Diurnal Fluctuation in Tissue Nitrate Concentration of Field-grown Leafy Greens at Two Latitudes

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Abstract. Tissue nitrate (NO_3) concentration (TNC) in leafy greens generally decreases with increasing light intensity and photoperiod in controlled environment studies. Harvesting late in the day has been recommended as a way to produce leafy greens with lower TNC, although data from field research do not support this recommendation. This study investigated the effect of time of day of harvest on TNC in lettuce (*Lactuca sativa* L.) and spinach (*Spinacia oleracea* L.) grown in the field during the summer at Pullman, WA (lat. 46° N) and Fairbanks, AK (lat. 64° N). Whole plants were sampled every 2 h on three separate, 24-h harvest dates at each latitude. Plants were dried, ground, and analyzed for $\text{NO}_3\text{-N}$. At the high-latitude location, TNC decreased linearly during the day (1000 to 2300 HR) on all three dates for spinach and one for lettuce. At the low-latitude location, TNC decreased linearly during the day (1000 to 1900 HR) on one date and increased linearly during the night (2000 to 0400 HR) on two dates for lettuce. The TNC (average 287 to 607 mg $\text{NO}_3\text{-N/kg}$ fresh weight for lettuce and 141 to 189 mg $\text{NO}_3\text{-N/kg}$ fresh weight for spinach) and magnitude of diurnal fluctuation (generally less than 25%) should not pose a human health risk regardless of when plants are harvested.

Leafy green vegetables are an important source of NO_3^- in the human diet (Maynard et al., 1976; Santamaria et al., 1999; Vogtmann et al., 1984). Vegetables with a tendency to accumulate high concentrations of NO_3^- include lettuce, spinach, Swiss chard (*Beta vulgaris* var. *cicla* L.), and radish (*Raphanus sativus* L.). These species are considered nitrophilic (Vogtmann et al., 1984) because of luxury nitrogen (N) consumption that may lead to its accumulation in excess of the amount required for maximum growth (Grindlay, 1997).

Plants grown under conditions of high light intensity and long photoperiod often have lower TNC (Maynard et al., 1976) attributable, in part, to light activation of nitrate reductase activity (Kaiser and Huber, 2001; Solomonson and Barber, 1990). In a global survey of field-grown vegetables, Roorda van Eysinga (1984) reported that several crops, including lettuce and spinach, grown in areas of high light intensity had lower TNC than those same crops grown in areas with low light intensity. Increased light intensity decreased TNC in spinach (Steingrover et al., 1986), oat (*Avena sativa* L.), rye (*Secale cereale* L.) (Chen and Ries, 1969), and corn (*Zea mays* L.) (Hageman et al., 1961) seedlings. Leaf blade $\text{NO}_3\text{-N}$ concentration in spinach grown in a controlled environment at 145 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ during the light period increased from ≈ 1100 to 2300 mg $\text{NO}_3\text{-N/kg}$ fresh weight during the dark period when sampled every 2 h (Steingrover et al., 1986). Cantliffe (1972a) reported that spinach grown in a controlled environment under light levels of $\approx 120 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ had more than twice the TNC than those grown under ≈ 320

$\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Spinach TNC was reduced from 2000 to 700 mg $\text{NO}_3\text{-N/kg}$ fresh weight when light intensity was increased from 200 to 800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Proietti et al., 2004). Either increases in light intensity or longer photoperiods decreased TNC in wheat (*Triticum aestivum* L.) (Harper and Paulsen, 1968). Cantliffe (1972b) found that the TNC of spinach grown for 12 h in light of $\approx 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was 22% lower than spinach exposed to 6 h of light at the same intensity.

To lower TNC in leafy greens grown for human consumption, researchers have recommended harvesting crops near the end of the photoperiod (Cantliffe, 1972a; Maynard et al., 1976; Steingrover et al., 1982). However, this recommendation is based on studies conducted in growth chamber or greenhouse environments. In a field study, Weightman et al. (2006) found no consistent trends in TNC when sampling field-grown lettuce at 3-h intervals from 0700 to 2200 HR. In fact, TNC was often lower at 0700 and 1000 HR compared with 1600 HR. The objective of this study was to determine the influence of time of day of harvest on TNC in summer-grown lettuce and spinach at two latitudes with different light intensities and photoperiods.

Materials and Methods

Study sites and preparation. One study site was located at the Washington State University (WSU) Tukey Organic Research Farm (lat. 46°43' N, long. 117°10' W) in a Palouse silt loam (fine-silty, mixed, superactive, mesic Pachic Ultic Haploxeroll). The other site was located at the University of Alaska Fairbanks (UAF) Experiment Farm (lat. 64°86' N, long. 147°68' W) in a Tanana mucky silt loam (coarse-loamy, mixed, superactive, subgelic Typic Aquiturbels). Both sites featured a randomized complete block experiment design with four replications. Plot size at WSU was 1.2 m wide $\times$ 7.6 m long (9.1 m²) with five rows of plants. Plot size at UAF was 0.6 m wide $\times$ 6.1 m long (3.7 m²) with three rows of plants.

At WSU, compost was applied at ≈ 56 kg total N/ha and incorporated with a tractor-driven tiller. At UAF, a bed shaper-cultivator created raised beds. Compost was not available at the UAF site. Fishmeal was applied at WSU (8N–1.3P–0K; Peaceful Valley Farm Supply Inc., Grass Valley, CA) and at UAF (9N–2P–0K; Pro-Pell-It!; Marion Ag. Services, Inc., Aurora, OR) at 168 kg total N/ha. The fishmeal was broadcast by hand and incorporated with a rake to a depth of 5 cm several days before seeding or transplanting at both sites.

Soil samples were taken with a 2-cm diameter probe at depths of 0 to 15 cm, 15 to 30 cm, and 30 to 60 cm after compost application at WSU but before planting or fishmeal application at both sites. Soil at both sites was sampled again immediately after the last harvest. Separate samples were collected from each replication with two soil cores combined for each replication. Samples for both sites were sent to a commercial soil

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testing laboratory and analyzed for KCl-extractable $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ by colorimetric analysis in a continuous flow analyzer, pH (1:10 soil:water ratio by glass electrode), and organic matter by dry combustion.

'Cracoviensis' lettuce and 'Giant Winter' spinach were selected based on performance in previous research conducted at the WSU site (Ott et al., 2008). At WSU, both crops were seeded directly into soil on 13 May 2009 at 20 cm within-row spacing and rows spaced 20 cm apart. After emergence, plants were thinned to one per space. One line of drip tape irrigated each row twice a week at ≈ 2.5 cm/irrigation. At UAF, plant spacing was 20 cm within the row and 25 cm between rows. Lettuce was planted in a greenhouse on 16 May 2009 and transplanted into the field on 9 June. As a result of problems with greenhouse germination, spinach was direct-seeded on 9 June 2009. Two lines of drip tape were used to irrigate three rows of plants five times a week (≈ 0.5 cm/irrigation). At both locations, weeds were controlled by hand removal. Light intensity was measured every 15 min from planting through harvest using a photosynthetically active radiation (*PAR*) sensor (Hobo S-LIA-M003; Onset, Bourne, MA) and recorded with a data logger (Hobo Micro Station S-LIA-M003, H21-002; Onset).

Tissue sampling and processing. Tissue samples were collected every 2 h during three separate, 24-h periods at each location. Harvests always started at 1000 HR and ended at 0800 HR the next day. At WSU, two plants were collected from each plot at each harvest on 12 June, 18 June, and 24 June 2009. At UAF, harvest dates were 2 July, 7 July, and 10 July 2009. As a result of a limited number of plants, only one plant was harvested per plot on each date at UAF. Lettuce plants had bolted by the third harvest at UAF.

At harvest, plants were cut ≈ 3 cm below the soil surface, placed in labeled plastic bags, and transported from the field to the laboratory within 1 h. Roots and damaged leaves were removed from plants. Plants were rinsed with deionized water to remove soil, blotted dry with paper towels, and weighed. Samples were placed in paper sacks, dried at 80 °C for at least 48 h, and weighed again for data correction.

Oven-dried tissue was ground using a mortar and pestle. To extract $\text{NO}_3\text{-N}$, 0.4 g of ground tissue was transferred to a 50-mL vial to which 40 mL of 0.025 M $\text{Al}_2(\text{SO}_4)_3$ extracting solution was added. If 0.4 g was not available, all of the ground tissue was transferred and the exact weight recorded. Vials were capped and placed on an oscillating shaker for 30 min. The solution was filtered through a Whatman #2 paper and refrigerated until analysis.

Nitrate concentration was measured using an ion selective nitrate electrode (Orion Model 9700 BN Ionplus; Thermo Electron Corporation, Waltham, MA). The electrode was calibrated according to manufacturer recommendations using standards with concentrations of 1.4, 14, and 140 mg $\text{NO}_3\text{-N/L}$ in the same matrix solution as the tissue extracts. Approximately 30 mL of 20 °C extract was

poured into a 100-mL beaker with a magnetic stir bar, and the extract was stirred at the same rate as the standards. Electrode drift was monitored by reading the midrange standard every six samples. The electrode was recalibrated if the reading deviated by 10% or greater from the standard concentration.

Whole-plant TNC on a fresh weight basis was calculated and plotted as a function of time for each harvest at each site. Data were analyzed using the PROC MIXED procedure in SAS (SAS Inst., Cary, NC). Because harvests occurred on separate days at each site, data were analyzed separately by site. Linear regression analysis (PROC REG) was used to test for the presence of significant trends in TNC over time. The night period was defined as *PAR* 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ or less. Because plant sampling started at 1000 HR each day at both locations, for the purposes of regression analysis, the day period was defined as 1000 to 2300 HR for Fairbanks and 1000 to 1900 HR for Pullman. Night periods were 2400 to 0500 HR at Fairbanks and 2000 to 0400 HR at Pullman.

Results

Available N in the top 60 cm ($\text{NO}_3 + \text{NH}_4$ at 0- to 30-cm depth, NO_3 at 30- to 60-cm depth) of soil was not significantly different between sites and averaged 61 kg N/ha before fish meal application and 136 kg N/ha after the third harvest (Table 1). The average maximum light intensity was 1480 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at UAF and 1940 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at WSU. The UAF site had ≈ 5 h of 0 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\cdot\text{d}^{-1}$ *PAR* (Fig. 1), although there was always sufficient light to see throughout the 24-h sampling period. The WSU site had ≈ 8 h of 0 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}\cdot\text{d}^{-1}$ *PAR* (Fig. 2). Weather conditions were mostly clear on all three harvest days at UAF. At WSU, clouds increased after 1200 HR on 12 June and 18 June 18 partly cloudy. Sky conditions generally are reflected in *PAR* measured at each location (Figs. 1 and 2).

Average fresh weight yield was 65 and 73 g/plant for lettuce at UAF and WSU, respectively. Average fresh weight yield was 9 and 57 g/plant for spinach at UAF and WSU, respectively. The low fresh weight yield for spinach at UAF was as a result of direct seeding less than 1 month before harvest started. Average TNC at UAF was 607 mg $\text{NO}_3\text{-N/kg}$ fresh weight for lettuce and 189 mg $\text{NO}_3\text{-N/kg}$ fresh weight for spinach. At WSU, average TNC was 287 mg $\text{NO}_3\text{-N/kg}$ fresh weight for lettuce and 141 mg $\text{NO}_3\text{-N/kg}$ fresh weight for spinach.

At each site, lettuce consistently had higher TNC than spinach. At UAF, there were no significant main effects of harvest time or interaction between species and harvest time on TNC. TNC decreased linearly during the day (1000 to 2300 HR) on all three dates for spinach and one for lettuce (Fig. 1). At WSU, there was no significant main effect of harvest time or interaction between species and harvest time for the 12 to 13 June sampling. For the remaining two harvests, there was a significant main effect of harvest time and interaction between time and species ($P \leq 0.05$). Tissue $\text{NO}_3\text{-N}$ concentration decreased linearly during the day (1000 to 1900 HR) on one date and increased linearly during the night (2000 to 0400 HR) on two dates for lettuce (Fig. 2).

Discussion

In principle, our results support the recommendation to harvest late in the day to produce leafy greens with lower TNC (Cantliffe, 1972a; Maynard et al., 1976; Steingrover et al., 1982). However, although in the majority of cases TNC decreased linearly with time during the day and increased at night, the magnitude (slope) of change was relatively small with maximum diurnal changes of less than 25% compared with controlled environment studies in which TNC varied by as much as 100% to 300% diurnally or under different light intensities (Cantliffe 1972a; Proietti et al., 2004; Steingrover et al., 1986). In contrast, in field-grown lettuce Weightman et al. (2006) also reported that TNC did not follow a diurnal pattern if plants were sampled at 3-h intervals from 0700 to 2200 HR, and TNC was often lower at 0700 and 1000 HR than at 1600 HR. Although our results indicate some diurnal change in the TNC of lettuce and spinach, the trends are not as apparent as those from controlled-environment studies in which a clear diurnal pattern in TNC is reported (Maynard et al., 1976; Steingrover et al., 1986).

Light intensity and duration in this field study were considerably higher than in controlled environment studies for which clear diurnal changes in TNC are reported. For example, Cantliffe (1972a) reported that spinach grown in a controlled environment under light levels of $\approx 120 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ had more than twice the TNC compared with those grown under $\approx 320 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Spinach TNC was reduced from 2000 to 700 mg $\text{NO}_3\text{-N/kg}$ fresh weight when light intensity was increased from 200 to 800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Proietti et al., 2004). The controlled environment light intensities are well below the maximum *PAR*

Table 1. Pre-fertilization and post-harvest soil test data for study sites at the University of Alaska, Fairbanks (UAF) and Washington State University (WSU), Pullman.

	Depth (cm)	$\text{NO}_3\text{-N}$ (kg·ha ⁻¹)		$\text{NH}_4\text{-N}$ (kg·ha ⁻¹)		pH		Organic matter (%)	
		UAF	WSU	UAF	WSU	UAF	WSU	UAF	WSU
Pre-fertilization	0–30	24.7	14.3	13.5	16.6	7.53	6.67	5.57	3.60
	30–60	42.6	10.1						
Post-harvest	0–30	70.3	77.3	8.1	34.5	6.93	6.25	5.86	3.75
	30–60	42.6	40.4

Fig. 1. Variations in photosynthetically active radiation (*PAR*) and tissue nitrate concentration (*TNC*) in lettuce and spinach sampled over three 24-h periods at the University of Alaska (*UAF*) in Fairbanks, AK. Lines indicate significant ($P \leq 0.05$) linear relationships between time and *TNC* for the light period. An insufficient number of data points exist for linear analysis of *TNC* over time during the dark period.

Fig. 2. Variations in photosynthetically active radiation (*PAR*) and tissue nitrate concentration (*TNC*) in lettuce and spinach sampled over three 24-h periods at Washington State University (*WSU*) in Pullman, WA. Lines indicate significant ($P \leq 0.05$) linear relationships between time and *TNC* for the period indicated.

values (1400 to $1900 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; Figs. 1 and 2) measured during summer production at the two latitudes where our study was conducted, which may partially explain the lower *TNC* in our studies. Also, Cantliffe (1972b) reported that *TNC* of spinach grown for 12 h in light of $\approx 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was 22% lower than spinach exposed to 6 h of light at the same intensity, even with relatively high N fertilizer rates. It is possible that with the longer photoperiods and higher light intensities that were observed in this field study and that occur in the summer at northern latitudes, there was not a sufficient dark period for plants to accumulate nitrate and produce the clear diurnal patterns observed in greenhouse and growth chamber research.

Because harvests did not occur on the same days between the *WSU* and *UAF* sites, it is not possible to draw direct comparisons between the two latitudes. Overall, *TNC* in lettuce appeared to be higher at *UAF* than at *WSU* although plant size and soil N conditions were similar between sites. Differences in *TNC* between sites were generally small for spinach although plants were much smaller at *UAF* as a result of direct seeding. The longer photoperiod associated with the higher latitude at *UAF* did not appear to result in

lower *TNC* than at the lower latitude, perhaps because light intensity was lower at *UAF* than at *WSU*. At *UAF*, bolting of lettuce by the 10 July harvest resulted in highly erratic *TNC* for this sampling date (Fig. 1). At *WSU*, the presence of cloud cover on 12 June and 18 June (*PAR* data, Fig. 2) may be responsible for the absence of clear linear trends in *TNC* during those days for either species at this site. These lower (12 June) or erratic (18 June) light intensities may have reduced nitrate reductase enzyme activity and impacted the linear trends in *TNC*, which were more common on sunny days.

Overall, the *TNC* measured in this study was much lower than in controlled environment research and other field studies. A significant factor influencing the *TNC* in leafy vegetables is fertilizer supply and N availability (Guadagnin et al., 2005; Maynard et al., 1976). Fertilizer from organic sources typically has a lower N availability than inorganic N fertilizer (Gaskell and Smith, 2007) and may be responsible for low *TNC* observed in this study.

The European Commission has set limits on acceptable concentrations of NO_3 in leafy greens depending on crop, harvest time, and method of cultivation (Santamaria, 2006).

According to the European Commission, fresh lettuce and spinach grown in summer under field conditions have maximum permissible nitrate levels of $2500 \text{ mg NO}_3/\text{kg}$ fresh weight ($565 \text{ mg NO}_3\text{-N}/\text{kg}$ fresh weight). In our study, average *TNC* ranged from 287 to $607 \text{ mg NO}_3\text{-N}/\text{kg}$ fresh weight for lettuce and 141 to $189 \text{ mg NO}_3\text{-N}/\text{kg}$ fresh weight for spinach. These concentrations were well below the European Commission limits for spinach and exceeded the limits for lettuce only at *UAF* on one harvest date.

Spinach generally had lower *TNC* than lettuce in this study. This is contrary to research indicating that spinach has higher *TNC* than lettuce (Muramoto, 1999; Roorda van Eysinga, 1984; Santamaria et al., 1999). Amr and Hadidi (2001) measured slightly higher *TNC* in lettuce than in spinach. Additionally, in a 10-year study in which spinach and lettuce samples were collected from retail markets in Sweden, average *TNC* was $600 \text{ mg NO}_3\text{-N}/\text{kg}$ fresh weight for lettuce and $400 \text{ mg NO}_3\text{-N}/\text{kg}$ fresh weight for spinach (Merino et al., 2006). However, there is considerable cultivar variation within a species of leafy greens (Cantliffe, 1973; Maynard and Barker, 1974; Ott et al., 2008). For example, when Barker et al. (1974) evaluated 18 cultivars of spinach, they found

that some cultivars had TNC that was twice as high as other cultivars. Differences in TNC between lettuce and spinach grown in this study were as likely the result of the cultivars selected as the species themselves.

Conclusions

Our results indicate that although TNC of summer field-grown leafy greens may decline linearly and gradually over time during the day and increase at night, diurnal variation is not as consistent nor is the magnitude of change as great as that reported in controlled-environment studies. Higher light intensities and longer photoperiods during the summer at these latitudes may explain the difference between this field study and controlled-environment research. The TNC and magnitude of diurnal fluctuation should not pose a human health risk regardless of when plants are harvested under these conditions.

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