Korean J. Soil Sci. Fert. Vol.52, No.1, pp.11-19, 2019

Korean Journal of Soil Science and Fertilizer

Article

https://doi.org/10.7745/KJSSF.2019.52.1.011
pISSN : 0367-6315 eISSN : 2288-2162

Effect of Cold Stress on the Content of Minerals and Water Soluble Vitamins in Spinach (*Spinacia oleracea*)

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Received: January 12, 2019
Revised: February 26, 2019
Accepted: February 27, 2019

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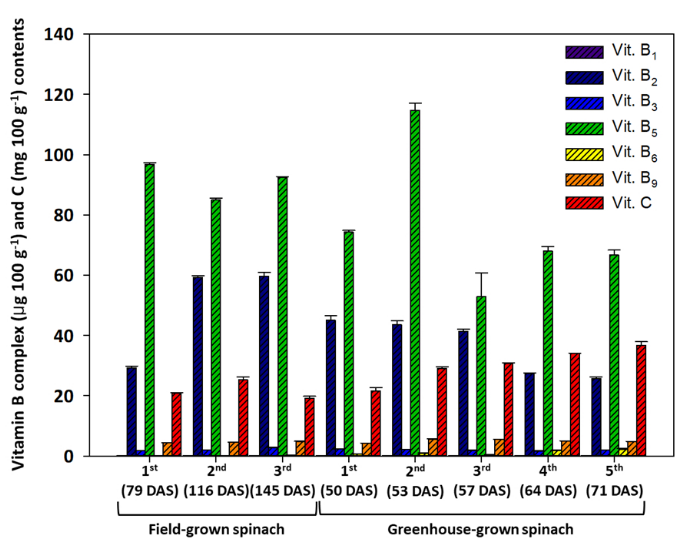
ABSTRACT

To assess the effect of cold stress on the levels of minerals (C, N, P, K, Ca, Mg, Mn, Zn, Cu and Fe) and water soluble vitamins (B1, B2, B3, B5, B6, B9 and C) in spinach, an experiment was set up under greenhouse conditions and compared to field results. Levels of macro and micronutrients, except iron in spinach were unaffected by growth conditions and cold stress. The abundance of water soluble vitamins were most pronounced in greenhouse grown plants exposed to 14 and 21 days of cold stress. Significant increases in vitamin C (from 22.73 to 34.47 mg 100 g^{-1} dry matter) accumulation in stressed plants were observed, thus highlighting its role in plant defense mechanisms. Results indicate that cultivation of leafy vegetables in a greenhouse with low temperature (4 to 7°C) exposure for a shorter period (14 to 21 days) before harvest can improve the nutritional quality.

Keywords: Cold stress, Essential nutrients, Greenhouse, Spinacia oleracea, Vitamins



Water soluble vitamin (WSV) contents of field- and greenhouse-grown spinaches.

Introduction

Of all the green leafy vegetables, spinach (*Spinacia oleracea*) is the most important source of nutrients (Citak and Sonmez, 2009). On the basis of FAO statistics (FAO, 2011), nearly 20,793,353 tons of spinach were produced around the world in 2011 and it is arguably the first or second most nutritious vegetable. However, from a global perspective, the nutritional quality of spinach tends to vary with time. Furthermore the cultivation of this vegetable in controlled facilities such as plant factories/greenhouses or application of cold stress on the root area may help resolve such problems and substantially enhance its nutritional quality (Kitano et al., 2008). Spinach prefers a cool climate and preferably between 2 and 24°C and it is not unusual that this vegetable can withstand cold stress (Steindal et al., 2015). Generally, cold stress activates cold tolerance/acclimatization processes in plants involving many biochemical and physiological changes involving the production of some of the nutritionally relevant compounds. These include, for example, minerals and vitamins (ascorbic acid and pantothenic acid) with antioxidant properties that protect the plant cells from damage (Becana et al., 1998; Proietti et al., 2009). Accumulation of such health-promoting nutrients elevates the overall nutrient quality of crops and their enhanced intake by humans will pave the way to benefits of good health through eating spinach.

On the other hand, greenhouses allow crop production to take place through the regulation of a growth environment. Under such conditions, local stress may be applied to plants in order to specifically evaluate the effect of certain environmental stresses on plant nutritional quality, which could be complicated in open field conditions (Ito et al., 2014). Such research will be a step forward to the successful development of a new, cost-benefit, efficient crop cultivation practice in a protected environment. It could harness the potential benefits of external environmental stimuli such as cold stress which could possibly pave the way for the accumulation of nutritionally and biologically important nutrients with plant defense mechanisms. The result is the production of high quality vegetables where less power is consumed. To date no studies have been published that investigate differences due to the impact of cold stress on the nutritional quality of spinaches produced in artificially controlled environments. The present study aimed to see how the levels of macro and micronutrients including water soluble vitamins in spinach are influenced by cold stress in a greenhouse trial using an open-field experiment as the control study (no cold stress).

Materials and Methods

Plant material and experimental set-up Spinach (*Spinacia oleracea* cv. Jeoncheonhu, Sagyejul, Namdongcho and Mustang) was conventionally grown between October 2014 and February 2015 in a field located at Namhae-gun, Gyeongsangnam-do, South Korea (34° 46'N, 127° 57'E). Soil at this site had the following characteristics: pH 7.1, EC 0.36 ds m^{-1}, total carbon (C) 1.86%, total nitrogen (N) 0.16% and available phosphorous (P) 215.87 mg kg^{-1}. The land was prepared in a randomized manner consisting of three replicates. Basal application of N (urea), P (super phosphate) and K (potassium chloride) fertilizers were given at a rate of 10, 5.9 and 7.9 kg ha^{-1}.

One dose of K fertilizer (4 kg ha^{1}) was top dressed after one month of sowing. The spinach received N fertilizer as top-dress at a rate of 5 kg ha^{1} month^{-1} until the end of the harvest season. The field temperature fluctuated between 19.5 and -2.5°C throughout the cropping period. The first harvest was implemented when the leaf was over 7 cm in length, which was on the 79th day after sowing (DAS). Successive harvests were made on the 116th and 145th DAS.

A greenhouse pot trial was designated to test the effect of short-term cold stress treatment on the nutritional quality of spinach. Briefly, spinach (*Spinacia oleracea* cv. Sagyejul) was planted in pots and maintained in a high tunnel greenhouse located at the Gyeongsang National University Experiment Station, Jinju, South Korea (35° 12'N, 128° 02'E) between December 2014 and February 2015. Soil collected from the site where the field study was conducted was used for the greenhouse trial. Fertilizers were applied following the same schedule and rates as adopted for the field study. Greenhouse temperature was maintained at 10 to 15°C. The first harvest when the leaf length was more than 7 cm occurred on the 50th DAS. After the first harvest, cold stress was given to spinach by opening the roll-up side of the greenhouse, due to which the temperature declined steeply and fluctuated between 4 and 7°C for rest of the cropping period. The vegetable was harvested on the 3rd (53rd DAS), 7th (57th DAS), 14th (64th DAS) and 21st (71st DAS) days after cold stress.

Sample collection and chemicals On each harvest day, samples were picked, packed in a polyethylene bag and taken to the laboratory for analysis. In the laboratory, the spinach was hand-washed to remove any adhering soil particles, freeze dried, ground to a fine powder in liquid nitrogen, vacuum packed in small bags and stored at -20°C until further chemical assays. All chemicals and solvents used in this experiment were of analytical grade and obtained from Sigma-Aldrich, South Korea. All solutions were prepared with distilled water. The prepared solutions were stored at 4°C until further use.

Analysis of minerals Total concentrations of C and N were analyzed by Automated True Macro Element Analyzer (TruMAC; Leco) using a 0.20 g sample. About 0.50 g of the sample was acid digested using 10 ml of $HClO_4$:H_2O:H_2SO_4 (9:5:1; v/v/v). After digestion, samples were diluted and the volume was increased to 100 ml using distilled water. Filtered samples (using Whatmann no. 2 filter paper) were used to determine the concentrations of total P, K, Ca (calcium), Mg (magnesium), Fe (iron) and Cu (copper) using Inductively Coupled Plasma Optical Emission Spectrometry (OPTIMA 5300DV; PerkinElmer).

Determination of water soluble vitamins Water soluble vitamins were determined using the method employed by Santos et al. (2012) with some modifications. Nearly 0.1 g was extracted with extraction solution (1.4 ml of 10 mM ammonium acetate + 1.3 ml methanol containing 0.05% butylated hydroxytoluene + 100 µl of 50 ng ml^{-1} hippuric acid) in an ultrasound bath (Power Sonic 410, Power-Sonic Corp., US) for 5 min. Samples were centrifuged at 15,000 rpm for 5 min, filtered (through a 0.45 mm syringe filter) and analyzed in an Accela High Performance Liquid Chromatography (HPLC) system (Thermo Scientific, US) equipped with a diode array detector (DAD) (Thermo Scientific, US). Atlantis dC18 column (4.6 × 100 mm, 5 mm particle size) was used. About 10 mM ammonium acetate solution (pH 4.5) served as mobile phase A, methanol with 0.1% acetic acid as mobile phase B and methanol with 0.3% acetic acid as mobile phase C in different gradients as shown in Table S1. The flow rate was 0.2 ml min^{-1}. DAD recorded the spectra from 200 to 680 nm. Column and autosampler compartments were thermostated at 20 and 5 ºC, respectively. Quantification was based on an internal standard curve.

Statistical analyses Statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc.). Duncan's multiple range test (DMRT) was performed at the 0.05 probability level for making treatment mean comparisons. Graphs were generated through SigmaPlot software version 10.0 (Systat Software Inc.).

Results and Discussion

The type of spinach cultivar (Jeoncheonhu, Sagyejul, Namdongcho and Mustang) was found to have no significant ($p \leq 0.05$) effect on the parameters investigated in the field study (data not shown) thus confirming earlier reports (Amr and Hadidi 2001) that any differences between the cultivars of the same species are insignificant. Hence, only the consolidated data (means) of all the four field-grown spinach cultivars are given and compared with the greenhouse-grown spinach cultivar Sagyejul.

Essential macro and micronutrient contents of field- and greenhouse-grown spinaches Mineral contents of the spinach plants evaluated in this study are given in Table 1. In general, spinach contained a large amount of essential minerals/nutrients (440.26 to 459.56 g kg^{-1}) and is suited as a valuable foodstuff for supplying minerals. No significant ($p \leq 0.05$) difference existed between the total mineral content of the field- and greenhouse-grown spinaches. Also, cold stress did not exhibit a noticeable increase in the total mineral content of the greenhouse-grown spinaches. Observed slight increase in the nutrient contents of greenhouse-grown spinaches with cold stress for a shorter period (3-7 days) might be related to higher protein, osmotically active metabolites and certain enzymes synthesis, namely of antioxidative mechanisms imparting cold tolerance to plants (Fortunato et al., 2010). With increased cold stress (≥ 14 days), all of the investigated minerals except Fe declined. Witnessed decline is attributed to slow metabolism or reduced photosynthetic activity (Ramalho et al. 2013). The proportional increase in Fe content with increased cold stress exposure could be due to the release of catalytic Fe from proteins endowed with antioxidant defenses to cope with low temperature (Becana et al. 1998).

Looking at the macronutrients, C content was between 381.53 to 412.50 g kg^{-1} and significantly ($p \leq 0.05$) the highest level was found in the greenhouse-grown spinach exposed to nearly 7 days of cold stress. The N, P and K levels of spinach plants (g kg^{-1}) ranged from 31.22 to 57.68, 5.16 to 6.90 and 0.52 to 1.03, respectively. These values inclined to be higher in greenhouse-grown spinach exposed to 3 days of cold stress, however, they did not differ significantly ($p \leq 0.05$) from the field-grown spinach. The N and P results of this study did agree with

Table 1. Mineral composition of field- and greenhouse-grown spinaches.

Harvest	Primary macronutrients ($g\ kg^{-1}$ dry matter)				Secondary macronutrients ($mg\ kg^{-1}$ dry matter)		Micronutrients ($mg\ kg^{-1}$ dry matter)				Total essential minerals ($g\ kg^{-1}$ dry matter)
	C	N	P	K	Ca	Mg	Cu	Fe	Mn	Zn	
Field-grown spinach											
1st (79 DAS)	398.9	49.03	6.77	1.02	97.3	85.2	8.2	140.3	33.2	113.0	456.2
2nd (116 DAS)	383.8	57.68	6.55	1.03	121.1	96.4	9.2	108.1	55.0	76.4	449.5
3rd (145 DAS)	402.0	45.95	5.16	0.71	125.9	82.1	6.9	91.4	22.1	62.5	454.2
Greenhouse-grown spinach											
1st (50 DAS)	388.2	49.16	6.90	0.98	87.4	77.1	8.1	121.7	32.3	80.3	445.7
2nd (53 DAS)*	381.5	55.13	6.50	1.00	124.6	94.5	9.7	102.2	47.0	80.3	444.6
3rd (57 DAS)*	412.5	40.48	5.52	0.67	104.2	71.0	7.3	101.7	28.3	78.3	459.5
4rth (64 DAS)*	402.6	31.22	5.54	0.52	66.9	50.9	6.8	121.8	28.5	72.8	440.2
5th (71 DAS)*	405.2	35.93	5.56	0.54	75.8	60.4	7.9	247.8	27.3	79.8	447.8
$LSD_{0.05}$	13.4	5.73	0.15	0.08	18.1	6.8	2.3	55.2	7.7	23.1	10.8

*- exposed to cold stress.

findings of relevant studies. Citak and Sonmez (2009) pointed out that spinach contains an average of 16.8 to 43.7 $g\ kg^{-1}$ of N and 2.1 to 8.0 $g\ kg^{-1}$ of P, respectively. Another study carried out by Peyvast et al. (2008) showed that the N and P contents of spinach were between 32.3 to 37.2 and 2.7 to 7.1 $g\ kg^{-1}$, respectively. However, the observed K content was nearly 10-fold less than those reported elsewhere (Citak and Sonmez 2009; Ramalho et al. 2013). The Ca and Mg content in 1 kg of spinach leaves were 66.97 to 125.89 and 50.87 to 96.35 mg, respectively. Citak and Sonmez (2009) and Peyvast et al. (2008) observed similar values in their studies for Ca, however, the ranges they reported were 100-fold higher for Mg. This may be due to the intense application of organic manures that renders abundant nutrients to plants, subsequently increasing the plants' ability to produce more chlorophyll (Citak and Sonmez 2009) where Mg is the central atom.

The micronutrients results of this study were on par with those of previous studies (Gulser 2005; Peyvast et al. 2008; Citak and Sonmez 2009). Among the microelements, Cu ranged from 6.80 to 9.67 $mg\ kg^{-1}$ in both the field- and greenhouse-grown spinaches. The Mn and Zn contents of spinach were found to be between 22.10 to 55.04 and 62.54 to 113.02 $mg\ kg^{-1}$, respectively, and we can clearly observe that the field values were significantly ($p \leq 0.05$) higher than the greenhouse study values. The samples' Fe content was considerably higher in the greenhouse-grown spinach, and was observed from the spinach plants exposed to 21 days of cold stress. The current study highlights that spinach is a very useful Fe source.

Water soluble vitamin (WSV) concentrations of spinach in response to cold stress − greenhouse vs. field study A good separation of vitamin B1 (thiamin), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenate), vitamin B6 (pyridoxine), vitamin B9 (folate) and vitamin C (ascorbate) was achieved within 6 min with the developed method (Fig. S1). Retention times of thiamin, riboflavin, niacin, pantothenate, pyridoxine, folate and ascorbate were 2.83, 3.01, 3.18, 3.58, 3.91, 4.09 and 5.12 min, respectively. Identified WSV in spinach were quantified in the following concentration values (μg 100 g^{-1} dry matter): thiamin 0.08 to 0.23, riboflavin 25.7 to 59.7, niacin 1.73 to 2.83, pantothenate 68.0 to 114.7, pyridoxine 0.02 to 2.26, folate 4.37 to 5.61 and ascorbate 19160 to 36630 (Table 2). Nearly 0.16, 0.19, 0.07, 0.2, 190 and 56 mg thiamin, riboflavin, pantothenate, pyridoxine, folate and ascorbate were reported by Hounsome et al. (2008) in a 100 g fresh leaf of spinach. Another study (Santos et al. 2012) showed that the thiamin, riboflavin, niacin, pantothenate, pyridoxine, folate and ascorbate contents of spinach (μg 100 g^{-1} fresh weight) are in the following respective ranges: 194 to 244, 223 to 257, 167 to 179, 345 to 526, 8 to 13, 0.9 to 1 and 170 to 1440.

Cold stress and cultivation of the spinach plants in greenhouse conditions had a significant ($p \leq 0.05$) effect on its pantothenate, pyridoxine and ascorbate contents. Soon after cold stress, leaf content of pyridoxine and ascorbate started to rise and reached their maximum after 21 days, with a 3- and 1.7-fold increase with reference to those unexposed to cold stress (50 DAS), respectively. Plants exposed to stress have to counteract an increased population of oxygen reactive species to avoid cell components' experiencing oxidative damage. Spinach when exposed to low temperature could have undergone a profound modification of leaf metabolism, a process involving specifically the increased production of pyridoxine and ascorbate with the capacity to scavenge oxygen reactive species in the chloroplast and even more markedly in the leaf blade (Denslow et al. 2005; Proietti et al. 2009). For this reason we observed that increased cold stress exposure correspondingly resulted in increased pyridoxine and ascorbate contents in spinach. Ito et al. (2014) reported an increased ascorbate content of leafy vegetables with an ideal low temperature acclimatization. Johansen et al. (2016) found that short periods of low temperature did affect ascorbate content. Steindal et al. (2013) reported 16% higher ascorbate levels in vegetables exposed to low temperature (0-10°C) for a shorter period before harvest in a controlled environment. To the best of our knowledge,

Table 2. Water soluble vitamin (WSV) contents of field- and greenhouse-grown spinaches.

Harvest	B complex vitamins (μg 100 g^{-1} dry matter)						C vitamin/ascorbate (mg 100 g^{-1} dry matter)	Total WSV (mg 100 g^{-1} dry matter)
	Thiamin (B_1)	Riboflavin (B_2)	Niacin (B_3)	Pantothenate (B_5)	Pyridoxine (B_6)	Folate (B_9)		
Field-grown spinach								
1st (79 DAS)	0.14	29.3	1.87	96.8	0.19	4.37	20.8	20.9
2nd (116 DAS)	0.13	59.3	1.95	85.0	0.02	4.61	25.3	25.5
3rd (145 DAS)	0.22	59.7	2.83	92.4	0.21	4.87	19.2	19.3
Greenhouse-grown spinach								
1st (50 DAS)	0.14	45.0	2.33	74.3	0.67	4.30	21.5	21.6
2nd (53 DAS)*	0.13	43.5	2.14	114.7	0.92	5.61	29.0	29.2
3rd (57 DAS)*	0.08	41.4	1.81	52.9	0.51	5.56	30.8	30.9
4rth (64 DAS)*	0.23	27.3	1.73	68.0	1.84	4.94	34.0	34.1
5th (71 DAS)*	0.23	25.7	2.01	66.7	2.26	4.84	36.6	36.7
$LSD_{0.05}$	0.03	9.0	0.36	19.1	0.49	0.33	4.8	4.8

* - exposed to cold stress.

there are no published research studies about the B complex vitamin concentrations of spinach or another vegetables in response to cold stress effect, though some of the B complex vitamins such as thiamine, pyridoxine and folate had been previously reported (Asensi-Fabado and Munne-Bosch 2010) to alleviate the effects of several environmental stresses, presumably by protecting the plant from oxidative damage. It is because, the antioxidant vitamins that have been of most attention in plants are carotenoids (vitamin A), ascorbate and tocochromanols (vitamin E) (DellaPenna and Pogsan 2006; Linster and Clarke 2008; Falk and Munne-Bosch 2010). Further, the total WSV contents of spinach was maximum for the greenhouse samples (31.04 to 34.57 mg 100 g^{-1} dry matter) that were exposed to cold stress for 14 and 21 days (64 and 71 DAS) than others including the field-grown ones. Thus, the results showed that mild cold stress for 14 to 21 days can be successfully used to enhance the important health-promoting WSV in spinach.

Conclusions

Our findings indicate that cultivation of spinach in controlled environments will enhance the nutritional quality of this vegetable. Not only greenhouse farming but also cold stress had notable effects on the nutritionally and functionally important compounds of spinach and is high useful. From an overall perspective, cultivation of leafy vegetables such as spinach in controlled environment/greenhouse with low temperature (4 to 7°C) exposure for shorter period (14 to 21 days) before harvest is suggested as one of the significant vegetable cultivation practice. It will lead to the production of high quality produce with requisite nutrients such as essential minerals and water soluble vitamins to sustain human life. Future studies can focus on the effect of freezing injury on the nutritional quality including polyphenols and fat soluble vitamins of greenhouse-grown spinaches. In such studies, antioxidant compounds can be also identified and quantified using HPLC which will provide an excellent opportunity to understand the important nutritionally relevant compounds involved in plant defense mechanisms.

Acknowledgement

This study was supported by the National Research Foundation of Korea grant funded by the Korean government (NRF-2015R1A6A1A03031413).

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Supplementary Material

Table S1. Gradient elution utilized on water soluble vitamins analysis using HPLC-DAD.

Time (min)	Mobile phase (%)		
	A (10 mM ammonium acetate, pH 4.5)	B (methanol with 0.1% acetic acid)	C (methanol with 0.3% acetic acid)
0	90	10	0
3	90	10	0
4	50	0	50
7	50	0	50
10	0	100	0
17	0	100	0
20	90	10	0
30	90	10	0

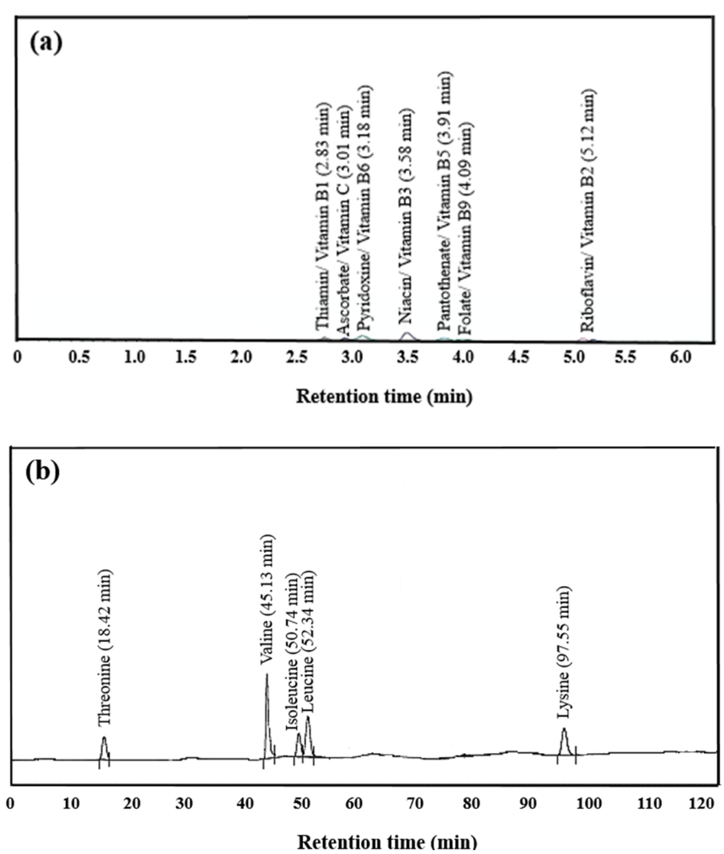

Fig. S1. Chromatogram of (a) water soluble vitamins and (b) essential amino acids examined in this study.