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Summer (Subarctic) versus Winter (Subtropic) Production Affects Spinach (*Spinacia oleracea* L.) Leaf Bionutrients: Vitamins (C, E, Folate, K_1, provitamin A), Lutein, Phenolics, and Antioxidants

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ABSTRACT: Comparison of spinach (*Spinacia oleracea* L.) cultivars Lazio and Samish grown during the summer solstice in the subarctic versus the winter solstice in the subtropics provided insight into interactions between production environment (light intensity), cultivar, and leaf age/maturity/position affecting bionutrient concentrations of vitamins (C, E, folate, K_1, provitamin A), lutein, phenolics, and antioxidants. Growing spinach during the winter solstice in the subtropics resulted in increased leaf dry matter %, oxidized (dehydro) ascorbic acid (AsA), α- and γ-tocopherol, and total phenols but lower reduced (free) AsA, α-carotene, folate, and antioxidant capacity compared to summer solstice-grown spinach in the subarctic. Both cultivars had similar bionutrients, except for higher dehydroAsA, and lower α- and γ-tocopherol in 'Samish' compared to 'Lazio'. For most bionutrients measured, there was a linear, and sometimes quadratic, increase in concentrations from bottom to top canopy leaves. However, total phenolics and antioxidant capacity increased basipetally. The current study has thus demonstrated that dehydroAsA, α-tocopherol, and γ-tocopherol were substantially lower in subarctic compared to subtropical-grown spinach, whereas the opposite relationship was found for antioxidant capacity, α-carotene, and folates (vitamin B_9). The observations are consistent with previously reported isolated effects of growth environment on bionutrient status of crops. The current results clearly highlight the effect of production environment (predominantly radiation capture), interacting with genetics and plant phenology to alter the bionutrient status of crops. While reflecting the effects of changing growing conditions, these results also indicate potential alterations in the nutritive value of foods with anticipated shifts in global climatic conditions.

KEYWORDS: *Spinacia oleracia, carotenoids, day-length, phytonutrients, phylloquinone, temperature, tocopherols, global climatic conditions*

INTRODUCTION

As consumers become more aware of how fruits and vegetables can improve their health, there has been a shift away from the conventional narrow focus on yield to a broader consumer-driven focus that includes quality related to human health bionutrients.[1,2] Fresh spinach (*Spinacia oleracea* L.) is one of the most commonly consumed vegetables and arguably one of the most nutritionally complete vegetables that provides at least 20% or more (100g fresh weight basis) of the recommended dietary intake of ascorbic acid (vitamin C), carotenoids: β-carotene (provitamin A) and lutein, folate (vitamin B_9), phylloquione (vitamin K_1) and α-tocopherol (vitamin E).[3] Vitamins C,[4] K,[5] E,[6] and carotenoids[7] in plants, and to some degree in humans, function as antioxidants, with K_1[8] acting as a redox factor and folate[9] as a methyl donor involved in one-carbon transfers. Each of these vitamins is found in the chloroplast serving together as part of the photosynthetic machinery.[10]

In developed countries, fresh spinach is available year-around, thanks to the ability to grow it at different times of the year in diverse locations, thereby assuring reliable, uninterrupted retail supply. How the wide differences in production location and growth conditions impact spinach leaf vitamins, phenolics and antioxidant capacity is still uncertain. Although the effects of genetics and environment have been examined, these studies have been conducted mostly in isolation. Previous single-factor studies have demonstrated genetic (cultivar) and environmental (production season) effects on vitamin C, phenolics and antioxidant capacity of spinach.[11,12] Other recent studies have also documented significant differential effects of leaf age and canopy position (bottom, middle and top leaves) on vitamins C, E, K_1, B_9, carotenoids and antioxidant capacity of spinach.[13] The goal of this study was to investigate the interactive effects of growth environment (subarctic versus subtropics) and plant canopy leaf location (bottom, middle and

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top) on the concentration of key vitamins (C, E, K_1, and folate-B_9), carotenoids, total phenolics, and antioxidant capacities of two spinach cultivars namely.

MATERIALS AND METHODS

Plant Materials and Field Production. Two widely grown commercial spinach (*S. oleracia* L.) cultivars, 'Lazio' (flat-leafed) and 'Samish' (semisavoy or crinkle-leafed), were grown at the USDA-ARS Subtropical Agricultural Research farm, following commercial production practices, in Weslaco, Texas, United States (26°N, 98°W, elevation 27 m) and at a commercial field adjacent to the Agri-Food Canada, Atlantic Food and Horticulture Research Center, following commercial production practices in Kentville, Nova Scotia, Canada (45°N, 64°W, elevation 35 m) similar to Weslaco, Texas production practices. In Nova Scotia, 'Lazio' and 'Samish' seeds were sown 31 May and 14 June, 2011 respectively and harvested July 14, 2011, and 'Lazio' and 'Samish' in Texas were sown 11 November, 2011 and harvested 28 and 27 January, 2012 respectively. In Texas and Nova Scotia, plants were grown on separate raised beds that were 30 m long, 1.0 m apart, 15 cm high, 30 cm wide at the surface, and 35 cm wide at the base. Irrigation was subsurface, using drip tape with 30 cm spacing between emitters and nominal discharge ratings of 102 L/h/30 m (T-Systems International, Inc., San Diego, CA). There were at least four randomized beds per cultivar with an established plant density of ca. 50 plants per meter per double-row bed. Basic environmental meteorological measurements of temperature and solar radiation (400−1100 nm) were made at each location during the respective growing seasons. Soil texture in Texas was a sandy loam and in Nova Scotia a loamy sand. At both locations, spinach was direct-seeded. At harvest, ten plants per replicate were cut and placed in an ice chest and returned to the lab within 1 h of harvest. Field harvest procedures and subsequent leaf separation were as previously reported in the literature.[13] After plant washing and removal of lower dried and senescent leaves, plants were surface disinfected by dipping in 0.3% sodium hypochlorite for 30 s, rinsing in distilled water and then separating leaves into bottom, middle and top as follows: the lowest (bottom) leaves (2−3 each) were kept, the next 3−4 leaves discarded, the subsequent 2−3 leaves collected as middle leaves, then the following 3−4 leaves discarded, keeping the 2−3 top leaves. Leaf tissue, after separation into bottom, middle and top canopy leaves were frozen in liquid nitrogen, stored at −80 °C prior to overnight shipment in dry ice, to Beltsville, MD for subsequent laboratory processing/analysis. In the lab, subsamples were lyophilized (Virtis Feeezemobile, The Vitis Co. Inc., Gardiner, NY) for dry matter determinations and the remaining leaf samples were used for bionutrient analyses as described below.

Texas-grown spinach, in Beltsville, MD, was subsampled into fresh-frozen tissue for ascorbic acid determination or lyophilized for dry matter content, and for all other bionutrient analyses described below. Freezing spinach to −80 °C and storage at −80 °C for less than six weeks does not impact ascorbic acid profiles.[14] Lyophilized, Texas-grown spinach was sent to Nova Scotia for carotene, tocopherol, and total antioxidant analyses. Nova Scotia-grown spinach was subsampled into fresh-frozen tissue for ascorbic acid determination or lyophilized for dry matter content. Lyophilized, Nova Scotia-grown spinach was sent to Beltsville, MD for total phenolics, folate and phylloquinone analyses as described below.

Plant Bio-Nutrient Analysis. *Compositional Analyses.* All chemicals and standards unless otherwise stated are high performance liquid chromatography (HPLC) grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Ascorbic Acid. Total ascorbic acid (TAsA) and free L-ascorbic acid (FAsA) were extracted from frozen spinach leaves and detected by HPLC as previously described for spinach by Hodges et al. [15] Briefly, 1.5 g of fresh frozen tissue was homogenized with 15 mL of 5% metaphosphoric acid for 30 s, in an ice bath, then sonicated using a probe for 30 s. Extracts were centrifuged at 4 °C for 15 min at 15000× g_n, 2 mL of the supernatant was removed and microfuged at 20817× g_n for 8 min. FAsA was extracted by vortexing 200 μL of extract with 1 mL of 150 mM phosphate/5 mM EDTA buffer pH 7.4, then 200 μL of H_2O. TAsA was extracted by vortexing 200 μL of extract with 1 mL of 150 mM phosphate in 5 mM EDTA buffer, pH 7.4, then 200 μL of 5 mM DTT. Both FAsA and TAsA were incubated in the dark for 15 min, 100 μL of 8.5% *O*-phosphoric acid was added and each solution was filtered through a 0.22 μm nylon syringe filter (Millipore, Bedford, MA). A standard curve using commercial L-ascorbic acid followed the same procedure as FAsA. Detection was by HPLC, using a C18 column (Luna 5 μ, 150 × 4.6 mm, Phenomonex, Torrance, CA) with an isocratic mobile phase of 100 mM phosphoric acid pH 3.0 at a flow rate of 0.6 mL/min was detected by a diode array detector at 243 nm. Concentrations of TAsA and FAsA were calculated by the use of an L-ascorbic acid standard curve (all $R^2 \geq 0.99$), and the difference between TAsA minus FAsA was equal to the concentration of dehydroascorbic acid (DAsA) and concentrations were expressed as mg/100 g fresh weight.

Carotenoids and Tocopherols. Carotenoids (α- and β-carotene, lutein) and tocopherols (α- and γ-tocopherols) were extracted from lyophilized tissue, and determined by HPLC as previously described for spinach by Lester at al[13,16] Briefly, samples (0.05 g of lyophilized tissue) were weighed into glass tubes and 7.5 mL of 1% butylated hydroxytoluene (BHT) in EtOH and 500 μL of the internal standard (86.82 μM *trans-β-apo*-8 carotenal) were added. Tissues were homogenized for 15 s then sonicated, using a probe for 30 s then flushed with N_2, capped and incubated at 70 °C for 15 min, then 180 μL of 80% KOH was added. Vials were flushed with N_2, capped and incubated at 70 °C for 30 min. Cooled samples were diluted with 3.0 mL of Milli-Q water (Millipore, Bedford, MA) and 3.0 mL of hexane/toluene solution (10:8 v/v), vortexed and equilibrated at room temperature for phase separation (10 min). The organic upper layer was removed, evaporated to dryness using N_2 at 30 °C. Extraction with hexane/toluene was repeated four times and the combined organic fraction was dried (N_2 at 30 °C). The dry residue was redissolved in 500 μL of 100% acetone, filtered through 0.2 μm nylon filters (Millipore Corp.). The constituent carotenoids and tocopherols were separated using a C18 column (Discovery 5 μ, 150 × 4.6 mm i.d., Sulpelco, Bellefonte, PA) and acetonitrile/ethanol (50:50 v/v) at a flow rate of 1.0 mL/min for 10 min. Detection was accomplished via a photodiode array detector connected to the HPLC. Absorbance was measured at 290 nm for tocopherols and 454 nm for carotenoids and were quantified using previously developed external calibration standard curves for each compound.

Folate. Folate, as 5-methyltetrahydrofolate, was extracted from lyophilized tissue, and determined by HPLC as previously conducted for spinach by Lester et al.[13] as described by Lester and Crosby.[17] Briefly, each 100 mg sample was homogenized in10 mL of 0.1 M potassium phosphate buffer pH 6.0, filtered through moistened Masslinn towels (Chicopee, New Brunswick, NJ), then centrifuged for 25 min at 40000× g_n at 21 °C. The supernatant was diluted to 50 mL, and 20 mL was then filtered through a solid phase extraction column containing 500 mg of styrene divinylbenzene (Alltech, Deerfield, IL) previously wetted with extraction buffer. Folates were eluted with 6 mL of 1N HCL containing 25% (v/v) acetonitrile into a vial containing 0.2 mL of triethylamine then reduced under N_2 to 5 mL and sealed with a Teflon-lined cap. Detection of folates was by HPLC, using a C18 column (Luna 5 μ, 150 × 4.6 mm, Phenomonex, Torrance, CA) with a programmable mobile phase of 0.1% formic acid and 0.1% formic acid in acetonitrile at a flow rate of 0.85 mL/min and detected by a fluorescence detector at 290 nm excitation and 350 nm emission with a photomultiplier of 12. An external standard, 5-methyltetrahydrofolate, was used to construct a standard curve and retention determinations. Results were expressed as μg/g dry weight.

Phylloquinone. Phylloquinone was extracted from lyophilized spinach leaves, under dim light, and determined by RP-HPLC as previously carried out for spinach by Lester et al.[13] as described by Booth et al.[18] Briefly, each 100 mg sample was homogenized with 10 mL of H_2O and 0.4 mL of 200 μg/mL menaquinone (internal standard, K_2) for 1 min, after which 15 mL of 2-propanol/hexane (3:2, v/v) was added. The sample was vortexed for 1 min and then centrifuged for 5 min at 1500× g_n at 21 °C. The upper (hexane) layer

Table 1. Environmental Differences between Spinach Cultivars Produced during the Subarctic Summer Solstice in Nova Scotia, Canada and during the Subtropic Winter Solstice in South Texas, USA

	summer solstice, subarctic		winter solstice, subtropical	
	Nova Scotia, Canada		South Texas, United States	
	'Lazio'	'Samish'	'Lazio'	'Samish'
elevation (meters)	35	35	27	27
average day length	15 h 10 min	15 h 17 min	10 h 48 min	10 h 48 min
sowing to harvest (days)	44	30	80	79
cumulative season light (kW/m^2)	225.6	161.0	162.3	149.6
avg. season temperature (°C)	16.1	17.0	18.4	18.0
avg. season max./min. temp (°C)	21.5/11.8	22.6/12.2	24.5/12.8	24.5/12.9

was transferred to a glass culture tube and dried under a stream of N_2. The residue was dissolved in 4 mL of hexane and then purified by loading 1 mL into a preconditioned silica gel column (4 mL of 3.5% ethyl ether in hexane, followed by 4 mL of 100% hexane). The loaded column was washed with 2 mL of hexane, and phylloquinone was eluted with 8 mL of 3.5% ethyl ether in hexane and the eluate evaporated on a water-jacketed heating block (Pierce Reacti-Therm, Pierce Chemical Co., Rockford, IL) at 40 °C under N_2. Phylloquinone was reconstituted in 2 mL of mobile phase (99% methanol and 1% 0.05 M sodium acetate buffer, pH 3.0) and filtered through a 0.22 μm nylon syringe filter (Millipore, Bedford, MA). Detection of phylloquinone was conducted by HPLC, using a C18 column (201TP, 5 μ, 150 × 4.6 mm, Grace, Deerfield, IL) with an isocratic mobile phase (described above) at a flow rate of 1 mL/min and detected by diode array at a wavelength of 270 nm. The phylloquinone (Vitamin K_1) concentration was quantified using the internal standard menaquinone (Vitamin K_2) based on peak area and expressed as μg/g dry weight.

Total Phenolics. Total phenolics were detected using the Fast Blue BB method of Medina[19,20] and modified herein for chlorophyll containing plant tissues. Total phenolics in 20 mg of lyophilized tissue were extracted with 10 mL of 80% MeOH containing 0.1% formic acid, in a Teflon-lined capped vial, vortexed, then sonicated with a probe for 30 s. Chlorophyll was removed by washing the extract solution and tissue with 4 mL of hexane, capped, vortexed and sonicated for 30 s then centrifuged at 6650× g_n for 5 min at 4 °C. The hexane wash was repeated two additional times for a total of 3 washes, and each hexane wash layer was removed and discarded. One milliliter of washed methanolic extract was combined with 1 mL of Milli-Q H_2O and microcentrifuged at 20817× g_n for 5 min. Total phenolics were determined in 0.5 mL of supernatant by adding 0.5 mL of Milli-Q H_2O, then 100 μL of 0.1% Fast Blue BB hemi zinc chloride, vortexed, then 100 mL of 5% NaOH, vortexed again, and allowed to react for 90 min at room temperature under normal light conditions with vortexing every 15 min. Total phenolics were determined spectrophotometrically at 420 nm, and quantified using a previously developed gallic acid standard curve in the range of 0−0.50 mg/mL Milli-Q H_2O, and expressed as total phenolics (mg gallic acid equiv/g dry weight). The calibration curve was linear in the range studied with a correlation coefficient of $r^2 = 0.999$.

Oxygen Radical Absorbing Capacity (Antioxidant Capacity). The oxygen radical absorbing capacity (ORAC, or antioxidant capacity) in 50−100 mg of lyophilized tissue was analyzed on a Fluoroskan Ascent FL microplate reader (Thermo Electron Corp. Vanataa, Finland) using 2,2′-azobis (2-amidinopropane) dihydrochloride (AAHP) as a peroxyl generator, fluorescein as the probe and 6-hydroxy-2,5,7,8-tetramethylchlorman-2-carbonic acid (Trolox), as previously used for spinach,[13] following the method of Prior et al.[21] and expressed as antioxidant capacity (μMol Trolox equiv/g dry weight).

Statistical Analyses. Data from leaf dry weight, plant bionutrient analysis, and total anti-oxidant determinates were subjected to analysis of variance (ANOVA) using the general linear model of SAS (SAS Institute Inc. Cary, NC). Means were compared using the "Least Square Means" procedure of SAS version 9.2. Only $P \leq 0.05$ significant differences are discussed unless stated otherwise. The experiment-wise analysis is based upon the ANOVA of the factorial design (cultivar × canopy leaf position × geographical location) by the general linear model of SAS version 9.2. By assigning an average numerical position on the main stem axis for bottom, middle and top canopy leaves for each geographical location, polynomial models could be used to determine if there were linear or quadratic increases or decreases in bionutrients based upon leaf position. The repeatability of all bionutrients and antioxidants was checked by conducting two injections of each replicate of each sample on the same day. In total, each cultivar × canopy leaf position × geographical location was represented by four (Texas) or five (Nova Scotia) replicates. The precision and sample stability were evaluated by running daily, either a standard curve comparison, or an internal and external standard with each sample throughout the study.[22]

RESULTS AND DISCUSSION

Environment. Spinach grown during the summer solstice in the subarctic was exposed to 43% more day length, resulting in 46% more daily irradiation light, than was spinach grown during the winter solstice in the subtropics (Table 1). In addition to the profound effects that a 19° difference in latitude provided, spinach grown during the winter solstice in the subtropics experienced higher season and average minimum and maximum air temperatures than spinach grown during the summer solstice in the subarctic. The two locations allowed for differences in solar angle of the sun, depth of the ozone column, and cloud dispersion of light during the respective growing seasons. These factors influenced the A+B ultraviolet light spectra (280−400 nm), which can have quantitative effects on spinach AsA, β-carotene, and lutein[2] and leaf antioxidant capacities.[23] These environmental differences also had a profound effect on plant growth rate between locations, which in turn affected dry matter content (Table 2); therefore, where appropriate, nutrients were expressed on a dry weight basis.

Production location, canopy leaf position and cultivar had significant effects on bionutrient profiles; however, effects varied as indicated by the interactive effects among these factors (Table 2).

Ascorbic Acid. Reduced or free ascorbic acid (FAsA) was generally higher in middle and top canopy leaves than in bottom canopy leaves for both cultivars grown in both the subarctic during the summer solstice and in the subtropics during the winter solstice (Figure 1A). Only 'Samish' demonstrated an environmental (day-length) effect with higher FAsA during the subtropic winter solstice. Oxidized or dehydroascorbic acid (DAsA) in both cultivars was dramatically impacted by day-length and canopy position (Figure 1B). 'Lazio' and 'Samish' grown during the subtropic winter solstice had 3- to 6-fold higher DAsA, respectively, in bottom and middle canopy leaves. Top canopy leaves had only 12−25%

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Table 2. Experiment-wise Analysis of Variance (ANOVA) for Location (sub-arctic, Nova Scotia, Canada and sub-tropic, Texas, USA), Cultivar ('Lazio' and 'Samish') and Leaf Canopy Position (bottom, middle, and top) for Spinach Leaf Dry Matter (DM), Total, Reduced (free) and Oxidized (dehydro) Ascorbate, Lutein, α- and β-Carotene, Folate, Phylloquinone (Vit. K_1), γ- and α-Tocopherol, and Total Phenols and Anti-oxidants[a]

		ascorbate (mg/100 g FW)			carotenoids (μg/g DW)					tocopherol (μg/g DW)			
source	leaf DM (%)	total	free	dehydro	lutein	α-carot	β-carot	folate (μg/g DW)	vit. K_1 (μg/g DW)	γ	α	total phenolics (mg gallic acid equiv/g DW)	anti-oxidants (μMol Trolox equiv/g DW)
location (Loc)	**	NS	**	**	NS	**	NS	**	NS	**	**	**	**
cultivar (Cv)	NS	NS	NS	*	NS	NS	NS	NS	NS	**	**	NS	NS
Loc X Cv	NS	NS	**	NS	NS	NS	NS	NS	**	**	**	NS	**
leaf position (Lp)	**	**	**	**	**	**	NS	*	**	**	**	**	NS
Loc X Lp	**	**	**	**	NS	**	**	**	*	NS	**	NS	**
Cv X Lp	**	NS	NS	**	NS	*	NS	NS	NS	*	**	NS	**
Loc X Cv X Lp	NS	**	NS	**	NS	NS	NS	NS	NS	**	NS	**	*
Polynomial fits for leaf canopy position by location:													
subarctic	–	L**	L**	L**	L**	Q**	L**, Q**	Q**	L**	L**	L*	L**	–
subtropic	L**, Q*	L**, Q**	L**, Q**	L**, Q**	–	L**, Q**	–	L**	L**	Q*	L**	–	L**

[a]Polynomial fits of responses to leaf age are shown for both locations. NS, *, ** = not significant and significant at $P = 0.05$ or $P = 0.01$, respectively. L, Q, and – represent linear, quadratic, or non-significant polynomial relationships, respectively.

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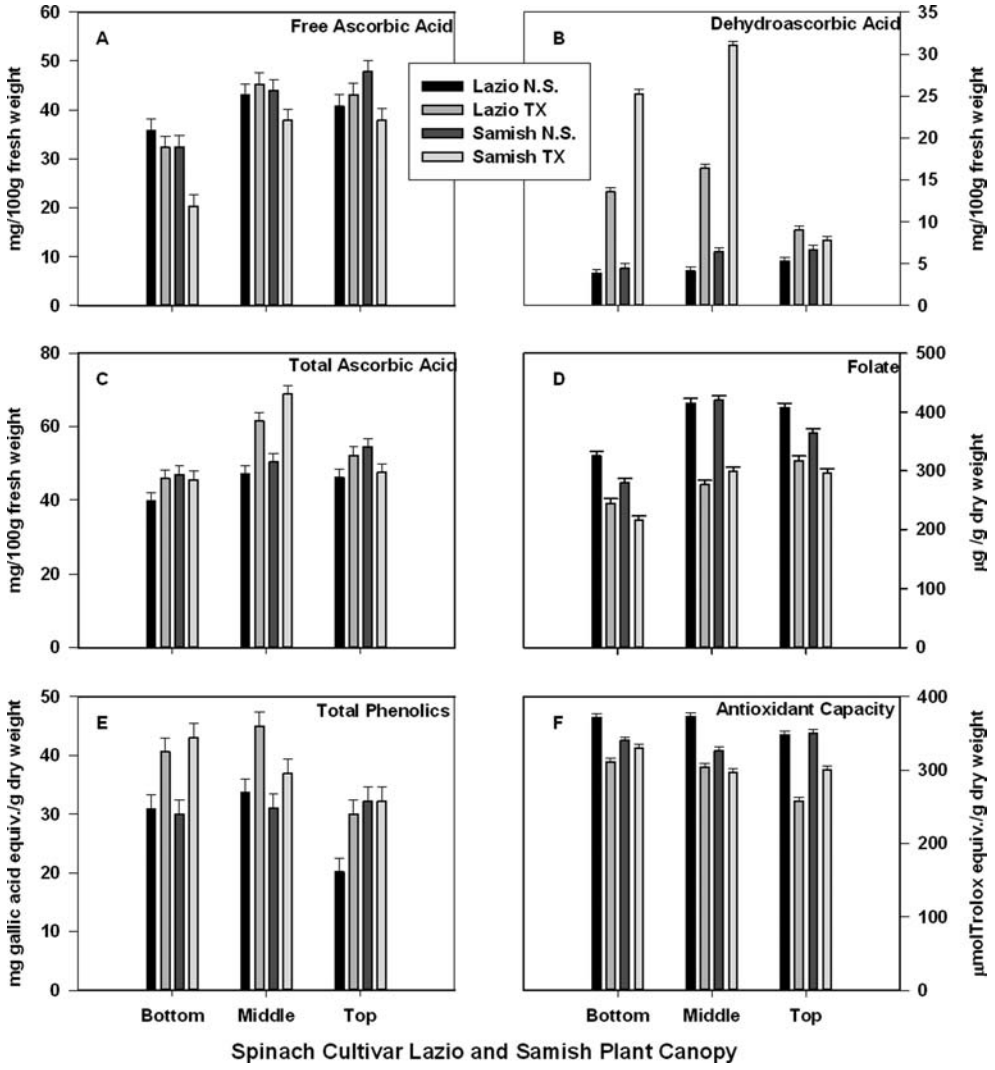

Figure 1. Spinach cultivars Lazio and Samish grown during the subarctic summer solstice in Nova Scotia, Canada (NS) and the subtropic winter solstice in Texas, United States (TX). Plant were separated by bottom, middle and top canopy leaves and assayed for vitamin C as (A) free ascorbic acid, (B) dehydroascorbic acid, and (C) total ascorbic acid, vitamin B_9 as (D) folates, (E) total phenolics, and Oxygen Radical Scavenging Capacity as (F) antioxidant capacity. Error bars represent the standard error of the mean (S.E.).

more DAsA for 'Samish' and 'Lazio', respectively. The impact of the subtropical winter solstice day-length also greatly influenced total ascorbic acid (TAsA) profiles, especially for 'Lazio' (Figure 1C). 'Lazio' grown during the subtropic winter solstice generally had higher TAsA levels at all canopy leaves, whereas 'Samish' had a mixed response, with no TAsA differences in bottom canopy leaves, substantially higher TAsA in middle canopy leaves, and less TAsA in top canopy leaves. This cultivar by canopy effect in TAsA compared to FAsA was also reflected in elevated DAsA concentrations during the subtropic winter solstice. DAsA normally makes up less than 10% of the TAsA,[24] but in this study it contributed as much as 50%. Higher levels of TAsA and FAsA in middle and top canopy versus bottom canopy leaves has been reported for spinach,[13] collard (*Brassica oleracia* L. convar. *Acephala* (DC.), mustard (*Brassica juneca* L.) and turnip (*Brassica rapa* L.) greens[25] and is expected to be higher in the upper canopy of rapidly growing leaves where higher levels of FAsA-generating enzymes are present.[26] The elevated concentration of DAsA, during the subtropical winter solstice indicated a more stressful environment as the enzyme ascrobate oxidase, which degrades FAsA to DAsA, is increased due to environmental stress.[27] One morphological response to elevated levels of DAsA is reduced plant growth;[28] thus, the lower total plant mass (data not shown) of subtropical winter solstice-grown spinach may be partially attributed to elevated DAsA concentrations.

Folate. Similar to FAsA responses, folate levels were higher in middle and top canopy than bottom canopy leaves for both cultivars and environments. Folate was higher in all leaves grown under the subarctic summer solstice and, except for middle canopy leaves, folate was higher in 'Lazio' than 'Samish' (Figure 1D). Higher levels of folate in middle and top canopy versus bottom canopy leaves has been previously reported for spinach,[13] collard, mustard, and turnip greens;[23] folate concentrations are expected to be higher in the upper canopy, as folate is synthesized only in mitochondria, which are abundant in rapidly growing leaves[29] and fruits.[17] Knowledge of folate biosynthesis and its regulation is fragmentary, as no coherent overall picture has emerged.[29] Folate concentrations seem to increase linearly with continuous light exposure, even in detached spinach leaves.[13] Soil type[17] and genetic[25] effects on folate synthesis and accumulation have also been reported.

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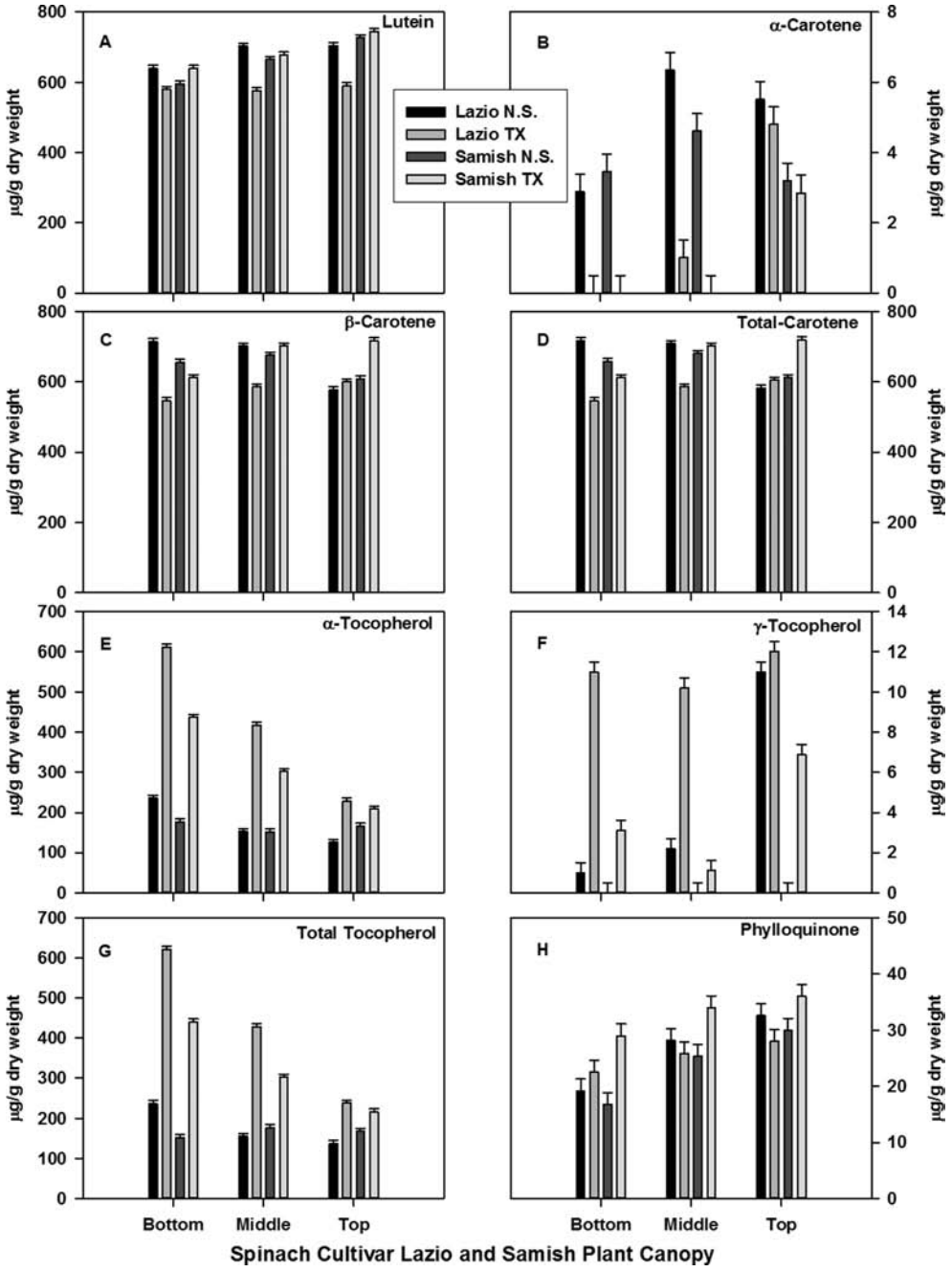

Figure 2. Spinach cultivars Lazio and Samish grown during the subarctic summer solstice in Nova Scotia, Canada (NS) and the subtropic winter solstice in Texas, United States (TX). Plant leaves were separated by bottom, middle and top canopy locations and assayed for (A) lutein, (B) α-carotene, (C) provitamin A as β-carotene, (D) total carotenes, (E) vitamin E as α-tocopherol, (F) γ-tocopherol, (G) total tocopherols and (H) vitamin K as phylloquinone. Error bars represent the standard error of the mean (S.E.).

Total Phenolics and ORAC (Antioxidant Capacity). Total phenolics (Figure 1E) were generally lower in top canopy leaves than in middle and bottom canopy leaves for 'Lazio' regardless of growing location, and for 'Samish' grown during the subarctic summer solstice. Similar observations have also been reported in black currant (*Ribes nigrum* L.) cultivars, whereby, higher total phenolics were in fruits grown at lower latitudes (subtropics) compared with higher latitudes[30] presumably due to radiation and temperature differences between these growth environments.

Total antioxidant concentrations also tended to be higher in bottom and middle versus top canopy leaves, but total antioxidants were always higher in all leaves, regardless of canopy location, or cultivar when grown during the summer solstice in the subarctic higher latitude. It has generally been observed that under high light conditions, plants tend to have higher antioxidant capacities and this helps protect against oxidative stress arising from light-dependent overproduction of reactive oxygen species (ROS).[24] This helps explain the observed trends in TAsA and FAsA levels seen in subarctic summer solstice-grown spinach leaves (Figure 1A).

Phenolics also are known to protect ascorbic acid from oxidative decomposition, but phenolics are generally weak scavengers.[31] Although subtropic winter solstice grown-spinach

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had higher total phenolics, likely resulting from the lower antioxidant levels, the higher total phenolic levels may not have been able to compensate for the lower antioxidant concentrations and were less effective in controlling oxidative decomposition of AsA, resulting in heightened DAsA levels.

Carotenoids. Lutein was generally higher in middle and top canopy leaves than in bottom canopy leaves for both cultivars under both growing environments (Figure 2A). However, this effect was weak in 'Lazio'. Lutein is one of two major carotenoids in spinach, with β-carotene being the other[13] and is usually found in higher concentrations in upper canopy leaves.[25]

Alpha-carotene was greatly influenced by growing location and to a lesser extent by canopy position (Figure 2B). Alpha-carotene was present in both cultivars, but with lesser concentrations in bottom than middle or top canopy leaves when grown during the subarctic summer solstice. When grown during the subtropic winter solstice α-carotene was either barely detectable in bottom or middle canopy leaves of both cultivars. Regardless of the large environmental effect, there was a cultivar effect as 'Lazio' had nearly a 2-fold higher α-carotene concentration than 'Samish'. In spinach leaves, α-carotene is generally present in much lower concentrations (0−6 μg/g dry weight) compared to β-carotene (575−725 μg/g dry weight) (Figure 2C). Nevertheless, the response of α-carotene in spinach to subarctic summer solstice versus subtropic winter solstice growing conditions is a notable, novel finding.

Beta-carotene, which accounted for nearly 100% of the total carotene in leaves (Figure 2D), was different in bottom versus middle or top canopy depending on growth environment. When grown during the subarctic summer solstice β-carotene was higher in bottom and middle canopy versus top canopy leaves. But when grown during the subtropic winter solstice β-carotene was lower in bottom canopy versus middle and top canopy leaves. Beta-carotene, like lutein, is usually found in higher concentrations in upper canopy leaves.[25] However, this study indicated the concentration of β-carotene in spinach leaves is confounded by cultivar, geographic location and leaf canopy position. This lack of a significant trend for any one of the aforementioned variables is likely due to β-carotene's role in plant photosynthesis, as it is more directly affected by light, and thus more dynamic, than most other plant derived bionutrients and has been shown to exhibit instability related to biochemical properties of the enzymatic pathway during early stages of plant development and in field environments.[25,32]

Tocopherols. Like β-carotene, α-tocopherol (Figure 2E) accounted for nearly 100% of the total tocopherols (Figure 2G). Alpha-tocopherol (the bioactive form of vitamin E) was higher in bottom canopy leaves and declined linearly in middle and top canopy leaves when grown in the subtropic winter solstice, and subtropical, winter solstice-grown spinach had 2 to 3-fold more α-tocopherol in bottom canopy leaves and up to 2-fold more in top canopy leaves than subarctic, summer solstice-grown spinach. Alpha-tocopherol concentrations are inversely related to growth rate, with the highest concentrations found in the "slower-growing" older leaves.[13] Also, spinach grown in the subarctic summer solstice showed little difference in α-tocopherol concentrations between any of the canopy leaves, a new finding.

Gamma-tocopherol was either detected or not detected based on cultivar leaf position and geographical location (Figure 2F). 'Samish' grown during the subarctic summer solstice had no γ-tocopherol, but when grown in the subtropic winter solstice γ-tocopherol was mostly found in the top canopy leaves. A similar trend was found with 'Lazio' but only when grown in the subarctic summer solstice. 'Lazio' grown in the subtropic winter solstice had the highest concentration of γ-tocopherol with nearly equal concentrations found in bottom, middle and top canopy leaves. Like α-carotene, γ-tocopherol is present in spinach leaves, but it is not a critical pool (0−12 μg/g dry weight) of tocopherol compared to α-tocopherol (175−625 μg/g dry weight) (Figure 2E). Nevertheless, the response of γ-tocopherol in spinach cultivars and canopy leaf position to subarctic summer solstice versus subtropic winter solstice growing conditions was a novel finding.

Phylloquinone. Phylloguinone was generally lower in bottom and middle canopy leaves than in top canopy leaves (Figure 2H). There was an environmental effect but it differed by cultivar. 'Samish' had higher phylloquinone when grown during the subtropic winter solstice, but "Lazio" had only higher levels in bottom canopy leaves under the subtropic winter solstice. 'Lazio's' middle and top canopy leaves showed no strong statistical phylloquinone concentration difference between the two environments.

Our data for lutein and phylloginone are consistent with previous findings that growth under high light conditions is generally associated with higher carotenoids, tocopherols and phylloquinone levels.[33] In contrast, Simkin et al.[34] found that higher carotenoids levels tomato (*Lycopersicon esculentum*) leaves were associated with shorter photoperiods; this could be due to shifts in resource (nutrients and photoassimilates) allocation patterns among components of the photosynthetic machinery.[35]

The general line of thought for the past 60 plus years[35] has been that plants exposed to higher light levels have higher nutritional concentrations. This, on the surface appears plausible especially when photosynthesis is dependent upon the presence of bionutrients: ascorbic acid, carotenes, folate, totopherols and phylloquinone for its biochemical functioning and photosynthesis is activated by light. The current study presents evidence that genetics, environment, and plant phenology either acting singly or interactively can alter the patterns of bionutrient concentrations in crops. TAsA was generally higher when grown during the subtropic winter solstice, whereas folate was higher when grown during the subarctic summer solstice and phylloquinone had a contrasting cultivar response to latitude and day-length. Leaf lutein concentration tended to increase from the bottom canopy, middle to the top, whereas total carotenoids showed little change by canopy location with both carotenoids affected little by latitude and day-length. In contrast, total-tocopherol concentrations were highest in the bottom canopy and lowest in the top with their response being more striking in plants grown in the subtropic winter solstice. This study provides a foundation of biochemical information that can be used by spinach breeders, growers, and consumers to help understand the impact of plant bionutrient responses due to changing environmental conditions.

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