

## Effect of different durations of root area chilling on the nutritional quality of spinach

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**Abstract:** It has been proposed that cold acclimatization of the plant body enhances the nutritional value of leaf vegetables; however, little is known about the optimal duration of cooling required to enhance the quality of factory-processed vegetables. The ideal low temperature acclimatization period increases ascorbic acid and sugar content without increasing the nitrate ion concentration for a given root area. Here, we investigated the ascorbic acid and sugar contents in spinach with a given root area subject to chilling for different durations; 2, 4, 5, 6, and 7 days. Ascorbic acid and sugar content levels remained stable at the beginning of chilling; however, after 6 days, the levels of both parameters doubled compared to those in the control (no chilling). In comparison, nitrate ion concentration gradually decreased with increasing acclimatization period. Spinach plants acclimatized for 7 days showed similar results as those acclimatized for 6 days. Our results show that the optimal duration for root area chilling to produce high quality spinach is 6 days, when controlling the temperature of the nutrient solution under plant factory conditions.

*Keywords:* ascorbic acid, environment control, nitrate ion concentration, plant factory, sugar content

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### 1. INTRODUCTION

Consumers are becoming increasingly concerned about the nutritional value and components of the foods they eat; consequently, a cultivation technology needs to be established whereby the produced vegetables contain high-functional ingredients. Generally, subjecting the plant body to a certain level of stress has been found to have a beneficial effect on the amount of positive compounds in plants, as well as improving their taste. Studies have assessed this stress response to enhance the nutritional value of vegetables (Kitano et al., 2008). Plant factories are closed agricultural facilities, with artificially controlled environments. This design allows the cultivation of plants through the regulation of the growth environment. Hence, under such conditions, local stress may be applied to the plant body to specifically evaluate the effect of certain stressors, which may be complicated under open culture conditions. Therefore, plant factories represent suitable environments for research related to the effect certain stimuli on plants, to ultimately produce high-quality vegetables. However, plant factories also have certain limitations due to the high costs of facility design and operation. Therefore, a cultivation technology by which high-quality vegetables at low power consumption under plant factory conditions needs to be established.

Spinach is a popular nutritious leaf vegetable and is rich in ascorbic acid (Vitamin C), which is perceived as a beneficial compound for human health. However, the nutritional value of spinach varies across the year. For instance, because of the increased rate of growth of spinach during the summer, nutritionally beneficial compounds decrease, whereas

nutritionally detrimental compounds increase, such as nitrate ions (Tamura et al., 2004; Fujiwara et al., 2005). The cultivation of spinach in controlled facilities, such as plant factories, may be used to resolve such problems. For instance, studies have shown that applying cold stress on the root area enhances the nutritional quality of this vegetable (Kitano et al., 2008). 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. MATERIALS AND METHODS

#### 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 set up with a hydroponic system. The control cultivation panel conditions were set to a 14 h photoperiod, a daytime photosynthetic active photon flux density (PPFD) of 200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 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 Figure 1. First, 60 plants were grown under the above control condition for 20 days (Fig.2A). On the 21<sup>st</sup> day, as shown in Figure 2B, 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. 2C), 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. 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.

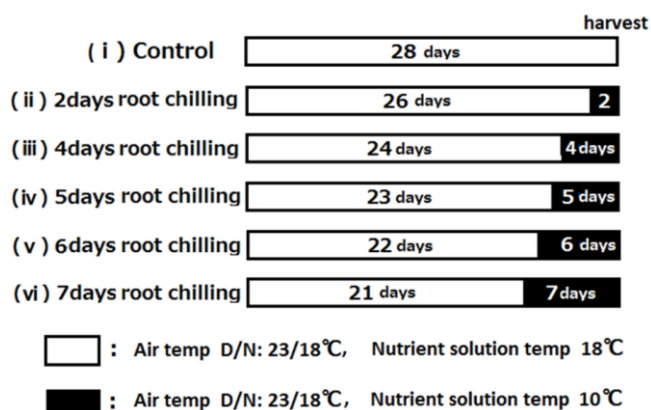


Fig.1 Schematic diagram of 6 experimental conditions

## 2.2 Determination 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 (2002) 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 (Tatebe, 1995).

## 2.3 Determination of Sugar Content

Sugar content was measured using a Brix meter (POTSDTM1; Thanko, Tokyo, Japan), following the method reported by Shishido (2005). Leaf stems 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 for measuring the sugar content.

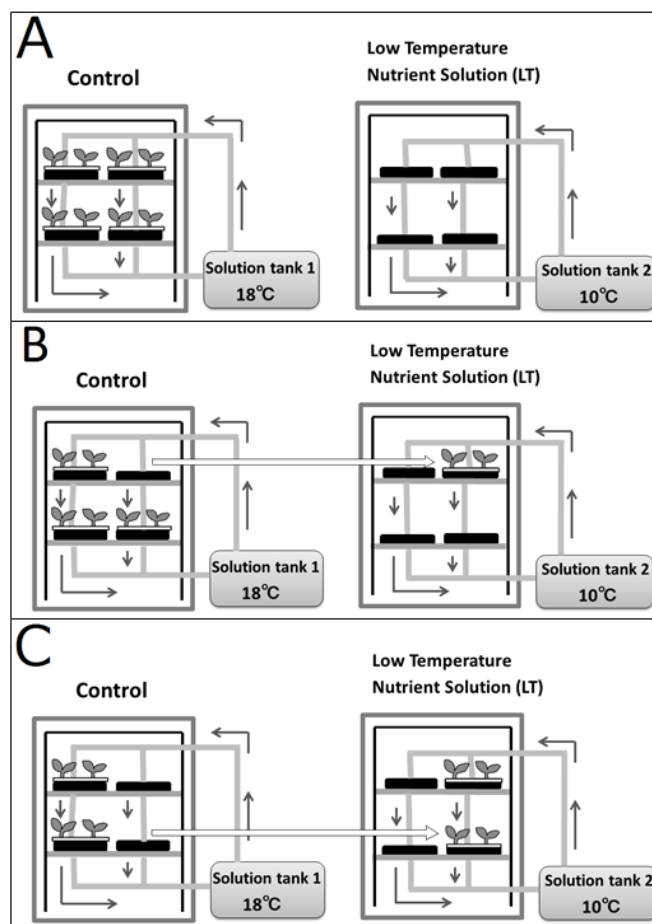


Fig. 2 Schematic diagram of the spinaches cultivation experiment.

## 2.4 Sampling and Analysis

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

## 3. RESULTS AND DISCUSSION

The physical appearances of spinach under each condition are shown in Figure 3. 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.

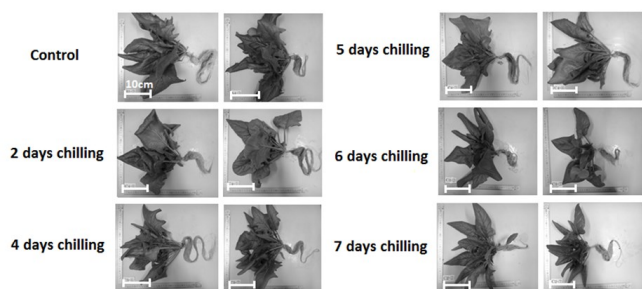


Fig. 3. Physical appearances of spinach treated with the different durations of root area chilling of 10 °C before the harvest. Control ( no chilling) are grown where nutrient solution temperature remained at 18 °C

Figure 4 shows the effects of low temperature at the root area on the fresh weight (Fig. 4A), dry matter (Fig.4B), and leaf area (Fig.4C) 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.

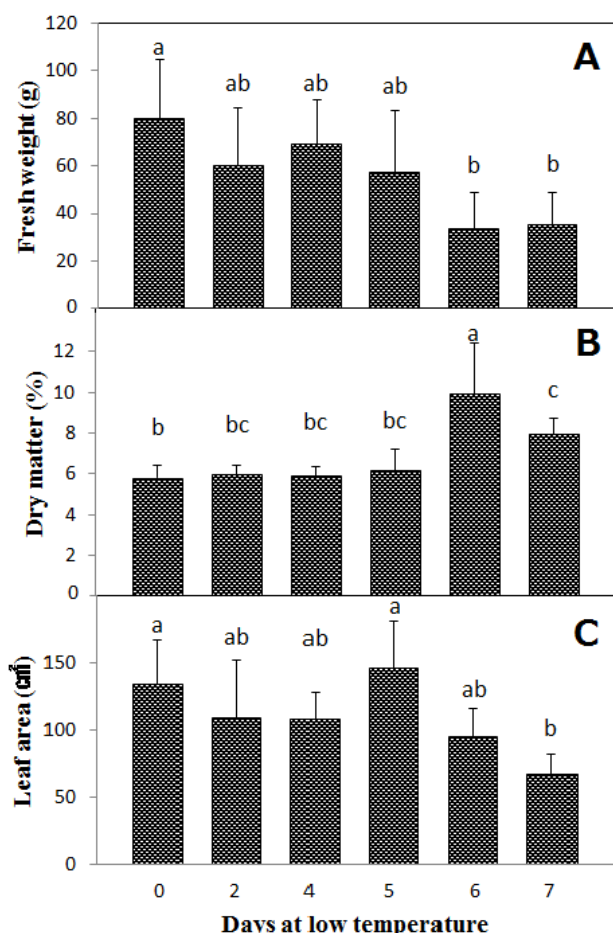


Fig.4 Effect of root area acclimatization to low temperature on the fresh weight (A), dry matter (B), and leaf area (C).

Figure 5 shows the effect of low temperature on spinach root area with respect to ascorbic acid content (Fig. 5A), nitrate

ion concentration (Fig. 5B), and sugar content (Fig. 5C). Ascorbic acid content remained stable until 5 days of acclimatization, and then exhibited a 100% increase by the 6<sup>th</sup> day. No significant difference in ascorbic acid content was observed between the 6<sup>th</sup> and 7<sup>th</sup> 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 6<sup>th</sup> 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.

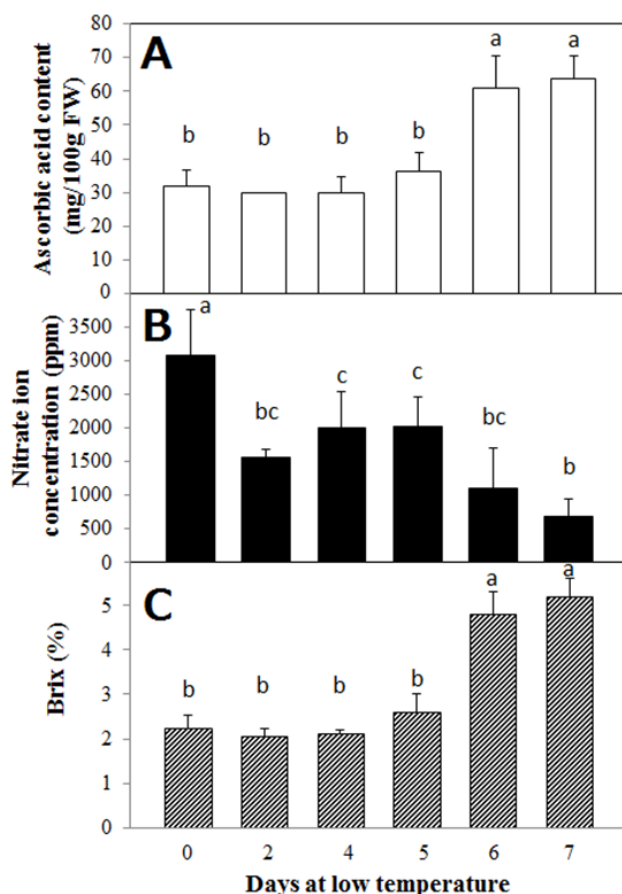


Fig. 5 Ascorbic acid (A), nitrate ion concentration (B), and brix (C) in spinach given the different durations of root area chilling of 10 °C before the harvest

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 6<sup>th</sup> day (Fig.6A, 6C). The nitrate ion concentration decreased within 5 days after chilling (Fig.6B).

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 (Fennell and Marekhardt, 1998). The suppression of water absorption by the roots causes osmotic adjustment and antioxidant functions (Kitano et al., 2008; Koda et al., 2003).

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 (1997) indicated that the ability for photosynthetic CO<sub>2</sub> assimilation at saturating irradiance and saturating CO<sub>2</sub> 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. (1992) 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<sub>2</sub>ase, Sed 1,7-P<sub>2</sub>ase, and sucrose-phosphate synthase, with the highest level of activation occurring by the 6<sup>th</sup> 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 (Schoner et al., 1990). 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 (2007) supposed.

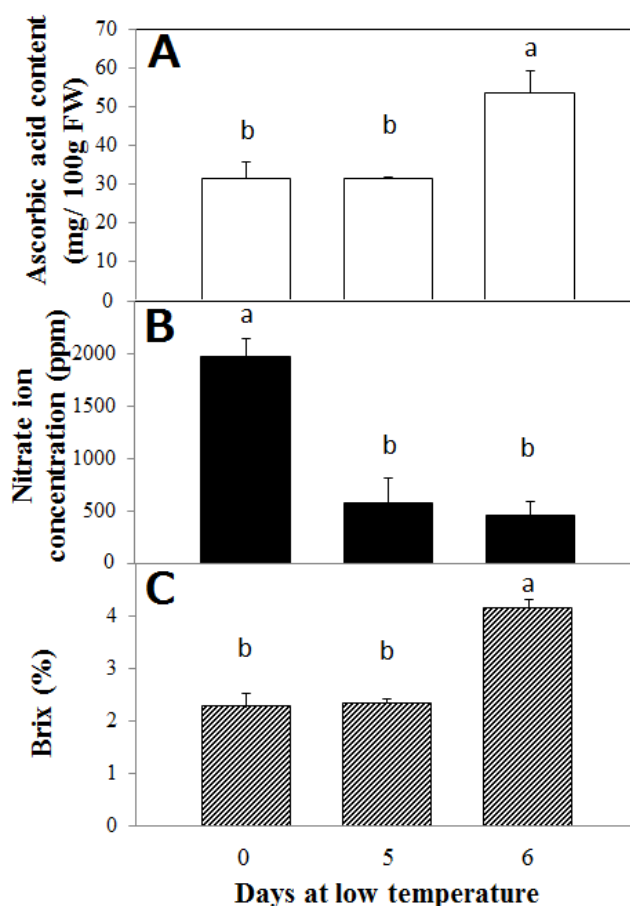


Fig.6 Ascorbic acid (A), nitrate ion concentration (B), and brix (C) in spinach given the different durations of root area chilling of 10 °C before the harvest in the repeated testing.

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. (2009) and Kitano et al. (2008). Sugiyama et al. (1992) and Davies (1986) 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  $\beta$ -carotene and  $\alpha$ -tocopherol increased after chilling (Kato et al., 1994). 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. (2006) 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 (2007) and Bergquist (2006) 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.

#### 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.