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RESEARCH ARTICLE

Variety-specific responses of lettuce grown in a gravel-film technique closed hydroponic system to N supply on yield, morphology, phytochemicals, mineral content and safety

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Abstract

Utilization of nitrogen (N) element is a common practice used to reach profitable yields in horticultural crops and N supply can be used as a tool to manipulate the enhancement of phytochemicals and minerals in vegetable crops to address consumer-oriented quality production. Hence the study was aimed to investigate the effect of N application on leaf morphology, ascorbic acid content, phenolic acids, flavonoids, mineral content, and nitrate residues in three lettuce varieties (two green leafy lettuce, Multigreen 1 and Multigreen 3; one red leafy lettuce, Multired 4) grown in a closed hydroponic system (gravel-film) at harvest. Nitrogen was applied as ammonium nitrate (NH_4NO_3) at six different concentrations of 0, 60, 90, 120, 150 and 180 mg L^{-1}. The results obtained during 2015 and 2016 seasons were similar and demonstrated variety dependent responses with respect to different N application rates. Multigreen 3 was more sensitive to N supply and showed higher amount of nitrate residue at harvest. Variety Multired 4 was less sensitive to N supply followed by Multigreen 1. Although N supply at 120 mg L^{-1} improved the yield and the number of leaves in Multigreen 3, overall 90 mg L^{-1} can be recommended for these lettuce varieties to improve the yield and the accumulation of ascorbic acid content, phenolic acids mainly caffeic, caftaric acids, quercetin (the important flavonoid in lettuce), and Fe and Mn contents. Furthermore, the concentration of 90 mg L^{-1} improved the antioxidant property (FRAP and $ABTS^+$) and reduced the nitrate accumulation, ensuring safe food for consumers.

Keywords: fertilizer application, fresh weight, phenolic acids, antioxidant property, flavonoids, Fe, Mn, food safety

1. Introduction

Lettuce (*Lactuca sativa* L.) is one of the most consumed fresh leafy vegetables across the world in salad mixes and as sandwich fillings. It contains antioxidant phytochemicals including caffeic acid and its derivatives, flavonols (quercetin and kaempferol derivatives), vitamins and carotenoids and low caloric content (Nicolle 2004; Stefanelli *et al*. 2010; Becker *et al*. 2015). Consumers are becoming aware of the health benefits of vegetables and the consumption of vegetables has been trending upwards (Van der Merwe

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et al. 2016). Important quality attributes in horticultural crops include visual quality and taste which are directly influenced by nitrogen (N) availability (Stefanelli *et al*. 2010). Thus, N application can be used to manipulate lettuce leaf quality (Becker *et al*. 2015) by improving foliage growth (yields) and phytonutrient content (Stefanelli *et al*. 2010).

The gravel-film technique (GFT) is a popular recirculating hydroponic system in South Africa for the cultivation of leafy vegetables with very short cycles, by commercial growers, for improving yields and quality at harvest and to improve the shelf life during marketing (Maboko and Du Plooy 2013). On the other hand, the N supply in the GFT (at the root zone) can affect the growth morphology (plant height, leaf size and number), phytochemical and mineral content and quality attributes such as colour and shape in lettuce varieties (Daniele *et al*. 2013).

Since lettuce is consumed in fresh form, high rates of N application especially in nitric form are associated with negative health related issues (Addiscott and Benjamin 2004; Qadir *et al*. 2017). On the other hand, water resources contaminated with nitrate can result in euthrophication and limit the availability of drinking water (Daniele *et al*. 2013). Thus, N application need to be balanced in order to avoid over fertilization and to reduce the cost of N application. Therefore, a well-balanced N fertilization is important in order to produce higher yield without compromising the quality of the vegetables (Daniele *et al*. 2013). Another approach is to reduce the rate of N application by planting varieties that are efficient in the use of N (Rather *et al*. 1999).

Nitrogen application at 200 kg ha^{-1} negatively affected dry matter, sugar and vitamin C content in crisphead lettuce (Poulsen *et al*. 1995). However, lettuce fertilization treatments with high N content caused a reduction in phenolic acid content, antioxidant capacity (Stefanelli *et al*. 2011; Becker *et al*. 2015), mineral content (Pitura and Michałojć 2015) and anthocyanins (Becker *et al*. 2015). Marketing lettuce is not only related to the fresh weight and overall leaf quality, but it is also associated with phytochemicals, minerals and vitamins and permitted levels of nitrate and nitrite in the fresh leaves (Konstantopoulou *et al*. 2010). The red lettuce varieties possess higher composition of caffeic acid derivatives (chicoric acid, chlorogenic acid and caffeoylmalic acid), flavonoids (cyanidin, quercetin and luteolin) than the green varieties (Becker *et al*. 2015). Red lettuce varieties contain higher concentration of anthocyanin (Becker *et al*. 2015). Studies have demonstrated an increase in chlorophyll content with decreasing phytochemical content with increased N supply (Qadir *et al*. 2017). Furthermore, interaction effect between N fertilizer application and genotypes affect phytochemicals and nitrate accumulation in lettuce (Becker *et al*. 2015). Generally N supply at 120 mg L^{-1} is recommended commercially for the GFT growers to fertilize lettuce. However, it is important for growers to know the lettuce varieties that are more or less sensitive to N supply. Also, by planting less sensitive lettuce varieties, growers can save the cost used for N fertilizer by using lower concentration of N application and deliver safe lettuce that comply with food safety requirements. At the same time, limited information is available on the influence of N supply on physiological response of green and red lettuce varieties grown in GFT. Although the commercially recommended N supply is 120 mg L^{-1}, this concentration should not negatively influence the biosynthesis of ascorbic acids and phenolic compounds that have numerous health benefits. Therefore, this study envisaged to investigate the effect of N application on: i) fresh leaf weight, leaf area, number of leaves; ii) ascorbic acid content; iii) phytochemicals (chlorogenic acid, caftaric acid, caffeic acid, chicoric acid, kaempferol, quercetin, myrcetin, and epicatechin); iv) chlorophyll and anthocyanin; v) mineral elements; and vi) nitrate residue at harvest on three leafy lettuce varieties (Multigreen 1, Multigreen 3 and Multired 4) grown for fresh salad packs in a closed hydroponics system.

2. Materials and methods

2.1. Plant materials

Three lettuce varieties (two green curly Mutigreen 1 and Multigreen 3, and one red oak Multired 4) obtained from Starke Ayres (Pty) Ltd., Kaalfontein, South Africa, were sown in 200 cavity polystyrene trays using Hygromix® and vermiculite. After 28 days, seedlings were transplanted in 24 hydroponic tables (gravel-film technique) and each table contained 10 plants per cultivar with 20 cm×15 cm plant spacing under 40% white shade-net structure during winter seasons (June to July) in 2015 and 2016. The cultivars were fertilized with six different concentrations of ammonium nitrate (NH_4NO_3) (as the source of N) of 0, 60, 90, 120, 150 and 180 mg L^{-1}. The treatment combinations were replicated four times and laid out in a split-plot design. A set of 24 tanks filled with 1 000 L of nutrient solution were used for the gravel-film technique culture. The nutrients were applied in g 1 000 L^{-1} of water: calcium chloride ($CaCl_2$) (67 mg Ca L^{-1} and 132 mg Cl L^{-1}), monopotassium phosphate (MKP, 45 mg P L^{-1} and 57 mg K L^{-1}), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (44 mg Mg L^{-1} and 57 mg S L^{-1}), potassium sulfate (K_2SO_4) (118 mg K L^{-1} and 50 mg S L^{-1}), potassium chloride (KCl) (65 mg K L^{-1} and 59 mg Cl L^{-1}) and Hidrospoor (1.74 mg Fe L^{-1}, 0.36 mg Mn L^{-1}, 0.22 mg Zn L^{-1}, 0.024 mg Cu L^{-1}, 0.46 mg B L^{-1} and 0.04 mg Mo L^{-1}). The pH of the nutrient solution was maintained between 6.0 and 6.5 using phosphoric acid for all treatments.

2.2. Morphological parameters

Lettuce were harvested at 30 days after transplanting and fresh and dry leaf weight, leaf number and leaf area were recorded using the method described previously by Mahlangu *et al*. (2016). Fresh weight (FW) was taken by weighing the lettuce leaves using an electronic balance. Leaf area meter (LI-3100 area meter, LiCor, Lincoln, NE, USA) was used to measure the leaf area.

2.3. Biochemical analysis

Lettuce leaves were subjected to biochemical analysis after harvest. The analysis included the determination of ascorbic acid, phenolic acids (chlorogenic acid, caftaric acid, caffeic acid, chicoric acid) and flavonoids ((isorhamnetin, quercetin, myricetin, epicatechin, anthocyanin (red lettuce)), total chlorophyll (green lettuce), antioxidant properties (ferric antioxidant power (FRAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay). Determination of ascorbic acid was performed as previously described by Mahlangu *et al*. (2016) using 5 g of fresh leaves. For the quantification of phenolic acids and flavonoids, 5 g of freeze dried samples were extracted in 16 mL of methanol-water-formic acid mixture (25:24:3, v/v) using an ultrasonic extraction devise as described previously by Ntsoane *et al*. (2016). The phenolic acids, flavonoids and anthocyanins were quantified at wavelengths of 330, 350 and 368 nm, respectively (Ntsoane *et al*. 2016). Antioxidant properties (FRAP and $ABTS^+$) were determined using 0.4 g of fresh leaves (Mampholo *et al*. 2016) and expressed as Trolox equivalent antioxidant capacity (mg TEAC 100 g^{-1} FW).

2.4. Mineral analysis and nitrate concentrations

After harvest, lettuce leaves were oven dried at 70°C for 48 h and thereafter ground and sieved to quantify the leaf N, P, K, Ca, Mg, Zn and Fe contents using the ICP OES (inductively coupled plasma optical emission spectrometry) according to Mahlangu *et al*. (2016). The nitrate concentration in the leaves were determined according to the method described in AOAC (1995) and adopting the procedure described by Konstantopoulou *et al*. (2010) from 10 plants per replicate from each treatment.

2.5. Statistical analysis

The 2-year data were subjected to analysis of variance in GenStat® (ver. 11.1, VSN International Ltd., Hemel Hempstead, UK). If interactions were significant, they were used to explain results. If interactions were not significant, means were separated using Fisher's protected *t*-test least significant difference. The polynomial model procedure was tested in GenStat®.

3. Results

Fresh and dry leaf weight, leaf number, leaf area, ascorbic acid, phenolic acids and flavonoids and antioxidant activity were affected by N rate during 2015 and 2016. Interactions between year and N application rate (Y×N), year and variety (Y×V) and year by nitrogen application by plant variety (Y×N×V) were not significant (data not shown).

Relationships between N application rate and morphological parameters (leaf weight, leaf area and leaf number), ascorbic acid, phytochemicals (phenolic acids and flavonoids), antioxidant activities, and mineral composition for the three varieties were illustrated by polynomial models and the specific regressions differed in level of significance and degree of association.

3.1. Morphological parameters

This study showed that optimum fresh leaf weight (yield) can be obtained with concentrations of 90, 120 and 150 mg L^{-1} (Fig. 1-A). Among the three varieties, Multigreen 3 showed higher fresh leaf weight while the red lettuce, Multired 4, showed lower fresh weight (Fig. 1-A).

It was possible to identify the lettuce varieties that were more responsive, based on the regression coefficients (Table 1). The N concentrations influenced fresh leaf weight of Multigreen 3 compared to Multigreen 1, and fresh leaf weight was strongly improved in these two varieties with N application (Fig. 1-A; Table 1). The red leafy lettuce, Multired 4, showed poor response to N application rates for the improvement of leaf weight (Fig. 1-A; Table 1).

Leaf area was higher in Multired 3 than in Multigreen 1 and Multigreen 4 (Fig. 1-B). However, the response of Multigreen 1 was higher than Multigreen 3 and it clearly demonstrated that the leaf area of Multigreen 1 can be improved with increased N supply up to 150 mg L^{-1} (Table 1). In Multigreen 3, leaf area increased with N supply up to 120 mg L^{-1} and thereafter a declining trend was noted with increasing N application rates.

It is evident from Fig. 1-C that leaf number was higher in Multigreen 3 and it demonstrated a strong response to increasing N application rates (Table 1). Although Multired 4 showed less leaf number, the response to increasing N supply was moderate (Fig. 1-C; Table 1). Although the response to leaf area was very strong to increasing N supply in Multigreen 1, a weak response to leaf number

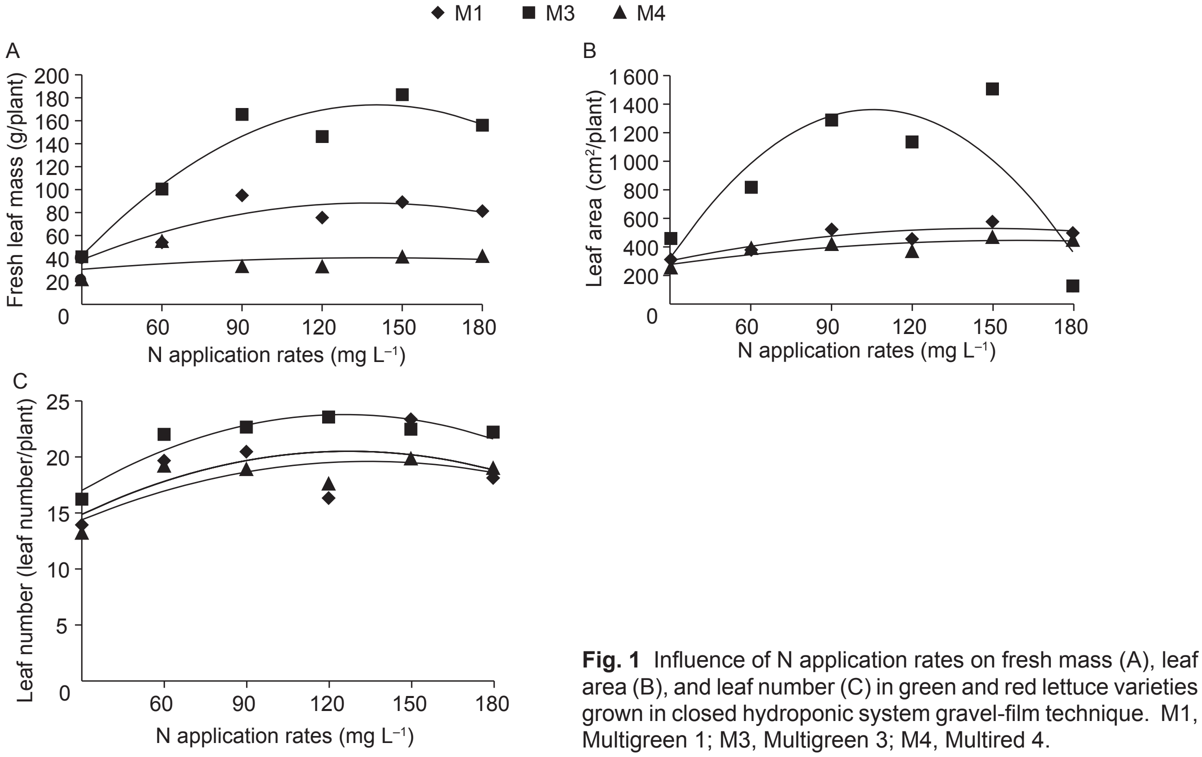

Fig. 1 Influence of N application rates on fresh mass (A), leaf area (B), and leaf number (C) in green and red lettuce varieties grown in closed hydroponic system gravel-film technique. M1, Multigreen 1; M3, Multigreen 3; M4, Multired 4.

Table 1 Regression equations for the morphological parameters in leafy green and red lettuce varieties grown in closed hydroponic system gravel-film technique and fertilized with N concentrations of 0–180 mg L^{-1}

Morphological parameter	Regression equation	R^2
Leaf fresh mass (g/plant)		
Multigreen 1	$y=-3.7964x^2+34.718x+7.9$	0.79
Multigreen 3	$y=-9.5089x^2+89.021x-37.08$	0.93
Multired 4	$y=-0.7429x^2+6.9x+24.4$	0.12
Leaf area (cm^2/plant)		
Multigreen 1	$y=-14.773x^2+145.24x+169.81$	0.80
Multigreen 3	$y=-163.09x^2+1148.8x-659.59$	0.72
Multired 4	$y=-8.9554x^2+95.645x+187.151$	0.75
Leaf number (leaf number/plant)		
Multigreen 1	$y=-0.5357x^2+4.5486x+10.83$	0.40
Multigreen 3	$y=-0.6654x^2+5.5798x+12.072$	0.89
Multired 4	$y=-0.4295x^2+3.8534x+10.935$	0.65

was noted (Fig. 1-B and C). It is evident from this study that all three varieties showed different trends for increasing N application rate for the three morphological parameters. However, overall N application rates beyond 120 to 150 kg ha^{-1} negatively affected the morphological parameters (Fig. 1-A–C).

3.2. Ascorbic acid and chlorophyll content

Within the three lettuce varieties, red var. Multired 4 showed the highest and green var. Multigreen 1 showed the lowest ascorbic acid content (Fig. 2-A). Ascorbic acid content in the three lettuce varieties demonstrated a decrease with higher N availability (>90 mg L^{-1}) (Fig. 2-A). All three lettuce varieties revealed a strong negative response (Multigreen 1, R^2=0.98; Multigreen 3, R^2=0.90; Multired 4, R^2=0.96) to increasing rates of N supply (Table 2).

Chlorophyll content in both green leafy lettuce varieties Multigreen 1 and Multigreen 3 was strongly influenced by N application rates (Table 2). Chlorophyll content was lower at the lower N application of 60 mg L^{-1} and increased gradually with N supply and revealed higher chlorophyll content at 90 to 120 mg L^{-1} in Multigreen 3, and thereafter the levels declined at higher concentrations (150 and 180 mg L^{-1} of N) (Fig. 2-B). However, in Multigreen 1, the chlorophyll content increased linearly with increasing rates of N application (Fig. 2-B) and a strong positive response existed between the total chlorophyll content and nitrogen application rates (Table 2).

3.3. Phytochemicals and antioxidant activity

Concentrations of chicoric, chlorogenic, caftaric, and caffeic acids were remarkably higher in red var. Multired 4 (Fig. 3-A–D) with chicoric acid being the predominant phenolic acid (Fig. 3-A). Within the three lettuce varieties, Multigreen 1 contained the lowest concentrations of chlorogenic, caftaric and chicoric acids (Fig. 3-A–D). It is evident from this study that N supply poorly influenced the chichoric acid accumulation in the three varieties in this study (Table 2).

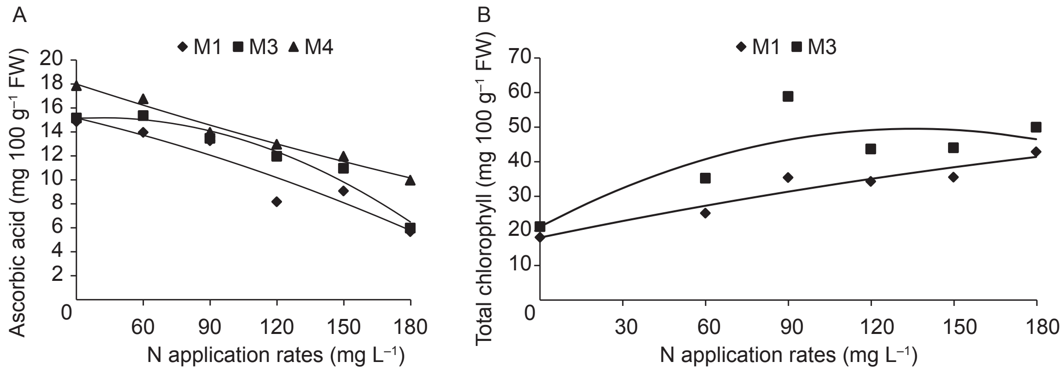

Fig. 2 Influence of N application on ascorbic acid (A) and total chlorophyll content (B) in lettuce varieties grown in closed hydroponic system gravel-film technique. M1, Multigreen 1; M3, Multigreen 3; M4, Multired 4.

Table 2 Regression equations for the bioactive compounds and antioxidant properties in leafy green and red lettuce varieties grown in closed hydroponic system gravel film technique and fertilized with N concentrations of 0–180 mg L^{-1}

Bioactive compounds	Regression equation	R^2	Bioactive compounds	Regression equation	R^2
Ascorbic acid			Quercetin		
Multigreen 1	y=0.0482x^2–1.9061x+19.89	0.98	Multigreen 1	y=–0.0323x^2–0.3429x+5.857	0.66
Multigreen 3	y=–0.1089x^2–1.1175x+16.43	0.90	Multigreen 3	y=–0.143x^2+0.5773x+7.439	0.39
Multired 4	y=–0.4x^2+1.0657x+14.52	0.96	Multired 4	y=0.0832x^2–2.8516x+22.172	0.50
Phenolic acids			Myrcetin		
Chicoric acid			Multigreen 1	y=0.0571x^2–0.3069x+1.25	0.76
Multigreen 1	y=–0.0038x^2+0.8348x+44.501	0.21	Multigreen 3	y=–0.0271x^2+0.1091x+1.378	0.39
Multigreen 3	y=0.0042x^2–1.188x+192.97	0.49	Multired 4	y=0.0159x^2–0.5475x+4.207	0.51
Multired 4	y=–0.0242x^2+3.4297x+618.75	0.38	Epicatechin		
Chlorogenic acid			Multigreen 1	y=0.0448x^2–0.3289x+1.353	0.25
Multigreen 1	y=–0.0012x^2+0.3601x+12.037	0.85	Multigreen 3	y=–0.0249x^2+0.0948x+1.3885	0.39
Multigreen 3	y=–0.0015x^2+0.274x+38.752	0.92	Multired 4	y=0.0169x^2–0.5353x+4.1098	0.50
Multired 4	y=–0.0139x^2+3.3043x+93.162	0.96	Anthocyanin		
Caftaric acid			Multired 4	y=–0.2232x^2+1.5576x+0.582	0.93
Multigreen 1	y=–0.0107x^2+1.7309x+89.396	0.79	Antioxidant properties		
Multigreen 3	y=–0.0068x^2+1.0563x+224	0.28	Antioxidant capacity ($ABTS^+$)		
Multired 4	y=–0.0045x^2–0.0901x+612.77	0.27	Multigreen 1	y=0.0313x^2–0.3358x+1.3489	0.93
Caffeic acid			Multigreen 3	y=0.05x^2–0.7215x+3.4277	0.96
Multigreen 1	y=–0.0022x^2+0.3915x+22.227	0.75	Multired 4	y=–0.7625x^2–5.5539x+128.77	0.80
Multigreen 3	y=–0.0027x^2+0.4064x+25.88	0.30	Antioxidant power (FRAP)		
Multired 4	y=0.0007x^2–2.05x+798.27	0.47	Multigreen 1	y=0.0607x^2–3.3864x+30.74	0.95
Flavonoids			Multigreen 3	y=2.2589x^2–25.055x+92.65	0.96
Isorhamnetin			Multired 4	y=–0.6911x^2–5.7111x+127.57	0.97
Multigreen 1	y=0.0882x^2–1.0272x+7.514	0.40	Chlorophyll content		
Multigreen 3	y=–0.1654x^2+0.6578x+8.794	0.39	Multigreen 1	y=0.0002x^2+0.1653x+18.073	0.92
Multired 4	y=0.1004x^2–3.3722x+26.114	0.51	Multigreen 3	y=–0.0015x^2+0.418x+21.216	0.69

However, N supply strongly influenced the accumulation of chlorogenic acid in all three lettuce varieties. In Multired 4, the chlorogenic acid accumulation decreased after 120 kg N ha^{-1} (Fig. 3-B). On the other hand, in the other two green lettuce varieties chlorogenic acid accumulation reached a plateau after the application of 120 kg N ha^{-1} (Fig. 3-B). Caffeic acid concentration was very low in the green lettuce varieties in this study (Fig. 3-D). Caftaric and caffeic acids in Multigreen 1 demonstrated a moderate strong response to N supply when compared to the other two lettuce varieties from 0 to 180 kg ha^{-1} (Fig. 3-C and D; Table 2). Overall, 60 kg N ha^{-1} showed the highest concentrations of caftaric acid in Multired 4 (Fig. 3-C and D) but caffeic acid remarkably declined after 60 kg N ha^{-1}. Furthermore, accumulation of both phenolic acids were favoured in Multigreen 3 between 60 to 120 kg N ha^{-1} (Fig. 3-C and D).

Isorhamnetin and quercetin were the predominant flavonoids in these three lettuce varieties and the red

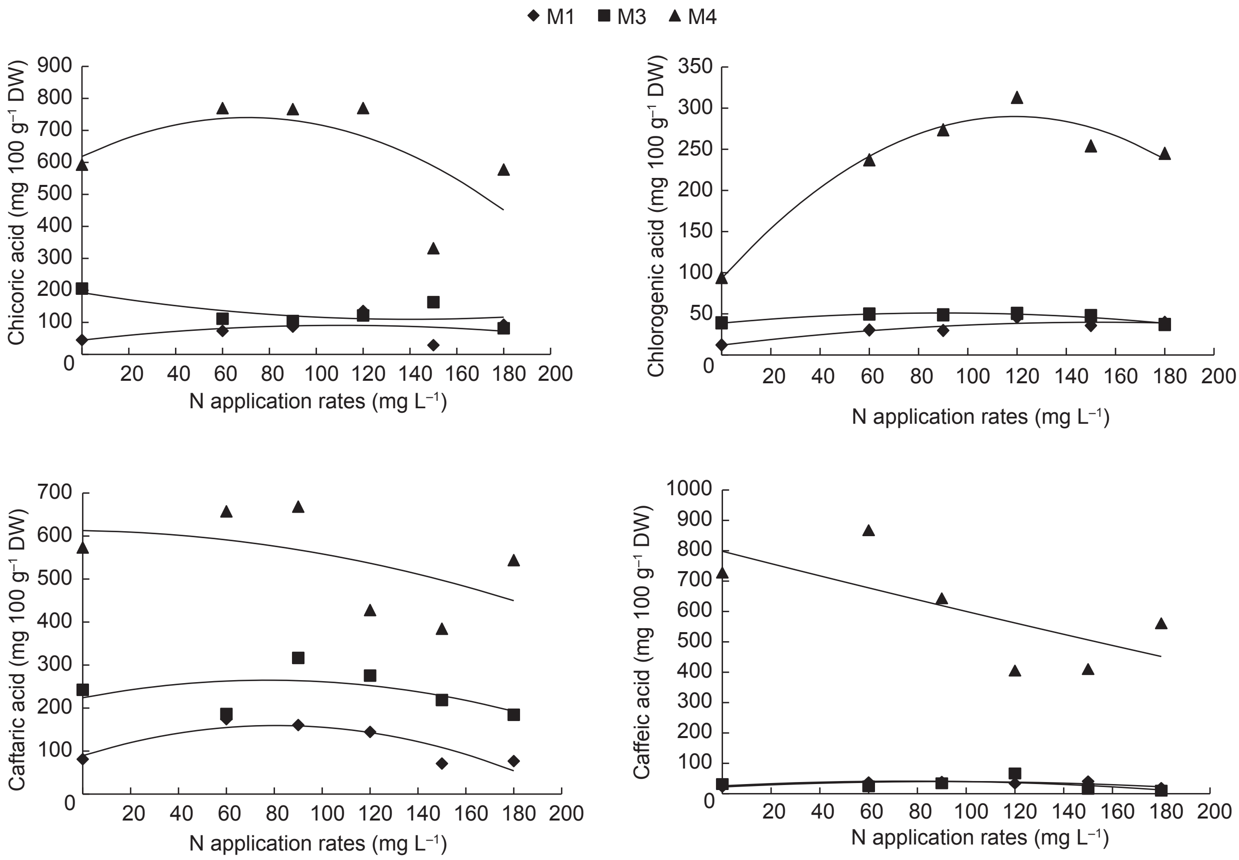

Fig. 3 Influence of N application rates on chicoric acid (A), chlorogenic acid (B), caftaric acid (C), and caffeic acid (D) contents in green and red lettuce varieties grown in closed hydroponic system gravel-film technique. M1, Multigreen 1; M3, Multigreen 3; M4, Multired 4. DW, dry weight.

lettuce Multired 4 demonstrated higher concentrations of flavonoids (Fig. 4-A–E). Biosynthesis of isorhamnetin and epicatechin in all three lettuce varieties poorly responded to the increasing N supply (Table 2). Although the biosynthesis of quercetin and myrcetin in Multired 4 and Multigreen 3 responded poorly with respect to the increasing N supply, a moderate response was noted in Multigreen 1 (Table 2). Nitrogen application after 60 kg ha^{-1} effected higher accumulation of flavonoids in red lettuce var. Multired 4 (Fig. 4-A–D). Biosynthesis of the major flavonoids, isorhamnetin and quercetin, were generally favoured between 60 to 90 kg N ha^{-1} in both the green varieties (Fig. 4-A and B). Furthermore, N application rates strongly influenced the concentration of anthocyanin in red leafy lettuce Multired 4 (Table 2). Anthocyanin concentration was higher at 90 and 120 mg L^{-1} N application and subsequently decreased with increasing N supply (Fig. 4-E).

Interestingly, a similar pattern was found with respect to antioxidant activity measured by $ABTS^+$ and FRAP regarding the higher rates of N application. Red lettuce Multired 4 showed the highest $ABTS^+$ and FRAP activities (Fig. 5-A and B). The higher N application rates at 150 and 180 mg L^{-1} reduced the antioxidant activity in all three lettuce varieties (Fig. 5-A and B). The N application rates revealed a strong influence on the antioxidant activity in all three lettuce varieties (Table 2) but the antioxidant activity was higher at the lower rates of N application (60 and 90 mg L^{-1}) (Fig. 5-A and B).

3.4. Mineral components

Leaf Ca and K did not show any response to different N application rates in lettuce (data not shown). In the green cultivars, the higher P levels were reached at 120 and 150 mg N L^{-1} and thereafter, a decline in P levels were noted at 180 mg N L^{-1} N (Fig. 6-A). But in red leafy lettuce Multired 4, the accumulation of P levels in the leaves did not differ with increasing rates of N supply of 120, 150 and 180 mg L^{-1} (Fig. 6-A). Although increased N supply favored the increase of Zn levels in all three lettuce varieties, a weak response was noted in this study (data not shown). However, all three lettuce varieties demonstrated a higher

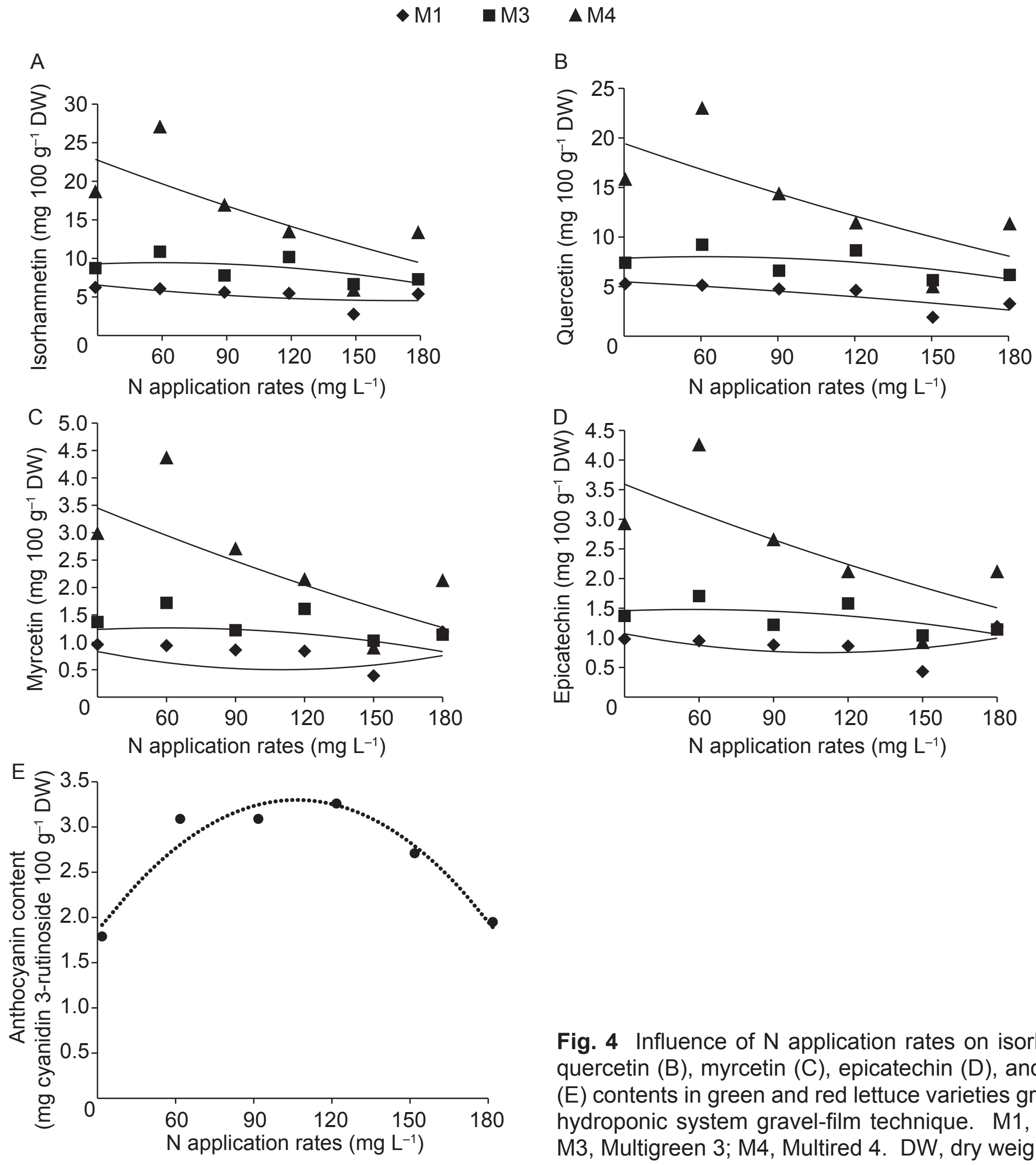

Fig. 4 Influence of N application rates on isorhamnetin (A), quercetin (B), myrcetin (C), epicatechin (D), and anthocyanin (E) contents in green and red lettuce varieties grown in closed hydroponic system gravel-film technique. M1, Multigreen 1; M3, Multigreen 3; M4, Multired 4. DW, dry weight.

response to accumulation of P with respect to N application rates (Table 3). Manganese content was higher in all three lettuce varieties at 90 and 120 mg N L^{-1} application rates and it gradually declined after 120 mg N L^{-1} (Fig. 6-B). Among the three lettuce varieties, Multigreen 3 demonstrated higher response to N supply (Fig. 6-B; Table 3).

Accumulation of Fe levels in Multired 4 was moderately influenced by N supply than in the green varieties (Table 3). However, in Multired 4 the highest Fe content was obtained at 90 to 120 mg N L^{-1} and thereafter, a declining trend was noted with increasing N supply (Fig. 6-D). Green lettuce Multigreen 1 revealed slightly higher Fe content than Multigreen 3. However, both green varieties had similar trends by showing an increase in Fe content with N supply from 60 to 120 mg L^{-1} and thereafter, reaching a plateau at 150 and 180 mg L^{-1} (Fig. 6-D). With respect to the mineral B accumulation in the leaves, N supply showed a minimum influence in the green var. Multired 3 compared to the other two varieties. Therefore, B content in varieties Multigreen 1 and Multired 4 can be improved by manipulating N supply (Fig. 6-D).

3.5. Nitrate content in the leaves

Generally the nitrate concentration increased with increasing N supply (60 to 180 mg L^{-1}) in all three varieties (Fig. 7; Table 3). The green var. Multigreen 3 tended to accumulate higher nitrate residue than the other two lettuce varieties (Fig. 7). However, the red lettuce var. Multired 4 demonstrated lower accumulation of nitrate with increasing N supply (Fig. 7). It is evident from this study that the nitrate levels were kept at 2.00 mg 100 g^{-1} DW at 90 mg N L^{-1} that

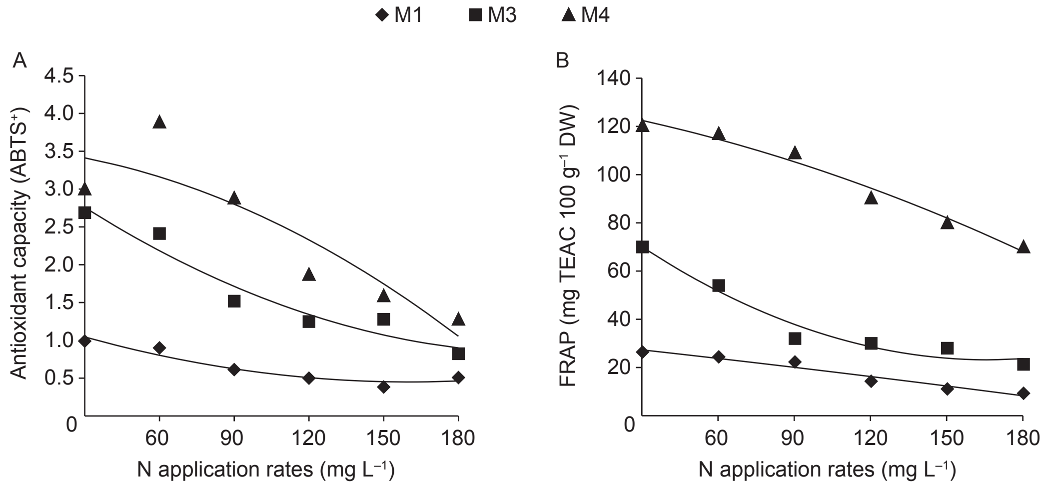

Fig. 5 Influence of N application rates on antioxidant capacity (ABTS+, A), and antioxidant power (FRAP, B) in green and red lettuce varieties grown in closed hydroponic system gravel-film technique. M1, Multigreen 1; M3, Multigreen 3; M4, Multired 4.

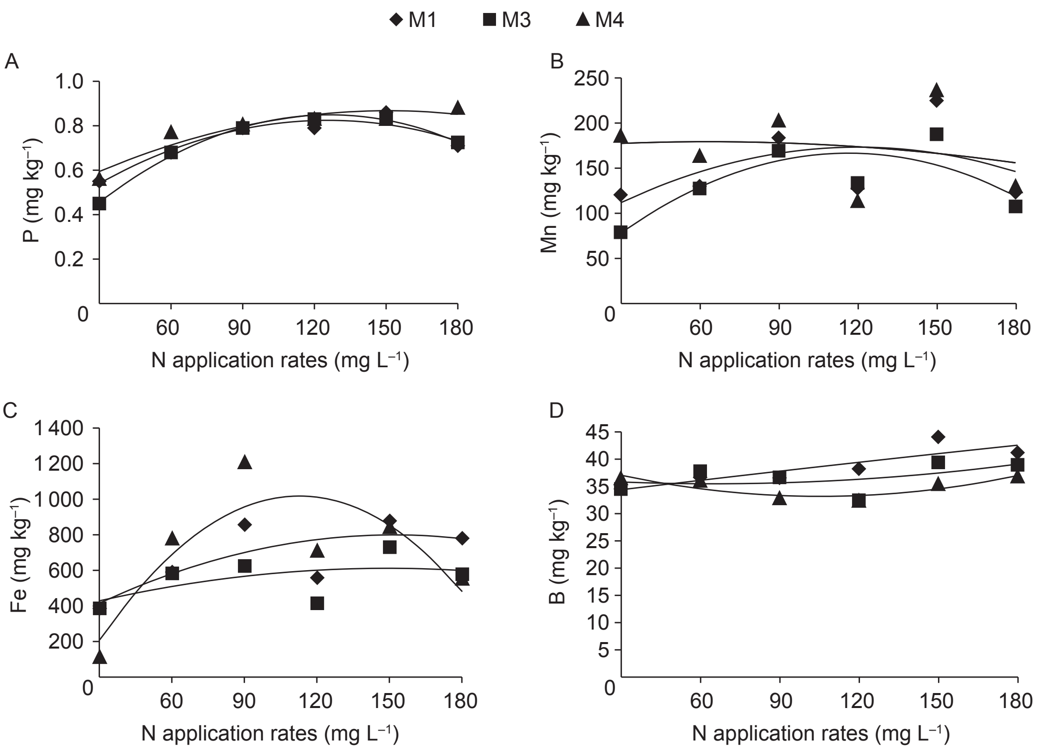

Fig. 6 Influence of N application rates on minerals concentration: P (A), Mn (B), Fe (C), and B (D) in red and green lettuce varieties grown in closed hydroponic system gravel-film technique. M1, Multigreen 1; M3, Multigreen 3; M4, Multired 4.

yielded the highest fresh leaf weight in Multired 4 (Fig. 7). In Multigreen 1 and Multigreen 4, the nitrate levels were 5.00 and 5.70 mg 100 g^{-1} DW at harvest respectively with 120 mg N L^{-1} that also showed the highest fresh leaf weight (Fig. 7). The accumulation of N residue in the green lettuce varieties can be minimized without compromising the yields with 90 N mg L^{-1} (Figs. 1-A and 7).

4. Discussion

It is evident from this study that increased accumulation of bioactive compounds, antioxidant properties and mineral composition can be achieved without compromising the yield by selecting less N sensitive varieties that can use N effectively. N application generally improved leaf area

Table 3 Regression equations for the minerals and nitrate content in leafy green and red lettuce varieties grown in closed hydroponic system gravel-film technique and fertilized with N concentrations of 0–180 mg L⁻¹

Minerals	Regression equation	R^2
P		
Multigreen 1	$y=-0.0379x^2+0.3198x+0.1755$	0.99
Multigreen 3	$y=-0.0279x^2+0.2333x+0.336$	0.92
Multired 4	$y=-0.0168x^2+0.1691x+0.442$	0.89
Mn		
Multigreen 1	$y=-6.8071x^2+54.573x+63.92$	0.28
Multigreen 3	$y=-10.602x^2+82.43x+6.39$	0.68
Multired 4	$y=-1.5375x^2+6.4682x+172.53$	0.04
Fe		
Multigreen 1	$y=-23.164x^2+234.65x+205.16$	0.59
Multigreen 3	$y=-11.571x^2+115.05x+325.74$	0.30
Multired 4	$y=-106.51x^2+799.74x-483.44$	0.72
B		
Multigreen 1	$y=-0.022x^2+1.7895x+32.615$	0.76
Multigreen 3	$y=0.2441x^2-1.0542x+36.619$	0.27
Multired 4	$y=0.6139x^2-4.3138x+40.752$	0.76
Nitrate content (mg 100 g⁻¹ DW)		
Multigreen 1	$y=-0.1308x^2+2.2291x-1.6955$	0.90
Multigreen 3	$y=-0.1448x^2+2.0448x-0.3498$	0.84
Multired 4	$y=0.249x^2-0.7984x+2.0334$	0.89

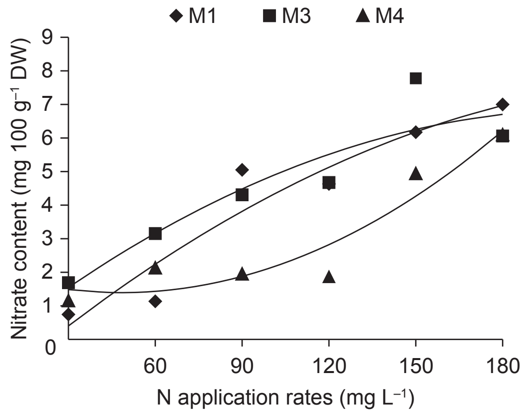

Fig. 7 Influence of N application rates on nitrate content in the leaves in red and green lettuce varieties grown in closed hydroponic system gravel-film technique. M1, Multigreen 1; M3, Multigreen 3; M4, Multired 4.

and attributed increased chlorophyll content due to the effective use of photosynthetically active radiation (Tei *et al*. 2002). However, the morphological response of the green lettuce varieties to different N supply rates differed from the reports of Mahlangu *et al*. (2016) where, Lollo Bionda Lobi leafy lettuce grown in non-circulating hydroponic system (0 to 180 mg N L⁻¹) failed to show any increase in fresh weight although the form of N source was NH_4NO_3. In this study, fresh weight in the green varieties showed strong correlations with increasing N supply (Table 1). However, discrepancies in results could be due to the type of growing system used or it can also be due to the genotype.

In red lettuce varieties, photosynthetically produced carbon molecules are used more for the production of phenolic compounds than for the use of plant growth and fresh weight (Becker *et al*. 2015). This relates to the C/N shift theory that shows a shift to carbon based metabolites under N deficiency for the biosynthesis of phenolic acids and flavonoids (Becker *et al*. 2015). The reports of Becker *et al*. (2015) further confirmed that the activity of phenylalanine ammonia-lyase that is responsible for the biosynthesis of polyphenol propanoids are higher at lower N concentrations, which further explains the observed increased phenolic acids (caffeic acid, caftaric acid) and flavonoids (isorhamnetin, quercetin, myricetin and epicatechin) and lower fresh leaf weight in var. Multired 4 in this study. Slight divergence in the trends were observed with the biosynthesis of phenolic acids in green varieties (Multigreen 1 and Multigreen 3) with respect to N application rates in this study and the reports of Becker *et al*. (2015). The lettuce genotypic differences could have attributed to these observed differences. Increasing N application rates were shown to reduce phenolic acids and flavonoids in butterhead lettuce *cv*. Egery (Qadir *et al*. 2017). However in this study the response to increasing N supply with regards to the accumulation of different phenolic acids and flavonoids composition differed (Figs. 3-A–D and 4-A–E).

N supply affects leaf chlorophyll content (Mahlangu *et al*. 2016) and in this study N application rates between 90 to 120 mg L⁻¹ were sufficient to maintain the chlorophyll content to carry out photosynthesis effectively in both green cultivars (Fig. 2-B). This observation coincides with the findings of Mahlangu *et al*. (2016) on the green lettuce cultivar Lollo Bionda Lobi grown in a non-circulating hydroponics system where 100 to 120 mg N L⁻¹ was reported to be sufficient to maintain photosynthesis. However in cultivar Lollo Bionda Lobi, the relationship model between the applied N rates and the chlorophyll content fitted linearly (Mahlangu *et al*. 2016) whereas in Mulitigreen 1 and Multigreen 3, they showed a polynomial model (Fig. 2-B). The observed discrepancies in chlorophyll content could be due to the lettuce genotypic differences.

Increased rates of N applications have been reported to reduce the ascorbic acid content in different fruit and vegetable crops (Freyman *et al*. 1991; Lee and Kader 2000). On the contrary, Chiesa *et al*. (2009) reported that high N (150 kg ha⁻¹) increased ascorbic acid content in lettuce. Variation in accumulation of ascorbic acid content with respect to different rates of N applications varied with the findings of Mahlangu *et al*. (2016) where the ascorbic acid concentration was higher at 100 to 120 mg N L⁻¹. In this study, the highest ascorbic acid content was obtained at 60 mg L^{-1} in both green and red varieties (Fig. 2-A) and as shown by Mahlangu *et al*. (2016) it declined with increasing rates of N application. The observed divergences with regards to the N application rates and ascorbic acid content in lettuce can be explained due to the type of N source and application methods. Type of fertilizers such as ammonium nitrate (Sady *et al*. 2010) or urea (Leja *et al*. 2007; Sady *et al*. 2010) was shown to increase the ascorbic acid content compared with ammonium sulphate (Sady *et al*. 2010). Abd El-Rehem and Abd El-Baky (2009) demonstrated that the use of ammonium nitrate reduced the ascorbic acid content with higher application rates. IM and FNB (2000) recommended daily intake (RDI) for an adult male and female is 90 and 75 mg of ascorbic acid. It is evident in this study that 100 g of Multired 4 provides 17 mg of ascorbic acid at 60 mg N L^{-1} and similarly 100 g of Multigreen 1 and Multgreen 3 contained 14 and 15.5 mg of ascorbic acid, respectively.

Phenolic acids, flavonoids and ascorbic acid are known as antioxidants and play a major role in human nutrition. Stefanelli *et al*. (2011) reported that the changes in total phenol content and the antioxidant power (FRAP) revealed similar trend with increased rates of N applications and that the highest FRAP activity and total phenolic content was attained at 400 mg N L^{-1}. Heimler *et al*. (2007) established a positive correlation between total phenolic content and antioxidant capacity in 5 out of 10 lettuce cultivars. The trend in antioxidant activity in this study differed from the findings of Mahlangu *et al*. (2016), which showed higher activities at 120 mg L^{-1}. In this study in all three cultivars the antioxidant power (FRAP) and capacity ($ABTS^+$) were higher at lower N concentrations (60 mg L^{-1}) and decreased with higher rates of N application (120 mg L^{-1}) (Fig. 5-A and B). It is noteworthy that this observation correlates well with the trends shown by ascorbic acid and flavonoids (Figs. 2-A and 3-A–D). Differences in nitrate accumulation between the green lettuce and red lettuce varieties were noted in this study and a similar observation was reported by Becker *et al*. (2015). There is discrepancy between the findings of this study and the reports of Becker *et al*. (2015) with regard to increasing nitrate content in the leaves with increasing rate of N application. Becker *et al*. (2015) stated that the nitrate residue did not increase steadily with increasing N concentration in the nutrient solution. Permitted nitrate content for greenhouse lettuce is set at 4 500 mg kg^{-1} of fresh weight when grown from 1 October to 31 March and at 3 000 mg kg^{-1} from 1 April to 30 September by the European Community (ESFA 2008; Becker *et al*. 2015). Although the nitrate levels in the leaves were calculated on a dry weight basis in this study, the contents were much lower than the levels set by the European Community. It can be recommended to use less N sensitive varieties to promote the production of phytochemicals and to reduce accumulation of nitrates in a controlled production system.

Reports of Stefanelli *et al*. (2011) stated that Ca increased while B, Mg, Mn, and Zn significantly decreased with increasing N application rates. In this study N application rates helped to improve the mineral composition (Fe, B and P) in the leaves and different accumulation trends were noted with respect to the different cultivars with the red cultivar responding well. Petropoulos *et al*. (2016) showed the influence of N application on Mn and Zn in lettuce leaves (Petropoulos *et al*. 2016). However, in this study, N application influenced the accumulation of Mn in Multigreen 3 (Fig. 6-B). An increase in minerals especially Fe, and Mn that are an important source of micro elements in lettuce will add value by enhancing the nutritional value in the food chain (consumer) (Lynch and Stoltzfus 2003).

5. Conclusion

This study confirms the use of manipulation of agro-fertilization practice to improve bioactive compounds and minerals without marginalizing yields. It is recommend to use the red lettuce var. Multired 4 with Multigreen 1 or Multigreen 3 in salad packs in order to meet the nutritional requirements of the consumers. Nitrogen concentration of 90 mg L^{-1} can be recommended for red lettuce (Multired 4) and green lettuce (Multigreen 1 and Multigreen 3) for gravel-film production to improve bioactive compounds and minerals while obtaining sufficient yields.

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