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Tavakoli, Fatemeh [et al.]. «Signaling function of NH4+ in the activation of Fe-deficiency response in cucumber (Cucumis sativus L.)». *Planta*, Vol. 260 (July 2024), art. 53 DOI 10.1007/s00425-024-04480-5

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Signaling function of NH_4^+ in the activation of Fe-deficiency response in cucumber (*Cucumis sativus* L.)

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Acknowledgments This work was supported by the University of Tabriz. Partial financial support was provided by the Serbian Ministry of Science, Technological Development and Innovation (contract no. 451-03-47/2023-01/200053) and the Spanish Ministry of Science, Innovation and Universities project PID2019-104000RB-I00.

Main conclusion NH_4^+ is necessary for full functionality of reduction-based Fe deficiency response in plants

Abstract

Nitrogen (N) is present in soil mainly as nitrate (NO_3^-) or ammonium (NH_4^+). Although the significance of a balanced supply of NO_3^- and NH_4^+ for optimal growth has been generally accepted, its importance for iron (Fe) acquisition has not been sufficiently investigated. In this work, hydroponically grown cucumber (*Cucumis sativus* L. cv. Maximus) plants were supplied with NO_3^- as the sole N source under −Fe conditions. Upon the appearance of chlorosis, plants were supplemented with 2 mM NH_4Cl by roots or leaves. The NH_4^+ treatment increased leaf SPAD and the HCl-extractable Fe concentration while decreased root apoplastic Fe. A concomitant increase in the root concentration of nitric oxide and activity of FRO and its abolishment by an ethylene action inhibitor, indicated activation of the components of Strategy I in NH_4^+-treated plants. Ammonium-pretreated plants showed higher utilization capacity of sparingly soluble $Fe(OH)_3$ and higher root release of H^+, phenolics, and organic acids. The expression of the master regulator of Fe deficiency response (*FIT*) and its downstream genes (*AHA1*, *FRO2*, *IRT1*) along with *EIN3* and *STOP1* was increased by NH_4^+ application. Temporal analyses and the employment of a split-root system enabled us to suggest that a permanent presence of NH_4^+ at concentrations lower than 2 mM is adequate to produce an unknown signal and causes a sustained upregulation of Fe deficiency related genes, thus augmenting the Fe acquisition machinery. The results indicate that NH_4^+ appears to be a widespread and previously underappreciated component of plant reduction-based Fe deficiency response.

Keywords Ethylene, FIT, Foliar Application, Iron Deficiency, NH_4^+ Signaling, STOP1

Abbreviations CS: Citrate synthase; EIN3/EIL1: ETHYLENE INSENSITIVE 3 & ETHYLENE INSENSITIVE 3-LIKE 1; FIT: FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR; FRO2: FERRIC REDUCTION OXIDASE 2; IRT1: IRON-REGULATED TRANSPORTER 1; MDH: malate dehydrogenase; PAL: Phenylalanine ammonia lyase; PEPC: Phosphoenol pyruvate carboxylase; STOP1: SENSITIVE TO PROTON RHIZOTOXICITY 1

Introduction

Iron (Fe) deficiency is a major nutritional disorder in plants primarily resulting from the scarce solubility of Fe compounds such as oxides and oxyhydroxides, leading to low availability in the soil solution (Römheld and Nikolic 2006). Iron is required by several enzymes involved in the synthesis of chlorophyll; thus, a low Fe supply induces immediate interveinal chlorosis, particularly in the young leaves (Cakmak et al. 2023).

Plants developed various adaptive mechanisms to cope with Fe deficiency that coordinate to increase the acquisition of sparingly soluble Fe from the soil. In this context, two main strategies have been identified: dicots and nongraminaceous monocots employ 'Strategy I' or 'reduction-based strategy,' while graminaceous monocots operate with the 'Strategy II' or 'chelation-based strategy' (Pavlovic and Nikolic 2018; Cakmak et al. 2023). In a reduction-based mechanism, Fe^{3+} is reduced by FERRIC REDUCTION OXIDASE 2 (FRO2) at the plasma membrane before being transported across the membrane by IRON-REGULATED TRANSPORTER 1 (IRT1) (Ivanov et al. 2012; Li et al. 2023). In Arabidopsis, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) coordinates the Fe-deficiency response in the root epidermis for Fe acquisition (Bauer et al. 2007). Therefore, AtFIT physically interacts with subgroup Ib BASIC HELIX–LOOP–HELIX (bHLH) transcription factors to activate the transcription of *AtFRO2* and *AtIRT1* (Yuan et al. 2008).

Iron deficiency response in 'Strategy I' plants is additionally associated with enhanced activity of plasma membrane proton pumps (H^+-ATPase) AHA1 and AHA2, leading to the acidification of rhizosphere and increasing Fe^{3+} solubility (Molnár et al. 2023), release of organic acids (e.g. malate, citrate, succinate) (Abadía et al. 2002) and phenolic compounds (Rodríguez-Celma et al. 2013; Schmid et al. 2014) particularly coumarins (Fourcroy et al. 2014) contributing to the mobilization of Fe in the rhizosphere. From organic acids, citrate is not only involved in the Fe^{III} chelation and hindering its precipitation in the rhizosphere (Molnár et al. 2023) but is a potent chelator of Fe^{III} in the xylem (Rellán-Álvarez et al. 2010) and has a crucial role in plant Fe homeostasis under deficiency conditions. Considering a high content of negatively charged carboxyl groups in the root apoplast, this compartment serves as a cation sink and is an excellent storage pool for Fe (Liu et al. 2023). Phenolics are likely the major players in the reutilization of Fe pool in the root apoplast, contributing directly to the remobilization and re-utilization of apoplastic root Fe through chelation- and reduction-based effects (Jin et al. 2007; Pavlovic et al.

2013). In addition to the effect on the expression of *FRO2* and *IRT1*, FIT positively regulates the synthesis and excretion of phenolic substances such as coumarin (Schmid et al. 2014).

Ethylene, a gaseous plant hormone, is well known to be involved in Fe deficiency signaling (García et al. 2010). As a positive signal, ethylene is produced upon Fe deficiency, and through transcription factors ETHYLENE INSENSITIVE 3 and ETHYLENE INSENSITIVE 3-LIKE 1 (EIN3/EIL1) directly interact with and stabilize FIT (Lingam et al. 2011; Riaz and Guerinot 2021). Nitric oxide (NO) has also been proposed as a Fe-deficiency signal that, like ethylene, is a positive regulator of Fe-deficiency response (Graziano and Lamattina 2007) and acts on FIT both through transcription (through S-nitrosoglutathione, GSNO) (Kailasam et al. 2018) and posttranslation (stabilization) of FIT (Meiser et al. 2011).

The expression of genes encoding the downstream components of Strategy I, e.g., *FRO*, *IRT,* and *AHA*, could also be augmented upon exogenous application of ethylene (Romera and Alcántara 2004) and NO donors (Koen et al. 2012) in Fe-deficient plants. This evidence implies that application of factors eliciting a rise in the native components of Fe signaling pathway enhances the Fe-deficiency responses and increases the plant's ability to reuse internal sparingly soluble Fe resources.

Nitrogen (N), as an essential macronutrient, is predominantly taken up as nitrate (NO_3^-) or ammonium (NH_4^+) ions. It is well established that the highest plant biomass is achieved under a combination of NO_3^- and NH_4^+ in the culture media. A balanced ion uptake, easier intra- and extra-cellular pH homeostasis, and facilitated regulation of carbon and N assimilation contribute to higher plant performance under a combinative supply of NO_3^- and NH_4^+ (Hawkesford et al. 2023). Ammonium-based fertilizers, especially ammonium sulphate, are widely used as N source in alkaline soils due to its acidifying effect favoring the availability of Fe and other micronutrients. Nevertheless, the contribution of Fe acquisition to the advantages of balanced N nutrition either in field-grown plants or in hydroponics has not been sufficiently addressed. Several studies showing the advantage of NH_4^+ over NO_3^- in the uptake and root-shoot translocation of Fe and its mobility in the leaf apoplast (Nikolic and Römheld 2003; Zhu et al. 2018; Zhu et al. 2019) used NH_4^+ as the sole N source and compared the results with the sole NO_3^- medium. Ammonium as the exclusive N source causes various ionic, redox, metabolic, and hormonal imbalances (Shilpha et al. 2023). This may also indirectly affect plant response to Fe

deficiency, and such effects may not represent the conditions with balanced levels of both N forms required for optimum growth.

We hypothesize that NH_4^+ acts as a signaling molecule that independently of its acidifying effect, contributes to the plant's Fe acquisition machinery by augmenting the response to Fe deficiency. In this context, here we report the effects of the supplementation of a NO_3^--based medium with a low NH_4^+ concentration (2 mM) on the root Fe acquisition properties in Fe-starved plants. Using both root and leaf NH_4^+ applications in a pH-buffered medium, we show that NH_4^+ is necessary for full functionality of reduction-based Fe deficiency response in plants.

Materials and methods

Plants cultivation and treatments

Seeds of cucumber (*Cucumis sativus* L. cv. Maximus) plants were germinated on moist perlite in dark for four days. Young seedlings were transferred to the light conditions and irrigated with 50% Hoagland nutrient solution (Johnson et al. 1957) (pH 5.8). Seven-day-old seedlings were transferred to aerated 50% hydroponic medium and precultured for further one week. Thereafter, similar-sized plants were transferred into 1 L plastic pots containing full-strength Hoagland nutrient solution with exclusion of NH_4^+ source (Online Resource S1).

Two different Fe treatments including adequate (+Fe; 20 µM) and deficient (–Fe) supply were assigned randomly to the pots. For –Fe treatments, Fe was completely withdrawn from the nutrient solutions. Four days after starting the –Fe treatments and simultaneous with appearance of the chlorosis in the young leaves, different NH_4^+ treatments were started for both +Fe and –Fe plants (Online Resource S1). Three levels of NH_4^+ treatment were considered including without ($-NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) applied either to the nutrient solution (root application, $+NH_4^+R$) or sprayed on the leaves (leaf application, $+NH_4^+L$). For avoiding leaf damage, the leaf application was performed gradually using 1 mM NH_4Cl solution without addition of any surfactant while avoiding direct light. A sum of 2 mmol NH_4 was applied to each plant weekly. The nutrient solution was buffered with 2.0 mM $CaCO_3$ (with daily adjustment at pH 5.8) unless otherwise stated in the text. The concentration of N in the nutrient solution was kept at 14.0 mM for both $-NH_4^+$ and $+NH_4^+$ plants (Online Resource S1).

Plants were grown under controlled environmental conditions with a temperature regime of 25/18 °C (day/night) and with a photoperiod of 14/10 h (light/dark), a relative humidity of 50–

60% and at a photon flux density of about 400 μmol m^{-2} s^{-1} provided by fluorescent lamps. Nutrient solutions were replaced every week.

Plants harvest and determination of biomass

Plants were harvested two weeks after different NH_4^+ treatments. After determination of Spectral Plant Analysis Diagnostic (SPAD index) in the young leaves using a chlorophyll (Chl) meter SPAD-502 device (Minolta Camera Co., Osaka, Japan), the shoot and roots were separated, rinsed with distilled water and blotted dry on filter paper. After measurement of fresh weight (FW), the samples were oven-dried (at 70 °C for 48 h) and used for determination of dry weight (DW).

Temporal analyses of NO and FRO activity

Plants were cultivated under –Fe conditions with three NH_4^+ levels ($-NH_4^+$, $+NH_4^+R$ and $+NH_4^+L$) as described above. The concentration of NO and the activity of FRO in the 2-3 cm from the root tips were measured daily starting from day two after NH_4^+ treatments continued for 14 days according to the methods described below.

Split root experiment

The root system of 10-day-old cucumber plants was split into two equal halves by gentle root separation in water. Each of the two halves was placed in a 1 L container filled with aerated half-strength Hoagland nutrient solution for healing the root damages. Two different Fe treatments (+Fe and –Fe) were applied on two-week-old plants for four days then +Fe plants were treated with two NH_4^+ levels as $-NH_4^+$ and $+NH_4^+R$ while –Fe plants were treated both through roots ($+NH_4^+R$) and leaves ($+NH_4^+L$). For root application, one side of the split-root system was treated ($+NH_4^+R+$) while the other half left untreated ($+NH_4^+R-$). The nutrient solution was buffered with 2.0 mM $CaCO_3$ with daily adjustment at pH 5.8. The activity of FRO and the concentration of NO were determined for 14 days in the roots of experimental plants.

Application of ethylene action inhibitor

Plants response to silver thiosulfate (STS), an inhibitor of ethylene action, was studied in an independent experiment. Plants were grown under –Fe conditions as described above then the freshly prepared STS at 400 µM was applied to the roots simultaneous with starting NH_4^+ treatments in the pH-buffered nutrient solution (2 mM $CaCO_3$, pH 5.8). The root activity of FRO, the changes in the medium pH and SPAD readings of the young leaves were determined one week after starting the NH_4^+ and STS treatments.

Study of concentration and exudation of organic acids and phenolics, rhizosphere acidification and activity of related enzymes

Plants that were previously cultivated under different Fe supply levels (+Fe and –Fe) for four days in NH_4^+-free medium, were treated with two ($-NH_4^+$, $+NH_4^+R$) and three levels of NH_4^+ ($-NH_4^+$, $+NH_4^+R$ and $+NH_4^+L$) in +Fe and –Fe plants, respectively in the pH-buffered medium (2.0 mM $CaCO_3$) as described above. The activity of enzymes involved in the synthesis of organic acids and phenolic compounds was determined in the roots two weeks after starting NH_4^+ treatment.

Estimation of root acidification capacity: Root capacity for rhizosphere acidification (H^+ release) was estimated one week after different NH_4^+ treatments according to the method described by Sadeghzade (et al. 2021). Briefly, the plants were transferred to pH-buffered (1 mM MES at pH 5.8) fresh nutrient solution of the respective Fe treatments but without NH_4^+ under growth chamber conditions in the light. After 8 h, the nutrient solutions were brought to volume and titrated to pH 8.0 using a KOH standard. The proton release was calculated on the root FW basis (Sadeghzade et al. 2021).

Collection of root exudates: Collection of root exudates was carried out one and two weeks after NH_4^+ treatment. For this purpose, roots of the intact plants were rinsed with 0.5 mM $CaCl_2$ for 30 min and placed in 50 mL of aerated 0.5 mM $CaCl_2$ during 3 h in the dark under growth chamber conditions to collect root exudates. Root exudate samples were freeze-dried and stored at −20 °C until analysis. Analysis of phenolics in the root exudate was performed in the first collection while organic acids were analyzed in both time points. After the second collection of exudates, 2-cm long root tips were excised, weighed and immediately frozen in liquid N and after freeze-drying were stored at −20 °C until analysis of organic acids and total phenolics. $+Fe+NH_4^+$ plants were not analyzed for organic acids in the root extract or exudate.

Fe mobilization experiment

For evaluation of the effect of NH_4^+ pretreatment on the Fe mobilization in the rhizosphere, Fe-deficient plants that were treated for one week with three NH_4^+ treatment levels ($-NH_4^+$, $+NH_4^+R$ and $+NH_4^+L$) in the pH-buffered medium (2.0 mM $CaCO_3$), were transferred to $-NH_4^+$ medium supplemented with 50 µM insoluble Fe ($+Fe(OH)_3$) with daily pH adjustment. After three days, plants were photographed and the leaf Chl concentration was determined in the young leaves. The synthesis of $Fe(OH)_3$ was performed using the reaction between $FeCl_3$ and KOH at a molar ratio of 1:3 over 30 min with continuous stirring. The mixture was centrifuged at 5000 g and the colloidal $Fe(OH)_3$ was washed with distilled water for three times and collected.

Nitric oxide determination

Nitric oxide (NO) concentrations were determined in the 20-mm long root tips by colorimetric method using Griess reagent (Sun et al. 2003). After extraction with 100 mM phosphate buffer (pH 7.4) and centrifugation, NO concentration was determined in a reagent mixture containing sulphaniliamide and N-(1-naphthyl) ethylenediamine (NED) at 520 nm. Standard curve was produced by $NaNO_2$ in the range of 0–5 µM and NO concentration was calculated as nmol g^{-1} FW.

Visual detection of NO in the root tips was carried our using 4,5-diaminofluorescein-2 diacetate (DAF-2DA) (Sigma). The root tips of approximately 20 mm from the apex were cut and incubated in 10 µM DAF-FM diacetate prepared in 10 mM Tris-HCl (pH 7.4) at 25 °C for 1 h in the dark then rinsed twice with the buffer to remove excess fluorophore. As negative control, the root tips were pre-incubated with 400 µM NO scavenger, cPTIO (2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) (Sigma) in darkness for 2 h at 25 °C, before adding the fluorophore (Sandalio et al. 2008). The roots were mounted in distilled water on the slides and subjected to observations using fluorescence microscope (OPTIKA, B-500TiFL, Italy) equipped with excitation B filter (EX 450 – 490, DM 495, EM 500 – 550). Pictures were taken with the same exposure time for all treatments (20 s), using a digital camera connected to the microscope (OPTIKAM PRO COOL 5 Camera, 4083.CL5).

Assay of enzymes activities

Activity of enzymes was determined in the 2-3 cm from the root tips. The activity of ferric chelate reductase (FRO, EC 1.16.1.17) was determined in the presence of bathophenathroline dissulfonic acid (BPDS) and $Fe^{III}EDTA$ in darkness for 1 h at 25 °C. Reduction rates were determined as formation of a red $Fe^{II}(BPDS)_3$ complex by measuring the absorbance at 535 nm against blanks (without roots) and using an extinction coefficient of 22.14 mM^{-1} cm^{-1} for calculation (Hajiboland et al. 2020).

Determination of activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was carried out in an assay buffer containing 25 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM DTT, 1 mM $KHCO_3$ and 3 U mL^{-1} lactate dehydrogenase (LDH, 1.1.1.27). The activity was monitored by disappearance of NADH at 340 nm over 3 min using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH (Ashton et al. 1990). Malate dehydrogenase (MDH, 1.1.1.37) activity was determined using oxaloacetic acid as substrate and monitoring the decrease of NADH at 340 nm (Mukerji and Ting 1968). The activity of citrate synthase (CS, EC 2.3.3.1) was determined according to Srere (1967). The activity was assayed by monitoring the reduction of acetyl-CoA to CoA which was measured following the reduction of 5 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoic acid (TNB) with CoA at 412 nm. The activity of enzyme was calculated using an extinction coefficient of 13.6 mM^{-1} cm^{-1} for TNB (Srere 1967).

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity was assayed as the amount of trans-cinnamic acid formed from L-phenylalanine in the reaction medium during 30 min at 30 °C and its concentration was determined at 290 nm and calculated using its extinction coefficient of 9630 M^{-1} cm^{-1} (Jun et al. 2018).

Assay of organic acids and phenolics

The concentration of organic acids in the root tips was determined after extraction in 5% H_3PO_3 using HPLC system (LC-10 AT, Shimadzu Corporation). The samples of root exudates were extracted with distilled water, filtered and directly injected to the HPLC (Neumann and Römheld 1999). Phenolics in the root extract and exudate samples were extracted for three times with 70% aqueous methanol at 4 °C in the dark. Total soluble phenolics in the supernatant were determined at 750 nm using Folin-Ciocalteu reagent and gallic acid as standard (Blainski et al. 2013).

Determination of Fe

The young leaves and the roots of whole plants were collected and divided into two equal parts that were used either for total Fe concentration or the concentrations of HCl-extractable Fe (leaf) and apoplastic Fe (root). Oven-dried leaf samples were extracted with 1 M HCl solution for 24 h by continuous shaking then the Fe concentration was determined in the filtrates by atomic absorption spectrometry (AAS) (Başar 2003). The root apoplastic Fe was determined using the Bienfait's method described elsewhere (Pavlovic et al. 2013). Total Fe was determined after digestion of samples in a muffle furnace at 500 °C for 8 h. After resolving in the 0.5 M HCl, the Fe concentration was determined by AAS (AA-6300, Shimadzu, Japan).

Gene expression analyses

Plants were grown for four days under +Fe and –Fe treatments in NH_4^+-free medium then the Fe-deficient plants were treated with three levels of NH_4^+ ($–NH_4^+$, $+NH_4^+R$ and $+NH_4^+L$) in the pH-buffered medium (2.0 mM $CaCO_3$) as described above. Plants were harvested one and two weeks after NH_4^+ treatment for gene expression analyses.

The total RNA was extracted from the root tissues using a CTAB-based method (Japelaghi et al. 2011). First-strand cDNA was synthesized from 2 µg of total RNA from each sample using the PrimeScript™ RT reagent kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. Gene expression analysis was carried out with Bio-Rad SsoAdvanced™ Universal SYBR® Green Supermix (2X) and specific primers (Online Resource S2). Real-time PCR was performed on PowerAmp96™ Real-Time PCR System (Kogene Biotech) with the following thermal cycling parameters: 30 s at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 30 s). PCR reactions were done in triplicate, including no-template controls. The results were analyzed with PowerAmp96 series software and the relative gene expression of the target was calculated with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Actin and F-box were used as internal control genes. Sequence data from this article can be found in the GenBank/EMBL data libraries and EST database (http://www.icugi.org/) under accession numbers given in Online Resource S2.

Experimental design and statistical analyses

The experiments were undertaken using completely randomized block design with four independent biological replicates. Pairwise multiple comparison of means was performed by Tukey's test at $P < 0.05$ using Sigma Stat software version 3.5.

Results

Shoot growth of plants supplied with sufficient Fe did not respond to NH_4^+ application, while dry biomass of roots significantly increased by leaf NH_4^+ application. Under –Fe conditions, however, both shoot and root biomass increased with NH_4^+ (Fig. 1a, b). The leaf SPAD reading was significantly decreased under Fe deficiency conditions, while NH_4^+ treatment increased this parameter even in +Fe plants (Fig. 1c). Similar to the SPAD data, the leaf Chl (*a*+*b*) concentrations were significantly increased by NH_4^+ not only in Fe-deficient plants but also in Fe-sufficient ones (Online Resource S3). Although the leaf application of NH_4^+ on the SPAD readings and Chl (*a*+*b*) concentration was significantly more effective than root application at harvest (Fig. 1c, Online Resource S3), the recovery by root NH_4^+ application was faster (Online Resource S4) and was already detectable from the second day after the NH_4^+ treatment.

The leaf HCl-extractable Fe was increased by NH_4^+ applied through roots and leaves, while total leaf Fe concentration was not significantly affected by NH_4^+ treatments (Fig. 2a, b). In contrast, the Fe concentration in the root apoplast was significantly decreased under NH_4^+ treatment along with total Fe concentration (Fig. 2c, d).

To study the NH_4^+-mediated activation of Fe acquisition mechanisms in the roots, fluorescence imaging was employed for NO localization in the roots of Fe-deficient plants. Results showed an apparent NO accumulation in the root tips of NH_4^+-treated plants, while much less NO was accumulated in the absence of NH_4^+ and could not be detected under the same exposure time (20 ms) (Fig. 3).

A temporal analysis was conducted to uncover the effect of NH_4^+ treatment on the root FRO activity and NO concentration (Fig. 4). Application of NH_4^+ both through roots and leaves increased the root NO levels and FRO activity. The FRO activity reached its maximum 4-6 days after NH_4^+ treatment (8-12 days after beginning of Fe starvation) (Figs 4b, d) while the maximum amount of NO was achieved later, 8-10 days after starting NH_4^+ treatment (12-14 days after –Fe treatment) (Figs 4a, c).

A similar effect of root and leaf NH_4^+ application on the NO level and FRO activity implies a systemic effect of NH_4^+ on the root response to Fe. Therefore, an independent experiment was carried out in a split-root system using both +Fe and –Fe plants. The lowest NO concentration was observed in +Fe plants in the absence of NH_4^+ (+Fe–NH_4^+), while supplementation of +Fe plants with NH_4^+ (+Fe+NH_4^+R) caused a considerable rise in the NO accumulation (Fig. 5a). Like +Fe plants, the lowest NO level in –Fe plants was detected without NH_4^+ (–Fe–NH_4^+). However, it remained significantly higher than the +Fe counterparts (+Fe–NH_4^+). Ammonium supplementation of –Fe plants through leaves (–Fe+NH_4^+L) and roots (–Fe+NH_4^+R+ and –Fe+NH_4^+R–) significantly increased the NO accumulation in the root tips throughout the two-week assay period (Fig. 5a). Root application was more effective in increasing NO concentration in the treated side of the root (–Fe+NH_4^+R+), particularly towards the end of the experiment. The NO concentration in the roots of +NH_4^+L plants was less than the treated side of +NH_4^+R (–Fe+NH_4^+R+) plants but higher than that observed in the untreated side of the +NH_4^+R plants (–Fe+NH_4^+R–) (Fig. 5a).

The activity of FRO remained low in +Fe plants irrespective of the NH_4^+ treatment. In Fe-deficient plants, the activity of FRO was higher than +Fe plants but remained much lower than the NH_4^+-treated counterparts. The root application was more effective in improving root FRO activity even in the untreated side of roots (–Fe+NH_4^+R–) but sharply decreased towards the end of the assay period. In contrast, the leaf application showed less fluctuation and reached the root application treatment levels at the end of the assay period (Fig. 5b).

For investigation of the involvement of ethylene in NH_4^+-mediated root responses, –Fe plants were treated with STS simultaneously with NH_4^+ treatments. The FRO activity was significantly lower in +STS plants than –STS ones (Fig. 6a). Ammonium lowered the pH of the medium despite buffering with 2.0 mM $CaCO_3$ and daily adjustment. The effect of STS, however, was not statistically significant (Fig. 6b). Although the young leaves were green in NH_4^+-treated plants in the absence of STS, they were strongly chlorotic in +STS plants and the leaf SPAD in +NH_4^+ plants did not differ with –NH_4^+ ones (Fig. 6c, d).

The release rate of H^+ from the roots was considerably higher under –Fe conditions and was further elevated by NH_4^+ treatment. Root application was more effective than leaf application in releasing H^+ to the medium (Fig. 7a). Similar to H^+ release, the phenolic exudation was higher in –Fe plants compared with +Fe ones. Ammonium treatment through roots,

however, did not influence the release of phenolics under these conditions. At the same time, leaf NH_4^+ application significantly increased this parameter (Fig. 7b). The concentration of phenolics in the root extract was significantly decreased under Fe deficiency conditions but increased with NH_4^+ supplementation through roots and remained unaffected from NH_4^+ applied to the leaves. The root phenolic concentration of +Fe plants was decreased by NH_4^+ treatment (Online Resource S5).

To examine the influence of NH_4^+-mediated modifications in the rhizosphere chemistry on the Fe mobilization in the rooting medium, –Fe plants were treated with NH_4^+ through roots and leaves for one week and then were transferred to $-NH_4^+$ medium with supply of 50 µM sparingly-soluble $Fe(OH)_3$ (pH 5.8). Three days after $Fe(OH)_3$ resupply, leaf chlorosis almost completely disappeared in NH_4^+-treated plants (Fig. 7c). This was not accompanied by a significant difference in the leaf Chl (*a*+*b*) concentration between both application modes (Fig. 7d).

The activity of PAL was not significantly affected by Fe deficiency but increased by NH_4^+ both in leaf and root applications to –Fe plants (Table 1). The activity of PEPC decreased under Fe deficiency conditions, while NH_4^+ treatment increased its activity slightly. The activity of MDH was not changed by Fe deficiency but was increased by root NH_4^+ application. Contrastingly, the activity of CS, increased under Fe deficiency conditions but decreased by NH_4^+ treatment both in the +Fe and –Fe plants (Table 1).

The concentrations of oxalic acid and malic acid decreased under Fe deficiency conditions while the citric acid concentration increased; succinic acid concentrations remained unaffected under Fe starvation (Fig. 8a, d, g, j). The NH_4^+ treatment did not affect oxalic acid and malic acid levels but decreased the concentration of citric acid (Fig. 8a, d, g). Root application of NH_4^+ caused a significant increase in the root concentration of succinic acid (Fig. 8j). The root exudation of oxalic acid (Fig. 8b, c) and citric acid (Fig. 8h, i) was not affected by Fe starvation in either of the two analyzed time points, while the exudation of malic acid (Fig. 8e) and succinic acid (Fig. 8l) increased in the first and second-time points, respectively. The NH_4^+ treatment did not influence or decrease the exudation of organic acids except for citric acid. Leaf application of NH_4^+ increased the citric acid exudation up to 230-fold (Fig. 8h, i).

The expression of the critical genes responsible for plants' Fe responses was investigated under Fe deficiency without and with NH_4^+ treatment. The expression levels (fold changes) of

AHA1, *FRO2,* and *IRT1* were elevated by Fe starvation at both analyzed time points (Fig. 9). Root supply of NH_4^+ to –Fe plants increased the expression of *FRO2* but did not change the expression of *AHA1* and decreased the expression of *IRT1*. However, the expression levels of *AHA1* and *IRT1* remained considerably higher than in +Fe plants at both time points. Leaf application of NH_4^+ consistently increased the expression level of *AHA1*, *FRO2,* and *IRT1* at both analyzed time points (Fig. 9). In contrast to *AHA1*, *AHA2* was not upregulated under Fe deficiency conditions; instead its expression decreased at the second time point. Foliar NH_4^+ decreased *AHA2* expression at the first time point in –Fe plants, but root NH_4^+ supply slightly enhanced *AHA2* expression at the second time point (Online Resource S6).

The expression level of *EIN3* was higher under Fe starvation, and the NH_4^+ treatments elevated this parameter at both analyzed time points without significant difference between the two application modes (Fig. 10a, b). The expression of *FIT* was increased under Fe deficiency conditions, and it was further elevated by NH_4^+ treatment; the leaf NH_4^+ application was significantly more effective than the root NH_4^+ application (Fig. 10c, d). Similarly, the expression of *STOP1* was increased in Fe-starved plants. However, a significant effect of NH_4^+ treatment in –Fe plants was observed only by root NH_4^+ application (Fig. 10e, f).

Discussion

In addition to being the source of reduced N required for amino acid structure and cellular components, NO_3^- and NH_4^+ also are signaling molecules regulating gene expression and downstream regulatory pathways (Guan 2017). This signaling role of NO_3^- regulates seed germination, seedling establishment, leaf growth, shoot branching, root system architecture, and flowering time (Vidal et al. 2020). More limited is the evidence for a signaling role of NH_4^+. Most transcriptomic and proteomic studies in this context have been conducted with NH_4^+ as the sole N source or under high NH_4^+ concentrations that induce NH_4^+ toxicity responses in plants (Shilpha et al. 2023). In the few studies working with low NH_4^+ concentrations (1-3 mM) as the sole N source, 'ammonium-specific' gene sets have been identified (Patterson et al. 2010), and distinct phosphorylation patterns have been observed for proteins with signaling functions, transcription factors, and transporters (Engelsberger and Schulze 2012). However, poor information on NH_4^+-specific responses in NO_3^--supplied plants is available under nutritional stress conditions, including Fe deficiency.

In this study, pH-buffering of the nutrient medium could not completely hamper NH_4^+-mediated increase of H^+ abundance in the root apoplast. However, NH_4^+ also mediated numerous biochemical and molecular changes that are characteristic for Fe-starvation responses. This indicates that NH_4^+, in addition to root apoplast acidification, favors tolerance to Fe deficiency by enhancing the inherent responses of plants to Fe deficiency.

The involvement of NO and FRO in the NH_4^+-mediated improvement of Fe nutrition

Nitric oxide mediates the Fe deficiency response in Strategy I plants as a signaling molecule that increases rapidly in the roots of plants grown under Fe-deficient conditions (Graziano and Lamattina 2007). Both DAF staining (Fig. 3) and direct biochemical assay of NO (Figs 4a, c, 5a) showed NH_4^+-mediated NO accumulation in Fe-deficient plants. At the same time point (four days after NH_4^+ treatment), NO could not be detected in the absence of NH_4^+ using the same exposure time (Fig. 3b) or was significantly lower than $+NH_4^+$ plants in the direct assay (Fig. 4a, c). However, the pattern of increase in the endogenous level of NO in Fe-deficient plants was different from that of FRO activity (Figs 4, 5) suggesting the lack of a direct and robust association between NO level and FRO activity. Recent evidence highlighted the contribution of S-nitrosoglutathione (GSNO) instead of NO to the regulation of Fe deficiency responses (Kailasam et al. 2018; Romera et al. 2023). Determining GSNO was not within the scope of this study. However, the fact that NO and GSNO have different lifetimes and different time courses of abundance after Fe starvation (Romera et al. 2023), may indirectly explain the absence of a strong correlation between temporal curves of NO levels and FRO activity in our work.

Ammonium did not restrict NO production in Fe-deficient plants (Fig. 5a). However, the enhanced NO level was not associated with an elevated activity of FRO (Fig. 5b) or the expression of *FIT* (Fig. 10c, d) in Fe-sufficient plants. It is known that Fe is a negative regulator of gene expression that acts at sites downstream to the site of action of NO and controls the expression of proteins involved in its uptake at the transcriptional and post-transcriptional levels (Brumbarova and Bauer 2005). When Fe is available, FIT is degraded through poly-ubiquitination by haemoerythrin RING-type E3 ligase BRUTUS-LIKE PROTEINS (BTSLs) (Hindt et al. 2017; Riaz and Guerinot 2021). Thus, posttranslational control of FIT is critical to prevent excess Fe uptake. Fe-sufficient plants are not capable of accumulating *FER* (*FIT*), *FRO1*,

and *IRT1* transcripts in response to GSNO application in tomato (Graziano and Lamattina 2007) or by NO-inducing treatments (putrescine) in Arabidopsis (Zhu et al. 2016).

There is evidence of the effect of NO on plants' Fe homeostasis independent from the Fe acquisition machinery of reduction-based strategy. Nitric oxide is involved in increasing the availability of endogenous Fe, as was observed in maize (Graziano et al. 2002), and facilitates the delivery of Fe between and within plant cells through the formation of Fe–nitrosyl complexes (Vanin et al. 2004; Graziano and Lamattina 2005 2007) or a direct positive effect on chlorophyll biosynthesis (Zhang et al. 2006). Here, the NH_4^+-mediated increase in the higher SPAD readings and Chl concentration even in the +Fe plants (Fig. 1c; Online Resource S3) and higher concentration of leaf HCl-extractable Fe (Fig. 2a) was at least partly attributable to the direct role of NO in the increasing cellular Fe availability in the leaves.

The mechanism for NH_4^+-mediated NO production that could be converted to GSNO is obscure. Diamines (putrescine) and polyamines (spermidine and spermine) could be possibly involved. The accumulation of putrescine and spermidine by low NH_4^+ concentrations (1.0 and 5.0 mM) has been documented (Urra et al. 2022). The role of spermidine, spermine (Tun et al. 2006), and putrescine (Zhu et al. 2016) in triggering a NO burst has been demonstrated. Elevated activity of NR, as one of the critical NO producers in plants (Rockel et al. 2002), is another probable mechanism. However, the root activity of NR was not affected by NH_4^+ in cucumber plants (Online Resource S7). Although the cellular level of NH_4^+ was increased in the roots by NH_4^+ application (Online Resource S8), it could not be the triggering factor for NH_4^+-mediated elevation of Fe deficiency responses in this work because it could not explain the difference between two application modes in the –Fe plants or the Fe-deficiency response of $–NH_4^+$ plants (Online Resource S8). However, ethylene is a putative factor that likely mediates the NH_4^+ effect through transcriptional and posttranslational regulation of NO/GSNO biosynthetic genes, as discussed below.

The involvement of ethylene in the NH_4^+-mediated improvement of Fe nutrition

Ethylene and NO act in conjunction and positively regulate each other's production (Romera et al. 2023). Although ethylene or its precursors were not measured, the gene expression of its signaling component (Fig. 10) and the result of the application of STS (Fig. 6) that abolished the NH_4^+ effect suggest that NH_4^+ acted through ethylene. Ethylene is required for full-level FIT

accumulation and protects FIT from proteasomal degradation (Lingam et al. 2011). The mechanism for NH_4^+-mediated increase in ethylene production or signaling is obscure. Numerous observations revealed that both sub-optimal (Lingam et al. 2011) and supra-optimal supply of N as NO_3^- (10-40 mM) (Nandwal et al. 2000) or NH_4^+ (>10 mM) (Li et al. 2019) stimulate ethylene biosynthesis and/or signaling (Ma et al. 2023). Under moderate levels of N supply, however, sole NH_4^+ (5 mM) promotes while sole NO_3^- (5 mM) inhibits ethylene evolution by regulating ethylene biosynthetic genes (Chen et al. 2021). In this work, the expression of *EIN3* was additionally upregulated by NH_4^+ in –Fe cucumber plants (Fig. 10a, b), confirming the involvement of elevated endogenous ethylene in $+NH_4^+$ plants. In addition, a synergistic relationship observed between NO and ethylene synthesis and signaling (García et al. 2011; Romera et al. 2023) suggests that NH_4^+-mediated increase in the endogenous level and/or signaling of ethylene and NO were mutually augmented in cucumber plants.

Synthesis, concentration and release of phenolics in roots

Upregulation of genes encoding the phenolics biosynthetic enzymes such as PAL (Yang et al. 2010) and feruloyl-CoA 6'-hydroxylase (Rodríguez-Celma et al. 2013) and those encoding for efflux transporters, e.g., PLEIOTROPIC DRUG RESISTANCE 9 (PDR9), is regulated through the core pathway of FIT (Rodríguez-Celma et al. 2013; Schmidet al. 2014; García et al. 2021). Ethylene also regulates these genes through EIN3/EIL1 (García et al. 2021). In this work, an increase in the activity of PAL in Fe-deficient plants required NH_4^+, but there was no difference between leaf and root applications (Table 1). Nevertheless, a reciprocal relationship was observed for the content and release of phenolics: a significant increase in the root content (Suppl data) was observed only in $+NH_4^+$R plants, while the release of phenolics was enhanced only in $+NH_4^+$L ones. Higher activity of the upstream components governing phenolics exudation, e.g., FIT (Fig. 10c, d), could likely be responsible for higher phenolics release in $+NH_4^+$L compared with $+NH_4^+$R counterparts (Fig. 7b).

Synthesis, concentration and release of organic acids in roots

Modifications in the activity of PEPC and the tricarboxylic acid (TCA) cycle enzymes under Fe deficiency conditions have been frequently reported in diverse Strategy I plants (reviewed by Vigani 2012). Here, the activity of CS increased by Fe deficiency, which was in agreement with

other reports (López-Millán et al. 2009), while the activity of PEPC was decreased under these conditions that contrasted with the observations on various plant species, including cucumber (López-Millán et al. 2009). This is most likely related to the severe Fe deficiency at the time of analysis, i.e., 17 days after omitting Fe from the nutrient solution in cucumber plants compared to those used in other studies. In agreement with this assumption, both increase (López-Millán et al. 2009) and decrease (Rodríguez-Celma et al. 2011) in the activity of mitochondrial respiratory chain enzymes have been reported under Fe starvation, which has been attributed to different degrees of Fe deficiency in the studied plants (Vigani 2012). Irrespective of the mechanism, the changes in the activities of PEPC, MDH, and CS (Table 1) correlated well with the root contents of oxalate, malate, and citrate (Fig. 8) in Fe-deficient cucumber plants.

One or both NH_4^+ application modes caused an increase in the activities of PEPC and MDH, slightly or significantly, while decreasing the activity of CS (Table 1). The activation of PEPC and MDH by NH_4^+ was expected because of the frequently reported upregulation of TCA enzymes by NH_4^+ as the significant source of carbon skeletons for NH_4^+ assimilation (Foyer et al. 2011). Despite the different and unexpected response of CS activity to NH_4^+, it correlated well with the reduction of citrate tissue content in $+NH_4^+R$ plants. In $+NH_4^+L$ plants, however, lower citrate content in $+NH_4^+L$ plants was most likely the consequence of its higher release rate into the medium (Fig. 8), similar to that observed for phenolics (Fig. 7b and Online Resource S5).

The release of TCA cycle-related organic acids to the rhizosphere in Strategy I plants is crucial for the chelation and mobilization of Fe, particularly the sparingly soluble Fe^{III} in the rhizosphere, and making it accessible to plants (Abadía et al. 2002; Molnár et al. 2023). In contrast to the release mechanisms of phenolic compounds, the involvement of the FIT pathway in regulating the related efflux transporters, e.g., THE ALUMINIUM-ACTIVATED MALATE TRANSPORTERS (ALMT) and MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE), has not yet been demonstrated. The data, however, suggest a probable role for STOP1 (see below). In addition to the role of citrate in chelating Fe^{III} in the rhizosphere, its complex with Fe^{III}, as the dominant form of Fe moving in the xylem, hinders its precipitation during root-shoot Fe transport (Rellán-Álvarez et al. 2010). Citrate export into the xylem is mediated by FERRIC REDUCTASE DEFECTIVE 3 (FRD3) (Durrett et al. 2007). Although FRD3 is not downstream of FIT (Sivitz et al. 2012), its full induction under Fe deficiency requires ethylene (García et al. 2010).

Overall, the enhanced release of H^+, phenolics, and citrate by one or both NH_4^+ treatments could contribute to a significant reduction of Fe in the root apoplast observed in cucumber plants (Fig. 2c) and may also be of high significance for soil-grown plants.

The activation of Strategy I-related genes by NH_4^+

The expression levels of three Fe acquisition components of Strategy I, *AHA1*, *FRO2,* and *IRT1*, and that of upstream regulatory components, *EIN3* and *FIT*, all were expectedly upregulated under Fe deficiency, were additionally enhanced by NH_4^+ when applied to the leaves. The effect of root-applied NH_4^+ on the expression of *FRO2*, *EIN3,* and *FIT* was similar or less than the leaf application of NH_4^+, while *AHA1* expression was not affected, and root-applied NH_4^+ somewhat decreased that of *IRT1* compared with $-NH_4^+$ counterparts. The higher effect of leaf application compared with root application may be related to the gradual application of NH_4^+ that lasted almost the whole experiment period. At the same time, the applied NH_4^+ to the nutrient solution may be taken up and rapidly metabolized within the plant. A rapid and efficient amelioration of Fe-deficiency, as was reflected in faster re-greening of $+NH_4^+R$ compared with $+NH_4^+L$ plants (Online Resource 4), may also be in part responsible for the downregulation of *AHA1* and *IRT1* in $+NH_4^+R$ compared with $-Fe-NH_4^+$ plants. This assumption was also reflected in the faster and more potent effect of root NH_4^+ application in the elicitation of FRO activity observed in the split root system (Fig. 5b).

Apart from the difference between the two application modes, a higher expression rate mediated by NH_4^+, sustained even two weeks after NH_4^+ application (about 17 days after Fe starvation), despite considerable mitigation of leaf chlorosis in NH_4^+-treated plants compared with $-NH_4^+$ counterparts. Since the Fe was omitted entirely from the nutrient solution of –Fe plants, enhanced mobilization of Fe in the root apoplast (Fig. 2c) and increased Fe availability in the leaves (Fig. 2a) were most likely insufficient for activating Fe sensing molecules.

Irrespective of the difference between $+NH_4^+$ and $-NH_4^+$ plants, the elevated expression level of studied genes in –Fe plants lasted for over two weeks without considerable donwregulation, except for FRO. The expression of *FRO* was diminished in the second week of analysis (Fig. 10d), associated with the same descending trend of the FRO activity (Figs 4 and 5). The downregulation of *FRO2* was observed despite a continuously higher expression of *FIT*, the upstream regulatory molecule that coordinately regulates *FRO2* and *IRT1*. This suggests a

transcriptional control of *FRO* under a long-term Fe starvation that does not act for *IRT1*. A post-transcriptional control of *FRO2*, distinct from *IRT1*, has been postulated (Connolly et al. 2003), while a distinct control at the transcriptional level needs further confirmation.

The SENSITIVE TO PROTON RHIZOTOXICITY 1 (STOP1), a C2H2 zinc finger transcription factor, is constitutively expressed (Sawaki et al. 2009; Li and Tian 2023) and regulated mainly through post-transcriptional modifications (Godon et al. 2019). STOP1 has pleiotropic roles and acts mainly through CBL-INTERACTING PROTEIN KINASE 23 (CIPK23) (Tian et al. 2021), whose mutations (*lks*) render the plants hypersensitive to Fe deficiency (Tian et al. 2016). However, the interaction of STOP1 with Strategy I components has not been explored so far. The protein abundance of STOP1 is increased by 0.1-2.5 mM NH_4^+ when combined with phosphorus deficiency or low pH, while the expression of STOP1 is not affected by NH_4^+ (Tian et al. 2021). In this work, however, STOP1 showed enhanced expression level under Fe deficiency, further augmented by NH_4^+, suggesting a synergistic effect of NH_4^+ and Fe deficiency on its expression. This is the first report on the upregulation of *STOP1* expression under Fe deficiency and low levels of NH_4^+; however, probable post-transcriptional modifications of *STOP1* under the applied treatments in this work are obscure. Since the stability of STOP1 in the root tips requires Fe^{3+}/Fe^{2+} and H^+ ions (Godon et al. 2019), the increased availability/level of these ions by NH_4^+ observed in this study may also contribute to STOP1 stability under experimental conditions. The increased *STOP1* expression may be, at least partly, the factor responsible for the enhanced release rate of malate (in –Fe–NH_4^+ plants) and citrate (in +NH_4^+L ones) observed in this research. In Arabidopsis, STOP1 regulates the transcription of genes encoding malate (*AtALMT1*) and citrate (*AtMATE*) transporters (Sawaki et al. 2009).

Conclusion

Using a low NH_4^+ concentration applied to the plants previously and simultaneously grown in a NO_3-based medium indicates a signaling function of low NH_4^+ (2 mM) levels beyond its nutritional role, H^+ release effect, and/or toxicity of NH_4^+ when used as the sole N source.

The cellular NH_4^+ level in the leaves and roots was not correlated with the extent to which the root and leaf application of NH_4^+ affected the analyzed Fe deficiency markers, suggesting the contribution of the downstream molecule(s) generated upon NH_4^+ treatment in the leaves and roots that moved in the xylem and phloem and guarantee(s) an augmented and long-term

response to Fe deficiency. The nature of such signal is unknown, but NO/GSNO, ethylene, polyamines, or H^+ (through STOP1 or NO) are the probable candidates. The difference between root and leaf application and the results of temporal analyses suggested that a sustained upregulation of Fe-responsive genes needs the permanent presence of NH_4^+ with concentrations even lower than 2 mM.

Although the absence of any Fe source in the –Fe medium in this work did not allow a significant increase in the leaf Fe status, NH_4^+ application in soil-grown plants is expected to considerably improve the Fe acquisition and mobilization in the root apoplast and its transport into shoot. The results showed the superior effect of leaf spray of NH_4^+ over its root application because the former induces a long-lasting response that could be applied throughout the growth season without interaction with soil components and/or loss through microorganisms.

Declarations

Compliance with ethical standards

Conflict of Interest The authors have no competing interests to declare that are relevant to the content of this article.

Data Availability Statement Data will be available upon request.

Author contributions

Roghieh Hajiboland conceived the study, analyzed data and wrote the original draft; Miroslav Nikolic and Charlotte Poschenrieder conceived the study and reviewed and edited the manuscript; Fatemeh Tavakoli cultivated the plants, performed the elemental and biochemical analyses; Dragana Bosnic & Predrag Bosnic performed the gene expression analyses; Roser Tolra analyzed the organic acids. All authors read and approved the final manuscript.

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Table 1 The activity of phenylalanine ammonia lyase (PAL) (nmol mg^{-1} pr min^{-1}), phosphoenolpyruvate carboxylase (PEPC) (μmol mg^{-1} pr min^{-1}), malate dehydrogenase (MDH) (nmol mg^{-1} pr min^{-1}) and citrate synthase (CS) (nmol mg^{-1} pr min^{-1}) in the **root** tips of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated for four days without iron (–Fe) or with adequate supply of iron (+Fe) in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) through roots ($+NH_4R$) or leaves ($+NH_4L$) simultaneous with different Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for two weeks

		PAL	PEPC
+Fe	$–NH_4$	12.6±1.28 [c] *	698±137 [a]
	$+NH_4R$	13.7±0.85 [bc]	562±174 [ab]
–Fe	$–NH_4$	12.7±0.75 [c]	399±36 [b]
	$+NH_4R$	16.3±0.21 [a]	472±51 [ab]
	$+NH_4L$	14.9±0.42 [ab]	562±128 [ab]
		MDH	CS
+Fe	$–NH_4$	181±29 [b]	120±10 [b]
	$+NH_4R$	187±37 [b]	77±17 [c]
–Fe	$–NH_4$	148±31 [b]	172±19 [a]
	$+NH_4R$	326±26 [a]	79±12 [c]
	$+NH_4L$	151±44 [b]	72±29 [c]

* Data are mean ± SD (n = 4). Differences among data of each parameter indicated by different letters are statistically significant ($P < 0.05$)

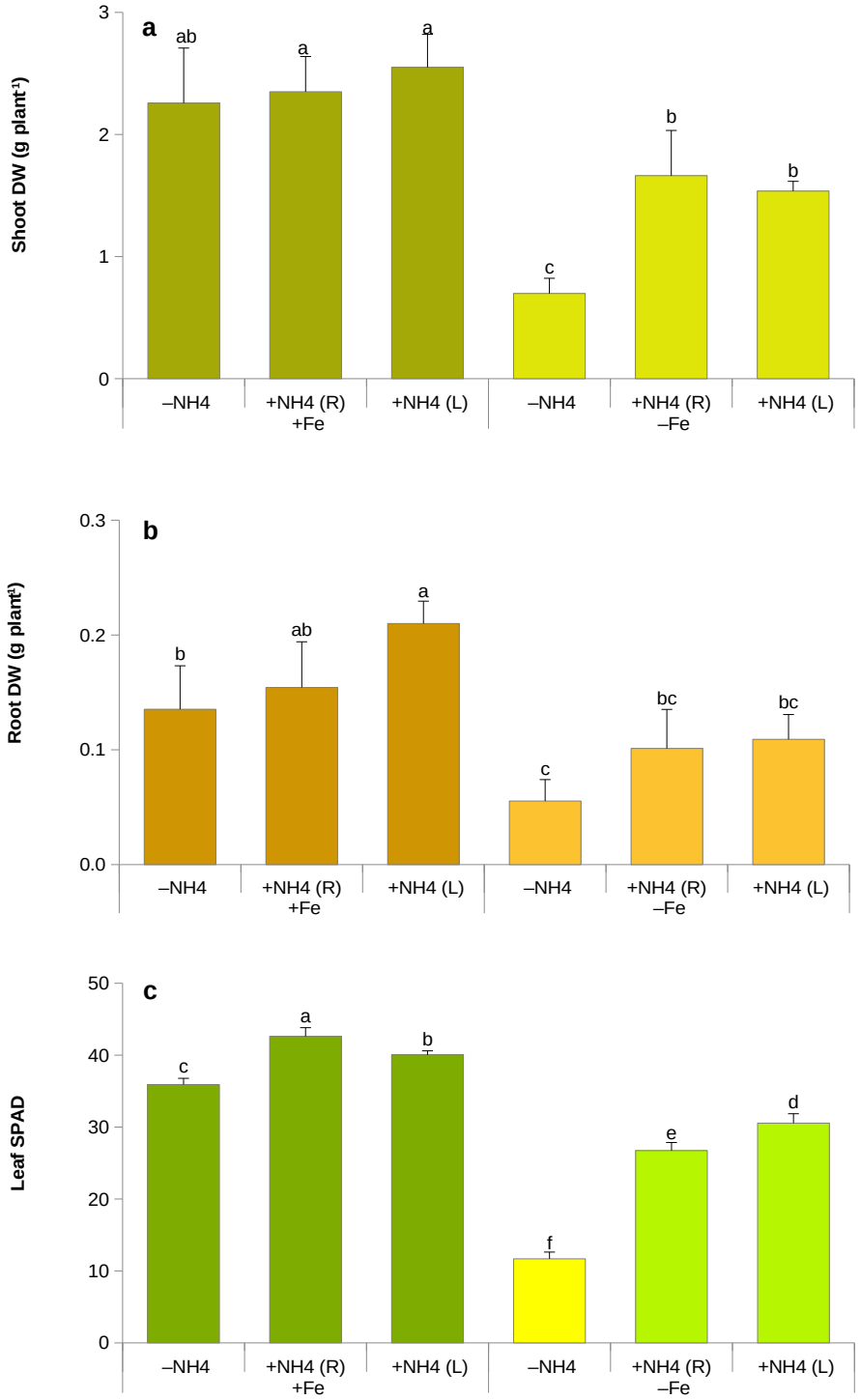


Fig. 1 Shoot (**a**) and root (**b**) biomass and leaf SPAD (**c**) in cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated for four days without iron (–Fe) or with adequate supply of iron (+Fe) in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) through roots ($+NH_4R$) or leaves ($+NH_4L$) simultaneous with different Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for two weeks. Data are mean ± SD (n = 4). Differences among bars indicated by different letters are statistically significant ($P < 0.05$)

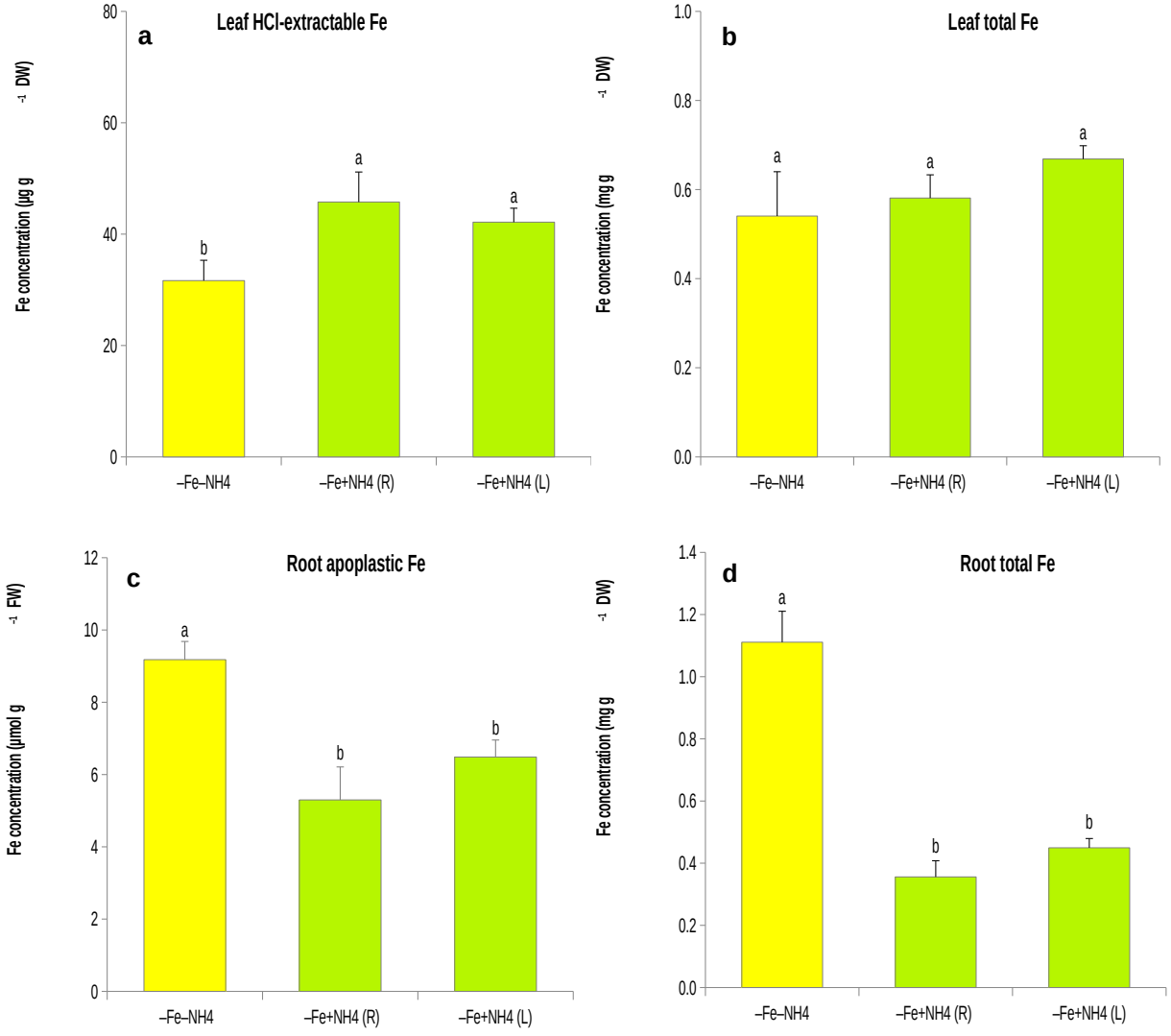


Fig. 2 The concentration of HCl-extractable Fe (**a**) and total Fe (**b**) in the young leaves, apoplastic Fe (**c**) and total Fe (**d**) in the roots of cucumber (*Cucumis sativus* L. cv. Maximus) plants grown for four days without Fe supply in the $–NH_4^+$ medium, then were treated with NH_4^+ applied through roots ($+NH_4R$), through leaves ($+NH_4L$) or left untreated ($–NH_4$) simultaneous with −Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for two weeks. Data are mean ± SD (n = 4). Differences among bars indicated by different letters are statistically significant ($P < 0.05$)

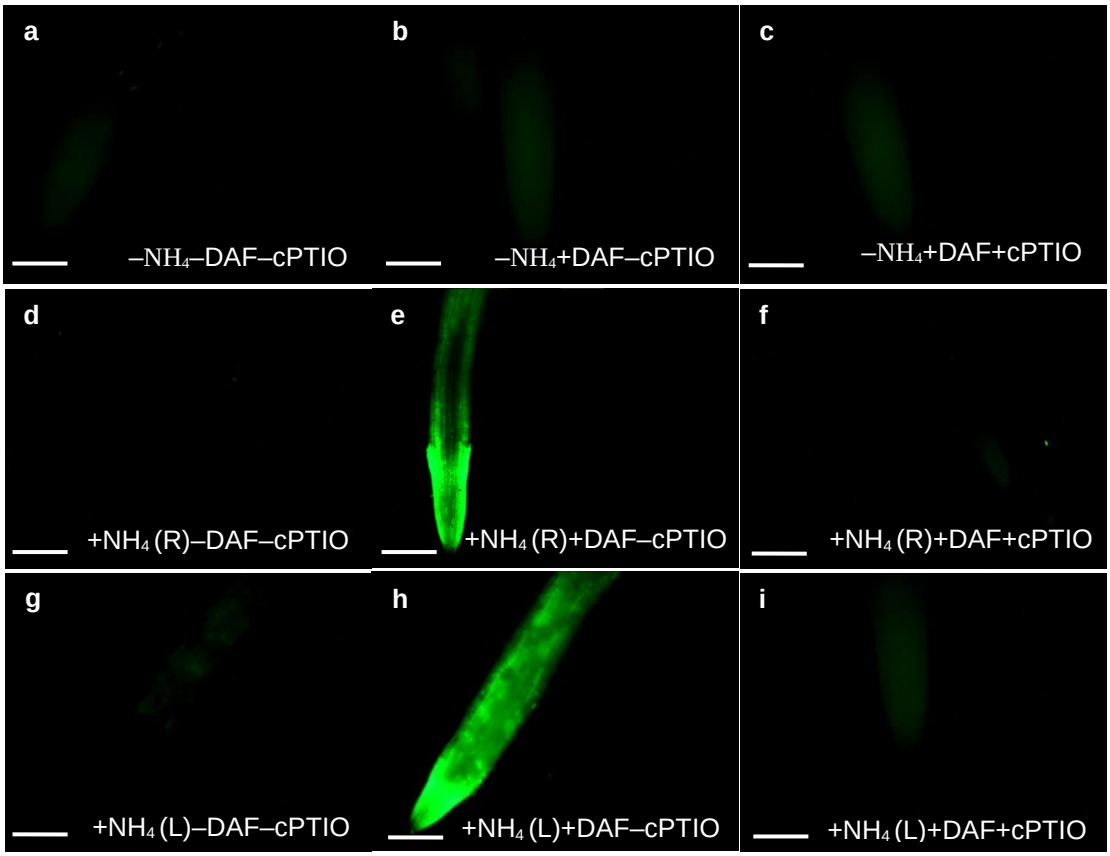


Fig. 3 Nitric oxide (NO) detection using DAF staining in the root tips of cucumber (*Cucumis sativus* L. cv. Maximus) plants grown for four days without Fe supply in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) (**a-c**) and with 2 mM NH_4^+ (as NH_4Cl) through roots ($+NH_4R$) (**d-f**) or leaves ($+NH_4L$) (**g-i**) simultaneous with –Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for four days. cPTIO (NO scavenger) was used as negative control (**c, f, i**). The exposure time was similar (20 ms) for all pictures. Scale bar indicates 200 µm

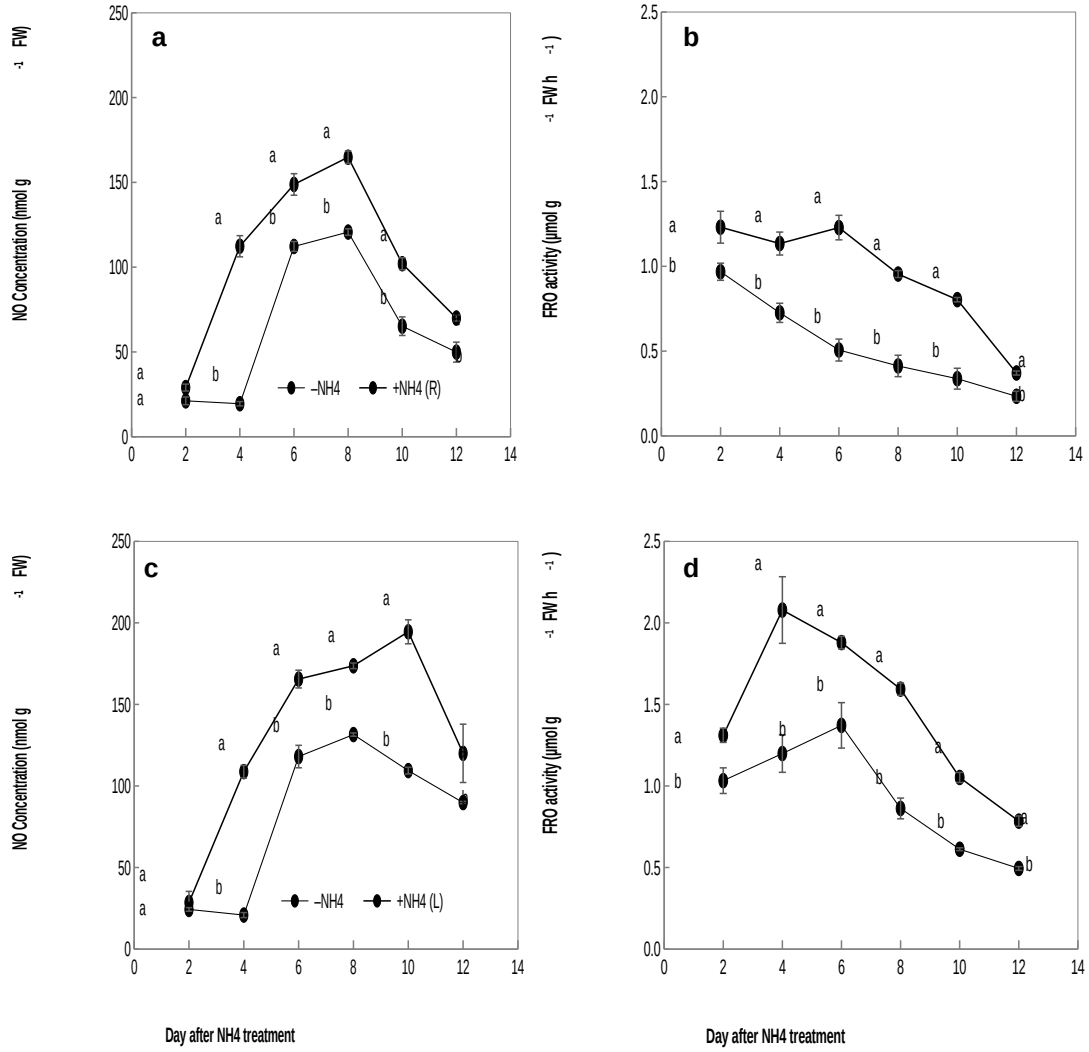


Fig. 4 Temporal changes in the root concentration of nitric oxide (NO) (**a, c**) and activity of ferric reductase oxidase (FRO) (**b, d**) in cucumber (*Cucumis sativus* L. cv. Maximus) plants grown for four days without Fe supply in the $–NH_4^+$ medium, then were treated with NH_4^+ applied through roots ($+NH_4R$), through leaves ($+NH_4L$) or left untreated ($–NH_4$) simultaneous with −Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for two weeks. In the leaf application treatment $–NH_4$ plants were sprayed with distilled water. Data are mean ± SD (n = 4). Differences between data of each time point indicated by different letters are statistically significant (t-test, $P < 0.05$)

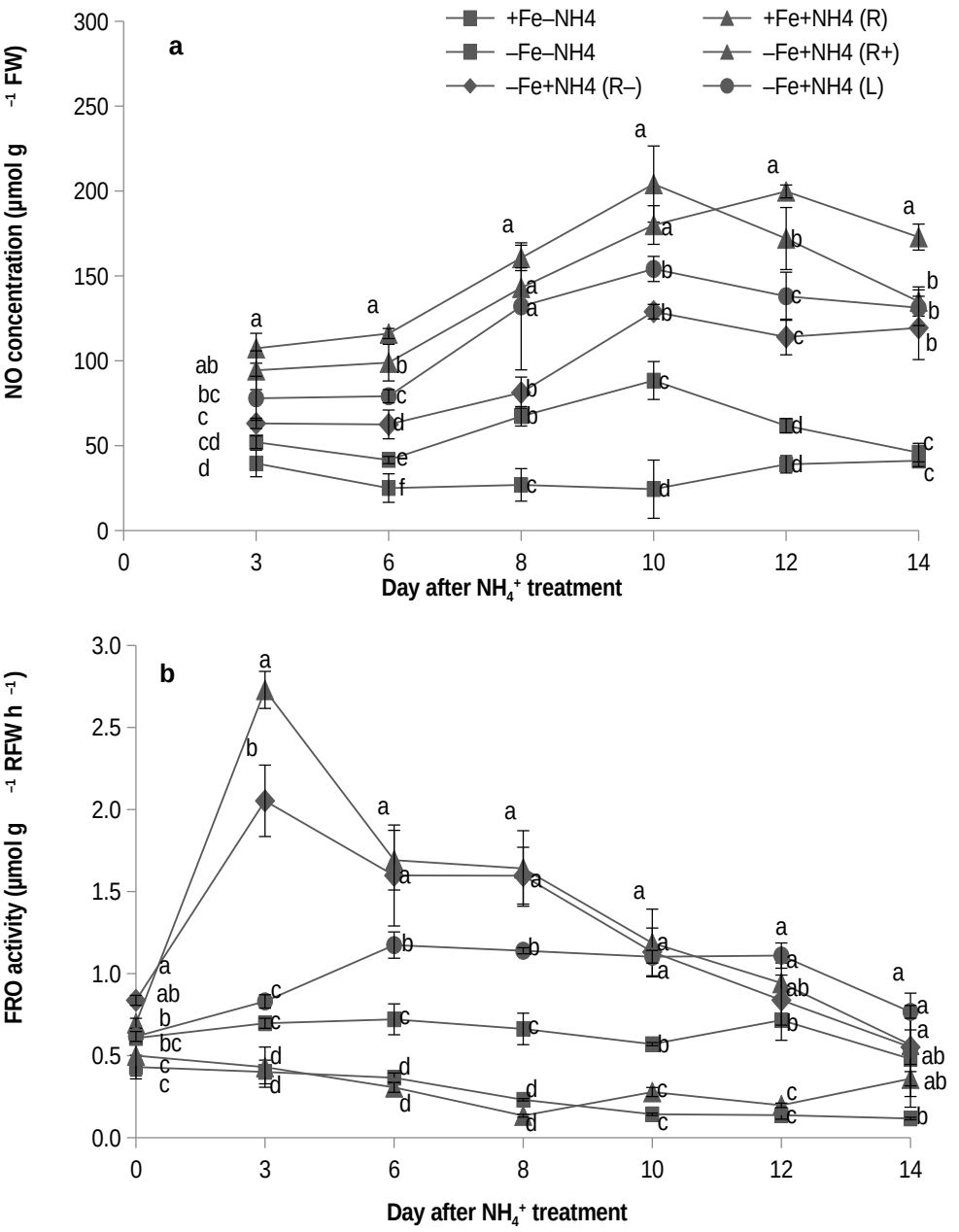


Fig. 5 Temporal changes in the root concentration of nitric oxide (NO) (**a**) and activity of ferric reductase oxidase (FRO) (**b**) in cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated under Fe-sufficient (+Fe) or Fe-deficient (–Fe) conditions in the $-NH_4$ medium, then were treated with 2 mM NH_4 (as NH_4Cl) through roots ($+NH_4R+$ and $+NH_4R–$), through leaves ($+NH_4L$) or left untreated ($–NH_4$) simultaneous with Fe treatments in a split-root system for two weeks. The treated root side was designated as $+NH_4R+$ while the un-treated root side was defined as $+NH_4R–$. Data are mean ± SD (n = 4). Differences among data of each time point indicated by different letters are statistically significant ($P < 0.05$)

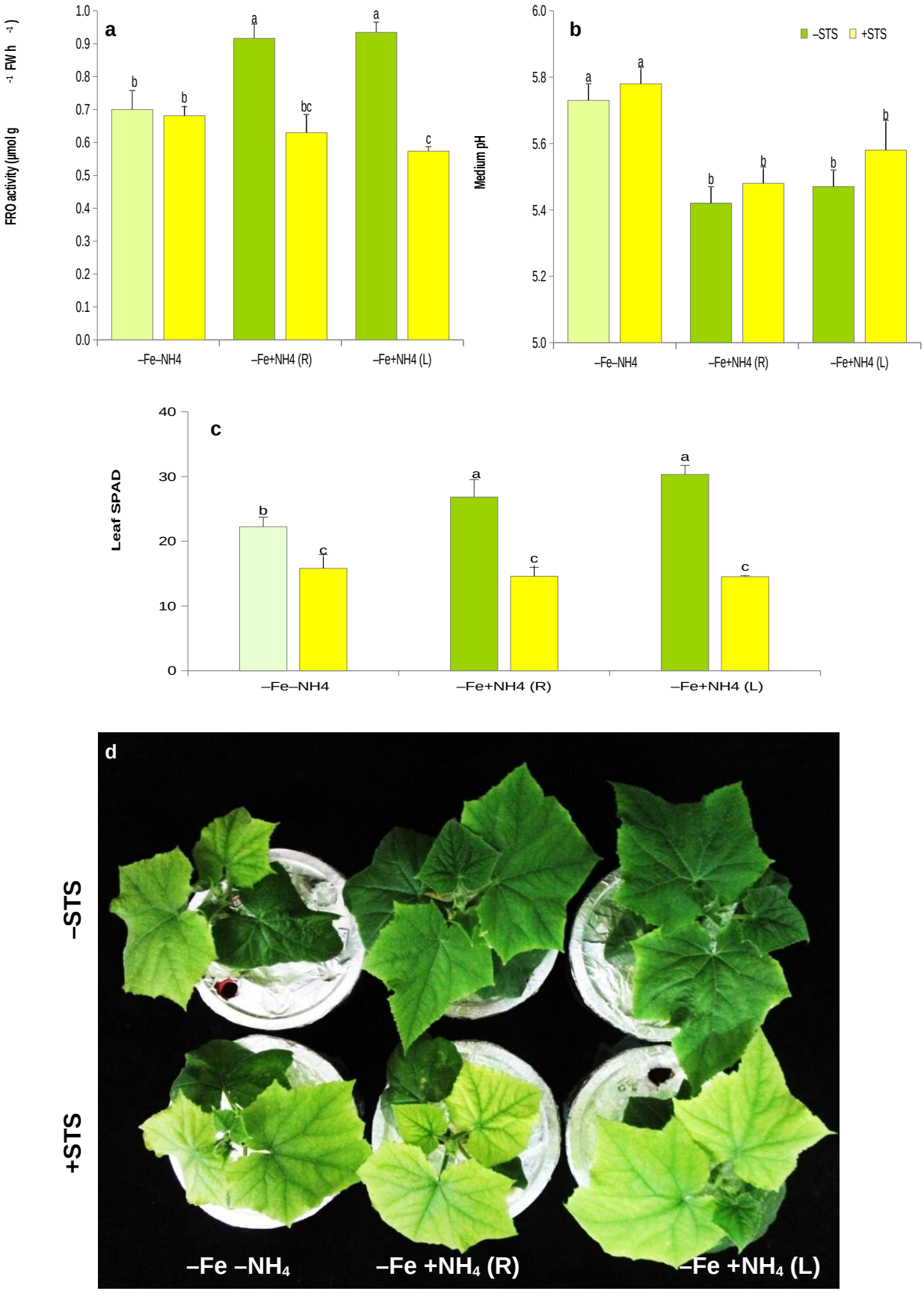
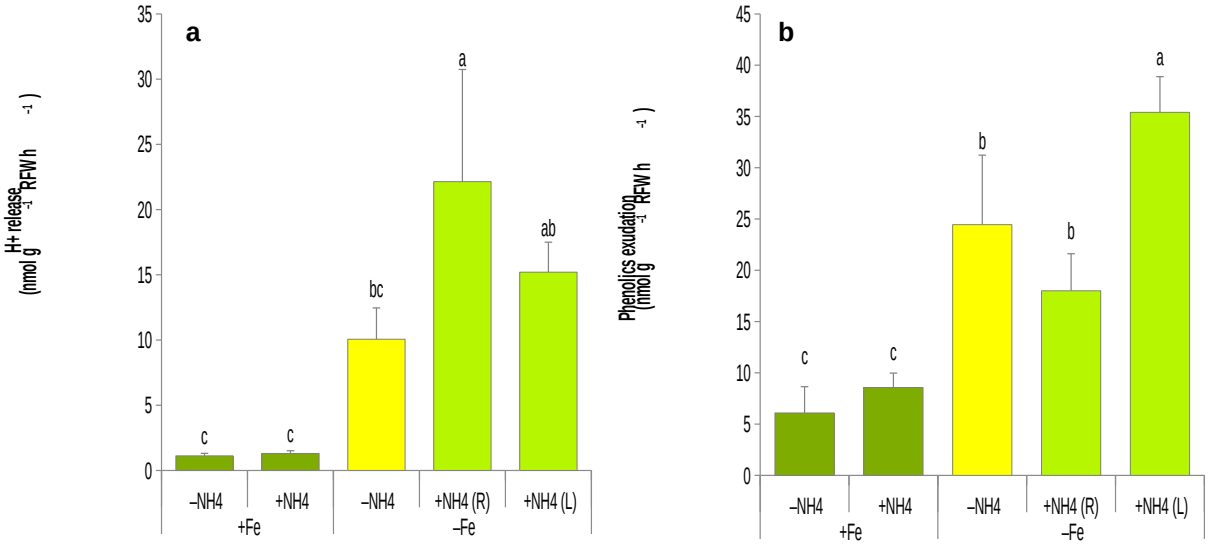
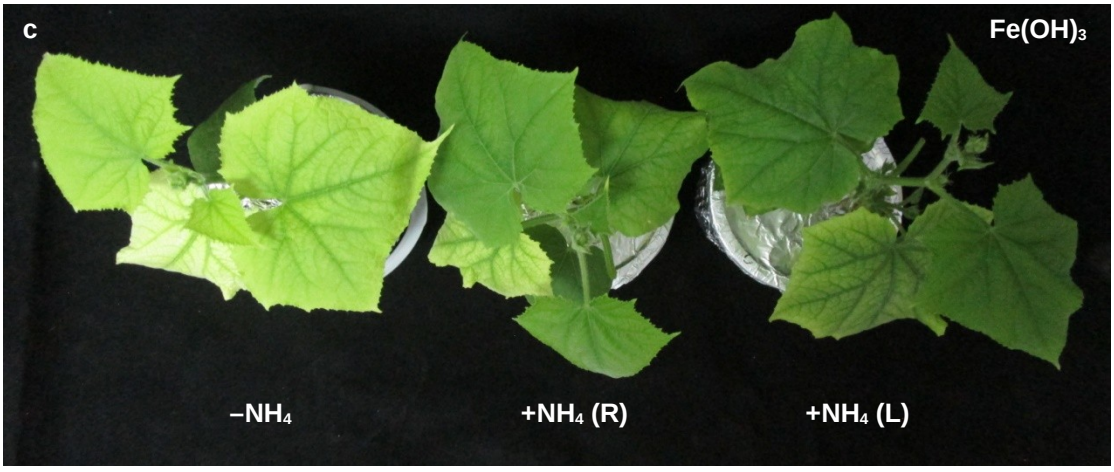

a
FRO activity (µmol g -1 FW h -1)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
b
b
a
bc
a
c
–Fe–NH4
–Fe+NH4 (R)
–Fe+NH4 (L)
b
Medium pH
6.0
5.8
5.6
5.4
5.2
5.0
–STS
+STS
a
a
b
b
b
b
–Fe–NH4
–Fe+NH4 (R)
–Fe+NH4 (L)
c
Leaf SPAD
40
30
20
10
0
b
c
a
c
a
c
–Fe–NH4
–Fe+NH4 (R)
–Fe+NH4 (L)
d
–STS
+STS
–Fe –NH4
–Fe +NH4 (R)
–Fe +NH4 (L)

Fig. 6 The root activity of ferric reductase oxidase (FRO) (**a**) daily reduction of pH in the medium (buffered with 2 mM $CaCO_3$ and adjusted daily to 5.8) (**b**) and leaf SPAD (**c**) in cucumber (*Cucumis sativus* L. cv. Maximus) plants (d) grown for four days without Fe supply in the $–NH_4^+$ medium, then were treated with NH_4^+ applied through roots ($+NH_4R$), through leaves ($+NH_4L$) or left untreated ($–NH_4$) in the absence or presence of 400 µM silver thiosulfate (STS) simultaneous with −Fe treatment for one week. Data are mean ± SD (n = 4). Differences among bars indicated by different letters are statistically significant ($P < 0.05$)


a
H+ release (nmol g-1 RFW h-1)
35
30
25
20
15
10
5
0
c
c
bc
a
ab
–NH4
+NH4
–NH4
+NH4 (R)
+NH4 (L)
+Fe
–Fe
b
Phenolics exudation (nmol g-1 RFW h-1)
45
40
35
30
25
20
15
10
5
0
c
c
b
b
a
–NH4
+NH4
–NH4
+NH4 (R)
+NH4 (L)
+Fe
–Fe


c
Fe(OH)3
–NH4
+NH4 (R)
+NH4 (L)

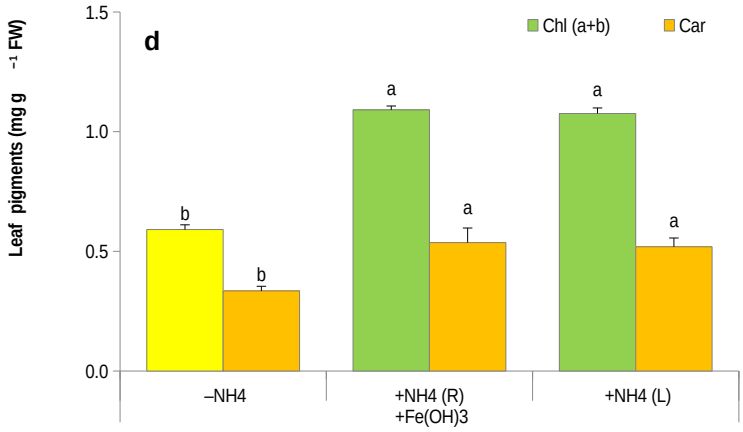

d
Chl (a+b)
Car
Leaf pigments (mg g-1 FW)
1.5
1.0
0.5
0.0
b
b
a
a
a
a
–NH4
+NH4 (R)
+Fe(OH)3
+NH4 (L)

Fig. 7 The root release rate of H^+ (**a**) and phenolic compounds (**b**) in cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated under Fe-sufficient (+Fe) or Fe-deficient (–Fe) conditions in the $-NH_4$ medium, then were treated with 2 mM NH_4 (as NH_4Cl) through roots ($+NH_4R$), through leaves ($+NH_4L$) or left untreated ($-NH_4$) simultaneous with Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for one week. Lower panel (**c**) shows the Fe-deficient plants cultivated for one week without or with NH_4^+ supply through roots ($+NH_4R$) or leaves ($+NH_4L$) in $CaCO_3$-buffered nutrient solution, then were transferred to the $-NH_4$ nutrient solution (pH 5.8) with supply of 50 μM sparingly soluble $Fe(OH)_3$ for three days. The leaf chlorophyll (*a*+*b*) and carotenoids (Car) concentrations (**d**). Data are mean ± SD ($n = 4$). Differences among bars indicated by different letters are statistically significant ($P < 0.05$).

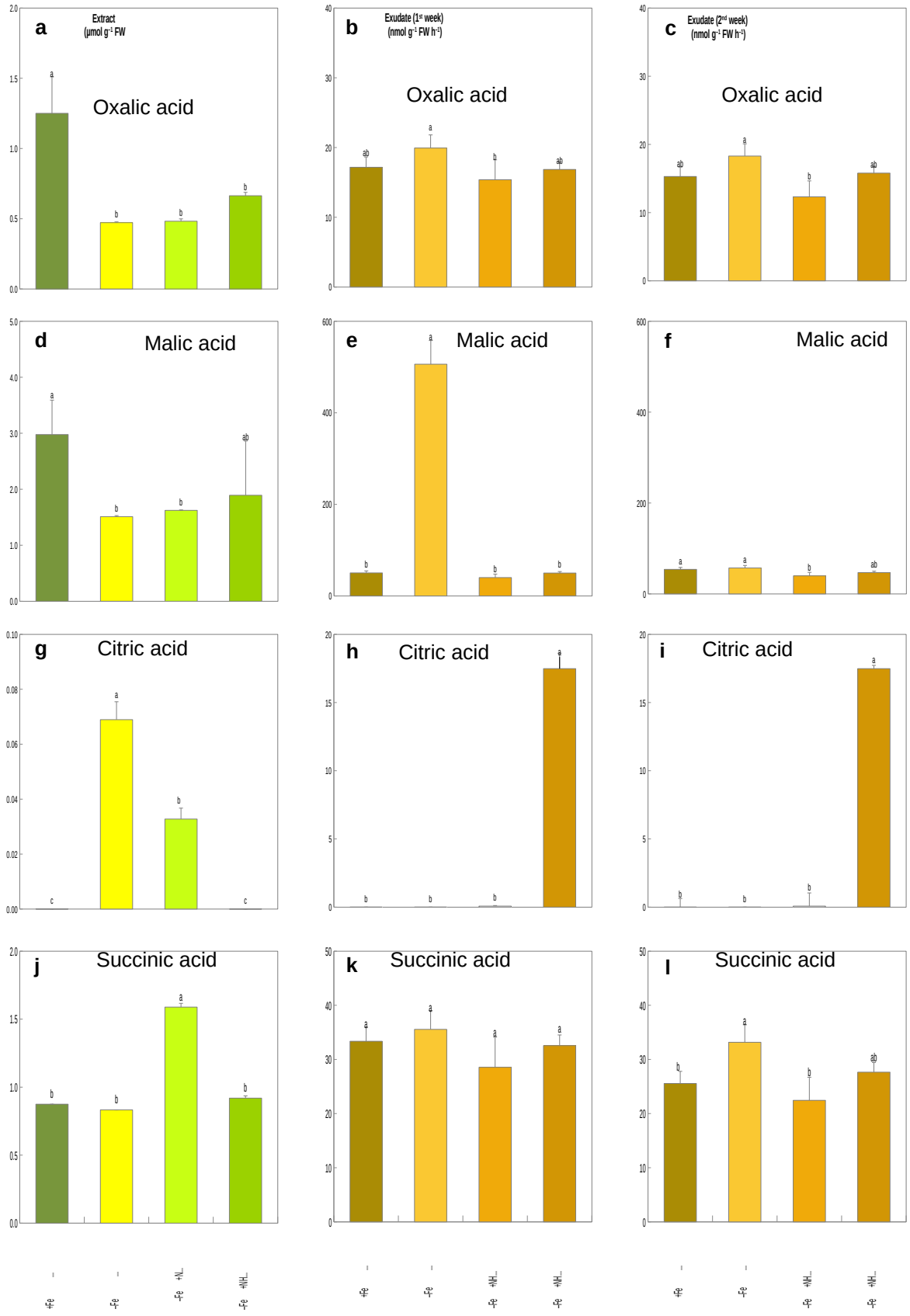

a
Extract
(μmol g-1 FW
Oxalic acid
b
Exudate (1st week)
(nmol g-1 FW h-1)
Oxalic acid
c
Exudate (2nd week)
(nmol g-1 FW h-1)
Oxalic acid
d
Malic acid
e
Malic acid
f
Malic acid
g
Citric acid
h
Citric acid
i
Citric acid
j
Succinic acid
k
Succinic acid
l
Succinic acid
+Fe
-Fe
+N
-Fe
+N
-Fe

Fig. 8 The concentration (μmol g^{-1} FW) of organic acids in the root tips (**a, d, g, j**) and exudation rate of the whole plant (nmol g^{-1} FW h^{-1}) (**b-c, e-f, h-i, k-l**) in cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated under Fe-sufficient (+Fe) or Fe-deficient (–Fe) conditions in the $-NH_4$ medium, then were treated with 2 mM NH_4 (as NH_4Cl) through roots ($+NH_4R$), through leaves ($+NH_4L$) or left untreated ($-NH_4$) simultaneous with Fe treatments. The exudates were collected and analyzed one and two weeks after NH_4 application while the root extract was analyzed only after two weeks NH_4 treatment. Data are mean ± SD (n = 4). Differences among bars indicated by different letters are statistically significant ($P < 0.05$)

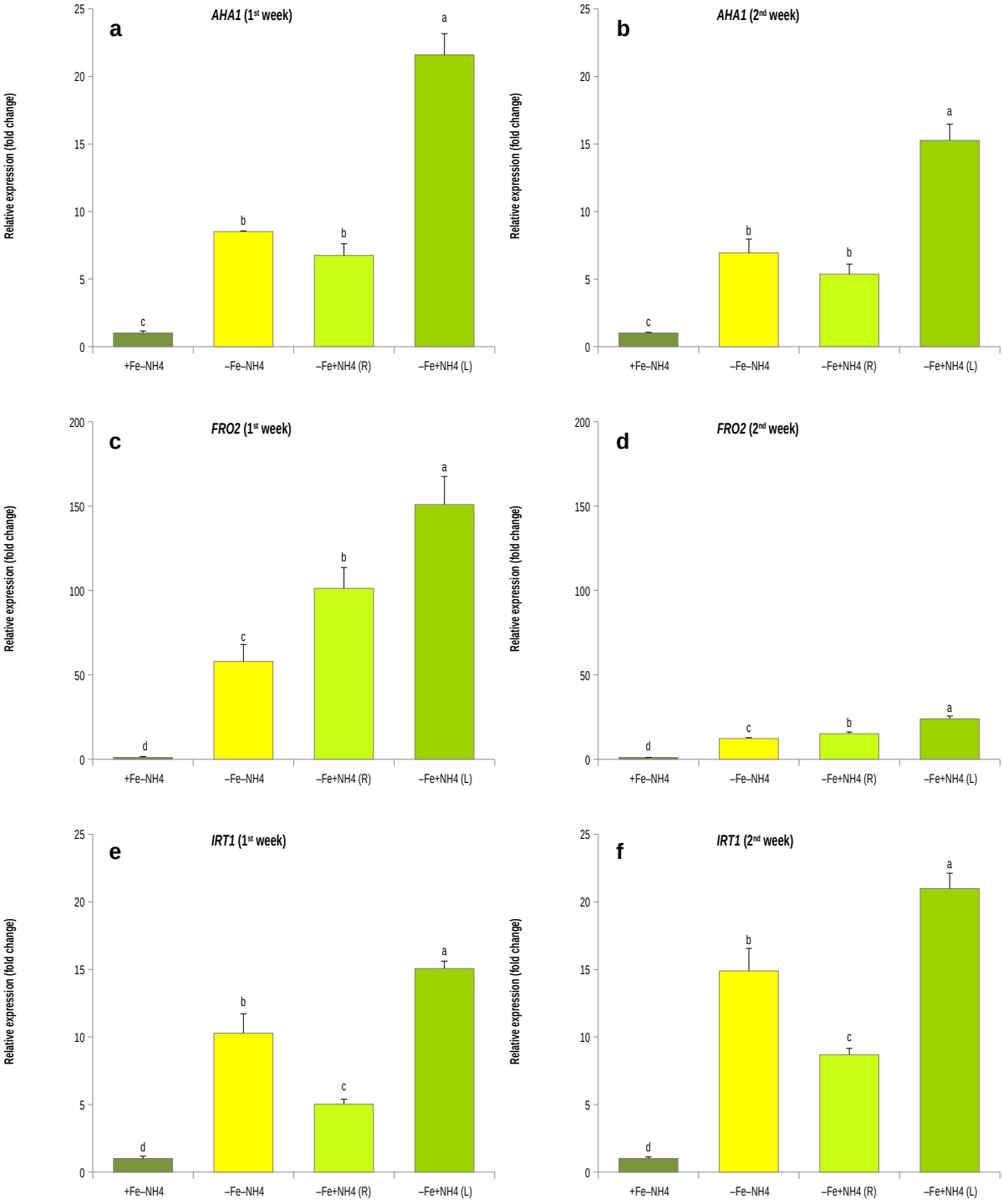


Fig. 9 The relative expression (fold change) of *AHA1*(**a, b**), *FRO2* (**c, d**) and *IRT1* (e, f) in the roots of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated under Fe-sufficient (+Fe) or Fe-deficient (–Fe) conditions in the $-NH_4$ medium, then were treated with 2 mM NH_4 (as NH_4Cl) for one or two weeks through roots ($+NH_4R$) or leaves ($+NH_4L$) or left untreated ($-NH_4$) simultaneous with Fe treatments. Data are mean ± SD (n = 4). Differences among bars indicated by different letters are statistically significant ($P < 0.05$)

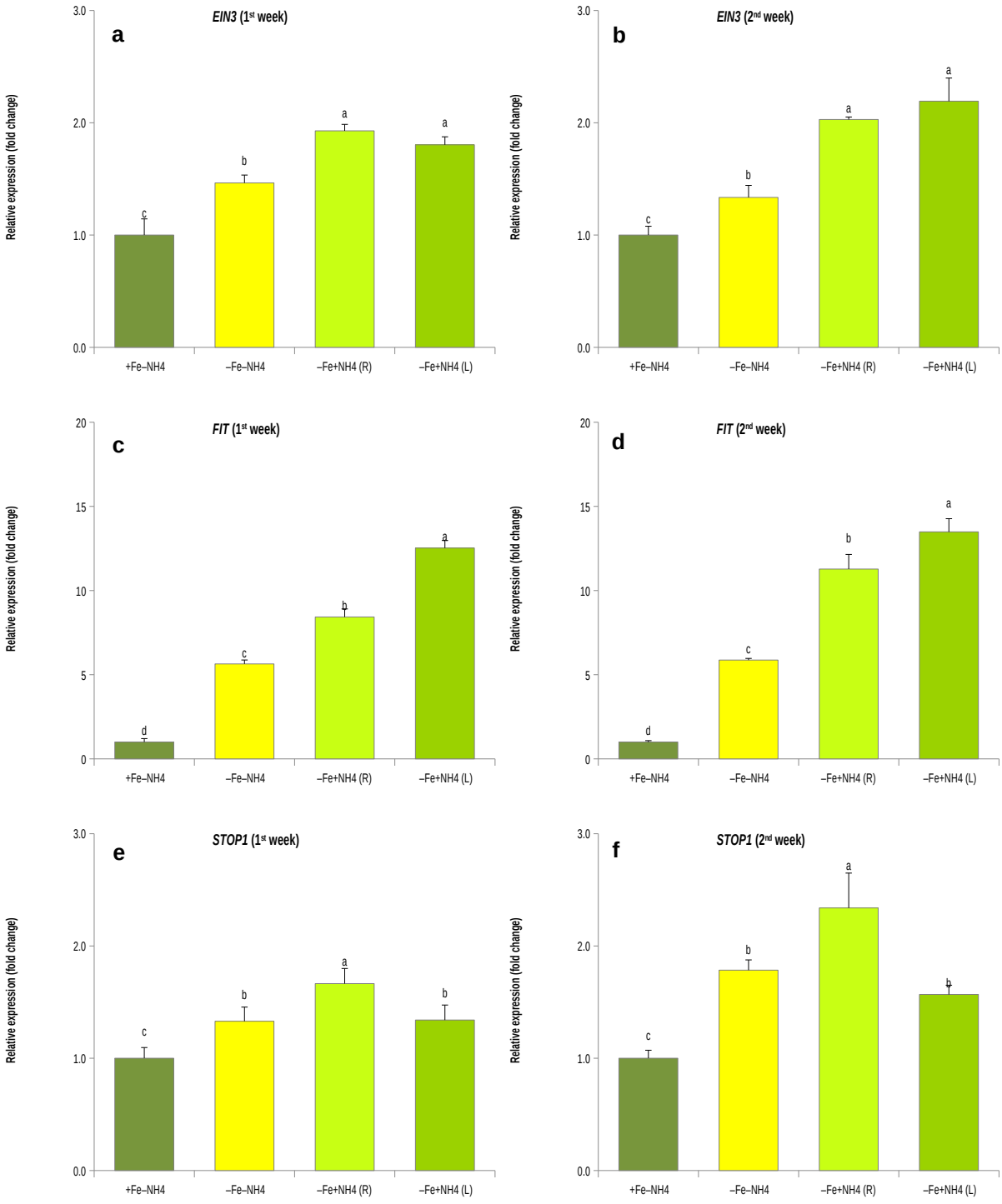


Fig. 10 The relative expression (fold change) of *EIN3* (**a, b**), *FIT* (**c, d**) and *STOP1* (e, f) in the roots of cucumber (*Cucumis sativus* L. cv. Maximus) plants plants cultivated under Fe-sufficient (+Fe) or Fe-deficient (–Fe) conditions in the $-NH_4$ medium, then were treated with 2 mM NH_4 (as NH_4Cl) for one or two weeks through roots ($+NH_4R$) or leaves ($+NH_4L$) or left untreated ($-NH_4$) simultaneous with Fe treatments. Data are mean ± SD (n = 4). Differences among bars indicated by different letters are statistically significant ($P < 0.05$)

Online Resources:

Online Resource S1 The concentrations of different salts (mM) applied to the medium, the total concentrations of nitrate (Nit) and ammonium (NH_4) (mM) and the total concentration of nitrogen (N), calcium (Ca), potassium (K) and phosphorus (P) in the nutrient solution. The pH was buffered at pH=5.8 using 2.0 mM $CaCO_3$. Hoagland solution (Johnson et al. 1957) was used as the base of nutrient solution composition and used as control treatment

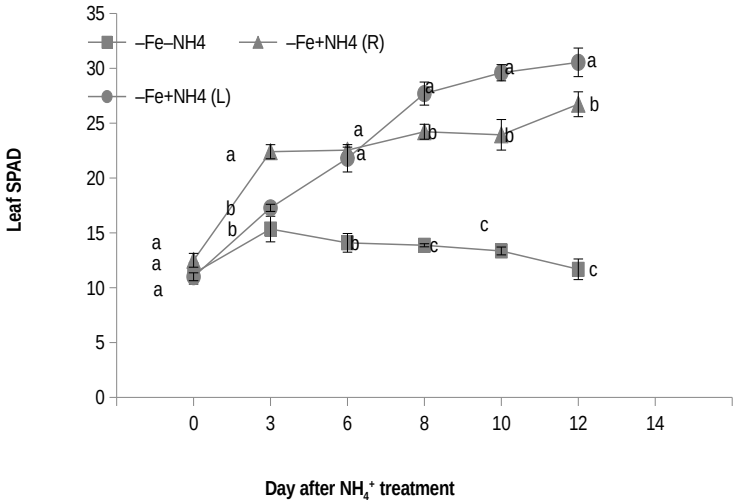
	Hoagland	– NH_4 medium	+ NH_4 medium
KNO_3	6.0	6.0	4.0
$Ca(NO_3)_2$	4.0	4.0	4.0
$NH_4H_2PO_4$	2.0	---	---
KH_2PO_4	---	2.0	2.0
NH_4Cl	---	---	2.0
KCl	---	---	2.0
Total NO_3^-	14.0	14.0	12.0
Total NH_4^+	2.0	0.0	2.0
Total N	16.0	14.0	14.0
Total K	6.0	8.0	8.0

Online Resource S2 Nucleotide sequences of primers used in this study

Gene name	NCBI GeneBank accession number	Sequence (5′ to 3′)
AHA1	AJ703810	F: GGATGGGCTGGTGTAGTTTG R: GGCGGTCTTGTTTTCCAGTA
AHA2	EU735752.2	F: GTGTGAATGATGCTCCTGCTT R: CAACACGGCACTAATGATAACG
FRO2	XM_004143566.3	F: CTTCCATGTTGGCATCTCCT R: AAGGAGGCGAGCAGAGAGTAG
		F: AAATAGCCCTGTTGCACTCG R: GATGCTGCCTTGTAATTTAGGG
EIN3	NM_001308862.1	F: TTAGGCGTTCCAGAAGATGG R: GAGATTTTGGTTCTCCGTGGT
FIT	XM_004145771.3	F: TCATCCATGGCGACAACTT R: CACCACACGATTCTCGATCA
STOP1	XM_004139657.3	F: CTCCTGCAATTCCTCTCGAC R: AAGCAATCGACCCAAAACTG
IRT1	AY590764.1	
ACT	AB010922	F: GCTGGCATATGTTGCTCTTG R: CGATGGTGATGACTTGTCCA
F-box	XM_031885920.1	F: ATCAGCGGAGAGATTTGAGTG R: ACAACCAGCAATCCAAGACC

Online Resource S3 The concentration (mg g^{-1} FW) of chlorophyll (Chl *a*+*b*) and carotenoids in the leaves of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated for four days without iron (–Fe) or with adequate supply of iron (+Fe) in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) through roots (+NH_4R) or leaves (+NH_4L) simultaneous with different Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for two weeks. Data are mean ± SD (n = 4). Differences among data of each column indicated by different letters are statistically significant ($P < 0.05$)

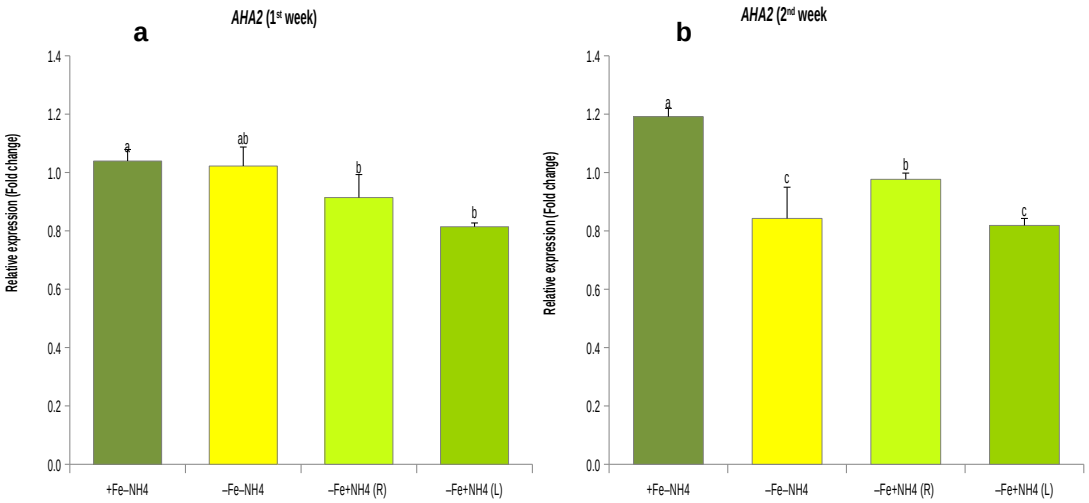
		Chl (*a*+*b*)	Carotenoids
+Fe	–NH_4	1.39±0.12 [b]	0.53±0.02 [b]
	+NH_4R	1.53±0.04 [a]	0.53±0.01 [b]
	+NH_4L	1.58±0.05 [a]	0.66±0.01 [a]
–Fe	–NH_4	0.59±0.04 [c]	0.38±0.01 [d]
	+NH_4R	1.35±0.08 [b]	0.45±0.01 [c]
	+NH_4L	1.59±0.09 [a]	0.54±0.01 [b]



Online Resource S4 The SPAD readings in the young leaves of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated for four days without iron (–Fe) or with adequate supply of iron (+Fe) in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) through roots ($+NH_4R$) or leaves ($+NH_4L$) simultaneous with different Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for 12 days. Data are mean ± SD (n = 4). Differences among data of each time point indicated by different letters are statistically significant ($P < 0.05$)

Online Resource S5 The concentration of total phenolic compounds (μg g^{-1} FW) in the root tips of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated for four days without iron (–Fe) or with adequate supply of iron (+Fe) in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) through roots ($+NH_4R$) or leaves ($+NH_4L$) simultaneous with different Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for two weeks. Data are mean ± SD (n = 4). Differences among data of each column indicated by different letters are statistically significant ($P < 0.05$)

		Phenolics
+Fe	$–NH_4$	182±5.2 [a]
	$+NH_4R$	141±4.7 [b]
–Fe	$–NH_4$	129±4.7 [c]
	$+NH_4R$	182±4.2 [a]
	$+NH_4L$	121±3.9 [c]



Online Resource S6 The relative expression (fold change) of *AHA2* in the roots of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated under Fe-sufficient (+Fe) or Fe-deficient (–Fe) conditions in the $-NH_4$ medium, then were treated with 2 mM NH_4 (as NH_4Cl) for one (**a**) or two weeks (**b**) through roots ($+NH_4R$) or leaves ($+NH_4L$) or left untreated ($-NH_4$) simultaneous with Fe treatments. Data are mean ± SD (n = 4). Differences among bars indicated by different letters are statistically significant ($P < 0.05$)

Online Resource S7 The activity of nitrate reductase (NR) * in the leaf and roots of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated for four days without iron (–Fe) in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) through roots ($+NH_4R$) or leaves ($+NH_4L$) simultaneous with −Fe treatment in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for one week. Data are mean ± SD (n = 4). Differences among data of each column indicated by different letters are statistically significant ($P < 0.05$)

Treatments		NR activity (nmol mg^{-1} pr min^{-1})	
		Leaf	Root
+Fe	$–NH_4$	10.5±0.26 [c]	56.2±2.30 [a]
	$+NH_4R$	13.7±0.57 [a]	55.6±2.41 [a]
	$+NH_4L$	11.9±0.47 [b]	51.9±3.42 [a]
–Fe	$–NH_4$	12.3±0.67 [ab]	51.8±4.69 [a]
	$+NH_4R$	12.6±0.31 [ab]	51.7±3.07 [a]
	$+NH_4L$	13.1±0.59 [a]	55.8±2.99 [a]

* In vivo NR (E.C. 1.6.6.1) activity was determined using the method described by Jaworski (1971). The samples were taken one day after replacement of nutrient solution, cut into 5–10 mm sections and placed in incubation buffer (100 mg tissue for 10 mL of buffer) containing 25 mM potassium phosphate buffer (pH 7.2), 25 mM KNO3, and 1% Triton X-100. The samples were infiltrated using vacuum (80 kPa). After 3 h, the vacuum was released and the samples were incubated at 30 °C in darkness for 1 h, then placed in a boiling water bath to stop the NR activity. The resulting nitrite was determined spectrophotometrically at 540 nm in a reaction mixture containing sulfanilamide and naphthylethylenediamine dihydrochloride (N-NEDA). Soluble proteins were measured with the method of Bradford using BSA as standard. Activity of NR was presented as nmol nitrite generation mg^{-1} protein min^{-1}

Online Resource S8 The concentration of NH_4^+ * in the leaf and roots of cucumber (*Cucumis sativus* L. cv. Maximus) plants cultivated for four days without iron (–Fe) or with adequate supply of iron (+Fe) in the $–NH_4^+$ medium, then were treated with different NH_4^+ levels including without ($–NH_4^+$) and with 2 mM NH_4^+ (as NH_4Cl) through roots (+NH_4R) or leaves (+NH_4L) simultaneous with different Fe treatments in the $CaCO_3$-buffered nutrient solution (2.0 mM, pH 5.8) for one week. Data are mean ± SD (n = 4). Differences among data of each column indicated by different letters are statistically significant ($P < 0.05$)

Treatments		NH_4^+ concentration (µmol g^{-1} FW)	
		leaf	Roots
+Fe	–NH_4	24.4±2.14 [c]	25.1±1.21 [cd]
	+NH_4R	69.8±3.35 [a]	60.1±3.69 [a]
	+NH_4L	47.3±2.48 [b]	28.7±1.93 [c]
–Fe	–NH_4	44.2±1.49 [b]	20.9±0.93 [d]
	+NH_4R	75.5±3.51 [a]	34.9±2.74 [b]
	+NH_4L	39.4±2.79 [b]	26.7±2.18 [c]

* Ammonium was extracted in 100 mM HCl then 500 µL chloroform was added and the samples were agitated for 10 min at 4°C. The aqueous phase was collected through centrifugation at 12,000 g for 10 min and incubated with 50 mg activated charcoal and were thoroughly mixed. After centrifugation at 20,000 g for 5 min in 4°C, concentration of NH_4^+ was determined in the supernatant using the indophenol blue method (Bräutigam et al. 2007)