Effects of light level, time of harvest and position within field on variability of tissue nitrate concentration in commercial crops of lettuce (Lactuca sativa) and endive (Cichorium endiva)

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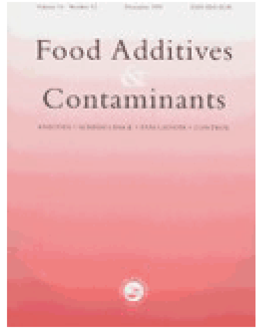

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Journal:	*Food Additives and Contaminants*
Manuscript ID:	TFAC-2005-276.R3
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	12-Dec-2005
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Methods/Techniques:	Survey
Additives/Contaminants:	Nitrate
Food Types:	Vegetables

Abstract

Seven commercial crops of lettuce and one crop of endive were sampled in order to study the variability in plant tissue nitrate concentration (TNC). Assuming that an appropriate sampling pattern was employed, 10 plants were sufficient to give an acceptable estimate of the mean TNC. Short-term shading (24-48 h) had no significant effects on mean TNC, unlike the increase in TNC known to occur following dull periods 10-14 days prior to harvest. The effect on TNC of time of day harvested was significant, but there was no obvious pattern of diurnal variation. Averaged over all experiments, the coefficient of variation for TNC was of the order of 35%. Increasing the sample size from 10 to 40 plants would only be expected to decrease the standard error of measurement of TNC from 16 to 12% of the mean, because of the underlying analytical error which would remain constant.

Keywords: lettuce, endive, nitrate, light, shading, variability, sampling directive, Lactuca sativa, Cichorium endiva

Introduction

Nitrate is naturally present in all vegetables. Green, leafy vegetables for example lettuce and spinach, contain the highest concentration, and are the major source of nitrate in the diet. The European Commission has set maximum limits for levels of nitrate in lettuce and spinach (Anon. 2005). The limits for tissue nitrate concentration (TNC) in fresh lettuce are as follows (in each case values are presented for crops grown either under cover, or in the open air respectively, on a fresh weight basis): 4500 and 4000 $mgNO_3^-$ kg^{-1} for non-Iceberg type lettuces harvested from 1 October to 31 March; 3500 and 2500 $mgNO_3^-$ kg^{-1} for non-Iceberg type lettuces harvested from 1 April to 30 September; 2500 and 2000 $mgNO_3^-$ kg^{-1} for Iceberg type lettuces (harvested at any time of the year).

Currently, nitrate analysis for monitoring purposes is based on a determination made on a bulked (composite) sample of typically 10 lettuce plants (Anon. 2005). This choice of sample size is based on values found in the sampling Directive 2002/63/EC (Anon. 2002a) for the official control of pesticide residues in, and on, products of plant or animal origin. For the purposes of monitoring contaminants other than nitrate, this sample size would only apply where the unit weight is less than 250 g. This weight covers all lettuce varieties except Iceberg types, which have a higher marketable head weight (Anon. 2001) and where normally five plants or 2 kg of fresh plant tissue would be sampled (Anon. 2002a). However, the regulations pertaining to nitrate stipulate that the minimum number of units per laboratory sample is 10 for Iceberg lettuce types (Anon. 2005). One of the aims of the present study was to determine whether the present method of bulking 10 plants is adequate to provide a representative sample of lettuce, and was part of a larger study designed to provide guidance on sampling lettuce and spinach for nitrate analysis (Weightman *et al*. 2005).

Before an optimum sample size can be estimated, the variance of the population must be known. However, at the outset of the study, there were no data available regarding the typical variability in TNC for commercially grown crops of lettuce, particularly outdoor crops. Moreover in designing sampling plans, it is important to understand the sources of any variability present.

It is known that there is considerable variation in TNC in lettuce and spinach, with typically reported ranges for protected crops (i.e. crops grown under cover) of 2000 - 5000 $mgNO_3^-$ kg^{-1} (mean 3300 $mgNO_3^-$ kg^{-1}) for lettuce, and 2600 - 4900 $mgNO_3^-$ kg^{-1} (mean 3900 $mgNO_3^-$ kg^{-1}) in spinach (van Eysinga 1984). Soil nitrate is known to influence TNC in lettuce (Richardson and Hardgrave 1992), and significant spatial variability in soil nitrate is known to exist in UK soils (Dampney *et al*. 1997).

Soil sampling protocols have been developed in order to address such variability (MAFF 1983), but there is relatively little information on development of plant sampling protocols (Needham and Harrod 1973; MAFF 1979). Current field sampling protocols for advisory purposes in the UK (e.g. Anon. 1991) are based on work carried out in the 1940s and 1950s (Lessells 1959, 1973; Boyd and Simpson 1956). Moreover much of the data on which the earlier studies are based are not publicly available, and where documents are available, relate principally to soil rather than plant sampling.

It has been demonstrated that winter-sown crops have generally higher TNC levels than summer crops in the same environment (Byrne *et al*. 2004), and that northern European crops have higher TNC levels than corresponding southern European crops (Anon. 2005). It is recognised that these differences can be due to both higher irradiance in summer, which tends to reduce nitrate, and also to higher growth rates which coincide with periods of high irradiance and warmer temperatures (Kanaan and

Economakis 1992). Burns (2000) suggested that shading in the 10-14 days prior to harvest would increase TNC in glasshouse lettuce, even when best agricultural practice is being followed. Therefore, current guidelines in UK crop assurance protocols suggest that growers avoid sampling lettuce during dull weather conditions (Anon. 2002b). However, the importance of short timescale variations in irradiance (e.g. 24 - 48 h) and how much these contribute to variability in TNC at sampling, is less clear.

Before nitrate can be incorporated into amino acids, it must be reduced by the enzyme nitrate reductase (NRD) in the plant, using light generated energy directly. There is therefore a tendency for nitrate levels to decline in light, causing a diurnal rhythm as reduction takes place (Carrasco and Burrage 1992). However, in contrast, Hardgrave (1994) showed that for three glasshouse lettuce experiments (in winter, spring and summer), time of day (am or pm) had no effect on plant nitrate residues. Moreover, in two studies with lettuce (one winter and one summer), Byrne *et al.* (2004) showed that time of day (08.00, 12.00 and 16.00 h) had no significant effects on TNC. In a further growth room study by the same authors, sampling on 9 occasions through the day, there was no significant temporal trend in TNC.

The present work reports the results of three separate studies to address sources of variability in TNC. In the first, two fields of lettuce were sampled, and TNC determined for individual lettuce heads for a relatively large sample size (60 plants). These data therefore allowed the testing of whether 10 plants would give an unbiased estimate of the mean, and also allowed assessment of the utility of various sampling patterns in the field. In the second study, the effect of short-term fluctuations in light level on TNC was investigated. The third study was carried out in order to examine the levels of TNC in lettuce through the day and to estimate their significance, in four different field situations. In each of these studies, determination of TNC of individual plants enabled further statistical analysis to be carried out. Based on analysis of variance (ANOVA) the significance of the effects of shading and time of harvest could be estimated, as well as the residual error, enabling estimates to be made of the standard error.

Materials and methods

Locations of crops

Seven commercial crops of lettuce (*Lactuca sativa* L.) and one crop of endive (*Cichorium endiva* L.) were studied. All crops were located in the Vale of Evesham, Worcestershire, UK. Experimental situations varied between field, glasshouse and polytunnel crops. Site and varietal details are shown in Table I. All agronomic decisions were made by the grower and represented standard commercial practice in that location. Harvest date was also decided by the grower when plants were judged to have reached marketable head weight.

[Insert Table I about here]

Experiment 1 – variability in TNC in commercial crops

Sixty lettuce plants were harvested at each of two commercial field sites, individually numbered according to their field positions, and measurements were made of the individual plant fresh weights and TNC levels. Two contrasting sites were chosen, with site A having more weeds and a more uneven crop than site B (based on visual assessment by the sampler), but both being representative of commercial practice. At site A, the lettuce variety was of a ‘Cos’ type which has long, upright leaves, and forms a loose head. At site B, an ‘Iceberg’ (or ‘Crisphead’) type of lettuce was grown, which forms a tight, crisp, white head. At both sites, plants were harvested from six adjacent beds, from within a larger field area. Actual areas harvested were 480 and 60 m^2 at sites A and B respectively.

Further analysis was carried out on the data, by retrospectively selecting 10 data points from the field layout as if they had been sampled by conventional W or X shaped sampling patterns. This selection was repeated twice for each sampling pattern at each site.

For each site, individual TNC values for 10 plants, selected from both W shaped and X shaped patterns were used to calculate means, by two different methods:

a). An estimate of the TNC which would have been measured on a pooled/composite sample of 10 lettuce heads, using a weighted mean, calculated as follows;

TNC of composite sample = $(TN_1+TN_2…TN_{10})/(PW_1+PW_2….PW_{10})$, where,

TNn = amount of nitrate-N (mg) in an individual lettuce head,

TNn= TNCn*PWn/1000 for n = 1….10, and

TNC is tissue nitrate concentration (mg kg^{-1}); PW is plant fresh weight (g) and n = 1 to 10.

b). From the individual TNC values measured, the arithmetic mean, and associated standard error (SE) and coefficient of variation (CV) were calculated.

Experiment 2 – short term effects of shading on TNC

The aim of this experiment was to assess the effect of short term (24 h) periods of shading on TNC in lettuce and endive crops. Differential treatments were achieved by placing shade cloth over the plants in the experimental area using the design described below. Prior to starting the experiment, the amount of light transmitted through the shade material was determined using a 'sunfleck' ceptometer (Decagon) placed above and below the shading material. The ceptometer gave an instantaneous measurement of photosynthetically active radiation (PAR), and observations of 10 repeated measurements showed that the material absorbed 85% (SD=3.1) of the incident PAR. This level of shading would be representative of incident light on a dull day, with full cloud cover.

The experiment was repeated four times in separate commercial crops (Table I). The plants in shading treatment 1 were shaded for 24 hours, then left unshaded for the remainder of the experiment (24 – 48 h), while treatment 2 remained unshaded throughout the experiment (total duration 48 h). Ten plants were taken from each shading treatment at each of two harvests, either (a) after 24 h, or (b) after 48 h.

At each site, there were four replicates of each treatment, arranged within the commercial crop, and 40 plants were harvested in total (10 plants for each shading x harvest treatment combination). Plants were weighed and then frozen prior to nitrate analysis.

Analysis of variance (ANOVA) was used to estimate the significance of the various treatment effects and to partition the total variance, thereby allowing assessment of the magnitude of the residual variation (or residual error). The treatments were studied in a fully factorial design and the results subjected to ANOVA, where the main treatment effects (shading and harvest timing each with 1 df) and their interactions (each with 1 df) were compared to the residual error (df=36).

Experiment 3 – magnitude of diurnal variation in TNC

Plants from commercially grown crops of lettuce were harvested from two sites each (Table I). Samples were harvested over a 24 h period on six occasions, at 3 h intervals (times; 07.00, 10.00, 13.00, 16.00, 19.00 and 22.00 h). On the day prior to sampling, 60 plants were numbered in the field, and randomly sampled the following day. At each sampling occasion, 10 plants were harvested, plant fresh weights were recorded, and individual plants bagged and frozen, prior to analysis.

Each data set was analysed separately by ANOVA in order to assess the significance of time of harvest during the day, assuming that each timing had equal variance. The main treatment effect (harvest timing with 5 df) was tested for significance against the residual error (variation between plants within harvests; 54 df).

Analytical methodology

Tissue nitrate concentration was determined using high performance liquid chromatography with UV detection, following hot water extraction of nitrate from the plant tissue. The method was reported by Farrington (2001) based on British Standard BS EN 12014-2: 1997, and meets the requirements of EC document VI/4800/96 and Council Directive 93/99. The relative repeatability of the method, estimated from duplicate analyses was 10.5%.

Results and discussion

Experiment 1

Appreciable variability in lettuce head weights was measured within a site. Reinink and Eenink (1988) examined diverse genotypes of lettuce and found significant differences in TNC, although they noted that these were confounded with differences in plant weight between varieties. Smaller lettuce plants tend to have higher TNC levels. However, in the present study, sorting data on the basis of the plant weight showed no significant differences in mean TNC for plants above, or below the mean plant weight in each case at p=0.05: Site A; 933 (SE 93.8, n=27) *vs* 760 (SE 68.3, n=33) mgNO_3^- kg^{-1} for plants <170g and >170g respectively, and site B; 1486 (SE 128.9, n=29) *vs* 1226 (SE 91.4, n=31) mgNO_3^- kg^{-1} for plants <300g and >300g respectively. Since plant weights had relatively little effect on TNC within a site and plant fresh weights were distributed evenly around the mean, the effect of head weight was not considered further.

The distribution of individual plant TNC within the two sites was not uniform, and areas of more than six continuous sampling positions were identified, which had individual TNC lower than the site mean, at both sites. Table II shows the grand mean TNC (n=60) for each site, and the mean TNC's based on groups of 10 plants. All analyses described here were based on arithmetic means. The grand means differed significantly between the two sites, with site B being highest.

[Insert Table II about here]

Having a detailed field plan allowed assessment of different options for estimating TNC within the field, based on standard sample numbers (n=10). In the first example, plants were grouped by plot order (Table II). This would be representative of a sampling pattern whereby 10 plants were sampled contiguously along a row. There was appreciable within site variation, with plots 41-50 at Site A having significantly higher mean TNC levels than plots 11-20, 21-30 and 31-40 at p=0.05. More importantly, certain groups of 10 plants, gave mean TNC values which were significantly different to that of the grand mean. For example, at site A, plots 21-30 had significantly lower mean TNC than the grand mean, whereas plots 41-50 had mean significantly higher mean TNC than the grand mean at p=0.05. Similarly for site B, the mean TNC for plots 11-20 was significantly lower than that of plots 21-30, and also of the grand mean at p=0.05. This spatial variability in plant TNC probably reflects underlying variation in soil mineral nitrogen within the field, but also differences in individual plant growth in response to localised areas of soil compaction, or variability in soil pH.

For plants sampled along the row, the coefficient of variation had an approximate two-fold range between plant groups within a site (31-56% for Site A and 20-67% for Site B).

Next, rather than using plot order, individual plant TNC data was allocated to 6 groups of 10 plants chosen at random. Each of these randomisations was repeated 10 times at each site (data not presented here for the sake of brevity). In all cases, the mean and the median were very similar (838 *vs* 840 for Site A and 1352 *vs* 1333 mgNO_3^- kg^{-1} for Site B). There was some evidence from these randomised data that the SE increased as TNC levels increased within a site. However, it should be noted that coefficients of variation tended to be lower at Site B, with the higher mean TNC.

The estimates of the TNC for bulked/composite samples gave values which in three out of eight cases, appeared to be outside the confidence limits of their respective grand means. However, considering the greatest deviation of a pooled value from the grand mean was equal to 16.5% of the grand mean, and the analytical error represents *c.* 10.5% of the mean, a deviation of this magnitude can be considered acceptable. The estimates of TNC based on arithmetic means calculated here using data from either X or W sampling patterns, gave values which were not significantly different from one another, or significantly different from the grand means for TNC, at their respective sites at p=0.05 based on their confidence intervals (Table III).

[Insert Table III about here]

Spatial variability in NO_3^- is appreciable in most UK soils and this means that sampling protocols need to be designed robustly in order to obtain a reliable estimate of the mean. The data confirm that the choice of 10 plants, sampled randomly using an X or W shaped sampling pattern, provided acceptable measures of the overall mean, for the two fields of lettuce sampled in this study. For vegetable crops harvested from narrow beds, it is assumed that a number of beds being harvested on the same day could be pooled to make one block, and also harvested using similar shaped sampling patterns.

Experiment 2

In the short term shading experiment the main treatment effects on TNC were small. Averaged across both times of harvest, shading had no significant influence on plant TNC (Table IV). However, in two of the four trials (experiments E and F), the overall effect of time of harvest on plant TNC was significant (means averaged across the two shading treatments).

[Insert Table IV about here]

In addition to the main treatment effects, there were significant interactions noted between shading and harvest treatments in experiments C and F ($p<0.01$ and $p<0.05$ respectively; Table IV). In both these experiments, shading in the 0-24 h immediately prior to harvest (timing a) increased TNC. However, there was no significant difference in TNC between shaded and unshaded treatments 24 h after the shading treatments had been removed (timing b). These interactions were only significant in two out of four cases. Given that the shading treatment employed here was relatively severe compared to average light levels experienced on a dull day under normal conditions, it is unlikely that these short term effects on shading will be of importance in commercial practice. These results support the conclusions of Byrne *et al.* (2001) who found that extending daylength with supplementary light for four days prior to harvesting lettuce had no significant effect on plant nitrate levels.

Experiment 3

There were no significant effects of time of harvest on plant fresh weight (data not presented), but a significant effect on TNC ($p<0.05$ at both sites; Table V). At both sites, harvest at 07:00 and 13:00 h gave the lowest TNC levels and at 16:00 h gave the highest TNC levels. The sampling at 16:00 h gave TNC values significantly higher than those at the previous sampling at 13:00 h, based on the SED.

[Insert Table V about here]

Although the time of day the plants were harvested was shown to have a significant effect on variability in TNC values in lettuce, there were no consistent temporal trends. It should also be noted that the differences recorded between sampling times would not solely be due to environmental effects, but also included some variability associated with sampling (e.g. operator, speed of transfer of samples to cold storage etc).

However, studies on lettuce by Reinink (1991), Hardgrave (1994) and Byrne *et al*. (2004) all failed to detect a significant relationship between TNC and time of harvest within the day. It is most likely that any true diurnal variation due to nitrate uptake and cycling of NRD, is masked by variation due to transient wilting in high light and temperature conditions (which would tend to increase TNC during the day) and differences in overall growth rate of the plant.

Summary of data from experiments 1-3 and effect of increasing sample size on error estimates

The data from these experiments are summarised in Table VI as mean, SE and CV. Burns (2000) showed that CVs for hydroponic and soil-grown glasshouse in winter were low in the range 8-17% and higher in summer soil-grown lettuce (44%). Based on the data in Table VI, a similar range was seen in the present study and the average CV was *c*. 35%. Based on a population with a mean TNC of 1000 mgNO_3^- kg^{-1}, variation of this magnitude would imply that increasing the sample size from 10 to 40 plants would decrease the SE from 12 to 6% of the mean. However, within the estimates of residual variation, is also contained the analytical, or measurement error, which has been estimated at 10.5%. Therefore, however large the sample size becomes, there is a point beyond which the standard error cannot decrease any further, and it is likely that the SE would not fall below *c*. 12% of the mean without any reduction in the analytical error.

[Insert Table VI about here]

Conclusions

The studies were designed to quantify the relative importance of short-term effects of irradiance and diurnal variation, and to gain an estimate of the residual variation in TNC in commercial lettuce crops.

It is concluded that short-term differences in light levels on mean TNC are relatively small, compared to the differences in mean TNC seen between harvest at different times of the year, or shading 10-14 days prior to harvest (Burns 2000). Although the time of day at which plants are harvested appeared to influence TNC levels, there was no trend in TNC with time which could be used as a basis for improving advice regarding time of sampling during the day.

Since a sample of 10 lettuce plants, assuming they are harvested using an appropriate random (e.g. W or X shaped) sampling pattern gives a reasonable estimate of the grand mean, it would appear that 10 is an acceptable sample size for surveillance purposes. However, similar data have not been collected at other points in the supply chain e.g. from packhouses or warehouses, where such sampling patterns may not be appropriate and where the possibility of non-compliance can result in serious financial penalties to the grower.

Acknowledgements

The authors would like to thank the commercial growers for generously allowing harvesting of their crops during the course of this study, and Ms Jo James from ADAS Rosemaund for carrying out sampling. The financial support of the UK Food Standards Agency is gratefully acknowledged.

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Table I. Site, variety details and harvest dates for lettuce and endive crops sampled in 2003.

Site code	Site type	Species	Variety	Date harvested
Experiment 1				
A	Field	Lettuce	Goodison	16th September
B	Field	Lettuce	Iceberg	16th September
Experiment 2				
C	Tunnel	Endive	Glory	15th April
D	Glasshouse	Lettuce	M'lady	16th April
E	Field	Lettuce	Little Gem	29th May
F	Field	Lettuce	Lobjoits	30th May
Experiment 3				
G	Field	Lettuce	Pinnochio	4th July
H	Field	Lettuce	Pinnochio	17th July

Table II. Mean TNC levels, with associated standard errors and coefficients of variation, for either Cos or Iceberg type lettuce plants sampled in experiment 1, each based on either 60 plants, or 10 plants chosen in successive plot order.

Grouping	Mean TNC ($mgNO_3^- kg^{-1}$)	SE	CV (%)
Grand means		*Site A (Cos)*	
Plots 1-60	838	57.1	52.8
		Site B (Iceberg)	
Plots 1-60	1352	79.3	45.4
Ranked by plot order			
		Site A (Cos)	
Plot 1-10	771	135.2	55.5
Plot 11-20	684	99.8	46.1
Plot 21-30	575	57.1	31.4
Plot 31-40	681	107.0	49.7
Plot 41-50	1329	182.0	43.3
Plot 51-60	987	97.7	31.3
		Site B (Iceberg)	
Plot 1-10	1448	243.1	53.1
Plot 11-20	832	180.0	68.5
Plot 21-30	1775	206.4	36.8
Plot 31-40	1537	168.3	34.6
Plot 41-50	1001	64.5	20.4
Plot 51-60	1348	107.8	25.3

Table III. TNC estimated for a pooled (composite) sample of 10 heads, and the corresponding arithmetic mean from TNC values of individual lettuce heads, with its associated standard errors and coefficients of variation, for lettuce plants sampled in experiment 1. Each estimate is based on 10 plants selected using a W or X shaped sampling pattern, compared to the grand mean for each site.

Sampling pattern	N*	Pooled TNC† ($mgNO_3^- kg^{-1}$)	Mean TNC ($mgNO_3^- kg^{-1}$)	SE	LCL‡	UCL‡	CV (%)
				Site A			
W1	10	969	1016	212.3	592	1140	66.1
W2	10	733	716	114.9	486	945	50.8
X1	10	886	929	168.0	593	1265	57.2
X2	10	976	952	105.3	741	1163	34.9
Grand Mean	60		838	57.1	723	952	52.8
				Site B			
W1	10	1440	1402	198.5	1005	1799	44.8
W2	10	1400	1421	215.9	989	1853	48.0
X1	10	1192	1204	216.1	772	1636	56.8
X2	10	1388	1359	249.2	860	1857	58.0
Grand Mean	60		1352	79.3	1193	1511	45.4

*, Number of observations.
†, Weighted mean, estimated from the individual fresh weights and TNC values, bulked to give a pooled/composite sample.
‡, LCL & UCL = Lower and upper confidence limits respectively at p=0.05.

Table IV. Tissue nitrate concentrations in early or late season lettuce grown with or without shading, at two harvest timings either (a), during shading or (b) 24 hours after removal of shading material.

Site code	Time of season	Shading treatment	Tissue nitrate concentration ($mg\ NO_3^- kg^{-1}$)	
			Harvest timing	
			(a)	*(b)*
C	Early	*Unshaded*	1505	2390
		Shaded	2158	1939
		Harvest*Shade SED	235.6	(**)[†]
		Harvest timing means	1831	2164
		Harvest SED	166.6	(ns)
			(a)	*(b)*
D	Early	*Unshaded*	1680	1747
		Shaded	1915	1871
		Harvest*Shade SED	245.7	(ns)
		Harvest timing means	1797	1809
		Harvest SED	173.7	(ns)
			(a)	*(b)*
E	Late	*Unshaded*	886	818
		Shaded	1259	714
		Harvest*Shade SED	208.8	(ns)
		Harvest timing means	1072	766
		Harvest SED	147.6	(*)
			(a)	*(b)*
F	Late	*Unshaded*	562	535
		Shaded	815	457
		Harvest*Shade SED	106.7	(*)
		Harvest timing means	688	496
		Harvest SED	75.4	(*)

[†]Significance of effects (df = 36); **, p<0.01; *, p<0.05; ns, not significant.

Table V. Diurnal variation in tissue nitrate concentration for lettuce at two sites.

Site code		Tissue nitrate concentration ($mg\ NO_3^-\ kg^{-1}$)					
		Time (h)					
		7:00	10:00	13:00	16:00	19:00	22:00
G	Mean	1983	2173	1812	2511	2138	2155
	SED	197.4	(*)†				
H	Mean	2591	2896	2627	3111	2950	2701
	SED	179.7	(*)				

†Significance of effects (df = 54); *, $p<0.05$.

Table VI. Summary of mean TNC, with associated standard errors and coefficients of variation from Experiments 1-3.

Site code	Tissue nitrate concentration Grand mean ($mgNO_3^- kg^{-1}$)	SE	CV (%)
A	838	57.1	52.8
B	1352	79.3	45.4
C	1998	166.6	26.3
D	1803	173.7	30.5
E	919	147.6	50.8
F	592	75.4	40.3
G	2129	139.6	20.7
H	2813	127.0	14.3