

Carotenoid pigments in kale are influenced by nitrogen concentration and form

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Abstract: The objective of these experiments was to investigate the effects of N rate and form on the accumulation of lutein, β -carotene and chlorophyll pigments in the leaf tissues of kale. Winterbor, Toscano and Redbor kale cultivars were greenhouse grown using nutrient solution culture. In the first study, N treatments were 6, 13, 26, 52 and 105 mg L⁻¹ at a constant 1 NH₄-N:3 NO₃-N ratio. On a fresh weight basis, plant pigment concentrations (lutein, β -carotene and chlorophylls) were not affected by N rate. When calculated on a dry weight basis, however, carotenoid pigments increased linearly in response to increasing N rate. In a second study, N rate was held constant at 105 mg L⁻¹ and N form was changed as follows: 100% NH₄-N:0% NO₃-N, 75% NH₄-N:25% NO₃-N, 50% NH₄-N:50% NO₃-N, 25% NH₄-N:75% NO₃-N and 0% NH₄-N:100% NO₃-N. Increasing NO₃-N in nutrient solutions from 0 to 100% resulted in increases in both lutein and β -carotene concentrations. Increases in carotenoid concentrations would be expected to increase the nutritional value of kale. Therefore N management should be considered in crop production programmes designed to increase the concentrations of nutritionally valuable carotenoids.

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INTRODUCTION

Plant foods have been utilised for thousands of years for their therapeutic properties. One class of vegetable crops with high nutritional and medicinal value is the *Brassica* genus, members of which contain high levels of available nutrients, anticarcinogenic glucosinolates and beneficial carotenoids. Understanding the unique metabolic pathways of *Brassica* phytochemicals and how they can be enhanced through cultural practices has become increasingly important. Nitrogen can alter plant composition more than any other element¹ and is often the most limiting nutrient affecting plant growth in cropping systems.² Plants take up inorganic N as ammonium (NH₄⁺) and nitrate (NO₃⁻) from mineralised organic matter or inorganic fertilisers; however, optimal plant growth is achieved with combined N form fertility.² Although luxuriant fertility levels may influence mineral content and crop quality, there is limited information on the effect of N on the carotenoid content of vegetables.³ Studies involving N fertilisation should therefore investigate both physical and chemical quality factors.⁴

Carotenoids are C₄₀ isoprenoid polyene plant compounds which are divided into two groups: the oxygenated xanthophylls such as lutein (3R,3'R,6'R- β , ϵ -carotene-3,3'-diol) and zeaxanthin (3,3'R- β , β -carotene-3,3'-diol) and the hydrocarbon carotenes such as

β -carotene (β , β -carotene) and α -carotene (6'R, β , ϵ -carotene).⁵ Carotenoids are bound to the specific chlorophyll-carotenoid binding protein complexes of PSI and PSII within the thylakoid membranes.^{6,7} In plants, carotenoids function in light harvesting, quenching of excited triplet chlorophyll, scavenging of singlet oxygen, excess energy dissipation and structural stabilisation.⁸ Vegetable crops are primary sources of carotenoids in the diet, and cruciferous vegetables, including subspecies of *Brassica oleracea* L., are relatively abundant sources.⁹ Dietary intake of carotenoids is associated with reduced risk of lung cancer and chronic eye diseases, including cataract and age-related macular degeneration.^{10–12} Moreover, consumption of a variety of vegetables providing a mixture of carotenoids is more strongly associated with disease reduction than individual carotenoid supplements.^{10,11}

Green leafy vegetables are rich in dietary carotenoids, and kale (*B. oleracea* L. var. *acephala* DC) ranks highest among all vegetable crops for reported lutein and β -carotene concentrations.¹³ Combined lutein/zeaxanthin levels in the leaves of kale are reported to range from 147 to 395 μ g g⁻¹ fresh tissue, while levels of β -carotene range from 28 to 145 μ g g⁻¹ fresh tissue.^{13,14} However, the authors caution that the carotenoid values reported were based

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on limited sample sizes and do not account for differences in crop genetics or variability in growing and harvesting conditions.¹⁴ Screening fresh leaf tissue of leafy *B. oleracea* cultivars resulted in a lutein range of 48–134 $\mu\text{g g}^{-1}$ and a β -carotene range of 38–100 $\mu\text{g g}^{-1}$.¹⁵ Carotenoid accumulation is also influenced by physiological and biochemical attributes as well as environmental growth factors such as light, temperature and fertility.^{3,9}

Fruits and vegetables are primary sources of carotenoids in the human diet, and their consumption is associated with disease reduction.¹⁶ Dietary intake of lutein and β -carotene can reduce the risk of eye diseases and lung cancer in humans.¹¹ Kale ranks highest among all vegetable crops for reported lutein and β -carotene concentrations.^{13,14} Nitrogen rate and form can influence plant growth and development and alter pigment accumulation. Therefore two separate studies were initiated to assess the influences of N rate and form on (1) plant biomass production and (2) the accumulation patterns of lutein, β -carotene and chlorophyll pigments in the leaf tissues of three different kale cultivars. Different kale cultivars were evaluated to assess the genetic variability previously reported for carotenoid accumulation.¹⁵

MATERIALS AND METHODS

Plant culture

For each study, Winterbor, Toscano and Redbor kale cultivars (Johnny's Selected Seed, Winslow, ME, USA) were seeded into rockwool growing cubes (Grodan A/S, Hedehusene, Denmark). The cubes were supplied with bottom heat (23 °C) and greenhouse grown (22 °C day/14 °C night set temperatures) for 2 weeks under natural photoperiods in Durham, New Hampshire (latitude 43° 8'N). Nutrients were applied as needed with 200 mg L⁻¹ Peter's 20N-6.9P-16.6K water-soluble fertiliser (The Scotts Company, Marysville, OH, USA).

Nitrogen rate study

Seeds of each cultivar were sown on 4 October 2001. Kale plants were transferred on 18 October 2001 to 37.9 L hydroponic containers (Rubbermaid Inc., Wooster, OH, USA) each containing 30 L of modified Hoagland's nutrient solution.¹⁷ Five plants of each of the three cultivars were placed in 2.2 cm holes at a spacing of 10.6 cm × 9.5 cm on each container lid. Elemental concentrations of the nutrient solutions were as follows (mg L⁻¹): K, 117.3; Ca, 80.2; Mg, 24.6; S, 32.0; Fe, 0.5; B, 0.25; Mo, 0.005; Cu, 0.01; Mn, 0.25; Zn, 0.025. The nutrient solutions were aerated via plastic tubing connected to an air blower (VB-007S, Sweetwater, Ft Collins, CO, USA). Plants were grown under increasing N treatment concentrations of 6, 13, 26, 52 and 105 mg L⁻¹. The N form ratio was kept constant at 1 NH₄-N:3 NO₃-N. The P level varied slightly with the N rate, amounting to 93, 91, 89, 86 and 80 mg L⁻¹ for the N treatments

of 6, 13, 26, 52 and 105 mg L⁻¹ respectively. The experimental design was a split plot, with N rate treatment as the main plot and kale cultivar as the subplot. Each treatment was replicated four times, and solutions were replaced every 2 weeks throughout the experiment to refresh the solution to the initial nutrient concentrations.

Plants were harvested on 29 November 2001. At harvest, shoot and root tissues were separated; five plants in each treatment/cultivar combination were bulked and fresh weight (FW) was determined. Plant tissues were washed with soap (Aquet, Bel-Art Products, Pequannock, NJ, USA) and rinsed with deionised water. Shoot tissues were equally divided into two groups. One shoot tissue group was placed in paper bags and dried at 45 °C for no less than 72 h, at which time dry weight (DW) was calculated. The other shoot tissue group was placed in plastic freezer bags and stored in a freezer (-80 °C) prior to lyophilisation.

NH₄-N:NO₃-N study

On 6 February 2002, seeds of each kale variety were started and plant culture was as described above. On 22 February 2002, plants were transferred to the hydroponic containers. Plants were acclimatised in nutrient solutions with the following elemental concentrations (mg L⁻¹): NO₃-N, 98.0; NH₄-N, 7.0; P, 15.3; K, 117.3; Ca, 80.2; Mg, 24.6; S, 32.0; Fe, 0.5; B, 0.25; Mo, 0.005; Cu, 0.01; Mn, 0.25; Zn, 0.025. The nutrient solutions were aerated as described above. On 7 March 2002, treatments were initiated and consisted of N at 105 mg L⁻¹ supplied as (1) 100% NH₄-N:0% NO₃-N, (2) 75% NH₄-N:25% NO₃-N, (3) 50% NH₄-N:50% NO₃-N, (4) 25% NH₄-N:75% NO₃-N and (5) 0% NH₄-N:100% NO₃-N. The experimental design and concentrations of the other essential elements were similar to those of the N rate study described above. Plants were harvested on 6 April 2002 and handled as described above.

Carotenoid and chlorophyll determination

Tissue extraction

After harvest, plant samples from each study were lyophilised for 72 h (6 L FreeZone, LabConCo, Kansas City, MO, USA). Freeze-dried tissues were combined with ~50 g of dry ice in a household food chopper (Handy Chopper Plus, Black & Decker, Towson, MD, USA). Macerated tissues were placed in 20 mL scintillation vials and CO₂ gas was vented prior to storage at -20 °C. Pigments were extracted and separated using established procedures.^{15,18} A 0.10 g subsample was rehydrated with 0.8 mL of deionised water and placed in a water bath at 40 °C for 20 min. After incubation, 0.8 mL of the internal standard ethyl- β -8-apo-carotenoate (Sigma Chemical Co., St Louis, MO, USA) and 2.5 mL of tetrahydrofuran (THF) stabilised with 25 mg L⁻¹ 2,6-di-*tert*-butyl-4-methoxyphenol (BHT) were added. The sample was homogenised in tissue-grinding

tubes (Potter-Elvehjem, Kontes, Vineland, NJ, USA) using ~25 insertions with a pestle attached to a drill press (Craftsman 15 inch Drill Press, Sears, Roebuck and Co., Hoffman Estates, IL, USA) set at 540 rpm. During homogenisation the tube was immersed in an ice bath to dissipate heat. The tube was then placed in a clinical centrifuge for 3 min at 500 × *g*. The supernatant was removed and the sample pellet was resuspended in 2.0 mL of THF and homogenised again with the same extraction technique. The extraction procedure was repeated two more times until the supernatant appeared colourless. The combined supernatants were reduced to 0.5 mL using nitrogen (N-EVAP 111, Organomation Inc., Berlin, MA, USA) at 40 °C, then 2.5 mL of MeOH and 2.0 mL of THF were added to the sample prior to high-performance liquid chromatography (HPLC) analysis.

HPLC analysis

An Agilent 1100 HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, CA, USA) was used for pigment separation.¹⁵ All samples were analysed for carotenoid and chlorophyll compounds using an RP C-18, 80 Å, 3.0 µm, 300 mm × 4.6 mm column (Adsorbosphere HS, Alltech, Deerfield, IL, USA) fitted with a 7.5 mm × 4.0 mm, 5.0 µm guard column (All Guard C-18, Alltech). The column was maintained at 16 °C using a thermostatted column compartment. The eluents used were (A) 75% acetonitrile, 20% methanol, 5% hexane, 0.05% BHT, 0.013% triethylamine (TEA) and (B) 50% acetonitrile, 25% THF, 25% hexane, 0.013% TEA. The flow rate was 0.7 mL min⁻¹ and the gradient was 100% A for 30 min, 50% A/50% B for 2 min, 100% B for 2 min and 50% A/50% B for 2 min. The eluent composition was returned to 100% A for 2 min and the column was equilibrated for 10 min prior to the next injection. Eluted carotenoid and chlorophyll compounds from a 20 µL injection were detected at 452, 652 and 665 nm and data were collected, recorded and integrated using Agilent 1100 HPLC ChemStation software. Peak assignment was performed by comparing retention times and line spectra obtained from photodiode array detection with authentic standards (lutein from Carotenature, Lupsingen, Switzerland; β-carotene, chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) from Sigma Chemical Co.). Plant pigment data are presented on both FW and DW bases.

Statistical analysis

Data were analysed using the PROC GLM analysis of variance (ANOVA) procedure of the SAS v9.1 software package (SAS Institute, Cary, NC, USA). The relationship between experimental dependent variables and N treatments was determined by regression analysis. Orthogonal polynomials were used to study changes associated with N treatments by

partitioning the sums of squares into components associated with linear and quadratic terms.¹⁹

RESULTS AND DISCUSSION

Nitrogen rate study

Biomass production

Kale shoot tissue fresh weight (STFW) responded significantly to N rate ($P \leq 0.001$) and cultivar ($P \leq 0.001$). Increases in N rate from 6 to 105 mg L⁻¹ resulted in STFW increases of 317, 310 and 268% for Winterbor, Toscano and Redbor respectively. Linear increases in STFW were observed for each kale cultivar as the N supply increased (Table 1). The highest accumulation of STFW for each kale cultivar occurred at the N treatment of 105 mg L⁻¹. Shoot tissue dry weight (STDW) also responded significantly to N rate ($P \leq 0.001$) and cultivar ($P = 0.012$). Increases in STDW as the N rate increased from 6 to 105 mg L⁻¹ were 138, 140 and 151% for Winterbor, Toscano and Redbor respectively. Linear increases in STDW were observed for each kale cultivar in response to increasing N rate in nutrient solutions (Table 1).

Plant pigment concentrations expressed on an FW basis

Accumulations of lutein, β-carotene, Chl *a* and Chl *b* in fresh leaf tissue of kale were not affected by N rate (Table 2). However, a significant amount of variation in the pigments was associated with differences among kale cultivars (lutein, $P \leq 0.001$; β-carotene, $P \leq 0.001$; Chl *a*, $P \leq 0.001$; Chl *b*, $P \leq 0.001$). Significant differences in carotenoid and chlorophyll pigment concentrations exist among *B. oleracea* cultivars.^{15,20}

Although the effects of N supply on plant growth are well understood, knowledge of the effect of N rates on protein, vitamin and phytonutrient contents in crop plants is limited. A published review

Table 1. Mean fresh (FW) and dry weight (DW) of shoot tissues (g per plant) of kale cultivars grown under increasing nitrogen rates in nutrient solution culture

Nitrogen (mg L ⁻¹)	Biomass for cultivars ^a					
	Winterbor		Toscano		Redbor	
	FW	DW	FW	DW	FW	DW
6	56.5	7.6	52.6	8.2	80.1	10.1
13	109.2	13.2	100.9	13.1	127.5	15.5
26	135.4	15.2	138.1	16.1	207.6	22.8
52	233.8	22.1	153.4	15.1	234.5	21.2
105	236.11	18.1	215.9	19.7	294.9	25.4
Contrast ^b						
Linear	***	***	***	**	***	***

^a Composition of sampled leaf blade tissues of four replications, five plants each.

^b Significance for linear contrasts for N fertility rates: *** $P \leq 0.001$; ** $P \leq 0.01$. Regression equations for each significant response: Winterbor STFW = $-9.0 + 48.4T$; Toscano STFW = $18.4 + 37.9T$; Redbor STFW = $28.0 + 53.6T$; Winterbor STDW = $6.2 + 3.0T$; Toscano STDW = $7.0 + 2.5T$; Redbor STDW = $8.1 + 3.6T$.

Table 2. Mean pigment concentrations expressed on a fresh weight basis ($\mu\text{g g}^{-1}$ FW) in leaf tissues of kale cultivars grown under increasing nitrogen rates in nutrient solution culture

Nitrogen (mg L^{-1})	Pigments ^a			
	Lutein	β -Carotene	Chl a	Chl b
<i>Winterbor</i>				
6	76.2	54.5	1147.2	335.0
13	81.3	55.1	1149.7	328.2
26	87.1	63.8	1375.7	361.9
52	78.6	59.8	1287.9	324.0
105	77.0	60.9	1149.7	290.3
Contrast ^b				
Linear	NS	NS	NS	NS
<i>Toscano</i>				
6	110.1	75.6	1546.2	493.2
13	114.7	73.2	1747.4	538.6
26	109.0	73.7	1695.5	487.0
52	105.2	60.1	1447.4	437.7
105	96.1	73.8	1482.1	442.0
Contrast ^b				
Linear	NS	NS	NS	*
<i>Redbor</i>				
6	68.7	54.4	1051.6	316.9
13	79.6	55.6	1005.3	329.5
26	79.8	56.6	1192.1	337.6
52	70.1	54.5	1043.5	297.9
105	73.2	61.2	1244.7	328.6
Contrast ^b				
Linear	NS	NS	NS	NS

^a Composition of four replications, five plants each.

^b Significance for linear contrasts for N fertility rates: * $P \leq 0.05$; NS, non-significant. Regression equation for significant response: *Toscano* Chl b = $54.0 - 2.0T$.

of the literature concluded that high rates of N fertilisers usually decrease vitamins and phytonutrients and increase the amount of tissue $\text{NO}_3\text{-N}$.²¹ Several studies demonstrate increased carotene content under increasing N fertilisation for members of the Brassicaceae and Chenopodiaceae families.^{22–24} A linear increase in lutein/zeaxanthin and β -carotene was reported in parsley (*Petroselinum crispum* Nym.) when grown under increasing N rates in nutrient solution culture.²⁵ Carrot (*Daucus carota* L.) carotenoid concentrations responded quadratically to increasing N rates, being maximal at an N rate of 160 kg ha^{-1} . However, increasing N fertility rates have been reported to decrease carotene content in carrots.²⁶ Although trends were not significant in the current study, carotenoid compounds in kale tended to be reduced with increasing N fertility levels (Table 2). Nitrogen rates that produce maximum biomass do not ensure maximal lutein or β -carotene production in kale.

Plant pigment concentrations expressed on a DW basis

Because of the use of dried material in the phytonutritional supplement industry, effort was made to calculate carotenoid accumulation on a dry weight basis. The moisture content of kale leaf tissue

Table 3. Mean carotenoid pigment concentrations expressed on a dry weight basis ($\mu\text{g g}^{-1}$ DW) and moisture content (%) in leaf tissues of kale cultivars grown under increasing nitrogen rates in nutrient solution culture

Nitrogen (mg L^{-1})	Pigments ^a		Moisture ^b
	Lutein	β -Carotene	
<i>Winterbor</i>			
6	585.8	400.6	86.6
13	677.9	470.6	88.3
26	792.2	551.0	88.7
52	862.2	631.0	90.5
105	962.9	731.0	92.3
Contrast ^c			
Linear	***	***	***
<i>Toscano</i>			
6	688.0	470.6	84.1
13	882.7	560.6	87.1
26	848.0	630.4	88.4
52	1052.4	622.0	90.5
105	1068.0	791.1	90.9
Contrast ^c			
Linear	**	***	***
<i>Redbor</i>			
6	528.6	360.3	87.4
13	663.3	470.3	88.0
26	717.0	520.1	89.1
52	778.8	602.0	90.9
105	915.4	721.0	91.5
Contrast ^c			
Linear	***	***	***

^a Composition of four replications, five plants each.

^b Percentage of moisture in leaf tissues. Regression analysis performed on transformed data using \sin^{-1} .

^c Significance for linear contrasts for N fertility rates: *** $P \leq 0.001$; ** $P \leq 0.01$. Regression equations for each significant response: *Winterbor* lutein = $0.5 + 0.1T$; *Toscano* lutein = $0.6 + 0.1T$; *Redbor* lutein = $0.4 + 0.01T$; *Winterbor* β -carotene = $0.3 + 0.1T$; *Toscano* β -carotene = $0.4 + 0.1T$; *Redbor* β -carotene = $0.3 + 0.1T$; *Winterbor* % moisture = $67.1 + 1.3T$; *Toscano* % moisture = $65.5 + 1.5T$; *Redbor* % moisture = $67.9 + 1.0T$.

responded significantly to N rate ($P \leq 0.001$) and cultivar ($P = 0.005$). Linear increases in leaf tissue moisture were observed for each kale cultivar in response to increasing N rates (Table 3). Changes in plant moisture content can result under increased N rates. Increases in total yield of celery (*Apium graveolens* L. var. *dulce* Pers.) were reported as N rates increased from 30 to 90 kg ha^{-1} , but there were decreases in plant dry matter content over the increasing N treatments.²⁷ However, greater increases in tissue water content in the perennial grass *Molinia caerulea* L. occurred under low N level ($0.1 \text{ mol m}^{-3} \text{ NH}_4\text{NO}_3$) than under higher N level ($5 \text{ mol m}^{-3} \text{ NH}_4\text{NO}_3$).²⁸

Lutein concentration in kale leaf tissue expressed on a DW basis responded significantly to N rates ($P \leq 0.001$) and cultivar ($P \leq 0.001$). β -Carotene expressed on a DW basis also responded significantly to N rates ($P \leq 0.001$) and cultivar ($P = 0.011$). Each cultivar displayed significant linear increases in lutein

and β -carotene concentrations when expressed on a DW basis as a result of increasing N rate. Lutein in the leaf tissues increased by 63, 55 and 62% as the N rate increased from 6 to 105 mg L⁻¹ for Winterbor, Toscano and Redbor respectively. As the N rate increased from 6 to 105 mg L⁻¹, β -carotene in the leaf tissues increased by 82, 68 and 100% for Winterbor, Toscano and Redbor respectively (Table 3).

Increasing N rates in nutrient solution culture increased kale leaf tissue biomass. Changes in lutein and β -carotene concentrations were measured for each kale cultivar when the leaf tissue pigments were expressed on a DW basis. The highest lutein and β -carotene accumulation in kale dry leaf tissue occurred at the N treatment of 105 mg L⁻¹. Therefore this rate was chosen to investigate the effect of different N form ratios on carotenoid production in kale leaf tissue.

Nitrogen form study

Biomass production

Kale STFW responded significantly to N form ($P \leq 0.001$), cultivar ($P \leq 0.001$) and the interaction of N form and cultivar ($P \leq 0.001$). Linear increases in STFW were observed for each kale cultivar as the % of NO₃-N in nutrient solutions increased (Table 4). Highest accumulations for STFW occurred at 25% NH₄-N:75% NO₃-N for Winterbor and Redbor, while maximum biomass for Toscano occurred at 0% NH₄-N:100% NO₃-N. Kale STDW responded significantly to N form ($P \leq 0.001$) and cultivar ($P \leq 0.001$). A small but significant portion of the variability in STDW was attributed to the interaction of N form and cultivar ($P = 0.012$). Linear increases in STDW were observed for each kale cultivar in response to varying N form in nutrient solutions from 100% NH₄-N:0% NO₃-N to 0% NH₄-N:100% NO₃-N.

Table 4. Mean fresh (FW) and dry weight (DW) of shoot tissues (g per plant) of kale cultivars grown under varying %NH₄-N:%NO₃-N in nutrient solution culture

%NH ₄ -N: %NO ₃ -N	Biomass for cultivars ^a					
	Winterbor		Toscano		Redbor	
	FW	DW	FW	DW	FW	DW
100:0	83.4	12.7	40.8	6.0	34.4	4.1
75:25	210.4	32.5	116.6	16.3	186.0	26.8
50:50	359.4	52.7	258.1	50.8	315.3	38.5
25:75	611.0	110.4	358.7	66.8	547.9	73.2
0:100	557.2	102.6	316.1	58.9	478.9	58.2
Contrast ^b						
Linear	***	***	***	***	***	***
Quadratic	NS	NS	*	NS	*	*

^a Composition of sampled leaf blade tissues of four replications, five plants each.

^b Significance for linear contrasts for N fertility forms: *** $P \leq 0.001$; * $P \leq 0.05$; NS, non-significant. Regression equations for each significant response: Winterbor STFW = $-32.5 + 132.97$; Toscano STFW = $-14.5 + 77.97$; Redbor STFW = $-44.6 + 120.57$; Winterbor STDW = $-15.1 + 25.87$; Toscano STDW = $-6.5 + 15.57$; Redbor STDW = $-3.0 + 14.77$; Redbor STDW = $-35.5 + 39.77 - 3.97^2$.

Maximum STDW accumulation occurred at 25% NH₄-N:75% NO₃-N for all cultivars in the study (Table 4).

Although the highest biomass production of zucchini squash (*Cucurbita pepo* L. var. *melopepo* Green Magic) has been measured when N form was 100% NO₃-N,²⁹ increases in plant growth in response to combined NH₄-N and NO₃-N over single NO₃-N supply are frequently observed. Improved tomato (*Lycopersicon esculentum* Mill.) growth in nutrient solution culture resulted under a ratio of 1 NH₄-N:10 NO₃-N over NO₃-N alone.³⁰ Yields of hydroponic tomatoes were higher at 25% NH₄-N:75% NO₃-N fertility than at 100% NO₃-N alone.³¹ Yield of sweet pepper (*Capsicum annuum* L. Grossum Group) was greatest in nutrient solution culture when 30% of N was supplied as NH₄-N.³² It has been theorised that low biomass production under NH₄-N is the result of a decrease in availability of carbon skeletons, resulting from NH₄⁺ detoxification.²⁹ When NH₄-N is absorbed in excess, the carbon skeletons required for incorporation of NH₄⁺ into organic N come at the expense of plant growth.³³ The effects of N form treatments on the kale yields in the current study follow previously reported trends.

Plant pigment concentrations expressed on an FW basis

Lutein concentration in the kale cultivars responded significantly to N form ($P \leq 0.001$), cultivar ($P \leq 0.001$) and the interaction of N form and cultivar ($P = 0.011$). Lutein concentrations in fresh leaf tissues ranged from a high of 125.0 $\mu\text{g g}^{-1}$ for Toscano under 0% NH₄-N:100% NO₃-N to a low of 48.0 $\mu\text{g g}^{-1}$ for Winterbor under 100% NH₄-N:0% NO₃-N (Table 5). Lutein and violaxanthin values ranged from 71.0 to 114.0 $\mu\text{g g}^{-1}$ in two field-grown kale cultivars.²⁰ Violaxanthin is a diepoxide xanthophyll that is converted to zeaxanthin by the enzyme violaxanthin deepoxidase under light stress.³⁴ Lutein was measured at 186.3 $\mu\text{g g}^{-1}$ in kale of unknown culti-type and growing conditions at a local market in Germany, but no zeaxanthin was detected.³⁵ The greatest increase in lutein in fresh leaf tissues for all three cultivars occurred as N form changed from 100% NH₄-N:0% NO₃-N to 75% NH₄-N:25% NO₃-N, with lutein levels increasing only slightly as the % of NH₄-N in the nutrient solutions decreased over the remaining treatments. This indicates that NO₃-N is required to maximise lutein production in kale.

Significant linear increases in lutein in response to increasing NO₃-N in nutrient solutions were found for Toscano, while Winterbor leaf tissue lutein responded quadratically to increasing NO₃-N (Table 5). Varying N form in nutrient solutions from 100% NH₄-N:0% NO₃-N to 0% NH₄-N:100% NO₃-N increased the lutein concentration by 155, 73 and 39% for Toscano, Winterbor and Redbor respectively. Higher accumulations of lutein in kale leaf tissues would increase dietary nutritional contributions. Therefore the effect of N form on lutein production in kale should

Table 5. Mean pigment concentrations expressed on a fresh weight basis ($\mu\text{g g}^{-1}$ FW) in leaf tissues of kale cultivars grown under varying % $\text{NH}_4\text{-N}$:% $\text{NO}_3\text{-N}$ in nutrient solution culture

% $\text{NH}_4\text{-N}$: % $\text{NO}_3\text{-N}$	Pigments ^a			
	Lutein	β -Carotene	Chl <i>a</i>	Chl <i>b</i>
<i>Winterbor</i>				
100:0	48.0	10.2	355.6	437.0
75:25	82.0	14.1	540.3	726.7
50:50	82.6	22.4	729.6	711.0
25:75	83.3	22.6	740.3	681.0
0:100	83.2	30.0	644.5	689.2
Contrast ^b				
Linear	*	**	*	NS
Quadratic	*	NS	*	*
<i>Toscano</i>				
100:0	48.8	14.3	400.2	443.2
75:25	80.6	16.6	843.7	736.4
50:50	124.1	24.4	1348.4	1246.0
25:75	119.8	37.4	1764.5	1105.1
0:100	125.0	43.5	1710.0	1065.0
Contrast ^b				
Linear	***	***	***	**
Quadratic	*	NS	NS	**
<i>Redbor</i>				
100:0	51.0	15.0	535.7	408.0
75:25	73.1	19.2	824.1	649.5
50:50	67.6	22.6	628.2	559.2
25:75	72.0	28.2	823.0	584.0
0:100	66.0	34.0	604.0	493.8
Contrast ^b				
Linear	NS	**	NS	NS
Quadratic	NS	NS	NS	NS

^a Composition of four replications, five plants each.

^b Significance for linear and quadratic contrasts for N fertility forms: *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; NS, non-significant. Regression equations for each significant response: Winterbor lutein = $2.2 + 3.57 - 0.57T^2$; Toscano lutein = $4.3 + 1.9T$; Winterbor β -carotene = $0.6 + 0.5T$; Toscano β -carotene = $0.3 + 0.8T$; Redbor β -carotene = $1.0 + 0.5T$; Winterbor Chl *a* = $38.1 + 7.5T$; Winterbor Chl *b* = $22.5 + 29.27 - 4.17T^2$; Toscano Chl *a* = $15.6 + 35.37T$; Toscano Chl *b* = $45.3 + 15.7T$.

be considered in plant improvement programmes emphasising carotenoid accumulation.

β -Carotene concentration in the kale cultivars responded significantly to N form ($P \leq 0.001$). Concentration of leaf tissue β -carotene increased linearly in response to increasing $\text{NO}_3\text{-N}$ in nutrient solutions for each cultivar tested (Table 5). β -Carotene concentrations in the fresh leaf tissues ranged from a high of $43.5 \mu\text{g g}^{-1}$ for Toscano under 0% $\text{NH}_4\text{-N}$:100% $\text{NO}_3\text{-N}$ to a low of $10.2 \mu\text{g g}^{-1}$ for Winterbor under 100% $\text{NH}_4\text{-N}$:0% $\text{NO}_3\text{-N}$. As the form of N in nutrient solutions changed from 100% $\text{NH}_4\text{-N}$:0% $\text{NO}_3\text{-N}$ to 0% $\text{NH}_4\text{-N}$:100% $\text{NO}_3\text{-N}$, β -carotene concentrations increased by 200, 214 and 130% for Winterbor, Toscano and Redbor respectively. The form of N in nutrient management should therefore be considered in phytonutrient programmes designed to increase β -carotene content in kale. β -Carotene values have been reported to range from 38.0 to $73.0 \mu\text{g g}^{-1}$

for field-grown kale.^{9,20,35} The lower β -carotene values measured in this study may be the result of N form treatments or cultivar genetics.⁹

Chlorophyll was found in much higher concentrations than carotenoids in the kale leaf tissue (Table 5). Values for Chl *a* and Chl *b* were within previously reported ranges for kale.^{15,18} Chlorophyll *a* concentration responded significantly to N form ($P \leq 0.001$), cultivar ($P \leq 0.001$) and the interaction of N form and cultivar ($P = 0.013$). Chlorophyll *b* concentration responded significantly to N form ($P \leq 0.001$), cultivar ($P \leq 0.001$) and the interaction of N form and cultivar ($P \leq 0.001$). Maximum Chl *a* values occurred at 25% $\text{NH}_4\text{-N}$:75% $\text{NO}_3\text{-N}$ for all kale cultivars. Chlorophyll *a* concentration increased linearly for Toscano and increased, then decreased quadratically for Winterbor in response to increasing $\text{NO}_3\text{-N}$ in nutrient solutions. A quadratic trend, first increasing, then decreasing, was observed for Chl *b* in Winterbor and Toscano in response to increasing $\text{NO}_3\text{-N}$. Similar to data in the current study, increases in leaf greenness of tomato, estimated by chlorophyll Soil Plant Analysis Development (SPAD), occurred as $\text{NH}_4\text{-N}$ was increased from 0 to 25%.³¹ In contrast to the current study, Chl *a* and *b* contents in sunflower (*Helianthus annuus* L.) leaves were statistically higher under 100% $\text{NH}_4\text{-N}$ in nutrient solution culture than under 100% $\text{NO}_3\text{-N}$.³³

In plants, N is an important component of proteins and biomolecules such as chlorophyll. Significant positive correlations occurred between lutein and Chl *a* and *b* and between Chl *a* and Chl *b* at 100% $\text{NH}_4\text{-N}$:0% $\text{NO}_3\text{-N}$. At 75% $\text{NH}_4\text{-N}$:25% $\text{NO}_3\text{-N}$, both lutein and Chl *a* correlated with Chl *b*. Tissue N concentration correlated with lutein, Chl *a* and Chl *b* at 50% $\text{NH}_4\text{-N}$:50% $\text{NO}_3\text{-N}$. Correlations were observed for the carotenoids and chlorophylls at each of the 50% $\text{NH}_4\text{-N}$:50% $\text{NO}_3\text{-N}$, 25% $\text{NH}_4\text{-N}$:75% $\text{NO}_3\text{-N}$ and 0% $\text{NH}_4\text{-N}$:100% $\text{NO}_3\text{-N}$ treatments, with the exception of β -carotene and Chl *b* at 0% $\text{NH}_4\text{-N}$:100% $\text{NO}_3\text{-N}$. Similarities in behaviour of carotenoids and chlorophylls have been reported for various crop species.^{36,37} Chlorophyll pigments were found to correlate highly with total carotenoid levels in the leaves of Swiss chard (*Beta vulgaris* L.).³⁸ Positive correlations have been reported between total chlorophyll and carotenoid contents in tobacco (*Nicotiana tabacum* L.) leaves.³⁶ Statistical differences in carotenoid and chlorophyll contents in response to KNO_3 , NH_4Cl and NH_4NO_3 N forms in solution culture in 16- and 24-day-old corn (*Zea mays* L. var. *rugosa* Bonif.) seedlings suggest that the metabolisms of these two pigments are interrelated.³⁹ Correlations between carotenoid and chlorophyll accumulation among *B. oleracea* cultivars has recently been observed. Therefore it may be possible to use chlorophyll pigmentation, or degree of green colouration, to make an assessment of total carotenoid content in leafy vegetable crops.¹⁵

Table 6. Mean carotenoid pigment concentrations expressed on a dry weight basis ($\mu\text{g g}^{-1}$ DW) and moisture content (%) in leaf tissues of kale cultivars grown under varying $\% \text{NH}_4\text{-N}:\% \text{NO}_3\text{-N}$ in nutrient solution culture

%NH ₄ -N: %NO ₃ -N	Pigments ^a		Moisture ^b
	Lutein	β -Carotene	
<i>Winterbor</i>			
100:0	471.2	60.3	84.6
75:25	821.0	70.4	84.5
50:50	821.0	120.6	85.3
25:75	832.2	100.5	82.0
0:100	841.4	120.7	81.7
Contrast ^c			
Linear	**	NS	*
Quadratic	*	NS	NS
<i>Toscano</i>			
100:0	498.0	80.2	85.2
75:25	810.7	90.5	87.0
50:50	1243.0	100.5	81.1
25:75	1252.8	160.9	81.6
0:100	1251.9	190.9	81.5
Contrast ^c			
Linear	***	*	*
Quadratic	*	NS	NS
<i>Redbor</i>			
100:0	511.3	90.5	88.3
75:25	731.4	100.5	85.5
50:50	681.7	150.8	87.6
25:75	720.8	160.9	86.6
0:100	661.4	190.8	87.9
Contrast ^c			
Linear	NS	NS	NS
Quadratic	NS	NS	NS

^a Composition of four replications, five plants each.

^b Percentage of moisture in leaf tissues. Regression analysis performed on transformed data using \sin^{-1} .

^c Significance for linear and quadratic contrasts for N fertility forms: *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; NS, non-significant. Regression equations for each significant response: Winterbor lutein = $0.5 + 0.17T$; Toscano lutein = $0.4 + 0.27T$; Toscano β -carotene = $0.04 + 0.037T$; Winterbor % moisture = $86.1 - 0.87T$; Toscano % moisture = $87.1 - 1.37T$.

Plant pigment concentrations expressed on a DW basis

The moisture content of kale leaf tissue responded significantly to N form ($P = 0.03$) and cultivar ($P \leq 0.001$). Linear increases in kale leaf tissue moisture content were observed for Winterbor and Toscano in response to increasing $\text{NO}_3\text{-N}$. Lutein concentration in kale leaf tissue expressed on a DW basis responded to N form ($P \leq 0.001$) and cultivar ($P \leq 0.001$), and β -carotene also responded significantly to N form ($P \leq 0.001$) and cultivar ($P = 0.02$). Lutein in the leaf tissues expressed on a DW basis increased by 78, 155 and 30% as the amount of $\text{NO}_3\text{-N}$ increased in nutrient solutions for Winterbor, Toscano and Redbor respectively. As the amount of $\text{NO}_3\text{-N}$ increased, β -carotene in the leaf tissues expressed on a DW basis increased by 100, 137 and 111% for Winterbor, Toscano and Redbor respectively (Table 6).

Nitrogen affected biomass and carotenoid pigment production in kale leaf tissues. Kale leaf tissue biomass increased with increasing N rates, but it also increased with increasing $\text{NO}_3\text{-N}$ in nutrient solutions when the level of N was kept constant at 105 mg L^{-1} . Carotenoid pigments in the fresh kale leaf tissue did not respond to changes in N rates, but carotenoid pigments did increase with increasing N rates when expressed on a DW basis. Increases in carotenoid pigments, expressed either on an FW or a DW basis, resulted for some kale cultivars when $\text{NO}_3\text{-N}$ was increased in nutrient solutions. Changes in N rate and form in the media resulted in changes in lutein and β -carotene concentrations in the leaf tissues of different kale cultivars. Therefore it will be important to understand the influence of N management in production systems designed to increase the concentrations of nutritionally important carotenoids in kale.

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