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Effect of iron supply and nitrogen form on growth, nutritional status and ferric reducing activity of spinach in nutrient solution culture

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Abstract

This study was carried out in order to give some information that could improve spinach nutritional status and productivity. In this paper, the effect of two N forms (N was added either as 100% nitrate or as 80% nitrate and 20% ammonium) and three Fe levels (0 μM Fe; 20 μM FeEDDHA; 3 μM FeEDDHA + 10 mM $NaHCO_3$) on the growth, chlorosis symptoms and shoot nutrient element accumulation was studied in spinach plants (var. Viroflay), grown in hydroponics; six treatments and three harvests (at about 20 days interval each, until plants reached their commercial size) were applied in total. The results indicated that under conditions of Fe sufficiency (20 μM Fe), mixed N nutrition induced higher production of dry matter as well as improved Fe, Mn and Zn plant nutritional status. In plants grown under Fe deprivation (0 μM Fe), shoot Fe concentration was not significantly affected by the N form until the end of the experiment despite mixed N nutrition induced higher dry matter production up to harvest 2; plants grown under Fe deprivation and with mixed N nutrition presented also higher shoot Mn and Zn concentration. Under conditions of high concentration of bicarbonates and low level of Fe (3 μM Fe + 10 mM $NaHCO_3$), the N form had not a significant influence on total dry matter production whereas shoot Fe and Mn accumulation in 100% NO_3-fed plants was found to be significantly reduced compared to mixed N nutrition; regardless of the N form, those plants presented the least dry matter production, highest intensity of leaf chlorosis as well as highest root ferric reducing activity compared to plants grown under Fe deprivation.

Keywords: Fe-deficiency; Lime-induced chlorosis; N form; Nutrient elements interactions; Spinach

1. Introduction

It is well known that nutrient availability to people is primarily determined by the output of foods produced by agricultural systems. Importantly, plant foods provide most of the nutrients that feed the developing world; agriculture must change in ways that closely link food production to human health and nutritional requirements.

Specifically, it is also well known that nutrient elements in the leaves of spinach are always important to human health (Welch, 2002); spinach as a dietetic nutrient has long been the object of many investigations.

Unexceptionably, nitrogen plays a pivotal role in the inorganic nutrition of plants and hence in determining growth. The form of N supply, to a great extent, controls the uptake ratio of cations and anions and thus, influences dry matter production and root rhizosphere and apoplastic pH (Mengel, 1995; Marschner, 1997). It has repeatedly been reported that micronutrients interactions with N occur frequently due to change in the nutrient solution pH with the addition and uptake of NH_4-N and NO_3-N; when more NO_3-N is applied, rhizosphere pH increases whereas when more NH_4-N is absorbed, rhizosphere pH decreases. Specifically, Fe nutrition of plants can be significantly affected by N form because of the aforementioned changes in the rhizosphere and apoplastic pH and the uptake ratio of cations and anions (Mengel et al., 1994).

Fe chlorosis as a matter of simple deficiency is seldom the case because Fe availability is often restricted by the limited solubility of Fe oxides in aerobic environments or by elevated concentration of nitrate and bicarbonate on calcareous soils (Mengel, 1995). Both Fe level and N source in plants growth medium are involved in multiple interactions with other elements; the effects of interactions are expressed in different ways, including uptake phenomena and biochemical reactions (positive and negative synergisms, competition, protection, etc.); some interactions are the result of Fe deficiency and

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others are the cause of Fe deficiency, directly or indirectly; for example, it has been observed that ammonium-N has induced Fe deficiency because of the increased uptake of P (Wallace et al., 1992). In crops whose commercial yields are the leaves, such as lettuce, spinach, endive, cabbage, etc., a great number of studies have been done on the influence of N fertilization (rate and form) on yield, nitrate accumulation and ion composition (Cantliffe, 1972a,b; Barker et al., 1974; Wang and Tadashi, 1997; Santamaria and Elia, 1997; Wang and Li, 2004; Simonne et al., 2001). Evaluating the effect of nitrogen form under different iron levels in the growth medium on nutrient element accumulation in leafy vegetables could provide some useful information for improving their nutritional status. In this work, we started studying the impact of N form under varied Fe levels in the growth medium on growth characteristics, mineral nutrition and physiological responses in spinach.

2. Materials and methods

2.1. Plant culture

In November 2002, after soaking in 1 mM $CaSO_4$ overnight, seeds of the smoothly leaved cultivar Viroflay of *Spinacia oleraceae* L. were germinated and grown in sand culture for 10 days, receiving half strength the Long Ashton nutrient solution for macronutrients and full for micronutrients; in that precultured period 10 μM FeEDDHA were added to the nutrient solution. When the mean initial fresh weight of plants was 175.0 mg, 144 plants were selected in total to start the experiment. The seedlings were transplanted to individual 4 L plastic pots (one seedling per pot), filled with medium grade silica sand and placed in a glasshouse without supplementary heating and lighting; the mean temperature from 22 to 30 November 2002 was 19.6 °C, from 1 to 31 December 2002 11.6 °C and from 1 to 22 January 2003 14.3 °C. The pots were arranged in a completely randomised block factorial design with eight replicates, three Fe levels, two N ratios, at three growth stages corresponding to three harvests. Six treatments (I–VI) were applied in total; the six relevant nutrient solutions consisted a combination of three Fe levels (0 μM Fe, 20 μM FeEDDHA, 3 μM FeEDDHA Fe + 10 mM $NaHCO_3$ + 0.5 g $CaCO_3$ L^{-1} nutrient solution) and two N ratios (N was added either as nitrate, 100% NO_3-N, or as nitrate and ammonium with a ratio 80% NO_3-N:20% NH_4-N, but the same total N concentration). Each plant was irrigated three times daily with 0.08 L of the modified Long Ashton nutrient solution. In the treatments (I, III, V) with 100% NO_3-N (14 mM NO_3-N), the nutrient solution consisted of: 5 mM $Ca(NO_3)_2$, 4 mM KNO_3, 1.3 mM $MgSO_4$, 2 mM KH_2PO_4, whereas in the treatments (II, IV, VI) with 80% NO_3-N:20% NH_4-N (11.2 mM NO_3-N:2.8 mM NH_4-N) the nutrient solution consisted of: 5 mM $Ca(NO_3)_2$, 1.2 mM KNO_3, 1.3 mM $MgSO_4$, 2 mM KH_2PO_4, 0.9 mM $(NH_4)_2SO_4$, 1 mM $NH_4H_2PO_4$, 2.8 mM KCl (Zornoza and Gonzalez, 1998). The concentration of micronutrients was: 100 μM NaCl, 30 μM H_3BO_3, 10 μM $MnSO_4$, 2 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.5 μM $(NH_4)_6Mo_7O_{24}$. Fe concentration in the nutrient solution of treatments I, II was: 0 μM; in treatments III, IV: 20 μM and in treatments V, VI: 3 μM Fe plus 10 mM $NaHCO_3$ and 0.5 g $CaCO_3$ L^{-1} of nutrient solution. The nutrient solution of the treatments V and VI refers an imitation effort of calcareous soils solutions. The nutrient solution pH of the treatments with 0 or 20 μM Fe-EDDHA was buffered with 1 M NaOH to 6.00 whereas that of treatments with 3 μM Fe + 10 mM $NaHCO_3$ + 0.5 g $CaCO_3$ L^{-1} to 7.50.

Plants were harvested on days: 18 (harvest 1), 42 (harvest 2) and 61 (harvest 3) from the beginning of the treatments, and separated to the shoot (upper plant) and root; the root system was washed carefully, three times with deionised water. The fresh weights of the upper plant and root were taken at each harvest. Then, the plant material was dried to constant weight in a forced draught air oven at 80 °C, weighed, wet-ashed (Kjeldahl method) and dry-ashed in a furnace at 550 °C. The concentration of N and P was determined by using the indophenol-blue and molybdenum-blue method in the wet digest, respectively; K was measured with flamephotometry and Ca, Mg, Fe, Mn, Zn, Cu using a Varian A220 atomic absorption spectrometer, in the dry digest.

2.2. Chlorosis

The mean value of the chlorosis score of every pair of leaves of each experimental plant was recorded at each harvest using a one to five rating scale (1 = normal green leaves; 5 = severe chlorosis with necrotic spots) (Wei et al., 1994).

2.3. Root ferric chelate reductase activity

At the second and third harvest, the ferric chelate reductase activity (FC-R) of the roots was determined with total root systems of intact plants according to the protocol described by Romera et al. (1991).

2.4. Statistics

Each value represents the average of eight replicates except that of ferric chelate reducing activity of the roots (four replicates). Significant differences in mean values between treatments were evaluated by the ANOVA, LSD test at $P < 0.05$.

3. Results

3.1. Chlorosis score

Chlorosis symptoms appeared first on the seventh day after the beginning of the treatments, in the young leaves of plants grown with high bicarbonates and N as 80% NO_3-N:20% NH_4-N whereas plants grown with high bicarbonates and N as 100% N-NO_3 showed the highest score at the end of the experiment. At harvest 3, which was coincident with the end of the experiment, there were some plants grown without Fe (0 mM Fe) with whatever N form, with no visual symptoms of Fe deficiency; as regards the main effect of Fe levels, the mean chlorosis score of plants grown without Fe was 1.20, that of plants grown with high

Table 1
The chlorosis score of spinach plants grown in treatments with different Fe levels and N ratios (mean ± S.E., $n = 8$)

Treatment	Chlorosis score		
	Harvest 1	Harvest 2	Harvest 3
Without Fe			
I. Nr[a] 100/0	1.16 ± 0.13 b	1.78 ± 0.52 b	1.22 ± 0.49 a
II. Nr[a] 80/20	1.03 ± 0.09 ab	1.54 ± 0.28 ab	1.18 ± 0.19 a
With 20 μM Fe			
III. Nr[a] 100/0	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a
IV. Nr[a] 80/20	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a
3 μM Fe + NaHCO$_3$			
V. Nr[a] 100/0	1.53 ± 0.28 c	2.96 ± 0.85 c	3.81 ± 0.72 c
VI. Nr[a] 80/20	2.25 ± 0.33 d	2.82 ± 0.32 c	2.89 ± 0.29 b

Chlorosis score: 1 = normal green; 5 = severe chlorosis with necrotic spots. The values followed by different letters within a column are significantly different at $P > 0.05$.

[a] Nr: NO$_3$-N/NH$_4$-N.

bicarbonates 3.35, whereas that of plants grown with Fe 1.00; regarding the main effect of N form, the NO$_3$-N fed plants showed significantly higher chlorosis score than plants grown with both N forms. At harvests 1 and 2, chlorosis scores of plants grown with 0 mM Fe and N as 100% NO$_3$-N were significantly higher than those of plants grown with 20 mM Fe, with whatever N form; there were no significant differences between the relevant treatments at harvest 3 (Table 1).

3.2. Plant growth

According to ANOVA results for whole plants dry weight, the Fpr of interaction 'Fe levels × N forms' at harvests 2 and 3, were found statistically significant ($P = 0.041$, 0.046, respectively); at harvest 1 the relevant Fpr was found 0.057.

At harvest 2, plants grown with 0 μM Fe and N as 100% NO$_3$-N showed significantly lower total dry weight compared to plants grown with 0 μM Fe and N as 80% NO$_3$-N:20% NH$_4$-N, as well as, lower total dry weight compared to plants grown with Fe, with whatever N form. The mean total dry weights of plants grown whether without Fe (0 μM Fe) and N as 80% NO$_3$-N:20% NH$_4$-N or with 20 μM Fe, with whatever N form, were not significantly different. At harvest 1, the variation of the total dry weight of plants among the six treatments, was similar to that of harvest 2. At harvest 3, plants grown with 20 μM Fe and N as 100% N-NO$_3$, showed significantly lower total dry weight compared to plants grown either with 20 μM Fe and N as 80% NO$_3$-N:20% NH$_4$-N or without Fe (0 μM Fe), with whatever N form (Table 2). There was not a significant differentiation of the mean total dry weight of plants grown whether with 20 μM Fe and N as 80% NO$_3$-N:20% NH$_4$-N or without Fe (0 μM Fe), with whatever N form (Table 2). Plants grown with high concentration of bicarbonates with whatever N form, produced the significantly least dry matter compared to plants grown with 20 μM Fe or without Fe (0 μM Fe) at all the three harvests; those plants also showed the highest chlorosis score.

According to ANOVA results for the mean relative growth rate (RGR) of plants, the Fpr of interaction 'harvests × Fe levels × N forms' was found statistically significant ($P = 0.0002$); in particular, the RGR of plants grown with Fe deprivation (0 μM Fe) and N as 100% N-NO$_3$ at harvest 2, was found to be 16% lower (significantly decreased) compared to the relevant RGR at harvest 1 but through harvest 3 there was no further decrease. The RGR of plants grown with 20 μM Fe and N as 100% N-NO$_3$ at harvest 2, was significantly lower (13%) compared to the relevant RGR at harvest 1, whereas the relevant RGR through harvest 3 was further significantly lower (15%) compared to that at harvest 2 (Fig. 1).

At both harvests (2 and 3), the Root/Shoot ratio (on dry weight basis) of plants under stress caused by high bicarbonates and N as 100% N-NO$_3$ was significantly restricted compared to plants grown in all other treatments (Table 2). At harvest 3, regarding the N form (main effect), the root/shoot ratio of plants grown with N as 100% N-NO$_3$ was significantly lower than that of plants with N with both forms.

3.3. Root ferric chelate reducing activity

At harvests 2 and 3, plants grown with high bicarbonates in the nutrient solution reduced significantly higher quantities of Fe^{3+} (per gram of root fresh weight) compared to plants grown

Table 2
The dry weight (mg plant^{-1}) of whole spinach plants grown in treatments with different Fe levels and N ratios and their root/shoot DW ratio (mean ± S.E., $n = 8$)

Treatment	Whole plant DW (mg)			Root/shoot DW ratio		
	Harvest 1	Harvest 2	Harvest 3	Harvest 1	Harvest 2	Harvest 3
Without Fe						
I. Nr[a] 100/0	126 ± 32 ab	785 ± 107 b	3517 ± 735 a	0.194 ± 0.09 b	0.173 ± 0.03 a	0.230 ± 0.06 ab
II. Nr[a] 80/20	152 ± 25 a	993 ± 283 a	3374 ± 858 a	0.170 ± 0.05 b	0.172 ± 0.04 a	0.181 ± 0.03 b
With 20 μM Fe						
III. Nr[a] 100/0	154 ± 33 a	1136 ± 214 a	2685 ± 541 b	0.260 ± 0.10 ab	0.213 ± 0.03 a	0.248 ± 0.03 a
IV. Nr[a] 80/20	154 ± 36 a	1064 ± 194 a	3479 ± 680 a	0.177 ± 0.05 b	0.195 ± 0.03 a	0.208 ± 0.05 ab
3 μM Fe + NaHCO$_3$						
V. Nr[a] 100/0	97 ± 19 bc	350 ± 86 c	674 ± 164 c	0.217 ± 0.08 ab	0.121 ± 0.02 b	0.117 ± 0.06 c
VI. Nr[a] 80/20	71 ± 22 c	274 ± 108 c	1065 ± 349 c	0.308 ± 0.17 a	0.178 ± 0.05 a	0.141 ± 0.03 b

The values followed by different letters within a column are significantly different at $P > 0.05$.

[a] Nr = NO$_3$-N/NH$_4$-N.

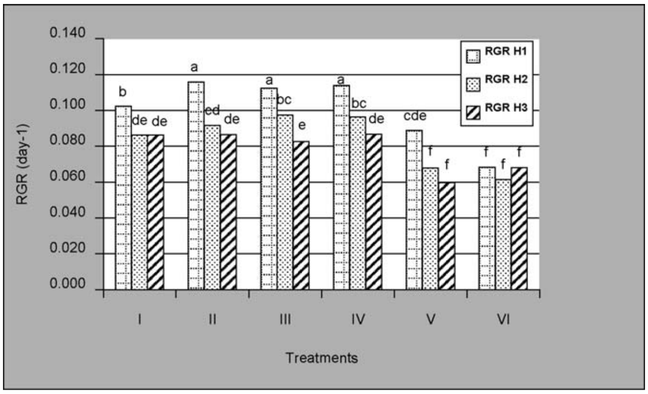

Fig. 1. The mean RGR (day^{-1}) of whole spinach plants (FW) grown in six treatments with three Fe levels (treatments I, II: 0 μM Fe; treatments III, IV: 20 μM Fe; treatments V, VI: 3 μM Fe + 10 mM $NaHCO_3$) and two N ratios (treatments I, III, V: 100% NO_3-N; treatments II, IV, VI: 80% NO_3-N:20% NH_4-N) in the nutrient solution, at harvest 1 (H1), harvest 2 (H2) and harvest 3 (H3). The values followed by different letters are significantly different at $P > 0.05$ (means, $n = 8$).

with or without Fe, regardless of the N form (significant main effect of 'Fe level') (Table 3). There was also found to be a significant positive correlation between the chlorosis score and the reduced Fe^{2+} quantities by plants grown either at harvest 2 ($r = 0.59$, $P = 0.004$) or at harvest 3 ($r = 0.62$, $P = 0.001$).

3.4. Elemental concentration

The results of the concentrations of the nutrient elements determined are summarized in Tables 4–7. Data concerning the mentioned main effects of 'Fe levels' and 'N forms' on nutrient elements concentration are not shown. Because of the little dry matter produced by each plant at harvest 1, every sample analyzed consisted of the unified dry matter of four plants; in that way there were only two replicates at harvest 1.

3.4.1. Nitrogen–phosphorus–potassium–calcium–magnesium

Regarding the shoot N concentration, the interaction between 'Fe levels and N forms' was not found significant in anyone harvest. At harvest 2, the main effect of Fe levels was that plants

Table 3
Root ferric chelate reducing activity of intact spinach plants grown in treatments with different Fe levels and N ratios at harvests 2 and 3 (mean ± S.E., $n = 4$)

Treatment	Root ferric chelate reductase activity (nmol Fe^{2+} h^{-1})			
	Harvest 2		Harvest 3	
	$plant^{-1}$	g^{-1} root FW	$plant^{-1}$	g^{-1} root FW
Without Fe				
I. Nr[a] 100/0	73.4 ± 14.6 b	78.8 ± 32.5 a	102.6 ± 27.5 a	21.5 ± 10.5 a
II. Nr[a] 80/20	86.5 ± 16.0 ab	76.5 ± 61.0 a	92.4 ± 16.9 ab	17.0 ± 4.6 a
With 20 μM Fe				
III. Nr[a] 100/0	84.3 ± 14.3 ab	40.7 ± 13.9 a	85.5 ± 9.8 ab	19.6 ± 10.6 a
IV. Nr[a] 80/20	97.8 ± 17.6 a	68.3 ± 42.0 a	111.3 ± 22.0 a	15.2 ± 4.9 a
3 μM Fe + $NaHCO_3$				
V. Nr[a] 100/0	89.2 ± 7.8 ab	330.5 ± 142.6 b	63.8 ± 12.3 b	127.1 ± 91.5 b
VI. Nr[a] 80/20	83.6 ± 22.0 ab	303.6 ± 295.9 b	68.9 ± 22.3 b	61.8 ± 41.8 a

The values followed by different letters within a column are significantly different at $P > 0.05$.

[a] Nr = NO_3-N/NH_4-N.

Table 4
Shoot Ca, Mg concentration (g kg^{-1} DW) of spinach plants grown in treatments with different Fe levels and N ratios (mean ± S.E., $n = 2$ (harvest 1), $n = 8$ (harvests 2 and 3))

Treatment	Shoot Ca concentration (g kg^{-1} DW)			Shoot Mg concentration (g kg^{-1} DW)		
	Harvest 1	Harvest 2	Harvest 3	Harvest 1	Harvest 2	Harvest 3
Without Fe						
I. Nr[a] 100/0	11.2 ± 0.2 a	13.3 ± 1.9 a	13.3 ± 2.0 b	6.6 ± 0.5 b	9.6 ± 1.3 b	9.0 ± 1.2 cd
II. Nr[a] 80/20	10.1 ± 0.5 ab	9.5 ± 1.9 b	11.3 ± 1.6 bc	6.8 ± 1.1 b	11.8 ± 5.2 b	8.0 ± 1.1 d
With 20 μM Fe						
III. Nr[a] 100/0	9.9 ± 0.4 b	7.4 ± 2.0 bc	8.8 ± 1.2 de	7.2 ± 0.1 b	10.3 ± 2.2 b	9.7 ± 0.5 c
IV. Nr[a] 80/20	8.2 ± 0.3 c	6.6 ± 0.8 c	7.6 ± 1.3 e	6.2 ± 0.2 b	12.2 ± 1.3 b	8.8 ± 0.6 cd
3 μM Fe + $NaHCO_3$						
V. Nr[a] 100/0	10.3 ± 0.7 ab	13.7 ± 4.4 a	17.5 ± 3.8 a	9.2 ± 1.0 a	20.2 ± 5.6 a	18.9 ± 3.3 a
VI. Nr[a] 80/20	10.2 ± 0.4 ab	12.1 ± 2.6 a	10.3 ± 1.3 cd	6.8 ± 0.1 b	17.4 ± 2.3 a	13.1 ± 1.0 b

The values followed by different letters within a column are significantly different at $P > 0.05$.

[a] Nr = NO_3-N/NH_4-N.

Table 5
Shoot Fe, Mn concentration (mg kg^{-1} DW) of spinach plants grown in treatments with different Fe levels and N ratios (mean ± S.E., $n = 2$ (harvest 1), $n = 8$ (harvests 2 and 3))

Treatment	Shoot Fe concentration (mg kg^{-1} DW)			Shoot Mn concentration (mg kg^{-1} DW)		
	Harvest 1	Harvest 2	Harvest 3	Harvest 1	Harvest 2	Harvest 3
Without Fe						
I. Nr[a] 100/0	128.0 ± 17.7 a	81.1 ± 15.3 c	66.9 ± 8.0 b	111.9 ± 17.1 a	122.8 ± 37.8 bc	108.0 ± 33.4 b
II. Nr[a] 80/20	127.6 ± 5.0 a	82.8 ± 5.5 c	66.6 ± 3.8 b	126.3 ± 1.6 a	169.5 ± 44.4 a	225.6 ± 33.9 a
With 20 μM Fe						
III. Nr[a] 100/0	127.3 ± 13.2 a	89.7 ± 25.5 c	70.6 ± 7.2 b	89.3 ± 2.0 b	94.6 ± 38.9 c	79.6 ± 14.1 c
IV. Nr[a] 80/20	141.0 ± 27.8 a	111.8 ± 10.9 b	80.5 ± 5.4 a	107.4 ± 11.0 ab	144.2 ± 15.5 ab	200.2 ± 30.1 a
3 μM Fe $NaHCO_3$						
V. Nr[a] 100/0	127.6 ± 9.0 a	89.6 ± 10.3 c	66.1 ± 9.6 b	16.9 ± 0.2 c	9.1 ± 3.0 d	6.0 ± 1.2 d
VI. Nr[a] 80/20	162.4 ± 24.4 a	141.6 ± 23.3 a	91.3 ± 4.6 a	18.2 ± 2.5 c	13.7 ± 2.7 d	13.7 ± 6.7 d

The values followed by different letters within a column are significantly different at $P > 0.05$.
[a] Nr = NO_3-N/NH_4-N.

Table 6
Shoot Zn, Cu concentration (mg kg^{-1} DW) of spinach plants grown in treatments with different Fe levels and N ratios (mean ± S.E., $n = 2$ (harvest 1), $n = 8$ (harvests 2 and 3))

Treatment	Shoot Zn concentration (mg kg^{-1} DW)			Shoot Cu concentration (mg kg^{-1} DW)		
	Harvest 1	Harvest 2	Harvest 3	Harvest 1	Harvest 2	Harvest 3
Without Fe						
I. Nr[a] 100/0	189.0 ± 1.8 b	136.2 ± 26.6 b	143.2 ± 25.2 c	10.5 ± 1.9 b	9.2 ± 4.4 b	10.2 ± 3.7 b
II. Nr[a] 80/20	253.5 ± 26.4 a	182.7 ± 57.6 a	272.6 ± 64.0 a	13.4 ± 0.5 a	11.3 ± 2.3 b	15.0 ± 1.6 a
With 20 μM Fe						
III. Nr[a] 100/0	135.4 ± 4.3 c	98.9 ± 31.5 c	95.1 ± 14.2 d	11.2 ± 0.7 a	9.8 ± 2.0 b	13.8 ± 0.9 a
IV. Nr[a] 80/20	165.5 ± 5.4 bc	143.6 ± 15.4 b	218.3 ± 48.4 b	10.9 ± 0.5 a	11.1 ± 0.5 b	14.2 ± 1.1 a
3 μM Fe + $NaHCO_3$						
V. Nr[a] 100/0	94.8 ± 11.8 d	66.9 ± 12.1 d	73.0 ± 9.9 d	11.5 ± 1.3 a	11.8 ± 1.3 a	15.6 ± 1.9 a
VI. Nr[a] 80/20	106.8 ± 19.9 cd	81.6 ± 25.2 cd	82.4 ± 9.0 d	12.8 ± 0.9 a	14.7 ± 4.3 a	15.1 ± 1.2 a

The values followed by different letters within a column are significantly different at $P > 0.05$.
[a] Nr = NO_3-N/NH_4-N.

grown with 0 μM Fe presented significant lower shoot N concentration than plants grown either with 20 μM Fe or with high bicarbonates. Besides, the root N concentration of plants grown with both forms of N, at harvest 3, was significantly higher than that of plants with 100% NO_3-N (main effect). The main effects of 'Fe levels' and 'N forms' on shoot P concentration were found significant; plants supplied with both forms of N presented higher shoot P concentration than plants with 100% NO_3-N whereas plants grown with high bicarbonates presented the smallest shoot P concentration compared to plants grown with 20 μM Fe or without Fe. The root P concentration of plants grown with high bicarbonates was also significantly lower than

Table 7
Root Fe, Mn, Zn, Cu concentration (mg kg^{-1} DW) of spinach plants grown in treatments with different Fe levels and N ratios (mean ± S.E., $n = 8$), at harvest 3

Treatment	Root Fe, Mn, Zn, Cu concentration (mg kg^{-1} DW)			
	Fe	Mn	Zn	Cu
Without Fe				
I. Nr[a] 100/0	552 ± 183 d	155 ± 40 bc	184 ± 53 c	13.4 ± 2.2 d
II. Nr[a] 80/20	928 ± 443 cd	187 ± 49 b	475 ± 137 a	18.2 ± 4.6 c
With 20 μM Fe				
III. Nr[a] 100/0	871 ± 376 cd	208 ± 52 b	191 ± 40 c	19.8 ± 3.3 bc
IV. Nr[a] 80/20	1215 ± 186 bc	374 ± 79 a	338 ± 88 b	19.0 ± 3.7 c
3 μM Fe + $NaHCO_3$				
V. Nr[a] 100/0	2181 ± 932 a	75 ± 30 cd	94 ± 13 c	25.7 ± 5.0 b
VI. Nr[a] 80/20	1794 ± 324 ab	60 ± 5 d	99 ± 9 c	41.1 ± 1.4 a

The values followed by different letters within a column are significantly different at $P > 0.05$.
[a] Nr = NO_3-N/NH_4-N.

that of plants grown with 20 μM Fe or without Fe, regardless of the N form. Shoot K, Ca and Mg concentration of plants grown with high bicarbonates was significantly higher than that of plants grown with 20 μM Fe or without Fe, regardless of the N form; shoot K, Ca and Mg concentration in plants supplied with N as 100% NO_3-N was significantly higher than in plants supplied with both forms of N, regardless of the Fe level. At harvest 3, plants grown with high concentration of bicarbonates and N as 100% N-NO_3 presented the highest shoot K, Ca and Mg concentrations compared to all other treatments (significant interaction of 'Fe level × N form') (Table 4).

3.4.2. *Iron, manganese, zinc, copper*

According to ANOVA results, shoot Fe concentration of spinach plants grown without Fe (0 μM Fe) was found to be significantly lower than in plants with 20 μM Fe, regardless of the N form, whereas, regardless of the Fe level, shoot Fe concentration in plants grown with N with both forms was significantly higher than that of plants with 100% NO_3-N. Given that the Fpr of interaction 'Fe levels × N forms' for shoot Fe concentration at harvests 2 and 3 were also found to be statistically significant ($P < 0.05$), shoot Fe concentration of plants grown with 20 μM Fe and with both forms of N was significantly higher compared to that of plants grown either with 20 μM Fe and N as 100% NO_3-N or without Fe and with whatever N form (by 25% higher at harvest 2 and 17% at harvest 3). In plants grown with high bicarbonates, whose dry weight was the significantly least, Fe concentration in both shoots and roots, was higher than the relevant ones in plants with 20 μM Fe or without Fe, with whatever N form (Table 5). In every treatment, root Fe concentration was much higher than that of shoots; it should be mentioned that the method used does not differentiate between concentration into the symplasm and concentration into the apoplasm of the roots (Table 7).

The shoot Mn and Zn concentration of spinach plants grown with 0 μM Fe was significantly higher than those of plants grown with 20 μM Fe, and much higher than those of plants grown with high bicarbonates, regardless of the N form. Shoot Mn, Zn and Cu concentration in plants grown with both forms of N was significantly higher than that of plants with 100% NO_3-N, regardless of the Fe level. Because of the significant interaction between 'Fe levels × N forms' on shoot Mn and Zn concentration at harvest 3, plants grown with 0 μM Fe and both forms of N presented the significantly highest shoot Zn level (Table 6); plants grown with 20 μM Fe and 100% NO_3-N showed the lowest shoot Mn concentration compared to plants either with 20 μM Fe and mixed N nutrition or without Fe with whatever N form. Regarding the shoot Mn concentration of plants grown in the bicarbonates treatment with 100% NO_3-N was found to be below the Mn deficiency critical level for many plants (Table 5). Besides, plants grown with 0 μM Fe and with both forms of N presented the highest root Zn concentration whereas plants grown with 20 μM Fe and with both forms of N presented the highest root Mn concentration (Table 7).

Given that the interactions between 'Fe levels × N forms' for the mean shoot and root Cu concentration at harvest 3 were found significant, the shoot and root Cu concentration of plants grown without Fe and 100% NO_3-N were the lowest ones; those of roots of plants grown with high bicarbonates and with both forms of N, the highest (Table 6).

3.5. *Correlations*

There were found to be significant negative correlations between the chlorosis score and the total dry weight of plants at all the three harvests; the relevant correlation coefficients were: $r = -0.64^{***}$, $r = -0.74^{***}$, $r = -0.84^{***}$, respectively. Significant correlations between shoot dry weight and shoot P ($r = 0.48^{***}$), Ca ($r = -0.50^{***}$), Mg ($r = -0.37^{*}$) and Mn ($r = 0.45^{**}$) concentrations were found at harvest 2, whereas, at harvest 3, significant correlations between shoot dry weight and shoot P ($r = 0.60^{***}$), K ($r = -0.45^{***}$), Ca ($r = -0.36^{***}$), Mg ($r = -0.77^{***}$), Mn ($r = 0.76^{***}$) and Zn ($r = 0.65^{***}$) concentrations were also observed. ***Significant at $P > 0.001$, **significant at $P > 0.01$, *significant at $P > 0.05$.

4. Discussion

The results from this study showed that the interaction between different Fe levels and N forms in the nutrient solution had a significant effect on vegetative growth, nutrient concentration and distribution in spinach plants. Concerning the total growth results through harvest 2 (Table 2), spinach plants that were grown under conditions of sufficient Fe supply (20 μM Fe) did not produce significantly different shoot dry matter due to N form; however, in the case where spinach plants were grown under conditions of Fe deprivation (0 μM Fe) and supplied with N as 100% NO_3-N, they produced significantly lower shoot dry matter compared to plants without Fe but with mixed N nutrition, despite the absence of intense chlorotic symptoms (Table 1). However, at the end of the experiment, among the first four treatments (I–IV), the least dry matter was produced by the plants grown with sufficient Fe and 100% NO_3-N and not by the plants without Fe and 100% NO_3-N as had happened through harvest 2. This could be due to the fact that through harvest 2, the lack of Fe from the growth medium of plants caused the decrease of the rhizosphere pH, as well as, the increase of the root Fe^{3+} reducing capacity; those plant physiological responses might have increased Fe availability by remobilization of Fe compounds deposited either in the cell wall of root cells or at the growth medium from the relevant Fe quantities that had been added in the precultured period.

Despite the aforementioned variation of the vegetative growth among the three harvests, the shoot Fe concentration was significantly higher in plants grown with sufficient Fe and both forms of N compared either to plants with sufficient Fe and 100% NO_3-N or without Fe and whatever N form at all the three harvests; the shoot Fe concentration among the treatments with sufficient Fe and 100% NO_3-N and without Fe with whatever N form was not significantly differentiated; differences in Fe concentration were highly significant and indicated that Fe concentration increased with the addition of mixed N nutrition. Regardless of the N form, the Fe deprivation from the nutrient solution significantly decreased shoot and root Fe concentration, even before the appearance of intense chlorotic symptoms. Besides, spinach plants fed with sufficient Fe showed significantly higher root Fe concentration compared to plants without Fe, regardless of the N form.

At the end of the experiment, the interaction of varied Fe levels and N forms was found to be significant for shoot Ca, Mg, Fe, Mn, Zn and Cu concentration. Regarding the main effects, the N form in the growth medium significantly influenced the concentration of those plant nutrients determined; both forms of N nutrition enhanced the accumulation of P, Fe, Mn, Zn, Cu and reduced that of K, Ca and Mg; similar findings were documented by Clark et al. (2003) and Wallace et al. (1992) for several plant species. Contrary to our findings, Zornoza and Gonzalez (1998) found that the presence of 20% N as NH_4 reduced the uptake of Fe, Mn and Cu by the spinach cultivar Viroflay grown in continuously aerated nutrient solution. The high pH in the rhizosphere owning to NO_3-N supply reduced the shoot P concentration and suppressed markedly the Mn, Zn and Cu contents of plants (Tables 5 and 6); similar results were obtained by Savvas et al. (2003). The well-known effect of high rhizosphere pH on the uptake of P, Fe, Mn, Zn, Cu has also been referred to by Marschner (1997) whereas, in other uptake studies, it has been shown that NH_4^+ has an inhibitory effect on K^+, Mg^{2+} and Ca^{2+} uptake (Pill and Lambeth, 1977; Fageria, 2001).

The presence of high concentration of bicarbonates in the growth medium significantly reduced the growth of the whole spinach plants, caused the more intense chlorotic symptoms in their leaves and significantly increased the root Fe^{3+} reducing activity (Tables 1–3). The N form did not have a significant effect on the total dry matter production of those plants; however, at the end of the experiment, the root/shoot ratio (on dry weight basis) of plants with high bicarbonates and 100% NO_3-N was significantly restricted compared either to that of plants with high bicarbonates and with both forms of N or to that of plants grown with 20 μM Fe or without Fe with whatever N form; those plants that presented the smallest root proportion, presented also the most intense chlorotic symptoms. It has repeatedly been reported that a decreased root/shoot ratio has physiological disadvantages because it may not only decrease the quantity of nutrient element absorbing sites with a resultant lower source–sink ratio in the plant, but also renders higher the total requirement by increasing the proportion of shoot, in which most of the nutrient element of the plant is utilized. Similar results were taken by Wei et al. (1994) in relevant experiments with susceptible and resistant subclovers cultivars; the Root/Shoot weight ratio of those plants was increased with increasing chlorosis resistance; the resistant cultivars had higher root proportion than the susceptible ones under Fe-deficiency stress condition. Hajiboland et al. (2003) have also referred that rice tolerance to bicarbonate was closely associated with root dry weight and length.

Despite the fact that plants grown with high bicarbonates presented the most intense chlorosis and the lowest dry matter production, those plants presented significantly higher shoot and root Fe concentration compared to plants with 20 μM Fe or without Fe, regardless of the N form. According to Römheld (2000) various soil factors resulting in severe inhibition of root growth might be responsible for triggering a restriction in leaf expansion growth, which in turn elevates the Fe concentration in these chlorotic leaves as a consequence of the diminished dilution of Fe concentration; in our case, high bicarbonates in the growth medium caused the restriction of the root growth. Feeding plants with high bicarbonates in the nutrient solution resulted also in high root Fe levels. Regarding the N form, root Fe concentration of plants grown with mixed N nutrition was 20 times higher than the relevant shoot Fe concentration whereas this ratio for plants grown with 100% NO_3-N (those plants presented also the highest chlorosis score) was equal to 33 (Tables 5 and 7); these results indicated that at low pH (plants fed with the presence of NH_4-N) more Fe is translocated to the shoot whereas at high pH (plants fed with 100% NO_3-N) more Fe is immobilized in the root. Rivero et al. (2003) and Zou et al. (2001) also realized that Fe tended to accumulate more in the roots with the application of NO_3-N than with NH_4-N. Under such conditions, shoot Fe concentration of plants grown with high bicarbonates and N as 100% NO_3-N was found to be more reduced compared to plants with high bicarbonates and mixed N nutrition, even though the root Fe level was high; NO_3-N nutrition resulted in lower shoot Fe concentration due to inhibited uptake and translocation of Fe from roots to shoots as a consequence of high pH at the root surface. Kosegarten et al. (1998) had supported that this might indicate a lower utilization of leaf Fe by the plants grown with Fe and 100% NO_3-N whereas Nikolic and Römheld (1999) referred that Fe inactivation in the leaf apoplast could not be the primary cause of Fe deficiency chlorosis induced by high concentration of bicarbonate at the root surface. This is in agreement with results obtained later by the aforementioned researchers (Nikolic and Römheld, 2003) who found that high pH in the nutrient solution restricted uptake and shoot translocation of Fe, independently of N form.

The shoot Mn concentration in the treatment with high bicarbonates and N as 100% NO_3-N was found to be below the critical deficiency level for many plants. Besides the analytical data, the symptoms of the chlorotic mottling of the younger leaves of those plants looked more like manganese deficiency symptoms. These results imply that the restricted plant growth at these treatments should be due mainly to Mn deficiency, originating from too high pH levels in the rhizosphere. This is in agreement with Islam et al. (1980), who found that growth depression of various crop species at high solution pH was associated with nutrient deficiencies.

Certainly, the concentrations of other essential elements were significantly affected by the bicarbonate treatments; the shoot and root P, Mn, Zn concentration were found to be significantly decreased whereas the shoot K, Ca, Mg significantly increased. The nutritional disorder caused by high concentration of bicarbonates in the growth medium affected the inorganic composition of spinach plants differently, as compared to the lack of added Fe in the nutrient solution.

Shoot Mn and Zn concentrations in plants grown with Fe deprivation (0 μM Fe) and N with both forms, were significantly increased despite the fact that there were little or no Fe-chlorosis symptoms exhibited in those plants (Tables 1, 5 and 6). The lack of Fe in the nutrient solution was associated with some synergistic and antagonistic relationships between nutrient elements in the plants. These relationships were evident before the appearance of chlorotic symptoms; in this respect, they could be used as a diagnostic tool for detection of initial stages of Fe deficiency.

Regarding ferric chelate reductase activity, higher values were attained at harvest 2, possibly reflecting the Fe demand of growth; a decrease of the enzyme activity was observed in the subsequent period, at harvest 3; similar results were obtained by Agnolon et al. (2001). Regarding to the Fe levels, plants grown with high bicarbonates, presented higher ferric chelate reductase activity (per g root fresh weight) compared to plants with 20 μM Fe or without Fe at both harvests 2 and 3. The application of N form did not interfere with the ferric chelate reductase activity at harvest 2, whereas at harvest 3 plants fed with high bicarbonates and 100% NO_3-N showed approximately twice as much activity of the enzyme compared to plants receiving N with both forms (Table 3); those plants also presented the highest chlorosis score. Contrary to our findings, Zribi and Gharsalli (2002) found more restricted Fe^{3+} reduction capacity at high pH (100% NO_3-N fed plants) that led to higher Fe levels in the root apoplast and presumably interrupted Fe translocation to the shoot (Rosen et al., 1990; Mengel, 1995).

5. Conclusions

Nitrogen forms had a significant effect on biomass production and nutrient element accumulation in spinach plants (var. Viroflay) grown under varied Fe levels, in hydroponics. Under conditions of Fe sufficiency, mixed N nutrition (N added as 80% nitrate and 20% ammonium) induced higher production of dry matter, as well as, improved Fe, Mn and Zn plant nutritional status. At early stages of Fe deprivation, mixed N nutrition induced higher production of dry matter and higher shoot Mn and Zn concentration. Under conditions of high concentration of bicarbonates and low level of Fe (a mimic effort of calcareous soil solution), the N form had not a significant influence on total dry matter production; shoot Fe and Mn concentration was found to be significantly reduced in case that N was added as 100% NO_3. We hope that these results provide some information concerning possible direction for future research on the interdependence of absorbed plant nutrients and their accumulation in leafy vegetables in order to improve food production.

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