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

Ayana Ito and Hiroshi SHIMIZU

Future Co-creation Center, Daiwa House Industry Co., Ltd., Osaka 530-8241, Japan
Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

(Received November 7, 2019; Accepted April 18, 2020)

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.

Keywords: lipid peroxidation, low temperature stress, plant factory, root zone, value-added vegetables

INTRODUCTION

Plant factories are controlled-environment agricultural facilities that enable stable vegetable production year-round regardless of the weather conditions. Many researchers have investigated the production of high value-added vegetables in plant factories by controlling environmental conditions or giving certain stimuli, such as air temperature, root-zone temperature, water availability, salinity, ozone, and ultraviolet irradiation (Gazula et al., 2005; Chaves et al., 2009; Hikosaka et al., 2010; Bettaeb et al., 2011; Ito et al., 2013; Sudheer et al., 2016). Recent studies have shown that cold stress applied to the root area has a positive effect on the nutritional quality and produces a significant increase in the levels of highly functional plant constituents. For example, Chadirin et al. (2011a; 2011b; 2012) reported that spinach root chilling induced a significant increase in the levels of beneficial substances (such as sugars, ascorbic acid, and Fe) and a decrease in those of harmful substances (such as NO3− and oxalic acid). Furthermore, Ogawa et al. (2018) indicated that root chilling at 10°C for 6 days increased the levels of antioxidants, such as rosmarinic acid and luteolin, in red perilla.

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 (Asada, 2006). Under severe stress conditions, oxidative stress markers, including H2O2, 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 (2015) 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 (H2O2) and malondialdehyde (MDA), which are highly reactive molecules formed under oxidative stress. In that regard, several studies have reported that products of the oxidation of biomolecules cause cancer and liver disease (Nair et al., 2007; Li et al., 2015). Thus, it is necessary to consider the effects of environmental stress on the levels of oxidation byproducts, and not only antioxidant content, for the proper evaluation of the quality and functionality of stress-exposed vegetables. 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 to produce high quality vegetables under artificial environment.

Original Paper

Environ. Control Biol., 58 (4), 115-121, 2020
DOI: 10.2525/ecb.58.115

Vol. 58, No. 4 (2020)
MATERIALS AND METHODS

Plant materials and growth 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 hours (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 CO₂ 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; Zen-sui, 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).

Root chilling treatment
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. (2015). Spinach plants were grown under 24 different experimental conditions. First, all plants were grown under control conditions for 20 days in chamber 1 (Fig. 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. 1B). On the following day, a further 5 plants were transferred to the hydroponic system in chamber 2 (Fig. 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. 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 superox-

![Fig. 1](image1.png)

**Fig. 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. 2](image2.png)

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

| transplant | harvest |
|------------|---------|
| (i) Control | 28 days |
| (ii) 2 days chilling | 26 days |
| (iii) 4 days chilling | 24 days |
| (iv) 5 days chilling | 23 days |
| (v) 6 days chilling | 22 days |
| (vi) 7 days chilling | 21 days |

: Nutrient solution temperature 18°C
: Nutrient solution temperature 14, 10, 7, or 4°C
Ascorbic acid content was measured using a reflective photometer (RQflex10; 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 (2002) 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 determined following the method of Oki et al. (2001) with slight modifications. Briefly, samples were ground in liquid nitrogen and mixed with 50% (v/v) ethanol. The absorbance at 532 nm was recorded in a microplate reader. A drop of the filtrate was placed onto the Brix meter to estimate the total sugar content.

Quantification of lipoperoxidation

The degree of lipid peroxidation (MDA content) was determined using the thiobarbituric acid reactive substances (TBARS) method according to Hodges et al. (1999) 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 minutes, cooled quickly in running tap water, and centrifuged at 13,950×g for 15 minutes. 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.

Statistical 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.

RESULTS

Under the same solution temperature conditions, the fresh weight of the aerial parts of plants decreased as chilling duration increased (Table 1). For the same root chilling duration, the fresh weight increased as solution temperature increased. Ascorbic acid content increased with increasing chilling duration.
ing duration for all temperatures (Fig. 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); 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.

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. 5A), 5 days at 7°C (Fig. 5A) and 10°C (Fig. 5B), and 7 days at 14°C (Fig. 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. 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. 6A). Thereafter, SOD activity rapidly declined after 6 days of chilling, and slightly increased again at day 7. Similarly, at 14°C, SOD activity rapidly increased and reached its maximum value at day 2 (Fig. 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. 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. 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. 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.
A. ITO AND H. SHIMIZU

DISCUSSION

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 (Ito et al., 2015). 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 (Sakamoto and Suzuki, 2015). Such an increase in ROS levels leads to the activation of SOD (Fridovich, 1986), which shows the fastest response to ROS among other antioxidants and ROS-scavenging enzymes (Asada et al., 1973). Thus, the increase in ascorbic acid concentration, SOD activity, and DPPH scavenging activity observed in this study might 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 (Mano, 2012). 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 suggests 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 H₂O₂ to H₂O and O₂ (Nakano and Asada, 1981). Therefore, the demand for ascorbic acid is expected to increase as H₂O₂ 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 (Kato et al., 1995), and in spinach exposed to root cooling at 5°C for 2 weeks (Chadirin et al., 2011a). In another study, it was indicated that ascorbic acid content is controlled by feedback inhibition of synthesis and by turnover (Pallanca et al., 2000). It is, therefore, conceivable that the feedback system inhibited the linear increase in ascorbic acid with the duration of the root chilling.

According to 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 (Kondo et al., 2017). 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 (Sakamoto and Suzuki, 2015; Ogawa et al., 2018).

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. (2009) 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 (Tanaka et al., 1990) and broccoli flower buds stored at low temperature (Li et al., 2004). Qiu et al. (2008) 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 (2012) 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 (Niki, 2014). 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 (Mano, 2012). 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 (Ito et al., 2015), 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
ROOT CHILLING AND ANTIOXIDANTS

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 accumulated 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.

REFERENCES

Asada, K., Urano, M., Takahashi, M. 1973. Subcellular localization of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. Eur. J. Biochem. 36: 257–266.

Asada, K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol. 141: 391–396.

Betta ai, I., Hamrouni-Sellami, I., Bourgou, S., Limam, F., Marzouk, B. 2011. Drought effects on polyphenol composition and antioxidative activities in aerial parts of Salvia officinalis L. Acta Physiol. Plant. 33: 1103–1111.

Chadirin, Y., Hidaka, K., Takahashi, T., Sago, Y., Wajima, T., Niki, E. 2001b. Application of temperature stress to roots of spinach. I. Effect of the low temperature stress on quality. Environ. Control Biol. 49: 133–140.

Chadirin, Y., Hidaka, K., Sago, Y., Wajima, T., Niki, E. 2001b. Application of temperature stress to root zone of spinach. II. Effect of the high temperature pre-treatment on quality. Environ. Control Biol. 49: 157–164.

Chadirin, Y., Sago, Y., Hidaka, K., Wajima, T., Niki, E. 2012. Application of temperature stress to root zone of spinach. III. Effective method for short term application of low and high temperatures stresses to roots. Environ. Control Biol. 50: 199–207.

Chaves, M. M., Flexas, J., Pinheiro, C. 2009. Photosynthesis under drought and salt stress: regulation mechanisms form whole plant to cell. Ann. Bot. 103: 551–560.

Fridovich, I. 1986. Biological effects of the superoxide radical. Arch. Biochem. Biophys. 247: 1–11.

Ganazza, A., Kleinheinz, M. D., Streeter, J. G., Miller, A. R. 2005. Temperature and cultivar effects on anthocyanin and chlorophyll B in related Lolio Rosso Lettuce Cultivars. HorticScience 40: 1731–1733.

Hikosaka, S., Ito, K., Goto, E. 2010. Effects of ultraviolet light on growth, essential oil concentration, and total antioxidant capacity of Japanese mint. Environ. Control Biol. 48: 185–190.

Hodges, D. M., Delong, J. M., Forney, C. F., Prange, R. K. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207: 604–611.

Ito, A., Shimizu, H., Hiroki, R., Nakashima, H., Miyasaka, J., Ohdo, K. 2013. Effect of different duration of root area chilling on the nutritional quality of spinach. Environ. Control Biol. 51: 187–191.

Ito, A., Shimizu, H., Hiroki, R., Nakashima, H., Miyasaka, J., Ohdo, K. 2015. Quantitative relationship of the nutrient quality of spinach with temperature and duration in root area chilling treatment. Environ. Control Biol. 53: 35–42.

Kato, T., Aoki, K., Yamanishi, H. 1995. Effect of low temperatu