Research Article

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Corn salad (*Valerianella locusta* (L.) Laterr.) growth in a water-saving floating system as affected by iron and sulfate availability

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Abstract

BACKGROUND: Unbalanced nutrient availability causes disequilibrated plant growth, which can result in a worsening of harvested product quality, such as high nitrate content in edible tissues. To cope with this problem, improved knowledge of the mechanisms involved in nutrient acquisition and regulation is necessary. For this purpose the responses of acquisition mechanisms of N, Fe and S were studied as a function of Fe and S availability using two corn salad cultivars grown hydroponically, considering also aspects related to N metabolism.

RESULTS: The results showed that an increase in Fe or S availability enhanced nitrate uptake and assimilation, which in turn increased biomass production of leaves with lower nitrate content. In particular, high S availability exerted a positive effect (gene expression and functionality) both on the uptake and metabolism of N and on Fe acquisition mechanisms.

CONCLUSION: The data presented here show close interactions between N, S and Fe, highlighting that relevant improvements in yield and quality from soilless culture might also be obtained through appropriate adjustments of nutrient availability. In this respect, concerning the role of S in the acquisition mechanisms of N and Fe and in N metabolism, its level of availability should be taken into high consideration for equilibrated plant growth.

Keywords: *Valerianella locusta* (L.) Laterr.; nutrient availability; closed-loop floating system; nitrate; ^{59}Fe; $^{35}SO_4^{2-}$

INTRODUCTION

In many arid or semi-arid regions such as the Mediterranean area, water, owing to its scarcity, costs and quality is becoming an economically valuable resource,[1] which is leading to the replacement of traditional agriculture by cultivation systems with higher water-use efficiency. Among the various suitable solutions, protected cultivation and hydroponic culture can represent a valid alternative,[2] particularly closed-loop hydroponic systems where plants are fed by a recirculating nutrient solution in order to save water and avoid nutrient leaching, thus limiting further the environmental impact of such culture.[3] In addition, soilless cultural practices such as floating systems allow the harvesting of clean materials, with a consequent reduction in the washing treatments required prior to the packaging of fresh foods.

On the other hand, in a closed-loop system, where the nutrient solution is used for more than one culture cycle, the use of highly concentrated hydroponic solutions is required in order to guarantee an adequate nutrient supply for plants during the entire period of production. Minor fertiliser additions during the production cycle are planned, but only to supplement the nutrient depletion from the hydroponic solution due to plant uptake. The use of such a concentrated nutrient solution can often cause, particularly in crops whose commercial yields are the leaves, a worsening of harvested product quality, such as high nitrate content in edible tissues.[4] This problem has often been observed in soil-grown crops exposed to high levels of NO_3^--N fertilisation.[5] Moreover, it is widely accepted that limiting nitrate consumption is a desirable preventive measure, as its intake at high level is considered a health risk factor, although this has not yet been demonstrated epidemiologically.[6] Thus in 2002, in order to protect public health, the European Commission amended EC Regulation No. 194/97 and adopted EC Regulation No. 563/2002, which set

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maximum nitrate levels in edible tissues of lettuce (fresh lettuce grown under cover and harvested from October to March) and spinach (fresh spinach harvested from September to March) plants at 4500 and 3000 mg kg^{-1} fresh weight (FW), respectively.

In addition to NO_3^--N availability in the growth medium, it has been widely demonstrated that nitrate accumulation in plant tissues is influenced by many other environmental factors, including growing period, air temperature, light intensity and timing of harvest.[7,8] However, it has also been shown that nutritional imbalances such as limited availability of Fe or S in the growth medium may cause nitrate accumulation in leaf tissues of cucumber or spinach, respectively.[9,10] There are many other cases, particularly within the Brassicaceae family, where suboptimal S nutrition results in high accumulation of nitrate in plant tissues.[11,12] These findings suggest that an insufficient and/or unbalanced supply of some nutrients (in these cases Fe or S) with respect to others dissolved in the nutrient solution could result in the perturbation of N uptake and metabolism, leading to the accumulation of nitrate in plant tissues. The different levels of nutritional requirements among plant species used for crop production represent a particularly difficult challenge when standard conditions for optimal availability of nutrients in the growth medium are to be ensured in order to obtain adequate and balanced plant growth. This aspect is particularly relevant for soilless cultural practices based on hydroponic solutions, where the availability of dissolved nutrients is closely linked to their chemical speciation. In particular, it is well known that the availability of Fe, which is widely used in hydroponics in the form of chelates with synthetic organic compounds, could be much lower than that estimated on the basis of the salt dissolved. In fact, concerning the pH-dependent stability of different Fe complexes[13] and the ligand affinity to other cations,[14,15] the availability of the micronutrient may be strongly affected by a change in pH or by the presence of other ions. Sulfur availability can also be easily limited by the precipitation of calcium sulfate dihydrate from solution due to a heterogeneous nucleation process.[16]

In the present study, using two corn salad (*Valerianella locusta* (L.) Laterr.) cultivars grown in soilless culture with a floating system and employing a nutrient solution composition widely adopted in greenhouse cultivation, we evaluated plant yield (g m^{-2}) and quality (NO_3^- and nutrient contents, chlorophyll content in relation to the availability of Fe and SO_4^{2-} in the growth medium. Mechanisms of NO_3^-, SO_4^{2-} and Fe^{3+} acquisition by plant roots supplied with different levels of Fe^{3+} and SO_4^{2-} were also investigated at the physiological and molecular levels in relation to N metabolism.

EXPERIMENTAL

Plant material and growth conditions

Plants were grown hydroponically in a floating system composed of expanded polystyrene boards floating on a nutrient solution contained in rectangular pots (approximately 0.1 m^2 surface, 20 L capacity), with the same ratio between nutrient solution volume and plant number as that used in greenhouse cultivation. The trials were carried out in a growth chamber[17] under controlled climatic conditions: day/night photoperiod, 16/8 h; radiation, 220 μ Einsteins m^{-2} s^{-1}; day/night temperature, 25/20 °C; relative humidity (RH), 70–80%.

Corn salad (*V. locusta* (L.) Laterr.) seeds of cultivars 'Gala' and 'Eurion' (Dotto SpA, Mortegliano, UD, Italy), both widely used in northeastern Italy for greenhouse cultivation in soilless systems, were manually sown on boards (in holes arranged in rows at a distance of approximately 3 cm apart) with perlite (BPB Italia SpA, Milan, Italy). The boards were placed on rectangular pots containing 1 mmol L^{-1} $CaSO_4$ and left in the dark for 5 days at 27 °C and 90% RH for seed germination. After emergence, in order to obtain a density of 1800 plants m^{-2}, seedlings were counted and any excess removed. The boards were then transferred to rectangular pots containing an aerated nutrient solution composed of 3.5 mmol L^{-1} $Mg(NO_3)_2$, 3.984 mmol L^{-1} $Ca(NO_3)_2$, 1.5 mmol L^{-1} $(NH_4)_2SO_4$, 3.5 mmol L^{-1} KH_2PO_4, 3.75 mmol L^{-1} K_2SO_4, 0.5 mmol L^{-1} $CaSO_4$, 10 μmol L^{-1} $Mn(NO_3)_2$, 5 μmol L^{-1} $Zn(NO_3)_2$, 40 μmol L^{-1} H_3BO_3, 1 μmol L^{-1} $Cu(NO_3)_2$, 0.5 μmol L^{-1} $(NH_4)_6Mo_7O_{24}$ and 40 μmol L^{-1} Fe-ethylenediamine-di (o-hydroxy-o-methylphenylacetic) acid (o,oEDDHA) (adjusted to pH 6 with 1 mol L^{-1} KOH). This nutrient solution was chosen because it is widely used by horticultural farms in northeastern Italy for greenhouse cultivation of corn salad plants in soilless systems. In order to ensure the availability of Fe-chelate, the highly stable complex Fe(III)-o,oEDDHA was used,[13–15] which was prepared by mixing $FeCl_3$ with o,oEDDHA in a molar ratio of 1:1.1.[18]

The solution was aerated by bubbling, which also guaranteed its constant mixing. In order to evaluate the effect of Fe or SO_4^{2-} availability on corn salad yield and quality, in one-third of the pots (five pots) the nutrient solution was supplemented with more Fe(III)-o,oEDDHA to give a final Fe concentration of 100 μmol L^{-1}, while in another five pots the SO_4^{2-} content was increased to 15 mmol L^{-1} ($CaSO_4$). As control, the remaining five pots were maintained with the nutrient solution in its original composition without any supplementation. Because of the high content of nitrate in this hydroponic solution, the levels of SO_4^{2-} and Fe supplementation were chosen to adjust the NO_3^-/SO_4^{2-} or NO_3^-/Fe ratio to more equilibrated growth conditions in accordance with previous observations.[9,10,19] In order to assess the availability of nutrients in the nutrient solution, the concentration of ionic species was estimated using the geochemical program MINTEQA (Visual MINTEQ Ver. 2.61 of 29/12/2008 using Lindsay's revised databases[20,21]) from the total concentration of ions in the solution either before or after its supplementation with Fe or SO_4^{2-}. The nutrient solution was renewed every week after measuring the pH of the old one with a pH meter; thence root proton extrusion (mmol H^+) was evaluated as $[H^+] = (\log[H^+]_{initial} - \log[H^+]_{final}) \times$ volume of nutrient solution.[22] Samples of nutrient solution without any supplementary Fe or SO_4^{2-}, collected 2 days after preparation and filtered through a 0.2 μm filter, were analysed for their P, SO_4^{2-}, Ca, Mg and Fe contents. Inorganic P was quantified spectrophotometrically at 705 nm,[23] while SO_4^{2-} content was analysed turbidimetrically.[24] Calcium, Mg and Fe contents were analysed by inductively coupled plasma atomic emission spectroscopy (VISTA MPX, Varian, Turin, Italy).[25]

At the end of the hydroponic growing period (45 days), plants, with the exception of those at the board margins, were sampled, divided into roots and shoots and used for analytical determinations and uptake assays. The sampling from the board corresponded to ten rows of 25 cm length. Fresh mass and leaf area, determined using an LI-3100 area meter (LI-COR Inc., Lincoln, NE, USA), are presented per m^2 of floating board. Leaf chlorophyll contents were evaluated measuring on fully expanded young leaves the SPAD index values using a portable SPAD-502 meter (Minolta, Osaka, Japan). Nutrient (Ca, K, Mg, Cu, Fe, Mn, Zn and P) contents in leaf tissues were determined as described above, after digestion with H_2O_2. To determine total S concentration,

dried leaf samples were ashed in an oven at 600 °C.[26] The ash was dissolved in 10 mL of 3 mol L^{-1} HCl and filtered through Whatman No. 42 paper. In contact with $BaCl_2$ a $BaSO_4$ precipitate is formed that is determined turbidimetrically.[24]

Measurement of net NO_3^- uptake

Nitrate uptake by roots was assayed according to Pavlou *et al.*[9] with slight modifications. Excised roots (*ca* 0.8 g FW) of corn salad plants were rinsed briefly in 0.5 mmol L^{-1} $CaSO_4$ and subsequently immersed in 20 mL of a continuously aerated uptake solution containing 1 mmol L^{-1} KNO_3 and 0.5 mmol L^{-1} $CaSO_4$ (pH 6) at 25 °C. NO_3^- depletion from the uptake solution was measured over 10 min by removing 0.2 mL aliquots every 2 min.

The concentration of NO_3^- in samples was determined according to Cataldo *et al.*[27] with slight modifications, by mixing the 0.2 mL aliquots with 0.8 mL of 50 g L^{-1} salicylic acid in conc. H_2SO_4. After 20 min of incubation at room temperature, 19 mL of 2 mol L^{-1} NaOH was added. The samples were cooled to room temperature and their NO_3^- content was determined spectrophotometrically by measuring the absorbance at 410 nm. The net NO_3^- uptake rate was calculated by linear regression analysis and expressed as µmol NO_3^- g^{-1} root dry weight (DW) h^{-1}.

Measurement of SO_4^{2-} (^{35}S) uptake

The capability of the root apparatus to absorb $^{35}SO_4^{2-}$ was assessed according to Astolfi *et al.*[28] with slight modifications. Excised roots sampled as described above were washed with water. After 30 min the roots were transferred to beakers containing 20 mL of a freshly prepared, continuously aerated micronutrient- and SO_4^{2-}-free uptake solution. Sulfate ($^{35}SO_4^{2-}$, specific activity 2.1 kBq µmol^{-1} $^{35}SO_4^{2-}$) was added at 0.6 mmol L^{-1} concentration and the uptake period was 30 min. Thereafter the root tissues were transferred to an ice-cold desorption solution containing 0.6 mmol L^{-1} $CaSO_4$ and 10 mmol L^{-1} 2-[*N*-Morpholino]ethanesulfonic acid (MES)-KOH (pH 6) for 30 min. The roots were then oven dried at 60 °C, weighed, mineralised with 100 mL L^{-1} H_2O_2 at 60 °C and suspended in 1 mol L^{-1} HCl for ^{35}S determination by liquid scintillation counting. The $^{35}SO_4^{2-}$ uptake rate was expressed as µmol $^{35}SO_4^{2-}$ g^{-1} root DW h^{-1}.

Measurement of ^{59}Fe uptake from Fe-o,oEDDHA

The capability of the root apparatus to absorb ^{59}Fe from ^{59}Fe(III)-o,oEDDHA was assessed according to Cesco *et al.*[29] with slight modifications. Excised roots were washed with 0.5 mmol L^{-1} $CaSO_4$ for 30 min and then transferred to beakers containing 20 mL of a freshly prepared, continuously aerated micronutrient-free uptake solution comprising 0.7 mmol L^{-1} K_2SO_4, 0.1 mmol L^{-1} KCl, 2 mmol L^{-1} $Ca(NO_3)_2$, 0.5 mmol L^{-1} $MgSO_4$, 0.1 mmol L^{-1} KH_2PO_4 and 10 mmol L^{-1} MES-KOH (pH 6). ^{59}Fe-o,oEDDHA, prepared by mixing $^{59}FeCl_3$ with o,oEDDHA in a molar ratio of 1 : 1.1 (specific activity 114 kBq µmol^{-1} Fe), was added to give a final Fe concentration of 100 µmol L^{-1}. To limit photochemical reduction of the micronutrient in the uptake solution supplemented with the Fe source,[30] the beakers were covered with black plastic foil during the entire experiment. The uptake solution was buffered at pH 6 with 10 mmol L^{-1} MES-KOH and the uptake period was 30 min. Thereafter the root tissues were transferred to a freshly prepared ^{59}Fe-free nutrient solution for 10 min to remove excess ^{59}Fe at the root surface and then harvested. Root apoplastic ^{59}Fe pools were removed by 1.2 g L^{-1} sodium dithionite and 1.5 mmol L^{-1} 2,2′-bipyridyl in 1 mmol L^{-1} $Ca(NO_3)_2$ under bubbling N_2.[31] The roots were then oven dried at 80 °C, weighed, ashed at 550 °C and suspended in 1 mol L^{-1} HCl for ^{59}Fe determination by liquid scintillation counting. The ^{59}Fe uptake rate was expressed as µmol ^{59}Fe g^{-1} root DW h^{-1}.

Fe(III)-o,oEDDHA reduction by intact roots

To assess the root capacity to reduce Fe(III)-o,oEDDHA,[32] excised roots were incubated in the dark at 25 °C for 60 min in 20 mL of an aerated solution containing 0.5 mmol L^{-1} $CaSO_4$, 0.5 mmol L^{-1} 4,7-di(4-phenylsulfonate)-1,10-phenanthroline (bathophenanthrolinedisulfonate; BPDS), 10 mmol L^{-1} MES-KOH (pH 6) and 100 µmol L^{-1} Fe(III)-o,oEDDHA. Thereafter the absorbance of the solution at 535 nm was measured at intervals of 15 min and the amount of Fe(III) reduction was calculated from the concentration of the Fe(II)-$BPDS_3$ complex formed, using an extinction coefficient of 22.1 L mmol^{-1} cm^{-1}.

Determination of NO_3^- content in leaf tissues

Cell juices of leaf samples were prepared by thawing the leaf tissues followed by centrifugation at 10 000 × *g* for 15 min.[9] The concentration of NO_3^- in leaf samples was determined spectrophotometrically according to Cataldo *et al.*[27] with slight modifications, as described above.

Gene expression analysis

Root and leaf samples were collected, frozen immediately in liquid nitrogen and conserved at −80 °C until further processing. RNA extractions were performed using an Invisorb Spin Plant RNA kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. A 1 µg aliquot of total RNA (checked for quality and quantity using a spectrophotometer, followed by migration in agarose gel) from each sample was retrotranscribed using 1 pmol of Oligo $d(T)_{23}VN$ (Sigma Aldrich, Milan, Italy), 15 U of Prime RNase Inhibitor (Eppendorf, Hamburg, Germany) and ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) for 1 h at 42 °C according to the manufacturers' instructions. After RNA digestion with 1 U of RNase A (USB, Cleveland, OH, USA) for 1 h at 37 °C, gene expression analyses were performed by adding 0.1 µL of the cDNA to FluoCycle™ SYBR Green (20 µL final volume; Euroclone, Pero, Italy) in a DNA Engine Opticon Real-Time PCR Detection system (T_m = 58 °C; Biorad, Hercules, CA, USA).

Primers were designed on Expressed Sequence Tag (EST) from *Lactuca sativa* (accession numbers indicated in parentheses) and were as follows: *LATS* gene (TC21916) CAATCGGGTTAGGGTTGATG and CCTCCATGCTCCGATAAAAA; *ST* gene (TC27557) TGGAAAAGAAGGGTGTCGAG and CATTCGGTTGCAGAAGATCA; *FRO* gene (BQ862965) GAGAAGGCCCATGATCGTTA and TCCATAGAGAGCTGCCGATT; *NR* gene (TC24977) ACACCAACAAGCACCATTGA and GCGACATGATTCCTCCATTT; and, as housekeeping gene (*alpha-tubulin TUA*; TC28003), CCACCGACTGGACTGAAAAT and CCTAAACATCGCCGTGAACT. Triplicates were performed in three independent experiments. Analyses of real-time results were performed using Opticon Monitor 2 software (Biorad) and R (Version 2.10.1; http://www.r-project.org/) with the qPCR package (Version 1.1–8; http://www.dr-spiess.de/qpcR.html).[33] Efficiencies of amplification were calculated according to the authors.[33] Polymerase chain reaction (PCR) efficiencies were 76.7, 69.5, 75.5, 68.9 and 78.4% for *LATS, ST, FRO, NR* and *TUA* genes respectively. Statistical validation (*t* test) was performed using SigmaPlot 11.0 (Systat Software, Chicago, IL, USA), considering the differences in PCR efficiency and setting the *alpha-tubulin* gene expression for the control treatment equal to unity in the analysed tissue.

Statistical analysis

Each experiment was repeated three times. The significance of differences between means was calculated using the *t* test ($P < 0.05$; $N = 3$) with SigmaPlot 11.0 (Systat Software).

RESULTS

The analysis of the nutrient solution composition with the MINTEQA speciation program, using a pH value fixed at 6 (corresponding to the initial pH value of the hydroponic solution) and a temperature of 20 °C, showed that the predicted availability of nutrients at chemical equilibrium was slightly different from that expected on the basis of the amounts of dissolved salts. According to MINTEQA, only Ca^{2+}, Mg^{2+}, SO_4^{2-} and PO_4^{3-} can undergo partial precipitation. In particular, some Ca and Mg phosphates and sulfates can potentially form, causing, via their precipitation, a decrease in the availability of nutrients in their soluble forms (Ca^{2+}, Mg^{2+} and SO_4^{2-}, *ca* −20%; PO_4^{3-}, *ca* −30%). In order to evaluate whether the predicted precipitation of dissolved nutrients was actually occurring in the nutrient solution, the four nutrients that exhibited a precipitation pattern were determined analytically in the nutrient solution 2 days after its preparation, having previously filtered the samples through a 0.2 µm filter. The results confirmed, with the exception of SO_4^{2-}, a decrease in availability of these nutrients at levels comparable to those predicted by the MINTEQA speciation program (Ca^{2+}, −22%; Mg^{2+}, −15%; PO_4^{3-}, −22%). The analysis with the geochemical model of the nutrient solution containing a higher amount of Fe (100 *vs* 40 µmol L^{-1} Fe^{3+} in the control nutrient solution) showed that the supplemental addition of Fe did not significantly change the concentrations of the dissolved nutrients. On the other hand, the supplemental addition of SO_4^{2-} (15 *vs* 6 mmol L^{-1} in the control nutrient solution) slightly influenced the solubility equilibria of Ca^{2+} and Mg^{2+} towards a higher degree of sulfate precipitation in comparison with phosphate, but without significant modifications in the overall availability of these nutrients in solution. In all cases, Fe was completely complexed by o,oEDDHA in solution; also, soluble Cu was completely present in its complexed form with o,oEDDHA.

Table 1 reports the values of yield, expressed as fresh corn salad leaves per m^2 of board, obtained from *V. locusta* (L.) Laterr. plants after 45 days of hydroponic cultivation with the floating system. The results showed that 'Gala' and 'Eurion' plants grown in hydroponic solution without any supplemental addition of Fe(III)-o,oEDDHA or SO_4^{2-} exhibited commercial yields of 1178 and 1043 g m^{-2} respectively. While SPAD index values were similar between the two cultivars, levels of leaf nitrate content detected in 'Eurion' were higher than those measured in 'Gala', confirming what was previously observed by the authors in greenhouse experiments. When corn salad plants were grown for 45 days in hydroponic solution with increased content of Fe or SO_4^{2-}, a different response to the treatments was observed between the two cultivars. In 'Gala', supplemental addition of Fe(III)-o,oEDDHA (100 µmol L^{-1}) to the nutrient solution caused a pronounced increase in yield, measured as either amount or surface area of leaves (+36%), and resulted in a higher SPAD index (+4%) and a lower nitrate content (−8.5%) than those measured in control plants exposed to 40 µmol L^{-1} Fe-o,oEDDHA. When plants of this cultivar were grown hydroponically with 15 mmol L^{-1} SO_4^{2-}, marketable yield (+39%) and leaf area (+42%) were higher than those of control plants (6 mmol L^{-1} SO_4^{2-}). In the case of 'Eurion' the edible leaf tissues produced by plants cultivated at an SO_4^{2-} availability of 15 mmol L^{-1} showed a significant decrease in the amount of accumulated nitrate (−18%). In contrast to 'Gala' plants, the supplemental availability of Fe or S in the nutrient solution did not significantly modify the yield, SPAD index and nitrate content of leaves produced by 'Eurion' plants.

Leaf nutrient concentrations at harvest are reported in Table 2. In comparison with plants grown in nutrient solution without any supplemental addition of Fe(III)-o,oEDDHA or SO_4^{2-} (control), the presence of 100 µmol L^{-1} Fe(III)-o,oEDDHA in the growth medium caused a significant decrease in the amounts of Cu and Mg measured in the leaves of both cultivars. The use of a higher level of o,oEDDHA in this treatment, which resulted in the complete complexation of Cu in the hydroponic solution, could be at least partly involved in the limited Cu accumulation in plant tissues. Moreover, only in 'Gala' plants a significant increase in leaf Fe levels were recorded. Plants exposed to 15 mmol L^{-1} SO_4^{2-} in the nutrient solution exhibited an increase in the levels of Cu accumulated in the leaves of both cultivars. Conversely, the levels of Mg and Mn in the leaves of both cultivars were significantly lowered by the treatment with 15 mmol L^{-1} SO_4^{2-}. Furthermore, hydroponic cultivation with 15 mmol L^{-1} SO_4^{2-} did not modify the level of Fe accumulated in the leaves of 'Eurion' but increased it in 'Gala' plants.

In order to evaluate the functionality of NO_3^-, Fe(III)-chelate and SO_4^{2-} acquisition mechanisms operating at the root level of corn salad plants grown in the three nutrient solutions, uptakes of NO_3^- (Fig. 1), ^{59}Fe from Fe(III)-o,oEDDHA (Fig. 2) and $^{35}SO_4^{2-}$ (Fig. 3) were measured using excised roots from plants of both cultivars at the harvested stage. The results showed that growing 'Gala' plants in the presence of 100 µmol L^{-1} Fe(III)-o,oEDDHA induced a rise in their capacity to take up NO_3^- (+31%); on the contrary, as expected, the higher micronutrient availability caused a significant decrease (−32%) in the capability to acquire ^{59}Fe. In these plants the capability to take up SO_4^{2-} was unaffected by the treatment (100 µmol L^{-1} Fe). Furthermore, in this cultivar the presence of 15 mmol L^{-1} SO_4^{2-} in the nutrient solution favoured the development of higher uptake rates of NO_3^- (+23%) and ^{59}Fe (+29%) but limited (−26%) the capacity to take up $^{35}SO_4^{2-}$, as expected. When 'Eurion' plants were grown in hydroponic solution with 100 µmol L^{-1} Fe(III)-o,oEDDHA, their roots exhibited an increase in NO_3^- uptake capacity (+32%), while the $^{35}SO_4^{2-}$ uptake rate was unaffected by the higher micronutrient availability. As expected, also in this cultivar the higher micronutrient availability caused a significant decrease (−58%) in the capability to acquire ^{59}Fe. The presence of 15 mmol L^{-1} SO_4^{2-} in the nutrient solution caused an increase (+36%) in the capacity to take up NO_3^- by roots of 'Eurion' plants but had no effect on the ^{59}Fe and $^{35}SO_4^{2-}$ uptake rates.

In roots excised from plants of both cultivars, Fe(III)-chelate reductase activity was also evaluated (Fig. 4A). 'Gala' plants supplied with 100 µmol L^{-1} Fe(III)-o,oEDDHA showed an Fe(III)-chelate reducing activity of 1.70 µmol Fe(II) g^{-1} root DW h^{-1}, which was much lower (−42%) than that measured in control plants grown with 40 µmol L^{-1} Fe(III)-o,oEDDHA. The presence of 15 mmol L^{-1} SO_4^{2-} in the nutrient solution induced in the roots of this cultivar a rise in activity, which reached an even higher value (+36%) than that of control plants exposed to the hydroponic solution without any supplemental addition of nutrients. When 'Eurion' plants were grown in nutrient solution with 100 µmol L^{-1} Fe-o,oEDDHA, the excised roots exhibited a lower Fe(III)-chelate reducing activity (−24%) than that of control plants exposed to 40 µmol L^{-1} Fe-o,oEDDHA (2.75 µmol Fe(II) g^{-1} root DW h^{-1}). The

Table 1. Effect of increased availability of Fe-EDDHA or SO_4^{2-} on leaf yield and area of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown for 45 days in nutrient solution (NS) with floating system. SPAD index values, nitrate contents and photographs of leaves of three plants are also shown

Cultivar	Gala			Eurion		
Growth condition	Control	100 µmol L^{-1} Fe	15 mmol L^{-1} SO_4^{2-}	Control	100 µmol L^{-1} Fe	15 mmol L^{-1} SO_4^{2-}
Leaf yield (g FW m^{-2})	1178 ± 106B	1607 ± 125A	1643 ± 102A	1043 ± 59B	1105 ± 101B	1251 ± 158B
Leaf area (m^2 m^{-2})	46.4 ± 4.2C	63.1 ± 5.9AB	65.8 ± 3.8A	42.4 ± 5.2C	45.7 ± 3.6C	53.3 ± 6.0AB
SPAD index	35.9 ± 0.8B	37.5 ± 0.5A	36.5 ± 0.7AB	35.1 ± 0.9B	35.8 ± 0.7B	36.1 ± 0.8B
NO_3^- content (mg kg^{-1} leaf FW)	3821 ± 151B	3520 ± 134C	3118 ± 284C	4169 ± 192A	3881 ± 113AB	4153 ± 205AB
						

Plant growth conditions: control, NS with 40 µmol L^{-1} Fe-EDDHA and 6 mmol L^{-1} SO_4^{2-}; 100 µmol L^{-1} Fe, NS with 100 µmol L^{-1} Fe-EDDHA and 6 mmol L^{-1} SO_4^{2-}; 15 mmol L^{-1} SO_4^{2-}, NS with 40 µmol L^{-1} Fe-EDDHA and 15 mmol L^{-1} SO_4^{2-}. Data are mean ± standard deviation of three independent experiments. Different letters within a row denote statistically significant differences among samples (*t* test, $P < 0.05$).

Table 2. Nutrient concentrations in leaves of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown as described in Table 1

Cultivar	Gala			Eurion		
Growth condition	Control	100 µmol L^{-1} Fe	15 mmol L^{-1} SO_4^{2-}	Control	100 µmol L^{-1} Fe	15 mmol L^{-1} SO_4^{2-}
Ca (g kg^{-1} leaf DW)	1.81 ± 0.10B	2.87 ± 0.32A	1.84 ± 0.21B	2.17 ± 0.25B	1.81 ± 0.15B	1.68 ± 0.19B
K (g kg^{-1} leaf DW)	17.1 ± 1.3AB	15.5 ± 0.9B	16.5 ± 1.3AB	16.6 ± 1.9AB	15.3 ± 1.0B	18.1 ± 1.2A
Mg (g kg^{-1} leaf DW)	0.70 ± 0.09BC	0.53 ± 0.05C	0.57 ± 0.06C	1.05 ± 0.11A	0.78 ± 0.14B	0.62 ± 0.05BC
S (g kg^{-1} leaf DW)	13.1 ± 0.8C	14.2 ± 1.4BC	15.2 ± 1.0B	20.0 ± 2.2A	22.6 ± 2.8A	21.1 ± 1.1A
P (g kg^{-1} leaf DW)	27.7 ± 1.9A	27.8 ± 1.3A	26.6 ± 2.7A	21.5 ± 1.4B	19.9 ± 2.8B	19.4 ± 3.1B
Cu (mg kg^{-1} leaf DW)	0.81 ± 0.09C	0.48 ± 0.05D	1.38 ± 0.11AB	1.11 ± 0.17B	0.67 ± 0.06C	1.52 ± 0.14A
Fe (mg kg^{-1} leaf DW)	82.4 ± 4.2B	97.4 ± 8.3A	95.3 ± 7.1A	88.0 ± 6.1AB	86.40 ± 9.9AB	80.7 ± 7.1B
Mn (mg kg^{-1} leaf DW)	55.4 ± 2.9B	54.7 ± 5.3B	38.1 ± 6.2C	69.8 ± 5.1A	70.1 ± 8.3A	55.2 ± 3.4B
Zn (mg kg^{-1} leaf DW)	18.3 ± 1.9A	19.7 ± 1.3A	19.8 ± 2.1A	19.1 ± 1.6A	18.3 ± 2.1A	19.7 ± 1.8A

Data are mean ± standard deviation of three independent experiments. Different letters within a row denote statistically significant differences among samples (*t* test, $P < 0.05$).

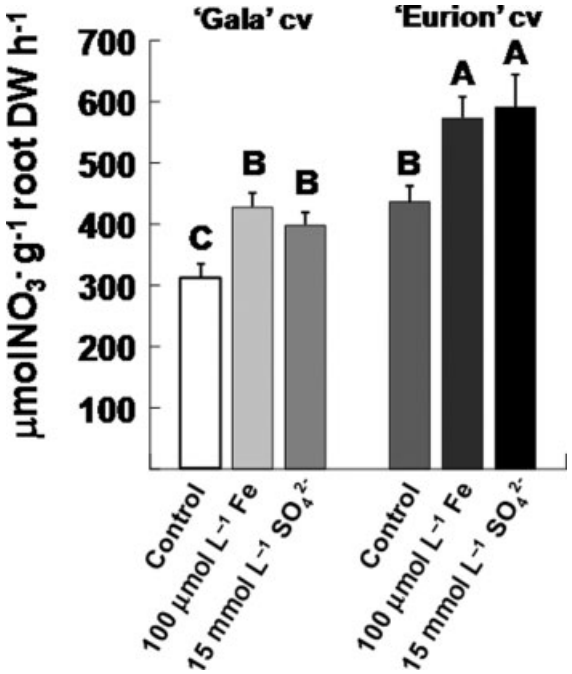

Figure 1. Uptake of NO_3^- by roots of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown for 45 days in nutrient solution (NS) with floating system. Plant growth conditions: control, NS with 40 µmol L^{-1} Fe- EDDHA and 6 mmol L^{-1} SO_4^{2-}; 100 µmol L^{-1} Fe, NS with 100 µmol L^{-1} Fe-EDDHA and 6 mmol L^{-1} SO_4^{2-}; 15 mmol L^{-1} SO_4^{2-}, NS with 40 µmol L^{-1} Fe-EDDHA and 15 mmol L^{-1} SO_4^{2-}. Net nitrate uptake was measured spectrophotometrically as depletion from a solution containing 1 mmol L^{-1} NO_3^-. Data are mean ± standard deviation of three independent experiments. Different letters denote statistically significant differences among samples (*t* test, $P < 0.05$).

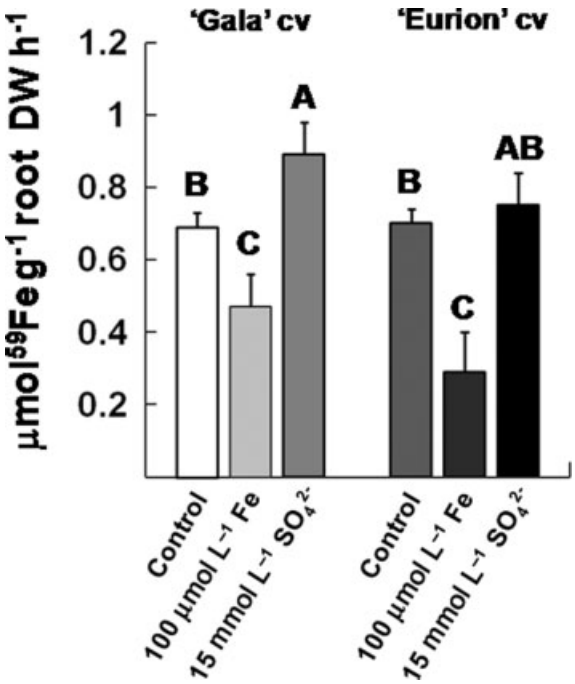

Figure 2. Uptake of ^{59}Fe by roots of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown as described in Fig. 1. Iron uptake was determined at pH 6 using ^{59}Fe (Fe-EDDHA, final Fe concentration of 100 µmol L^{-1}) tracers. Data are mean ± standard deviation of three independent experiments. Different letters denote statistically significant differences among samples (*t* test, $P < 0.05$).

use of 15 mmol L^{-1} SO_4^{2-} in the nutrient solution for growing 'Eurion' plants did not affect this activity.

Changes in the pH of the nutrient solution were determined weekly during the whole period of growth, and thereafter the root capability to release protons into the external medium was calculated. The data presented in Fig. 4B show that roots of both cultivars, when plants had been grown without any supplemental addition of Fe or SO_4^{2-}, were able to extrude protons into the outer medium, leading to partial acidification of the nutrient solution. In 'Gala' plants, this capability was significantly decreased by exposure to 100 µmol L^{-1} Fe(III)-o,oEDDHA or 15 mmol L^{-1} SO_4^{2-} in the nutrient solution. In contrast, these nutrient levels did not modify the root capability to extrude protons in 'Eurion' plants.

In order to evaluate the transcriptional regulation of membrane proteins involved in NO_3^-, Fe and SO_4^{2-} acquisition, the transcript abundance of *LATS* (coding for a low-affinity NO_3^- transporter), *FRO* (coding for an isoform of plasma membrane Fe(III)-chelate reductase) and *ST* (coding for a high-affinity SO_4^{2-} transporter)

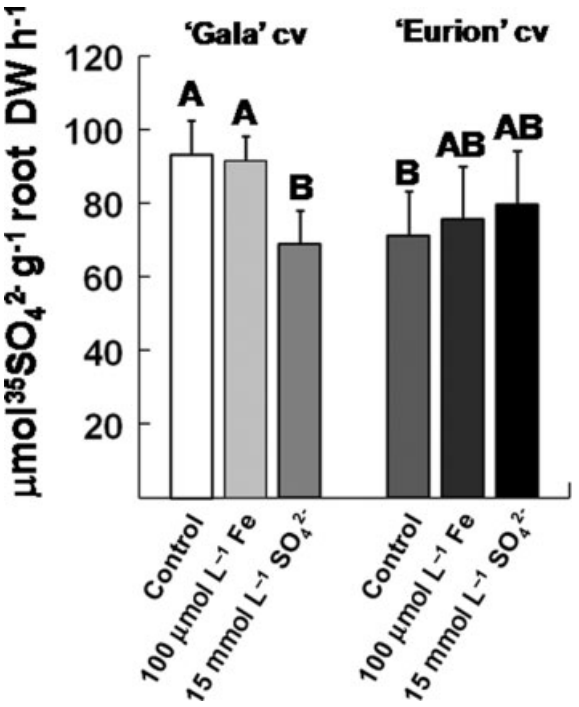

Figure 3. Uptake of $^{35}SO_4^{2-}$ by roots of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown as described in Fig. 1. Sulfate uptake was determined using ^{35}S (SO_4^{2-}, final S concentration of 600 µmol L^{-1}) tracers. Data are mean ± standard deviation of three independent experiments. Different letters denote statistically significant differences among samples (*t* test, $P < 0.05$).

genes was analysed in roots of corn salad plants grown in the three nutrient solutions. The results reported in Fig. 5 show that, as compared with control plants, those supplied with 100 µmol L^{-1} Fe(III)-o,oEDDHA or 15 mmol L^{-1} SO_4^{2-} exhibited a significant increase in the expression levels of *LATS* gene, irrespective of the cultivar considered. In contrast, the transcript abundance of *FRO* gene was unaffected in roots of 'Eurion' and decreased in those of 'Gala' on exposure to 100 µmol L^{-1} Fe(III)-o,oEDDHA; furthermore, this treatment did not modify the expression levels of *ST* gene. When plants were grown at 15 mmol L^{-1} SO_4^{2-}, for both cultivars the expression of *ST* gene was significantly decreased as compared with its transcript levels determined in control plants, while the transcript abundance of *FRO* gene was unchanged by the higher availability of SO_4^{2-}. Evaluation of the expression levels of *NR* (coding for an NO_3^- reductase) gene was also performed and showed that higher NO_3^- uptake by roots was accompanied by an up-regulation of the gene in both cultivars (Fig. 6).

DISCUSSION

The nitrate content in leafy vegetables for human nutrition is an important characteristic of quality, and the presence of this anion at high levels in plant tissues is considered to be a serious threat to human health. This problem of nitrate accumulation in edible plant tissues is not restricted to field-grown crops; it has also been observed in soilless culture where the roots come into contact with nutrients exclusively via the hydroponic solution. Even in these culture conditions the problem may be ascribed, at least in part, to an inadequate or unbalanced supply of Fe and S in the hydroponic composition. For these reasons, in the present study, corn salad (*V. locusta* (L.) Laterr., cultivars 'Gala' and 'Eurion') plants grown in soilless culture with a floating system were used to evaluate the contribution of Fe or SO_4^{2-} availability to the levels of nitrate in edible tissues, considering also, at physiological and molecular levels, the functionality of mechanisms involved in NO_3^-, SO_4^{2-} and Fe^{3+} acquisition at the root level.

In the present work, Fe was supplied chelated with a synthetic ligand (o,oEDDHA) known for its ability to form Fe-chelate with high binding stability,[14] which ensured the availability of Fe at the desired concentration (40 or 100 µmol L^{-1}). In fact, the low solubility of Fe inorganic forms at the pH of the nutrient solution employed here[34] and selected for its extensive use in greenhouse cultivation is well known. Furthermore, *in silico* analyses with a

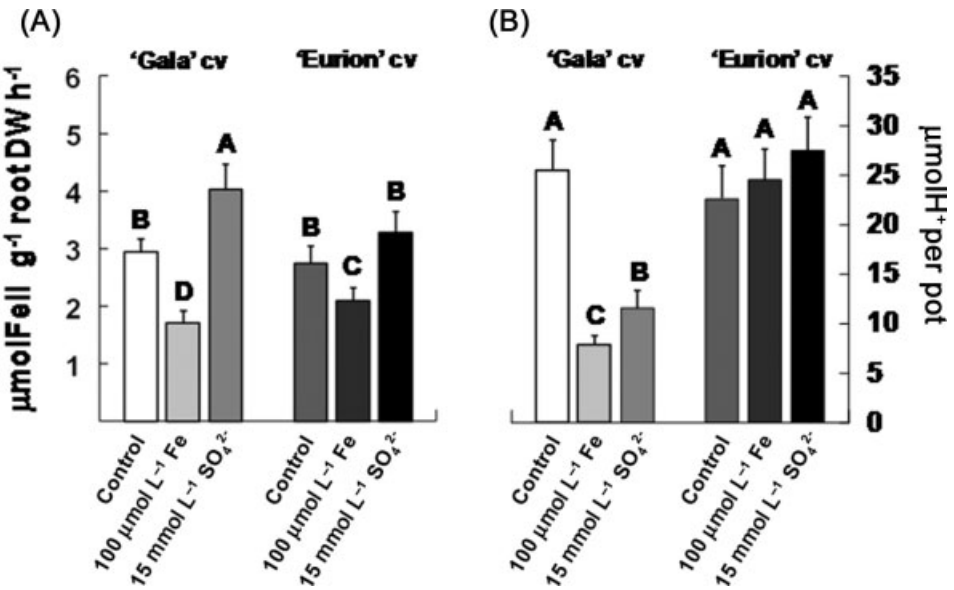

Figure 4. (A) Fe(III)-chelate reducing activity and (B) H^+ extrusion into nutrient solution by roots of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown as described in Fig. 1. Data are mean ± standard deviation of three independent experiments. Different letters denote statistically significant differences among samples (*t* test, $P < 0.05$).

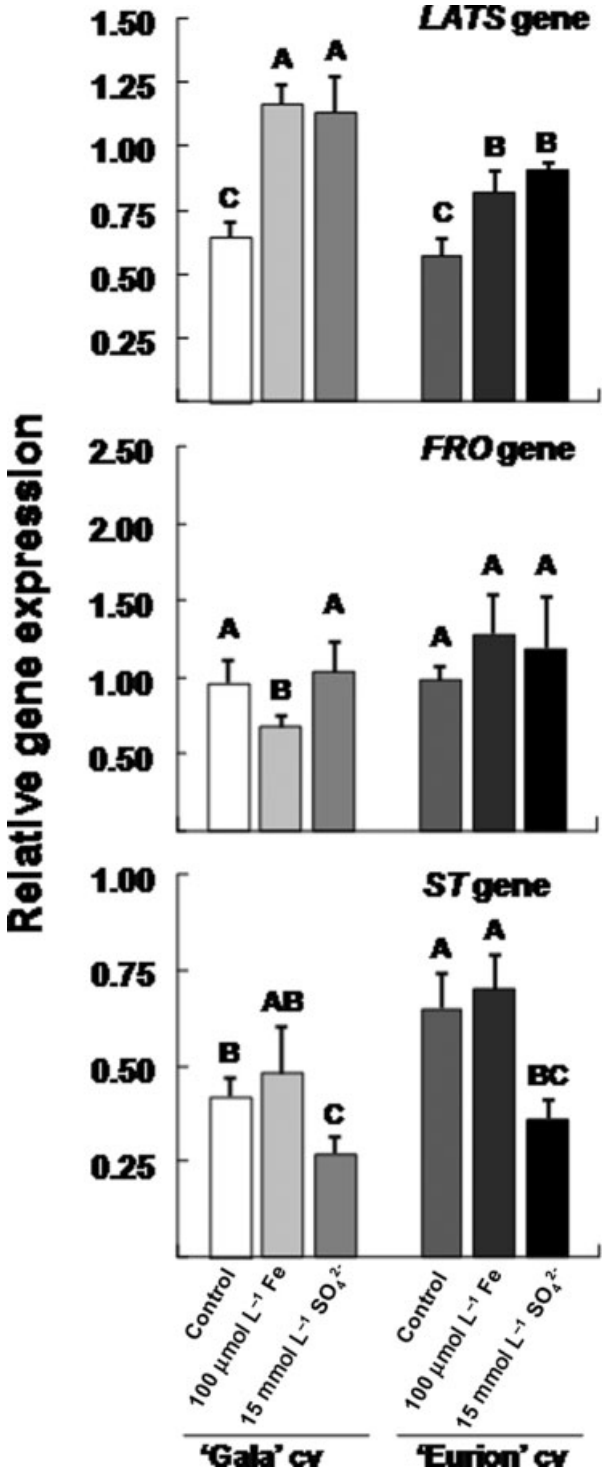

Figure 5. Real-time RT-PCR analyses of *LATS* (coding for a low-affinity NO_3^- transporter), *FRO* (coding for an isoform of plasma membrane Fe(III)-chelate reductase) and *ST* (coding for a high-affinity SO_4^{2-} transporter) gene expression in roots of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown as described in Fig. 1. Gene mRNA levels were normalised with respect to the housekeeping gene *alpha-tubulin*; relative changes in gene expression were calculated on the basis of expression levels of *alpha-tubulin* in roots of control plants. Data bars represent mean ± standard deviation of transcript levels from two independent experiments run in triplicate.

geochemical model revealed that the increase in Fe or SO_4^{2-} concentration in the hydroponic solution did not modify the availability of other dissolved nutrients.

The results presented in this paper show that the levels of nitrate accumulated in 'Gala' leaves were significantly reduced by increasing the Fe supply from 40 to 100 µmol L^{-1} in the nutrient solution (Table 1). This evidence suggests that the micronutrient concentration normally used in the hydroponic solution (40 µmol L^{-1}) was not sufficient to meet the nutritional needs of 'Gala' plants, inducing a latent Fe deficiency. In fact, in these plants, as compared with those grown in the presence of 100 µmol L^{-1} Fe-o,oEDDHA, in addition to the higher nitrate accumulation,[9] there was a lower content of chlorophyll, a smaller leaf area and a smaller leaf biomass accumulation (Table 1), which is consistent with the nutritional disorder symptoms often observed in Fe-deficient plants.[34] This point of view is further supported by results obtained by studying at physiological and molecular levels the mechanisms of ^{59}Fe, nitrate and $^{35}SO_4^{2-}$ acquisition operating at the root level. Corn salad plants, like other dicots, acquire Fe from the external medium via a mechanism called Strategy I,[35] which involves an obligatory reduction of ferric ion prior to membrane influx of Fe^{2+}. The enhanced activity (Fig. 4A) and transcript abundance (Fig. 5) of Fe(III)-chelate reductase together with the increased capability to take up the micronutrient from the outer medium (Fig. 2) when the roots of 'Gala' plants were supplied with 40 µmol L^{-1} Fe(III)-o,oEDDHA are in agreement with the development of the Fe-deficient response,[36] the level of which is finely tuned by the amounts of Fe supply.[19] Furthermore, the highest rate of proton extrusion into the nutrient solution by these plants (Fig. 4B), leading to acidification of the external medium, clearly indicates an attempt to increase Fe solubility via the H^+-promoted dissolution of barely soluble Fe forms, as observed at the rhizosphere of Fe-deficient plants grown in soil. The low rate of net nitrate uptake by roots measured at this level of Fe (40 µmol L^{-1}) (Fig. 1) could also contribute to the decrease in pH of the root external medium,[19] which depends, in addition to the activation of plasma membrane H^+-ATPase,[37] on the increase in the ratio of cation/anion uptake typically observed in Fe-deficient plants.[38] This latter phenomenon also explains the high contents of Mg, K and Cu observed in the leaf tissues of these plants (Table 2). It is interesting to note that the creation of a pH shift towards acidic values at the apoplastic face of the plasma membrane could also optimise the Fe(III)-chelate reductase activity of the root plasma membrane. Also, the high levels of SO_4^{2-} uptake rate (Fig. 3), maintained at least partly by a high expression of the gene encoding the SO_4^{2-} transporter (Fig. 5), could reflect in these plants a greater demand for S to cope with the Fe shortage, similar to that observed in roots of other plant species when subjected to an Fe-deficient condition.[25,39] All these data taken together corroborate the idea that, under the growth conditions used in this study, 40 µmol L^{-1} Fe-o,oEDDHA could not be sufficient to meet the nutritional needs for proper growth of 'Gala' plants, inducing in their roots the typical response to micronutrient shortage and leading at the leaf level to nitrate accumulation due to the altered N metabolism.[9] This conclusion is supported by the low transcript abundance of NO_3^- reductase determined in the roots of these plants (Fig. 6). However, these conclusions cannot be generalised, as they are dependent on the genetic material used. In fact, 'Eurion' plants, although showing the adaptive response to micronutrient deficiency when grown at 40 µmol L^{-1} Fe (higher Fe(III)-chelate reductase activity (Fig. 4A) and ^{59}Fe uptake rate (Fig. 2) and lower nitrate uptake rate (Fig. 1)), seem to take only

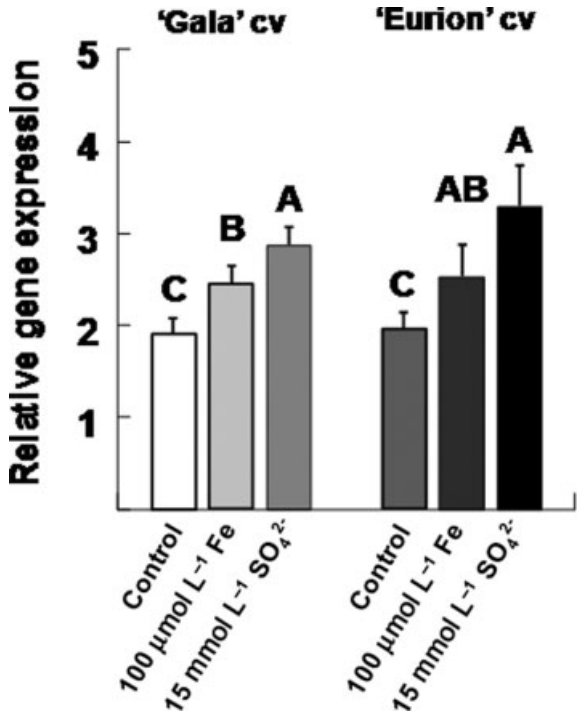

Figure 6. Real-time RT-PCR analyses of *NR* (coding for an NO_3^- reductase) gene expression in roots of two cultivars ('Gala' and 'Eurion') of corn salad (*Valerianella locusta* (L.) Laterr.) grown as described in Fig. 1. Gene mRNA levels were normalised with respect to the housekeeping gene *alpha-tubulin*; relative changes in gene expression were calculated on the basis of expression levels of *alpha-tubulin* in roots of control plants. Data bars represent mean ± standard deviation of transcript levels from two independent experiments run in triplicate.

partial advantage of the increased Fe availability (100 µmol L^{-1}) in the nutrient solution. Indeed, the results reported here show that in these plants the recovery of the previously described root physiological functions altered by the Fe limitation does not reflect changes in N metabolism able to reduce leaf nitrate contents (Table 1), despite the up-regulation in the gene expression of nitrate reductase (Fig. 6). Thus for this cultivar it is possible to envisage the involvement in the phenomenon of some other factor playing a greater role than the level of Fe availability in the hydroponic solution.

When SO_4^{2-} availability in the growth medium was increased to 15 mmol L^{-1}, the amount of nitrate accumulated in 'Gala' leaves was consistently lowered and a marked increase in the yielded biomass occurred (Table 1). This indicates that plants of this cultivar clearly benefited from the greater SO_4^{2-} availability. Based on these results, it can therefore be argued that the S amount provided in the hydroponic solution often used in greenhouse cultivation (6 mmol L^{-1}) is insufficient or, relative to nitrate, unbalanced for 'Gala' plants. In this regard it is interesting to note what has been demonstrated in S-deprived spinach plants,[10] where a disruption of N metabolism and nitrate uptake occurred, leading in turn to a huge increase in the anion content in young leaves. Our results show a lower root ability to take up nitrate (Fig. 1) and an increased capacity for S uptake (Fig. 3) in 'Gala' plants when fed with 6 mmol L^{-1} SO_4^{2-}, similar to the findings of Clarkson *et al.*[40] in S-starved cereal plants. The recovery of these altered physiological activities (low nitrate and high sulfate uptake rates) in 'Gala' plants when grown at 15 mmol L^{-1} SO_4^{2-} (Figs 1 and 3), which was concomitant with changes in transcript abundance of the genes coding respectively for the two transporters (Fig. 5), corroborates the hypothesis that, in our conditions, 6 mmol L^{-1} SO_4^{2-} is inadequate to meet plant S requirements. In addition, these results confirm the close interaction of S with N, which turns a lack of one into a limitation of the uptake (Fig. 1) and assimilation (Fig. 6) of the other.[41] Fismes *et al.*[42] argued that the interactions between S and N are synergistic at optimum rates and antagonistic at excessive levels of one of them. Furthermore, it is noteworthy that the low rates of net nitrate uptake recorded at 6 mmol L^{-1} SO_4^{2-} could also contribute to the acidification of the root external medium observed in these plants (Fig. 4B). All these data taken together highlight that the relevance of appropriate S availability to improve N-use efficiency by cultivated plants, as already noted for field crops,[42] is also applicable to soilless culture. It is interesting to stress the widely diffused opinion that improved yields without concomitant increases in resource inputs such as water and fertilisers will be required to ensure sustainable world crop production in the future.[43]

In addition to what is observed for N, interactions between S and Fe nutrition have also been demonstrated recently in several species of cultivated plants,[26,28,39,44,45] indicating that the levels of S availability in the growth medium can consistently affect the functionality of Fe acquisition mechanisms, particularly when they are induced by Fe deficiency. The results presented here show that, when SO_4^{2-} availability was 6 mmol L^{-1}, 'Gala' plants exhibited levels of Fe(III)-chelate reductase (Fig. 4A) and rates of ^{59}Fe uptake (Fig. 2) lower than those recorded in plants fed with high levels of SO_4^{2-} (15 mmol L^{-1}). It has been demonstrated that S deficiency limits the capacity of tomato plants to cope with Fe shortage by preventing the induction of Fe(III)-chelate reductase and by limiting the activity and expression of the Fe^{2+} transporter.[25] It is interesting to note that in plants grown at 15 mmol L^{-1} SO_4^{2-} the enhanced levels of these two activities were able to ensure, despite the marked increases in leaf biomass accumulation, Fe contents in the leaf tissues comparable to or even greater than those measured with the other treatments (Table 2). This evidence suggests that corn salad plants can take advantage of high S availability and conversely that, under the growth conditions used in this study, 6 mmol L^{-1} SO_4^{2-} in the hydroponic solution is not sufficient to ensure adequate acquisitions of N and Fe, which in turn results in limited growth and a possibly unhealthy food product. However, as observed previously, the plant response to different levels of SO_4^{2-} availability seems to depend on the cultivar employed. In fact, in contrast to 'Gala', 'Eurion' plants with higher SO_4^{2-} availability did not change significantly in terms of either the physiological activities of the roots (Fe(III)-chelate reductase, proton extrusion and ^{59}Fe and $^{35}SO_4^{2-}$ uptakes) or the leaf parameters considered here, including nitrate contents (Table 1). This pattern was evident even though net nitrate uptake rates and the transcript abundance of the root nitrate reductase gene were increased (Fig. 6). Thus for this cultivar it could be assumed that factors other than SO_4^{2-} availability may be involved to a larger extent in quality and yield.

CONCLUSIONS

The data presented here showed close interactions between N, S and Fe, which are responsible for synergistic or antagonistic effects depending on their reciprocal availability levels in the growth medium. In particular, an unbalanced availability of S with respect to N can alter the functionality of nitrate uptake and assimilation as well as mechanisms involved in Fe acquisition. For these reasons, levels of S availability should be taken into high

consideration for equilibrated plant growth, also considering the high availability of S fertilisers and their low cost. In addition to appropriate adjustments of nutrient availability, identification of specific cultivars suitable for soilless culture can contribute to obtaining improvements in yield and quality of plants produced with this water-saving growth system.

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