Research Article

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Texture, composition and anatomy of spinach leaves in relation to nitrogen fertilization

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Abstract

BACKGROUND: The postharvest quality and shelf life of spinach are greatly influenced by cultural practices. Reduced spinach shelf life is a common quandary in the Salinas Valley, California, where current agronomic practices depend on high nitrogen (N) rates. This study aimed to describe the postharvest fracture properties of spinach leaves in relation to N fertilization, leaf age and spinach cultivar.

RESULTS: Force–displacement curves, generated by a puncture test, showed a negative correlation between N fertilization and the toughness, stiffness and strength of spinach leaves ($P > 0.05$). Younger leaves (leaves 12 and 16) from all N treatments were tougher than older leaves (leaves 6 and 8) ($P > 0.05$). Leaves from the 50 and 75 ppm total N treatments irrespective of spinach cultivar had higher fracture properties and nutritional quality than leaves from other N treatments ($P > 0.05$). Total alcohol-insoluble residues (AIR) and pectins were present at higher concentrations in low-N grown plants. These plants also had smaller cells and intercellular spaces than high-N grown leaves ($P > 0.05$).

CONCLUSION: Observed changes in physicochemical and mechanical properties of spinach leaves due to excess nitrogen fertilization were significantly associated with greater postharvest leaf fragility and lower nutritional quality.

Supporting information may be found in the online version of this article.

Keywords: leaf texture; cell wall; spinach; nitrogen; leaf anatomy

INTRODUCTION

The postharvest quality of leafy vegetables is influenced by environmental factors including light, temperature, mineral nutrient and moisture availability.[1] There have been numerous reports describing changes in phytonutrient content and postharvest shelf life of spinach according to specific growing conditions.[1–3] However, few reports have described the textural properties of leafy greens other than lettuce.[4–7]

Leaf mechanical properties for some monocot and eudicot crops have been described, and their overall description has been linked to anatomical characteristics which in turn dictate the instrumentation utilized in the description.[5,8–11] In monocots, the tensile strength test is ideal to describe leaf fracture properties because of the parallel orientation of veins along the longitudinal axis of the leaf.[8–10] In contrast, the punch test has been used to describe eudicot leaf properties, because with this test the resistance to crack propagation originates from a combination of compressive and shear strength forces that is distributed along pinnate or palmate venation patterns. The punch test technique utilizes a rounded probe that distributes the applied force homogeneously across a given area.[8,9,12,13] From the tensile strength and punch tests, force–displacement curves are generated and used to derive the mechanical properties of the material under evaluation; these include toughness, stiffness, strength and displacement of the probe.[10,13]

Leaf mechanical properties, anatomy and composition are also influenced by changes in nutrient availability.[6,14–17] Current agronomic practices for economical spinach cultivation and marketing in California depend on consistent crop management practices together with optimal water and plant nutrient supply.[18,19] As consumer demands for fresh spinach increase, the pressure to raise yields is one factor contributing to an industry-wide tendency for over-fertilization with nitrogen (N). These practices have been associated with reduced cell wall strength due to rapid growth, diminished macro- and micronutrient absorption and greater allocation of N to cell wall.[20–22] All of these physiological correlations may have a negative effect on the mechanical properties of leaves, especially since leaf strength has been linked to extended quality retention and greater resistance to damage.[6,8,21,23,] The major aim of this study was to describe objectively the postharvest fracture properties of spinach leaves in relation to nitrogen fertilization, leaf age and cultivar. Additionally, this study sought to understand the influences of nitrogen fertilization on leaf anatomy and cell wall composition and how changes in these intrinsic but mutable characteristics affect the postharvest fracture properties of spinach leaves.

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EXPERIMENTAL

Growing conditions

Spinach seeds (*Spinacia oleracea* L. cv. Whale and Bolero) were sown into a 50:50 coir–vermiculate medium with a moisture content of 3:1 (soil:water ratio), kept in the dark at 20 °C for 24 h and then moved into greenhouse conditions. Plants were transplanted into the hydroponic system inside a 5 cm wide coir cone for support when they had five fully expanded leaves. The hydroponic system consisted of 7.5 L buckets, with each holding four plants with 7.5 cm between plants. Leaves 6–8 (older leaves) and 12–16 (younger leaves) on each plant were tagged to facilitate sampling. Temperature, relative humidity (RH) and photosynthetically active radiation (PAR) were recorded at plant level during each experiment. Photoperiod varied between 13 and 15 h according to the growing season, which extended from May to December in 2006, 2007 and 2008. Temperature ranged between 15 and 28 °C night/day, average RH was 65% and average PAR was 350 µmol m^{-2} s^{-1} (LI-190 quantum sensor, Licor Inc., Lincoln, NB, USA).

Treatments consisted of five nutrient solutions, each with a different N content (50, 75, 150, 200 or 250 ppm total N) with a constant nitrate to ammonium ratio of 80:20. The reference treatment was 150 ppm based on recommendations from Koike *et al*.[18] The pH was adjusted every 2 days to the target pH of 5.8 and once per week the nutrient solutions were fully replaced. All nutrient solutions had the following composition: P, 1 mmol L^{-1}; K, 5.5 mmol L^{-1}; Ca, 2.1 mmol L^{-1}; Mg, 1 mmol L^{-1}; S, 1.1 mmol L^{-1}; Fe, 16.8 mmol L^{-1}; Mn, 0.5mmol L^{-1}; B, 15.0 mmol L^{-1}; Cu, 0.4 mmol L^{-1}; Zn, 2.0 mmol L^{-1} and Mo, 0.3 mmol L^{-1}. To avoid depletion of oxygen in the root zone, air was bubbled through the root zone and nutrient solution using a dual diaphragm air pump (3 PSI, General Hydroponics, Sebastopol, CA, USA). Plants were harvested at dawn by hand and stored in plastic Ziploc® freezer bags inside an ice chest to maintain temperature near 2–4 °C. Leaves 6–8 and 12–16 were weighted, wrapped with moist paper towel and placed inside separate bags to maintain initial turgor pressure,[10] and stored at 0 °C for 24–48 h before evaluations began.

Leaf mechanical properties

Leaves 6, 8, 12 and 16 (counting from order of appearance of the leaf on the plant) from each N treatment were analyzed using the punch test to assess the leaf fracture properties. This procedure involves forcing a probe of known cross-sectional area through a section of a leaf as described by Read and Sanson.[10] The punch test was conducted at room temperature using the texture analyzer TAXT.T2 from Stable Microsystems (Godalming, UK). The protocol designed took into consideration the recommendations of Choong *et al*.,[8] Aranwela *et al*.[9] and Read and Sanson.[10]

The test involved using a 6 mm ball probe to penetrate the leaf at a pre-test speed of 2 mm s^{-1}, a test speed of 1 mm s^{-1} as the probe contacted the leaf and a post-test speed of 10 mm s^{-1}. Each leaf was placed between two clamped metal plates with coinciding holes (area of 0.95 cm^2) to keep the leaf flat. The probe moved a standard distance of 8 mm. The clearance between the probe and the hole in the plates was 0.15 mm. From this test a force–displacement graph for each randomly selected spinach leaf was generated and the fracture properties (1) toughness, (2) stiffness, (3) maximum force to puncture the leaf and (4) the displacement of the probe necessary to fracture each leaf were recorded. Toughness was measured as the area under the force–displacement curve, stiffness as the slope of that curve and the maximum force as the force needed to fracture the leaf. Only leaves with a weight identical to that recorded at harvest were used in the evaluations, and a total of 15 leaves per N treatment and leaf age were analyzed unless otherwise stated.

Initial punch test measurements were performed at three locations (distal, median and proximal) on leaves 6 and 12 (Fig. 1S) to establish the reproducibility of the test and whether a predictable test for leaf break failure while handling could be identified. Measurements were made on the two sides of the leaf blade relative to the main vein, with the probe penetrating the leaf from the adaxial side. After these evaluations, the distal left or right section of the leaves was selected as the final location for the assessment of spinach leaf fracture properties. In all evaluations the punch test was evaluated on blade tissue without conspicuous veins. The average distance between measurements was 3 cm.

Leaf anatomical analysis

Cross-sections from leaves 6, 8, 12 and 16 were used to compare variation in anatomy according to leaf age and N treatment. Light microscopy images of 10 replicates per N treatment for older (leaves 6 and 8) and younger leaves (leaves 12 and 16) were digitally captured. From the distal right side of the leaf (the location where texture measurements on other leaves of the same age were made), a 1 × 1 cm piece was cut with a razor blade. Each piece was held in a slit made in a piece of baby-cut carrot, and cross-sections of 100–150 µm were made with a vibrating microtome (Vibratome Series 1000 plus). Each section was placed on a glass slide with a drop of distilled (DI) water, protected with a cover glass, and photographed using an Olympus U-PMTVC camera on a BH-2 microscope at 4× and 10× objective magnification. Leaf and epidermis thickness, cell length, cell number, and thickness of the palisade and spongy mesophyll tissues were determined by analyzing printed photographs.[24] Cell length was determined by selecting at random and measuring 10 palisade cells from the printed images.

Cell wall analysis

Old (leaf numbers 6–8) and young (leaf numbers 12–16) spinach leaves were used for analysis of total cell wall (alcohol-insoluble residue, AIR), total pectin and non-cellulosic neutral sugars. Five replicates of 20 g fresh weight (FW) per leaf age per nitrogen treatment were used in the analyses. A method modified from Campbell *et al*. was used for cell wall extraction.[25] Tissue was finely chopped and placed in a beaker with 250 mL of 95% ethanol for gentle homogenization (6000 rpm) (IKA T25 Ultra-Turrax, Wilmington, NC, USA). Samples were then boiled in ethanol for 30 min, vacuum filtered through glass filter paper, and washed three times with a total volume of 200 mL of each solvent: ethanol, chloroform–methanol (1:1 v/v) and acetone. The washed AIR fraction (no chlorophyll remaining) was then placed in 50 mL Falcon tubes at 37 °C for drying and storage. For pectin analysis, duplicate 10 mg AIR fractions of each sample were placed in a test tube with a magnetic stirrer. The tubes were then placed in an ice bath and 3 mL sulfuric acid was added (in 1 mL aliquots). After acid hydrolysis, 3 mL nanopure water was added in 1mL aliquots. The solution was then transferred to a 50 mL Falcon tube and the final volume was adjusted to 20 mL by adding nanopure water. This fraction is known as soluble alcohol insoluble residue (SAIR). From this solution 200 µL aliquots were used for pectin analysis following a modified spectrophotometric method.[26] Pectin measurements

reported in this study are an approximation from uronic acid measurements and do not include the rhamnose, galactose and arabinose components. The non-cellulosic neutral sugars were analyzed by a modified spectrophotometric method described by Dubois *et al*.[27] in which the Anthrone reagent (Fisher Scientific, Loughborough, UK) was used to estimate hemicellulose and cellulose present in the SAIR.

The specific neutral sugar composition of the AIR was analyzed from triplicates of each replicate using 4 mg AIR hydrolyzed with 0.5 mL of 2M trifluoroacetic acid containing 100 mg mL^{-1} of *myo*-inositol as an internal standard,[28] and converted to alditol acetates,[29] for gas chromatographic–mass spectrometric analysis. Aliquots of the derivatized samples were injected into a gas chromatograph (HP model 6890) fitted with a 30 m × 0.25 mm DB-23 capillary column (J&W Scientific, Folsom, CA, USA) and a mass selective detector (HP model 61098A). Temperature in the injector was 250 °C, and a linear oven temperature gradient (initial temperature 160 °C, 0 min; the oven increased at 4 °C min^{-1} to 250 °C) was used to improve separation. The different alditol acetates were identified on the basis of their mass spectra following the procedure described by Vicente *et al*.[30] for arabinose (Ara), galactose (Gal), rhamnose (Rha), fucose (Fuc), xylose (Xyl), mannose (Man), and glucose (Glc).

Tissue analysis (macro- and micro-nutrients)

Spinach AIR was submitted for analysis at the Analytical Lab of the University of California, Davis. Total calcium, boron, total nitrate, ammonium and starch contents were determined by standard methodology (http://anlab.ucdavis.edu/analyses/plant) from leaves that had been oven dried at 70 °C for 4 days. Nitrate and ammonium were extracted from dry leaf tissue with 2% acetic acid. Nitrate quantification was determined by zinc reduction followed by conductimetric analysis, while ammonium was determined by diffusion conductivity.[31] For individual sugars (glucose, sucrose and fructose), 0.3 g oven-dried spinach was homogenized for 1.5 min at 1300 rpm. From the homogenate an aliquot of 2 mL was centrifuged for 30 min at 1400 rpm in Eppendorf tubes.

The supernatant was filtered using a 0.45 µm PVDF Phenex syringe filter. High-performance liquid chromatographic (model SPD-10AVP, Shimadzu, Kyoto, Japan) conditions were as follows: column Phenomenex Luna 5µ Amino 100A 250 × 4.6 µm i.d., flow rate 1.0 mL min^{-1}, mobile phase acetonitrile 80%, injection volume 10 µL, oven temperature 40 °C. Sugars were analyzed with a low-temperature evaporative light-scattering detector (ELSD-LT, Shimadzu).

Statistical analysis

A randomized complete block design was used in all greenhouse studies (total = 4). Replicates for all analyses corresponded to the greenhouse replicates. One-way analysis of variance with mean separation by Tukey test at $\alpha = 0.05$ was used to determine whether there were significant differences between treatments and to examine the interactions between leaf age and nitrogen treatments. SAS statistical software (SAS Institute, Inc., Cary, NC, USA) was used.

RESULTS

Growth conditions and macro-nutrient composition of spinach leaves

The agronomic parameters recorded in this study included yield (Table 1), dry weight percentage, leaf area (Table 2) and relative growth rate (data not shown). Spinach plants showed normal growth under all experimental conditions, but differences in total mass, leaf area and dry weight were correlated with changes in total N (Tables 1 and 2). N fertilization modified the macro- and micro-nutrient composition of spinach plants and these differences were generally consistent across all experiments (Table 1). There was an incremental gain in leaf nitrate as the total N supply increased. The highest ammonium concentration was measured in leaves from the 250 ppm total N treatment. Leaves from all treatments except low N (50 ppm) exceeded the recommended limit of 2500 ppm for total leaf nitrate at harvest (Table 1).[3]

Incremental increases in ammonium concentration as part of total N supplied to the plant did not significantly reduce the nitrate accumulation in the plant. Higher plant boron and calcium concentrations were measured for N treatments greater than 150 ppm total N. For individual leaves, boron concentrations decreased with increasing N fertilization irrespective of leaf age. In older leaves, increasing calcium concentration was correlated with N treatments greater than 150 ppm total N, but no clear pattern was observed for younger leaves (Table 1). In all experiments, the

Table 1. Macro- and micro-nutrient composition at harvest of spinach leaves according to N treatment and leaf age

Treatment	Yield (g per plant)	NO_3 (ppm) Plant	NH_4 (ppm) Plant	B (ppm)			Ca (%)		
				Plant	L6	L12	Plant	L6	L12
cv. Whale									
50 ppm	28d	2275d	121d	45c	N/A	N/A	0.6b	N/A	N/A
75 ppm	39c	4028c	146c	55b	156a	85a	0.8a	1.5a	1.0a
150 ppm	51b	10 440b	201b	60a	87b	57b	0.9a	1.1b	0.9a
250 ppm	58a	13 950a	256a	63a	103b	47b	1.1a	1.2b	1.0a
cv. Bolero									
75 ppm	33c	4278b	120b	53b	N/A	N/A	0.7b	N/A	N/A
150 ppm	44b	8254a	166a	78a	N/A	N/A	1.0a	N/A	N/A
250 ppm	49a	8751a	169a	74a	N/A	N/A	0.9a	N/A	N/A

$n = 8$ repetitions per treatment; N/A, no analysis.
Plant: refers to all individual leaf blades (no petioles) within a single plant.
Different letters indicate significant differences by Tukey test, $\alpha = 0.05$.

Table 2. Anatomical characteristics of spinach leaves (cvs Whale and Bolero) according to leaf age and N treatment

	Palisade cell length (μm)[a]		Number of palisade layers[b]		Leaf dry weight (%)		Leaf area (cm^2)[1]	
Treatments	Leaf 6	Leaf 12	Leaf 6	Leaf 12	Leaf 6	Leaf 12	Leaf 6	Leaf 12
cv. Whale								
75 ppm	96c	80b	3–4	4	8.0a	8.2a	82b	44c
150 ppm	143b	77b	2–3	3	6.5b	7.8b	106a	59b
250 ppm	164a	102a	2	2–3	6.2b	7.3c	134a	73a
cv. Bolero								
75 ppm	120b	97b	2–3	3	7.0a	8.3a	128c	90b
150 ppm	112b	121a	2–3	3	6.6a	8.0a	164b	127a
250 ppm	139a	128a	2–3	3	6.9a	7.8a	189a	123a

[a] $n = 10$ cross-sections from independent leaves per treatment.
[b] The number of cell layers in the palisade was not evaluated statistically.
For a given cv., different letters indicate significant differences by Tukey test, $\alpha = 0.05$.

200 and 250 ppm total N treatments were associated with the highest leaf mass yields (data not shown) and leaf areas (Table 2). However, 200 and 250 ppm total N plants also had the lowest percentages of dry weight among the N treatments (Table 2).

Leaf mechanical properties according to water loss, leaf age, location within the leaf, N treatment and cultivar

The influence of leaf water loss on fracture properties of spinach leaves was determined by maintaining spinach leaves at room temperature (20 °C and 70% RH) for 1, 2 and 4 min following post-collection storage (24–48 h at 0 °C wrapped in a moist paper towel in a plastic bag). As expected, water loss by the leaves increased over time; however, losses up to 4.3% caused no significant reduction in the measureable fracture properties of spinach leaf 12 (see supporting information Table 1S). Similar patterns were seen for leaf 6 (data not shown), although greater variability was observed.

Previous observations on commercially packaged spinach indicated that the distal portion of the leaves was the most often damaged (data not shown). Consequently, the leaf fracture properties at various leaf ages were determined from three locations within the leaf 6 and 12 for cv. Whale (Fig. 1 and supporting information Fig. 1S). Water loss in these evaluations was not significantly different ($P > 0.05$) within leaf ages and N treatments and was similar to those values reported in supporting information Table 1S (cv. Whale) for the 4 min treatment. For leaves 6 and 12, the maximum force necessary to puncture the leaf decreased when N concentrations exceeded 75 ppm (Fig. 1 and supporting information Fig. 1S). This pattern was also observed for toughness and for the displacement of the probe before and after penetration. The distal portion of leaf 6 from any N treatment consistently had the lowest maximum puncture force. However, for leaf 12, fracture properties were similar for all blade locations but differed between the 75 and 250 ppm total N treatments. In summary, greater forces were required to puncture younger leaves than older leaves (Figs 1 and 2).

The overall relationship between N fertilization and the mechanical properties of spinach (cv. Whale) irrespective of leaf age was characterized by a negative correlation between these parameters. Maximum puncture force, toughness and stiffness had the highest correlation coefficients of all four mechanical properties evaluated (−0.70, −0.67 and −0.61, respectively). Further analyses of other leaf ages (8 and 16) and N treatments (50 and 200 ppm) revealed a strong negative correlation between leaf fragility and N concentration irrespective of leaf age (Fig. 2). In these leaves, as well as with leaves 6 and 12, the younger leaves were less fragile than older leaves. Greater leaf fragility was observed in treatments with N concentration greater than 75 ppm. Correlation coefficient analysis indicated that the best descriptors of the leaf fracture properties of spinach according to N concentration were maximum puncture force and toughness, followed by stiffness.

The displacement of the probe necessary to puncture the leaf was not highly correlated with N concentration, despite leaves 6, 8, 12 and 16 (Fig. 2) showing a distinct reduction in the displacement of the probe associated with increased N in the nutrient solution. Spinach cultivar also had a significant influence on the fracture properties of leaves normalized for developmental age (Fig. 3). Leaf 12 from cv. Whale had greater toughness, stiffness and maximum puncture force than the same leaf from cv. Bolero, irrespective of N treatment. For leaf 6, differences between cultivars were only observed between the 75 and 250 ppm total N treatment. Overall, leaves from the 75 ppm treatment from both cultivars and leaf ages were tougher than leaves from the 150 and 250 ppm total N treatments (Fig. 3). N nutrition had a similar effect on the fracture properties of both cultivars; however, the overall effect was less pronounced on cv. Bolero.

Anatomy of spinach leaves according to leaf age, N treatment and cultivar

N fertilization levels significantly affected the anatomy of spinach leaves (Figs 4 and 5). The upper and lower epidermal layers of spinach leaves were each one cell layer thick. Numerous minor veins occurred throughout the entire leaf and were enclosed by bundle-sheath cells. Mesophyll was differentiated into palisade parenchyma and spongy parenchyma (Figs 4 and 5). The palisade generally consisted of two to four cell layers, depending on the N treatment (Table 2 and Fig. 5) with more or less isodiametric cells in the low-N treatment and elongated cylindrical cells in the high-N treatments. The spongy mesophyll consisted of one or two uneven layers of semi-branched cells with more extensive intercellular spaces with N concentrations greater than 75 ppm (Fig. 5).

Leaves from plants grown with N concentrations greater than 75 ppm had larger cells, with fewer cells in the palisade layers and greater leaf area (Table 2). Greater leaf area was observed for older

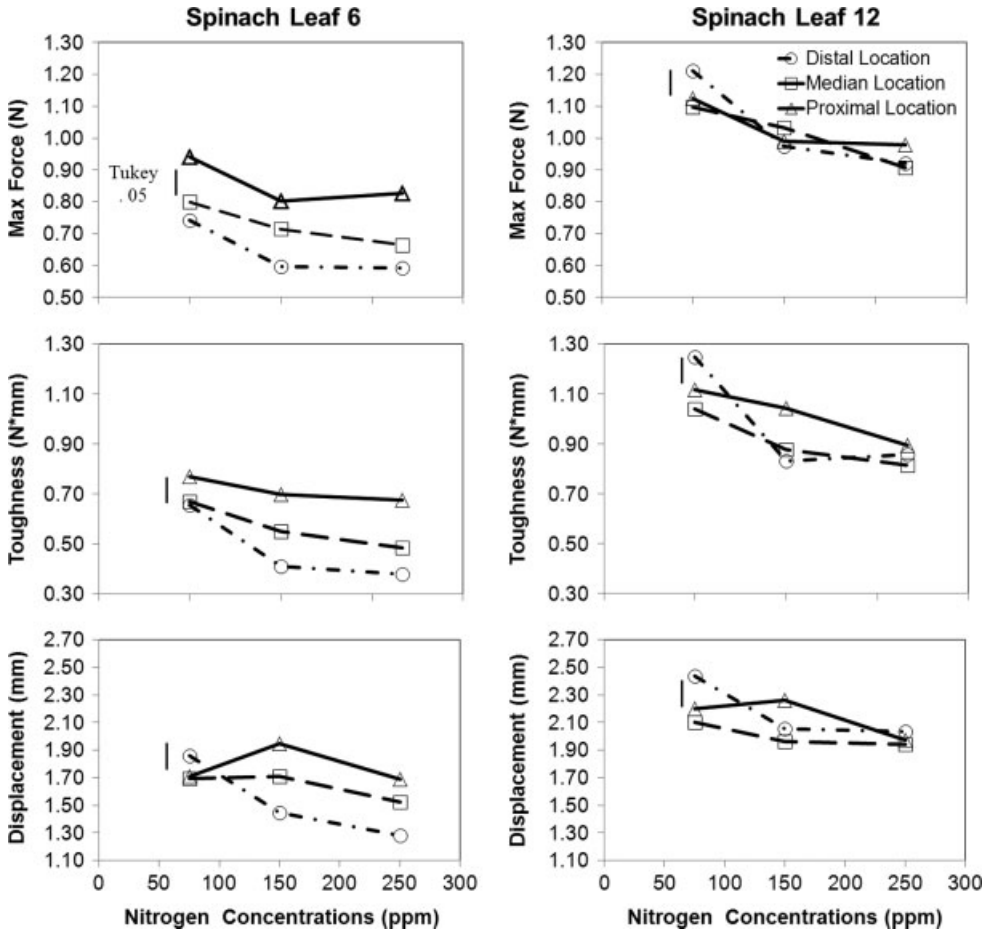

Figure 1. Mechanical properties of spinach leaves 6 and 12 (cv. Whale) according to location within the leaf. Values represent averages of six leaves per nitrogen treatment at three different locations within the leaf. Within each location two measurements were made, one on either side of the main leaf vein. Time elapsed between measurements averaged 3.8 min. During measurement, water loss per nitrogen treatment for leaf 6 was 3.51%, 4.33% and 4.01%; while for leaf 12 water loss was 3.33%, 4.10% and 3.95%, respectively. Vertical bars indicate significant differences by Tukey test, $P < 0.05$.

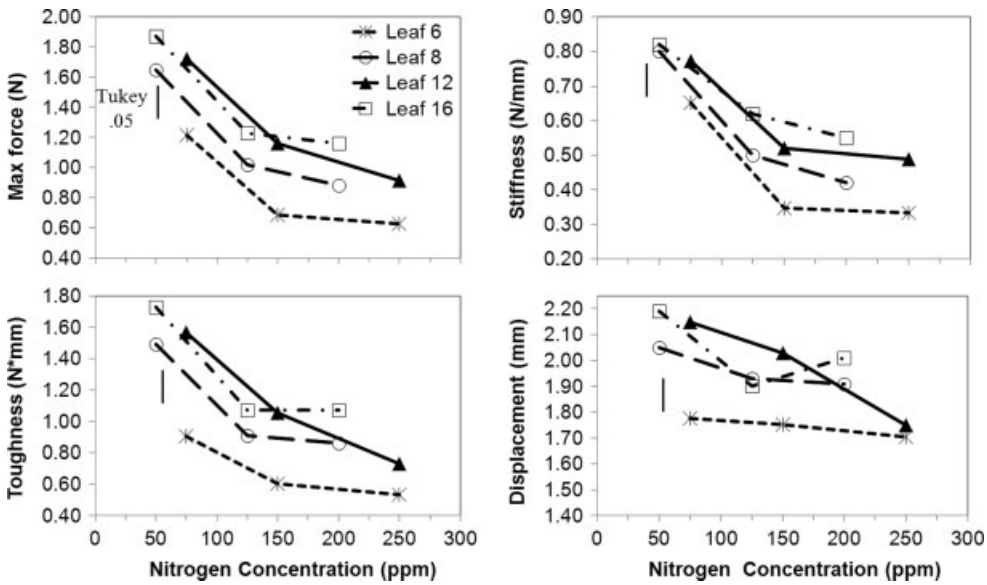

Figure 2. Mechanical properties of spinach leaves (cv. Whale) according to leaf age and nitrogen treatment. Values represent the average of 12 repetitions per treatment. Vertical bars indicate significant differences by Tukey test, $P < 0.05$. Measurements were made on the distal right or left section of the leaf.

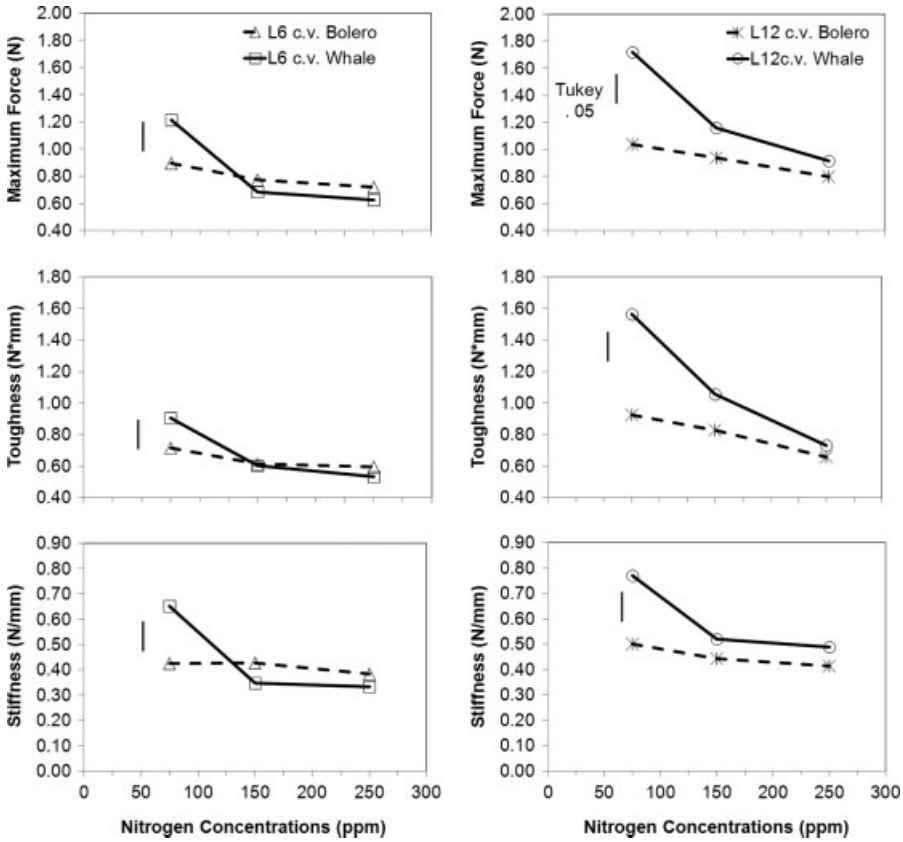

Figure 3. Mechanical properties of spinach leaves according to leaf age, nitrogen treatment and spinach cultivar. Values represent averages of 12 repetitions per treatment. Vertical bars indicate significant differences by Tukey test, $P < 0.05$. Measurements were made on the distal right or left section of the leaf.

leaves; however, cv. Bolero had larger and more lobed leaves than cv. Whale (Table 2; see supporting information Fig. 1S). Leaves 6 and 12 from cv. Whale grown under high-N treatments were on average 35% thicker (cross-sectional dimension) than those from low-N-treated plants. Leaves from high-N treatments had more extensive intercellular air spaces (Figs 4 and 5A). However, similar leaf thickness was found for leaf 6 from cv. Bolero in all N treatments. Leaf 12 from this cultivar was thinner than leaf 6, and differences in leaf 12 total leaf thickness were only observed between the lowest and highest N treatments.

Greater anatomical differences were observed in older leaves (leaves 6 and 8) than younger leaves when the effects of low and high N doses were compared, with the greatest effect in cv. Whale. Older cv. Whale leaves had greater leaf thickness than cv. Bolero, with both palisade and spongy mesophyll layers with increased thickness (Figs 4 and 5). Younger leaves from both cultivars were similar in total thickness, and in the thicknesses of the palisade and spongy mesophyll layers (Fig. 4). Epidermal thickness was similar between leaf ages, N treatments and cultivars, with the adaxial epidermal layer thicker than the abaxial epidermis (Figs 4 and 5).

For both spinach cultivars, average adaxial epidermal thickness for leaf 6 was 27 µm, while average abaxial thickness was 22 µm. For leaf 12, the average thickness of both epidermal layers was 18 µm (Figs 4 and 5).

Cell wall and plant constituents according to leaf age, N treatment and cultivar

The cell wall content and composition of spinach leaves varied with leaf age and N treatment and followed similar trends between and within cultivars (Table 3). Greater cell wall content was determined for leaves 6 and 12 from the 75 ppm total N treatment (Table 3) from both cultivars as well as for leaves 8 (5.65 mg AIR g^{-1} FW) and 16 (6.05 mg AIR g^{-1} FW) from the 50 ppm treatment (cv. Whale). Pectin concentration was also higher for these low-N treatments than for plants from both cultivars treated with 150 or 250 ppm total N. Neutral sugars were also present at higher concentrations for leaves 6 and 12 from the 75 ppm total N treatment (Tables 3) compared to 150 and 250 ppm total N treatments and leaf 16 (9.9 mg glucose equivalents g^{-1} FW) from the 50 ppm treatment. This trend was not observed for leaf 8 from the 50 ppm N treatment (8.1 mg glucose equivalents g^{-1} FW). Differences in starch concentration among N treatments from cv. Whale were negligible (data not shown).

AIR calcium concentrations from cv. Whale were only different between the 75 and 250 ppm total N treatments for younger leaves (leaf 12), while no differences were detected for leaf 6 (Table 3). Analysis of the concentrations of individual spinach cell wall (AIR) monosaccharides revealed that arabinose and galactose were also present at higher concentrations in leaves from the 75 ppm total N treatment. These leaves also had higher pectin content. Higher arabinose concentrations were determined for younger leaves, while galactose content was higher for cell walls of older leaves irrespective of N treatment. The remaining cell-wall associated monosaccharides were present at similar mol% concentrations between leaf ages and N treatments (Fig. 6).

Increases in N concentrations in the hydroponic solution were associated with decreased total sugar content in the leaves. Total soluble sugars in metabolically active pools and sucrose and

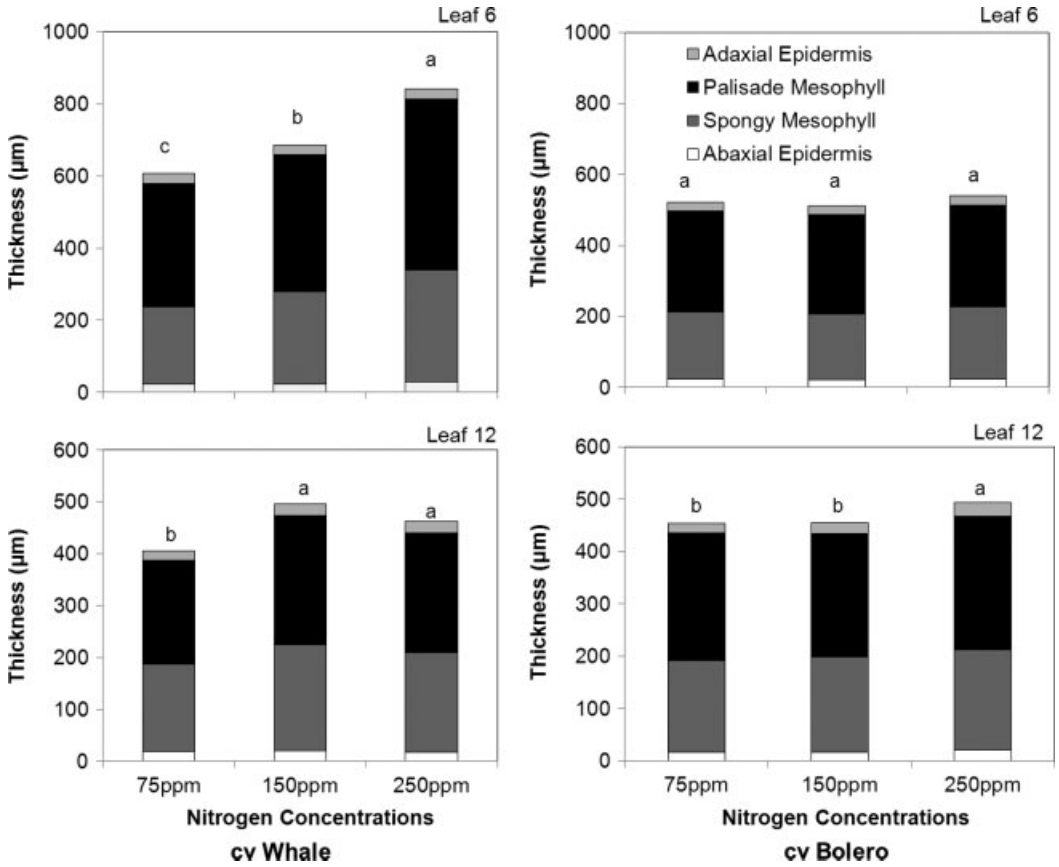

Figure 4. Anatomical features of spinach leaves of different ages from cvs Whale and Bolero as affected by growth in three different N concentrations. Values represent averages of 10 repetitions per treatment. Different letters indicate significant differences by Tukey test, $P < 0.05$.

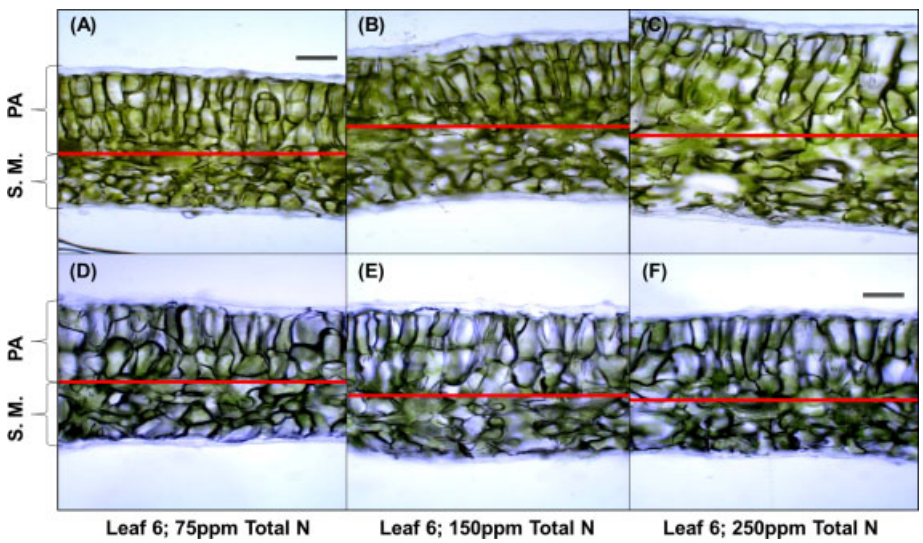

Figure 5. Light micrographs of spinach leaf cross-sections, from plants grown under different total nitrogen treatments (75, 150 and 250 ppm total N): (A, B, C) leaf 6 of cv. 'Whale'; (D, E, F) leaf 6 of cv. 'Bolero'. Scale bar = 100 µm. Adaxial epidermis location is towards the top of the page. Horizontal black line indicates the boundary between palisade mesophyll (PA) and spongy mesophyll (SP).

fructose decreased significantly with N concentrations greater than 75 ppm, while glucose totals showed no clear differences between low- and high-N grown plants. Sucrose was the predominant simple sugar at harvest for all N treatments, while fructose and glucose were present at much lower concentrations (Table 4).

Total chlorophyll and its components (chlorophylls *a* and *b*) increased as N concentrations in the nutrient solutions increased. Chlorophyll *a* concentrations were significantly higher than chlorophyll *b* (Table 4) but the ratio of chlorophyll *a* to chlorophyll *b* remained constant despite the changes in total N input (data not shown). As with chlorophyll content, carotenoid

Table 3. Alcohol-insoluble residues (AIR), pectin and non-cellulosic neutral sugars of spinach leaves (cv. Whale) at harvest according to leaf age and N treatment

	AIR[a] (mg AIR g^{-1} FW)		Pectin[a] (μg GalA g^{-1} FW)		Non-cellulosic neutral sugars[a,b] (μg Glc equivalents g^{-1} FW)		Ca (%AIR)[c]	
Treatment	L6	L12	L6	L12	L6	L12	L6	L12
cv Whale								
75 ppm	6.2a	6.9a	9920a	11 628a	14 290a	17 569a	1.3a	0.8b
150 ppm	5.8b	6.3a	7556b	7846b	10 536b	12 944b	1.5a	0.9b
250 ppm	5.3c	5.6b	6702b	7600b	10 769b	12 500b	1.2a	1.5a
cv Bolero								
75 ppm	6.1a	6.2a	12 258a	12 802a	29 048a	31230a	N/A	N/A
150 ppm	4.8b	5.6b	6694b	7433c	16 778b	17 818c	N/A	N/A
250 ppm	5.0b	5.8b	7143b	8446b	16 134b	18 316b	N/A	N/A

[a] n = Six repetitions per treatment and cultivar.
[b] Data are from anthrone colorimetric assays with spectrophotometer readings evaluated based on a Glc standard curve. Data presented here are qualitative only. Specific cell wall sugar contents are presented in Fig. 6.
[c] Calcium measurements are averages of three repetitions per treatment for cv. Whale. N/A, no analysis.
L6 = leaf 6; L12 = leaf 12, GalA = galacturonic acid; Glc = glucose.
Different letters indicate significant differences by Tukey test, $\alpha = 0.05$.

concentrations increased with increases in total N supply to the plants (Table 4).

DISCUSSION

Postharvest management for spinach focuses mainly on achieving optimal temperature control, preventing water loss, and minimizing mechanical damage during harvest and processing. Substantial variation in losses due to mechanical damage has been empirically described and tends to follow seasonal patterns (personal observation). Our main goal was to objectively describe the spatial variation in texture of spinach leaves according to location within the leaf blade. Further associated aims focused on describing impacts on sensory and nutritive quality parameters. The anticipated textural mosaic in individual leaves and across plant populations was compared among different N fertilization treatments (Fig. 2), spinach cultivars (i.e. a limited set of genetic variation) (Fig. 3) and leaf ages (Figs 1, 2 and 3).

Each harvest was performed before dawn, a time identified by Steingröver *et al.*[32] to be the stage in which spinach plants reach their maximum turgor, fresh weight and highest nitrate and phytonutrient levels. None of the plants grown under these experimental conditions displayed physiological disorders; all leaves and roots looked normal. As expected, increases in nitrogen fertilization in the hydroponic solution increased yield.[32–34] However, in the treatments that led to higher yields, leaf nitrate concentrations exceeded the maximum limit permitted by ECR-563/2002.[35] Spinach cultivar also had a significant influence on the amount of nitrate accumulated in the plants, highlighting the importance of selecting cultivars that have a lower propensity for nitrate accumulation, to improve their nutritional quality.[3]

To our knowledge there have been limited systematic attempts to describe the texture profile of leafy greens and herbs. Simmone *et al.*[36] determined that sensory panelists were able to differentiate between lettuces cultivated with nitrate- or ammonium-based fertilizers and gave higher crunchiness values for calcium-nitrate treated plants than for matched treatments with potassium nitrate. Our objective physical measurements indicated that spinach cultivated with high N, irrespective of N form, was less brittle (less crunchy, lower stiffness) than plants grown with more limited N availability despite having similar calcium concentrations in young and old leaves.

Toole *et al.*[5] and Newman *et al.*[6] described the mechanical properties of lettuce in relationship to cultivar differences and agronomic conditions, respectively. Both found strong correlations between these parameters and lettuce texture. Under our experimental conditions, the mechanical profiles of spinach leaves cultivated with a range of five N concentrations were distinct for each N treatment. Low-N grown plants (50 and 75 ppm total N) presented the highest values for leaf toughness, stiffness and maximum force when compared to the plants given high N, irrespective of leaf age and cultivar. These results are also in agreement with observations reported for lettuce by Newman *et al.*[6]

Leaf age also had a significant influence on these parameters; younger leaves were tougher and higher forces were needed for leaf puncture. Location within the spinach leaf blade likewise had a significant effect on leaf fracture properties. Lower maximum force to break as well as reduced leaf toughness was evident for the distal region of the plants in high-N grown plants irrespective of leaf age. These results confirm our visual observations of the origin of fragmented leaf pieces inside commercial spinach bags and coincide with the spatial zone of normal cell expansion in the distal leaf region.[37]

The effects of excess N fertilization on leaf anatomy included changes in cell size and shape, and the development of more extensive intercellular air spaces between veins, presumably due to excessive growth or expansion.[15,20,22,38] Under our experimental conditions, closely spaced, generally isodiametric cells were observed in low-N treated plants and these leaves had higher maximum force, toughness and stiffness. Our results are in agreement with reports by Wagstaff *et al.*[39] and Clarkson *et al.*[40] that associated changes in cell structure with differences in elastic properties of two lettuce cultivars during storage.

Both spinach cultivars showed similar responses to high and low N fertilization in terms of their fracture properties. However, cv. Whale for leaves 6 (only for the lowest N treatment) and 12 had higher values for each mechanical property criterion (stiffness,

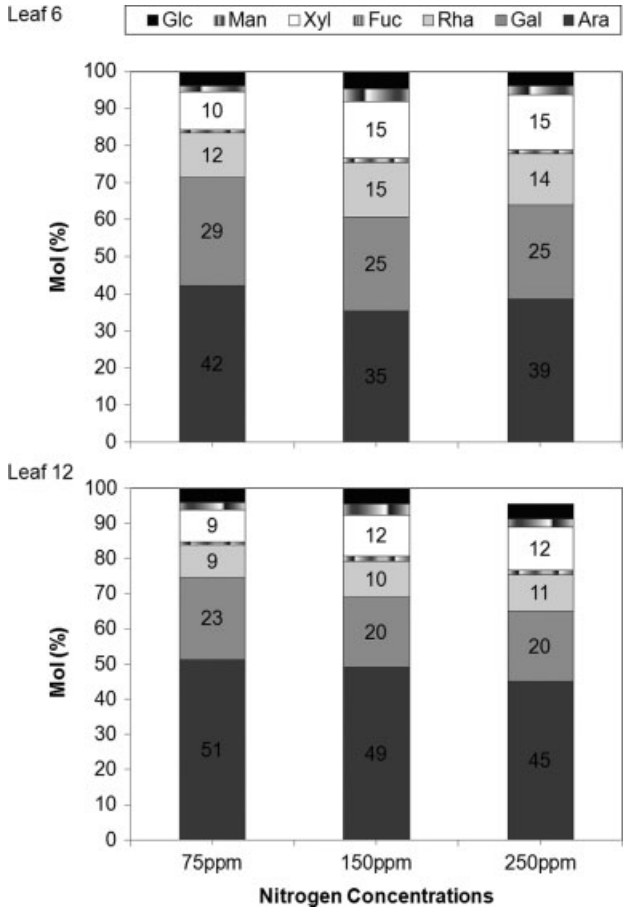

Figure 6. Monosaccharide composition of alcohol-insoluble residues of spinach leaves 6 and 12 according to nitrogen fertilization treatment. Values represent averages of three repetitions per leaf age and nitrogen treatment with two subsamples within treatments. Ara = arabinose; Gal = galactose; Rha = rhamnose; Fuc = fucose; Xyl = xylose; Man = mannose; Glc = glucose. Significant differences between the 75 ppm and 250 ppm treatments were only found for the Ara and Gal sugars for both leaf ages, as determined by Tukey test, $P < 0.05$.

toughness, maximum force) than did cv. Bolero. These results are in agreement with those summarized by Sams,[14] Wright *et al.*[15] and Schopfer,[13] who highlighted the direct influence that genetic factors have on leaf mechanical properties and the influence of the environment on the manifestation of those properties.

Read and Sanson[10] reported that one of the major difficulties in describing the fracture properties of leaves was the presence of veins across the leaf blade, while Choong *et al.*[8] highlighted the importance of epidermal thickness on leaf mechanical properties. All our punch test evaluations were done in regions of leaf blades where no major veins were present (Fig. 1 and supporting information Fig. 1S). Despite having a thinner epidermis, younger leaves had higher fracture properties than older leaves, suggesting that other anatomical and compositional parameters besides epidermal thickness are important in determining outcomes.

The influence of applied N concentrations on tissue boron and calcium accumulation was evaluated because of the significant effect these two mineral nutrients have on cell wall properties. Calcium is important in cell wall structure and metabolism because of its relationship with soluble pectin polysaccharides and the properties of the Ca-pectin gels.[41] It was expected that greater Ca concentrations would be present on those leaves with higher pectin content; however, this was not the case. Although our plant and individual leaf Ca estimations cannot identify where in the cells calcium was located, the AIR calcium assessments do identify Ca concentrations capable of influencing textural measurements. This observation could not be corroborated since no clear pattern in AIR Ca concentrations between N treatments was observed.

Unlike calcium, boron moves through the plant by passive flow, allowing little to no remobilization after it has been fixed,[42] at least in plant species that transport assimilated carbon as sucrose.[43] Overall increases in the total N supplied to the plant reduced individual leaf boron concentrations but not total plant boron content. It has been estimated that more than 90% of the boron in plant tissues is associated with the cell wall. Our results showed that high boron content in tissue was associated with incremental gains in plant dry weight (Tables 2 and 3) and reduced foliar nitrate content (Table 1).[42,44]

Boron has been reported to be involved in formation of rhamnogalacturonan II (RG-II) pectin dimer complexes, which are important for cell wall rigidity.[41] Reductions in boron content were positively correlated with reduced pectin content and lower leaf toughness and stiffness in spinach supplied with N concentrations greater than 75 ppm total N. Similar results have been reported by Brown and colleagues[45,46] for squash, tobacco, celery, tomato and wheat. Additionally, loosening of cell wall polymer interconnections could be due to increased cell wall pore sizes, weakening of the RG-II dimer networks and cleavage of the xyloglucan (XyG) that holds cellulose fibrils close together.[42,47] With high N supply, these changes could result in larger intercellular spaces and cells, thereby altering leaf anatomy and consequently the fracture properties of spinach.

In the present study, spinach leaves were kept fully turgid after harvest and before mechanical or structural parameters

Table 4. Chlorophyll, carotenoid and monosaccharide concentrations of spinach leaves cv. Whale at harvest according to N fertilization

Treatment	Chl. *a* (mg g^{-1} FW)	Chl. *b* (mg g^{-1} FW)	Total chl. (mg g^{-1} FW)	Carotenoid (mg g^{-1}FW)	Monosaccharides (mg g^{-1} DW)		
					Sucrose	Fructose	Glucose
50 ppm	0.9b	0.3b	1.2b	0.4b	40.8a	9.3b	10.0b
75 ppm	0.9b	0.4b	1.3b	0.4b	40.4a	10.4a	N/A
150 ppm	1.0b	0.4b	1.4b	0.5a	13.1	8.8b	11.1a
250 ppm	1.4a	0.6a	2.0a	0.6a	12.0b	8.0c	10.6a

$n = 10$ repetitions per treatment.
Spinach dry weight 10.1a, 9.0b, 7.6c and 6.2d (order of appearance reflects N treatments organized from low to high concentration).
Different letters indicate significant differences between treatments by Tukey test, $\alpha = 0.05$.

were evaluated. Despite recording up to 4% water loss between and within treatments during texture measurements, differences in the mechanical properties were mainly due to differences in cell wall composition and anatomical characteristics and were best explained by the correlation coefficients of maximum force and toughness. These results are not in agreement with those reported by Newman *et al*.,[6] which correlated loss of turgidity in lettuce leaves with reduced tensile strength.

Dale,[37] Nevins *et al*.[48] and Reiter *et al*.[49] reported that for grass, common bean (*Phaseolus vulgaris* L.) and *Arabidopsis*, vein incidence, leaf lifespan and cell wall composition (pectin and neutral sugar levels) varied with N fertilization. Similar observations were made under our experimental conditions for spinach. Recently, Wagstaff *et al*.[47] reported that modifications of cell wall properties in lettuce during storage were due to differences in XyG endotransglucosylase activity, which in turn modified lettuce anatomy, leaf strength and cell wall rigidity. Although XyG concentrations were not estimated in our study, neutral sugar determinations most likely included all the XyG present in the cell wall. The highest concentrations of neutral sugars in leaves were from the low-N treatments and coincided with high pectin content. Both conditions could be critical factors that increase leaf toughness since, as suggested by Brown *et al*.[42] and Cosgrove,[50] cell wall structure and rigidity depend on the level of coating of XyG and pectin on cellulose microfibrils.

Additionally, significant differences in arabinose and galactose concentrations were observed between young and old leaves within the same N treatment and between the low and high N fertilization. Fry[51] reported that there was a strong correlation between the concentrations of these two monosaccharides and pectin polymers in the spinach cell walls, a finding that is consistent with our results and with current cell wall models.[50] Accumulation of sugars and other carbohydrates in plants has been associated with increased sucrose and starch synthesis. Increments in sugar content also reduce the number of carbon skeletons available for the tricarboxylic acid cycle (TCA) and amino acid synthesis. Consequently, plants produce and accumulate lower nitrogenous compounds including chlorophylls and proteins.[52,53] Similar results were observed for low-N grown spinach, which accumulated high sugar concentrations and low chlorophyll and carotenoids. Our results follow trends similar to those reported by Okazaki *et al*.[34] in which the metabolic pathway of starch and sucrose synthesis was downregulated by an increased supply of nitrate to spinach plants.

CONCLUSIONS

N fertilization was shown to affect spinach (cv. Whale and Bolero) leaf anatomy, texture, and cell wall composition. Increasing N reduced the leaf fracture properties of spinach, making the harvested leaves more fragile, easier to break and leading to reduced storage life expectations. This phenomenon may be attributable to differences in total cell wall content, cell wall composition, leaf anatomy, and macro- and micro-nutrient concentrations between low- and high-N grown plants. Our results also highlight the importance that agronomic practices can have on the overall structure and composition of this leafy green vegetable and the negative effects of excessive N fertilization on leaf fragility and nutritional quality. Furthermore, our findings illustrate the fine balance that exists between C and N assimilation and partitioning within the plant, where dry weight and carbohydrates are constantly used to support cell enlargement in the small but fast-growing spinach plants. In this study, temperature and relative humidity conditions were held relatively constant during the cropping cycle, as was the nitrate : ammonium ratio. However, in field production of spinach, environmental conditions are constantly fluctuating and ammonium-based fertilization dominates agricultural practices. Variations in dry and wet conditions accompanied by fog and changes in N availability could significantly alter the C : N balance, which in turn may further modify leaf anatomy and texture. Thus it will be important in future studies to evaluate cultivation practices including light availability, other spinach cultivars and fertilization regimes to determine the relationship of our results to varying field conditions. Additionally, further analysis of the spinach cell wall constituents is of importance to elucidate which specific matrixes influence the primary wall structure and leaf texture. Overall, improved understanding of how N fertilization affects leaf structure and textural properties may lead to production modifications in spinach that will affect sensory and nutritional quality as perceived and received by consumers.

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Supporting information

Supporting information may be found in the online version of this article.

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