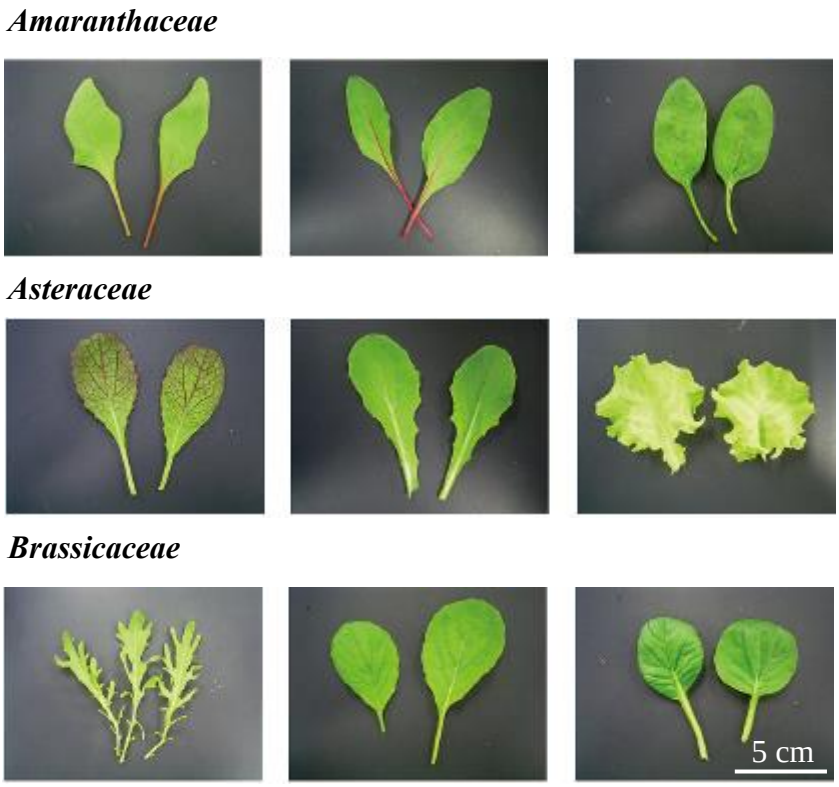


Fig. 1-1. Appearance of *Amaranthaceae*, *Asteraceae*, and *Brassicaceae* baby-leaf families.

Source: modified from Takahama et al., 2019

Fig. 1-2. The utilization of baby-leaf as salad (left) and edible ornamental (right).

Amaranth (*Amaranthus tricolor* L.), one of the *Amaranthaceae* plants, is a nutritious leafy vegetable and used medicinally as well as for food due to the bioactive compounds, including anthocyanin, betalain, ascorbic acid, β-carotene, phenolic, and flavonoids (Sarker et al., 2020; Sarker and Oba, 2018; Asao and Watanabe, 2009). Since seed, sprout, microgreen, and baby-leaf or baby-green are all edible varieties of the amaranth product (Fig. 1-3).

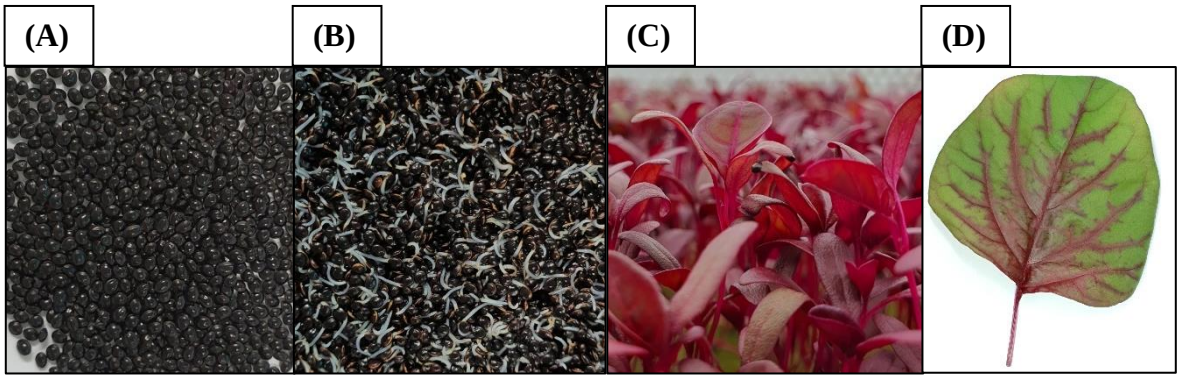


Fig. 1-3. The variety of amaranth products, including (A) seed, (B) sprout, (C) microgreen, and (D) baby-leaf.

High-quality amaranth baby-leaf is very prominent, so innovative cultivation and treatment are considered necessary to be established. Environmental factors, including light (Meas et al., 2020), temperature (Godar et al., 2015), and salinity (Omami and Hammes, 2006), influence amaranth leaf quality; these are considered abiotic stress, which has positive and negative impacts. As a result, rational application of abiotic stress may be beneficial. Although baby-leaf amaranth product has been established in the market, short-term application of abiotic stress for enhancing a quality especially bioactive compounds without causing an abnormal appearance has not been revealed yet. Among the well-known abiotic stresses, ultraviolet radiation and root-zone temperature were examined as prospective, fast-acting, and efficient abiotic stresses. Furthermore, several studies on using both as abiotic stressors for enhancing bioactive compounds in various plant models suggest that this may be a promising strategy for achieving research aims.

1.1.3. Ultraviolet-B (UV-B) radiation

By the wavelength, UV radiation (200–400 nm) is categorized as UV-C (200–280 nm), UV-B (280–315 nm), and UV-A (315–400 nm) (Vázquez and Hanslmeier, 2005) (Fig. 1-4). UV-B and UV-C radiation have higher energies compared to that of UV-A radiation; thus, UV-A, in general, take longer for altering plant quality (Moreira-Rodríguez et al., 2017). Irradiation with UV causes cell damage as a consequence of the production of reactive oxygen species (ROS) in the cell matrix (Mackerness, 2000). Among the UV irradiation reports, UV-B is more enunciated, and physiological response regarding plant quality is well investigated. Thus, UV-B irradiation for improving plant quality, especially bioactive compounds, has been developed and implemented in various plant production systems. In general, UV-B improves phenolics, flavonoids, antioxidants, and product shelf-life, while reduces photosynthesis and growth, those depending on the dosage, plant spices, and developmental stage.

There are two possible responses underling UV-B defense mechanisms, including specific and non-specific responsive elements. Specific pathways regulate these mechanisms via UV receptor proteins, phytochromes, and cryptochromes (Nagy and Schafer, 2000), and non-specific pathway regulation occurs via the alteration of ROS in the cell matrix (Escobar-Bravo et al., 2017). However, high fluence UV-B radiation over the biological threshold induces excess ROS in the cell matrix, and these ROS disrupt the cell redox balance and induce cell death (Mackerness, 2000). Several bioactive compounds are produced in plant cells via ROS defense mechanisms (Escobar-Bravo et al., 2017; Rácz and Hideg, 2021); therefore, UV-B irradiance could be used to enhance the bioactive compound concentration in plant. UV-B has beneficial and detrimental effects on plant quality and development; thus, the application of UV-B is necessary to carefully optimize alleviating the adversely effect.

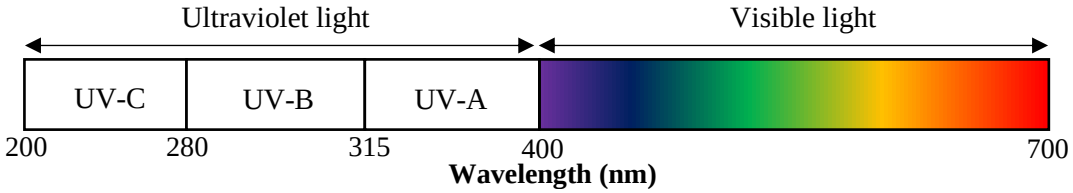


Fig. 1-4. The categorization of electromagnetic wavelength at ultraviolet and visible radiation.

1.1.4. Root-zone temperature

Root-zone temperature (RZT) refers to the root surrounding temperature, which is one of the abiotic stresses potentially enhancing plant bioactive compounds (Calleja-Cabrera et al., 2020). RZT is generally controlled by adjusting growth media temperature using various thermal chambers or controllers. Recently, hydroponic RZT studies have been carried out by controlling nutrient solution temperature using an electric heating and cooling device. The relevance of RZT in plant development and the synthesis of bioactive compounds was studied concerning its role in nutrient and water absorption (Qiuyan et al., 2012). Root membrane fluidity and aquaporin activity, a protein involved in water absorption, are affected by RZT levels (Carvajal et al., 1996; Maurel et al., 2015). As a result of these changes, calcium channel and lipid signaling are activated, affecting intracellular calcium ions (Mittler et al., 2012). When some thermal responsive mechanisms are altered, unstable oxidative products such as reactive oxygen species (ROSs) accumulate (Raja et al., 2017). Several bioactive compounds, including flavonoids and ascorbic acid, are synthesized and utilized to minimize ROS damage by ROS neutralizing activity. Signal molecules are delivered from the root to the shoot requesting a sufficient substrate for bioactive compounds biosynthesis, which causes nutrient and biomass losses from the shoot (Heckathorn et al., 2013). Root physiology and activity, as well as shoot growth, are both affected by RZT treatment; thus, suitable RZT controlling is a challenge for enhancing bioactive compounds while retaining a satisfactory quality of the shoot.

Even while high RZT treatment can enhance plant bioactive compounds, it may have a detrimental effect on the product's appearance and shelf life because of an increase in respiration rate (Chun et al., 1994; Falah et al., 2010; Masarirambi et al., 2018). For the low RZT, bioactive compounds in plants are also increased, while the end product's water and mineral content is decreased due to low water and nutrients transfer from the root (Calatayud et al., 2008; Nxawe et al., 2009).

According to the reviewed studies, a plant's bioactive compound, yield, and appearance were all affected by RZT treatment at low and high levels. The production purpose may dictate whether low or high levels of RZT are used.

1.2 Research objectives and structure of thesis

Baby-leaf amaranth product has been established in the market; nevertheless, commercial index and production procedure for high-quality has not yet been releveled. It is tough to conceive a novel approach for producing high-quality baby-leaf amaranth without unraveling these two concerns. To overcome the bottle neck challenge, the present research was conducted under the following aims:

1. To study the preliminary production of baby-leaf amaranth (Chapter 2)
2. To establish the quality index for production of baby-leaf amaranth (Chapter 2)
3. To obtain an appropriate treatment for enhancing bioactive compounds using UV-B (Chapter 3) and cooling RZT (Chapter 4)

The preliminary studies, including preliminary production and quality indexing were performed prior to subsequent studies for outlining baby-leaf amaranth's expected quality; then, two abiotic stress treatments were established under the defined standard. The experiments were carried out in the orientation shown in Fig. 1-5.

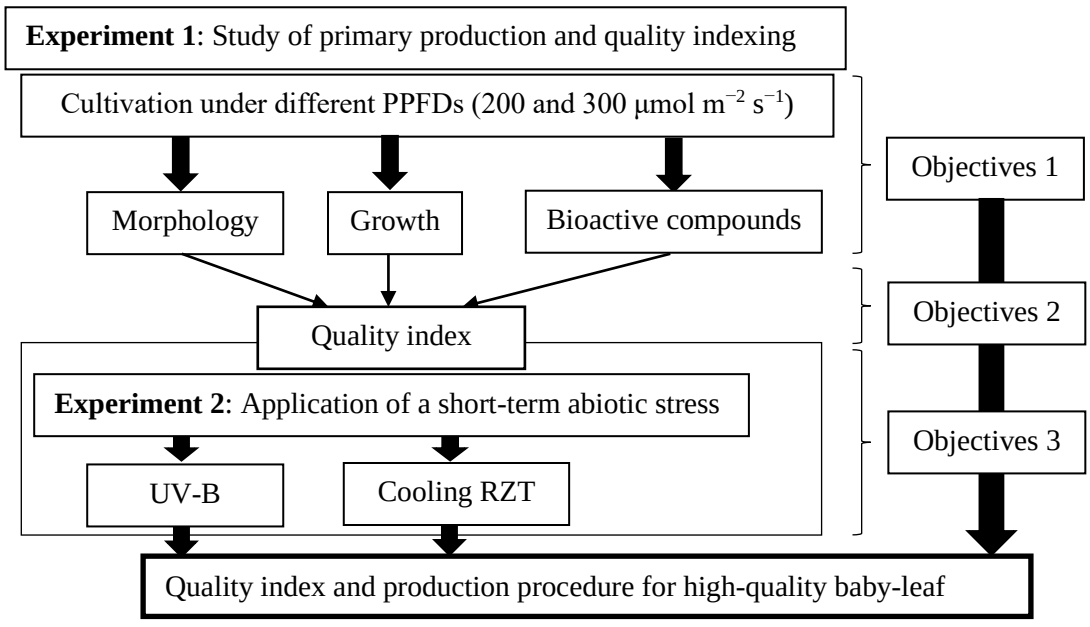


Fig. 1-5. Schematic diagram of experimental design according to research objectives.

CHAPTER 2

A preliminary production and quality indexing of baby-leaf amaranth

2.1 Study of the baby-leaf amaranth preliminary production

2.1.1 Introduction and objective

Baby-leaf amaranth products have been established on the market; there is currently no commercial procedure for producing them. In practice, the production aim related to the quality index serves as the manufacturing guild line; as a result, the quality index is necessary prior to establishing a production procedure. Similar to the production procedure, there is no commercial quality index for baby-leaf amaranth. A quality index for baby-leaf spinach, a member of the *Amaranthaceae* family, was suggested by Takahama et al. (2019), which included appearance, color, leaf size, leaf area, and bioactive compounds. It is preferable to have an elliptical, sagittate, or oblong-shaped leaf with a length of 8 cm and a leaf area of 10 to 15 cm^2. Any blemishes, yellowing, tear-offs, or incidents of pest infestations are not acceptable for appearance purposes. This quality index may be relevant to baby-leaf amaranth; however, no scientific study has been published on manufacturing high-quality baby-leaf amaranth based on this quality index. Thus, the preliminary production is crucial for collecting biochemical and physiological data for the provision of the quality index.

2.1.2 Materials and methods

2.1.2.1 Plant material and cultivation condition

Amaranthus tricolor (red amaranth) seeds were purchased from Nakahara Seed Product Co., Ltd. (Fukuoka, Japan). The seeds were germinated in the dark at an air temperature of 20ºC. The germinated seeds were transferred to a polyurethane sponge (M urethane; M Hydroponics Laboratory Co. Inc., Aichi, Japan) in a 1.2 L polypropylene cultivation container.

Once the seedlings had reached a height of 2.0–3.0 cm and development of pairs of true leaves was observed, they were transplanted into an 8 L cultivation container (Sun Box #16; SANKO Co. Ltd., Tokyo, Japan). A half-strength Otsuka A formulation (OAT house A treatment; OAT Agrio Co. Ltd., Tokyo, Japan) with an electrical conductivity of 0.10–0.12 S m^{-1} and pH 6.3–6.5 was used as a nutrient solution, and the seedlings were irradiated with white LED lamps (LD40S-N/19/21; Panasonic Corp., Osaka, Japan). The seedling cultivation conditions are shown in Table 2-1.

Table 2-1. *Amaranthus tricolor* L. seedling cultivation conditions.

Environmental factor	Setting value
PPFD (μmol m^{-2} s^{-1})	200
Light period (h)	16
Air temperature (ºC)	25/20
Relative humidity (%)	70
CO_2 concentration (μmol mol^{-1})	1,000

PPFD: photosynthetic photon flux density.

2.1.2.2 Measurement of leaf length, area, morphology, and colorimetric parameters

Thirteenth seedlings with 4−5 true leaves (63 leaves in total) were harvested approximately 31 days after sowing. The leaf length of an individual leaf was measured using a vernier caliper. The morphology of the leaf was shown in Fig. 2-1. The mobile camera with a 4.30 mm autofocus lens was used with a fixed setting: lens aperture of f/1.5, ISO 100, and white balance were fixed at 5,000 K. The integration time was adjusted to 1/125 s for all samples. An acquired image was segmented from the background and resized to 1,000 × 800. Fiji, an open-source image analyzer plugin in ImageJ software (Schindelin et al., 2012), was used to measure leaf area.

Leaf surface colorimetric parameters were measured using a color difference meter (CR-20; Konica Minolta, Inc., Tokyo, Japan). The built-in function of the meter averaged the measurements from five points on each leaf to determine L, a, and b by averaging data from five different locations on each leaf.

2.1.3 Results and discussion

The leaf length distribution as a histogram ranged from 2.0 to 10.0 cm among 63 amaranth leaves (Fig. 2-2). The mean and median of leaves length were 5.3 cm, and the mode was 4.5 cm (n=10, 15.87% of population). The histogram indicated that the high-frequency leaf length was distributed between 3.5− 6.5 cm. The comparison of leaf length distribution and leaf morphology demonstrated that amaranth leaf at a particular length has an acceptable shape based on Takahama et al.'s (2019) study.

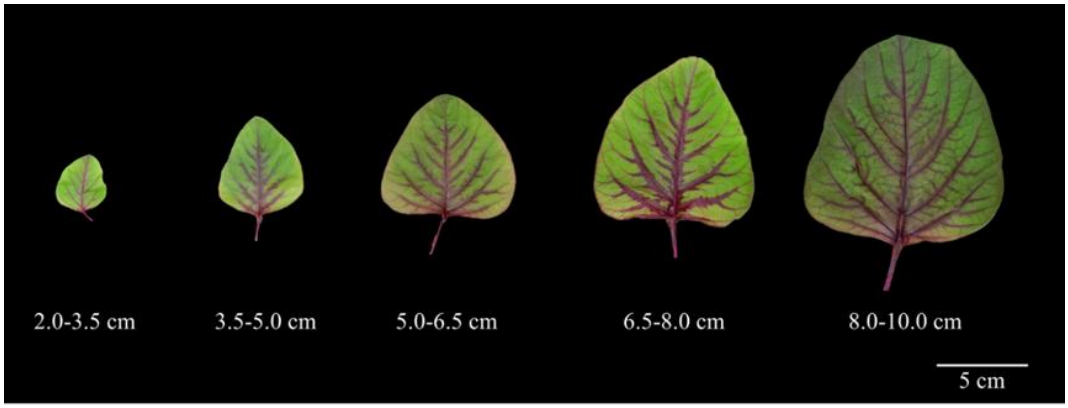


Fig. 2-1. Morphology of amaranth leaves in different sizes (31 DAS).

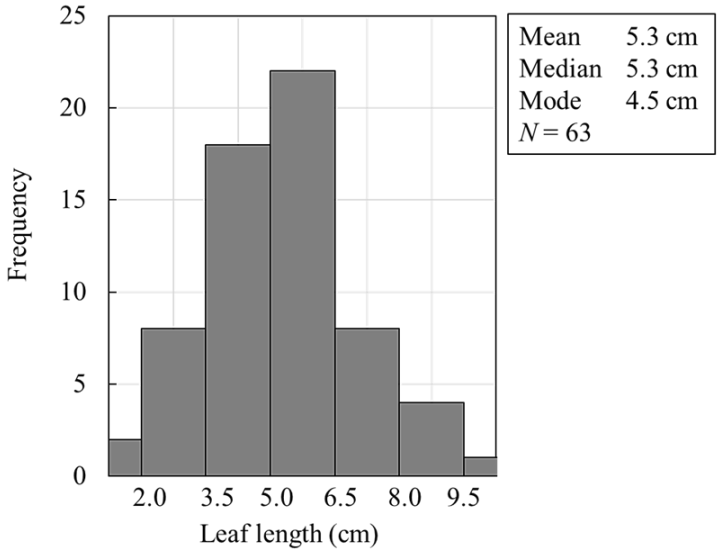


Fig. 2-2. Statistical analysis of leaf size distribution in amaranth leaf cultivated under white LED lamps (31 DAS).

The mean, median and mode values of 63 leaves are shown.

The relationship of leaf length and leaf area of amaranth leaf is shown in Fig. 2-3. The linear relationship from Fig. 2-3 (R^2 = 0.959) provided a leaf length of approximately 3.6−4.6 cm, matched to the recommended leaf area for *Amaranthaceous* baby-leaf that is 10-15 cm^2. This tentative leaf length also matched to leaf length distribution histogram (Fig. 2-2). In practical, a strictly individual leaf area measurement is almost impossible; therefore, a leaf length of approximately 4.0−5.0 cm will be set for further experiment.

It was proposed to use an index based on projected leaf appearance to control leaf quality in the following experiments (Table 2-1). Results from this experiment were merged with those from Takahama et al. (2019) to establish a distinctive morphology (Table 2-2). Leaves with uneven surfaces, discoloration, and yellowing will be rejected in further studies.

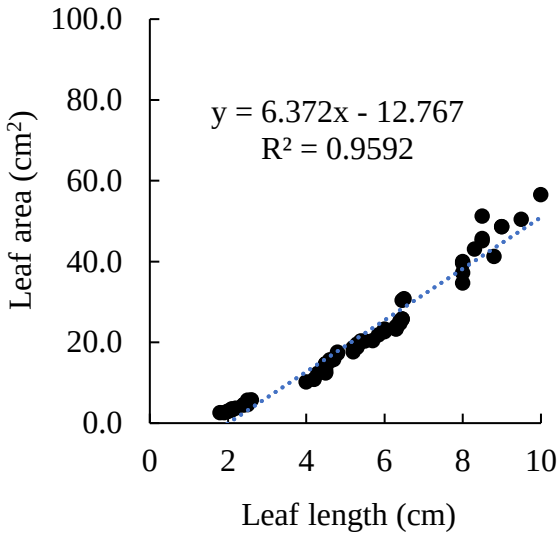


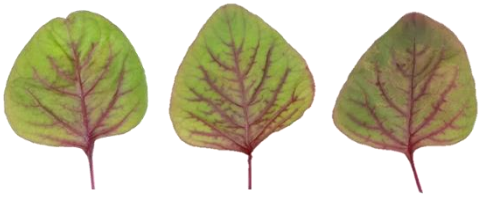
Fig. 2-3. The relationship between leaf area and leaf length of amaranth leaves.

Table 2-1 Expected qualities of amaranth leaf for further experiment.

Quality index	Expected quality or quantity
Leaf morphology and appearance	No indication of leaf disorderes[1]
Color	L* 26.0−31.0, a*>3.0, and b*<8.0
Leaf length (cm)	Approximately 4.0−5.0

[1] Uneven surfaces, discoloration, and yellowing

Table 2-2. Characteristics and acceptability of amaranth leaf morphology.

Characteristic	Leaf morphology	Acceptability
Healthy		Accept
Uneven surface		Reject
Discolored leaf		Reject
Yellowing		Reject

2.2 Effect of PPFD on growth and quality of baby-leaf amaranth

2.2.1 Introduction and objective

The intensity of biological active radiation of plants (300 – 800 nm) strongly influences crop physiology and biochemistry. For most vegetable crops, even a slight increase or decrease in photosynthetic photon flux density (PPFD) causes significant changes in morphology, physiology, and quality. Root, stem, and leaf dry matter and photosynthetic rate are all reduced under low PPFD (Yang et al., 2017). These alterations reduce the plant nutrients, water, and photosynthetic products, reducing crop yields (Wu et al., 2017). As it turns out, a minor increase in PPFD leads to significant changes in plant growth and physiology, resulting in the activation of biological processes (Wu et al., 2016). Plant bioactive compounds, including phenolics and flavonoids, substantially increase under a high fluctuant of PPFD, avoiding photo-damage (Ghasemzadeh et al., 2010; Pérez-López et al., 2018). Elevated PPFD may be beneficial for producing a good quality of leaf with high bioactive compounds; however, under circumstances of high PPFD, plants grow shorter, with thicker leaves and larger stem diameter, in general (Chabot and Chabot, 1977). The thickness and hardness play a crucial role in eating quality affecting consumer acceptability (Werthmann et al., 2015).

Gao et al. (2021) investigated the effect of PPFD on growth and bioactive compounds in broccoli seedlings. The results showed that PPFD 70 μmol m^{-2} s^{-1} under 12 h light period provided a higher bioactive compound while PPFD 50 μmol m^{-2} s^{-1} under the same light period provided a higher fresh weight, dry weight, and moisture content. The results implied that the suitable PPFD for improving growth and bioactive compounds of broccoli seedlings were different. Meas et al. (2020) reported that bioactive compounds of amaranth at early seedlings was higher in PPFD approximately 300 μmol m^{-2} s^{-1} under 16 h light period compared to 130, 180, 230 μmol m^{-2} s^{-1}. However, there was no significant difference in fresh weight between the treatments, suggesting that, within a particular range, PPFD does not influence the growth of amaranth seedlings. The review suggested that the optimal PPFD for enhancing seedling growth and bioactive compounds may be differed.

However, the report above focused on the early seedling stage (with pair of true leaves), which is relatively younger than the baby-leaf stage (4−5 true leaves); thus, the PPFD of 130 µmol m^{-2} s^{-1} may not be suitable growth. PPFD in a plant factory with artificial light can be controlled up to 500 µmol m^{-2} s^{-1} (Ke et al., 2022); however, PPFD of 200 µmol m^{-2} s^{-1} is typically used. As a result of this review, the influence of two PPFDs (200 and 300 µmol m^{-2} s^{-1}) on the growth, morphology, and bioactive compounds of amaranth leaves according to quality index was investigated.

2.2.2 Materials and methods

2.2.2.1 Plant material and cultivation condition

Plant material and cultivation were the same as in 2.1.2.1. The seedling was irradiated at different PPFDs of 200 and 300 µmol m^{-2} s^{-1} with white LED lamps (LD40S-N/19/21, Panasonic, Japan). The common cultivation condition except for PPFD was the same as in 2.1.2.1 (Table 2-1). The harvesting days for 200 and 300 µmol m^{-2} s^{-1} was approximately 31 and 27 DAS, respectively.

2.2.2.2 Yield and leaf morphology

Fifthly to fifthly-two amaranth leaves (leaf length 4.0−5.0 cm) were taken from 15 uniform seedlings and immediately freeze dried (FDU-1110, EYELA, Japan) for further analysis. Fresh and dry weights of an individual leaf were measured using a digital balance (IUW-200D, Sefi, Japan). The morphology of seedling was taken in the way same as in 2.1.2.2 and shown in Fig. 2-4.

2.2.2.3 Leaf lightness (L), redness (a*) and blueness (b*)*

Colorimetric parameters, including L*, a*, and b*, of the leaf surface were determined using a color difference meter (CR-20; Konica Minolta, Inc., Tokyo, Japan) as mentioned in 2.1.2.2.

2.2.2.4 Chlorophyll and carotenoid concentration determination

The pigments from amaranth leaves were extracted using the method described by Meas et al. (2020), with slight modifications.

In brief, powdered leaf samples (5 mg) were combined with 1 mL of 80% acetone (v/v) and homogenized for 30 minutes at 10ºC using a 40 W ultrasonic machine (ASU-2; AS ONE Corporation, Tokyo, Japan). The samples were incubated overnight at 4ºC and then centrifuged at 15,300 × g for 10 min at 4ºC using a centrifuge (MX-307, TOMY Seiko Co., Ltd., Japan).

The final volume of supernatant was adjusted to 1 mL using 80% acetone (v/v). A spectrophotometer (V-750; JASCO Corp., Japan) was used to measure the absorbance of each sample (A) at 470, 645, and 663 nm. Total chlorophyll, carotenoid, and β-carotene concentrations (conc.) were calculated using the following equations, as described by Sarker and Oba (2020):

Chlorophyll a conc. (mg g^{-1} DW) = $(12.21A_{663} - 2.81A_{645}) \times (V/W)$

Chlorophyll b conc. (mg g^{-1} DW) = $(20.13A_{645} - 5.03A_{663}) \times (V/W)$

Total chlorophyll conc. (mg g^{-1} DW) = Chlorophyll a conc. + Chlorophyll b conc.

Carotenoid conc. (mg g^{-1} DW) = $[1{,}000A_{470} - 3.27\text{Chl. a} - 104\text{Chl. b}/229] \times (V/W)$

where A_λ is the absorbance at λ (nm), *V* is the volume of the extract (mL), *W* is the sample weight (g), and *Chl. a* and *Chl. b* is chlorophyll a and b concentrations, respectively.

2.2.2.5 Betalain concentration determination

Five milligrams of freeze-dried plant tissue were mixed with 1 ml of cold 80% methanol (v/v) with 50 mM ascorbic acid and then homogenized using A 40 W ultrasonic machine (ASU-2, As one, Japan) for 10 minutes at 10ºC. The sample was incubated overnight at 4ºC and centrifuged (15,300 g at 4ºC) for 10 min (MX-307, TOMY, Japan).

The final volume of supernatant was adjusted to be 1 ml by 80% methanol (v/v). A spectrophotometer (V-750, Jasco, Japan) was used to quantify the β-cyanin and β-xanthin at 540 and 475 nm, respectively. The concentration was calculated as follows:

$$C = [A \times (M/\varepsilon) \times \text{dilution factor} \times V \times 1{,}000]/W$$

where *C* is the concentration of β-cyanin or β-xanthin (mg g^{-1} DW); *A* is the absorbance at 540 or 475 nm, *M* is the molecular weight of betanin (550.47 g mol^{-1}) or indicaxanthin (308.0 g mol^{-1}), ε is the molar extinction coefficient of betanin (6.2×10^4 M^{-1}) or indicaxanthin (4.8×10^4 M^{-1}), *V* is the extracted volume (L), and *W* is the sample weight (g).

The data were calculated as milligrams betanin equivalent per gram of dry weight for β-cyanins and milligrams indicaxanthin equivalent per gram of dry weight for β-xanthins.Betalain was quantified by combining of β-cyanin and β-xanthin concentration as described by Sarker and Oba (2020).

2.2.2.6 Anthocyanin concentration determination

The determination of anthocyanin concentration was carried out using a procedure modified from Mancinelli and Schwartz (1984). Five grams of freeze-dried plant tissue were mixed with 0.5% chloroform and homogenized for 10 minutes at 10ºC with an ultrasound. The sample was centrifuged (15,300 g at 4ºC) for 5 min and the supernatant was then discharged.

The residues were collected and mixed with 1 mL of a 1% HCl-methanol (v/v) solution and incubated overnight at 4ºC. The incubated samples were centrifuged at 15,300 × g for 30 min at 4ºC. The absorbance was measured at 530 and 657 nm using the V-750 spectrophotometer.

An appropriate dilution of cyanidin-3-glucoside (C3G) was used as the standard. The anthocyanin concentration was calculated using the equation from Stanciu et al. (2009), as follows:

$$A = A_{530} - 0.25 \times A_{657}$$

$$C = [A \times (M/\varepsilon) \times \text{dilution factor} \times V \times 1{,}000] / W$$

where A_λ is the absorbance, C is the anthocyanin concentration as C3G equivalents (mg C3G g^{-1} DW), M is the molecular weight of C3G (485 g mol^{-1}), ε is the molar extinction coefficient of C3G (25.74 M^{-1}), V is the extracted volume (L), and W is the sample weight (g).

2.2.2.7 Total phenolic concentration determination

The extraction of samples for determination of total phenolic concentration was carried out based on the procedure described in Sarker and Oba (2020).

Powdered leaf samples (5 mg) were mixed with 1 mL of cold 90% methanol (v/v) and then homogenized for 30 min at 10ºC using ultrasound. The samples were incubated overnight at 4ºC.

The incubated samples were centrifuged at 15,300 × g for 10 min at 4ºC. The total phenolic concentration determination was based on the principle of the Folin-Ciocalteu reaction described in Jiménez-Aguilar and Grusak (2017). The extracts were mixed with 0.2 N of Folin-Ciocalteu reagent and 1 mol L^{-1} Na_2CO_3 in a ratio of 1:5:4 in a 1.5 mL polypropylene tube. The solutions were mixed well by vortexing and then centrifuged at 15,300 × g for 5 min at 4ºC. The absorbance of the solutions was measured at 760 nm using the V-750 spectrophotometer. An appropriate dilution of gallic acid were used to create a standard curve. Total phenolic concentration was expressed as gallic acid equivalents (mg GAE g^{-1} DW).

2.2.2.8 Total flavonoid concentration determination

The extraction of samples for total flavonoid concentration determination was carried out based on the procedure described in Sarker and Oba (2020). Powdered leaf samples (5 mg) were mixed with 1 mL of cold 90% methanol (v/v) and then homogenized for 30 min at 10ºC using ultrasound. The samples were incubated overnight at 4ºC. The incubated samples were centrifuged at 15,300 × g for 10 min at 4ºC.

Total flavonoid concentration determination was based on the principle of the aluminum chloride reaction. The extract was mixed with 5% $NaNO_2$ (w/v), 10% aluminum chloride (w/v) solution, and 1 mol L^{-1} NaOH at a ratio of 5:1.5:2:1.5 in a 1.5 mL polypropylene tube. The mixture was incubated for 10 min and then centrifuged at 15,300 × g for 5 min at 4ºC. Absorbance was measured at 410 nm using the V-750 spectrophotometer. An appropriate dilution of rutin were used to create a standard curve. The total flavonoid concentration was expressed as rutin equivalents (mg RTE g^{-1} DW).

2.2.2.9 Ascorbic acid concentration determination

Powdered leaf samples (10 mg) were combined with 1 mL of 5% meta-phosphoric acid (w/v) and mixed well by vortexing for 1 min. The incubated samples were centrifuged at 15,300 × g for 5 min at 4ºC.

Ascorbic acid determination was carried out using Reflectoquant® ascorbic acid test strips (Merck, Germany) and a reflectometer (RQflex®, Merck, Germany). Ascorbic acid concentration was expressed as mg g^{-1} DW.

2.2.2.10 Total antioxidant capacity determination

The total antioxidant capacity was determined using the method described by Miller and Rice-Evans (1996). Powdered leaf samples were mixed with 1 mL of cold 90% methanol (v/v) and then homogenized for 30 min at 10ºC using ultrasound. The samples were incubated overnight at 4ºC.

The incubated samples were centrifuged at 15,300 × g for 10 min at 4ºC. The extract was diluted 10-fold using 90% methanol (v/v) and then mixed with 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich, St. Louis, MO, USA) at a ratio of 1:10. The reduction in absorbance at 740 nm within 1 min was measured using the V-750 spectrophotometer. An appropriate dilution of Trolox were used to create a standard curve. Total antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (mM g^{-1} DW).

2.2.2.11 Statistical analysis

An experimental data was processed using statistical package for the social science (SPSS software, version 24, USA). Mean and standard error (SE) of measured parameters will be analyzed by analysis of variance (ANOVA). The mean of parametric data was compared using Student *t*-test where $p < 0.05$, * and $p < 0.01$, ** were used as significant level.

2.2.3 Results and discussion

Amaranth seedlings under different PPFDs have distinct morphology, growth, and biochemical characteristics. The color and appearance of the leaves were crucial in distinguishing between seedlings with PPFDs of 200 and 300 μmol m^{-2} s^{-1}. However, alterations in intracellular bioactive molecules, either related or unrelated to the appearance, may be responsible for these changes. Because of this, for the decision whether to employ low or high PPFD, growth, morphological and biochemical parameters were taken into consideration.

2.2.3.1 Growth and morphology

The leaf exposed to PPFD of 200 μmol m^{-2} s^{-1} appeared green and had a smooth leaf surface, whereas a full-red and uneven leaf surface was observed in the leaf exposed with PPFD at 300 μmol m^{-2} s^{-1} (Fig. 2-4).

Fig. 2-4. Morphology of amaranth leaves cultivated under different PPFDs: 200 (31 DAS) and 300 (27 DAS) μmol m^{-2} s^{-1}.

Several defensive mechanisms are activated in response to a high PPFD, including alterations in photoprotective pigments such as carotenoid and anthocyanin (Yu et al., 2021). Consequently, seedlings exposed to a PPFD of 300 μmol m^{-2} s^{-1} showed a complete red coloration on their projected leaf surface, suggesting that they were undergoing photo-stress (Kitazaki et al., 2018). A variety of baby-leaf products can be produced from the same cultivar if the quality criterion is met by amaranth red or green leaves. However, the leaf under PPFD of 300 μmol m^{-2} s^{-1} had a considerable hard surface (data not shown), which affects the eating quality.

For PPFDs of 200 and 300 μmol m^{-2} s^{-1}, the harvesting days were 31 and 27 DAS, respectively (Table 2-3). The increased PPFD provides more energy for growth and development (Larsen et al., 2020); hence, the harvest day of seedlings grown at 300 μmol m^{-2} s^{-1} may be shorter than that of seedlings grown at 200 μmol m^{-2} s^{-1}. The shorter production time means cheaper production costs that might be regarded from a business perspective. The fresh weight of amaranth leaves under PPFDs of 200 and 300 μmol m^{-2} s^{-1} was not statistically different (Table 2-3); however, dry weight was significantly different.

Table 2-3. Amaranth leaf fresh weight, dry weight, dry matter ratio (DMR), number of leaves and harvesting day under different PPFDs.

PPFD (μmol m^{-2} s^{-1})	Fresh weight (g)	Dry weight (g)	DMR	Number of leaves	Harvesting day (DAS)
200	0.30 ± 0.03	0.026 ± 0.002	0.096 ± 0.002	3.5 ± 0.13	31 ± 1
300	0.28 ± 0.09	0.032 ± 0.001	0.111 ± 0.003	3.3 ± 0.12	27 ± 1
Significant	NS	*	**	NS	NP

The mean and standard error for amaranth leaf fresh weight, dry weight, and dry matter ratio (n=50−52), for number of leaves (n=15) and for harvesting day (n=3) are shown. The asterisk indicates a significant different between treatments using Student *t*-test (*, $p<0.05$ and **, $p<0.01$). NS and NP indicate no statistical significance and not performed, respectively.

In general, the higher PPFD provides more energy for growth and development; the fresh and dry weights of leaves under the higher PPFD may be greater than those of the lower PPFD (Larsen et al., 2020; Ke et al., 2022). The higher PPFD leads to enhanced photosynthesis, which results in an increase in the leaf dry weight (Bhuiyan and van Iersel, 2021) however, that also results in increased transpiration (Pieruschka et al., 2010), which may result in a decrease in the leaf fresh weight. Because of this, the dry matter ratio of the leaf under PPFD at 300 μmol m^{-2} s^{-1} was higher than that under PPFD at 200 μmol m^{-2} s^{-1}, implying that there was a greater percentage of solid portion to liquid portion existent. The higher solid content in leaves influences texture profile considering hardness (Mureșan et al., 2022).

There were no significant differences in leaf number between PPFD 200 and 300 μmol m^{-2} s^{-1} (approximately 4 leaves), demonstrating that PPFD of 300 μmol m^{-2} s^{-1} may be reached the biological threshold. In general, the fluctuated PPFD reached biological threshold leads to fewer leaves because photosynthetic products were utilized to synthesize photoprotective compounds, such as carotenoids, flavonoids, and ascorbic acid, instead of generating new leaf (Bhuiyan and van Iersel, 2021). Since plants have a lower tendency to produce new leaves, it instead thickens the existing ones (Feng et al., 2019). An investigation of the previous leaf surface observation and dry matter ratio indicated a hard surface confirming this indication.

Table 2-4 shows the leaf colorimetric parameters, including L*, a*, and b*, that determine leaf appearance. The L* value of the leaf cultivated at PPFD of 200 μmol m^{-2} s^{-1} was significantly higher than that of 300 μmol m^{-2} s^{-1}. The brighter leaf surface coloration in Fig. 2-4 is consistent with a higher L* value. The leaves' redness and yellowness were altered by PPFD of 300 μmol m^{-2} s^{-1}, suggesting that this light level can enhance the leaves' redness while decreasing the leaves' yellowness. Reduced yellowness is a positive indicator of accomplishing the appearance quality index's criteria (Tables 2-1 and 2-2). In addition, the leaf that visually to be red has a* higher than 10.0 and b* lower than 5.0, whereas the leaf that visually to be green has a* ranging between 2.0− 9.0 and b* higher than 5.0 (data not shown).

Table 2-4. The lightness (L*), redness (a*) and blueness (b*) values of amaranth leaf under different PPFDs.

PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	L*	a*	b*
200	30.8 ± 0.2	3.1 ± 0.1	8.5 ± 0.3
300	27.7 ± 0.4	15.8 ± 0.5	1.1 ± 0.1
Significant	**	*	**

The mean and standard error for amaranth leaf L*, a*, and b* values (n=50−52) are shown. The asterisk indicates a significant different between treatments using Student *t*-test (*, $p<0.05$ and **, $p<0.01$).

Amaranth leaf color is generally involved with leaf pigments such as chlorophyll, carotene, betalain, and anthocyanin (Shaker and Oba, 2020). However, this finding could not directly discuss the pigment concentration owing to colorimetric parameters reflected only surface characteristics; however, pigment concentration reflected the outer and inner matrix of the leaf. Therefore, the determination of leaf pigment is crucial for a thorough study of the effect of PPFD on leaf quality.

2.2.3.2 Photosynthetic pigments

Amaranth leaf pigments exhibited a significant different between leaves of seedlings under different PPFDs (Table 2-5). Leaf chlorophyll concentration was significantly higher (1.0−1.3 times) when the plant was exposed to higher PPFD (300 $\mu mol\ m^{-2}\ s^{-1}$). Chlorophyll is required to function in the photosynthetic apparatus and process properly. On the one hand, an increase in these concentrations indicates that the leaf under PPFD of 300 $\mu mol\ m^{-2}\ s^{-1}$ had higher chances of receiving light, thus synthesizing more photosynthetic pigment to capture more light (Li et al., 2011).

Table 2-5. Total chlorophyll (Chl conc.), carotenoid (Car conc.), betalain (Bet conc.), and anthocyanin (Ant conc.) concentrations in leaves of amaranth seedlings under different PPFDs.

PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	Chl conc. ($mg\ g^{-1}$ DW)	Car conc. ($mg\ g^{-1}$ DW)	Bet conc. ($mg\ g^{-1}$ DW)	Ant conc.[1] ($mg\ g^{-1}$ DW)
200	7.30 ± 0.18	2.42 ± 0.06	2.49 ± 0.09	8.12 ± 0.83
300	8.87 ± 0.32	2.82 ± 0.08	5.17 ± 0.22	26.28 ± 2.57
Significant	*	*	**	**

Data are shown as the mean ± standard error of the fifteen biological replicates. The asterisk indicates a significant different between treatments using Student *t*-test (*, $p<0.05$ and **, $p<0.01$). [1] Anthocyanin concentration is expressed as cyanindin-3-glucoside equivalents. DW: dry weight.

On the other hand, photosynthetic pigments are required in high amounts under high PPFD condition for capturing more light energy and producing more photosynthates, which are necessary for photoprotective compounds and antioxidants biosynthesis (Zhao et al., 2017). The PPFD of 300 $\mu mol\ m^{-2}\ s^{-1}$ may not be attained above the biological threshold of amaranth seedlings. Notably, plants that are subjected to long-term photo-stress would undergo irreversible photo-damage, in which photosynthetic pigments are deteriorated and eventually decrease in concentration (Sanches and Válio, 2006); however, this tendency is not found in the present experiment.

Carotenoids, photoprotective compounds in plants, in leaves irradiated PPFD of 300 $\mu mol\ m^{-2}\ s^{-1}$ was 1.0−1.2 times higher concentration compared to that PPFD of 200 $\mu mol\ m^{-2}\ s^{-1}$. The increase in carotenoid concentration may be related to its critical role in reducing photo-damage caused by high PPFD levels (Demming-Adams, 1990). The portion of the light is generally utilized in photosynthesis; thus, the excess light is perpetrated in photoreactions with various biomolecules, referred to as photooxidation. This process results in the formation of reactive oxygen species (ROS) radicals, which are harbored by an oxidative chain reaction, causing cell damage and, consequently, death (Malnoë, 2018).

A highly sensitive molecule with a strong light capturing tendency such as carotenoids is required to circumvent this (Muzzopappa and Kirilovsky, 2020). However, the crucial process addressing high PPFD in amaranth may not only be carotenoid, as compared to betalain and anthocyanin.

2.2.3.3 Bioactive compounds

The concentrations of anthocyanin and betalain in leaves cultivated at a PPFD of 300 μmol m^{-2} s^{-1} were 3.0−3.3 and 2.0−2.2 times higher, respectively, than those of 200 μmol m^{-2} s^{-1}. This incidence demonstrates that ROS is generated in seedling leaves under high PPFD, and antioxidant mechanisms are then activated to prevent severe damage from ROS. Anthocyanin contributes to the antioxidant system by scavenging superoxide radicals and hydrogen peroxide (Kovinich et al., 2014) and by blocking excessive light or ultraviolet radiation (Cooney et al., 2018); as a result, anthocyanin is accumulated in the epidermis and adjacent mesophyll cells (Kytridis and Manetas, 2006; Moustaka et al., 2018). In complement to anthocyanin, betalain, tyrosine-derived pigments, are responsible for cellular radical scavenging. A considerable antioxidant capacity provided by betalain in plants exposed to various abiotic stresses, such as light, temperature, and salinity, has long been recognized (Chen et al., 2018; Davies et al., 2018).

Even though betalain changed less than anthocyanin, its antioxidant capacity is comparable to ascorbic acid, rutin, catechin, and β-carotene in the same amount (Slimen et al., 2017). Resultantly, it can be inferred that even a slight increase in betalain may be sufficient to counteract the ROS generated by high PPFD.

The result implied that leaves exposed to PPFD of 300 μmol m^{-2} s^{-1} might suffer from light stress; hence, other antioxidants, including phenolic, flavonoid, and ascorbic acid were propelled for addressing this circumstance (Table 2-6).

Table 2-6. Total phenolic (Phl conc.), flavonoid (Flv conc.), and L-ascorbic acid (Asc conc.) concentrations and total antioxidant capacity (TAC) in leaves of amaranth seedlings under different PPFDs.

PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	Phl conc.[1] ($mg\ g^{-1}$ DW)	Flv conc.[2] ($mg\ g^{-1}$ DW)	Asc conc. ($mg\ g^{-1}$ DW)	TAC[3] ($mg\ g^{-1}$ DW)
200	11.06 ± 0.81	6.99 ± 0.60	8.85 ± 0.29	9.59 ± 1.04
300	30.83 ± 2.35	20.09 ± 0.99	9.14 ± 0.24	26.15 ± 0.89
Significant	**	**	NS	**

Data are shown as the mean ± standard error of the fifteen biological replicates. The asterisk indicates a significant different between treatments using Student *t*-test (*, $p<0.05$ and **, $p<0.01$). NS indicates no statistical significance. [1] Total phenolic concentration is expressed as gallic acid equivalents. [2] Total flavonoids concentration is expressed as rutin equivalents. [3] Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

PPFD affects plant growth and development, and secondary metabolites assist plants in responding to stress under the inappropriate PPFD. Non-enzymatic antioxidants in plants, such as phenolic, flavonoid, and ascorbic acid, have long been regarded as crucial for regulating cellular homeostasis (Escobar-Bravo et al., 2017). This study found that PPFD of 300 $\mu mol\ m^{-2}\ s^{-1}$ resulted in an approximately 3-folds increase in leaf phenolic and flavonoid concentrations compared to 200 $\mu mol\ m^{-2}\ s^{-1}$. This incidence corresponds to a total antioxidant capacity of leaves under PPFD of 300 $\mu mol\ m^{-2}\ s^{-1}$, which increased 3-folds compared to 200 $\mu mol\ m^{-2}\ s^{-1}$. Antioxidants such as phenolic, flavonoid, carotenoid, betalain, and anthocyanin may increase the antioxidant capacity shown in amaranth leaves (Tables 2-5 and 2-6).

However, ascorbic acid did not significantly differ between the two PPFDs, remaining at 8.9−9.2 $mg\ g^{-1}$ DW. Ascorbic acid, in general, is a first-line antioxidant capable of scavenging reactive oxygen species (ROS) and thereby inhibiting oxidative damage; hence, it will be immediately utilized prior to synthesize other antioxidants (Davey et al., 2000).

Under high PPFD circumstances, it is possible that ascorbic acid may be utilized early and maintained after more stable antioxidants such as phenolic and flavonoid are synthesized (Ntagkas et al., 2018). Even though ascorbic acid did not exceed the influent high PPFD response in this study, it is necessary to maintain other redox balances, such as the ascorbate-glutathione cycle (Hasanuzzaman et al., 2019) and suppressing lipid oxidation (Laguerre et al., 2007). Under continuous high PPFD, phenolic and flavonoids are generated progressively (Graham, 1998), coinciding with leaf thickening to block excess light (Feng et al., 2019).

On the one hand, high PPFD promoted the biosynthesis of phenolic and flavonoids; on the other hand, it accelerated the utilization of sugar in leaves as a substrate. Furthermore, it may be extrapolated that leaves cultivated under long-term exposure to high levels of PPFD (300 μmol m^{-2} s^{-1}) may have lower sugar content than leaves grown under lower PPFD. While this experiment did not investigate sugar content, some studies have found that long-term light stress increases bioactive compounds and reduces sugar content in plants (Chen et al., 2019; Proietti et al., 2021).

2.3 Conclusions

Amaranth seedling's leaf quality characteristics under a controlled environmental system were studied in the preliminary production. This retrieval of data provides valuable information. Inappropriate cultivation of the leaves resulted in yellowing, discoloration, uneven surface, and a hard surface that impaired eating quality. Rejecting criteria included these instances, and a preliminarily established quality index served as a guide for the subsequent studies.

The alterations in intracellular bioactive compounds and the quality of the baby-leaf are response events for elevated PPFD. The results of this experiment demonstrate that cultivating amaranth seedlings at a high PPFD (300 μmol m^{-2} s^{-1}) resulted in increased dry weight, bioactive compounds, and shorter harvest days (Tables 2-3 and 2-5), as well as complete red coloration (Table 2-4; Fig. 2-4), but also a hard and uneven surface, which are unexpected characteristics. At lower PPFD (200 μmol m^{-2} s^{-1}), green, smooth-surfaced leaves appeared, but they were less concentration of bioactive compounds and required a longer harvest time. Using varied PPFDs for seedling cultivation reveals a district characteristic that is both beneficial and detrimental to the quality of baby-leaf amaranth; hence, a rational management and treatment strategy could be applied to achieve production targets. As a result of these findings, the following study issue was "how to produce high bioactive compounds baby-leaf amaranth according to quality index?".

Therefore, a short-term application of abiotic stress may be possible to attain necessity. Applying an optimal level of abiotic stress will enhance the plant's bioactive compounds, and the optimal treatment duration may be employed to avoid a detrimental impact. Pre-harvest treatment with powerful abiotic stress may enhance the bioactive components in amaranth baby-leaf without impairing the quality index. Short-term application of two abiotic stresses, UV radiation (Chapter 3) and root-zone temperature (Chapter 4) control, will be used for accounting research purposes in the following studies.

CHAPTER 3

Enhancement of bioactive compounds in baby-leaf amaranth using short-term application of UV-B irradiation

3.1 Preliminary study and effect of recovery period after UV radiation

3.1.1 Introduction and objective

Biotic and abiotic stresses have been utilized to enhance bioactive compounds in plants, with ultraviolet (UV) irradiation being one of the most potent abiotic stresses employed for this purpose. UV-C (200–280 nm), UV-B (280–315 nm), and UV-A (315–400 nm) are three distinct types of UV radiation (Vázquez and Hanslmeier, 2005). UV-B and UV-C radiation have greater energy than UV-A radiation. There is a direct correlation between UV light exposure and cell damage resulting from reactive oxygen species (ROS) being produced within the cell matrix (Mackerness, 2000). Numerous bioactive compounds are synthesized in plant cells as a result of ROS defense mechanisms to avoid cell damage (Escobar-Bravo et al., 2017; Rácz and Hideg, 2021); hence, UV irradiation may be employed to increase the content of bioactive compounds in baby leaves. Plant growth, morphology, and bioactive compound concentration have been studied concerning the impacts of abiotic stressors, such as UV radiation. An abnormal cultivating environment generally has adverse effects on the quality and growth of plants (Raja et al., 2017). The short-term exposure to abiotic stress may not have the same deleterious effects and may be advantageous for enhancing bioactive constituents without causing an aberrant appearance.

Moreira-Rodrguez et al. (2017) found that the total phenolic and flavonoid concentrations in broccoli sprouts exposed to UV-B irradiation are significantly greater than those in sprouts exposed to UV-A irradiation at the same intensity and duration. As a result of its higher energy, UV-B radiation necessitates a shorter treatment duration than UV-A radiation. The results also showed that some phenolic compounds increased in concentration 2 to 24 hours after exposure to UV-A or UV-B radiations.

The recovery period may influence the biosynthesis of bioactive compounds. The expression of genes is delayed following UV-B irradiation, as shown in several investigations. Clayton et al. (2018) found that 12 and 36 hours after UV-B irradiation, the PAL gene in *Marchantia polymorpha* was upregulated 2.5 and 3.1-folds. PAL (CsPAL10) in cucumber has been shown to be upregulated to its maximal expression level 24 hours following UV-B irradiation (Qian et al., 2019). The activation of UV-B-specific receptors and stress-responsive elements is more seem at post-irradiation, resulting in delayed UV-B responses, but this propensity is not observed immediately after UV-B irradiation (White and Jahnke, 2002; Lee et al., 2021).

Accordingly, a study on the effects of the recovery period after UV-B irradiation on the quality of baby-leaf amaranth might be designed to avoid a misleading result from an impropriate harvest.

3.1.2 Materials and methods

3.1.2.1 Plant material and cultivation condition (please see section 2.1.2.1)

3.1.2.2 UV-B treatments

The six amaranth seedlings were subjected to short-term UV-B treatments once they had developed four to five true leaves, approximately 31 days after sowing. UV-B lamps (λ_{max} = 310 nm; TL 20W/01 RS; Philips, Hamburg, Germany) were installed in adjustable frames beneath white LED lamps to perform the treatments. UV-B intensity was measured at plant canopies using a spectroradiometer (USR-45DA; Ushio Inc., Tokyo, Japan), and was adjusted using aluminum foil partially wrapped around the UV-B lamp. The spectral radiant flux distribution is shown in Fig. 3-1. The same PPFD of 300 μmol m^{-2} s^{-1} was used to irradiate the seedlings in all the UV-B treatments[1].

Authors note[1] According to our previous experiment (section 2.2), UV-B treatment and increasing PPFD may synergistically enhance target bioactive compounds. Therefore, we used PPFD at 300 μmol m^{-2} s^{-1} for both UV-B treated seedlings and the control during treatment period.

Three levels of UV-B radiation (3, 6, and 9 W m^{-2}) were applied to the seedlings for 8 h. The treated seedlings were separated into two groups: those sampled immediately after UV-B irradiation (0 h recovery period) and those sampled 24 h after treatment (24 h recovery period; Fig. 3-2).

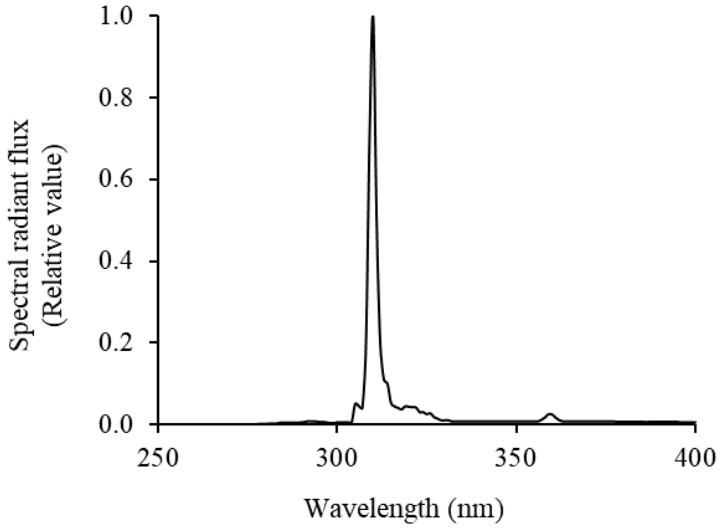


Fig. 3-1. Spectral radiant flux distribution of the ultraviolet B lamp used to irradiate amaranth seedlings.

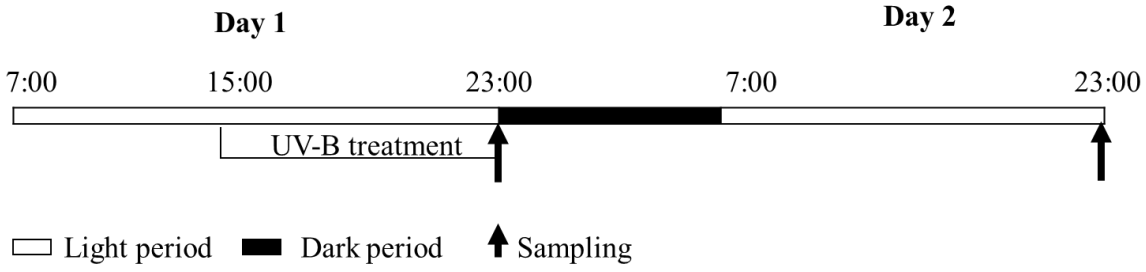


Fig. 3-2. Treatment period and sampling times for ultraviolet (UV) treatments of amaranth seedlings. The UV-B treatments included irradiation at intensities of 0, 3, 6, and 9 W m^{-2} for 8 h. Sampling was conducted twice at 0 and 24 h after UV-B treatment. Seedlings were irradiated at the same photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}.

3.1.2.3 Yield and leaf morphology

Four or five amaranth leaves of length 4.0–5.0 cm were harvested from four uniform seedlings and immediately freeze-dried (FDU-1110; Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at -80ºC for further analysis. The leaves of each seedling were cold-ground before biochemical analysis, and the fresh and dry weights of individual leaves were determined using a digital balance.

The morphology of the seedlings was recorded visually using a mobile camera. Leaf morphology, length, and colorimetric parameters (please see section 2.2.2.2 and 2.2.2.3) were carefully examined according to the quality index described in Tables 2-1 and 2-2.

3.1.2.4 Chlorophyll and carotenoid concentration determinations
(Please see section 2.2.2.4)

3.1.2.5 Betalain concentration determination (Please see section 2.2.2.5)

3.1.2.6 Anthocyanin concentration determination (Please see section 2.2.2.6)

3.1.2.7 Total phenolic concentration determination (Please see section 2.2.2.7)

3.1.2.8 Total flavonoid concentration determination (Please see section 2.2.2.8)

3.1.2.9 Ascorbic acid concentration determination (Please see section 2.2.2.9)

3.1.2.10 Total antioxidant capacity determination (Please see section 2.2.2.10)

3.1.2.11 Statistical analysis

The experimental data were processed using the Statistical Package for the Social Sciences (SPSS software, version 24, USA). The means of the measured parameters at the same UV-B irradiation level for different recovery periods were compared using the Student's *t*-test. Tukey's honestly significant difference (Tukey's HSD) test was performed to compare the means of the measured parameters among the treatments.

3.1.3 Results and discussion

It has been demonstrated that UV-B influents plant physiological and morphological characteristics, including growth, bioactive compounds, and appearance (Fina et al., 2017).

The growth retardation by reducing in leaf area and biomass, leaf discoloration, and leaf thickening are among the most prevalent UV-B-related detrimental effects (Robson et al., 2015).

3.1.3.1 Growth and morphology

This finding demonstrated that the short-term UV-B irradiation at intensities of 3, 6, and 9 W m^{-2} for 8 h did not have a deleterious effect on leaf quality (Table 3-1). Fresh and dry weights of control and treatment seedlings were not statistically different, ranging from 0.28 to 0.31 g and 0.029 to 0.032 g, respectively. The control and UV-B treated leaves exhibited no significant different in dry matter ratio (0.099 −0.116) as consequence to fresh and dry weights. It can be inferred that UV-B onset and 24h-recovery period are no shown detrimental effect on amaranth leaf fresh and dry weight, and dry matter ratio.

This finding is consistent with the Moreira-Rodríguez et al. (2017) study, in which UV-B radiation was applied to broccoli sprouts for 24 hours before harvesting, and influence on biomass was not observed. UV-B irradiation did not affect the biomass of *Indigofera tinctoria* L. seedlings 48 h after the treatment, but a significant loss in biomass was found at 96 h after the UV-B treatment due to a reduction in photosynthetic pigments (Ravindran et al., 2010). As a result, growth retardation is observed in the plant after UV-B irradiation; thus, short-term UV-B irradiation with a recovery period may be used to confine the adversely prevalent UV-B irradiation demonstrated in this study. UV-B treatment causes the leaf abnormality, as reported in many studies (Yao and Liu, 2006; Nascimento et al., 2015); nevertheless, defects in the quality index (Table 2-2), including discoloration, uneven surface, and yellowing on the leaf surface, were not observed in this research (Fig. 3-3). Leaf colorimetric parameters were shown in Table 3-2. The negative effect of UV-B may be related to the dose and post-irradiation recovery period. Oxidation occurs after UV-B irradiation, generating ROS and disrupting photosynthetic apparatus and cellular homeostasis. These all-occurrences force the plant to activate defense mechanisms, synthesizing several bioactive compounds to neutralize ROS by utilizing sharing substrate with the growing process (Cavinato et al., 2017).

Table 3-1. Amaranth leaf fresh weight (Leaf FW, A), dry weight (Leaf DW, B), and dry matter ratio (DMR, C) at 0 and 24 h after treatment with different short-term ultraviolet B (UV-B) irradiation intensities for 8 h.

(A)

UV-B intensity ($W\ m^{-2}$)	Leaf FW (g)		*t*-test [x]
	0 h	24 h	
0	0.28 ± 0.03	0.29 ± 0.02	0.902^{NS}
3	0.28 ± 0.02	0.31 ± 0.01	0.316^{NS}
6	0.29 ± 0.01	0.31 ± 0.01	0.286^{NS}
9	0.30 ± 0.02	0.30 ± 0.03	0.630^{NS}

(B)

UV-B intensity ($W\ m^{-2}$)	Leaf DW (g)		*t*-test
	0 h	24 h	
0	0.029 ± 0.005	0.030 ± 0.004	0.900^{NS}
3	0.032 ± 0.004	0.030 ± 0.005	0.854^{NS}
6	0.033 ± 0.009	0.035 ± 0.008	0.852^{NS}
9	0.030 ± 0.001	0.033 ± 0.002	0.620^{NS}

(C)

UV-B intensity ($W\ m^{-2}$)	DMR		*t*-test
	0 h	24 h	
0	0.099 ± 0.010	0.103 ± 0.023	0.838^{NS}
3	0.116 ± 0.026	0.116 ± 0.008	0.966^{NS}
6	0.103 ± 0.022	0.103 ± 0.023	1.000^{NS}
9	0.101 ± 0.011	0.101 ± 0.004	1.000^{NS}

Data are shown as the mean ± standard error of the four biological replicates.

[x] The means of a measured parameter for the two different recovery periods were compared using the Student's *t*-test. [NS] indicates no significant difference. The means within the columns are not significantly different.

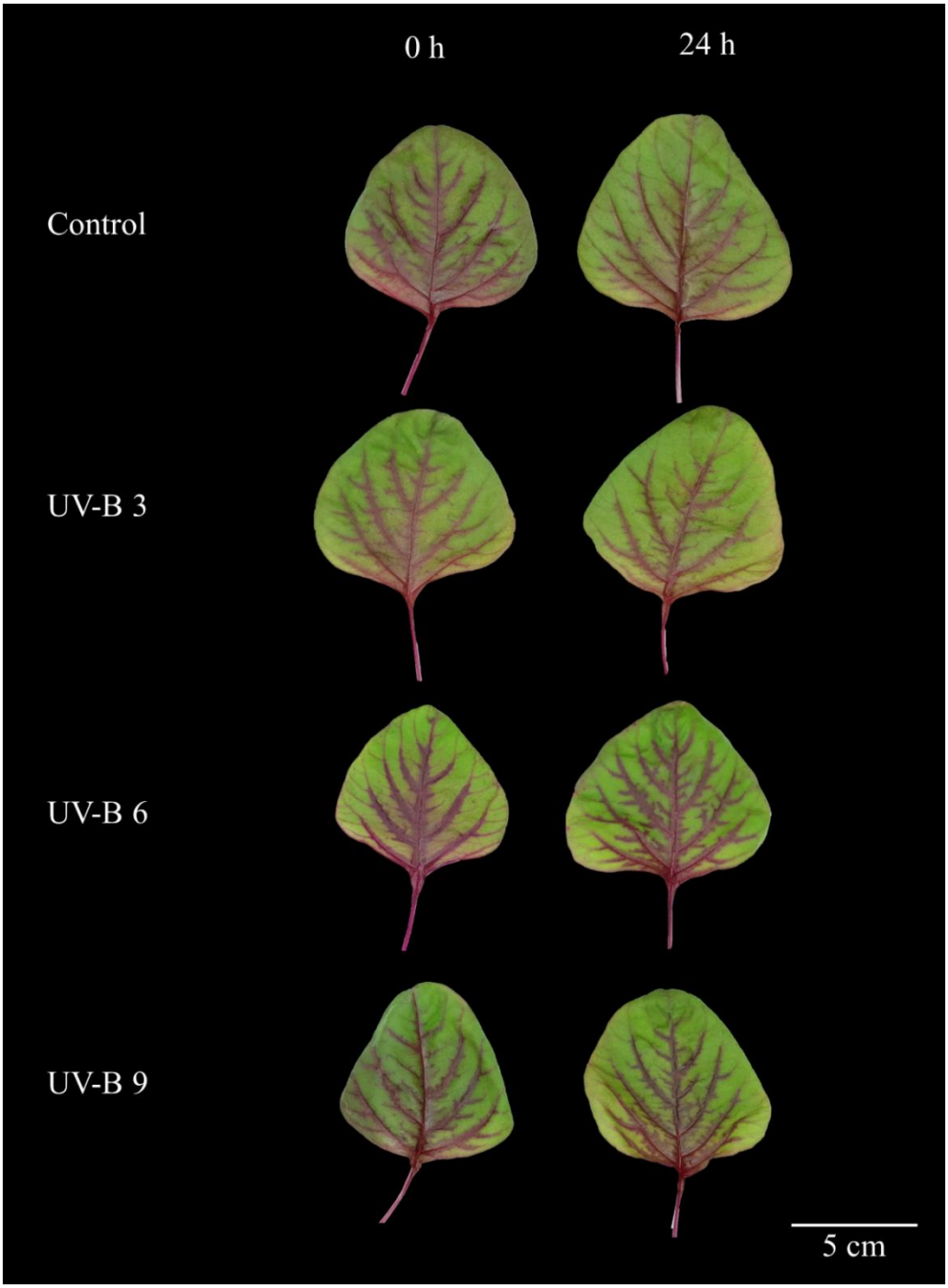


Fig. 3-3. Morphology of amaranth leaves of seedlings treated with different ultraviolet B (UV-B) irradiation intensities and recovery periods (0 and 24 h). The UV-B intensities: 3, 6, and 9 W m^{-2} are indicated as: UV-B 3, UV-B 6, and UV-B 9, respectively.

Table 3-2. Amaranth leaf lightness (L*, 0 = black and 100 = white; A), redness (a*, a* > 0 = trend to be red and a* < 0 = trend to be green; B), and blueness (b*, b* > 0 = trend to be yellow and b* < 0 = trend to be blue; C) at 0 and 24 h after treatment with different short-term ultraviolet B (UV-B) irradiation intensities for 8 h.

(A)

UV-B intensity ($W\ m^{-2}$)	L*		*t*-test [x]
	0 h	24 h	
0	30.85 ± 0.90	30.15 ± 1.65[a]	0.723[NS]
3	31.38 ± 1.93	27.45 ± 2.47[b]	0.002[*]
6	30.40 ± 1.45	26.60 ± 1.15[b]	0.006[*]
9	30.68 ± 1.91	27.66 ± 1.28[b]	0.039[*]
Significant	NS	**	

(B)

UV-B intensity ($W\ m^{-2}$)	a*		*t*-test
	0 h	24 h	
0	3.38 ± 0.24	3.65 ± 0.24[c]	0.504[NS]
3	3.40 ± 0.22	4.85 ± 0.29[b]	0.007[*]
6	3.30 ± 0.21	6.65 ± 0.27[a]	0.001[*]
9	3.65 ± 0.59	6.66 ± 0.31[a]	0.018[*]
Significant	NS	**	

(C)

UV-B intensity ($W\ m^{-2}$)	b*		*t*-test
	0 h	24 h	
0	9.55 ± 0.92	9.63 ± 0.64[a]	0.949[NS]
3	9.75 ± 0.63	7.35 ± 0.55[b]	0.008[*]
6	9.57 ± 0.76	7.78 ± 0.91[b]	0.001[*]
9	8.85 ± 0.81	6.28 ± 0.98[c]	0.001[*]
Significant	NS	**	

Data are shown as the mean ± standard error of the four biological replicates.

[x] The means of a measured parameter for the two different recovery periods were compared using the Student's *t*-test. [NS] indicates no significant difference. Means within columns were compared using Tukey's HSD at a significance level of $p<0.01$, **.

3.1.3.2 Photosynthetic pigments

As aforementioned, the adverse effect of UV-B may be related to the tendency to disrupt the photosynthetic apparatus; hence, the relevance of photosynthetic pigments in amaranth leaves may confirm this assumption. Chlorophylls and carotenoids, photosynthetic pigments, were not significantly different among the treatments at 0 h after UV-B irradiation (Fig. 3-4). Consequently, the total chlorophyll and carotenoid concentrations in leaves irradiated with UV-B were 1.2−1.5 and 1.3–1.5 times higher, respectively, after the 24 h recovery period. Chlorophylls and carotenoids, photosynthetic pigments, were not altered in Arabidopsis or sweet basil leaves at 0 hours after UV-B irradiation, but these changes were only observed 24–72 hours after irradiation (Sztatelman et al., 2015; Mosadegh et al., 2019). The incident shows that photosynthetic pigments were not affected by UV-B irradiation onset; it was affected in the post-irradiation period. UV-B induces ROS formation in the cell-matrix, which tends toward oxidizing chloroplast, the lipid compartment, resulting in the degradation of photosynthetic pigments. However, no such incident was observed in this study, implying that there are some antioxidants or defense mechanisms that subsidize oxidative elements. Thus, more photosynthetic pigments are needed to supply enough substrates to produce bioactive compounds after UV-B radiation as part of the UV-B defense mechanism (Coley et al., 1985).

Carotenoids, especially, β-carotene, acts as a defensive signal under oxidative stress by degradation and formation of β-carotene-oxidative products, resulting in a reduction in β-carotene concentration (Yuan et al., 2015). It can be inferred that amaranth's proper defensive system compensated for the UV-B severity in this experiment, even at the UV-B irradiation onset or 24 h recovery period.

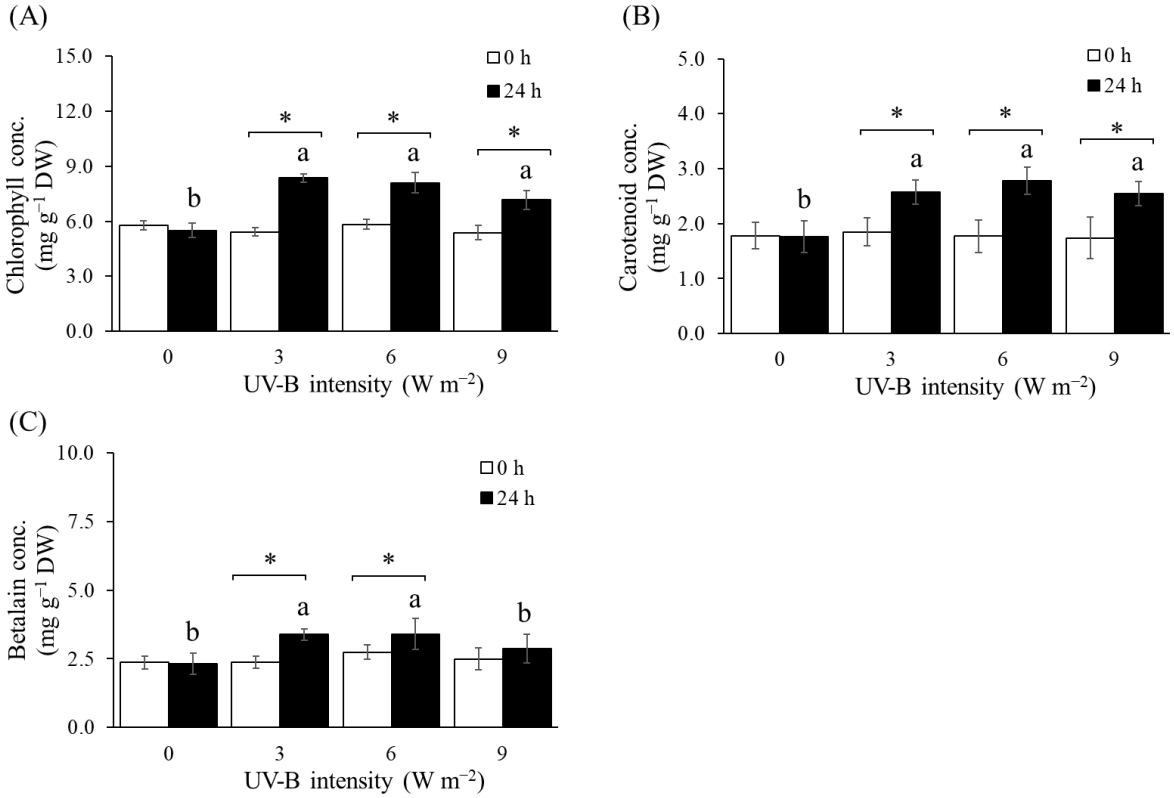


Fig. 3-4. Total chlorophyll (A), carotenoid (B), and betalain (C) concentrations (conc.) in the leaves of amaranth seedlings at 0 and 24 h after treatment with different short-term ultraviolet-B (UV-B) irradiation intensities for 8 h.

Vertical bars indicate standard error (n = 4). Means of the measured parameters at the same UV-B intensity for the two recovery periods were compared using the Student's *t*-test. * indicates significant difference. Lowercase letters indicate significant difference in measured parameters at the same recovery period determined using Tukey's HSD test at $p < 0.05$. DW: dry weight.

3.1.3.3 Bioactive compounds

Betalain, a potent antioxidant found in plants, has the same trend as photosynthetic pigments. Betalain concentration was not significantly different between the control and treatment groups at 0 h following UV-B irradiation; however, it increased 1.1–1.3 times higher during the 24-hour recovery period (Fig. 3-4). Although betalain is not a UV-B-specific response element, it functions as an antioxidant responsible for oxidative stress; therefore, UV-B-induced ROS is possibly neutralized. Thus, the photosynthetic apparatus functions properly under oxidative stress, and as a result, it yields more betalain to maintain homeostasis status. The present study's finding of higher concentrations of photosynthetic pigments, including chlorophyll and carotenoids, might explain the necessity for the synthesis of bioactive compounds for maintaining plant homeostasis, especially under abiotic stress.

Abiotic stress, such as UV-B, damages cells by generating ROS, which the antioxidant system plays a role in prevention of this severity. Anthocyanin, phenolic, flavonoid, and ascorbic acid have been shown to be effective antioxidants in plants; hence, UV-B treatment may influence these compounds. Those compounds were affected by UV-B treatment as a result of the intensity and recovery period in this experiment (Fig. 3-5). At 24 hours recovery period, the anthocyanin concentration in UV-B irradiated leaves was 2.0–2.2 times higher than in the control leaves. During the 0 h recovery period, the total phenolic and flavonoid concentrations were not significantly different but were significantly higher (1.8–2.0 times) after the 24 h recovery period. The total phenolic and flavonoid compounds concentrations in the leaves treated with different UV-B intensities were not significantly different.

Many studies have reported that UV irradiation and blue light induce phenolic compounds, flavonoids, and anthocyanin biosynthesis (Guo and Wang, 2010; Tsurunaga et al., 2013). These compounds are considered UV protective compounds that occur abundantly in epidermal and subepidermal tissues to block UV irradiation (Tohge and Fernie, 2017). The leaves sampled immediately after the UV-B treatment showed no significant difference in bioactive compound concentrations between the control and UV-B treatments, suggesting there is no benefit in irradiating plants without a post-UV-B irradiation recovery period.

The ascorbic acid concentration in the control and UV-B irradiation groups showed significant differences in response to the 0 and 24 h recovery periods. The leaves irradiated at 9 W m^{-2} showed the lowest ascorbic acid concentration at 0 h after treatment, whereas the leaves irradiated at 6 W m^{-2} showed the highest ascorbic acid concentration after the 24 h recovery period. In addition, ascorbic acid increased in amaranth leaves 24 h after UV-B treatment. The total antioxidant capacity of the amaranth leaves was not significantly different among the treatments at 0 h after irradiation; however, by 24 h after irradiation, the total antioxidant capacity of the UV-B irradiated leaves had increased 1.3−1.5 times. The effect of a recovery period has also been investigated in plants irradiated with UV-A (Lee et al., 2019). These researchers applied UV-A radiation to kale at 30 W m^{-2} for five days and found that the total phenolic compound concentration increased during the 1−3 days post-irradiation recovery period.

Lee et al. (2021) showed that the concentration of hydrogen peroxide, a marker of ROS generation by UV-B, increased significantly 8 h after UV-B irradiation and reached its maxima at 24 h in *Brassica napus*. According to the study, ROS was not immediately produced after irradiating the plant. Furthermore, the concentrations of target bioactive components, including phenolics and flavonoids, increased significantly after the post-irradiation period, reaching their maxima at 24 h. In the present study, a recovery period of 24 h after UV-B treatment promoted the accumulation of bioactive compounds, probably due to a time lag between the expression of genes related to their biosynthesis pathways and their accumulation. Such a time lag has been reported in many studies (Clayton et al., 2018; Moreira-Rodríguez et al., 2017; Qian et al., 2019; Strid et al., 1994).

This finding inferred that 24 h recovery period after UV-B irradiation could enhance the bioactive compounds of baby-leaf amaranth. This work may be helpful for further research into the effects of UV-B on baby-leaf amaranth quality after irradiation, as no previous reports have discussed time course changes after UV-B exposure. However, it is unclear how baby-leaf amaranth could respond to shorter or longer recovery periods after UV-B irradiation; therefore, this area requires extensive further research.

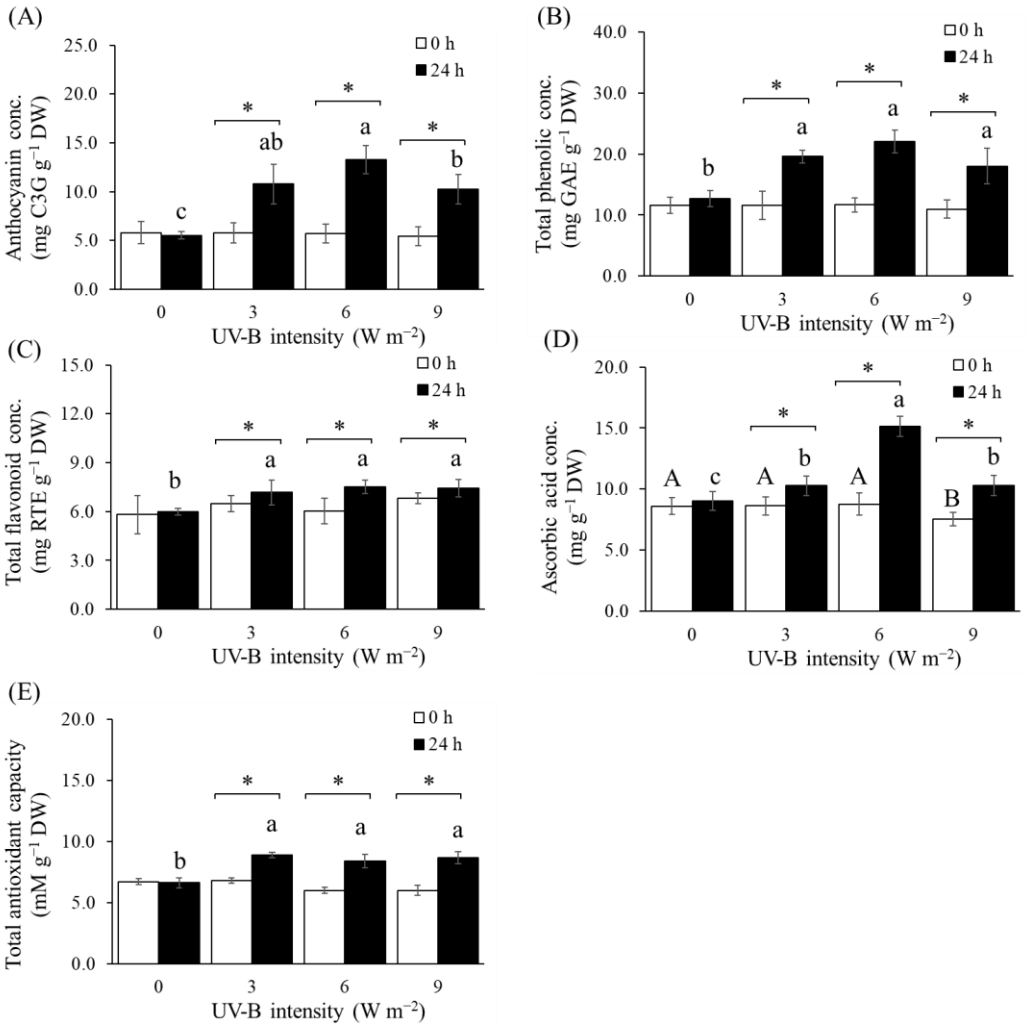


Fig. 3-5. Anthocyanin, total phenolic, total flavonoid, and ascorbic acid concentrations (conc.), and total antioxidant capacity of the leaves of amaranth seedlings at 0 and 24 h after treatment with different ultraviolet B (UV-B) irradiation intensities for 8 h. Vertical bars indicate standard error (n = 4). Means of the measured parameters at the same UV-B intensity for the two recovery periods were compared using the Student's *t*-test. * indicates significant difference. Uppercase and lowercase letters indicate significant differences at 0 and 24 h of the recovery periods, respectively, determined using Tukey's HSD test at $p < 0.05$. [1]Anthocyanin concentration is expressed as cyanindin-3-glucoside equivalents (C3G). [2]Total phenolic concentration is expressed as gallic acid equivalents (GAE). [3]Total flavonoid concentration is expressed as rutin equivalents (RTE). [4]Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

3.2 Effect of UV-B radiation intensity and irradiation period

3.2.1 Introduction and objective

UV-B treatment has been well shown to have both beneficial and detrimental effects. Many studies have investigated the effects of UV-B irradiation intensity or dosage level on growth, morphology, and bioactive compounds, including carotenoids, phenolics, glucosinolates, and chlorophylls (Goto et al., 2020; Zhao et al., 2020). UV-B-sensitive plants usually exhibit indications of cell and tissue damage on their leaves in discoloration, yellowing, and abnormal appearance, affecting product acceptably. Thus, UV-B treatment should be carefully examined.

UV-B might provide an effective short-term treatment to enhance bioactive compounds in baby-leaf thus, it should be designed based on irradiation intensity (W m^{-2}) and irradiation period (h). Goto et al. (2020) applied 3 W m^{-2} of UV-B to red perilla for three days. Son et al. (2020) applied 0.3-0.9 W m^{-2} of UV-A and B to lettuce for 23 days. Chen et al. (2018) applied 0.12 W m^{-2} of UV-B to *Prunella vulgaris* L for 15 days. From these studies, a UV-B irradiation intensity suitable for the high quality of young leaves can be considered, approximately 3 W m^{-2}.

In the previous experiment (section 3.1), UV-B intensities of 3, 6, and 9 W m^{-2} were applied to amaranth seedlings for 8 hours and increased bioactive compounds during a 24-hour recovery period without abnormality according to the quality index. It can be presumed that a difference in UV-B intensities with different irradiation periods may exhibit a district feature; thus, UV-B irradiation intensities of 3, 6, and 9 W m^{-2} with varying irradiation periods will be demonstrated in this experiment. An inappropriate UV-B dosage may cause the abnormality, but this investigation might be valuable for prospective investigation.

3.2.2 Materials and methods

3.2.2.1 Plant material and cultivation condition (please see section 2.1.2.1)

3.2.2.2 UV-B treatments

The six amaranth seedlings were subjected to short-term UV-B treatments once they had developed four to five true leaves, approximately 31 days after sowing. UV-B lamps (λ_{max} = 310 nm; TL 20W/01 RS; Philips, Hamburg, Germany) were installed in adjustable frames beneath white LED lamps to perform the treatments. UV-B intensity was measured at plant canopies using a spectroradiometer (USR-45DA; Ushio Inc., Tokyo, Japan), and was adjusted using aluminum foil partially wrapped around the UV-B lamp. The spectral radiant flux distribution is shown in Fig. 3-1. The same PPFD of 300 μmol m^{-2} s^{-1} was used to irradiate the seedlings in all the UV-B treatments.

Three levels of UV-B radiation (3, 6, and 9 W m^{-2}) were applied to the seedlings for 4, 8, 12, and 16 h. The treatment periods and sampling times are presented in Fig. 3-6. Control seedlings were irradiated under the same PPFD (300 μmol m^{-2} s^{-1}) without UV-B radiation for 16 h and sampled in the same way as the treatment seedlings.

3.2.2.3 Yield and leaf morphology (please see section 3.1.2.3)

3.2.2.4 Chlorophyll and carotenoid concentration determinations
(Please see section 2.2.2.4)
3.2.2.5 Betalain concentration determination (Please see section 2.2.2.5)
3.2.2.6 Anthocyanin concentration determination (Please see section 2.2.2.6)
3.2.2.7 Total phenolic concentration determination (Please see section 2.2.2.7)
3.2.2.8 Total flavonoid concentration determination (Please see section 2.2.2.8)
3.2.2.9 Ascorbic acid concentration determination (Please see section 2.2.2.9)
3.2.2.10 Total antioxidant capacity determination (Please see section 2.2.2.10)

3.2.2.11 Statistical analysis

The experimental data were processed using the Statistical Package for the Social Sciences (SPSS software, version 24, USA). Tukey's honestly significant difference (Tukey's HSD) test was performed to compare the means of the measured parameters among the treatments.

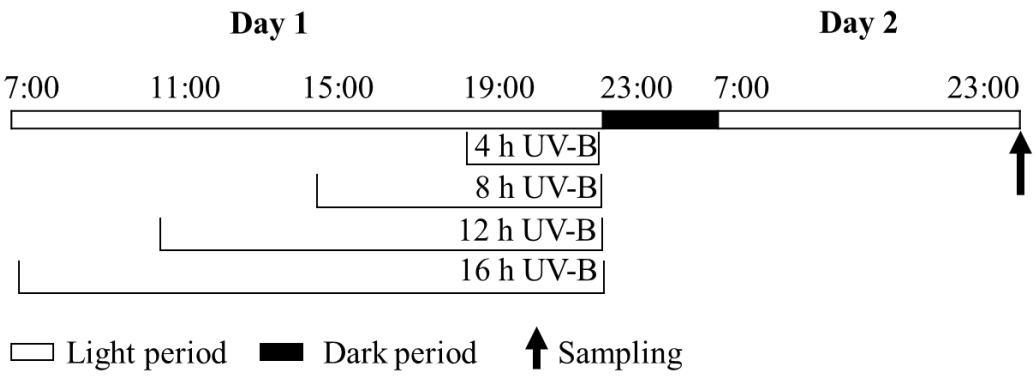


Fig. 3-6. Treatment period for ultraviolet (UV) treatments of amaranth seedlings. The UV-B treatment intensities were 0, 3, 6, and 9 W m^{-2}. Sampling was performed 24 h after the UV-B treatment. Seedlings were irradiated at the same photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}.

3.2.3 Results and discussion

3.2.3.1 Growth and morphology

The amaranth seedlings used in this study showed fresh and dry weights ranging from 0.29 to 0.33 and 0.031 to 0.040 g, respectively (Tables 3-3 and Fig. 3-7), and there were no significant differences in either the fresh or dry weights among the treatments. The dry matter ratio of the leaves ranged from 0.100 to 0.111.

Some of the leaves irradiated at 9 W m^{-2} for 8−16 h were decolored, and some of the leaves irradiated at 6 W m^{-2} for 16 h had an uneven leaf surface (Fig. 3-8). Reddish leaves without pigment deterioration were observed on seedlings irradiated at 6 W m^{-2} for 12 h. These leaves also showed signs of the development of uneven leaf surfaces.

The leaves irradiated at 3 W m^{-2} for 4−12 h showed leaf surface and color features similar to leaves of the control seedlings, except for the control seedlings irradiated for 16 h. Both leaf discoloration and leaf surface unevenness were considered to fall outside of the baby-leaf quality index (Tables 2-2 and 2-3). However, leaves with unexpected quality may contain beneficial constituents and be useful for other purposes.

Table 3-3. Amaranth leaf fresh weight (Leaf FW), dry weight (Leaf DW), and dry matter ratio (DMR) at 24 h after treatment with different short-term ultraviolet B (UV-B) irradiation intensities and periods.

Factors	Leaf FW (g)	Leaf DW (g)	DMR
UV-B intensity (W m^{-2})			
0	0.31 ± 0.01	0.032 ± 0.008	0.103 ± 0.017
3	0.30 ± 0.03	0.033 ± 0.006	0.109 ± 0.014
6	0.31 ± 0.04	0.034 ± 0.009	0.107 ± 0.031
9	0.32 ± 0.05	0.033 ± 0.006	0.104 ± 0.014
Irradiation period (h)			
4	0.32 ± 0.06	0.035 ± 0.006	0.111 ± 0.023
8	0.31 ± 0.04	0.033 ± 0.008	0.105 ± 0.026
12	0.32 ± 0.04	0.032 ± 0.004	0.100 ± 0.014
16	0.31 ± 0.05	0.033 ± 0.004	0.107 ± 0.014
A	NS	NS	NS
B	NS	NS	NS
A × B	NS	NS	NS

Data are shown as the mean ± standard error of the four biological replicates. Results of two-way analysis of variance for UV-B intensity (A), irradiation period (B), and their interaction (A × B) are shown. NS indicates no statistical significance. The means within the columns are not significantly different.

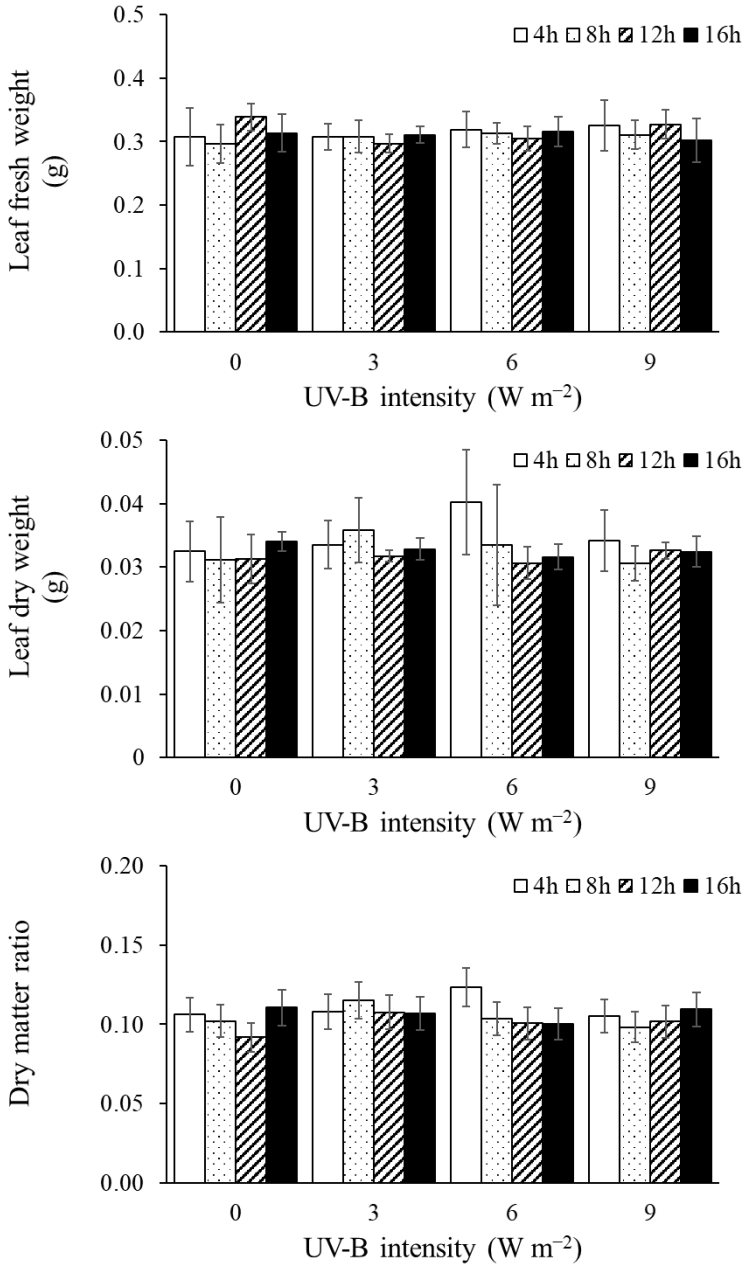


Fig. 3-7. Amaranth leaf fresh weight, dry weight, and dry matter ratio at 24 h after treatment with different short-term ultraviolet B (UV-B) irradiation intensities and periods.

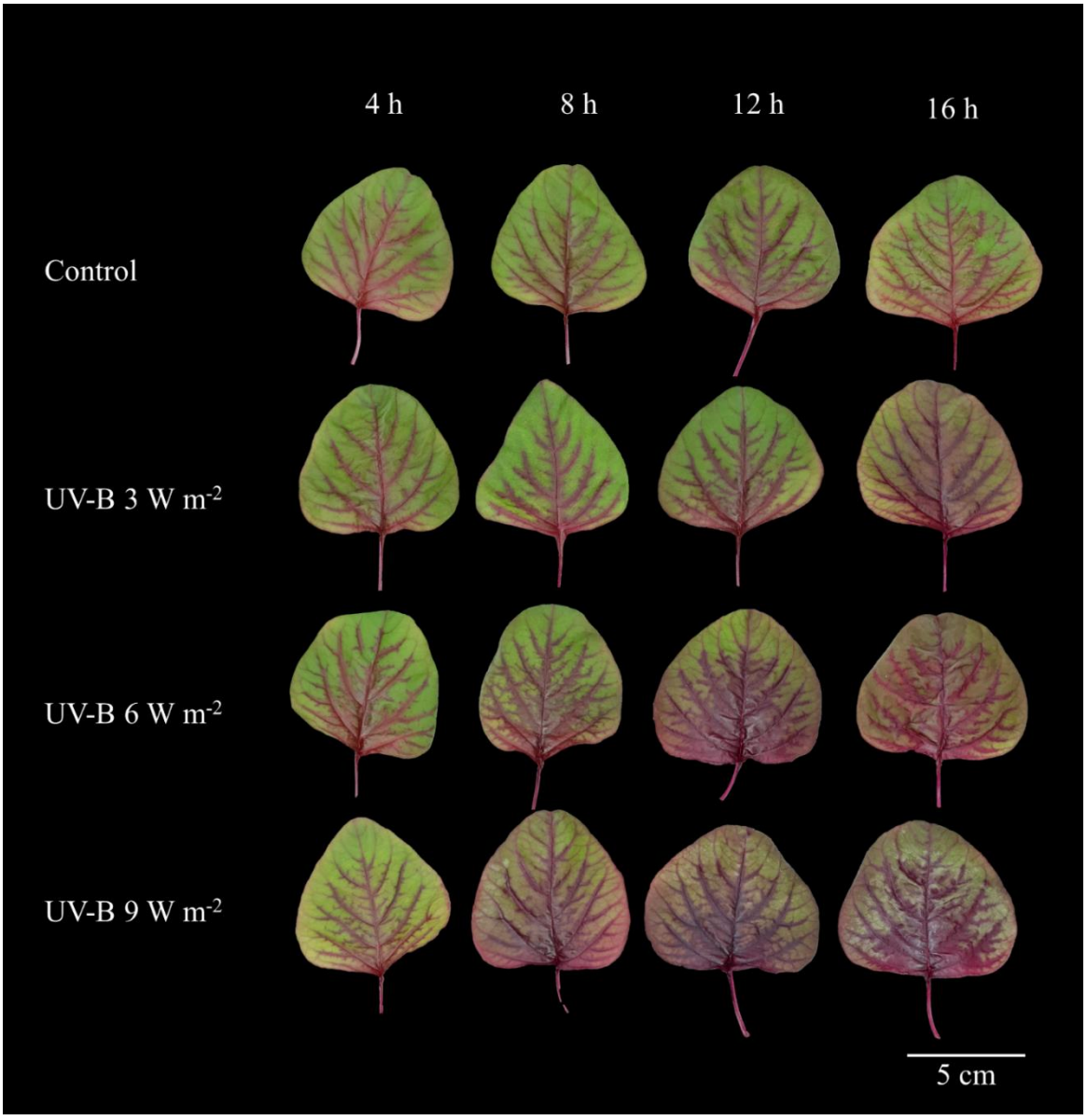


Fig. 3-8. Morphology of amaranth leaves of seedlings 24 h after short-term ultraviolet B (UV-B) treatments.

Irradiation with UV-B has been used to enhance plant bioactive compound accumulation by inducing plant defense mechanisms. Specific pathways regulate these mechanisms via UV receptor proteins, phytochromes, and cryptochromes (Nagy and Schafer, 2000), and non-specific pathway regulation occurs via the alteration of ROS in the cell matrix (Escobar-Bravo et al., 2017). However, high fluence UV-B radiation over the biological threshold induces excess ROS in the cell matrix, and these ROS disrupt the cell redox balance and induce cell death (Mackerness, 2000). Thus, the application of UV-B irradiation should be well-designed based on the irradiation intensity (W m^{-2}) and irradiation period (h). Leaf decoloring, surface changes, and necrosis indicate inappropriate UV-B irradiation conditions, including the intensity or exposure period (Hideg et al., 2013). In the present study, these symptoms were observed on the surfaces of leaves irradiated at 6 and 9 W m^{-2} for 12 and 16 h, and 8, 12, and 16 h, respectively (Fig. 3-7). Moreover, both the UV-B irradiation intensity and irradiation period played a role in changes in lightness, redness, and yellowness of amaranth leaves (Table 3-4). The findings of this study agree with other reports that UV-B irradiation can enhance leaf redness and reduce yellowness after treatment (Ota et al., 2017; Valenta et al., 2020).

Table 3-4. Amaranth leaf lightness (L*, 0 = black and 100 = white), redness (a*, a* > 0 = trend to be red and a* < 0 = trend to be green), and blueness (b*, b* > 0 = trend to be yellow and b* < 0 = trend to be blue) at 24 h after treatment with different short-term ultraviolet B (UV-B) irradiation intensities and periods.

Factors	L*	a*	b*
UV-B intensity (W m^{-2})			
0	30.24 ± 2.53[a]	3.50 ± 0.87[c]	9.48 ± 1.84[a]
3	27.46 ± 1.87[b]	4.99 ± 1.09[b]	3.99 ± 1.38[b]
6	26.37 ± 0.80[b]	6.54 ± 1.94[a]	3.50 ± 1.16[b]
9	26.54 ± 1.48[b]	6.21 ± 2.27[a]	3.16 ± 0.90[b]
Irradiation period (h)			
4	27.84 ± 1.78	2.48 ± 0.70[c]	5.27 ± 2.37
8	28.06 ± 2.22	5.37 ± 1.38[b]	4.98 ± 1.88
12	28.09 ± 3.05	6.09 ± 1.83[b]	4.91 ± 1.39
16	26.73 ± 2.09	7.31 ± 2.51[a]	4.96 ± 1.24
A	***	***	***
B	*	***	NS
A × B	**	**	NS

Data are shown as the mean ± standard error of the four biological replicates. Results of two-way analysis of variance for UV-B intensity (A), irradiation period (B), and their interaction (A × B) are shown. NS indicates no statistical significance. The asterisks indicate significance levels (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). Means within columns were compared using Tukey's HSD at a significance level of $p < 0.05$.

3.2.3.2 Photosynthetic pigments

Amaranth leaf color depends on pigments such as chlorophyll, β-carotene, carotenoids, betalain, and anthocyanin (Sarker and Oba, 2019). Both UV-B intensity and irradiation period significantly affected the total chlorophyll concentration of irradiated leaves ($p < 0.001$), whereas only UV-B intensity had a significant effect on the carotenoid, and betalain concentrations ($p < 0.001$; Table 3-5). The total chlorophyll concentration in leaves irradiated with UV-B at intensities of 3 and 6 W m^{-2} was significantly higher (1.2−1.5 times) compared to that in the leaves of the control seedlings (Table 3-6). However, the total chlorophyll concentration showed a reduction from 8.54 to 5.49 mg g^{-1} DW in the leaves irradiated at 9 W m^{-2} for 4 to 16 h.

In the present study, UV-B radiation had both enhancing and deleterious effects on photosynthetic pigments, especially chlorophyll (Table 3-6). The total chlorophyll concentration increased under UV-B irradiation at low intensities (3 and 6 W m^{-2}) during prolonged irradiation periods (4 to 16 h), whereas it decreased under high intensity (9 W m^{-2}) irradiance applied for 4 to 16 h. These results support those of Kong and Zheng (2020), who reported that UV-B irradiation at the appropriate level increases chlorophyll levels in leafy vegetables. However, deleterious effects of UV-B radiation on chlorophyll, β-carotene, and carotenoids have been observed under inappropriate treatment conditions (Hollósy, 2002). Findings of the present study showed no significant difference in carotenoid concentration among the UV-B treatment groups (Table 3-5). Carotenoid was significantly increased 24 h after irradiation with UV-B at 3, 6, and 9 W m^{-2} for a period of at least 4 h. In addition, there were no significant differences in carotenoid concentration among the UV-B treatments of different intensities.

Carotenoid increases may be linked to the vital role played by this pigment in light-harvesting systems, and the protection of photosynthetic pigments from high light and radiation, including UV light (Sankari et al., 2017). Similarly, Shen et al. (2017) have reported that the carotenoid concentration in tobacco leaves increased substantially 24 h after UV-B irradiation.

Table 3-5. Total chlorophyll (Chl conc.), carotenoid (Car conc.), and betalain (Bet conc.) concentrations in leaves of amaranth seedlings at 24 h after treatment with different short-term ultra-violet B (UV-B) irradiation intensities and periods.

Factors	Chl conc. (mg g^{-1} DW)	Car conc. (mg g^{-1} DW)	Bet conc. (mg g^{-1} DW)
UV-B intensity (W m^{-2})			
0	5.51 ± 0.83^{c}	1.75 ± 0.41^{b}	2.40 ± 0.34^{b}
3	8.28 ± 1.27^{a}	2.65 ± 0.37^{a}	3.07 ± 0.36^{a}
6	8.18 ± 0.89^{a}	2.83 ± 0.40^{a}	2.98 ± 0.29^{a}
9	6.89 ± 1.49^{b}	2.48 ± 0.46^{a}	2.80 ± 0.37^{a}
Irradiation period (h)			
4	7.29 ± 1.51	2.42 ± 0.64	2.78 ± 0.44
8	7.29 ± 1.49	2.39 ± 0.51	2.77 ± 0.38
12	7.26 ± 1.79	2.55 ± 0.59	2.79 ± 0.52
16	7.03 ± 1.73	2.34 ± 0.60	2.91 ± 0.35
A	***	***	***
B	NS	NS	NS
A × B	***	NS	NS

Data are shown as the mean ± standard error of the four biological replicates. Results of two-way analysis of variance for UV-B intensity (A), irradiation period (B), and their interaction (A × B) are shown. The asterisks indicate significance at $p < 0.001$, ***. NS indicates no statistical significance. Means within columns were compared using Tukey's HSD at a significance level of $p < 0.05$. DW: dry weight.

Table 3-6. Total chlorophyll concentration (Chl conc.) in leaves of amaranth seedlings at 24 h after treatment under different short-term ultraviolet B (UV-B) irradiation intensities and periods.

UV-B intensity ($W\ m^{-2}$)	Irradiation period (h)	Chl conc. ($mg\ g^{-1}$ DW)
Control		5.92 ± 0.98^{c}
3	4	6.52 ± 0.49^{b}
	8	8.48 ± 0.55^{a}
	12	9.16 ± 0.43^{a}
	16	8.95 ± 1.22^{a}
6	4	8.51 ± 0.77^{a}
	8	8.23 ± 1.05^{ab}
	12	8.23 ± 0.91^{ab}
	16	7.76 ± 1.05^{b}
9	4	8.54 ± 0.90^{a}
	8	6.94 ± 1.35^{b}
	12	6.63 ± 1.16^{b}
	16	5.49 ± 0.92^{c}

Data are shown as the mean ± standard error of the four biological replicates.

The means within the column were compared using Tukey's HSD at a significance level of $p < 0.01$.

DW: dry weight.

3.2.3.3 Bioactive compounds

The betalain concentration in leaves irradiated with UV-B was 1.2−1.5 times higher compared to that in leaves of the control. In the present study, the concentration of betalain, an indole derivative pigment, increased in leaves irradiated with UV-B (Table 3-5). However, betalain does not provide the UV-protective ability of amaranth (Adhikary et al., 2020).

It is likely that UV-B irradiation induced plant amino acid biosynthesis or bioconversion, especially of tyrosine, due to the upregulation of phenolic compounds (Frohnmeyer and Staiger, 2003). Tyrosine is utilized as a shared intermediate between phenolic compounds and betalain biosynthesis (Polturak et al., 2017). Therefore, in the present study, excess substrate may have increased betalain biosynthesis, accounting for the increase in betalain concentration in the amaranth leaves.

The total phenolic, flavonoid, anthocyanin, and ascorbic acid concentrations and the antioxidant capacity are shown in Fig. 3-9. Significant effects of both UV-B irradiation intensity and period on the bioactive compounds in the amaranth leaves were observed at $p < 0.05$−0.001. The anthocyanin concentration in leaves irradiated with UV-B at 3 and 6 W m^{-2} was 2.0−3.0 times higher when the irradiation period was increased compared to that in the control leaves.; however, the anthocyanin concentration in the control leaves was not affected by either UV-B irradiation intensity or period. A reduction in anthocyanin concentration from 9.93 to 5.07 mg C3G g^{-1} DW was observed in leaves irradiated with UV-B at 9 W m^{-2}.

Anthocyanin is a stress-responsive molecule synthesized in plants under biotic and abiotic stress conditions, including UV irradiation (Ebisawa et al., 2008). In the present study, the highest anthocyanin concentration was found in leaves irradiated with UV-B at 6 W m^{-2} followed by leaves irradiated at 3 W m^{-2} for irradiation periods of 4 to 16 h; whereas leaves irradiated with UV-B at 9 W m^{-2} showed lower anthocyanin concentrations (Fig. 3-9). The observed reduction in anthocyanin concentration at a higher UV-B intensity may be due to anthocyanin degradation as observed in perilla leaves under an unappropriated UV-B treatment (Ota et al., 2017).

Total phenolic and flavonoid concentrations were significantly higher in leaves irradiated with UV-B for longer irradiation periods. However, a reduction in the concentration of these compounds was found at 9 W m^{-2} with increasing irradiation period. In the present study, the total phenolic and flavonoid concentrations in amaranth leaves showed trends similar to that of anthocyanin (Fig. 3-9).

Although both phenolics and flavonoids are stable UV-protective compounds, they have limited stability under lighting or in oxidative environments (Ali et al., 2018; Diaconeasa, 2018). The findings of the present study indicated that the biosynthesis of phenolic compounds in baby-leaf amaranth depends on the intensity and period of UV-B irradiation.

The highest ascorbic acid concentrations were recorded in leaves irradiated at intensities of 3, 6, and 9 W m^{-2} for the 16, 8, and 4 h irradiation periods, respectively. The total antioxidant capacity of leaves irradiated with UV-B at 3 and 6 W m^{-2} increased from 7.01 to 10.87, and 7.82 to 10.47 mM g^{-1} DW, respectively, when the irradiation period was extended from 4 to 16 h. Irradiation with UV-B at 9 W m^{-2} reduced the total antioxidant capacity of leaves when the irradiation period was extended from 8 to 16 h. However, the total antioxidant capacity of UV-B-irradiated leaves at 3 and 6 W m^{-2} was higher compared to that of the leaves of the control.

Ascorbic acid, an essential vitamin, plays a vital role as a cellular antioxidant (Majer et al., 2014). Therefore, the increase in ascorbic acid observed in the present study showed a similar trend to that of antioxidant capacity in UV-B-irradiated leaves. Chen et al. (2019) reported that UV-A irradiation increased both anthocyanin and ascorbic acid concentrations in lettuce by altering ROS and receptors, and Ortega-Hernández et al. (2019) have found that ascorbic acid biosynthesis is highly related to L-galactono-γ-lactone dehydrogenase activity under UV-B treatment. The biosynthesis of ascorbic acid under UV-B treatment is probably regulated by specific receptors, such as UV RESISTANCE LOCUS 8 (UVR8) photoreceptor, and non-specific pathways related to ROS production. Findings of the present study showed that ascorbic acid in baby-leaf amaranth under low intensity UV-B irradiation (3 W m^{-2}) increased with the irradiation period (Fig. 3-9).

In contrast, ascorbic acid concentration under high-intensity UV-B treatments (9 W m^{-2}) increased initially and then decreased with prolonged treatment. During UV-B irradiation, ROS are produced in plant cells. Ascorbic acid is immediately liberated by ascorbate peroxidase to neutralize ROS to maintain a redox balance (Mittler, 2002).

The redox balance is generally maintained by many reducing agents, such as phenolic compounds, flavonoids, and ascorbic acid (Foyer and Noctor, 2005). When the phenolic compounds, flavonoids, and anthocyanins are reduced at high intensity UV-B, the redox balance is disturbed. It may be possible that ascorbic acid is required to maintain the redox balance, subsidizing the reduction in phenolics and flavonoids. Ascorbic acid may be required to maintain redox balance, thereby subsidizing the reduction of phenolics and flavonoids (Gill and Tuteja, 2010) or regenerating these compounds by ascorbic acid–flavonoid regenerative mechanism (de Souza and Giovani, 2004). The cell's antioxidant capacity is maintained if these bioactive compounds are maintained at an appropriate level. In the present study, it was evident that the antioxidant capacity increased substantially at 3 and 6 W m^{-2} with increasing irradiation period, whereas it was reduced at 9 W m^{-2} (Fig. 3-8). These results indicated that excessive UV-B irradiation had adverse effects on the quality of the baby-leaf amaranth, particularly at 9 W m^{-2} for a period of more than 4 h of irradiation.

Similar results were obtained for the leaves irradiated at 3 and 6 W m^{-2} for 16 and 8 h, respectively. This finding suggests the possibility that the same UV-B cumulative energy may result in the same response to UV-B irradiation.

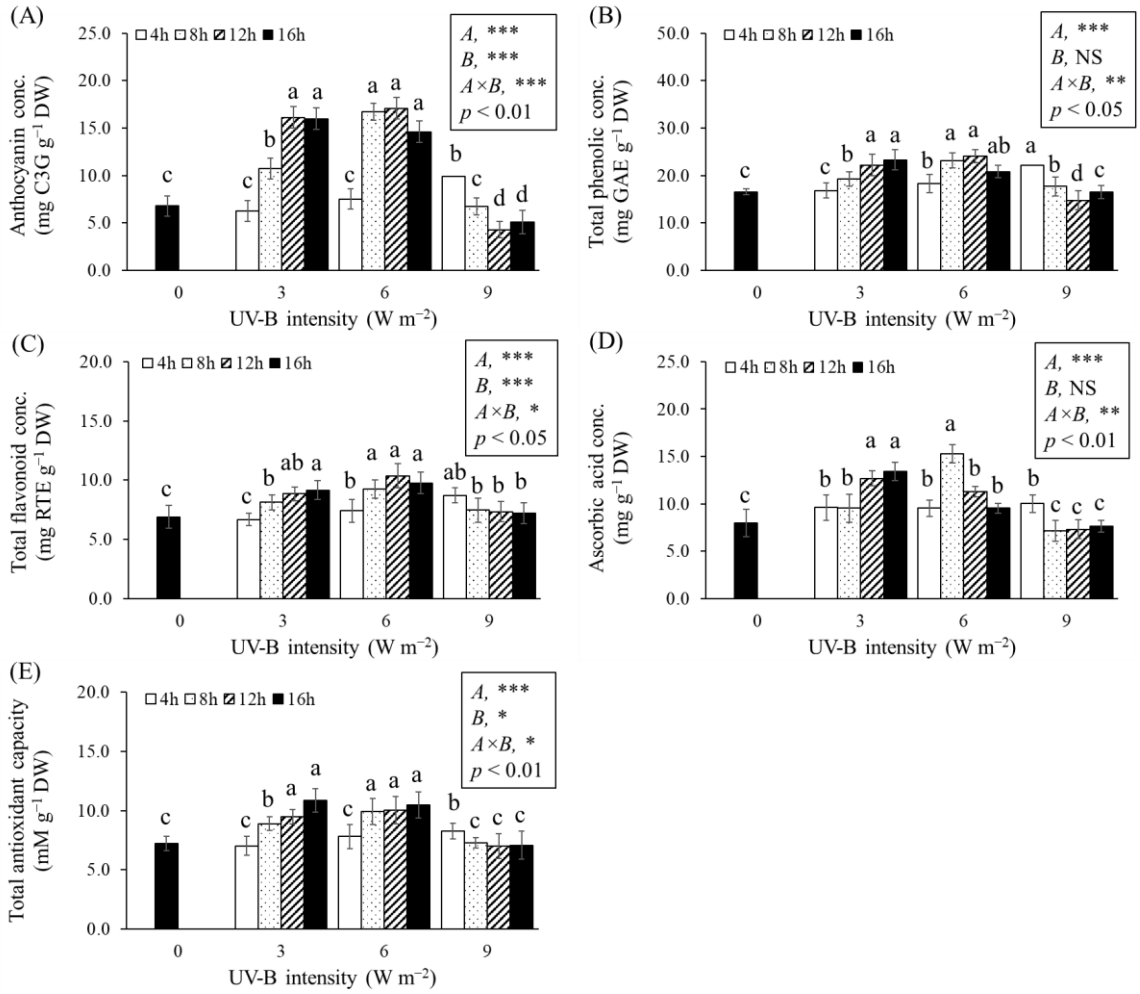


Fig. 3-9. Anthocyanin, total phenolic, total flavonoid, and ascorbic acid concentrations (conc.) and total antioxidant capacity of the leaves of amaranth seedlings at 24 h after treatment with different ultraviolet B (UV-B) irradiation intensities and recovery periods. Vertical bars indicate standard error (n = 4). Results of two-way analysis of variance of UV-B intensity (A), irradiation period (B), and their interaction (A × B) are shown. Asterisks indicate significance levels (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). NS indicates no statistical significance. Means were compared using Tukey's HSD test at $p < 0.05$ and $p < 0.01$. [1] Anthocyanin concentration is expressed as cyanindin-3-glucoside equivalents (C3G). [2] Total phenolic concentration is expressed as gallic acid equivalents (GAE). [3] Total flavonoid concentration is expressed as rutin equivalents (RTE). [4] Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

3.3 Effect of cumulative UV-B irradiation energy

3.3.1 Introduction and objective

UV-B irradiation, as previously mentioned, has long been recognized to improve crop quality. The intensity and period of UV-B irradiation should be considered while employing it, as UV-B radiation has both beneficial and detrimental effects. However, bioactive compounds are also influenced by the cumulative UV-B irradiation energy (Son et al., 2020). Some combinations (intensity period) show the same pattern as the previous section (3.2). Interestingly, seedlings received the same cumulative UV-B irradiation energy in this combination. According to Yoon et al. (2020), various cumulative UV-B irradiation energy has distinct effects on kale's bioactive compound accumulation. It is feasible that the same amount of cumulative UV-B irradiation energy will yield the same characteristic. This study should be able to figure out the best way to use UV-B based on cumulative energy design.

3.3.2 Materials and methods

3.3.2.1 Plant material and cultivation condition (please see section 2.1.2.1)

3.3.2.2 UV-B treatments

The six amaranth seedlings were subjected to short-term UV-B treatments once they had developed four to five true leaves, approximately 31 days after sowing. UV-B lamps (λ_{max} = 310 nm; TL 20W/01 RS; Philips, Hamburg, Germany) were installed in adjustable frames beneath white LED lamps to perform the treatments. UV-B intensity was measured at plant canopies using a spectroradiometer (USR-45DA; Ushio Inc., Tokyo, Japan), and was adjusted using aluminum foil partially wrapped around the UV-B lamp. The spectral radiant flux distribution is shown in Fig. 3-1. The same PPFD of 300 μmol m^{-2} s^{-1} was used to irradiate the seedlings in all the UV-B treatments.

Three levels of cumulative UV-B irradiation energies (130, 150, and 170 kJ m^{-2}) for each UV-B intensity (3, 6, and 9 W m^{-2}) were applied to the seedlings at the designed irradiation period as described in Table 3-7. This experiment aimed to investigate UV-B combinations, thus a control of 0 W m^{-2} was not included in the experiment.

The treatment combinations and sampling times are shown in Table 3-7 and Fig. 3-10, respectively.

Table 3-7. Ultraviolet B (UV-B) lighting conditions for the treatment of amaranth seedlings. The seedlings were irradiated under the same photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}.

Treatment	UV-B intensity ($W\ m^{-2}$)	Irradiation period		UV-B energy ($kJ\ m^{-2}$)
		hour	min	
130 kJ_3W	3	12	0	130
130 kJ_6W	6	6	0	130
130 kJ_9W	9	4	0	130
150 kJ_3W	3	13	55	150
150 kJ_6W	6	6	55	150
150 kJ_9W	9	4	40	150
170 kJ_3W	3	15	45	170
170 kJ_6W	6	7	55	170
170 kJ_9W	9	5	15	170

3.3.2.3 Yield and leaf morphology (please see section 3.1.2.3)

3.3.2.4 Chlorophyll and carotenoid concentration determinations
(Please see section 2.2.2.4)

3.3.2.5 Betalain concentration determination (Please see section 2.2.2.5)

3.3.2.6 Anthocyanin concentration determination (Please see section 2.2.2.6)

3.3.2.7 Total phenolic concentration determination (Please see section 2.2.2.7)

3.3.2.8 Total flavonoid concentration determination (Please see section 2.2.2.8)

3.3.2.9 Ascorbic acid concentration determination (Please see section 2.2.2.9)

3.3.2.10 Total antioxidant capacity determination (Please see section 2.2.2.10)

3.3.2.11 Statistical analysis

The experimental data were processed using the Statistical Package for the Social Sciences (SPSS software, version 24, USA). Tukey's honestly significant difference (Tukey's HSD) test was performed to compare the means of the measured parameters among the treatments.

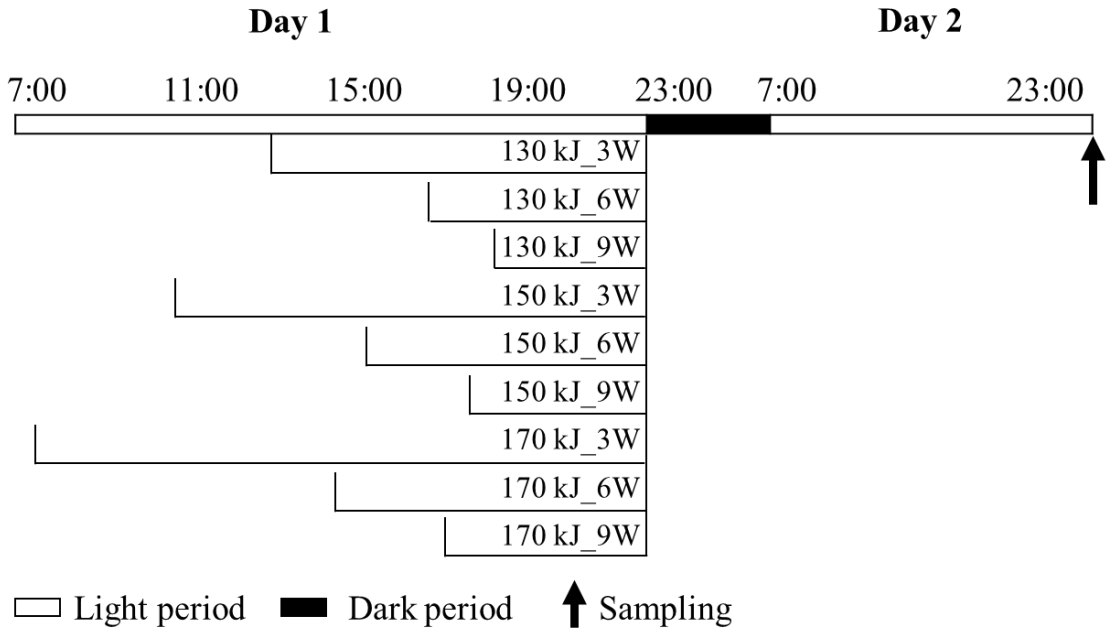


Fig. 3-10. Treatment period for different cumulative energy ultraviolet B (UV-B) treatments of amaranth seedlings.

Seedlings were irradiated at the same photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}.

3.3.3 Results and discussion

The appearance of the amaranth leaves is shown in Fig. 3-11, and the concentrations of the bioactive compounds and antioxidant capacity of the leaves are presented in Table 3-8.

3.3.3.1 Bioactive compounds

Because this experiment aimed to determine the effects of UV-B combinations, a control of 0 W m^{-2} was not investigated. The highest anthocyanin concentration was found in the leaves irradiated at 150 kJ m^{-2}, whereas there were no significant effects at 130 and 170 kJ m^{-2}.

Leaves irradiated with UV-B at 3 and 6 W m^{-2} did not show significant differences in anthocyanin concentration, which ranged from 15.74 to 16.82 mg C3G g^{-1} DW. However, the lowest anthocyanin concentration was found in the UV-B irradiated leaves at 9 W m^{-2}.

The total phenolic and flavonoid concentrations were not significantly different among the UV-B energies; however, the concentrations of both compounds in leaves irradiated at 3 and 6 W m^{-2} were significantly higher compared to the concentrations in leaves irradiated at 9 W m^{-2}. The ascorbic acid concentration in the leaves irradiated at 3 and 6 W m^{-2} was not significantly different, whereas it was lower in leaves irradiated at 9 W m^{-2}. A combination of the effect of cumulative UV-B irradiation energy and intensity was observed for the antioxidant capacity ($p < 0.01$). The highest antioxidant capacity was found in leaves irradiated at 6 W m^{-2}, at the cumulative energy of 150 kJ m^{-2} followed by leaves irradiated at 3 W m^{-2} at the same cumulative energies of 150 and 170 kJ m^{-2} cumulative energy of 150 kJ m^{-2} (Tables 3-8 and 3-9). However, there was no significant difference in the antioxidant capacity of irradiated leaves among the UV-B intensities at a cumulative energy of 130 kJ m^{-2}. The overall results indicated that UV-B irradiation intensity was the key factor for enhancing bioactive compounds in baby-leaf amaranth.

In the present study, the same cumulative UV-B irradiation energy promoted bioactive compound accumulation differently under different UV-B intensities. Amaranth leaves irradiated with UV-B at 3 and 6 W m^{-2} showed no significant differences in bioactive compound accumulation (Table 3-8), whereas leaves irradiated at 9 W m^{-2} showed a lower bioactive compound concentration at the same cumulative UV-B irradiation energy. The exact amount of cumulative UV-B irradiation energy at different UV-B intensities may be affected differently by the oxidative and antioxidative balance at a particular time (Considine and Foyer, 2014). During a stress response period, many bioactive compounds are utilized to act against ROS.

Thus, if higher amounts of ROS are generated created simultaneously, these bioactive compounds must be utilized faster. In the present study, the cumulative UV-B irradiation energy of 150 kJ m^{-2} at 6 W m^{-2} produced the highest concentrations of bioactive compounds in irradiated leaves, followed by the concentrations of compounds produced at an intensity of 3 W m^{-2} and at the same irradiation energies of 150 and 170 kJ m^{-2}.

In addition, the findings indicated that the irradiation period required at the cumulative UV-B irradiation energy of 150 kJ m^{-2} and intensities of 3 and 6 W m^{-2} was approximately 14 and 7 h, respectively, and 170 kJ m^{-2} of 3 W m^{-2} was 15 h. Practically, this extended treatment period may not be recommended from the perspective of the operating costs of a plant production facility.

In this study, the biomass of baby-leaf amaranth was not affected by short-term UV-B irradiation (Tables 3-1 and 3-3), but their leaf morphology and colorimetric parameters were altered (Tables 3-2 and 3-4; Figs. 3-3, 3-7, and 3-10). Adequate UV-B irradiation can improve leaf coloration and maintain chlorophyll content. Moreover, short-term UV-B irradiation did not affect β-carotene, carotenoid, or betalain concentrations in baby-leaf amaranth (Table 3-5 and Fig. 3-4). Nonetheless, the recovery period was essential for increasing the concentrations of phenolic compounds, flavonoids, anthocyanins, and ascorbic acid in amaranth leaves (Fig. 3-5). The results of the present study indicated that short-term UV-B irradiation did not affect the biomass of the baby-leaf amaranth; however, the leaf morphology and colorimetric parameters could be changed by UV-B irradiation, suggesting that irradiating amaranth seedlings with UV-B has both beneficial and adverse effects. An appropriate UV-B irradiation intensity can enhance leaf redness and maintain chlorophyll concentration in leaves during a 24 h recovery period. β-carotene, carotenoid, and betalain of baby-leaf amaranth were not affected by short-term UV-B irradiation. In addition, the recovery period was essential for improving phenolic compounds, flavonoids, anthocyanins, and ascorbic acid in amaranth leaves.

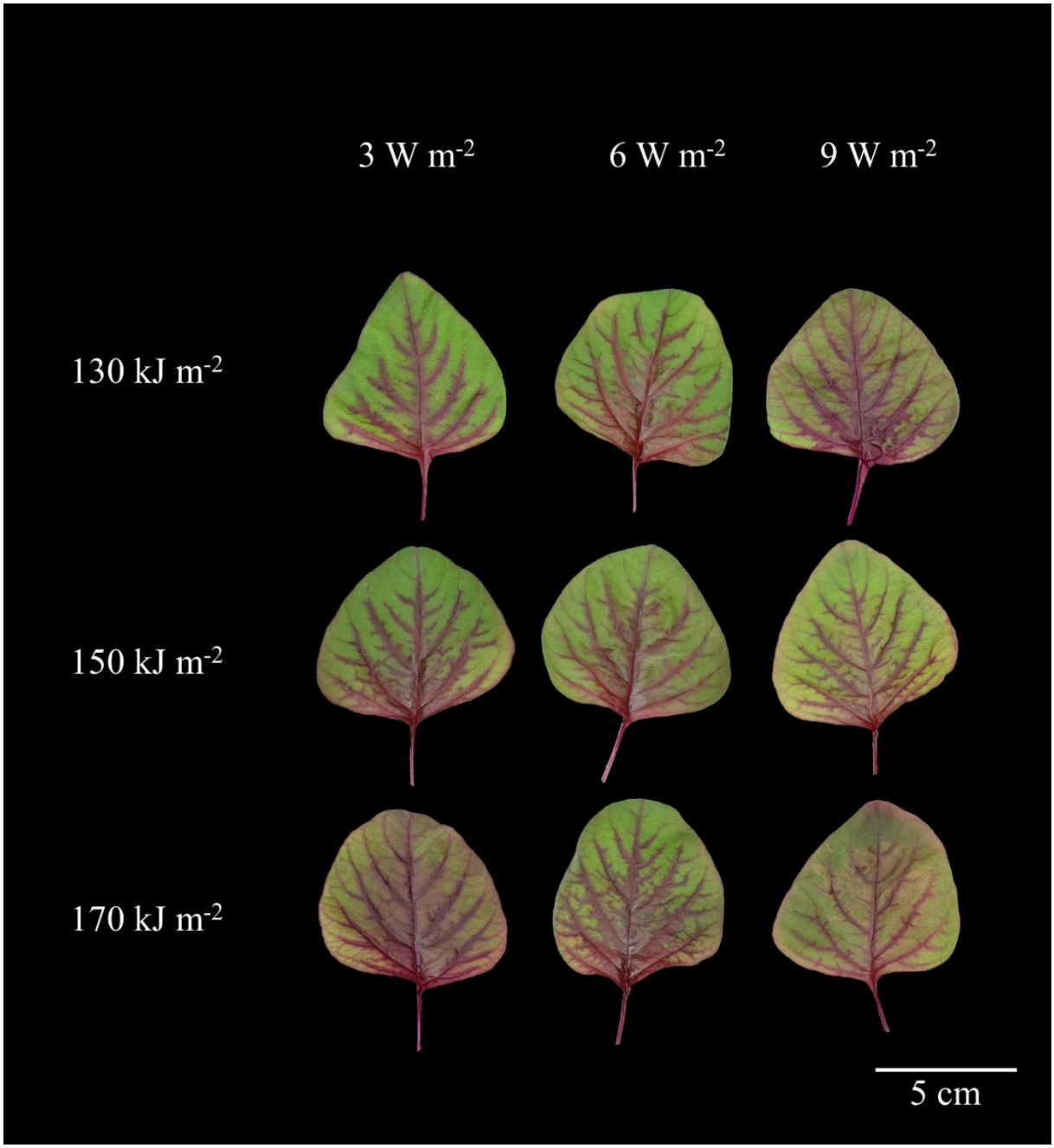


Fig. 3-11. Morphology of amaranth leaves 24 h after short-term UV-B treatments under different cumulative ultraviolet (UV-B) irradiation combinations of UV-B irradiation intensity and irradiation period.

Table 3-8. Anthocyanin (Ant conc.), total phenolic (Phl conc.), total flavonoid (Flv conc.), and ascorbic acid (Asc conc.) concentrations and total antioxidant capacity (TAC) of leaves of amaranth seedlings 24 h after treatment under different short-term ultraviolet B (UV-B) irradiation energies (UV-B energy) and intensities.

Factors	Ant conc.[1] (mg g^{-1} DW)	Phl conc.[2] (mg g^{-1} DW)	Flv conc.[3] (mg g^{-1} DW)	Asc conc. (mg g^{-1} DW)	TAC[4] (mM g^{-1} DW)
UV-B energy (kJ m^{-2})					
130	11.42 ± 4.47[b]	21.77 ± 1.96	7.70 ± 1.48	12.29 ± 2.48	8.07 ± 1.09[b]
150	17.71 ± 4.22[a]	22.06 ± 4.45	8.68 ± 2.62	13.44 ± 2.24	10.39 ± 1.64[a]
170	13.55 ± 4.42[b]	21.49 ± 3.27	7.91 ± 1.55	12.34 ± 3.54	8.51 ± 2.22[b]
UV-B intensity (W m^{-2})					
3	16.82 ± 3.52[a]	22.65 ± 3.08[a]	8.92 ± 1.91[a]	13.38 ± 2.07[a]	10.13 ± 1.56[a]
6	15.74 ± 4.33[a]	23.17 ± 3.10[a]	8.32 ± 2.22[a]	14.63 ± 1.83[a]	8.69 ± 2.48[b]
9	10.12 ± 4.50[b]	19.51 ± 2.63[b]	7.04 ± 1.22[b]	10.05 ± 2.20[b]	8.14 ± 1.11[b]
A	***	NS	NS	NS	***
B	***	**	*	***	***
A × B	NS	NS	NS	NS	**

Data are shown as the mean ± standard error of the four biological replicates. Results of two-way analysis of variance for UV-B intensity (A), irradiation period (B), and their interaction (A × B) are shown. The asterisks indicate significance levels (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). NS indicates no statistical significance. Means within columns were compared using Tukey's HSD at a significance level of $p < 0.05$. [1] Anthocyanin concentration is expressed as cyanindin-3-glucoside equivalents. [2] Total phenolic concentration is expressed as gallic acid equivalents. [3] Total flavonoids concentration is expressed as rutin equivalents. [4] Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

Table 3-9. Total antioxidant capacity (TAC) of leaves of amaranth seedlings 24 h after treatment under different short-term ultraviolet B (UV-B) irradiation energies (UV-B energy) and intensities.

UV-B energy ($kJ\ m^{-2}$)	Irradiation intensity ($W\ m^{-2}$)	TAC ($mM\ g^{-1}$ DW)
130	3	8.46 ± 0.61^{b}
	6	7.47 ± 1.06^{bc}
	9	8.27 ± 0.64^{b}
150	3	11.05 ± 1.42^{a}
	6	11.29 ± 1.58^{a}
	9	8.81 ± 0.59^{b}
170	3	10.87 ± 0.98^{a}
	6	7.33 ± 0.94^{c}
	9	7.34 ± 1.05^{c}

Data are shown as the mean ± standard error of the four biological replicates.

The means within the column were compared using Tukey's HSD at a significance level of $p < 0.01$.

Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

3.4 Conclusions

Our results suggest that UV-B irradiation at an intensity of 6 W m^{-2} and a cumulative energy of 150 kJ m^{-2} with a 24 h recovery period can enhance the bioactive compounds of baby-leaf amaranth without causing delayed growth and abnormal leaf appearance. To the best of our knowledge, this is the first study to report findings on the different responses of baby-leaf amaranth to UV-B irradiation, especially in relation to the cumulative UV-B irradiation energy and intensity.

CHAPTER 4

Short-term root-zone temperature regimes for increasing bioactive compounds of baby-leaf amaranth

4.1 Effect of cooling root-zone temperature

4.1.1 Introduction and objective

Plants have been treated with biotic and abiotic stresses to improve their bioactive compounds. Root-zone temperature (RZT) is one of the abiotic stresses potentially enhancing plant bioactive compounds (Calleja-Cabrera et al., 2020). The impact of RZT on growth and bioactive compounds was investigated in relation to its nutrition and water uptake functions in plants (Qiuyan et al., 2012). Low or high RZT induces water stress in plants via altering the fluidity of the root membrane and the activity of aquaporin, a protein involved in water absorption (Carvajal et al., 1996; Maurel et al., 2015). Calcium channel and lipid signaling are triggered as a consequence of these alterations, hence affecting intracellular calcium ions (Mittler et al., 2012). The calcium ion stimulates the activity of various temperature-sensitive elements, including calcium-dependent protein kinases and heat shock proteins. Additionally, reactive oxygen species (ROSs), unstable oxidative products are accumulated when certain thermal responsive mechanisms are altered (Raja et al., 2017). Numerous bioactive compounds that serve as ROS neutralizers, especially flavonoids and ascorbic acid, are synthesized and utilized to avoid ROS damage (Escobar-Bravo et al., 2017; Rácz and Hideg, 2021). From root to short, signal molecules are delivered to trigger a bioactive compound synthesis pathway. However, when the ROS generated by a low or high RZT exceeds a threshold level, cell death occurs (Mackerness, 2000). Furthermore, a signal molecule is delivered to the shoot, requesting shoot-to-root nutrition transport, resulting in nutrient and biomass losses from the shoot (Heckathorn et al., 2013). Therefore, the application of RZT treatment not only affects root physiology and activity but also affects shoot growth.

The RZT treatment is well-suited for hydroponic systems, ensuring a uniform nutrient solution temperature. Ogawa et al. (2018) demonstrated that a six-day RZT treatment at 10ºC enhanced the rosmarinic acid and luteolin concentrations of red perilla while decreasing the shoot fresh weight. For four weeks, cucumber plants were exposed to low RZT (10ºC), resulting in a decrease in leaves fresh weight and mineral content (Chung et al., 2002). In addition, the same treatment increased leaves soluble sugar concentration. Similarly, a short-term RZT treatment (7 days) at 10–15ºC enhanced total phenolic, anthocyanin, sugar, and antioxidant enzyme concentrations in red leaf lettuce while decreasing fresh weight (Sakamoto and Suzuki, 2015). The high-temperature RZT treatment (30ºC) increased the concentration of bioactive compounds but decreased their antioxidant activity and appearance. Nguyen et al. (2021) applied RZT treatments to coriander for 3 and 6 days at 15, 25, 30, and 35ºC. For six days, coriander treated under 15 or 35ºC yielded considerable concentrations of bioactive compounds, including ascorbic acid, chlorogenic acid, and carotenoids, while the highest biomass was found in those treated at 30ºC. Three days of treatment with coriander at 15ºC resulted in decreased total phenolic content, but the fresh weight is not significantly different from the control. Additionally, this study indicated that a particular RZT level and period might increase bioactive compounds differently.

Although high RZT treatment may enhance bioactive compounds in plants, it may have an adverse effect on the appearance and shelf life of the product due to an increase in respiration rate (Chun et al., 1994; Falah et al., 2010; Masarirambi et al., 2018). Consequently, low RZT levels increase bioactive compounds in plants but decrease the final product's water and minerals contents (Calatayud et al., 2008; Nxawe et al., 2009). These reviews concluded that RZT treatment at low and high levels had an effect on the bioactive compound, yield, and appearance of a plant. The manufacturing aim may determine whether a low or high level of RZT is employed. As a result, rational RZT management may increase the bioactive compounds in plants without adversely affecting growth and appearance.

The present research aimed to establish the short-term cooling RZT that could be designed to improve bioactive compound accumulation in baby-leaf amaranth without causing an abnormal appearance. The level of RZT and the period of treatment, and the combination of RZT at a different introducing period were investigated in particular.

4.1.2 Materials and methods

4.1.2.1 Plant material and cultivation condition (please see section 2.1.2.1)

4.1.2.2 Root-zone temperature treatment

The amaranth seedlings with four true leaves, approximately 27 days after sowing, were treated for RZT treatments. Six seedlings were transplanted in a hydroponic container (33 cm × 18 cm × 15 cm) for the treatment. The RZT was controlled using a handy cooler (TRL-107NHF, Thomas Kagaku Co., Ltd., Tokyo, Japan) with a temperature control device. Aeration was performed using air pumps and air stones to supply sufficient air to roots and to circulate the nutrient solution in each container. Seedlings were subjected to four different RZT treatments for 1, 3, 5, and 7 days at 5, 10, 15, and 20ºC. Four seedlings were harvested after 1, 3, 5, and 7 days of treatment.

The schematic diagram of RZT treatment shelf for amaranth seedling is illustrated in Fig. 4-1. The environmental conditions for RZT treatment were controlled as described in Table 4-1, except for the photosynthetic photon flux density (PPFD), which was set to 300 μmol m^{-2} s^{-1}. The seedlings in all treatments were exposed to the same aerial conditions, including air temperature (25/20ºC in light/dark periods). Control seedlings were cultivated under the same conditions as described above, and RZT was not controlled.

Temperatures of the air in the canopy and cultivation shelf were determined using a humidity and temperature recorder (TR-72wf, T&D Co., Ltd., Matsumoto, Japan) (Table 4-2). An infrared thermometer (830-T2, Testo, Inc.) was used to determine the surface temperature of the leaf and expanded polystyrene cultivation foam, whose emissivity was adjusted to 0.98 and 0.95, respectively (Chen, 2015; Krause and Nowo'swiat, 2020).

The temperature of the nutrient solution was determined at different areas using thermocouples from the temperature control device on a handy cooler.

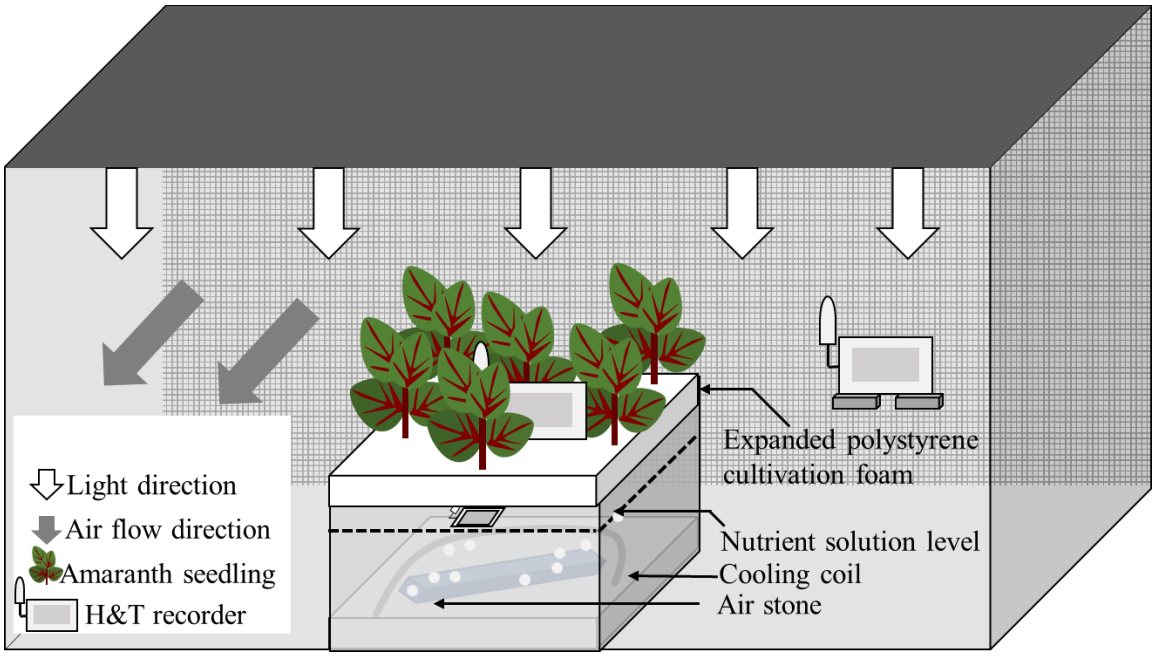


Fig. 4-1. Schematic diagram of RZT treatment shelf embedded in a close plant production system. H & T recorder: humidity and temperature recorder.

Table 4-1. *Amaranthus tricolor* L. seedling cultivation conditions.

Environmental factor	Setting value
PPFD (μmol m^{-2} s^{-1})	200
Light period (h)	16
Air temperature (ºC)	25/20
Relative humidity (%)	70
CO_2 concentration (μmol mol^{-1})	1,000

PPFD: photosynthetic photon flux density.

Table 4-2. The air temperature (AT), canopy temperature (CT), leaf surface temperature (LST), expanded polystyrene cultivation foam surface temperature (EPST), and nutrient solution temperature (NST) of RZT treatments.

Treatment	Period	Temperature (°C)				
		AT[1]	CT	LST	EPST	NST
Control	Light	24.82 ± 0.33	25.64 ± 0.39	25.44 ± 0.57	25.10 ± 0.46[a]	24.69 ± 0.62[a]
5/5°C		24.95 ± 0.34	25.59 ± 0.29	25.06 ± 0.54	22.65 ± 0.67[b]	5.01 ± 0.51[e]
10/10°C		24.95 ± 0.34	25.70 ± 0.39	25.25 ± 0.58	23.90 ± 0.57[ab]	10.03 ± 0.57[d]
15/15°C		24.89 ± 0.38	25.97 ± 0.52	25.13 ± 0.76	24.20 ± 0.71[a]	15.78 ± 1.09[c]
20/20°C		24.89 ± 0.38	25.85 ± 0.35	25.00 ± 0.71	24.80 ± 0.59[a]	20.00 ± 0.61[b]
Significant		NS	NS	NS	**	**
Control	Dark	19.34 ± 0.47	19.28 ± 0.43[a]	19.25 ± 0.77	19.85 ± 0.47[a]	19.83 ± 0.53[a]
5/5 °C		19.33 ± 0.48	18.90 ± 0.46[b]	19.18 ± 0.66	17.75 ± 0.49[b]	4.83 ± 0.26[d]
10/10 °C		19.33 ± 0.48	19.08 ± 0.44[a]	19.31 ± 0.48	19.65 ± 0.34[a]	9.62 ± 0.45[c]
15/15 °C		19.35 ± 0.49	19.18 ± 0.50[a]	19.53 ± 0.46	19.55 ± 0.64[a]	15.63 ± 1.03[b]
20/20 °C		19.35 ± 0.49	19.32 ± 0.50[a]	19.31 ± 0.63	19.80 ± 0.48[a]	19.84 ± 0.46[a]
Significant		NS	*	NS	**	**

Data are shown as the mean ± standard deviation. The AT and CT were recorded every minute for one day, from 8:00 to 21:59 for the light period (n = 3) and 00:00 to 6:59 for the dark period (n = 3). The LST of an individual leaf (n = 16) and the EPST (n = 10) were determined at 15:00 and 00:00, for light and dark periods, respectively. NST was measured during the light period (n = 3) at 15:00, 19:00, and 22:00 and during the dark period (n = 3) at 23:00, 00:00, and 1:00. NS indicates that there is no statistical significance.
[1] The RZT treatment of 5/5 and 10/10, and 15/15 and 20/20°C were coupling conducted at the same shelf therefore AT of those were measured at the same time. Means within columns of each period were compared using Tukey's HSD. The asterisks indicate significance levels (*, $p < 0.05$; **, $p < 0.01$). NS indicates no statistical significance.

4.1.2.3 Yield, leaf water content, and leaf morphology

Four or five younger amaranth leaves of length 4.0–5.0 cm were harvested from four uniform seedings, weighed, and subsequently freeze-dried (FDU-1110; Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at -80ºC for 24 hours before being stored at -30ºC for further analysis. Leaves fresh (FW) and dry (DW) weights were determined using a digital balance. The following equation was used to calculate the leaf water content (LWC) (Garnier and Laurent, 1994).

$$LWC\ (\%) = [(Leaf\ FW - Leaf\ DW)/Leaf\ FW] \times 100$$

The morphology of the leaf was recorded visually using a mobile camera. The leaves of each seedling were cold grounded before biochemical analysis.

4.1.2.4 Chlorophyll and carotenoid concentration determinations

(Please see section 2.2.2.4)

4.1.2.5 Betalain concentration determination (Please see section 2.2.2.5)

4.1.2.6 Anthocyanin concentration determination (Please see section 2.2.2.6)

4.1.2.7 Total phenolic concentration determination (Please see section 2.2.2.7)

4.1.2.8 Total flavonoid concentration determination (Please see section 2.2.2.8)

4.1.2.9 Ascorbic acid concentration determination (Please see section 2.2.2.9)

4.1.2.10 Total antioxidant capacity determination (Please see section 2.2.2.10)

4.1.2.11 Total sugar concentration determination

The sugar was extracted from leaf samples using a modified Hanson et al. (2006) extraction procedure. To summarize, powdered leaf samples (10 mg) were combined with 1 mL acetone and then homogenized for 30 minutes at 10ºC using ultrasound.

After 3 hours of incubation at 4ºC, the samples were centrifuged at 15,300 g for 5 minutes at 10ºC, and the supernatant was then discarded. The precipitate was mixed with 1 mL acetone, and the procedure described above was repeated about 2−3 times until the solution was clear. The decolored precipitate was combined with 0.5 mL of 80 % ethanol (v/v) and heated to 80ºC for 15 minutes in a hot air oven before cooling to room temperature.

The samples were centrifuged at 15,300 g for 5 minutes at 25ºC and the supernatant was transferred to a fresh tube. The sample tube was heated in a hot air oven at 80ºC until wholly evaporated and then re-dissolved in 1 mL distilled water. Total sugar concentration was determined as described by Dinssa et al. (2018) using the anthrone reagent. The anthrone reagent was prepared by dissolving 0.2 mg anhydrous anthrone in 98% sulfuric acid (v/v) in a 100 mL volumetric flask, resulting in a clear yellowish solution. The sample was diluted appropriately with distilled water (3−5 times dilution) and combined at a ratio of 1:9 with an anthrone reagent. The combination was carefully heated in a hot air oven at 90ºC for 10 minutes and then rapidly cooled in ice-cold water, resulting in a greenish-blue solution. The absorbance at 630 nm was determined using the V-750 spectrophotometer. A standard curve was created using an appropriate dilution of sucrose. The concentration of total sugar was expressed as mg g^{-1} DW.

4.1.2.12 Statistical analysis

The experimental data were processed using the Statistical Package for the Social Sciences (SPSS software, version 24, USA). Tukey's honestly significant difference (Tukey's HSD) test was performed to compare the means of the measured parameters among the treatments.

4.1.3 Results and discussion

4.1.3.1 Growth and morphology

Amaranth seedlings developed differently under various RTZs. The seedling under the control and RZT treatments at 20ºC had 7-8 leaves, in total at day 7, with 3-4 of those leaves were emerging new leaves (data not shown). The seedlings treated with RZT at 5, 10, and 15ºC showed minor leaf length and width changes and one emerging new leaf, resulting in five leaves by day seven. Younger leaves of length 4.0–5.0 cm were harvested and studied the leaf morphology under RZT treatments and the control as illustrated in Fig. 4-2. The visible redness of the leaves increased with the period of the RZT treatment. However, on the first day of treatment, seedlings treated with RZT at 5, 10, and 15ºC showed leaf wilting.

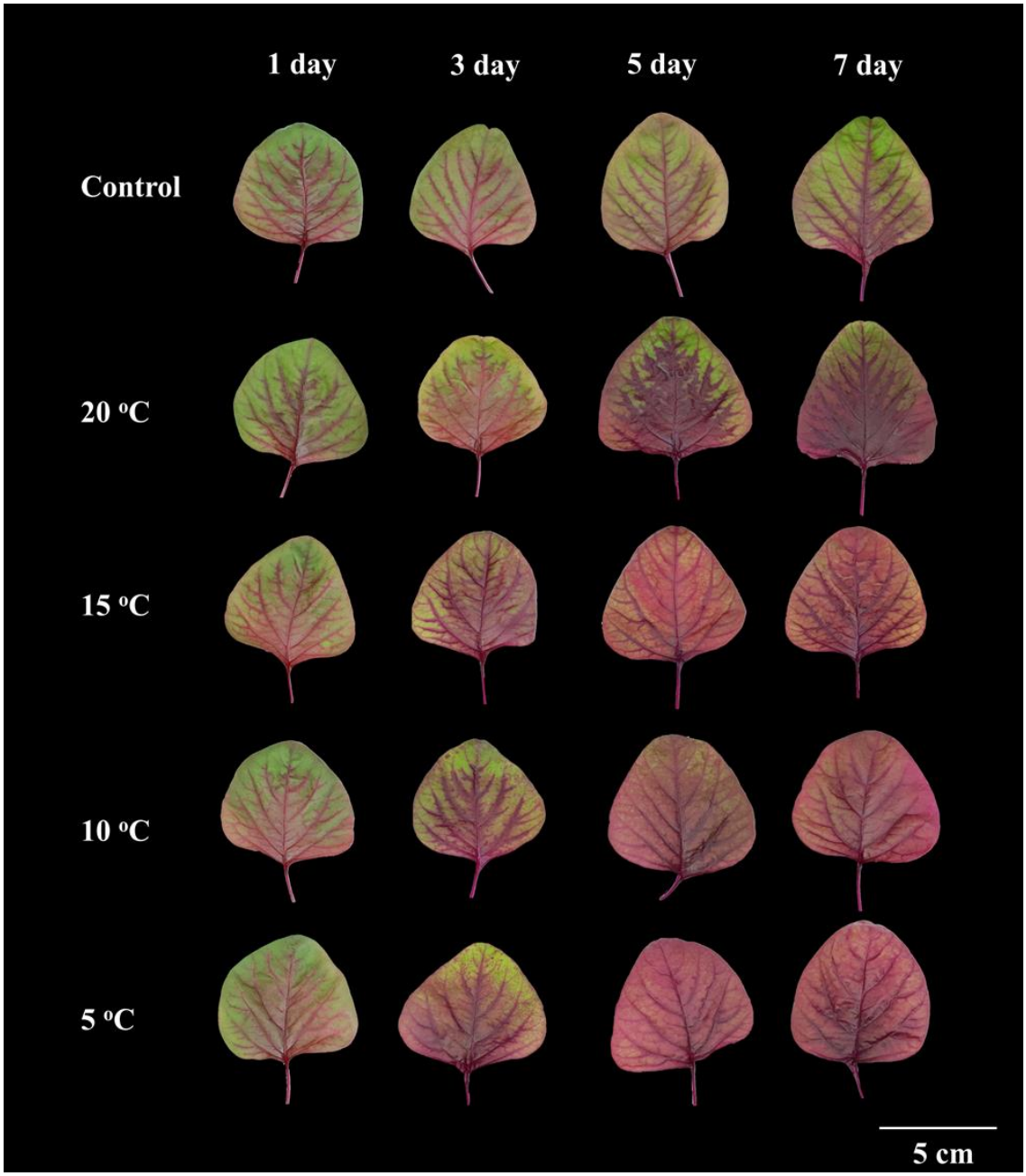


Fig. 4-2. Morphology of amaranth leaves of seedlings treated with different root-zone temperatures (RZT) and periods.

The RZT at day/night-time: 5/5, 10/10, 15/15, and 20/20ºC are indicated as: 5, 10, 15, and 20ºC.

The Amaranth leaf's fresh and dry weights ranged between 1.05 and 1.39 g and 0.12 and 0.21 g, respectively (Table 4-3). The RZT treatments of 5 and 10ºC significantly reduced leaf fresh weight compared to 15 and 20ºC. Three to seven days of treatment resulted in considerable increases in leaf fresh and dry weights, but one day of treatment resulted in a decrease in fresh weight. Interestingly, when the treatment period was extended from 3 to 7 days, the RZT treatments of 15 and 20ºC significantly increased leaf dry weight compared to the control (Table 4-3 and Fig.4-3).

Plant biomass is a growth indicator that reflects photosynthetic status, and stress; a decrease in plant biomass implies inadvertent cultivation or excessive stress. This study showed that the seedlings grown at RZTs of 5 and 10ºC had a substantial decrease in fresh weight, while seedlings treated at 20ºC had the highest fresh weight (Table 4-3). One-day treatment resulted in a significant reduction in fresh weight. Sakamoto and Suzuki (2015) found that treating red wave lettuce at RZT of 10ºC for seven days noticeably lowered the fresh weight of the lettuce. According to Nguyen et al. (2021), the fresh weight of coriander exposed to 15ºC RZT treatment decreased after six days but did not significantly change after three days. The RZT level and period demonstrated an influence of plant fresh weight on imbalance of root water uptake. Li et al. (2019) demonstrated that rice plants grown at RZT of 17.5ºC for two days increased malonaldehyde concentrations, reflecting an oxidative status in the root and shoot, and resulting in a decrease in shoot fresh and dry weights. It may be possible that the low RZT disturbed redox balance, generating an over flush ROS that necessitated neutralizing antioxidant enzymes or bioactive compounds (Escobar-Bravo et al., 2017). These actions need the energy to perform; hence, the rate of respiration is increased. Sugar as a carbon source is also used in respiration process and synthesis of bioactive compounds, which results in dry weight reduction.

Table 4-3. Amaranth leaves fresh weight (Leaf FW), dry weight (Leaf DW), and leaf water content (LWC) after treatment with different short-term root-zone temperatures (RZT) and periods.

Parameters	Leaf FW (g)	Leaf DW (g)	LWC (%)
RZT (ºC)			
5/5	1.05 ± 0.13^{c}	0.15 ± 0.04^{b}	85.68 ± 2.95^{b}
10/10	1.29 ± 0.10^{b}	0.16 ± 0.03^{b}	86.11 ± 3.16^{b}
15/15	1.36 ± 0.17^{ab}	0.19 ± 0.04^{a}	85.13 ± 2.68^{b}
20/20	1.39 ± 0.15^{a}	0.20 ± 0.05^{a}	88.81 ± 1.56^{a}
25/20	1.48 ± 0.13^{a}	0.16 ± 0.03^{b}	89.41 ± 0.99^{a}
Period (day)			
1	1.11 ± 0.14^{b}	0.12 ± 0.02^{c}	89.01 ± 1.27^{a}
3	1.28 ± 0.16^{a}	0.16 ± 0.03^{b}	87.15 ± 2.16^{b}
5	1.36 ± 0.21^{a}	0.19 ± 0.05^{a}	85.75 ± 3.02^{b}
7	1.33 ± 0.17^{a}	0.21 ± 0.03^{a}	83.83 ± 2.46^{c}
A	***	***	***
B	***	***	***
A × B	NS	**	**

Data are shown as the mean ± standard error of the four biological replicates. Results of two-way analysis of variance for RZT (A), period (B), and their interaction (A × B) are shown. NS indicates no statistical significance. The asterisks indicate significance levels (**, $p < 0.01$ and ***, $p < 0.001$). Means within columns were compared using Tukey's HSD at a significance level of $p < 0.05$.

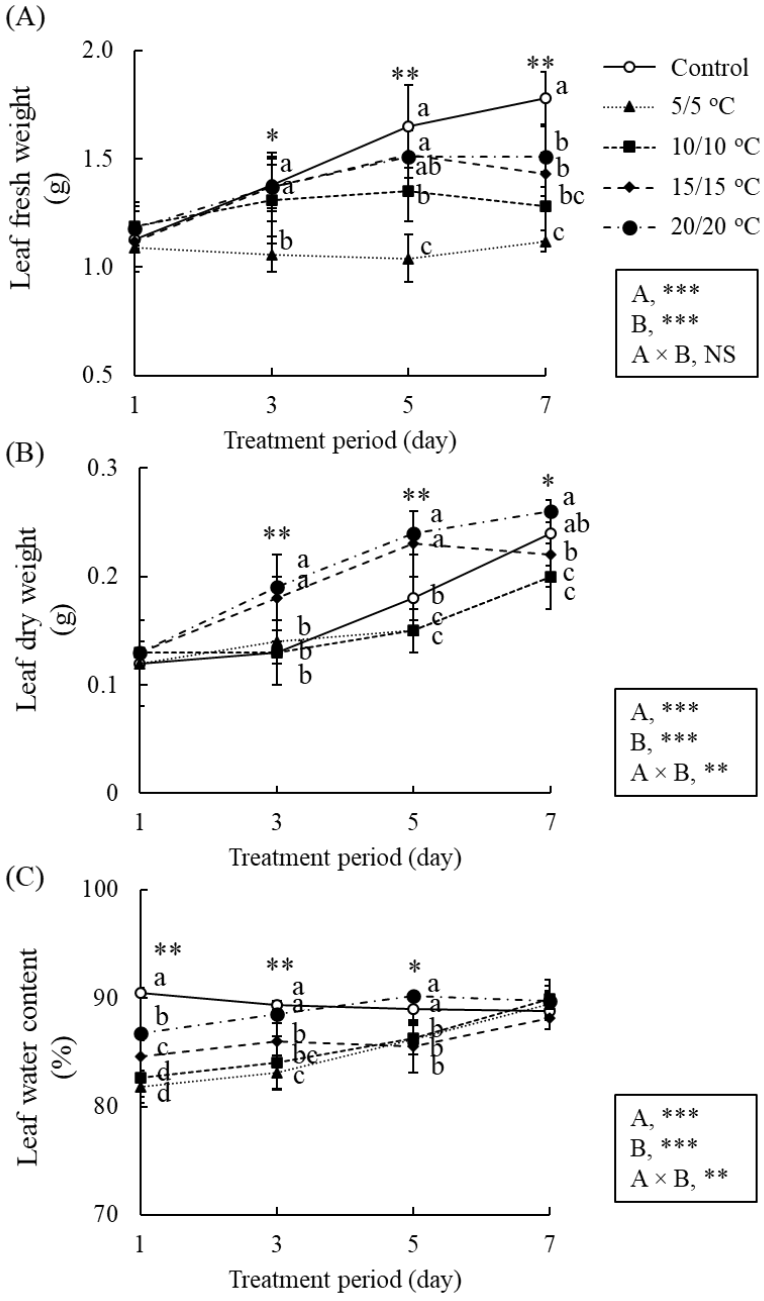


Fig. 4-3. Amaranth leaves fresh weight (A), dry weight (B) and leaf water content (C) after treatment with different short-term root-zone temperatures and periods. Vertical bar indicates standard error (n=4). Means were compared using Tukey's HSD at a significance level at $p < 0.05$, * and $p < 0.01$, **.

Due to the impediment to root water uptake, water is lost throughout the respiration process without being replenished by the root, resulting in a loss of shoot fresh weight (Calleja-Cabrera et al., 2020). However, in this study, the extended RZT treatment period increased leaves dry weight gradually; only the first day of treatment exhibited a decrease. It was considered that the RZT treatments used in this study were suboptimal ones and did not exceed amaranth's biological threshold, allowing the recovery process to proceed.

In this study, we examined leaf water content (LWC), a parameter for leaf water stress status in response to drought, temperature, and nutrient restriction (Heinen et al., 2009). The leaf water content of control seedlings was consistently between 88 and 90%, but seedlings under RZT treatments at 5, 10, and 15ºC resulted in a reduction in leaf water content since the first day of treatment as compared to that of the control. However, as the treatment period was prolonged, the leaf water content of seedlings treated with RZT at 5, 10, and 15ºC gradually increased. On the first day of treatment, RZT-treated leaves at 5, 10, and 15ºC showed a significant decrease in LWC (Fig. 4-3). The decrease in LWC implies an insufficient water supply, hence restricting photosynthesis since LWC ranges between 86 and 65 % (Zhou et al., 2021). Furthermore, a decrease in LWC affects the leaf's sensorial properties, particularly the appearance and texture regarding wilting (Garg et al., 2020). As a consequence of those mentioned above, a one-day RZT treatment may not be appropriate to be used prior to harvest. However, the extended RZT period resulted in a significant increase in LWC, while seedlings grown in control and at RZT of 20ºC exhibited minor changes in LWC (Table 4-3). Wang et al. (2021) found that plants grown in low water availability areas had lower LWC at first, but then increased to a sustainable level as the period was extended. Various physiological responses are triggered, in general, in order to maintain water levels in plants during water stress, including alteration of stomata conductance (Tari, 2003; Yu et al., 2015) proline biosynthesis (Sperdouli and Moustakas, 2014; Zegaoui et al., 2017), and the accumulation of photosynthesis-related pigments (Sanchez, et al., 1983; Mibei et al., 2016; Shah et al., 2017). As a result, reviews above are consistent with those of the following photosynthesis-related pigment’s results (Fig. 4-4 and Table 4-4).

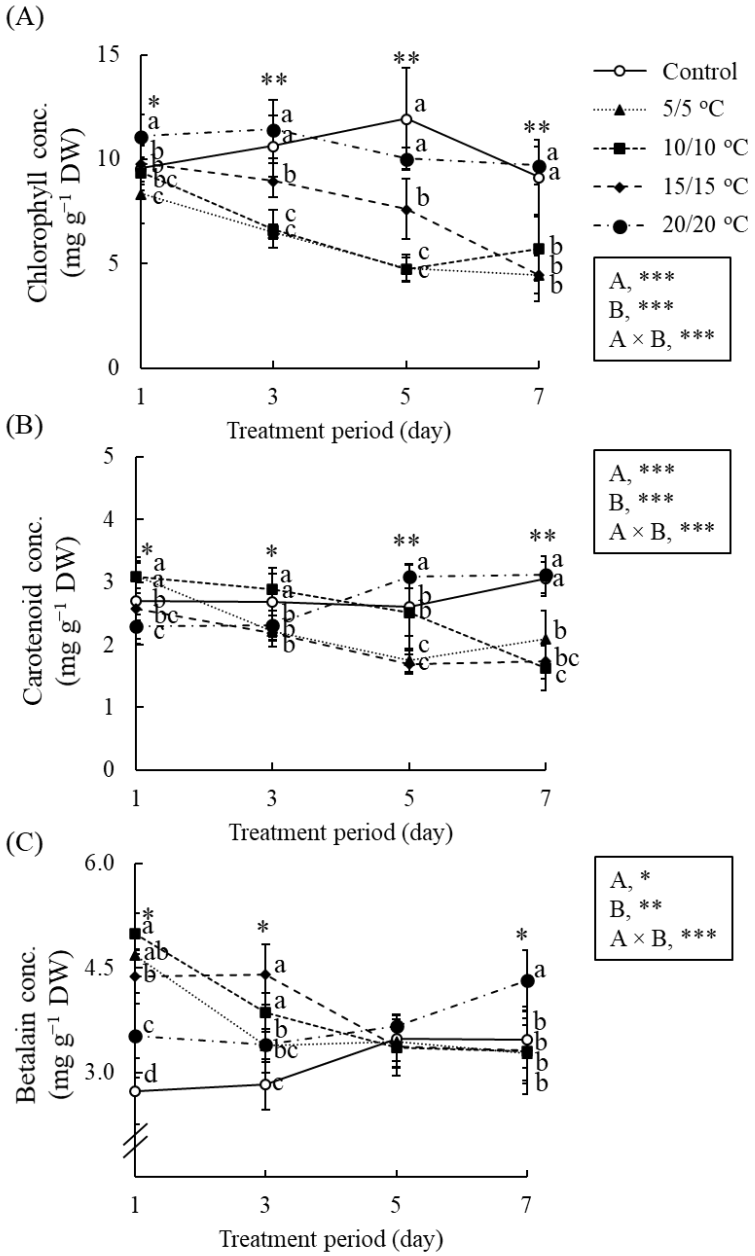


Fig. 4-4. Total chlorophyll (A), carotenoid (B), and betalain (C) concentrations (conc.) in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) and periods.

Results of two-way analysis of variance for RZT (A), period (B), and their interaction (A × B) are shown. The asterisks indicate significance levels (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). Vertical bar indicates standard error (n=4). Means were compared using Tukey's HSD at a significance level at $p < 0.05$, * and $p < 0.01$, **. Betalain concentration is expressed as β-cyanin and β-xanthin equivalents. DW: dry weight.

Table 4-4. Total chlorophyll (Chl conc.), carotenoid (Car conc.), and betalain (Bet conc.) concentrations in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) and periods.

Period (day)	RZT (ºC)	Chl conc. (mg g^{-1} DW)	Car conc. (mg g^{-1} DW)	Bet conc. (mg g^{-1} DW)
1	5/5	8.37 ± 1.44^{c}	3.10 ± 0.20^{a}	4.68 ± 0.07^{ab}
	10/10	9.38 ± 0.60^{bc}	3.08 ± 0.26^{a}	4.99 ± 0.29^{a}
	15/15	9.82 ± 0.89^{b}	2.57 ± 0.18^{bc}	4.38 ± 0.39^{b}
	20/20	11.12 ± 1.03^{a}	2.29 ± 0.19^{c}	3.53 ± 0.61^{c}
	Control	9.60 ± 1.07^{b}	2.70 ± 0.69^{b}	2.73 ± 0.48^{d}
	Significant	*	*	*
3	5/5	6.52 ± 0.35^{c}	2.22 ± 0.25^{b}	3.39 ± 0.24^{b}
	10/10	6.66 ± 0.91^{c}	2.89 ± 0.24^{a}	3.86 ± 0.28^{a}
	15/15	8.98 ± 0.81^{b}	2.18 ± 0.10^{b}	4.41 ± 0.43^{a}
	20/20	11.44 ± 1.39^{a}	2.31 ± 0.24^{b}	3.40 ± 0.41^{bc}
	Control	10.63 ± 1.46^{a}	2.68 ± 0.55^{a}	2.83 ± 0.36^{c}
	Significant	**	*	*
5	5/5	11.94 ± 0.64^{a}	1.75 ± 0.19^{c}	3.44 ± 0.38
	10/10	4.78 ± 0.56^{c}	2.52 ± 0.38^{b}	3.36 ± 0.41
	15/15	4.73 ± 1.44^{c}	1.69 ± 0.16^{c}	3.38 ± 0.30
	20/20	7.63 ± 0.50^{b}	3.09 ± 0.19^{a}	3.67 ± 0.16
	Control	10.05 ± 2.45^{a}	2.60 ± 0.69^{b}	3.49 ± 0.33
	Significant	**	**	NS
7	5/5	4.47 ± 0.89^{b}	2.09 ± 0.45^{b}	3.28 ± 0.40^{b}
	10/10	4.48 ± 1.54^{b}	1.63 ± 0.36^{c}	3.31 ± 0.47^{b}
	15/15	5.72 ± 1.28^{b}	1.73 ± 0.28^{bc}	3.32 ± 0.63^{b}
	20/20	9.15 ± 0.91^{a}	3.12 ± 0.30^{a}	4.32 ± 0.43^{a}
	Control	9.70 ± 1.79^{a}	3.05 ± 0.27^{a}	3.47 ± 0.41^{b}
	Significant	**	**	*

Data are shown as the mean ± standard error of the four biological replicates.

The means within the column were compared using Tukey's HSD at a significance level of $p < 0.05$, * and $p < 0.01$, **. Betalain concentration is expressed as β-cyanin and β-xanthin equivalents. DW: dry weight.

4.1.3.2 Photosynthetic pigments

Fig. 4-4 and Table 4-4 indicate that the RZT treatments and periods significantly affected amaranth leaf pigments. When the treatment period was extended from 1 to 7 days, the chlorophyll concentration in the leaves grown at RZTs of 5, 10, and 15ºC was 1.3–2.3 times lower than that in control and RZT of 20ºC. At one day of treatment, the seedlings grown at 5 and 10ºC yielded significantly more carotenoid than in control; however, the carotenoid concentration decreased with a longer treatment period. From one to seven days of treatment, the carotenoid concentration in the leaves grown at 20ºC gradually increased. Betalain concentrations in the seedlings grown at RZTs of 5, 10, and 15ºC increased dramatically 1.2–1.8 times on one day of treatment and gradually reduced when treatment period was extended to 7 days.

Chlorophyll and carotenoid influence amaranth development, stress response, photoprotection, food quality, and functional properties (Sarker and Oba, 2020). Chlorophyll is the primary photosynthetic pigment in plants, controlling their growth and development. Thus, a decreasing chlorophyll concentration in seedlings grown at RZTs of at 5, 10, and 15ºC may imply a deficient light-harvesting ability for photosynthesis (Fig. 4-4 and Table 4-4). Low RZT causes lipid oxidation in the membrane, resulting in ROS production, most notably in the plastid. This ROS subsequently oxidized the pigment, decreasing chlorophyll content (León-Chan et al., 2017). This decrease may have an effect on plant biomass, as described before, by lowering seedling fresh and dry weights under low RZT treatments. The findings corroborate those of Adebooye et al. (2010), who showed that a low RZT triggered photooxidation, decreasing in chlorophyll concentration in cucumber leaves. Furthermore, RZT treatment periods extended to 5–7 days at temperatures of 5, 10, and 15ºC resulted in a significant decrease in chlorophyll concentration. However, the seedlings in RZT treatment at 20ºC exhibited no significant difference from that in control. The changes in chlorophyll concentration, in general, depends on temperature, period, and plant spices. Thus, certain temperatures may not adversely affect plant photosynthetic status, as shown in Gazula et al. (2005) and Nguyen et al. (2021) studies.

Carotenoids are pigments accumulated in chloroplasts and crucial for photoprotection, capturing light, and stabilizing photosynthetic activities (Havaux, 1998). Carotenoids have a high antioxidant capability, scavenging singlet oxygen and peroxyl radicals (Stahl and Sies, 2003). Additionally, they neutralize electrically excited sensitizer molecules, preventing the generation of radicals and singlet oxygen (Young and Lowe, 2001). Carotenoids, in general, are synthesized in the same way as chlorophyll, indicating that they are a component of the photosynthetic machinery (Sagawa et al., 2016). In this experiment, carotenoid concentrations increased significantly in seedling leaves when exposed to RZTs of 5 and 10 ^{o}C at the first day but thereafter decreased by 30–40% when treatment period was extended. Under cold temperatures, the elongated hypocotyl 5, a protein that responds to cold and light stress, stabilized, but phytochrome interacting factor reduced antagonistically (Toledo-Ortiz et al., 2014). Both proteins interacted with the phytoene synthase enzyme regarding carotenoids biosynthesis under cold stress. However, carotenoids stability is dependent on the plant's age, leaf position, and phytohormones level. The low RZT suppresses phytohormones such as gibberellins, auxin, and cytokinin in root apical meristems, interfering with root-to-shoot hormonal transportation (Zhu et al., 2015; Müller and Munné-Bosch, 2021). This phenomenon affects plant photosynthesis through interactions between phytohormones and photosynthesis, which may be impaired under low RZT circumstances. When phytohormone levels are low, degradation of photosynthetic pigments such as carotenoids may occur (Cheminant et al., 2011). Consequently, as demonstrated above, the decline in carotenoid concentration after prolonged RZT treatment may be due to this.

4.1.3.3 Bioactive compounds

Betalain, a tyrosine-derived pigment, is required for homeostasis to be sustained in the presence of abiotic stress. Betalain accumulates in the epidermis, mesophyll, and guard cell of leaves (Jain and Gould, 2015; Zhou et al., 2021). In amaranth leaves, especially red leaves, has a higher betalain concentration as much as 2 to 5 times of green leaves (Liu et al., 2019). Betalain is involved in cold stress. Agarie (2015) reported that cold stress enhanced the concentration of betalain in *Suaeda japonica* leaves. As previously stated, low RZT induced ROS in plant cells, particularly chloroplasts.

Numerous cold-responsive bioactive compounds are synthesized to maintain the photosynthetic machinery. Betalain, rather than carotenoid, the photoprotective compounds, is strongly associated with cold stress (Li et al., 2019). The betalain concentration significantly increased in leaves under RZT treatments of 5, 10, and 15ºC and slightly reduced by maintaining level (Fig. 4-4 and Table 4-4). In general, similar to chlorophyll, the biosynthesis of betalain needs nitrogen as a backbone. Thus, the decrease in chlorophyll and the increase in betalain following RZT treatment might result from this. Jain and Gould (2015) showed that betalain concentrations increased 4-fold when exposed to water stress, but chlorophyll concentrations decreased dramatically. Under low RZT conditions, photosynthesis is restricted, depleted of resources, and the plant is forced to preserve a critical element for life. Chlorophyll may be less necessary in a resource-constrained environment with an excess of ROS, but betalain and carotenoid, antioxidative molecules, may be more needed (Polturak et al., 2016).

Anthocyanin has been commonly mentioned as plant stress-responsive bioactive molecules, acting ROS scavengers, and osmo-homeostatic agents (Stintzing and Carle, 2004). The anthocyanin concentration of seedlings subjected to RZT treatments were 1.3–2.3 times higher than that in control (Fig. 4-5 and Table 4-5). At three days of treatment, the anthocyanin concentration in the seedlings grown at 5 and 10ºC was significantly increased up to 16.23 and 15.65 mg cyanidin-3-glucoside g^{-1} DW, respectively.

The anthocyanin concentration of the seedlings at 15 and 20ºC gradually increased from one to seven-days of the treatment. Many literatures showed that RZT treatment significantly increases anthocyanin in leaves. For example, anthocyanin concentration increased in red leaf lettuce after being exposed to low RZTs at 10 and 15ºC for a certain period (Sakamoto and Suzuki, 2015). Similarly, the leaves of red perilla grown at RZT of 10ºC for six days exhibited a considerable increase in anthocyanin concentration compared to that in control (Ogawa et al., 2018). In this study, anthocyanin concentrations in amaranth leaves under RZT treatments responded variably depending on RZT level and period (Fig. 4-5 and Table 4-5).

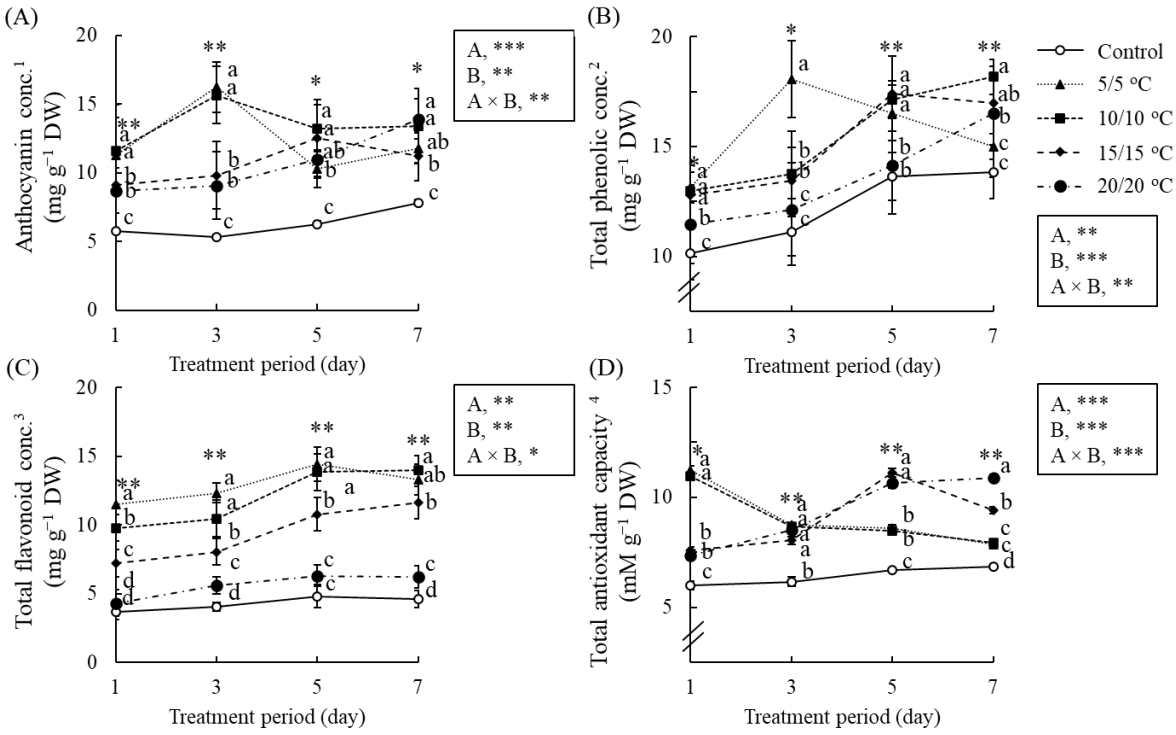


Fig. 4-5. Anthocyanin (A), total phenolic (B), and total flavonoid (C) concentrations and total antioxidant capacity (D) of leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) and period. Results of two-way analysis of variance for RZT (A), period (B), and their interaction (A × B) are shown. The asterisks indicate significance levels (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). Vertical bar indicates standard error (n=4). Means were compared using Tukey's HSD at a significance level at $p < 0.05$, * and $p < 0.01$, **. [1] Anthocyanin concentration is expressed as cyanindin-3-glucoside equivalents. [2] Total phenolic concentration is expressed as gallic acid equivalents. [3] Total flavonoid concentration is expressed as rutin equivalents. [4] Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

Table 4-5. Anthocyanin (Ant conc.), total phenolic (Phl conc.), and total flavonoid (Flv conc.) concentrations, and total antioxidant capacity (TAC) of leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) and period.

Period (day)	RZT (ºC)	Ant conc.[1] (mg g^{-1} DW)	Phl conc.[2] (mg g^{-1} DW)	Flv conc.[3] (mg g^{-1} DW)	TAC[4] (mM g^{-1} DW)
1	5/5	11.26 ± 2.76^{a}	13.14 ± 0.70^{a}	11.47 ± 1.79^{a}	11.23 ± 0.22^{a}
	10/10	11.59 ± 3.32^{a}	12.97 ± 1.36^{a}	9.78 ± 0.96^{b}	10.97 ± 0.16^{a}
	15/15	9.13 ± 2.05^{b}	12.77 ± 1.36^{a}	7.21 ± 0.99^{c}	7.60 ± 0.13^{b}
	20/20	8.68 ± 1.64^{b}	11.48 ± 1.79^{b}	4.31 ± 0.97^{d}	7.37 ± 0.21^{b}
	Control	5.76 ± 0.18^{c}	10.14 ± 1.19^{c}	3.68 ± 0.55^{d}	6.00 ± 0.20^{c}
	Significant	**	*	**	*
3	5/5	16.23 ± 1.84^{a}	18.06 ± 1.76^{a}	12.31 ± 0.72^{a}	8.74 ± 0.10^{a}
	10/10	15.65 ± 2.08^{a}	13.76 ± 1.93^{b}	10.46 ± 1.33^{a}	8.66 ± 0.07^{a}
	15/15	9.80 ± 2.45^{b}	13.44 ± 1.51^{b}	8.04 ± 0.97^{b}	8.04 ± 0.18^{a}
	20/20	9.05 ± 2.45^{b}	12.14 ± 2.12^{c}	5.60 ± 0.64^{c}	8.54 ± 0.14^{a}
	Control	5.34 ± 0.17^{c}	11.11 ± 1.52^{c}	4.05 ± 0.31^{d}	6.17 ± 0.21^{b}
	Significant	**	*	**	**
5	5/5	10.28 ± 1.38^{b}	16.53 ± 1.26^{a}	14.44 ± 1.24^{a}	8.61 ± 0.16^{b}
	10/10	13.22 ± 1.71^{a}	17.12 ± 0.86^{a}	13.83 ± 1.34^{a}	8.47 ± 0.02^{b}
	15/15	12.52 ± 2.78^{a}	17.40 ± 1.70^{a}	10.77 ± 1.21^{b}	11.12 ± 0.20^{a}
	20/20	10.99 ± 1.38^{ab}	14.14 ± 2.19^{b}	6.31 ± 0.80^{c}	10.67 ± 0.25^{a}
	Control	6.27 ± 0.22^{c}	13.64 ± 1.09^{b}	4.81 ± 0.84^{c}	6.69 ± 0.08^{c}
	Significant	*	**	**	**
7	5/5	11.79 ± 1.11^{ab}	15.01 ± 1.39^{c}	13.29 ± 1.10^{ab}	7.88 ± 0.10^{c}
	10/10	13.39 ± 2.70^{a}	18.17 ± 0.79^{a}	14.00 ± 1.01^{a}	7.95 ± 0.06^{c}
	15/15	11.22 ± 1.78^{b}	16.98 ± 1.40^{ab}	11.59 ± 1.18^{b}	9.41 ± 0.14^{b}
	20/20	13.91 ± 1.44^{a}	16.52 ± 2.11^{b}	6.21 ± 0.79^{c}	10.90 ± 0.13^{a}
	Control	7.82 ± 0.23^{c}	13.85 ± 1.23^{c}	4.62 ± 0.62^{d}	6.86 ± 0.11^{d}
	Significant	*	**	**	**

Data are shown as the mean ± standard error of the four biological replicates.

The means within the column were compared using Tukey's HSD at a significance level of $p < 0.01$. [1] Anthocyanin concentration is expressed as cyanindin-3-glucoside equivalents (C3G). [2] Total phenolic concentration is expressed as gallic acid equivalents (GAE). [3] Total flavonoid concentration is expressed as rutin equivalents (RTE). [4] Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

For instance, anthocyanin concentration dramatically increased 2 to 3 times compared to that in control after being exposed to 5 and 10ºC for three days, then declined by 15–20 % at day 5 and 7. The leaves under 15 and 20ºC treatments progressively developed anthocyanin with extended treatment period. This finding shows that RZT treatment of 5 and 10ºC may reach the biological low threshold temperature level of amaranth, trigging the biosynthesis of anthocyanin components by activating chalcone synthase, chalcone isomerase, and flavanone 3-hydroxylase (Liu et al., 2018). However, the reduction of anthocyanin, known as anthocyanin degradation, may begin after day 3 of RZT treatment. Under RZT, photosynthesis is limited, lacking sugar as an energy source, and plants are forced to recycle sugar to survive. Anthocyanin, in general, is present as a glycoside, connected with sugar may be targeted as an alternate sugar source. Thus, the deglycosylation by β-glucosidase and oxidation by polyphenol oxidase or peroxidase may potentially result in anthocyanin degradation (Oren-Shamir, 2009). The anthocyanin performs its function as a ROS scavenger similar to betalain. Both bioactive compounds may be used initially under RZT; afterward, additional stress-responsive components will continue to perform this function. Therefore, anthocyanin and betalain reduction were observed on day 5 and 7 (Figs. 4-4−4-5 and Tables 4-4−4-5)

At three days of treatment, the total phenolic concentration in the seedlings grown at 5ºC increased to 18.06 mg gallic g^{-1} DW and was the highest among RZT treatments. However, on seven days of treatment, total phenolic concentration in seedlings grown at 5ºC was slightly decreased, but the anthocyanin concentration increased at 10, 15, and 20ºC RZT treatments. The RZT treatments significantly increased flavonoids in amaranth leaves, except for 20ºC, which was not clearly different from the control. Extending the treatment periods from 1 to 7 days enhanced the leaf's flavonoid concentration, particularly in seedlings grown at RZTs of 5 and 10ºC on day 3 to 5. From day–1 to day–3, the antioxidant capacity of control seedlings was altered slightly, whereas that of seedlings treated with RZT was changed differently.

On the first day of treatment, both 5 and 10ºC RZT treatments showed the highest antioxidant capacity (11.23– and 10.97–mM g^{-1} DW, respectively), eventually reduced by 15–20%. Antioxidant capacity increased gradually over five days after start of RZT treatments at 15 and 20ºC.

The relevance of low temperature inducing flavonoids and phenolics in plants is significantly highlighted. Król et al. (2015) demonstrated that after a week of exposure to 10ºC, the total phenolic content of *Vitis vinifera* L. leaves increased. However, the cold-sensitive cultivar had a lower phenolic content. The data suggested that phenolic compounds may play a role in cold tolerance. As a result, after 30 days of long-term low-temperature stress at 15ºC, the accumulation of flavonoids and phenolic compounds in tomato leaves increased (Rivero et al., 2001). Rezaie et al. (2020) demonstrated that the low RZT induced transcription factors associated with phenylalanine ammonia-lyase and altered the expression of the cinnamate 4-hydroxylase gene, resulting in increased flavonoid and phenolic compound accumulation in sweet basil. These reviews corroborate this study's findings, revealing that seedlings exposed to RZT of 5ºC for three days significantly enhanced their total phenolic concentration (Fig. 4-5 and Table 4-5). Other RZT treatments resulted in a progressive increase in phenolic concentration. However, total flavonoid levels increased gradually when the period of RZT treatment was extended. The findings indicated that seedling leaves treated with RZT (5, 10, and 15ºC) exhibited a 3–5-folds increase in flavonoid concentration compared to controls.

When the treatment period was prolonged, the total phenolic and flavonoid concentrations rose substantially, showing that both are required to maintain homeostasis under low RZT. Cold-tolerance capacity varies according to plant species in terms of cold-responsive transcriptional factors; different mechanisms may be used differently depending on the magnitude and period of the cold stress.

Antioxidant capacity is the total amount of free radicals scavenged by a test solution used to determine a biological substance's antioxidant capacity (Rubio et al., 2016). Lee and Oh (2015) reported that the antioxidant capacity of kale treated with RZT of 4ºC significantly increases on the first day after treatment. Consequence to this finding, the antioxidant capacity of leaves exposed to RZTs of 5 and 10ºC increased dramatically by 1.5 to 1.8-folds compared to that in control on the first day (Fig. 4-5 and Table 4-5). The antioxidant capacity of seedling leaves treated at 15 and 20ºC increased gradually from day 1 to day 7. Plant ROS defense mechanisms are diverse and include both enzymatic and non-enzymatic responses. Both are used in accordance with the quantity of ROS and the physiological state of the plant (Das and Roychoudhury, 2014).

The antioxidant capacity of amaranth is strongly correlated with bioactive compounds classified as non-enzymatic antioxidants, such as anthocyanin, betalain, phenolic, flavonoids, and ascorbic acid (Sarker et al., 2020). However, only betalain and carotenoid increased in accordance with antioxidant capacity, while phenolic, flavonoids, and ascorbic acid exhibited a delayed response (Figs. 4-4−4-5 and Table 4-4−4-5). Carotenoid and betalain antioxidant molecules may act as the first line of defense against ROS damage in amaranth during RZT treatment. However, according to the reactivity and structure, phenolic and flavonoid compounds are considered secondary ROS scavenging mechanisms in plants (Fini et al., 2011). Additionally, delayed synthesis resulting from delayed gene expression in response to abiotic stress has been described in some literature (Clayton et al., 2018; Qian et al., 2019).

4.1.3.4 Nutrient-related compounds

The concentrations of nutrients-related compounds, such as sugar and ascorbic acid, were influenced by RZT levels and treatment periods (Fig. 4-6 and Table 4-6). On the first day of treatment, the total sugar concentration increased to 2.71 and 2.62 mg g^{-1} DW in leaves grown at 5 and 10ºC, respectively, whereas the control and RZT of 20/20ºC exhibited no significant change in sugar concentration. On day three of treatment, the highest β-carotene content (4.13 mg g^{-1} DW) was found at 20ºC. During prolonged RZT treatments, the β-carotene concentration declined by 15% to 30% compared to the control.

On day three, RZT treatments at 5 and 10ºC significantly increased ascorbic acid concentrations in amaranth leaves, but other RZT treatments marginally increased ascorbic acid concentrations when treatment period was extended.

Sugar imparts a sweet flavor to leafy vegetables, critical for consumer acceptance. Appropriately, a moderate low RZT increased the sugar content in leaves and enhanced the relative sweetness of leaves (He et al., 2020). Sugar accumulation was explored under low RZT conditions in various model plants, including red leaf lettuce, red perilla, and Chinese broccoli (Sakamoto and Suzuki, 2015; Ogawa et al., 2018; He et al., 2021).

Cooling RZT stimulates C-repeat binding factors, increasing starch hydrolysis to generate sucrose (Tarkowski and den Ende, 2015). Sucrose, the biological osmolant, also sustains osmotic pressure in the presence of restricted water due to low RZT.

Additionally, sucrose plays a critical role in maintaining homeostasis under low RZT stress by inducing the DELLAs family of transcription factors that regulate Myeloblastosis protein 75, which is required for anthocyanin biosynthesis (Valluru et al., 2008; Klemens et al., 2013). Sugar concentrations in seedlings grown at RZTs of 5 and 10^{o}C increased dramatically on the first day of treatment and then significantly decreased (Fig. 4-6 and Table 4-6). As a result, anthocyanin concentration increased on day three of treatment and remained stable thereafter (Fig. 4-5 and Table 4-5). This research demonstrates that low RZT may be used to enhance sugar concentration in amaranth leaves through anthocyanin accumulation, hence avoiding the adverse effects of low RZT stress.

Deng et al. (2019) also revealed that the sugar concentration in *Magnolia wufengensis* leaves increased during the first 24 hours of treatment and thereafter declined in proportion to the relative expression level of C-repeat binding factors. This mechanism is also connected to phytohormones, particularly gibberellin, which is regulated by the gibberellin oxidases family. Sucrose increases the activity of gibberellin oxidases; gibberellins are thus oxidized and reduced (Francisco et al., 2013). Gibberellin deficiency corresponds with chlorophyll and carotenoid stabilization (Cheminant et al., 2011). Thus, after one day, a decrease in chlorophyll and carotenoid concentrations were observed in amaranth leaves exposed to low RZTs for extended treatment periods of 5 and 7 days (Fig. 4-4 and Table 4-4).

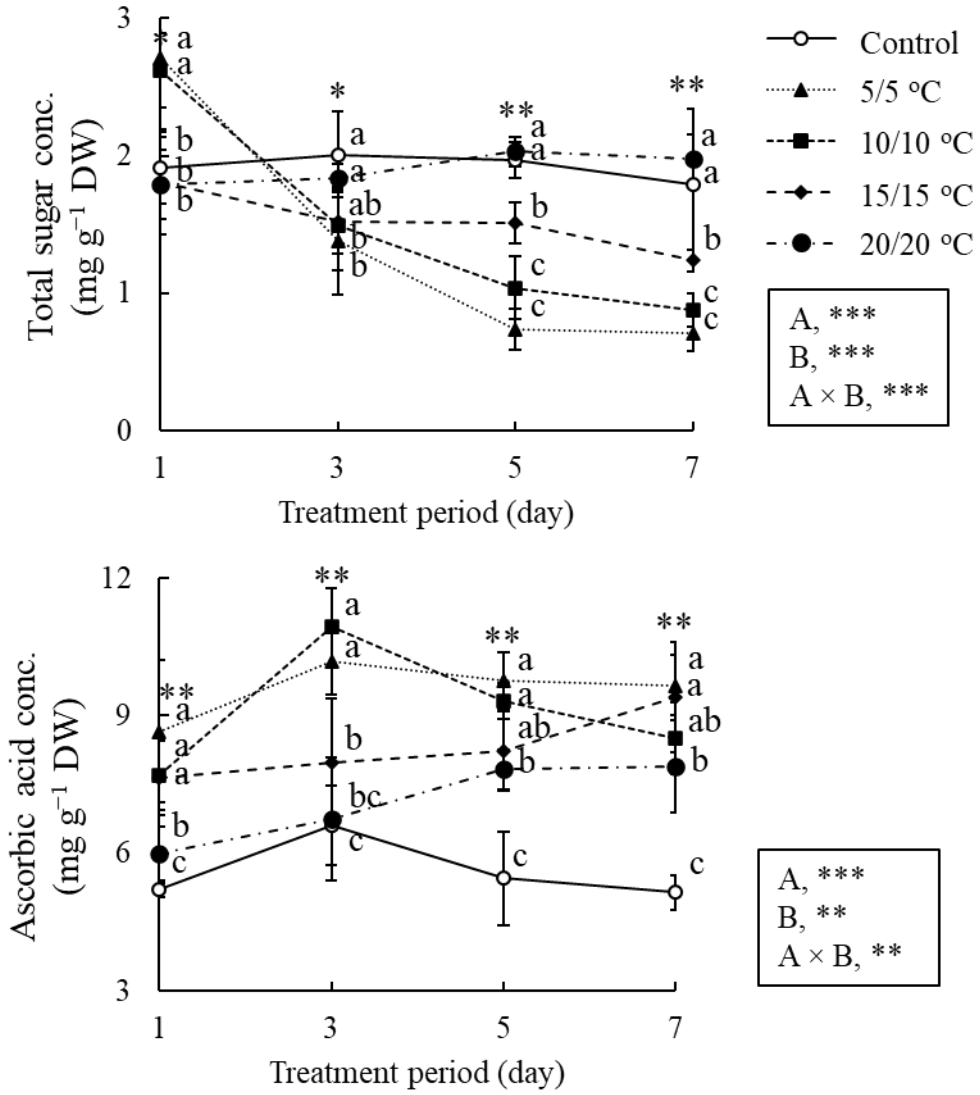


Fig. 4-6. Total sugar and ascorbic acid concentrations (conc.) in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) and periods.

Results of two-way analysis of variance for RZT (A), period (B), and their interaction (A × B) are shown. The asterisks indicate significance levels (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). Vertical bar indicates standard error (n=4). Means were compared using Tukey's HSD at a significance level at $p < 0.05$, * and $p < 0.01$, **. DW: dry weight.

Table 4-6. Total sugar and ascorbic acid concentrations (conc.) in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) and periods.

Period (day)	RZT (ºC)	Total sugar conc. (mg g^{-1} DW)	Ascorbic acid conc. (mg g^{-1} DW)
1	5/5	2.71 ± 0.57[a]	8.65 ± 1.55[a]
	10/10	2.62 ± 0.27[a]	7.70 ± 0.75[a]
	15/15	1.81 ± 0.38[b]	7.65 ± 0.81[a]
	20/20	1.79 ± 0.25[b]	6.00 ± 0.59[b]
	Control	1.91 ± 0.26[b]	5.20 ± 0.16[c]
	Significant	*	**
3	5/5	1.38 ± 0.39[b]	10.18 ± 0.73[a]
	10/10	1.49 ± 0.32[b]	10.93 ± 0.86[a]
	15/15	1.52 ± 0.23[ab]	7.97 ± 1.41[b]
	20/20	1.84 ± 0.10[a]	6.75 ± 1.33[bc]
	Control	2.01 ± 0.31[a]	6.60 ± 0.87[c]
	Significant	*	**
5	5/5	0.74 ± 0.15[c]	5.45 ± 0.62[c]
	10/10	1.04 ± 0.23[c]	9.77 ± 0.38[a]
	15/15	1.51 ± 0.15[b]	9.32 ± 0.87[a]
	20/20	2.03 ± 0.11[a]	8.23 ± 0.43[ab]
	Control	1.97 ± 0.13[a]	7.83 ± 1.03[b]
	Significant	**	**
7	5/5	0.71 ± 0.13[c]	9.66 ± 0.65[a]
	10/10	0.88 ± 0.12[c]	8.50 ± 0.78[b]
	15/15	1.24 ± 0.08[b]	9.40 ± 1.20[a]
	20/20	1.98 ± 0.17[a]	7.90 ± 1.01[b]
	Control	1.79 ± 0.55[a]	5.15 ± 0.38[c]
	Significant	**	**

Data are shown as the mean ± standard error of the four biological replicates.

The means within the column were compared using Tukey's HSD at a significance level of $p < 0.05$, * and $p < 0.01$, **. DW: dry weight.

For ascorbic acid, the cellular antioxidant increased significantly in leaves under 5ºC from day-1 and remained steadily until day-7, but it decreased slightly in leaves under 10ºCat day-7 (Fig. 4-6 and Table 4-6). Ascorbic acid is required to function as a scavenger in both enzymatic and non-enzymatic ROS defense mechanisms, implying that the ascorbic acid biosynthesis may continue under abiotic stress (Barnes et al., 2002). Ascorbic acid is a water-soluble vitamin that is critical for the immune system and as a cellular radical scavenger. Amaranth is a source of ascorbic acid, particularly for its leaves, containing 13 times the ascorbic acid content found in lettuce (Srivastava, 2011). The recommendation value of daily ascorbic acid intake for adult people is approximately 100 mg and a strategy for increasing ascorbic acid in vegetables has been often proposed in agriculture. This research discovered that ascorbic acid increased in seedlings subjected to RZT treatments of 5 and 10ºC after three days and thereafter declined slightly.

Subsequently, Ito and Shimizu (2020) observed that spinach (*Amaranthaceae*) exposed to 4ºC for 2–7 days enhanced ascorbic acid. Cheminant et al. (2011) reported that after two weeks of RZT treatment at 5ºC, the ascorbic acid content in spinach increased. Although research examined ascorbic acid in spinach, the response may differ based on the spices used. Ascorbic acid is a potent radical scavenger that acts through the ascorbate peroxidase-glutathione reductase pathway (Akram et al., 2017). It is more effective in scavenging hydrogen peroxide than catalase and peroxidase (Dolatabadian and Jouneghani, 2009). Ascorbic acid is produced from glucose, pectin, and myo-inositol; hence, a greater ascorbic acid level indicates increased utilization of those substances (Wheeler et al., 1998; Smirnoff et al., 2001). Due to the fact that these precursors have an effect on plant growth and development, long-term RZT treatment of amaranth may not be appropriate, as demonstrated by Figs. 4-3–4-6 and Tables 4-4−4-6.

4.2 Effect of cooling root-zone temperatures combination

4.2.1 Introduction and objective

Since each RZT level and period (section 4.1) demonstrated a distinct feature, a combination of varied RZT levels for three days before harvest may be performed to enhance the concentration of bioactive compounds. On the one hand, the RZT of 20 ºC may increase photosynthetic pigments, providing a precursor for bioactive compounds and nutrients; hence, two days will be set aside for this RZT. After 20ºC, RZTs of 5 or 10ºC will be performed for a day to improve the bioactive compounds and nutrients before harvest. On the other hand, RZTs of 5 and 10ºC may be firstly used for a day to enhance bioactive compounds and nutrients, followed by RZTs of 20ºC to gradually increase and maintain them till harvest.

4.2.2 Materials and methods

4.2.2.1 Plant material and cultivation condition (please see section 2.1.2.1)

4.2.2.2 Root-zone temperature treatment

The amaranth seedlings with four true leaves, approximately 27 days after sowing, were treated for RZT treatments. Six seedlings were transplanted in a hydroponic container (33 cm × 18 cm × 15 cm) for the treatment. The RZT was controlled using a handy cooler (TRL-107NHF, Thomas Kagaku Co., Ltd., Tokyo, Japan) with a temperature control device. Aeration was performed using air pumps and air stones to supply sufficient air to roots and to circulate the nutrient solution in each container. Seedlings were subjected to four different RZT treatments for 1, 3, 5, and 7 days at 5, 10, 15, and 20ºC. Four seedlings were harvested after 1, 3, 5, and 7 days of treatment.

The environmental conditions for RZT treatment were controlled as described in Table 4-1, except for the photosynthetic photon flux density (PPFD), which was set to 300 µmol m^{-2} s^{-1}.

The seedlings in all treatments were exposed to the same aerial conditions, including air temperature (25/20ºC in light/dark periods). Control seedlings were cultivated under the same conditions as described above, and RZT was not controlled. Seedlings were subjected to four RZT treatments (20T5, 20T10, 5T20 and 10T20). Table 4-4 shows the RZT treatment and period. Three days after treatment, four seedlings were harvested.

Table 4-4. Short-term root-zone temperature (RZT) conditions for the treatment of amaranth seedlings. The seedlings were irradiated under the same photosynthetic photon flux density of 300 μmol m^{-2} s^{-1} for 3 days.

Treatment	RZT treatment (day/night, ºC)		
	Day-1	Day-2	Day-3
20T5	20/20	20/20	5/5
20T10	20/20	20/20	10/10
5T20	5/5	20/20	20/20
10T20	10/10	20/20	20/20

4.2.2.3 Yield, leaf water content, and leaf morphology (please see section 4.1.2.3)

4.2.2.4 Chlorophyll and carotenoid concentration determinations

(Please see section 2.2.2.4)

4.2.2.5 Betalain concentration determination (Please see section 2.2.2.5)

4.2.2.6 Anthocyanin concentration determination (Please see section 2.2.2.6)

4.2.2.7 Total phenolic concentration determination (Please see section 2.2.2.7)

4.2.2.8 Total flavonoid concentration determination (Please see section 2.2.2.8)

4.2.2.9 Ascorbic acid concentration determination (Please see section 2.2.2.9)

4.2.2.10 Total antioxidant capacity determination (Please see section 2.2.2.10)

4.2.2.11 Total sugar concentration determination (Please see section 4.1.2.11)

4.2.2.12 Statistical analysis

The experimental data were processed using the Statistical Package for the Social Sciences (SPSS software, version 24, USA). Tukey's honestly significant difference (Tukey's HSD) test was performed to compare the means of the measured parameters among the treatments.

4.2.3 Results and discussion

4.2.3.1 Growth and morphology

The leaf was almost entirely red after three days of treatment with RZT, whereas the leaves of 20T5 and 20T10 were red with a partially green (Fig. 4-7); curiously, the entirely red leaf was observed on days 5 and 7 of the section 4.1. In this experiment, no wilting symptoms were observed in the leaf treated with RZT, whereas those treated with RZT at 5, 10, and 15^{o}C exhibited wilting in section 4.1. (Fig. 4-2). The leaf fresh weight of seedlings treated with RZT ranged between 1.04 and 1.09 g, which was significantly different from the control seedling (1.19 g) (Table 4-5). Leaf dry weight and dry matter ratio were not significantly different between seedlings treated with RZT and control seedlings. In this experiment, three days of RZT treatment had no significant effect on leaf water content. On day 3, however, the control seedling's leaf water content was lower than that in section 4.1. (Fig. 4-3).

While cooling the root zone can enhance bioactive compounds (Ogawa et al., 2018; Nguyen et al., 2021), it also affects leaf fresh weight, in general, by interfering with root water uptake (Maurel et al., 2015). Thus, one of the disadvantages of using cooling root-zone temperature is the reduction of leaf fresh weight (Nxawe et al., 2009). According to the result in Table 4-3, the RZT at 20^{o}C had no statistical difference in the leaf fresh weight compared to the control, and hence was employed in section 4.2 to avoid leaf fresh weight reduction. However, the notion may not be valid, since the results indicated that when seedlings were treated with a combination of RZTs, the leaf fresh weight was significantly reduced by 10−15 % compared to the control (Table 4-5).

Interestingly, the combination of RZTs was found to have a beneficial effect on plant by reducing water stress damage, as evidenced by the fact that the dry weight, and leaf water content were all not statistically different from the control group's results (Table 4-5). Those parameters reflect the stress status of the plant in regard to the restriction of water and nutrient balance (Calleja-Cabrera et al., 2020; Zhou et al., 2021).

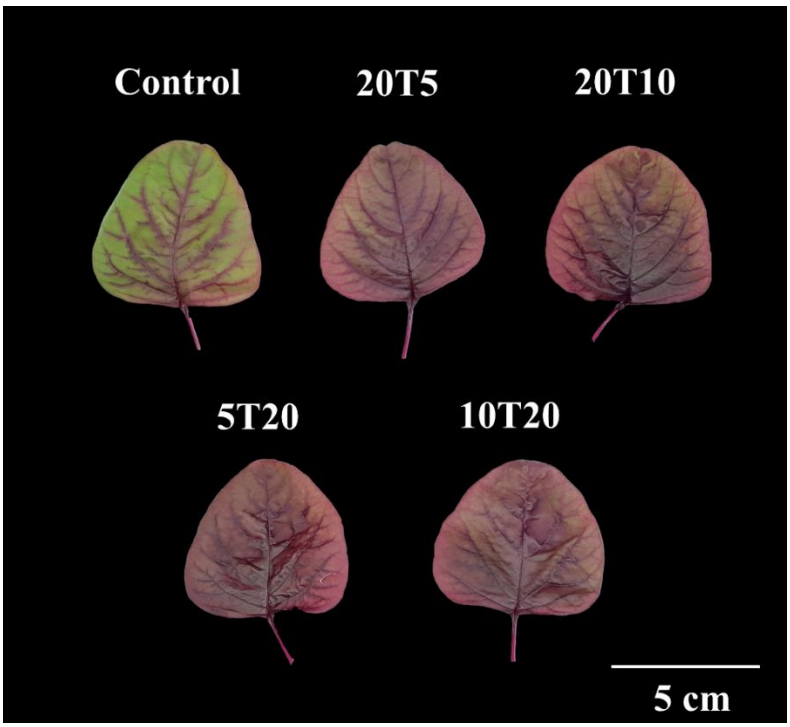


Fig. 4-7. Morphology of amaranth leaves of seedlings after treatment with different short-term root-zone temperatures (RZT) treatments for 3 days.
The seedlings that were subjected to two days of RZT at 20ºC followed by one day of RZT at 5 or 10ºC are designated as 20T5 and 20T10, respectively. Those seedlings that were treated at 5 or 10ºC for one day, then subjected at 20ºC for two days, are noted as 5T20 and 10T20.

Table 4-5. Amaranth leaves fresh weight (Leaf FW), dry weight (Leaf DW), and leaf water content (LWC) after treatment with different short-term root-zone temperatures (RZT) treatments for 3 days.

Treatment	Leaf FW (g)	Leaf DW (g)	LWC (%)
25/20 (control)	1.19 ± 0.07[a]	0.19 ± 0.01	84.80 ± 1.23
20T5	1.06 ± 0.01[b]	0.16 ± 0.02	85.14 ± 0.88
20T10	1.04 ± 0.02[b]	0.17 ± 0.01	85.25 ± 1.03
5T20	1.07 ± 0.06[b]	0.16 ± 0.02	85.45 ± 1.18
10T20	1.09 ± 0.02[b]	0.18 ± 0.02	83.18 ± 1.43
Significant	**	NS	NS

Data are shown as the mean ± standard error of the four biological replicates.

Means within columns were compared using Tukey's HSD. The asterisks indicate significance level (**, $p < 0.01$). NS indicates no statistical significance.

4.2.3.2 Photosynthetic pigments

RZT treatment had no effect on photosynthetic pigments, including chlorophyll and carotenoid, in this experiment (Table 4-6). The chlorophyll concentration in the leaf of seedlings treated with RZT ranged between 7.44 and 8.08 mg g^{-1} DW, which was not significantly different from the control. The carotenoid content of seedlings ranged from 2.41 to 2.53 mg g^{-1} DW. Betalain concentrations in seedlings treated with RZT increased 1.5 to 1.8-times compared to the control but did not differ significantly among RZT treatments (3.81 to 4.21 mg g^{-1} DW).

In section 4.1, chlorophyll may have been degraded by lipid oxidation (León-Chan et al., 2017) and reduced to a basal level for maintaining physiological processes under resource-constrained conditions with low RZT (Fig. 4-4). Notably, the finding in section 4.2 suggests that the combination of RZT had a beneficial effect on chlorophyll, despite the fact that there was no statistical difference between the RZT treatments and the control (Table 4-6). Chlorophyll may accumulate more when plants are under mild RZT stress, as shown in our experiment with a RZT temperature of 20ºC (Fig. 4-4). Then, no reduction in chlorophyll concentration was observed when RZTs of 5 and 10ºC were introduced (20T5 and 20T10, Table 4-6).

The mild cooling RZT of 15 to 20ºC at 25/20ºC of air temperature may promote chlorophyll accumulation, thereby increasing photosynthesis and thus bioactive compound or precursor biosynthesis (He et al., 2020). Thus, when seedlings are exposed to RZT, the precursors are promptly converted to cooled RZT-responsive compounds, and the resulting bioactive chemical compounds protect chlorophyll immediately from lipid oxidation. On the other hand, when RZTs of 5 and 10ºC were introduced prior to the application of 20ºC (5T20 and 10T20), the 20ºC may serve as a recovery condition; various bioactive compounds increased during the recovery period after RZT treatment (Lee and Oh, 2015).

RZT treatments in section 4.1 at 5 and 10ºC increased carotenoid concentrations for one day, then decreased over time (Fig. 4-4), however in this experiment, there was no significant difference between the RZT treatments and the control (Table 4-6). Carotenoid, as mentioned earlier, plays as ROS scavenger and photoprotective compounds (Stahl and Sies, 2003); under cooling RZT, in general, increase carotenoid concentration (Chadirin et al., 2011; Nguyen et al., 2020; He et al., 2021). Cooling RZT activates an elongated hypocotyl 5 (HY5), a switch for carotenoid biosynthesis (Catalá et al., 2011). However, many bioactive compounds, including flavonoids, are synthesized as controlled by this protein (Kim et al., 2017). In this experiment, RZT treatment may induce HY5, but carotenoid may not be a primary RZT stress-responsive compound. On the other perspective, oxidative products of β-carotene can promote carotenoid biosynthesis (Ramel et al., 2012). Carotenoids and chlorophyll share a similar biosynthetic pathway and crosstalk (Stanley and Yuan, 2019), which was observed and confirmed by this study (Table 4-6 and Fig. 4-4). We demonstrated that there was a different effect on carotenoids from the combination of RZTs in section 4.2 compared to section 4.1, where only a single RZT treatment was used.

Table 4-6. Total chlorophyll (Chl conc.), carotenoid (Car conc.), and betalain (Bet conc.) concentrations in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) treated for 3 days.

Treatment	Chl conc. (mg g^{-1} DW)	Car conc. (mg g^{-1} DW)	Bet conc. (mg g^{-1} DW)
25/20 (control)	9.71 ± 0.90	2.41 ± 0.15	2.51 ± 0.35[b]
20T5	7.44 ± 0.83	2.48 ± 0.28	3.95 ± 0.54[a]
20T10	7.55 ± 1.01	2.49 ± 0.70	3.94 ± 0.56[a]
5T20	8.08 ± 1.04	2.51 ± 0.49	4.21 ± 0.19[a]
10T20	7.78 ± 0.97	2.53 ± 0.31	3.81 ± 0.35[a]
Significant	NS	NS	**

Data are shown as the mean ± standard error of the four biological replicates.

Means within columns were compared using Tukey's HSD. The asterisks indicate significance level (**, $p < 0.01$). NS indicates no statistical significance. Betalain concentration is expressed as β-cyanin and β-xanthin equivalents. DW: dry weight.

4.2.3.3 Bioactive compounds

The anthocyanin concentration in seedlings treated with RZT ranged between 11.15 and 13.15 mg cyanidin-3-glucoside g^{-1} DW, which is approximately 1.3−1.5 times than that in the control (Table 4-7). In section 4.1, leaves treated with RZT treatments at 5 and 10ºC for 3 days had higher anthocyanin concentrations than leaves treated with RZT in section 4.2 (Fig. 4-5). However, in comparison to RZT at 20ºC for 3 days (section 4.1), a combination of RZT at 20ºC for 2 days and 5 or 10ºC for one day (section 4.2) enhanced anthocyanin concentration. Seedlings treated with RZT had a phenolic concentration 1.2–1.4 times than that of control seedlings. Three days of RZT treatment resulted in a significant increase in flavonoid concentration by 2.7–3.0 times of that in the control.

There were no significant differences among RZT treatments. The highest phenolic concentration (16.68 mg g^{-1} DW) was found in leaves treated with RZT 20T10, while the lowest was found in leaves treated with RZT 10T20. This finding is consistent with the antioxidant capacity of RZT-treated leaves, which was found to be highest in 20T10 (15.51 mM g^{-1} DW) and lowest in 10T20 (11.85mM g^{-1} DW), among RZT treatments.

Both phenolic concentration and antioxidant capacity of seedlings treated with RZT of 20T10 and 10T20 was significantly different. Interestingly, 20T10 and 10T20 were designed using the same RZT of 10 and 20ºC but with different periods, implying that a different RZT introducing period for 10ºC results in a different phenolic concentration and antioxidant capacity; in contrast, a different RZT introducing period for 5ºC showed a similar of those.

In this study, betalain, anthocyanin, phenolic, and flavonoid were found to function as bioactive compounds in response to cooling RZT (Tables 4-6 and 4-7, and Figs. 4-4 and 4-5). RZTs, either single or in combination, improve these concentrations at a certain period compared with controls. However, phenolic concentrations are affected by introducing 5 and 10ºC followed by or before 20ºC. Among RZT treatments, 10T20's leaves had the lowest concentration of phenolic compounds, the antioxidant capacity of 10T20's leaves was consequently reduced because of the lower phenolic concentration (Table 4-7). The antioxidant capacity of amaranth leaves, on the other hand, demonstrates the total ROS scavenging ability (scavenging, neutralizing, and radical reaction chain inhibition) (Rubio et al., 2016), which is highly correlated with betalain, phenolic, and flavonoid contents (Sarker and Oba, 2018; Sarker and Oba, 2020).

Table 4-7. Anthocyanin (Ant conc.), total phenolic (Phl conc.), and total flavonoid (Flv conc.) concentrations and total antioxidant capacity (TAC) of leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) treatments for 3 days.

Treatment	Ant conc.[1] (mg g^{-1} DW)	Phl conc.[2] (mg g^{-1} DW)	Flv conc.[3] (mg g^{-1} DW)	TAC[4] (mM g^{-1} DW)
25/20 (control)	8.11 ± 0.58^{b}	11.53 ± 0.58^{c}	4.10 ± 0.21^{b}	8.33 ± 0.97^{c}
20T5	13.15 ± 1.15^{a}	15.49 ± 1.13^{ab}	13.13 ± 0.93^{a}	13.45 ± 0.95^{ab}
20T10	13.14 ± 1.58^{a}	16.68 ± 0.80^{a}	12.00 ± 0.99^{a}	15.51 ± 1.22^{a}
5T20	12.61 ± 0.87^{a}	16.34 ± 0.83^{a}	12.45 ± 1.22^{a}	14.09 ± 1.08^{ab}
10T20	11.15 ± 1.04^{a}	14.40 ± 0.83^{b}	11.06 ± 0.94^{a}	11.85 ± 0.57^{b}
Significant	**	**	**	**

Data are shown as the mean ± standard error of the four biological replicates.

Means within columns were compared using Tukey's HSD. The asterisks indicate significance level (**, $p < 0.01$). NS indicates no statistical significance. [1] Anthocyanin concentration is expressed as cyanindin-3-glucoside equivalents. [2] Total phenolic concentration is expressed as gallic acid equivalents. [3] Total flavonoid concentration is expressed as rutin equivalents. [4] Total antioxidant capacity is expressed as Trolox equivalents. DW: dry weight.

4.2.3.4 Nutrients-related compounds

The effect of RZT treatments on nutrient-related compounds such as sugar and ascorbic acid is demonstrated in Table 4-8. The total sugar concentration in the leaves treated with 20T5 and 5T20 conditions was 2.43 and 2.53 mg g^{-1} DW, respectively. At the different RZT treatment introducing periods, RZT treatments of 5 or 10ºC for one day (20T5 and 5T20, and 20T10 and 10T20) resulted in increased sugar concentration, but RZT 5ºC yielded in a more effective increase in sugar concentration. Leaf sugar concentrations increased when RZT treatments were applied, supporting all of the proposed hypotheses in this study (Table 4-8).

In brief, leaves exposed to RZTs at 5 and 10ºC increased their sugar concentration by 1.5−1.6 times than that of the control on the first day, but this difference diminished over time. After day one, sugar concentration reduced, limiting the percussor necessary to synthesize bioactive compounds; thus, the reduction of those concentrations was observed (Figs. 4-4−4-6). In section 4.2, the sugar concentration of leaves under the combination of RZTs was maintained at a particular level; thus, bioactive compounds were maintained throughout the study due to no precursor limiting (Tables 4-6−4-8). Cooling RZT induces the starch degradation that results in sucrose, as previously described (Tarkowski and den Ende, 2015). This sucrose can then be hydrolyzed and used as a substrate for bioactive compounds or as a signal molecule that stimulates the biosynthesis of flavonoids (Klemens et al., 2013). Interestingly, the sugar concentration of leaves under the combination of 5ºC treatments (5T20 and 20T5) yielded more sugar than that of the combination 10ºC treatments (10T20 and 20T10) (Table 4-8). Both 5 and 10ºC may have reached a biological threshold, activating transcription factors that yield sugar, but 5 and 20ºC may function in synergy.

Both 5 and 10ºC RZTs can be used to increase ascorbic acid concentrations; however, the introducing period had a significant impact on the results. The combination of RZTs had a distinct effect on the ascorbic acid content in leaves treated with RZTs (Table 4-8). For 20T5 and 20T10, ascorbic acid concentrations were lower under the RZT at 5ºC than under the 10ºC. In comparison to other bioactive compounds found in RZT combinations, only ascorbic acid concentrations were significantly decreased in the leaves under 20T5. This finding implies that the RZT at 5ºC for one day prior to harvest may induce cold stress and produce ROS, for which ascorbic acid is required as a first-line scavenger, resulting in the reduction of that ROS concentration (Akram et al., 2017). However, leaves treated under 10T20 had a reduced ascorbic acid concentration, which was associated with a decreased phenolic concentration and antioxidant capacity.

In the instance of 10T20, it is probable that the application of RZT at 10ºC induces cold-stress and generates ROS, but the recovery period at 20ºC may have disguised the impact of stress, resulting in lower phenolic and ascorbic acid concentrations and antioxidant capacity than other combinations.

Table 4-8. Total sugar (sugar conc.) and ascorbic acid (Asc conc.) concentrations in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) treatments for 3 days.

Treatment	Sugar conc. (mg g^{-1} DW)	Asc conc. (mg g^{-1} DW)
25/20 (control)	1.24 ± 0.24^{c}	6.78 ± 0.40^{c}
20T5	2.53 ± 0.37^{a}	9.90 ± 0.38^{b}
20T10	1.84 ± 0.16^{b}	11.05 ± 0.71^{a}
5T20	2.43 ± 0.22^{a}	11.70 ± 0.84^{a}
10T20	1.83 ± 0.10^{b}	9.55 ± 0.51^{b}
Significant	**	**

Data are shown as the mean ± standard error of the four biological replicates.
Means within columns were compared using Tukey's HSD. The asterisks indicate significance level (**, $p < 0.01$). NS indicates no statistical significance. DW: dry weight.

If this hypothesis is correct, it may be described as pre-cooling RZT before cold-RZT, since pre-cooling prior to cold storage is comparable. The pre-cooling, in general, is the application of 10−20ºC of forced air to leafy vegetables in removing excess heat from the environment or metabolic process (Martínez and Artés, 1999). Numerous studies have shown that pre-cooling vegetables extend their shelf life by increasing bioactive compounds that function as related to radical scavengers and chelating agents (He et al., 2013; Garrido et al., 2015; Tian et al., 2016; Kongwong et al., 2019).

Thus, pre-cooling RZT (20ºC in this experiment), in contrast to the cold-RZT warning signal, may enhance bioactive compounds prior to cold-RZT (5 and 10ºC). On the other perspective, RZT at 20ºC is given after cold-RZTs (5 and 10ºC, 5T20 and 10T20); the RZT at 20ºC may not be considered the pre-cooling RZT but may be regarded as a recovery condition.

4.3 Conclusions

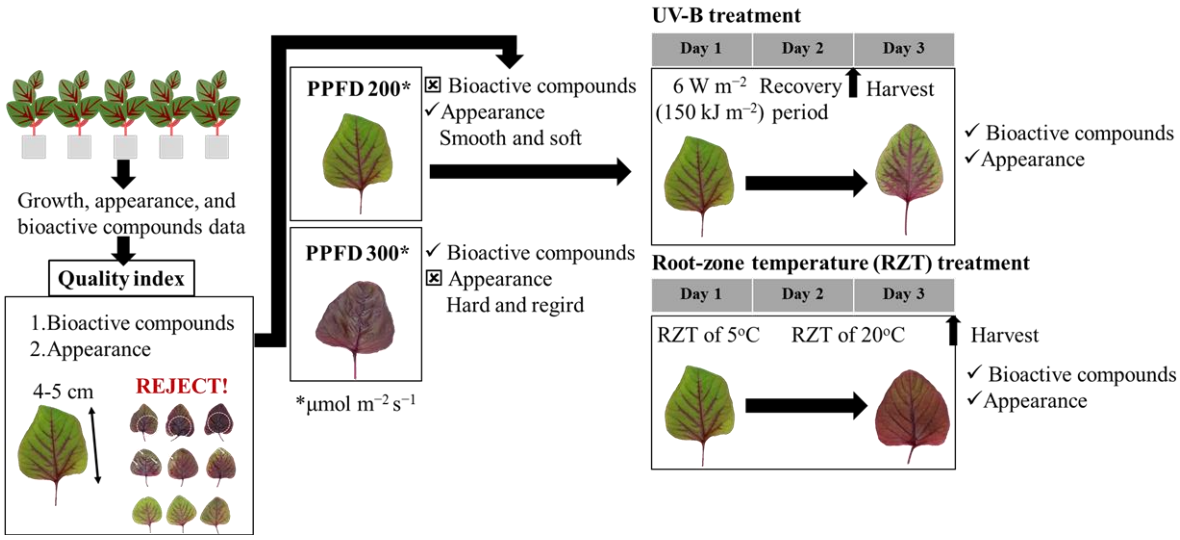
Our findings suggest that a one-day RZT combination at 5ºC followed by two-day at 20ºC before harvest can improve the target bioactive compounds and maintain nutrients in baby-leaf amaranth without affecting leaf appearance. Baby-leaf amaranth responded differently to different RZT combinations, and this is the first study to reveal findings on that. Further study is needed to show the relationship between pre-harvest treatment and post-harvest quality of baby-leaf amaranth since physiological processes in plants change over time, even after harvest.

CHAPTER 5

Conclusions and future work

5.1 Research outputs

In Chapters 3 and 4, the short-term treatments using UV-B irradiation and low RZT were applied to enhance bioactive compounds of baby-leaf amaranth without leaf abnormality. It was concluded that UV-B irradiation at 6 W m^{-2} with cumulative energy of 150 kJ m^{-2} and a 24 h post-irradiation recovery period could be an appropriate treatment to increase bioactive compounds in baby-leaf amaranth without causing the appearance abnormalities. For RZT treatment, the combination of RZT treatments at 5 and 10ºC for one day prior or followed by 20ºC for two days had varied effects on the growth and quality of amaranth leaves. After one day of RZT at 5ºC followed by two days of RZT at 20ºC, the highest concentration of bioactive compounds, nutrients, and antioxidant capacity was demonstrated in the leaves without impairing growth. Both treatments, two days of UV-B treatment and three days of cooling RZT treatment showed a significant characteristic in these studies, producing high-quality green and red baby-leaves, respectively. The findings could be used for the advancement of the baby-leaf industry.



5.2 Suggestion for future research

1. Validation of post-harvest quality

Because plant physiological conditions change continuously, even after harvest, further research is necessary to demonstrate the relation between pre-harvest treatment and post-harvest quality of baby-leaf amaranth.

2. Eating quality and consumer acceptably assessments

Although the particular baby-leaf product has more bioactive compounds with a good appearance, a market survey, customer acceptability test, and parameters related to eating quality should be researched further.

3. Pilot-scale manufacturing studies

In many cases, the absence of pilot-scale testing resulted in failure when attempting to perform the work on a larger scale. It is necessary to conduct translational research by demonstrating pilot-scale manufacturing to verify the study results. Pilot-scale manufacturing data will also be helpful in determining the economic viability of this investigation.

4. The combination of optimized UV-B and RZT treatments

The optimum combination of UV-B and RZT treatments at an appropriate time might improve more bioactive compounds. The question of whether combining the two optimal treatments would increase or decrease plant quality is an intriguing topic.

5. The time-course change after UV-B irradiation

Baby-leaf amaranth's bioactive compounds improve after 24 h of UV-B irradiation. Further experiments are needed to determine the time-course change from 0 h after UV-B irradiation.

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Supplementary data

β-carotene, a carotenoid, is referred to as the precursor of retinol (vitamin A). This compound can be converted to retinol in a 12:1 ratio, which means that 12 g of β-carotene may be converted to 1 g of retinol in the human body (Haskell, 2012). Due to its nutritional importance, beta-carotene concentration was determined in this study. Information on beta-carotene is included; however, it is placed in the supplements because it is unrelated to the plant science aspect of this study.

In this research, the concentration of β-carotene varied between 1.84 and 4.13 mg g^{-1} DW, providing approximately 150–345 μg of retinol g^{-1} DW of amaranth leaves.

Table S1. β-carotene concentration (conc.) in leaves of amaranth seedlings under different PPFDs.

PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	β -carotene conc. (mg g^{-1} DW)
200	2.83 ± 0.12
300	3.23 ± 0.07
Significant	*

Data are shown as the mean ± standard error of the fifteen biological replicates. The asterisk indicates a significant different between treatments using Student t-test (*, $p<0.05$ and **, $p<0.01$). DW: dry weight.

Table S2. β-carotene concentration (conc.) in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) treatments for 3 days.

Treatment	β-car conc. (mg g^{-1} DW)
25/20 (control)	2.73 ± 0.17
20T5	2.72 ± 0.32
20T10	2.81 ± 0.92
5T20	2.85 ± 0.41
10T20	2.76 ± 0.35
Significant	NS

Data are shown as the mean ± standard error of the four biological replicates.

Means within columns were compared using Tukey's HSD. The asterisks indicate significance level (**, $p < 0.01$). NS indicates no statistical significance. DW: dry weight. The seedlings that were subjected to two days of RZT at 20ºC followed by one day of RZT at 5 or 10ºC are designated as 20T5 and 20T10, respectively. Those seedlings that were treated at 5 or 10ºC for one day, then subjected at 20ºC for two days, are noted as 5T20 and 10T20.

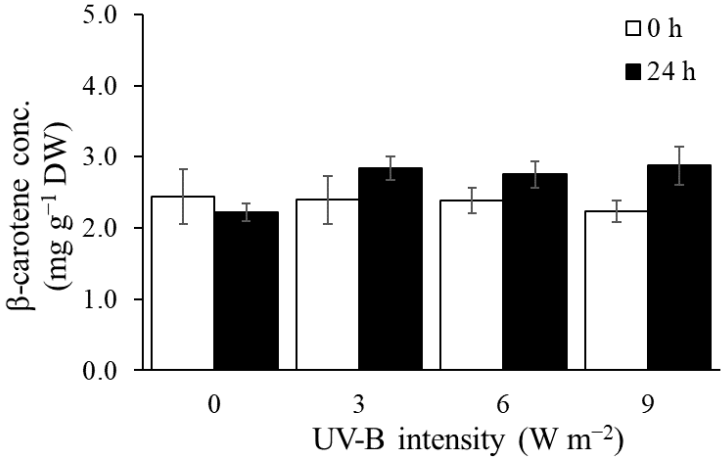

Fig. S1. β-carotene concentration (conc.) in the leaves of amaranth seedlings at 0 and 24 h after treatment with different short-term ultraviolet-B (UV-B) irradiation intensities for 8 h. Vertical bars indicate standard error (n = 4).
Means of the measured parameters at the same UV-B intensity for the two recovery periods were compared using the Student's t-test. * indicates significant difference. Lowercase letters indicate significant difference in measured parameters at the same recovery period determined using Tukey's HSD test at $p < 0.05$. DW: dry weight.

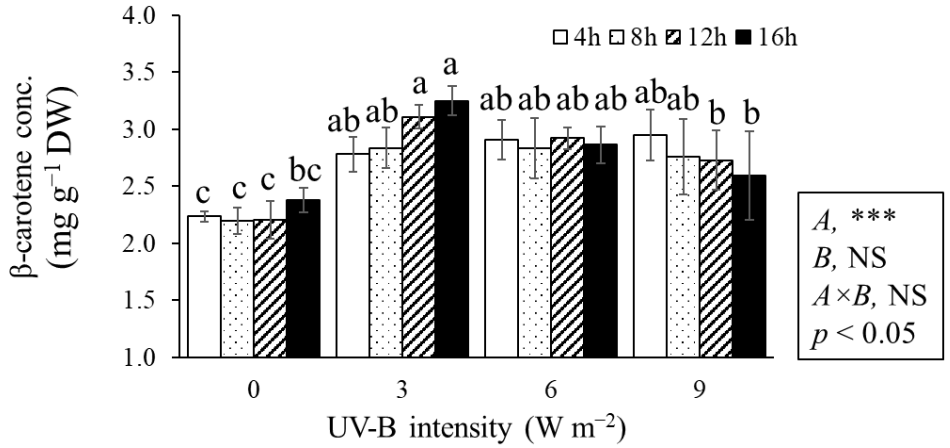

Fig. S2. β-carotene concentration (conc.) in the leaves of amaranth seedlings at 24 h after treatment with different short-term ultraviolet-B (UV-B) irradiation intensities and periods.
Vertical bars indicate standard error (n = 4). Results of two-way analysis of variance of UV-B intensity (A), irradiation period (B), and their interaction (A × B) are shown. Asterisks indicate significance levels (***, $p < 0.001$). NS indicates no statistical significance. Means were compared using Tukey's HSD test at $p < 0.05$.

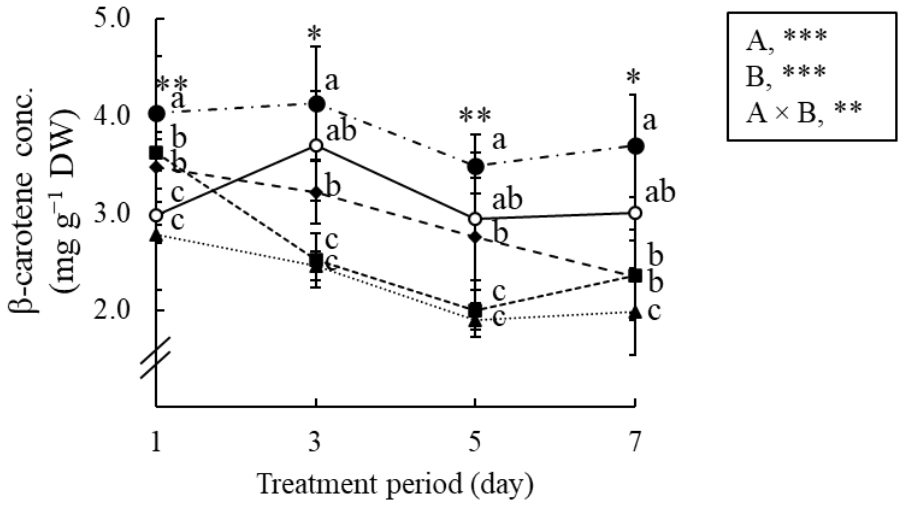

Fig. S3. β-carotene concentration (conc.) in leaves of amaranth seedlings after treatment with different short-term root-zone temperatures (RZT) and periods. Results of two-way analysis of variance for RZT (A), period (B), and their interaction (A × B) are shown. The asterisks indicate significance levels (**, $p < 0.01$, and ***, $p < 0.001$). Vertical bar indicates standard error (n=4). Means were compared using Tukey's HSD at a significance level at $p < 0.05$, *. DW: dry weight.

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“A journey of a thousand miles begins with a single step”

せんりのみちもいっぽから

Sincerely Yours,

Takon Wittayathanarattana