Analysis of Nutritional Components of Spinach Under Root Chilling Stress

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Chapter 1

Introduction

1.1 Background

In recent years, there has been an increasing concern regarding food insecurity due to the rapid growth of the world population. According to the United Nations, the population has been estimated to increase constantly, specifically in Asia and Africa, to reach 9 billion by 2050 as shown in Fig. 1-1[32]. Environmental destruction has been progressing in response to the fear of food shortage, as tropical rainforests are being cut down to bring a greater area under cultivation for food production [33]. The damage to biodiversity due to environmental destruction, such as the disappearance of rainforests, has resulted in global warming and frequent natural disasters. Furthermore, drinking water is scarce due to the massive amounts of water used for food production, and 30% of the world's population is already facing water stress due to its scarcity [21, 30].

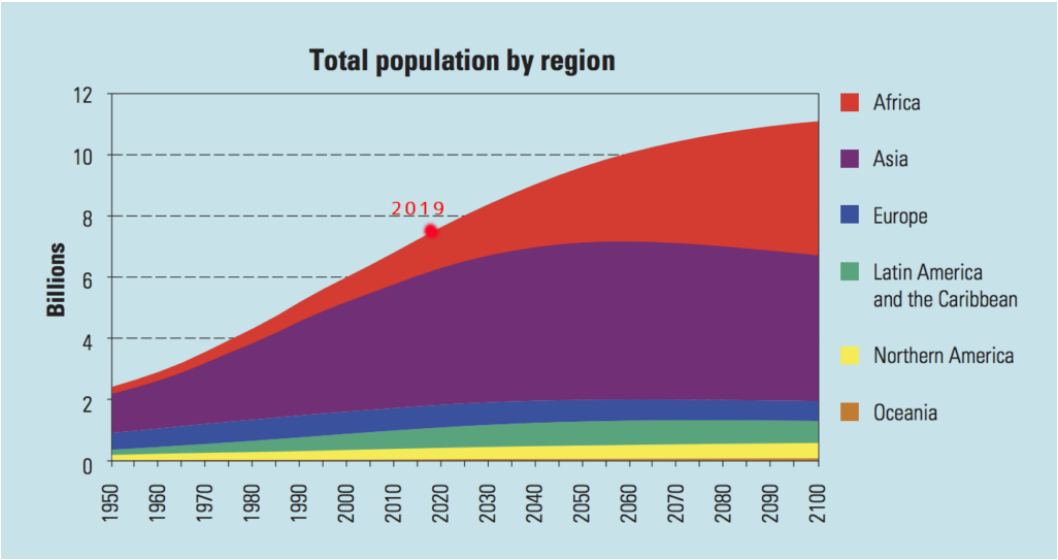

Fig. 1-1 World population prospects [31].

Meanwhile, Japan has a low calorie-based food self-sufficiency rate (Fig. 1-2), since Japan heavily relies on overseas import, except for rice [19]. Moreover, stable food production in agricultural fields has recently become difficult due to frequent abnormal weather conditions in Japan and other countries [10, 20]. Furthermore, the current average age of farmers in Japan is 67 years, and the aging of the working population and lack of successors have become serious problems in Japanese agriculture [18]. Hence, Japan is facing a severe problem of reducing domestic food production capacity while the food demand continues to increase worldwide. In this context, plant factories are attracting attention as a solution to the aforementioned problems.

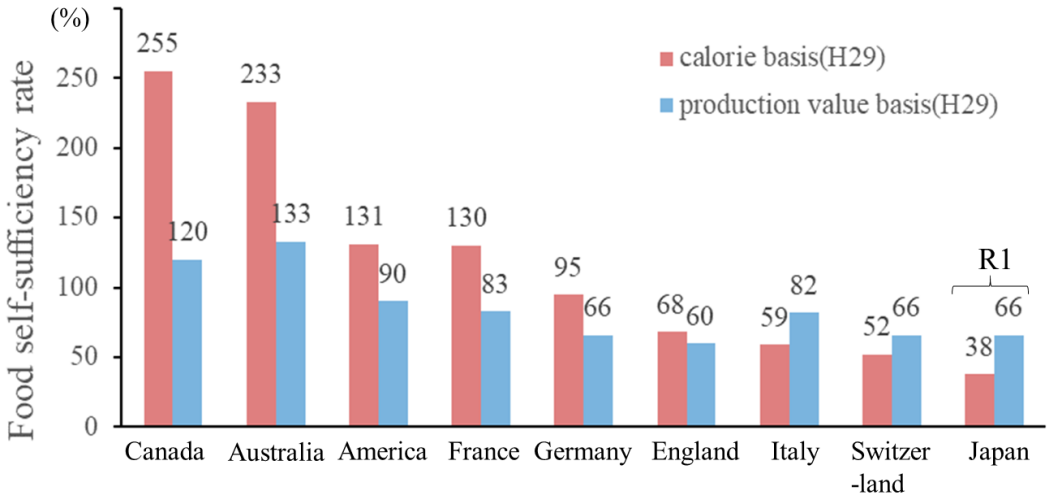

Fig. 1-2 Food self-sufficiency rate in Japan and other countries (partially modified by author) [19].

1.2 Plant factory

Plant factories are clean farming facilities that produce high-quality vegetables year-round. Moreover, pesticides are not used in these facilities because the plant growth environment is isolated from the external environment. Because the environment inside the plant factory is controlled automatically, the necessary work in the factory is light-load, thus, even aged people can work comfortably.

Plant factories are roughly divided into solar light plant factories and artificial light plant factories. A solar light plant factory is a cultivation facility that systematically produces vegetables using environmental control technology in a space that allows sunlight to pass through (Fig. 1-3). It is characterized by relatively low equipment costs and is suitable for cultivation of vegetables that require strong light and vegetables with high plant height. An artificial light plant factory is a cultivation facility that uses artificial light, such as LED, as a light source in a closed space and can control the cultivation environment to maintain the optimum conditions for plant growth (Fig. 1-4). As vertical cultivation is possible in this facility, the number of vegetables produced per unit area is high; therefore, it is suitable for the production of short leafy vegetables and seedlings.

Fig. 1-3　Solar light plant factory [17].

Fig. 1-4　Artificial light plant factory [15].

The design of plant factories allows plant cultivation in a controlled growth environment. Under such conditions, plants can be subjected to local stress to specifically evaluate the effects of certain factors, which may be difficult to study under field conditions. Therefore, plant factories provide suitable environments to study the effects of certain stimuli on plants, ultimately producing high-quality and value-added vegetables. Environmental factors such as light, temperature, fertilizer composition, and CO_2 concentration affect plant growth, morphogenesis, and secondary metabolism [16, 22, 23, 26]. In recent years, it has been reported that the growth of crops has been promoted by environmental control, especially in artificial plant factories. For instance, alternating irradiation with red and blue light using LED increased the fresh weight of leaf lettuce even when the total light intensity per day was the same as that with simultaneous irradiation [25]. Moreover, water with oxygenated micro/nanobubbles significantly promoted the fresh and dry weights and ascorbic acid content of leaf lettuce [14]. The application of these cultivation techniques has recently become a new research topic.

Many studies have investigated value-added vegetable production in plant factories by controlling environmental conditions or providing certain stimuli, such as air temperature, root-zone temperature, water availability, salinity, ozone, and ultraviolet radiation [1, 5, 8, 9, 24, 27]. Previous studies have reported that salinity stress increases the sugar and amino acid contents of tomatoes [34]. Additionally, it has been found that blue light or UV-B irradiation at night and the addition of sugar and salt to the hydroponic culture solution promoted anthocyanin accumulation in leaf lettuce [6, 28]. However, artificial light plant factories have certain limitations due to the high costs of facility construction and daily operation [15]. Therefore, a cultivation strategy for producing high-quality vegetables with low power consumption under plant factory conditions needs to be established.

Spinach is a popular nutritious leaf vegetable and is rich in ascorbic acid (i.e., vitamin C), which is a beneficial substance for human health. However, the nutritional value of spinach varies seasonally. For instance, because of the increased growth rate of spinach during summer, the levels of nutritionally beneficial compounds are depleted, whereas those of nutritionally detrimental compounds are elevated [7, 29]. Therefore, studies have focused on the cultivation of high-quality spinach in plant factories year-round. Previous studies have shown that spinach root chilling positively affects its nutritional quality, significantly enhancing the levels of beneficial substances (e.g., sugars, ascorbic acid, and ferrous ions) and suppressing the levels of harmful substances (e.g., nitrate ions and oxalic acid) [2, 3, 4]. However, the optimal conditions for root chilling

stress that can maximally enhance the nutritional value of spinach remain to be established.

1.3 Purpose of the study

The goal of this study was to establish a cultivation strategy for value-added spinach by applying root chilling stress. To achieve this goal, we evaluated (i) the changes in the nutritional value of spinach subjected to root chilling stress at a specific temperature; (ii) the triadic associations among the nutrient solution temperature during chilling treatment, chilling duration, and nutritional components of spinach; and (iii) the hypothesis regarding the mechanism underlying changes in the contents of nutritional components of spinach under root chilling stress.

Our results are anticipated to contribute toward establishing a cultivation program for producing high-quality and value-added vegetables with low power consumption under plant factory conditions.

1.4 Outline of the dissertation

Chapter 2 focuses on the analysis of the optimal root chilling duration required to enhance the quality of factory-produced spinach [11]. Previous studies have shown that the nutritional value of spinach increased by root chilling for 7 days; however, the mechanism underlying changes in nutritional value following root chilling treatment for less than 7 days remains unclear. We measured the contents of ascorbic acid, nitrate ions, and soluble sugars in spinach subjected to root chilling at 10°C for different durations (2, 4, 5, 6, and 7 days).

Chapter 3 outlines the analysis of triadic associations of nutrient solution temperature, chilling duration, and nutritional components of spinach [12]. This experiment was performed to determine the minimum chilling temperature and duration required to maximally enhance the nutritional value of spinach. We examined the contents of ascorbic acid, nitrate ions, and soluble sugars in spinach subjected to root chilling at various temperatures (4, 6, 10, and 14°C) for different durations (2, 4, 5, 6, and 7 days). Based on these results, we developed a relational expression for chilling duration and temperature.

Chapter 4 describes the analysis of changes in the contents of nutritional components of spinach following the root chilling treatment [13]. I hypothesized that changes in the contents of nutritional components following root chilling are due to

antioxidant reactions. To test this hypothesis, we assessed ascorbic acid content, superoxide dismutase activity, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity as proxies for antioxidant activity and malondialdehyde (MDA) content as an oxidative stress marker in spinach.

Finally, Chapter 5 summarizes the key conclusions of this study and offers suggestions for future research.

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Chapter 2

Effect of Different Durations of Root Area Chilling on the Nutritional Quality of Spinach

2.1 Introduction

Spinach is a green leaf-vegetable with health benefits because of its rich nutritional content. However, the nutrient quality of spinach varies over the year [9, 19]. Therefore, much research has been focused on cultivating high-quality spinach in plant factories year-round. Previous research has shown that cold stress to the root area of spinach has a positive effect on the nutritional quality, and produces a significant increase in beneficial substances (such as sugars and ascorbic acid) and a decrease in harmful substances (such as NO_3 and oxalic acid) [4, 5, 6]. However, the optimal duration of chilling that can maximally enhance the nutritional value of spinach is yet to be established. This study aimed to determine the optimal duration of low temperature acclimatization in the root area by which the ascorbic acid and sugar content was maximized without increasing the nitrate ion concentration.

2.2 Materials and methods

2.2.1 Plant materials and conditions

Spinach (Spinacia oleracea L. cv. 'Active') seeds were sown on water-soaked sponge. After 1 week, the germinated seedlings were transplanted to growth chambers (84 × 78 × 150 cm) set up with a hydroponic system (Fig. 2-1). The control cultivation panel conditions were set to a 14 h photoperiod, a day-time photosynthetic active photon flux density (PPFD) of 200 μmol $m^{-2}s^{-1}$, day and night air temperatures of 23 and 18°C, respectively, and a nutrient solution temperature of 18°C. The LED light (NE02-000089(01); Shibasaki, Saitama, Japan) was used as a light source. Spinach plants were

grown under 6 different experimental conditions as shown in Fig. 2-2.

First, 60 plants were grown under the above control condition for 20 days (Fig. 2-3A). On the 21st day, as shown in Fig. 2-3B, 10 plants were transferred to a different growth chamber, with a nutrient solution temperature of 10°C (low temperature nutrient solution: LT). Subsequent transferring of 10 plants each was made on the next day (Fig. 2-3C), 22 days after the experiment was initiated, followed by 2, 3, and 5 days later. Thus, spinach plant roots were exposed to LT conditions for 2, 4, 5, 6, and 7 days. Spinach plants transferred to LT were grown under the same conditions as those of control (no chilling) except the solution temperature. The temperature of the nutrient solution was controlled by a water temperature controller (ZC-700; Zensui, Osaka, Japan), and the nutrient solution pumped from a tank with a volume of 35 L (50 × 35 × 20 cm) was continuously circulated at 120 L h^{-1}. For all 6 conditions, spinach plants were grown by using a hydroponic technique called nutrient film technique (NFT). By using the NFT, large parts of the root are exposed to the air (thereby aerated), except for the part of the root that is submerged in culture solution. The experiment was conducted over a 28-day period, after which all spinach plants were harvested simultaneously. Five plants from each experimental condition were analyzed for the different components, while another 5 plants were used to measure the fresh weight, dry weight, and the leaf area of the largest leaf.

2.2.2 Quantification of ascorbic acid and nitrate ion content

Ascorbic acid content and nitrate ion concentration were measured using a reflection photometer (RQflex 10; Merck, Tokyo, Japan). Spinach was placed in a blender with 5% metaphosphoric acid and blended to a liquid form. The liquid was further diluted by adding 5% metaphosphoric acid, and solid components were removed using a centrifuge (Centrifuge 5415R; Eppendorf, Tokyo Japan). Tsukazawa [21] reported that both HPLC and RQflex produced very similar ascorbic acid content values; hence, correction was unnecessary. Nitrate ion content was measured in a manner similar to that for ascorbic acid, except that the spinach was blended using reverse osmosis water. Subsequent analysis procedures were the same as those for ascorbic acid. HPLC and RQflex measurements of nitrate ion concentration content in spinach were previously shown to be highly correlated [20].

2.2.3 Quantification of soluble solid content

Sugar content was measured using a Brix meter (POTSDTM1; Thanko, Tokyo, Japan), following the method reported by Shishido [17]. Leaf stems were collected from the leaf

with the maximal length and the facing leaf. Both stems were mashed using a muddler. A

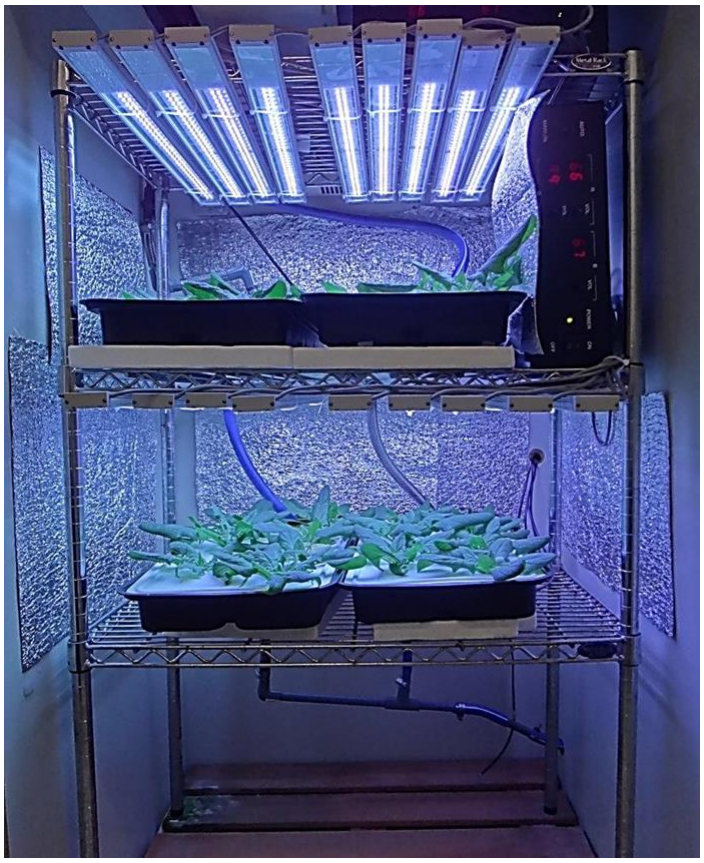

Fig.2-1　Spinach cultivated in a chamber

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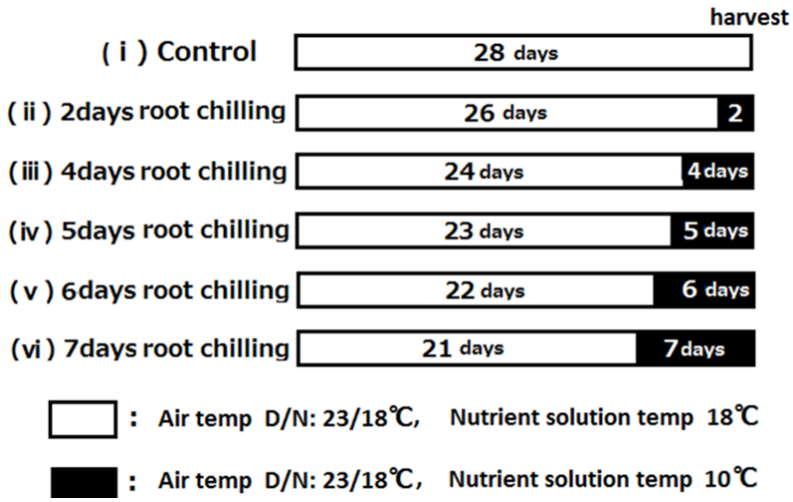

Fig.2-2　Schematic diagram of 6 experimental conditions. The experiment was conducted for 28 days, after which all spinach plants were harvested simultaneously.

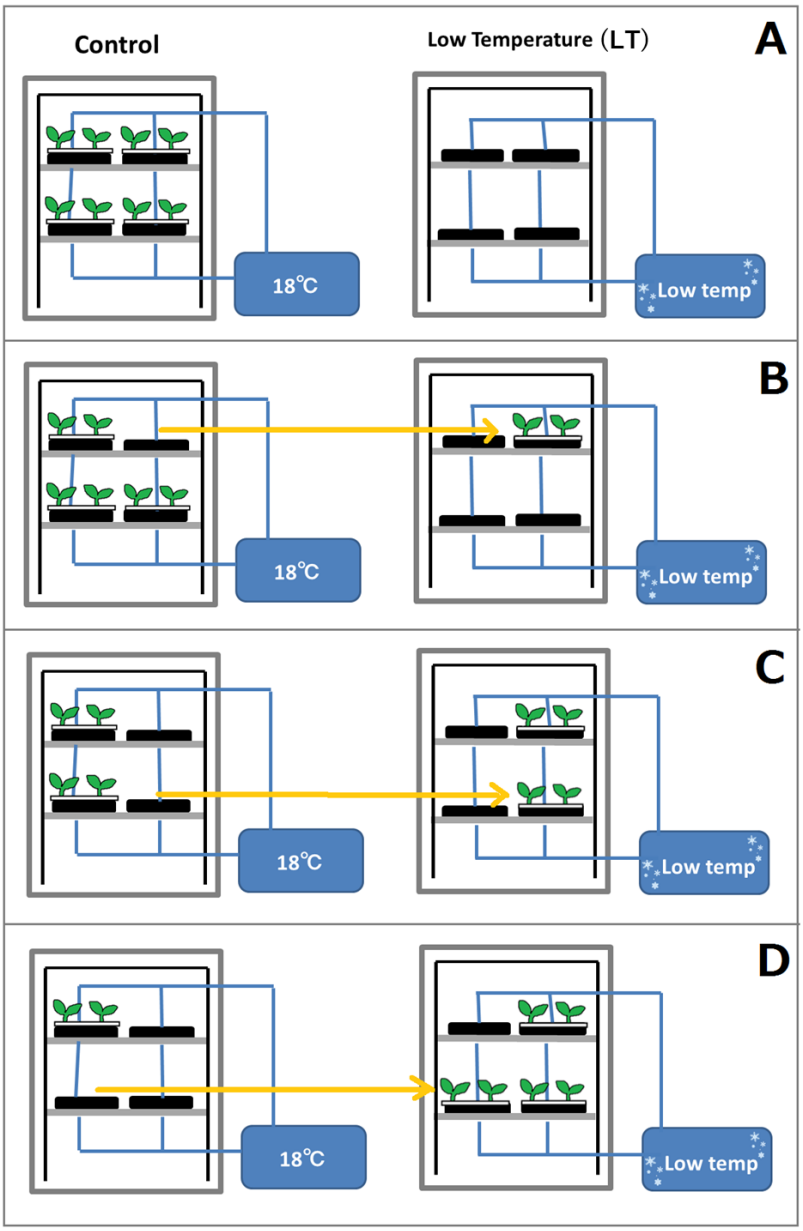

Fig. 2-3 Schematic diagram of the spinaches cultivation experiment. Spinach plants were grown under the above control condition for 20 days (A). On the 21st day, 10 plants were transferred to a different growth chamber, with a nutrient solution temperature of 10 °C (B). Subsequent transferring of 10 plants each was made on the next day (C), followed by 2, 3, and 5 days later. Thus, spinach plant roots were exposed to LT conditions for 2, 4, 5, 6, and 7 days.

drop of the filtrate was then placed onto the Brix meter for measuring the sugar content.

2.2.4 Sampling and analysis

The presented data for the growth parameters are the means of 5 replicates standard deviation (SD). Ryan's multiple comparison test was performed using software, and statistical significance was set at $P>0.05$.

2.3 Results and discussion

The physical appearances of spinach under each condition are shown in Fig. 2-4. Control plants appeared similar to plants acclimatized for 2 and 4 days. In contrast, plants acclimatized for 6 and 7 days tended to be shorter and flatter than that of the others. However, none of the plants exhibited any physiological disorder.

Figure 2-5 shows the effects of low temperature at the root area on the fresh weight (Fig. 2-5A), dry matter (Fig. 2-5B), and leaf area (Fig. 2-5C) of spinach. Root exposure to low temperature with increasing growth period resulted in a decrease in fresh weight and leaf area. In contrast, dry matter remained stable during the initial days of the experiment, but noticeably changed by 6 days of acclimatization.

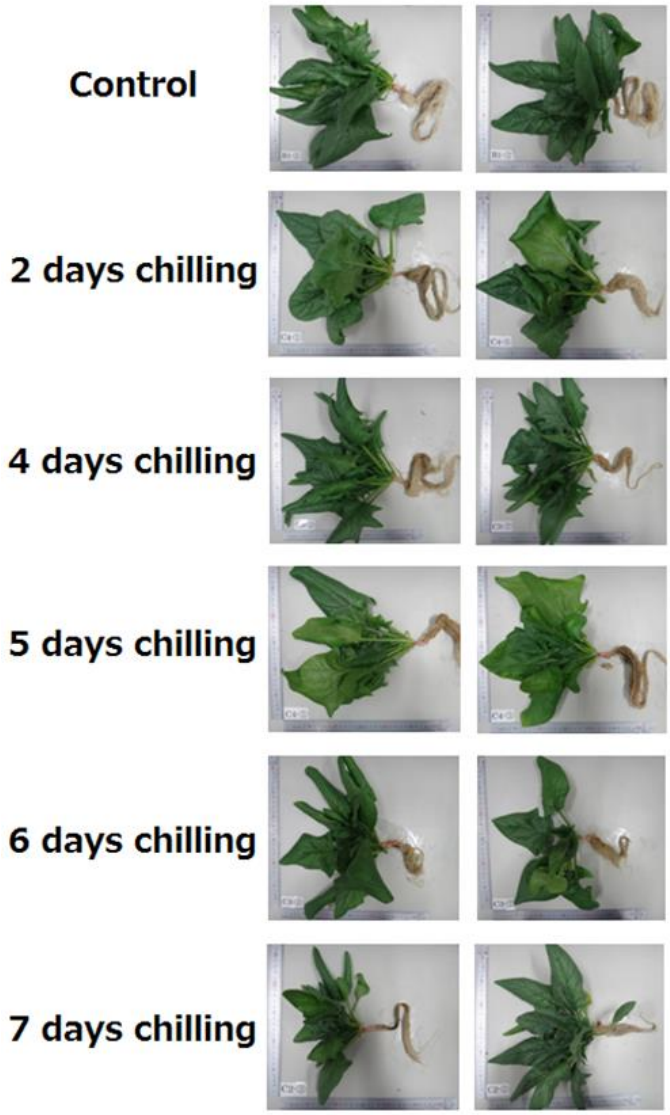

Fig. 2-4　Physical appearances of spinach treated with the different durations of root area chilling of 10°C before the harvest. Control (no chilling) are grown where nutrient solution temperature remained at 18°C.

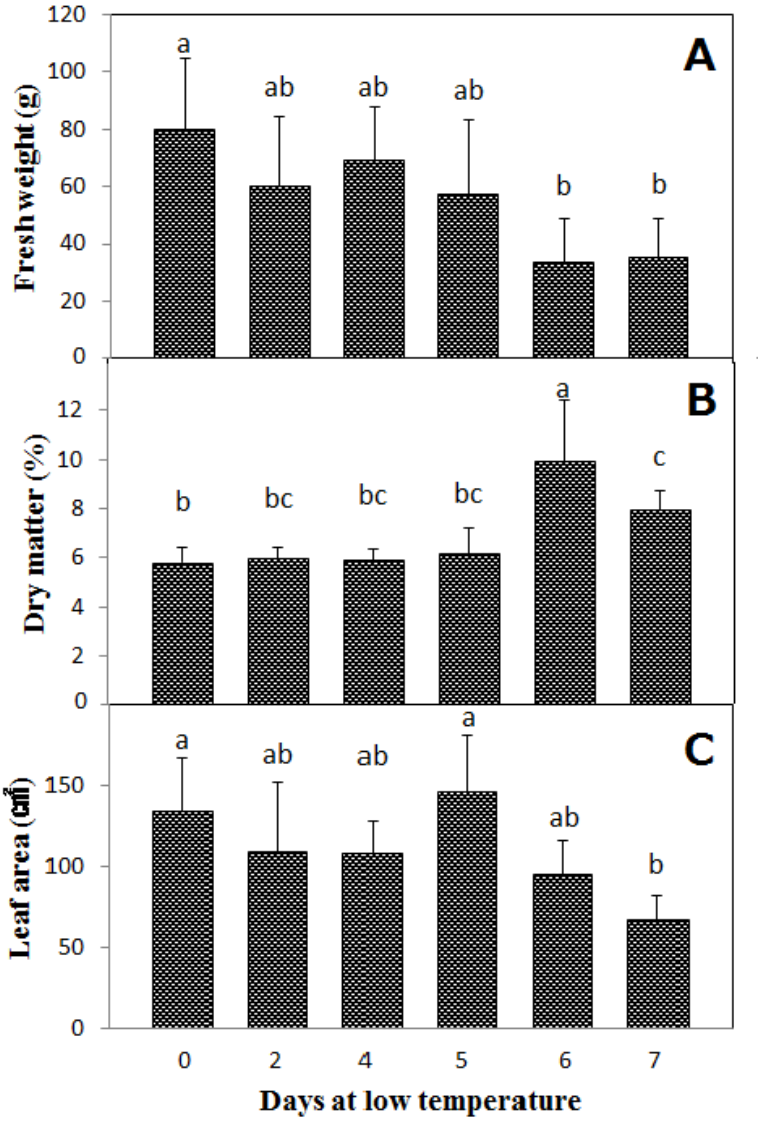

Fig.2-5 Effect of root area acclimatization to low temperature on the fresh weight (A), dry matter (B), and leaf area (C). Dry matter is defined as the value obtained by dividing dry weight by fresh weight, and is expressed in percentage. The vertical bars indicate the SD (*n*=5). Means with different letters within each panel are significantly different at the 5 % level by LSD.

Figure 2-6 shows the effect of low temperature on spinach root area with respect to ascorbic acid content (Fig. 2-6A), nitrate ion concentration (Fig. 2-6B), and sugar content (Fig. 2-6C). Ascorbic acid content remained stable until 5 days of acclimatization, and then exhibited a 100% increase by the 6th day. No significant difference in ascorbic acid content was observed between the 6th and 7th day of plant acclimatization. The nitrate ion concentration decreased within 2 days after the onset of chilling and was much lower than that of the control after 7 days of acclimatization. Sugar content showed a similar trend to that of ascorbic acid. No difference was observed in the sugar content until 5 days of acclimatization, after which it noticeably increased by the 6th day. Hence, spinach roots only needed 6 days to acclimatize to low temperature to increase the dry matter, ascorbic acid, and sugar content, with a simultaneous decrease in nitrate ion concentration. This observation indicates that root area required 6 days to acclimatize to low temperature to exert a beneficial effect on the spinach quality.

We conducted repeated testing focused on 5 and 6 days cold acclimatization that resulted in the rapid change in ascorbic acid and sugar content. It showed reproducible results that ascorbic acid and sugar content remained stable until 5 days of acclimatization, and then significantly increased by the 6th day (Fig. 2-7A, 2-7C). The nitrate ion concentration decreased within 5 days after chilling (Fig. 2-7B). The observed changes in the relative contents of different compounds in spinach may be primarily attributed to 2 plant functions. When the plant body is chilled, water absorption by the roots is suppressed. This phenomenon is caused by an increase in the viscosity of the nutrient solution and the reduced fluidity of the root cell membrane due to reduced activity of aquaporin, which is a protein that transports water across the membrane [8]. The suppression of water absorption by the roots causes osmotic adjustment and antioxidant functions [12, 13].

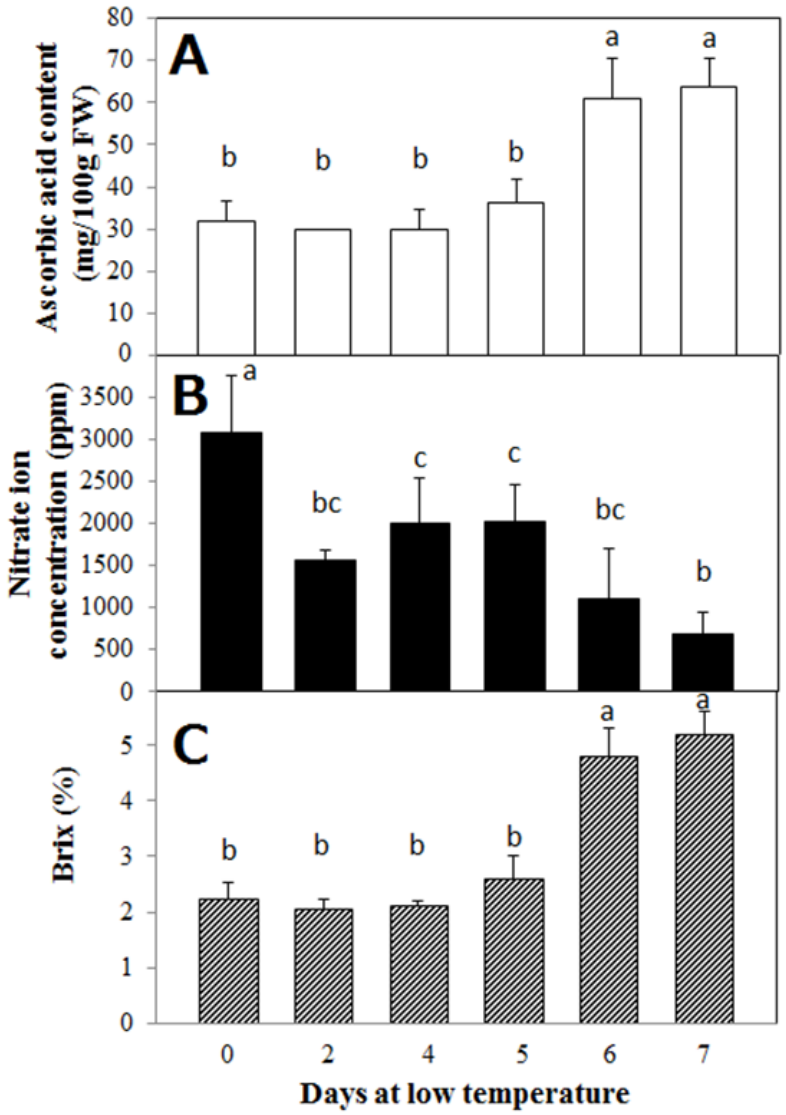

Fig. 2-6 Ascorbic acid (A), nitrate ion concentration (B), and brix (C) in spinach given the different durations of root area chilling of 10 °C before the harvest. The vertical bars indicate the SD (*n*=5). Means with different letters within each panel are significantly different at the 5 % level by LSD.

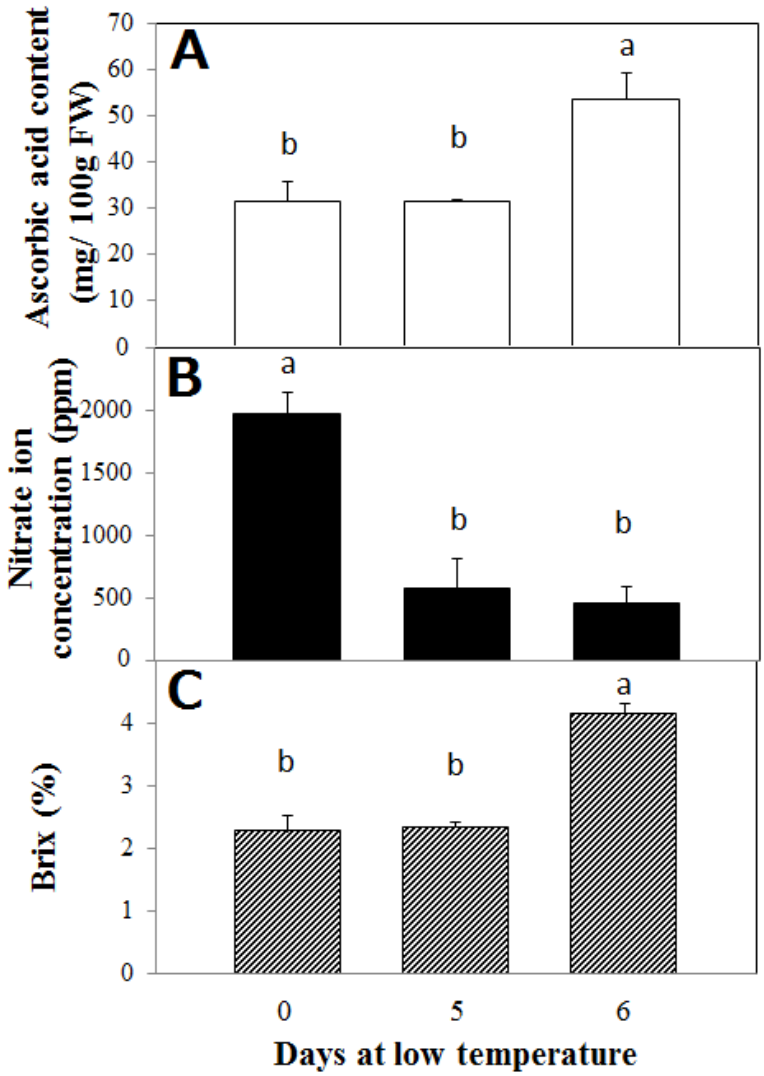

Fig. 2-7　Ascorbic acid (A), nitrate ion concentration (B), and brix (C) in spinach given the different durations of root area chilling of 10 °C before the harvest in the repeated testing. The vertical bars indicate the SD (*n*=5). Means with different letters within each panel are significantly different at the 5 % level by LSD.

Osmotic adjustment helps a plant that is subjected to low temperatures (including freezing) from dehydrating by maintaining the turgor pressure of the plant cell walls when water absorption is suppressed. Simultaneously, a synthetic enzyme produces a solute as a substitute to water, which protects the biogenic substances. This solute consists of sugar and its derivatives. This explains the increase in sugar content in the current experiment. Martindale and Leegood [14] indicated that the ability for photosynthetic CO_2 assimilation at saturating irradiance and saturating CO_2 increased significantly in leaves exposed to 10°C during a 10-day period, with the highest increase occurring after 6 days. Furthermore, the carbon flux of sucrose increased by nearly 2-fold. Holaday et al. [10] reported that spinach exposed to 10°C for 10 days after grown under warm conditions showed an increase in the total enzyme activity, including Rubisco, Fru1, 6-P_2 ase, Sed 1,7-P_2 ase, and sucrose phosphate synthase, with the highest level of activation occurring by the 6th day of chilling. Our results were consistent with those of previous studies.

Antioxidants remove active oxygen species. In general, active oxygen levels are increased under conditions of low temperature and excess light [16]. The accumulation of active oxygen in the body has a negative effect on both plant and human health. Ascorbic acid plays a significant role as an antioxidant that reduces the hydrogen peroxide decomposed by superoxide dismutase to form water. We assume that root area cooling increases ascorbic acid content in the plant body due to the generation of active oxygen.

In comparison, nitrate ion concentration decreased due to plant activity being sustained at low temperatures. Decreased water absorption by the roots suppressed the absorption of nitrate ions from the soil. However, the plant body must produce amino acids and synthesize proteins to sustain life activity; consequently, nitrate ions that have previously accumulated in plant body are consumed as a source of nitrogen. As a result, the nitrate ion concentration decreased with increased duration of root area cooling in the current experiment as Aoki [1] supposed. The results of this study showed that root area chilling of spinach increased ascorbic acid and sugar content, along with simultaneous decrease in nitrate ion concentration. These findings correspond to previously published results by Proietti et al. [15] and Kitano et al. [12]. Sugiyama and Hirooka [18] and Davies [7] suggested that the requirement for oxalic acid to adjust pH decreases with decreasing nitrate ion concentration. Therefore, through decreasing nitrate ion concentrations, oxalic acid content (which is also detrimental to human health) might be reduced in cold-acclimatized spinach. A number of studies have investigated the relationship of ascorbic acid and sugar content with the content of other useful compounds in cold-acclimatized spinaches, in which the content of carotene and -tocopherol increased after chilling [11].

Therefore, the content of other highly beneficial compounds might have also increased parallel to the increase in ascorbic acid and sugar contents in the current experiment.

Many researchers have investigated the relationship of antioxidant function with light. Bartoli et al. [2] found that high light intensity enhances the ability of plants to synthesize ascorbic acid; consequently, excess light serves as an effective environmental condition that increases the ascorbic acid content of the plant body. Therefore, it might be possible to produce higher-quality vegetables by growing plants under a combination of cold stress and high light stress.

Although we concluded that root area chilling for 6 days is sufficient to increase the nutritional value of spinach, we should examine the influence of other factors; growth stage to give cold stress and solution temperature at LT condition. Zhao [22] and Bergquist [3] reported that the antioxidant level depend on the growth stage, so plant cells having higher antioxidant capacity could produce more antioxidants, ascorbic acid when they are exposed to cold stress. Similarly, our results give the possibility that the nutritional quality of spinach is determined by the temperature of nutrient solution before harvest, suggesting that root area chilling for shorter duration than 6 days could be adequate to enhance the nutritional quality by lowering the solution temperature than 10°C. Further investigations are required to elucidate the best environmental condition to produce the high value-added spinach, and those studies are in progress.

This study did not conduct experiments in terms of enzyme and gene expression; hence, we were only able to infer the reasons for the response of spinach plants to root area chilling. Future research should focus on elucidating synthesis pathways and stress responses to better explain the results of the current study. Here, we investigated the relationship between the period of root area chilling and the increase in nutritional quality of spinach. Future studies should focus on determining the optimal low water temperature to enhance plant nutritional value, which would further contribute to the production of high-quality vegetables at low cost.

2.4 Conclusion

Here, we identified the optimal duration of spinach root area chilling to increase ascorbic acid and sugar content without increasing nitrate ion concentration. At the onset of root area chilling, ascorbic acid and sugar contents remained unchanged, while nitrate ion concentration immediately decreased. After 6 days of root area acclimatization to cold temperature, the ascorbic acid and sugar contents markedly increased, with no significant difference in content being observed between days 6 and 7 of chilling. Our results indicate

that 6 days of root area acclimatization to low temperature is sufficient to enhance the nutritional quality of spinach.

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Chapter 3

Quantitative Relationship of the Nutritional Quality of Spinach with Temperature and Duration in Root Area Chilling Treatment

3.1 Introduction

The research in the previous chapter has indicated that 6 days is the optimal duration for root area chilling to produce high quality spinach, when the temperature of the nutrient solution is controlled at 10°C under plant factory conditions [4].

Although root area chilling is known to enhance the nutritional quality of spinach, information remains limited about the relationship between chilling temperature, the duration of cold treatment, and the nutritional quality of spinach. By regulating the temperature of the culture solution below 10°C, a greater amount of valuable constituents may accumulate over a shorter duration (i.e., less than 6 days).

This study aimed to reveal the triadic relationship among nutrient solution temperature during chilling treatment, chilling duration, and the nutritional components of spinach. Specifically, changes in the content of ascorbic acid, sugar, and nitrate ions were calculated to evaluate the nutritional quality of spinach cultivated under different temperature conditions and timeframes. The results were used to quantify the minimum degree of cold stress required to enhance the nutritional value of spinach. This information is anticipated to contribute towards establishing cultivation technology that produces high-quality vegetables with lower power consumption under plant factory conditions.

3.2 Materials and methods

3.2.1 Plant materials and conditions

Spinach (Spinacia oleracea L. 'Active') seeds were sown on a water-soaked sponge made of flexible polyurethane foam. After one week, the germinated seedlings were transplanted to a hydroponic system in a growth chamber (KCLP-1000, Nippon Medical & Chemical Instruments Co., Ltd., chamber 1, Osaka, Japan). The cultivation conditions were set at a photoperiod of 14 h (06:00 h to 20:00 h), a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1} provided by LED lights (NE02-000089 (01); Shibasaki, Saitama, Japan) installed at 325 mm above the cultivation panel, a day/night air temperature regime of 23/18°C. The temperature of the nutrient solution was set at 18°C and was adjusted to an electric conductivity (EC) of 1.2 using Otsuka-A prescription (OAT Agrio Co., Ltd., Tokyo, Japan). Ten plants under each experimental condition were used. The temperature of the nutrient solution was controlled by a water temperature controller (ZC-700; Zensui, Japan), and the nutrient solution pumped from the tank was continuously circulated. The temperature of the rhizosphere of each cultivation panel was monitored by a thermo coupled sensor and data logger (CR-10X; Campbell Scientific, Logan, Utah, USA).

Spinach plants were grown under 24 different experimental conditions. First, all plants were grown under control conditions in chamber 1 (Fig. 3-1A). On the 21st day (as shown in Fig. 3-1B), 10 plants were transferred to a hydroponic system in a second growth chamber (chamber 2), in which the nutrient solution temperature was regulated at 4, 6, 10, and 14°C. On the following day, a further 10 plants were transferred to the hydroponic system in the second growth chamber (Fig. 3-1C), with another 10 plants being transferred 2, 3, and 5 days later. Thus, spinach plant roots were exposed to different low temperature conditions for the respective durations of 2, 4, 5, 6 and 7 days. In this procedure, the root area of spinach plants was subjected to cold stress for different durations. The experiment was repeated for all four temperature conditions (4, 6, 10, and 14°C). For all 24 experimental conditions, spinach plants were grown using the hydroponic "nutrient film technique" (NFT). When using the NFT, large parts of the root are exposed to the air, except for the root part that is submerged in the culture solution. The experiments of different chilling durations were conducted in succession at a constant temperature of the nutrient solution in chamber 2 to achieve the all experiment combinations mentioned above. The experiment was conducted over a 28-day period, after which all spinach plants were harvested simultaneously. Five plants from each experimental condition were analyzed for the different components, while another five

plants were used to measure the fresh weight, dry weight, and area of the largest leaf.

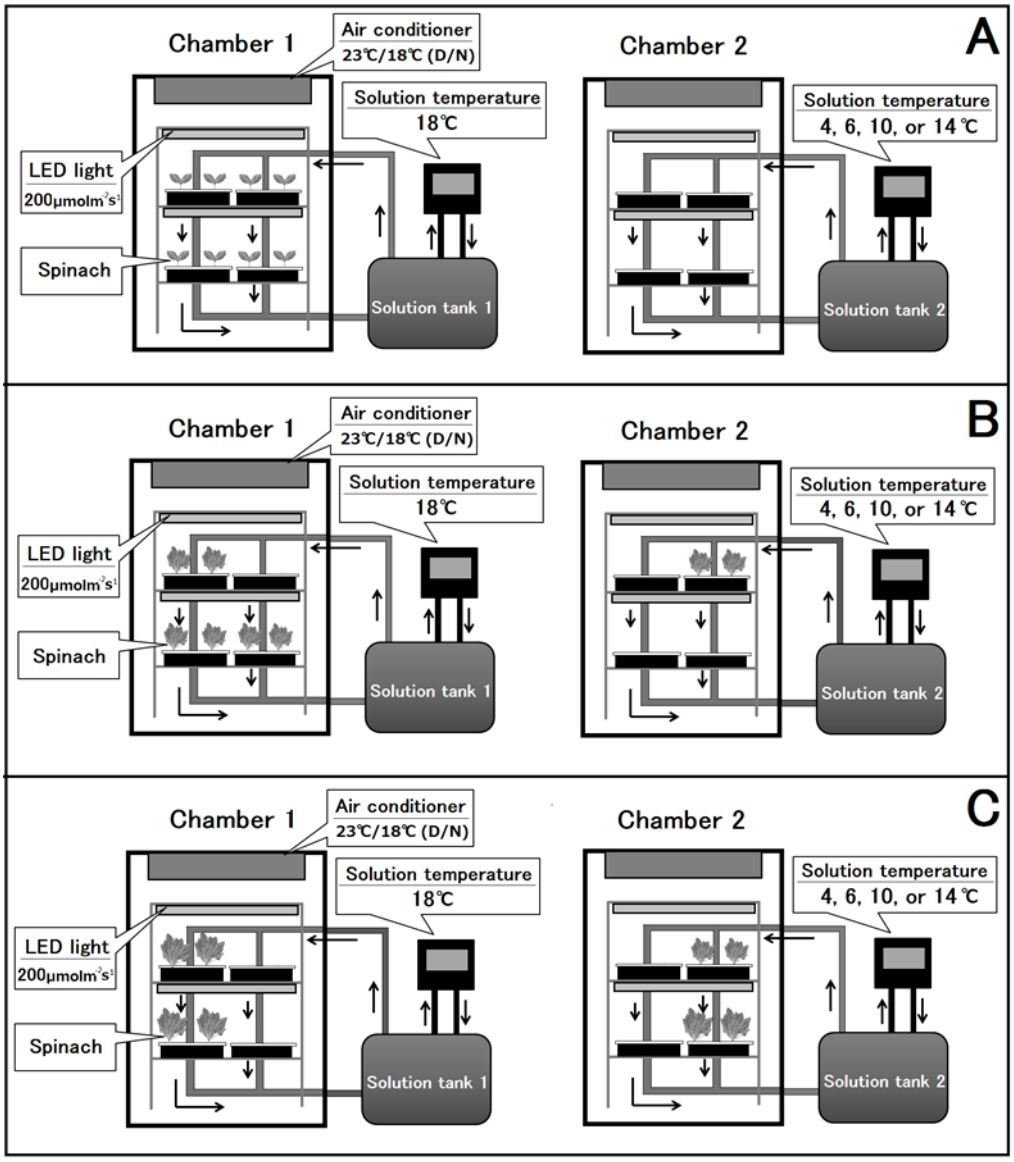

Fig. 3-1. Schematic diagram of the spinach cultivation experiment. Spinach plants were grown under control conditions in chamber 1 for 20 days (A). On the 21st day, 10 plants were transferred to chamber 2, where the temperature of the nutrient solution was regulated at 4, 6, 10, and 14°C (B). Further 10 plants were transferred the following day (C), and then after further 2, 3, and 5 days.

3.2.2 Quantification of ascorbic acid and nitrate ion content

Ascorbic acid content and nitrate ion concentrations were measured using a reflection photometer (RQflex 10; Merck, Tokyo, Japan). The spinach was placed in a blender with 5% metaphosphoric acid, and was blended to form a liquid. The liquid was further diluted by adding 5% metaphosphoric acid. The solid components were then removed by centrifugation (Centrifuge 5415R; Eppendorf, Tokyo, Japan). Tsukazawa [11] reported that both high performance liquid chromatography (HPLC) and RQflex produce very similar ascorbic acid content; hence, correction was unnecessary. Nitrate ion content was measured in a similar manner to that of ascorbic acid, except that the spinach was blended using reverse osmosis water. Subsequent analytic procedures were the same as those used for ascorbic acid. HPLC and RQflex measurements of nitrate ion content in spinach were previously shown to be highly correlated [10].

3.2.3 Quantification of soluble solid content

The measurement of total soluble solids in vegetables gives a fairly good indication of the sugar content. Soluble solid content was measured using a Brix meter (POTSDTM1; Thanko, Tokyo, Japan), following the method reported by Shishido [8]. Petioles were collected from the leaf with the maximal length and the facing leaf. Both stems were mashed using a muddler. A drop of the filtrate was then placed onto the Brix meter to measure the soluble solid content.

3.2.4 Sampling and analysis

The data presented for the growth parameters are the means of five replicates standard deviation (SD). Ryan’s multiple comparison test was performed with statistical significance accepted at $P>0.05$.

3.3 Results and discussion

The physical appearance of the spinach plants subjected to each of the 24 experimental conditions is shown in Fig. 3-2. At the same solution temperature, plants tended to be increasingly shorter and flatter with increasing duration of root chilling. For the same duration of chilling treatment, the size of spinach plants increased with increasing solution temperature. However, none of the plants exhibited any physiological disorders.

The effect of different levels of cold stress to the root area on the fresh weight of the aerial part, the dry matter percentage, and the leaf area of the largest leaf are shown

in Tables 3-1, 3-2, and 3-3, respectively. Under the same solution temperature conditions, the fresh weight and leaf area decreased with increasing chilling duration. For the same duration of root chilling, the fresh weight and leaf area increased with increasing solution temperature. In comparison, dry matter percentage showed the opposite trend. Under the same solution temperature conditions, the dry matter percentage increased with increasing chilling duration, except for the when the solution temperature was regulated at 14°C.

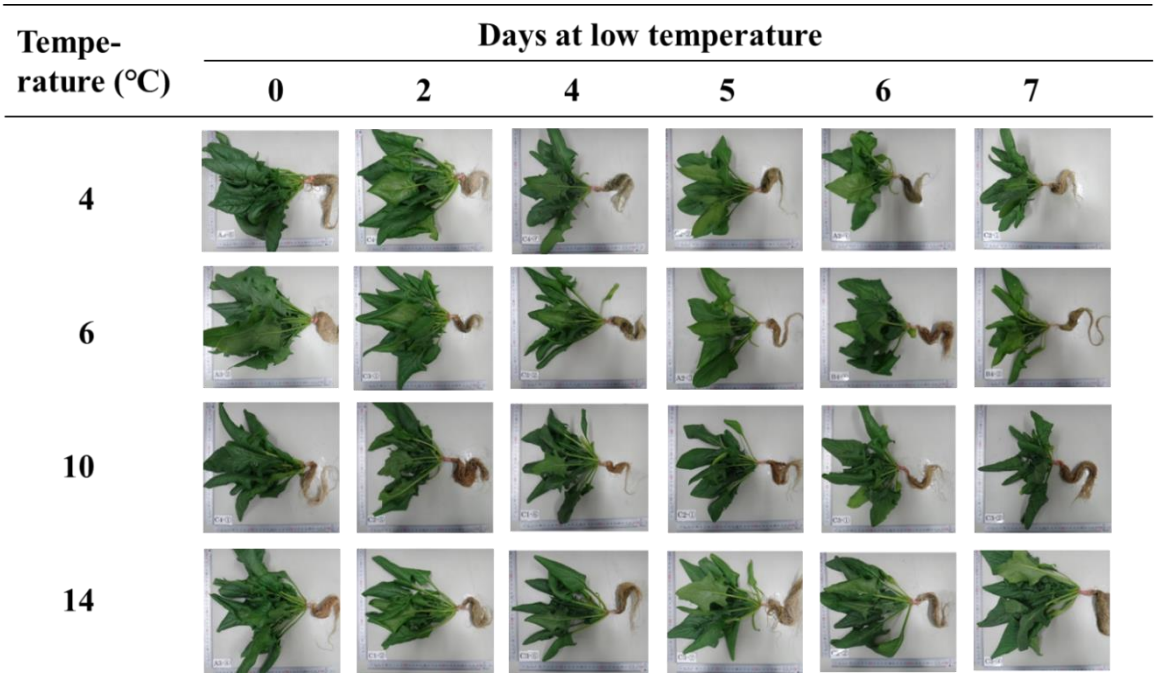

Fig. 3-2. Physical appearance of spinach plants subjected to different levels of chilling stress to the root area before harvest. Control (four pictures on the left), spinach plants were grown for 0 days at low temperature in the nutrient solution at 18°C.

Table 3-1 Effect of different levels of cold stress to the root area on the fresh weight (g) of the aerial part of the plant.

Temperature (℃)	Days at low temperature					
	0	2	4	5	6	7
4	76.4±9.70 a	78.2±17.6 a	35.3±16.1 b	26.8±5.63 b	30.0±10.1 b	28.3±6.35 b
6	72.8±28.1 a	55.7±16.5 ab	50.2±11.0 ab	30.7±4.74 b	29.0±9.07 b	25.6±5.61 b
10	79.4±25.4 a	60.3±24.1 ab	68.6±19.3 ab	57.0±26.5 ab	33.4±15.1 b	34.8±14.2 b
14	75.6±29.1 a	50.4±13.8 ab	43.6±13.7 b	46.4±14.3 ab	45.1±12.9 ab	38.8±10.5 ab

NB) Means ± standard deviations. Different lower case letters in each horizontal row denote significant differences by Ryan's multiple comparison test at $P < 0.05$ ($n = 5$).

Table 3-2 Effect of different levels of cold stress to the root area on the dry matter percentage (%).

Temperature (℃)	Days at low temperature					
	0	2	4	5	6	7
4	5.86±0.34 b	6.04±0.43 b	9.64±2.93 a	9.41±1.20 a	9.60±2.08 a	9.80±1.35 a
6	6.28±0.30 bc	5.63±0.20 c	6.56±0.61 bc	8.04±1.15 ab	7.46±0.96 bc	9.56±1.23 a
10	5.80±0.62 b	5.91±0.52 bc	5.89±0.43 bc	6.10±1.09 bc	9.85±2.55 a	7.91±0.81 c
14	6.01±0.35 n.s.	5.23±0.35	5.19±0.97	5.67±0.79	6.79±0.69	7.33±2.04

NB) Dry matter percentage is defined as the value obtained by dividing the dry weight by the fresh weight, and is expressed as a percentage. Means ± standard deviations. Different lower case letters in each horizontal row denote significant differences by Ryan's multiple comparison test at $P < 0.05$ ($n = 5$).

Table 3-3 Effect of different levels of cold stress to the root area on the leaf area (cm^2) of the largest leaf.

Temperature (℃)	Days at low temperature					
	0	2	4	5	6	7
4	143±37.0 ab	144±20.9 a	93.9±17.8 bc	71.5±8.88 c	72.0±11.1 bc	79.0±21.4 c
6	102±21.4 ab	121±20.9 a	124±24.2 a	95.6±9.98 ab	70.1±14.5 b	70.5±11.5 b
10	134±32.9 a	109±43.3 ab	108±20.4 ab	145±35.6 a	94.0±21.9 ab	66.0±15.9 b
14	115±35.0 n.s.	112±34.6	120±16.8	132±19.1	80.0±11.9	119±32.0

NB) Means ± standard deviations. Different lower case letters in each horizontal row denote a significant difference by Ryan's multiple comparison test at $P < 0.05$ ($n = 5$).

Figure 3-3 shows the effect of the duration of root area chilling on ascorbic acid content under each solution temperature. Under all solution temperature conditions, the ascorbic acid content increased with increasing chilling duration. The experimental duration that first showed a significant increase in ascorbic acid content compared to the control differed depending on the solution temperature. The chilling duration that first showed a significant change in ascorbic acid content was 4 days at 4°C, 5 days at 6°C, 6 days at 10°C, and 7 days at 14°C. The chilling duration required to increase ascorbic acid content tended to be shorter with decreasing solution temperature. Because ascorbic acid content might have already risen by 3 days of chilling at 4°C, an additional experiment was conducted.

The ascorbic acid content value after 3 days of root chilling at 4°C was 38.2 ± 3.96 (mg/100 g FW), and was not significantly different to the control (no chilling). Therefore, this confirmed that the duration of root area chilling first showed a significant increase after 4 days at 4°C. Figure 3-4 shows the effect of root area chilling duration on nitrate ion concentration for each solution temperature.

Under all temperature conditions, nitrate ion concentration declined significantly within 2 days of the onset of chilling. Nitrate ion concentration decreased with increasing chilling duration under the same solution temperature conditions. Figure 3-5 shows the effect of root area chilling duration on soluble solid content for each solution temperature. Soluble solid content showed a similar trend to that of ascorbic acid. The chilling duration at which soluble solid content first significantly increased compared to the control differed depending on the solution temperature of the root area chilling. As observed for ascorbic acid content, the chilling duration at which soluble solid content first significantly changed was 4 days at 4°C, 5 days at 6°C, 6 days at 10°C, and 7 days at 14°C. The chilling duration required for soluble solid content to increase tended to decline with decreasing solution temperature. Because soluble solid content might already have increased by 3 days of chilling at 4°C, an additional experiment was conducted. The Brix value after 3 days of root chilling at 4°C was 2.62 ± 0.20 (%), and was not significantly different from the control (no chilling). Therefore, we confirmed that the duration of root area chilling first showed a significant increase after 4 days at 4°C.

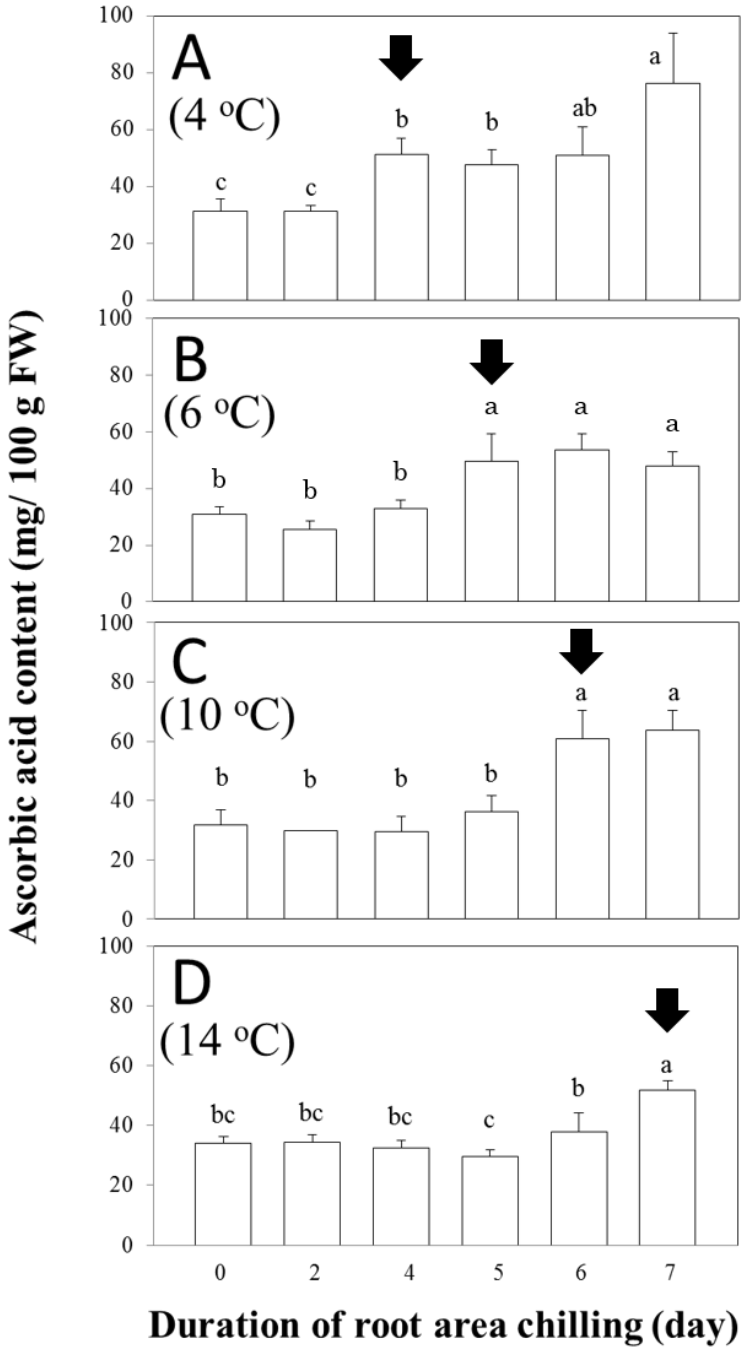

Fig. 3-3. Effect of the duration of root area chilling at 4°C (A), 6°C (B), 10°C (C), and 14°C (D) on the ascorbic acid content of spinach. The vertical bars indicate the SD (n = 5). Means with different letters within each panel are significantly different at the 5 % level by Ryan's multiple comparison test.

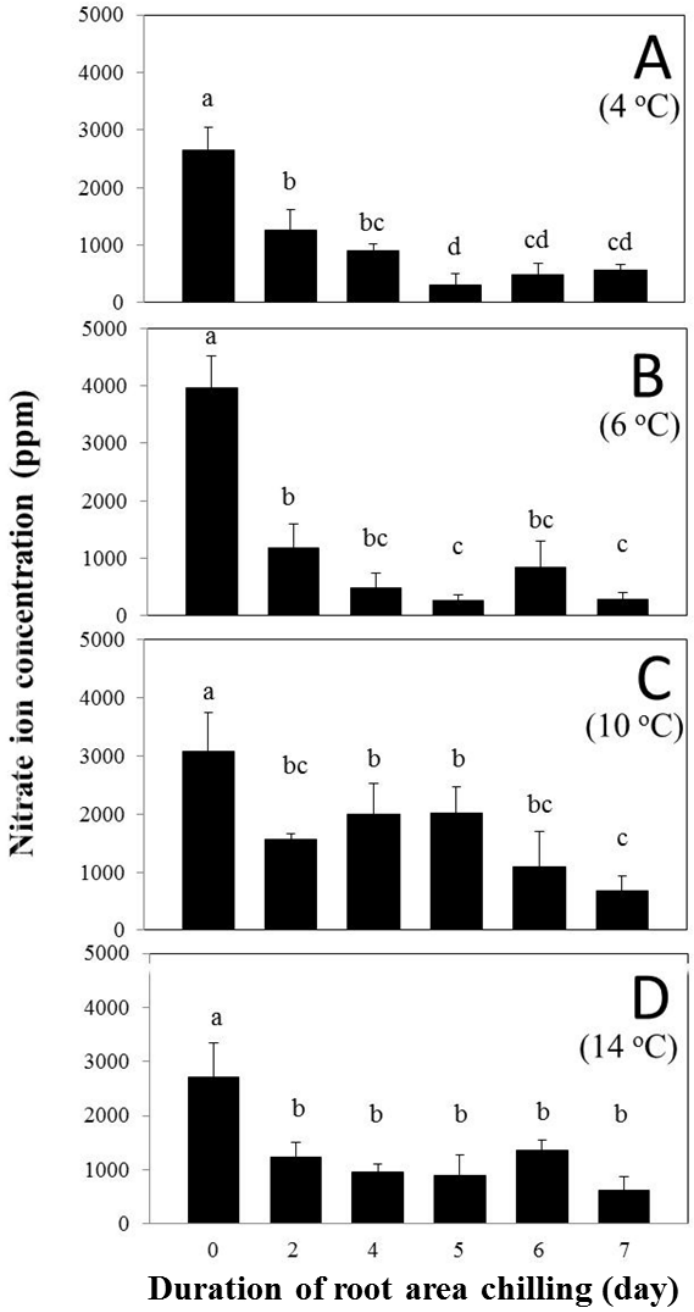

Fig.3-4. Effect of the duration of root area chilling at 4°C (A), 6°C (B), 10°C (C), and 14°C (D) on the nitrate ion concentration of spinach. The vertical bars indicate the SD (n = 5). Means with different letters within each panel are significantly different at the 5 % level by Ryan's multiple comparison test.

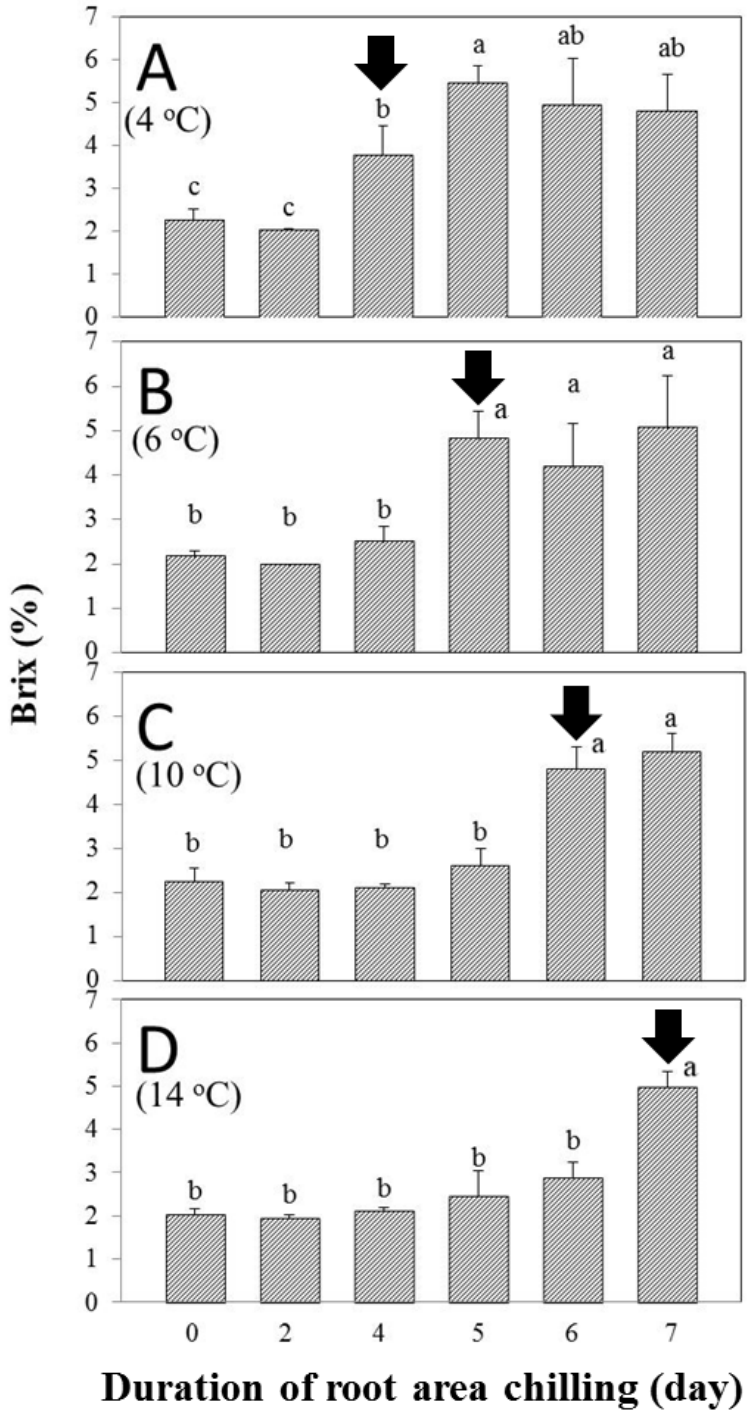

Fig. 3-5. Effect of the duration of root area chilling at 4°C (A), 6°C (B), 10°C (C), and 14°C (D) on the Brix of spinach. The vertical bars indicate the SD (n = 5). Means with different letters within each panel are significantly different at the 5 % level by Ryan's multiple comparison test.

When measuring the amount of ingredients contained in spinach at different levels of cold stress to the root area, ascorbic acid and soluble solid content exhibited a similar pattern (Figs. 3-3 and 3-5). For both compounds, a significant increase was observed 4 days after the initiation of root area chilling at 4°C, 5 days at 6°C, 6 days at 10°C, and 7 days at 14°C indicated by black arrows in Figs. 3-3 and 3-5. Figure 3-6 shows the relationship between solution temperature and the chilling duration at which a significant change in ascorbic acid and soluble solid content was first observed in the x - y plane. These data could be approximated to the line l_1 in Fig. 3-6, and were expressed as:

$$y = 0.288x + 3.05 \quad (1)$$

where x is the solution temperature and y is the duration of root area chilling. The triadic relationship between solution temperature, chilling duration, and the amount of each component was determined from the plane parallel to line l_2 , which was expressed as:

$$y = -3.47x \quad (2)$$

linel_2 is perpendicular to line l_1, and passes through the origin. line l_2 was defined as the "x-axis," with the intersection of line l_1 and line l_2 being the origin. The distance between line l_1 and each point was calculated.

The x-coordinate of each dataset was computed according to the following definition:

$$y \geq 0.288x + 3.05 \quad \Rightarrow X = -d \quad (3)$$

$$y \leq 0.288x + 3.05 \quad \Rightarrow X = d \quad (4)$$

Figures 3-7 and 3-8 show the replotting of each dataset on the x - y plane, where X is the value described in the above equations and Y is the ascorbic acid and soluble solid content, respectively. These two figures show that ascorbic acid and soluble solid content exhibited a sharp increase at the point $x = 0$. Thus, by adjusting x and y, so that $y \geq 0.288x + 3.05$, it is possible to produce high value-added spinach in plant factories.

We used the experimental results to develop the relational expression for solution temperature, the duration of root area chilling, and ascorbic acid and soluble solid content. When rhizosphere chilling is implemented by plant factories in Japan and other countries to improve the quality of spinach, the facilities should limit either the number of days spent at low temperatures or the lowest temperature of the solution. In such cases, the relational expression could be used to obtain the minimum solution temperature and the chilling duration required to improve nutritional quality, which would enhance the productivity of the plant factory.

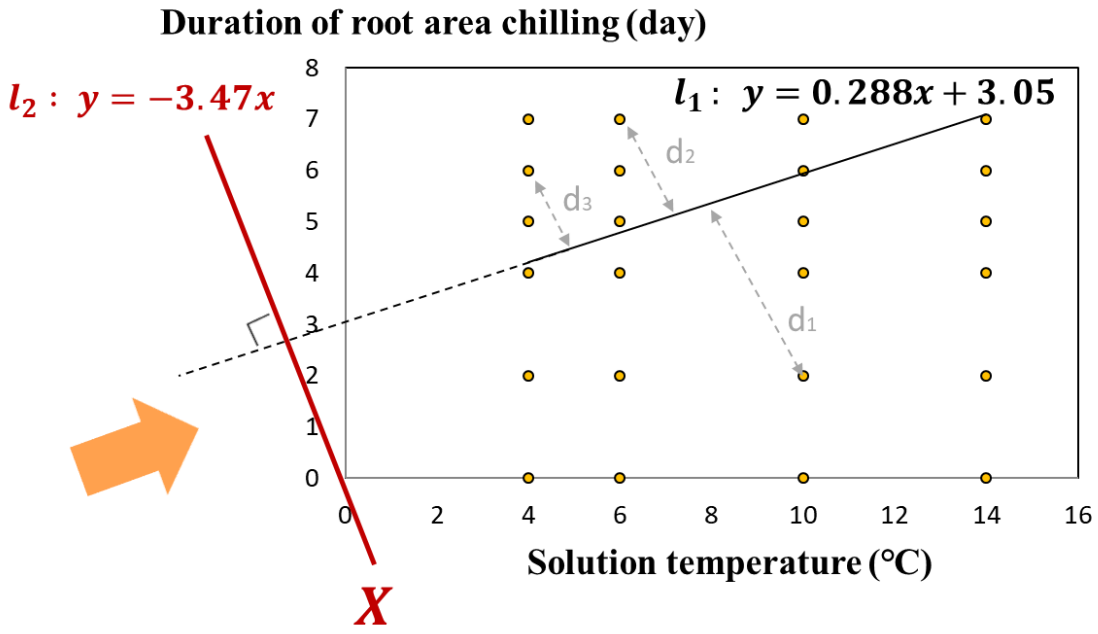

Fig. 3-6. The triadic relationship of solution temperature, chilling duration, and the amount of each component viewed from the plane panel parallel to line l_2.

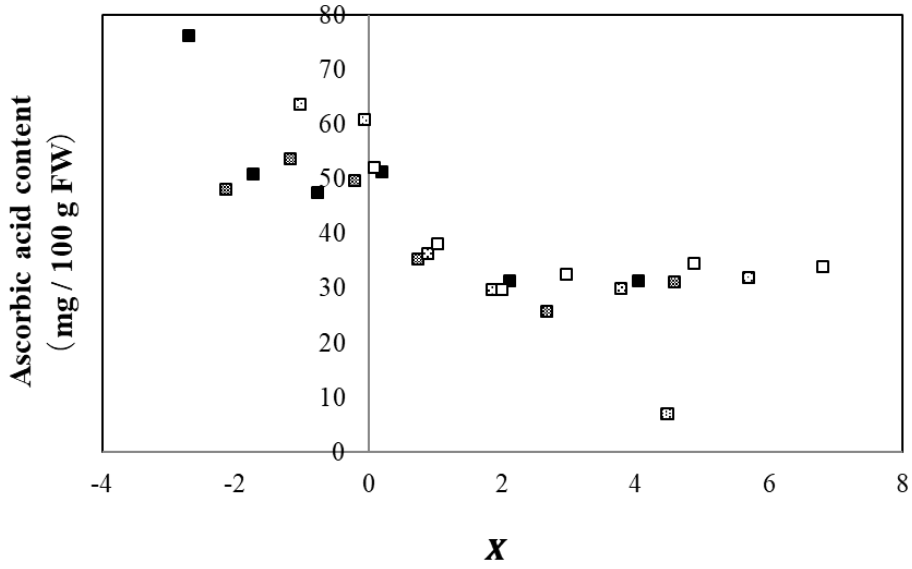

Fig. 3-7. Relationship between the *x*-value and the ascorbic acid content. *x* is the value determined from the distance between line l_1 in Fig. 3-6 and each data point.
Each symbol indicates a difference in the solution temperature during chilling treatment.

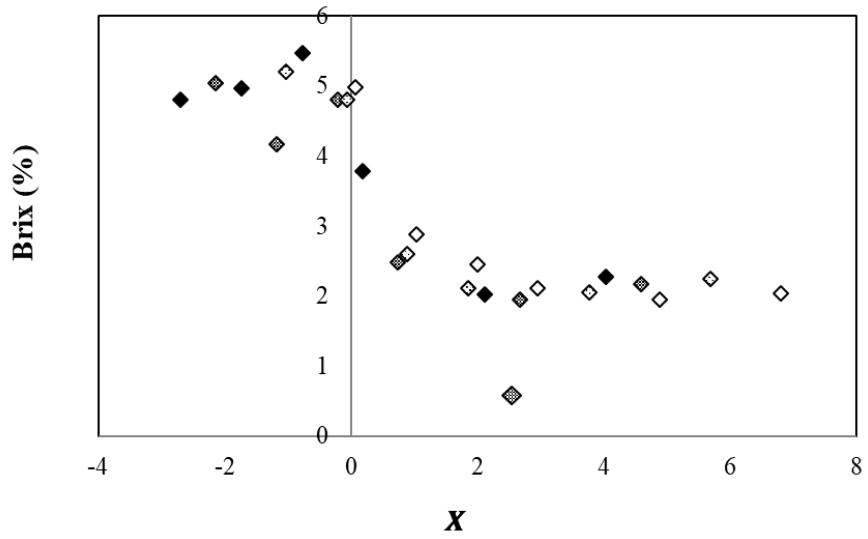

Fig. 3-8. Relationship between the *x*-value and Brix. *x* is the value determined from the distance between line l_1 in Fig. 3-6 and each data point.
Each symbol indicates a difference in the solution temperature during chilling treatment.

In our experiment, nitrate ion concentration declined significantly within 2 days of starting root area chilling (Fig. 3-4). In other words, root area chilling for 2 days is sufficient to decrease nitrate ion concentration only. When the plant body is chilled, water absorption by the roots is suppressed because of an increase in the viscosity of the nutrient solution and the reduced fluidity of the root cell membrane due to the reduced activity of aquaporin, which is a protein that transports water across the membrane [2].

The suppression of water absorption may cause the underground part of the root to respond directly to the change in thermal conditions, without the mediation of the above ground organ. Results from previous studies support the theory that plant roots have temperature sensors and that they regulate water absorption independent of the other parts of the plant. Hydroponically cultivated soybean continues to absorb nitrate ions at the same rate throughout the day, regardless of changes in the environmental conditions, such as light intensity and air temperature. This pattern of nitrate ion absorption was also observed in plants grown under continuous dark conditions or in root-resected aerial parts, and was found to be absent when the roots were maintained at a constant temperature [6]. These results indicate that roots might be able to differentiate between day and night, and that they modulate the absorption rate of nitrate ions as required [7]. In addition, a previous study reported that barley roots detect thermal changes via temperature sensors [9]. The temperature detecting-signal transduction mechanism of the plant body remains poorly understood. However, plant roots are likely to detect thermal changes independent of other organs; this information, along with changes in signal reception and photosynthetic products from the above-ground parts, enable them to regulate their response. Phototropin 1 is a blue light photoreceptor that is located in the top of the plant root, and contributes to root elongation, which induces drought tolerance [3]. If the sensor detects a change to the environment surrounding the plant root, it would be worthwhile enhancing the nutritional quality of the above-ground part by inducing stress or stimulation to the root area alone. Thus, future studies should not only focus on the vegetative response of plants under plant factory environmental conditions, but should also aim to elucidate the transduction mechanism between the aboveground and underground parts. This information could then be applied to establish novel and efficient cultivation technology based on these mechanisms.

The decrease in water absorption caused by cold stress to the root area leads to a decrease in the amount of nitrate ions that are absorbed from the roots. However, the plant body must produce amino acids and synthesize proteins to sustain life. Consequently, nitrate ions that have previously accumulated in the plant body as a source of nitrogen are used [1]. As shown in Fig. 3-4, the control plants (no chilling) at 6°C had the highest

nitrate ion concentration. Assuming that all nitrate ions contained in the plant body were used for the production of amino acids and the biosynthesis of proteins during chilling treatment, the fresh weight, dry matter percentage, and leaf area after chilling treatment would be expected to be highest under all other cultivation conditions. However, plants cultivated under all other conditions had higher fresh weight, dry matter percentage, and leaf area compared to those maintained at 6°C (Tables 3-1, 3-2, and 3-3). This result indicates that other mechanisms contribute to the decrease in nitrate ion concentrations after chilling.

Several studies have reported that plant roots are important for the short- and long-term storage of nitrogen. In particular, woody plants and bulb plants accumulate nitrogen in their roots during winter, and then transfer nitrogen from the root to the developing part in spring [5]. In our experiment, we did not measure the nitrate ion concentration contained in the root area after the chilling treatment. Therefore, we can only speculate on the changes in nitrate ion concentrations under cold stress. Nevertheless, our results indicate that the minimum amount of nitrate ions required to maintain life is regulated by the aboveground part of the plant, with only excess nitrate ions being stored in the root. From this study, several questions remain unanswered. The relational expression for solution temperature, the duration of root chilling, and ascorbic acid and soluble solid content developed in this study is only applicable if x (the solution temperature) is 4°C $\leqq x \leqq$ 14°C. When the temperature of the solution is regulated at less than 4°C, the change in ascorbic acid and soluble solid content remains uncertain. In addition, the relational expression derived in this study might differ depending on the growth stage at which the cold stress is applied. Here, we have limited application of cold stress to the period just before harvest. However, further research will be performed to study the application of cold stress at different growth stages, and its relationship with solution temperature, duration of root chilling, and soluble solid content. Furthermore, this triadic relationship has a histogram-based threshold, with at least 50 experimental conditions being required. Because only 24 experimental conditions were assessed in this study, we could not obtain the threshold value through the histogram. Further studies under various conditions are needed to elucidate the full relationship between root area temperature, duration of low temperature conditions, and the nutritional quality of spinach. The disadvantage of the cultivation technique that currently produces high value-added spinach with root area chilling is a decrease in the size and fresh weight of spinach plants at harvest. Thus, cold stress to the root area using solutions at lower temperatures for short durations may help improve productivity.

3.4 Conclusion

In this study, the triadic relationship between temperature of nutrient solution during root area chilling, duration of root chilling treatment, and the amount of nutritional compounds in spinach was investigated. Nitrate ion concentration significantly decreased within 2 days of the onset of chilling under all temperature conditions. Ascorbic acid and soluble solid content showed a similar trend, with a significant change occurring at a specific duration in each solution temperature, and the equation for the chilling duration and solution temperature was proposed. This study suggests an approach for producing high value-added spinach under factory conditions using the developed equation.

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Chapter 4

Effect of Temperature and Duration of Root Chilling on the Balance between Antioxidant Activity and Oxidative Stress in Spinach

4.1 Introduction

The research in the previous chapter has indicated that the production of high value-added spinach in plant factory using the equation for the chilling duration and solution temperature [6]. Moreover, we hypothesized that change in contents of nutritional compounds by root chilling are due to antioxidant reaction.

Several studies have reported that environmental stress accelerates the production of reactive oxygen species (ROS) in the plant body, which induces oxidative stress and triggers antioxidant pathways to manage them with the production of antioxidant molecules [2]. Under severe stress conditions, oxidative stress markers, including H_2O_2, lipid peroxides (LOOH), as well as lipid peroxidation-derived aldehydes, and oxidized proteins might accumulate in the plant body when ROS generation overcomes antioxidant capacity. For instance, Sakamoto and Suzuki [18] reported that although root chilling increases the levels of beneficial substances, such as anthocyanin, phenols, and ascorbic acid, it also increases the levels of harmful substances, such as hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), which are highly reactive molecules formed under oxidative stress. However, changes in antioxidant capacity and oxidative stress markers in vegetables under different root chilling temperatures and time frames have not been investigated yet.

Here, we measured ascorbic acid content, superoxide dismutase activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, as proxies of antioxidant activity, and MDA content, as an oxidative stress marker, in spinach. This study aimed to investigate the change in antioxidants and oxidized molecules in spinach under different levels of cold stress applied to the root area and to confirm that the above hypothesis is correct.

4.2 Materials and methods

4.2.1 Plant materials and conditions

Spinach (*Spinacia oleracea* L. 'Active') seeds were placed on a water-soaked polyurethane foam sponge. After one week, the germinated seedlings were transplanted to a hydroponic system in a growth chamber (KCLP-1000; Nippon Medical & Chemical Instruments, Osaka, Japan). The cultivation conditions were set at: photoperiod of 14 h (06:00 to 20:00), photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1} at the height of 3 cm from the cultivation panel by LED lights (NE02-000089(01); Shibasaki, Saitama, Japan), and day/night air temperature regime of 23°C/18°C. Relative humidity and CO_2 concentration were not controlled. The electric conductivity (EC) of the nutrient solution was controlled at 1.2 mS cm^{-1} using Otsuka-1/2 prescription (OAT Agrio; Tokyo, Japan). The temperature of the nutrient solution was controlled at 18±0.5°C by a water temperature controller (ZC-700; Zensui, Osaka, Japan), and the nutrient solution pumped from a tank was continuously circulated. The temperature of the rhizosphere of each cultivation tray was monitored with a thermometer (Tetra digital thermometer WD-1; Spectrum Brands Japan, Kanagawa, Japan).

Spinach plant roots were exposed to low temperatures (14°C, 10°C, 7°C, and 4°C) for different periods (2, 4, 5, 6 and 7 days) as described previously by Ito et al. [6]. Spinach plants were grown under 24 different experimental conditions. First, all plants were grown under control conditions for 20 days in chamber 1 (Fig. 4-1A). On the 21st day, 5 plants were transferred to a hydroponic system in chamber 2, in which the nutrient solution temperature was regulated at 4, 7, 10, and 14°C (Fig. 4-1B). On the following day, a further 5 plants were transferred to the hydroponic system in chamber 2 (Fig. 4-1C), with another 5 plants being transferred 2, 3, and 5 days later. Thus, spinach plant roots were exposed to different low temperature conditions for 2, 4, 5, 6 and 7 days. In this procedure, the root area of spinach plants was subjected to cold stress for different durations (Fig. 4-2). The experiment was repeated for all four temperature conditions (4, 7, 10, and 14°C). For all experimental conditions, spinach plants were grown using a hydroponic technique termed "deep flow technique" (DFT), where just part of roots near the stock are exposed to the air, except for the root part that is submerged in the nutrient solution. A 5.5 cm deep cultivation tray was used, and the water depth was 3.5cm. The experiment was conducted over a 28-day period, after which all spinach plants were harvested simultaneously. Five plants from each experimental condition were used to measure the fresh weight of aerial parts, and to analyze the levels of superoxide dismutase activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, and MDA in the

fourth and fifth fully expanded leaves. For the measurement of soluble solid content, petioles collected from the leaf with the maximal length and the facing leaf were used. For the measurement of ascorbic acid content, we used one of two halves of spinach plant removed two petioles of longest leaves and the fourth and fifth leaves.

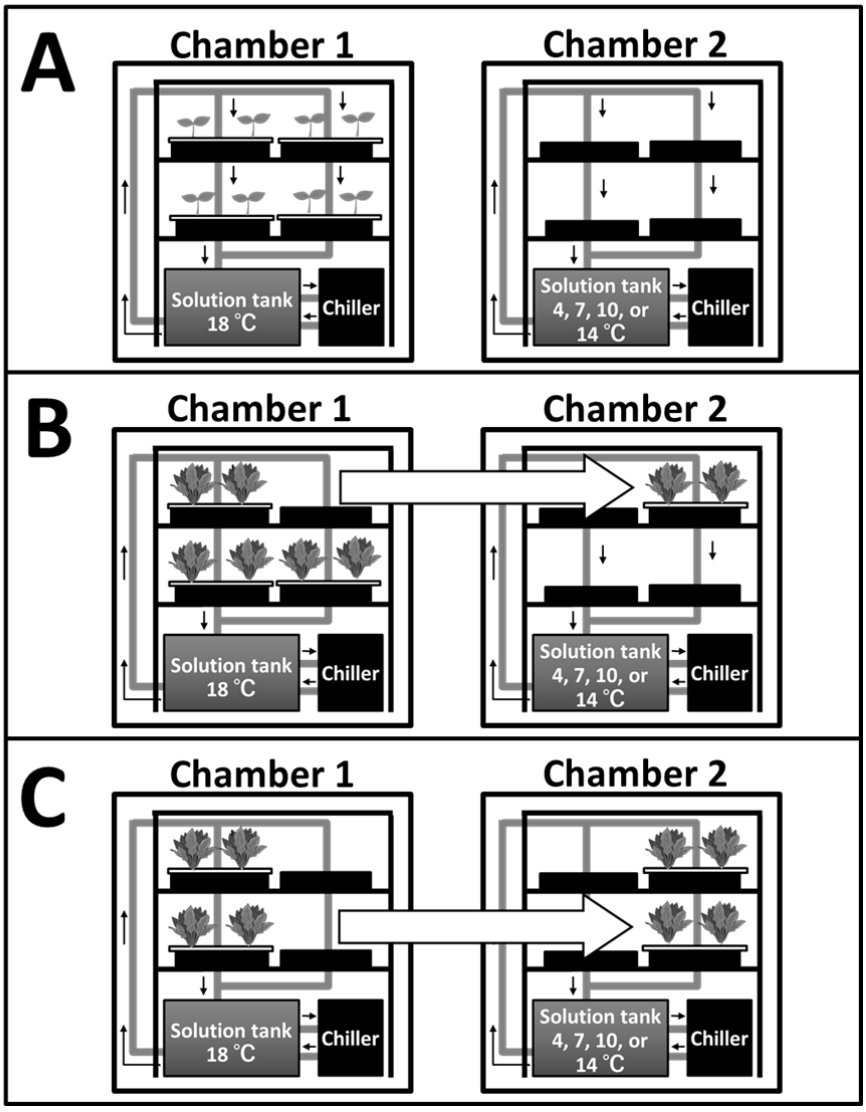

Fig. 4-1. Schematic diagram of the spinach cultivation experiment. Spinach plants were grown under controlled condition in chamber 1 for 20 days(A). On the 21st day, 5 plants were transferred to chamber 2, where the temperature of the nutrient solution was regulated at 4, 7, 10, and 14 °C(B). A further 5 plants were transferred the following day (C), and then after further 2, 3, and 5 days.

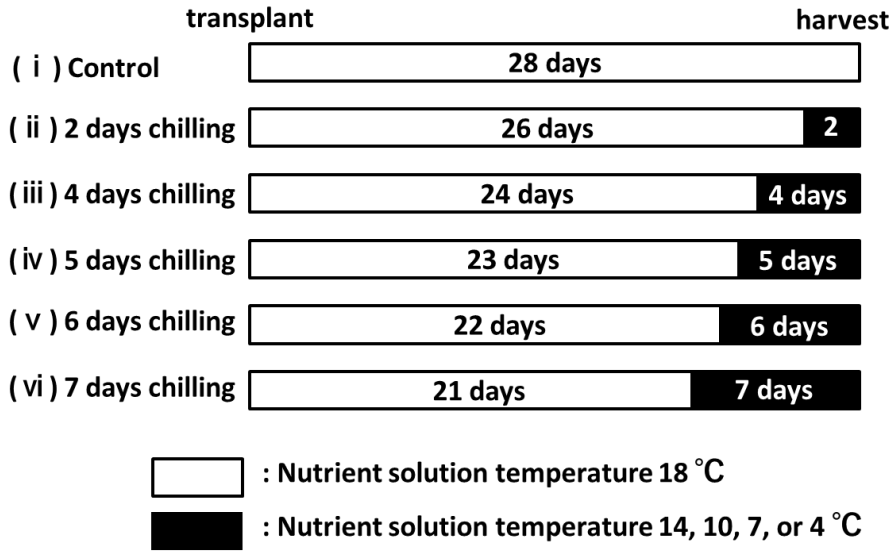

Fig. 4-2. Schematic diagram of experimental conditions. The experiment was conducted for 28 days, after which all spinach plants were harvested simultaneously.

4.2.2 Quantification of antioxidants

Ascorbic acid content was measured using a reflection photometer (RQflex 10; Merck, Tokyo, Japan). Spinach sample was placed in a blender with 5% metaphosphoric acid and was blended to form a liquid. The liquid was further diluted by adding 5% metaphosphoric acid. Insoluble particles were removed by centrifugation (Centrifuge 5415R; Eppendorf, Tokyo, Japan). Tsukazawa [21] reported that both high performance liquid chromatography (HPLC) and RQflex methods produce very similar results for ascorbic acid content; hence, correction was unnecessary. Analysis was performed three times per sample.

The ability of vegetable extracts to scavenge DPPH radicals was assessed according to the method of Oki et al. [15] with slight modifications. Briefly, samples were ground in liquid nitrogen and mixed with 50% (v/v) ethanol. Then, samples were centrifuged at 9,300 × *g* for 3 min at 4°C (Centrifuge 5415R; Eppendorf, Tokyo, Japan). A stock assay solution was prepared mixing 50 mL of 200 μM DPPH, 25 mL of 0.2 M morpholinoethanesulfonic acid (MES) buffer at pH 6.0, and 25 mL of distilled, deionized water. The stock assay solution was divided into aliquots (1450 μL) in microtubes. The reaction was initiated by adding 50 μL of vegetable extract supernatant. After incubation for 2 min in the dark at room temperature, the absorbance at 520 nm was measured in a microplate reader. Scavenging activity was expressed as micromoles of Trolox

equivalent/g of sample, which was calculated using a linear standard Trolox curve.

The capacity to scavenge superoxide radicals was quantified using a SOD Assay kit-WST (Dojindo Laboratories, Kumamoto, Japan) following the method established by Ukeda et al. [22]. Samples were ground in liquid nitrogen, mixed with 0.1 M phosphate buffer at pH 7.4, and were centrifuged as described previously. Aliquots (20 μL) of supernatants were mixed with 200 μL of water-soluble tetrazolium salt working solution. After incubation for 20 min at 37°C in an incubator, the absorbance at 450 nm was measured and SOD-like radical scavenging activity (unit/mg) was calculated by dividing the IC50 value of a standard SOD sample (unit/mL) by that of the sample (mg/mL).

4.2.3 Quantification of lipid peroxidation

The degree of lipid peroxidation (MDA content) was determined measuring the thiobarbituric acid reactive substances (TBARS) method according to Hodge et al. [5] with slight modifications. The reaction medium was composed of 4 mL of 20% (w/v) trichloroacetic acid aqueous solution, 1 mL of 0.67% (w/v) thiobarbituric acid (TBA) aqueous solution, and 2 mL of plant extract. The mixture was heated in a boiling water bath for 15 min, cooled quickly in running tap water, and centrifuged at 13,950 × *g* for 15 min. The clear supernatant was brought to 10 mL with distilled water. The absorbances at 532, 600, and 440 nm were recorded, and compared with those of a linear MDA standard curve. For the standard curve, MDA was dissolved in 100 mL of distilled, deionized water to produce a stock solution. Working standards were made by diluting the stock solution 1:999, 3:997, 5:995, and 10:990 with 80% (v/v) ethanol. The absorbance at 532 nm was recorded after adding TBA solution in the same way as the extracted sample. For both plant samples and the standard curve, a blank assay in the absence of TBA was performed in parallel.

4.2.4 Determination of soluble solid content

Soluble solid content was measured using a Brix meter (POTSDTM1; Thanko, Tokyo, Japan), following the method reported by Shishido [19]. Petioles were collected from the leaf with the maximal length and the facing leaf. They were mashed using a muddler. A drop of the filtrate was placed onto the Brix meter to estimate the total sugar content.

4.2.5 Sampling and analysis

Data are presented means of 5 replicates ± standard deviation (SD). Tukey's multiple comparison test was performed with the statistical significance at $P < 0.05$.

4.3 Results and discussion

Under the same solution temperature conditions, the fresh weight of the aerial parts of plants decreased as chilling duration increased (Table 4-1). For the same root chilling duration, the fresh weight increased as solution temperature increased.

Ascorbic acid content increased with increasing chilling duration for all temperatures (Fig. 4-3). The exposure time necessary to elicit a significant increase in ascorbic acid content compared with the control group depended on the solution temperature. Ascorbic acid content significantly increased after 4 days at 4°C, 5 days at 7°C, 6 days at 10°C, and 7 days at 14°C. The chilling duration required to increase ascorbic acid content tended to be shorter at lower solution temperatures. Sugar content, estimated as soluble solid content, showed a similar trend to that of ascorbic acid (Fig. 4-4); soluble solid content significantly increased after 4 days at 4°C, 5 days at 7°C, 6 days at 10°C, and 7 days at 14°C.

Table 4-1. Effect of different root chilling protocols on the fresh weight of aerial parts of spinach plants.

Temperature (°C)	Duration of root area chilling (day)					
	0	2	4	5	6	7
4	71.8±8.47 a	80.3±7.06 a	39.3±11.5 b	31.6±5.92 b	30.9±4.61 b	33.8±5.10 b
7	65.2±13.1 a	63.3±14.3 a	28.6±3.95 b	32.1±5.79 b	35.2±6.37 b	28.2±5.43 b
10	72.6±14.1 a	62.8±11.2 ab	56.1±9.57 ab	54.2±9.01 ab	51.9±10.2 ab	41.9±11.7 b
14	68.5±5.63 n.s.	60.1±10.3	56.4±17.8	69.8±12.8	59.0±13.8	56.0±7.36

NB) Means±standard deviation. Different lower-case letters in each horizontal row denote significant differences by Tukey's multiple comparison test at $P < 0.05$ (n=5).

n.s.: there were no significant changes among plants maintained at 14°C.

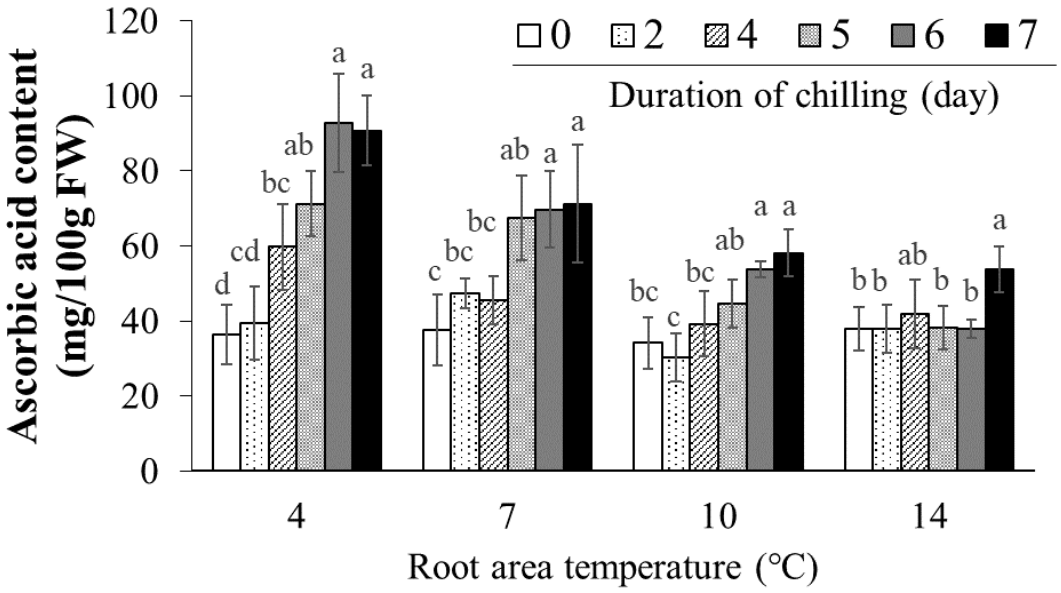

Fig. 4-3. Effect of root area chilling duration (0, 2, 4, 5, 6, and 7 days) at 4°C, 7°C, 10°C, and 14°C on the ascorbic acid content of spinach expressed as milligrams per 100 g of fresh weight (FW). Results are presented as means ± standard deviation (n = 5). Different letters indicate significant differences detected by Tukey's multiple comparison test ($P < 0.05$) within the same temperature. The effect of exposure time was analyzed separately for each temperature.

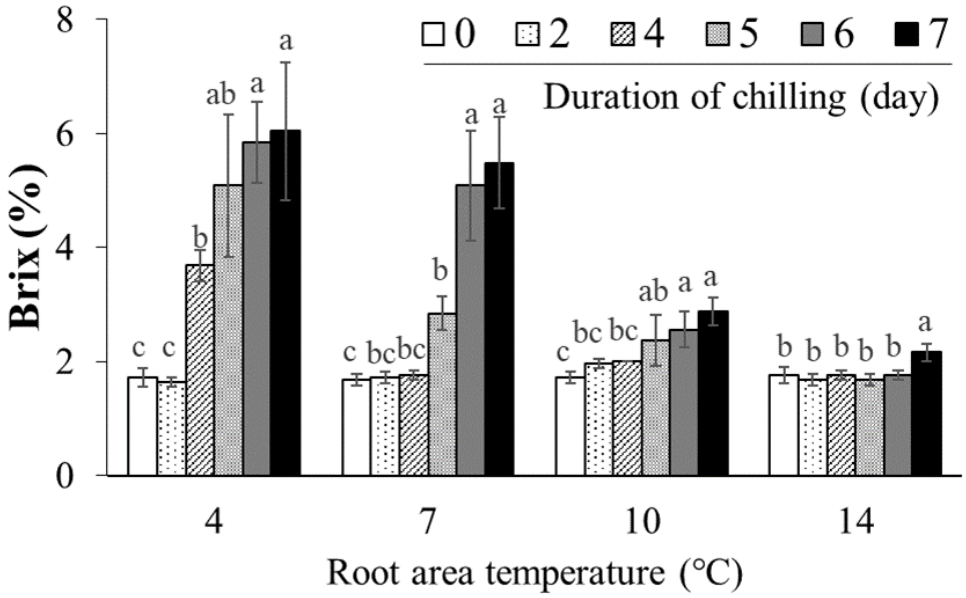

Fig. 4-4. Effect of root area chilling duration (0, 2, 4, 5, 6, and 7 days) at 4°C, 7°C, 10°C, and 14°C on the soluble solid content (measured as % Brix) of spinach. Results are presented as means ± standard deviation (n = 5). Different letters indicate significant differences detected by Tukey's multiple comparison test ($P < 0.05$) within the same temperature. The effect of exposure time was analyzed separately for each temperature.

Changes in DPPH scavenging activity were similar to those of ascorbic acid concentration within a relatively short time frame (2, 4, 5, and 6 days). The exposure time necessary to elicit a significant change in DPPH scavenging activity compared with the control group differed depending on the solution temperature. The capacity to scavenge DPPH significantly increased after 4 days at 4°C (Fig. 4-5A), 5 days at 7°C (Fig. 4-5A) and 10°C (Fig. 4-5B), and 7 days at 14°C (Fig. 4-5B). The time required to increase DPPH scavenging activity tended to be shorter at lower temperatures. An opposite trend was seen in plants exposed to root cooling at 4°C and 7°C for 7 days, when a slight decrease in DPPH scavenging activity compared with that at 6 days of chilling was observed (Fig. 4-5A).

When the root area was exposed to 7°C, SOD activity increased as chilling duration increased, reaching the highest levels after 5 days of chilling (Fig. 4-6A). Thereafter, SOD activity rapidly declined after 6 days of chilling, and slightly increased again at day 7. Similarly, at 4°C, SOD activity rapidly increased and reached its maximum value at day 2 (Fig. 4-6A). Thereafter, it decreased after 4 days of chilling, and increased again at day 7. However, a different trend occurred when plants were exposed to 10°C and 14°C (Fig. 4-6B). The changes in SOD activity in plants exposed to 10°C and 14°C resemble those in ascorbic acid content. Although SOD activity was not significantly different from the control group at the beginning of root cooling, a significant increase was observed after 7 days at 14°C and 5 days at 10°C (Fig. 4-6B).

The concentration of MDA in spinach exposed to root chilling was significantly affected at 4°C and 7°C, whereas no significant changes occurred at 14°C and 10°C (Fig. 4-7). At 7°C, MDA content was unchanged during the first 4 days of cold exposure, then it significantly increased at day 5. Thereafter, it decreased slightly and was not significantly different from the control group. Similarly, MDA content rapidly increased at day 5 of exposure to 4°C and decreased gradually thereafter.

In this study, we found that root area chilling induced significant changes in ascorbic acid content, SOD activity, and DPPH scavenging activity, whose levels are related to the ability to manage ROS. The results of ascorbic acid content showed reproducibility with the previous report [6]. Plant cells have endogenous antioxidants, such as ascorbic acid and glutathione, as well as an array of ROS-scavenging enzymes, such as SOD, to maintain low intracellular ROS levels. In a previous study, root area chilling increased the concentration of ROS in plant leaves [18]. Such an increase in ROS levels leads to the activation of SOD [4], which shows the fastest response to ROS among other antioxidants and ROS-scavenging enzymes [1]. Thus, the increase in ascorbic acid concentration, SOD activity, and DPPH scavenging activity observed in this study might

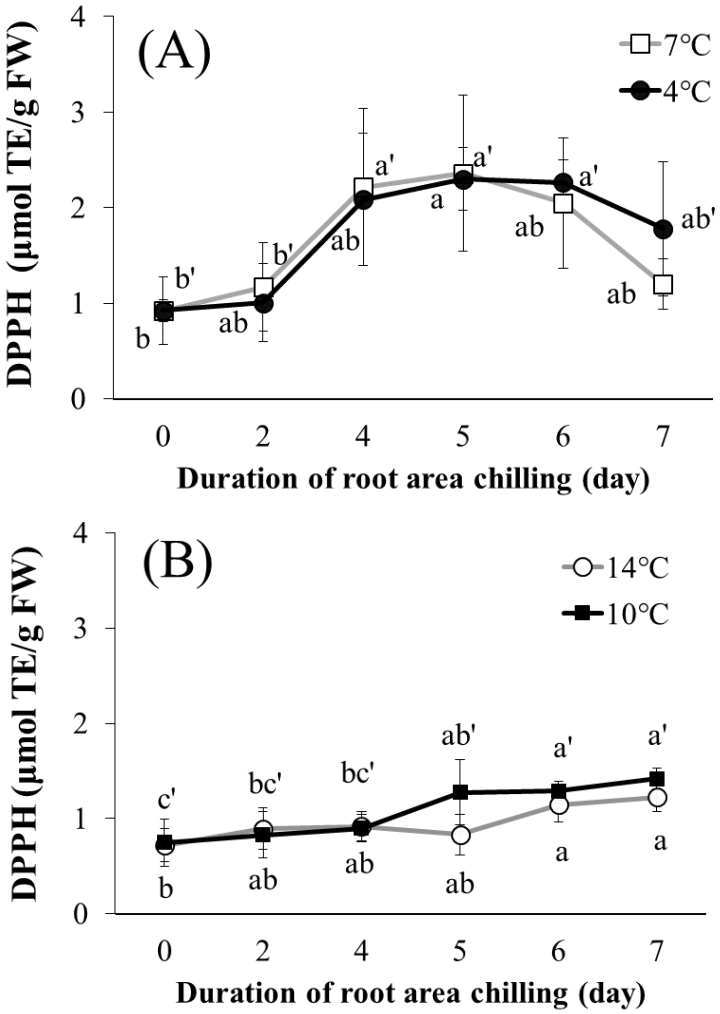

Fig. 4-5. Effect of root area chilling duration (0, 2, 4, 5, 6, and 7 days) at: (A) 4°C (filled circles) and 7°C (blank squares) and (B) 10°C (filled squares) and 14°C (blank circles) on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of spinach expressed as Trolox equivalents (TE) per gram of fresh weight (FW). Results are presented as means ± standard deviation (n = 5). Different letters indicate significant differences detected by Tukey's multiple comparison test ($P < 0.05$) within the same temperature. The effect of exposure time was analyzed separately for each temperature. Lower case letters without (7°C and 14°C) and with single apostrophes (4°C and 10°C) refer to differences between exposure times.

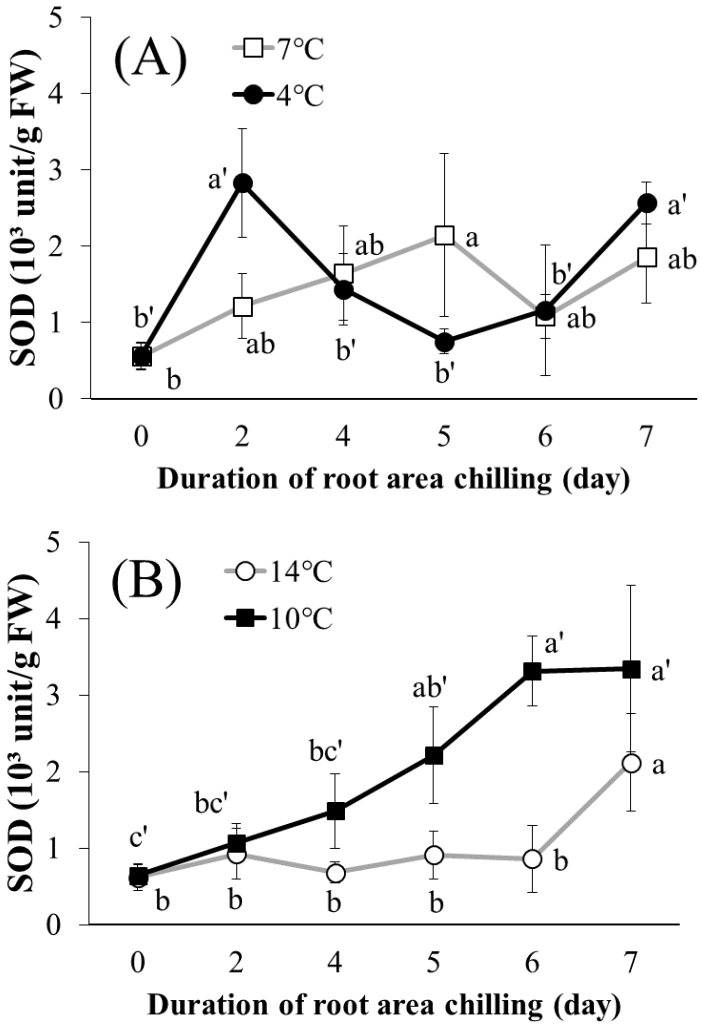

Fig. 4-6. Effect of root area chilling duration (0, 2, 4, 5, 6, and 7 days) at: (A) 4°C (filled circles) and 7°C (blank squares) and (B) 10°C (filled squares) and 14°C (blank circles) on the superoxide dismutase (SOD) activity of spinach expressed as unit per gram of fresh weight (FW). Results are presented as means ± standard deviation (n = 5). Different letters indicate significant differences detected by Tukey's multiple comparison test ($P < 0.05$) within the same temperature. The effect of exposure time was analyzed separately for each temperature. Lower case letters without (7°C and 14°C) and with single apostrophes (4°C and 10°C) refer to differences between exposure times.

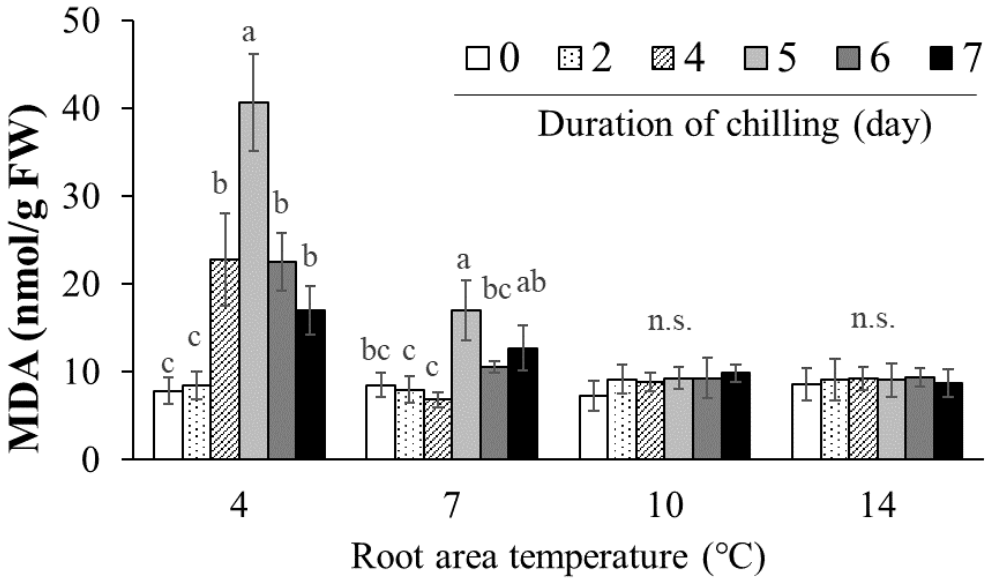

Fig. 4-7. Effect of root area chilling duration (0, 2, 4, 5, 6, and 7 days) at 4°C, 7°C, 10°C, and 14°C on malondialdehyde (MDA) content of spinach expressed as nmol per g of fresh weight (FW). Results are presented as means ± standard deviation (n = 5). Different letters indicate significant differences detected by Tukey's multiple comparison test ($P < 0.05$) within the same temperature. The effect of exposure time was analyzed separately for each temperature. n.s., there were no significant changes among different exposure times at either 10°C or 14°C.

be a response to the overgeneration of ROS triggered by root area chilling.

The excessive ROS production that surpasses the capacity of the antioxidant systems attacks cell membranes and oxidize phospholipids, resulting in the accumulation of highly reactive carbonyl species, such as MDA, which induce cell death and cancer [11]. Although there was little difference in the content of MDA in spinach exposed to root chilling at 10°C and 14°C, there were significant changes at 7°C and 4°C. This result suggest that phospholipids of cell membranes were oxidized in plants exposed to severe cold stress at 7°C and 4°C, probably because too much ROS was produced to be scavenged by antioxidants. On the other hand, ROS appeared to be kept at physiological levels under relatively mild cold stress conditions at 10°C and 14°C.

Ascorbic acid plays a role in the detoxification of H_2O_2 to H_2O and O_2 [12]. Therefore, the demand for ascorbic acid is expected to increase as H_2O_2 levels increase under severe and prolonged cold stress. However, the increase in ascorbic acid concentration in spinach exposed to root chilling was not linear, and ascorbic acid content was nearly unchanged after reaching a plateau. This change in ascorbic acid levels agrees with previous studies that investigated ascorbic acid concentration in spinach in a greenhouse during the winter [7], and in spinach exposed to root cooling at 5°C for 2 weeks [3]. In another study, it was indicated that ascorbic acid content is controlled by feedback inhibition of synthesis and by turnover [16]. It is, therefore, conceivable that the feedback system inhibited the linear increase in ascorbic acid with the duration of the root chilling.

The DPPH radical scavenging method measures radical removal capacity. It is suitable for the measurement of soluble antioxidants, such as ascorbic acid and glutathione, while it is unsuitable for lipophilic antioxidants, such as β-carotene and lycopene [8]. The significant increase in DPPH scavenging activity in spinach exposed to root chilling occurred within a shorter time frame than that for ascorbic acid. This result indicates that soluble antioxidants other than ascorbic acid were increased by root chilling, and the change in DPPH scavenging activity in spinach might reflect the antioxidant capacity of those components. Accordingly, significant increases in the levels of soluble antioxidants (anthocyanin, luteolin, and phenol) in plants have been observed upon the exposure of roots to low temperature [14, 18].

Both DPPH scavenging activity and MDA content in spinach exposed to root chilling at 4°C and 7°C decreased after reaching their maximum value. In that regard, Mano et al. [10] found that glutathione is the primary defense against LOOH and prevents its toxicity in plant cells while ascorbic acid does not play those roles. Therefore, it is possible that glutathione is responsible for the decrease in MDA levels, and the changes

observed during the first days of cold exposure reflect the consumption of glutathione as temperature decreases and exposure times increases. This explanation agrees with the decrease in DPPH scavenging activity after 7 days of chilling at 4°C and 7°C. However, further investigations are required to confirm this hypothesis because glutathione content was not measured in this study.

Superoxide scavenging activity followed an opposite trend to that of MDA content in spinach exposed to root chilling at 4°C. In previous studies, a similar behavior of SOD activity was observed in spinach exposed to drought stress [20] and broccoli flower buds stored at low temperature [9]. Qiu et al. [17] reported that the nonenzymatic glycation of SOD leads to the gradual inactivation of the enzyme, and glycated proteins are accumulated in the leaf of Arabidopsis and soybean under oxidative stress. Therefore, spinach exposed to root chilling could be under oxidative stress (evidenced by high MDA levels) because SOD was glycated and inactivated by the increased sugar content induced by environmental stress. In addition, Mano [11] found that reactive carbonyls, including MDA, have signaling functions that induce stress defense genes. Thus, it is possible that the accumulation of MDA after 5 days of root chilling at 4°C resulted in the activation of SOD, and MDA levels decreased after 7 days of chilling due to an enhancement in antioxidant defenses.

Studies have been conducted to reveal the effect of MDA on human health, and recent investigations have demonstrated that high concentration of MDA induces cancer and arteriosclerosis [13]. On the other hand, previous studies have shown that short-term intake of MDA at the level of nmol/g fresh weight might not be sufficient to harm human health [11]. Thus, the present result suggested that the spinach grown under the experimental conditions described in this paper has no harmful MDA content in the short term.

In the preceding study [6], it was reported that the ascorbic acid content increased with increasing chilling duration and the chilling duration required to show a significant increase was shorter with decreasing solution temperature according to the relational expression of solution temperature and chilling duration. In this study, it was revealed that severe cold stress to root area at 4°C and 7°C increase not only ascorbic acid and other antioxidants but also oxidized molecules. Furthermore, our results showed root chilling of 10°C or more for 7 days lead to the increase in the level of antioxidant compounds without accumulation of oxidized molecules. Our results underscore the need to pay attention not only to the content of antioxidants but also to that of other components that might affect human health when producing vegetables in artificial environments such as plant factories.

4.4 Conclusion

It has been proposed that cold stress applied to the root area promotes the production of reactive oxygen species and the increase in antioxidants levels in the plant body. However, changes in the balance between antioxidant activity and oxidative stress in plants under different levels of cold stress remain unexplored. Here, we assessed ascorbic acid content, superoxide dismutase activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, as proxies of antioxidant activity, and malondialdehyde (MDA) content, as an oxidative stress marker, in spinach. The root area was exposed to cold stress for 2, 4, 5, 6, and 7 days at various temperatures (4°C, 7°C, 10°C, and 14°C). Root chilling at 4°C and 7°C induced increases in ascorbic acid and DPPH scavenging levels, which were accompanied by the increase in MDA content, as cold exposure progressed. In contrast, root chilling at 10°C and 14°C increased antioxidant capacity without the increase in MDA concentration. The results of this study indicate that moderate cold stress applied to the root area of spinach could increase its antioxidant functions without accumulation of oxidative stress-related substances.

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Chapter 5

Conclusion

The purpose of this study was to establish a cultivation strategy for value-added spinach by exploiting the innate stress response of plants instead of using genetic engineering. Based on the analyses described in Chapters 2–4, the following three points were clarified.

In Chapter 2, we investigated the contents of ascorbic acid, soluble sugars, and nitrate ions in spinach subjected to root chilling at 10°C for different durations (2, 4, 5, 6, and 7 days) to identify the optimal duration of root chilling required to enhance the nutritional quality. While the contents of ascorbic acid and sugar remained stable at the beginning of chilling, these values became two times higher than the control values (no chilling) after 6 days. Meanwhile, nitrate ion content gradually decreased with increasing acclimatization period. Spinach plants acclimatized for 7 days showed similar results to those acclimatized for 6 days. These results indicate that the nutritional value did not change linearly with increasing chilling duration, and 6 days of acclimatization to chilling was sufficient to enhance the nutritional quality of spinach.

In Chapter 3, we examined the contents of ascorbic acid, nitrate ions, and soluble sugars in spinach subjected to root chilling at different temperatures (4, 6, 10, and 14°C) for various durations (2, 4, 5, 6, and 7 days) to reveal the associations of nutritional value of spinach with root chilling temperature and duration. The nitrate ion content significantly decreased within 2 days of chilling onset at all temperatures. The contents of ascorbic acid and soluble sugars followed a similar trend, both showing significant changes after 4 days at 4°C, 5 days at 6°C, 6 days at 10°C, and 7 days at 14°C. Moreover, we developed a relational expression for chilling duration and temperature, which could be used to obtain the minimum chilling temperature and duration required to maximally enhance nutritional quality. Based on our results, adjusting the chilling duration and temperature according to the relational expression would help plant factories produce value-added spinach even when a limited number of days can be spent at low temperatures or the lowest temperature.

In Chapter 4, we tested the hypothesis that the improvement in nutritive value following root chilling is due to the response of plants to oxidative stress. We assessed ascorbic acid content, superoxide dismutase activity, and DPPH scavenging activity as

proxies of antioxidant activity and MDA content as an oxidative stress marker in spinach. Root chilling at 4°C and 7°C increased ascorbic acid content and DPPH scavenging activity, ultimately increasing MDA content as cold exposure progressed. In contrast, root chilling at 10°C and 14°C increased antioxidant activity without increasing the resultant MDA content. The balance between antioxidant activity and oxidative stress in plants was altered under different degrees of cold stress; as such, moderate root chilling stress increased antioxidant activity without the accumulation of oxidative stress-related substances in spinach.

Therefore, value-added spinach can indeed be produced in plant factories by controlling environmental parameters and utilizing the innate stress responses of plants. Evaluating the association of contents of plant nutrient components with morphogenesis and environmental parameters would help in establishing cultivation methods for highly functional vegetables in the future.

Nonetheless, some issues remain to be solved in this research. The disadvantage of the proposed cultivation strategy for value-added spinach with root chilling is the decreased plant size and fresh weight at harvest, perhaps because oxidative stress induced by root chilling affects plant growth. Future studies on the signal transduction mechanisms of temperature sensing in plants and the effects of multiple environmental stress factors, such as light and temperature, will contribute to the development of practical cultivation strategies for vegetables with high nutritional value while maintaining the fresh weight.

List of Abbreviations

CO_2	carbon dioxide
DFT	deep flow technique
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	electrical conductivity
FW	fresh weight
HPLC	high performance liquid chromatography
H_2O_2	hydrogen peroxide
LED	light-emitting diodes
LOOH	lipid peroxides
LT	low temperature nutrient solution
MDA	malondialdehyde
NFT	nutrient film technique
NO_3^-	nitrate ion
PPFD	photosynthetic photon flux density
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TE	Trolox equivalents