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Modulation of photorespiration and nitrogen recycling in Fe-deficient cucumber leaves
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Highlights

- The effect of Fe deficiency on photorespiration has been little investigated.
- Photorespiration is affected by low Fe availability in cucumber leaves.
- Biochemical investigation revealed changes in peroxisome-enzymes.
- PR modulation involves the induction of N-recycling in Fe-deficient leaf.

Abstract

Low Fe availability affects plant production mainly by impairing the photosynthetic pathway, since Fe plays an essential role in chlorophyll synthesis as well as in the photosynthetic electron transport chain. Under these conditions, plant cells require the activation of protective mechanisms to prevent photo-inhibition. Among these mechanisms, photorespiration (PR) has been relatively little investigated in Fe-deficient plants.

The aim of this work was to investigate the effect of Fe deficiency on photorespiration by performing *in vivo* analysis in leaves as well as biochemical characterization of some PR-related enzyme activities in a peroxisome-purified fraction from cucumber leaves. Modelling of light response curves at both 21 and 2% pO₂ revealed a slowing down of PR under Fe deficiency. The activity of some PR-involving enzymes as well as the contents of glycine and serine were affected under Fe deficiency. Furthermore, nitrate reductase, the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle and hydroxypyruvate dehydrogenase isoform activities were differentially altered under Fe deficiency. The dataset indicates that, in Fe-deficient cucumber leaves, the modulation of PR involves the induction of some PR-related pathways, such as the photorespiratory N recycling and cytosolic photorespiratory bypass processes.

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Keywords

Fe deficiencyIron metabolismPhotorespirationPeroxisome

1. Introduction

Plant growth depends on the availability of mineral nutrients and, among them, iron (Fe) plays an important role because it is essential in several biological processes (Curie and Briat, 2003; Briat and Lobréaux 1997; Thomine and Vert, 2013; Vigani and Murgia, 2018). Among these, the photosynthetic and the respiratory electron transport chains are strongly affected by Fe deficiency, thereby decreasing the energetic support to the plant cell (Vigani et al., 2019).

Despite Fe-deficient leaves displaying a low photosynthetic rate, they are exposed to excess light energy per chlorophyll molecule, enhancing the risks of photoinhibition and photooxidative damage (Abadía et al., 1999; Andersson and Aro, 2001).

Several reports highlight that plants have different ways to reduce the burden of light to chloroplasts, thereby reducing both photoinhibition events and light-triggered chloroplast damage. These include morpho-anatomical changes, thermal dissipation, the xanthophyll cycle, adjustment of chlorophyll antennae size, the water-water cycle, PSI cyclic electron transport and rapid turnover of the D1 protein of PSII (Andersson and Aro, 2001; Kato et al., 2003).

Chlorophyll fluorescence is a useful tool to study the photosynthetic responses in plant species subjected to Fe deficiency, as revealed by previous studies in sugar beet (*Beta vulgaris* L.) (Belkhouja et al., 1998), peach (*Prunus persica* L.) (Molassiotis et al., 2006) and pear (*Pyrus communis* L.) (Morales et al., 2000a, Morales et al., 2000b). Analysis of chlorophyll fluorescence parameters in pear (Morales et al., 2000a), tomato (*Solanum lycopersicum* L.) (Donnini et al., 2003) and peach plants (Molassiotis et al., 2006) provided evidence of a general increase in non-photochemical quenching (NPQ) in leaves of Fe-deficient plants. Such findings indicate that there is an activation of photoprotective mechanisms, which in turn cause a reduction in maximum photochemical efficiency (Fv/Fm) and photochemical quenching of PSII (qp) along with a decrease of the actual quantum efficiency (Φ_{PSII}) and the intrinsic efficiency of PSII (Φ_{exc}) (Morales et al., 2000b).

In addition to the different mechanisms involved in dissipating the excess of reducing power and energy when CO₂ assimilation rate is limited, the photorespiratory cycle operating among chloroplasts, peroxisomes, mitochondria and cytosol, helps in protecting chloroplasts from photoinhibition and prevents plants from excessive accumulation of reactive oxygen species (ROS; Wingler et al., 2000; Sunil et al., 2019). Photorespiration (PR) can also dissipate excess reducing equivalents and energy either directly (using ATP, NAD(P)H and reduced ferredoxin) or indirectly (e.g., via alternative oxidase (AOX) and providing an internal CO₂ pool). Several authors have found that PR, which originated as a partner of oxygenic photosynthesis millions of years ago, is strictly linked to many pathways of plant metabolism (Timm et al., 2012, Hasunuma et al., 2010; Araújo et al., 2012; Fernie et al., 2013; Voss et al., 2013).

Photorespiration is, therefore, a complex metabolic process which involves four different cellular compartments. Among these, the impairment of both mitochondria and chloroplasts activities under Fe deficiency conditions has been studied and reviewed in Vigani et al. (2019). However, little information is available about the effect of Fe deficiency on peroxisome. The peroxisome is a small and single-membrane-delimited organelle in almost all eukaryotic cells. Recently, investigations have been carried out on the impact of peroxisome function on photosynthesis by screening over 150 *Arabidopsis* mutant lines defective in the expression of some genes encoding peroxisomal proteins (Li et al., 2019, Li et al., 2019). The authors demonstrated that impaired PR disturbs the balance of ATP and NADPH, leading to the accumulation of H₂O₂ that, in turn, activates the cyclic electron flow to produce ATP in order to compensate the imbalance of reducing equivalents. Besides the effect on the photosynthetic process, PR induction impacts on N metabolism. It is well known that carbon flux through chloroplast-peroxisome-mitochondria contributes to the so-called photorespiratory N cycle (Keys et al., 1978) which involves an exchange of amino groups between glycine (Gly) and serine (Ser), and the release of NH₄⁺ from Gly followed by its reincorporation into this amino acid via glutamine (Gln) and glutamate (Glu) (Keys et al., 1978). Despite evidence about the modulation of N metabolism under Fe deficiency which has been already reported (Borlotti et al., 2012; Vigani et al., 2017), the regulation of PR and of the photorespiratory N-cycle in Fe-deficient leaves have still been little studied.

Therefore, the aim of this work was to investigate the effect of Fe deficiency on photorespiration in cucumber leaves by characterizing some peroxisome-related enzyme activities and their biochemical link with N metabolism.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L. cv. Marketmore '76) were surface-sterilized and sown in Agriperlite, watered with 0.1 mM CaSO₄, allowed to germinate in the dark at 26 °C for 3 d, and then transferred to plastic boxes containing a nutrient solution with (+Fe) or without (-Fe) the addition of Fe(III)EDTA. The composition of the nutrient solution was as follows: 2 mM Ca(NO₃)₃, 0.75 mM K₂SO₄, 0.65 mM MgSO₄, 0.5 mM KH₂PO₄, 10 μM H₃BO₃, 1 μM MnSO₄, 0.5 μM CuSO₄, 0.5 μM ZnSO₄, 0.05 μM (NH₄)₂MoO₇ and 0.1 mM Fe(III)- ethylenediaminetetraacetic acid (Fe(III)-EDTA; when added). The pH was adjusted to 6.2 with NaOH. Aerated hydroponic cultures were maintained in a growth chamber with a day:night regime of 16:8 h and a photosynthetic photon flux density (PPFD) of 200 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) at the plant level. The temperature was 18 °C in the dark and 24 °C in the light. After 7 days of treatments, well-expanded leaves of plants were harvested and analyzed.

2.2. Gas exchange measurements

Gas exchange parameters were measured in leaves of plants at the end of the treatments using a portable infrared gas analyzer (LI-6400; Li-Cor, Lincoln, NE, USA). Measurements were performed at increasing light from 0 to 2500 μmol m⁻² s⁻¹ PPFD and at a leaf temperature of 25 °C, either with air (21% of O₂) or a synthetic air mixture containing <2% O₂ where N₂ replaced O₂. In both the cases, the airflow (300 μmol s⁻¹) was humidified by bubbling it in water of which the temperature was maintained lower than that of the leaf surface. When the synthetic air with <2% O₂ was utilized, O₂ from the bubbling solution was carefully removed by blowing the same synthetic air mixture for 10 min. Photosynthetic CO₂ assimilation (A₃₉₀), stomatal conductance (g_s), and the intercellular CO₂ concentration (C_i) were determined using the LI-6400 software. Quantum yield for CO₂ uptake was determined as the slope of the linear regression of CO₂ assimilation versus light intensity above 100 μmol photons m⁻² s⁻¹. The dark respiration R_d, was measured by covering the chamber with a black cloth at an ambient CO₂ concentration of 350 μmol mol⁻¹. In most cases the R_d values stabilized within 45 min.

The response of the net assimilation rate (A) to intercellular CO₂ concentration (A/C_i curve) is measured following the procedure reported in Guidi et al. (2017). The leaf was allowed to equilibrate inside the chamber for 10 min at ~390 μmol mol⁻¹ CO₂ concentration and saturating irradiance (~1800 μmol m⁻² s⁻¹). Once a steady-state was reached, the CO₂ concentration was decreased stepwise to 50 μmol CO₂ mol⁻¹ air. At the end of the measurements, the CO₂ concentration was increased stepwise to 1800 μmol mol⁻¹. Measurements were recorded when values of A, C_i and stomatal conductance did not vary at each step within the sequence. The maximum carboxylation rate at sub-stomata CO₂ concentration (V_{cmax}) was measured by fitting Farquhar's equation (Farquhar et al., 1980).

2.3. Analysis of chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed with a modulated light fluorimeter (PAM-2000 Walz, Effeltrich, Germany) on leaves of cucumber plants grown under control (+Fe) and Fe deficiency (-Fe) conditions for seven days. The F₀ was recorded in a 40 min dark-adapted leaves. The frequency of light pulses was programmed at 600 Hz to determine F₀ or 20000 Hz to record the kinetics of induction of fluorescence. The maximum fluorescence yield, F_m, was determined with a saturating pulse of 8000 μmol m⁻² s⁻¹ PAR for 800 ms. F₀ and F_m were subtracted and divided [(F_m-F₀)/F_m] to draw the maximum quantum efficiency of PSII photochemistry F_v/F_m. The current

fluorescence yield (Ft) and the maximum light-adapted fluorescence (Fm') were determined in the presence of an actinic illumination of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and then the PSII quantum efficiency for photochemistry in light conditions, Φ_{PSII} , was computed as the quotient $[(Fm' - Ft)/Fm']$ (Genty et al., 1989). The coefficient of photochemical quenching, qP, was calculated as $(Fm' - Ft)/(Fm' - F0')$ according to Schreiber et al. (1986). F0' measurements were carried out in the presence of far-red light (7 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in order to fully oxidize the PSII acceptor side. NPQ was calculated as $\text{NPQ} = (Fm - Fm')/Fm$ as reported by Bilger (1990). The apparent electron transport rate (ETR) was determined as $0.5 \times \Phi_{\text{PSII}} \times \text{PAR} \times 0.84$, where 0.5 accounted for the excitation of both PSII and PSI, and 0.84 represented the average value for leaf absorbance.

2.4. Isolation of peroxisomes from leaves

Isolation of peroxisomes from cucumber leaves was performed according to Reumann et al. (2007, 2009, 2014) with some modifications. Leaves from control (+Fe) and Fe-deficient (-Fe) plants were harvested 7 d after the start of the treatment and then gently ground in a buffer of high osmolarity [170 mM Tricine-KOH, pH 7.5, 1.0 M sucrose, 1% (w/v) albumin serum bovine (BSA), 2 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM KCl, and 1 mM MgCl₂] in the presence of protease inhibitors cocktail (0.1 mM PMSF, 0.2 mM benzamide and 0.2 mM ϵ -aminocaproic acid) using a mortar and a pestle. The suspension was filtered, and chloroplasts were sedimented at 5000 g (1 min). Approximately 20 mL of supernatant was loaded onto a Percoll density gradient prepared in Tris-EDTA (TE) buffer solution (20 mM Tricine-KOH, pH 7.5 and 1 mM EDTA) supplemented with 0.75 M sucrose and 0.2% (w/v) BSA overlaid by 36% sucrose (w/w) in TE buffer [top to bottom gradient composition: 3 mL of 15% Percoll, 9 mL of 38% Percoll, 2 mL each of a mixture of 38% Percoll, and 36% (w/w) sucrose at a ratio of 2:1 and 1:2, and 3 mL of 36% (w/w) sucrose in TE buffer]. The mixed Percoll-sucrose gradients were centrifuged for 12 min at 13,000 \times g and then 10–20 min at 27,000 \times g. Co-sedimentation of peroxisomes along with mitochondria and thylakoid membranes by differential centrifugation prior to isopycnic organelle separation increased irreversibly inter-organellar adhesion and peroxisome contamination. However, chloroplasts, thylakoid membranes, and mitochondria were retained mainly in the 15 and 38% (v/v) Percoll fraction near the top of the gradient, intact leaf peroxisomes crossed the Percoll layer and were recovered at the bottom, visible as a whitish diffuse organelle sediment. The peroxisome fractions at the bottom of the gradient of several Percoll gradients were combined, diluted in 36% (w/w) sucrose in TE buffer, washed several times in TE buffer and finally sedimented by centrifugation (30 min, 39,000 \times g) supplemented with protease inhibitors, and stored in appropriate aliquots, yielding the leaf peroxisomal fraction LP-P1. The enrichment in leaf peroxisomes was high, as determined by Western Blot analysis using catalase and glyoxylate oxidase as markers. Contaminating chloroplasts and mitochondria were estimated to comprise only 0.1 and 1.7% of the total extract, respectively (Reumann et al., 2007).

2.5. Enzymatic activity assays

A catalase (CAT) activity assay was performed according to the method described by Luck (1965). Leaves from control and Fe-deficient plants 7 d after the start of the treatment were ground in a mortar with the addition of liquid nitrogen, polyvinylpyrrolidone (PVPP) 10% (w/w leaves), and buffer in a ratio 1: 2.5 (w:v) (Buffer composition: Tris-HCl 220 mM-pH 7.4; MgCl₂ 1 mM; KCl 50 mM; Sucrose 250 mM; DTT 1 mM; PMSF (50 mg mL⁻¹ dimethyl sulfoxide, DMSO)). The suspension was filtered and then centrifuged at 12000 g for 30 min at 4 °C. The supernatant was recovered and then dialyzed overnight against diluted extraction buffer (approximately 1:50). Degradation of H₂O₂,

which reflects the catalytic activity of catalase was monitored by spectrophotometric analysis at 240 nm, 25 °C ($\epsilon_{240} = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) using Na-phosphate buffer 65 mM pH 7.2 as assay buffer.

Glycolate oxidase (GOX) activity was measured according to Macheroux et al. (1991) in an enzyme-coupled assay using horseradish peroxidase and o-dianisidine to remove hydrogen peroxide generated during oxidation of glycolate. A typical assay mixture contained $\mu 10 \text{ L}$ of horseradish peroxidase (1 mg mL^{-1}), $50 \mu\text{L}$ of o-dianisidine solution (8 mM, 20% Triton X-100), $10 \mu\text{L}$ of sodium glycolate (1 M), and $930 \mu\text{L}$ of 0.1 M potassium phosphate buffer, pH 8.3. The reaction was started by adding $10 \mu\text{L}$ of the GOX sample. Formation of the o-dianisidine radical cation, which reflects the catalytic activity of glycolate oxidase, was monitored by spectrophotometric analysis at 440 nm and at 25 °C ($\epsilon_{440} = 11,600 \text{ M}^{-1} \text{ cm}^{-1}$).

The hydroxypyruvate reductase (HPR) activity assay was measured as described previously by Schwitzguebel and Siegenthaler (1984) in a reaction medium containing 50 mM phosphate buffer (pH 6.2); 0.025% (v/v) TritonX-100; 1 mM KCN 0.2 mM NADH. The addition of 2 mM hydroxypyruvate started the reaction, and the oxidation of NADH was followed at 340 nm ($\epsilon_{340} = 6.22 \text{ M}^{-1} \text{ cm}^{-1}$).

For the superoxide dismutase (SOD) activity assay, leaf samples of control and Fe-deficient plants 7 d after the start of the treatment were ground in liquid N₂ with 10% (w/w) polyvinylpyrrolidone (PVPP) and homogenized in a medium containing: 50 mM K-phosphate buffer (pH 7.00), 1 mM EDTA, 0.05% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 1 mM Na-ascorbate and 0.50 mM phenylmethylsulphonyl fluoride (PMSF). After centrifugation at 12,000 g for 30 min at 4 °C, the supernatant was collected and dialysed overnight at 4 °C against 1:50 (v/v) diluted buffer. Superoxide dismutase activity was assayed according to the method of Scebba et al. (2003) at 560 nm, based on the enzyme's ability to inhibit the photoreduction of nitrobluetetrazolium (NBT). One enzyme unit was defined as the amount of enzyme inhibiting 50% of NBT photoreduction.

Enzymes related to N metabolism were assayed according to Borlotti et al. (2012). The nitrate reductase (NR, EC 1.7.1.1) activity was determined by using the colorimetric assay of nitrite performed according to Long and Oaks (1990). The glutamine synthase (GS, EC 6.3.1.2) activity was performed by monitoring the NADH formation according to Scheible et al. (1997); while the NAD(P)H-dependent glutamine-oxoglutarate aminotransferase (NAD(P)H-GOGAT, EC 1.4.1.14) activity was assayed according to Chen and Cullimore (1988).

2.6. Western blot analysis

Purified peroxisomes isolated from leaf of cucumber plants grown in the presence or the absence of Fe for 7 d, were loaded on a discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel, [3.75% (w/v) acrylamide stacking gel, and 10–15% (w/v) acrylamide separating gel] (according to the method of Laemmli, 1970).

Electrophoretic transfer to nitrocellulose membrane filters (Sigma, Milan, Italy) was performed in 48 mM Tris, 39 mM glycine, 0.0375% SDS and 20% (v/v) methanol for 1.5 h at room temperature at 0.8 mA cm^{-2} . After blotting, the membrane was incubated for 1 h in PBS-TB blocking buffer [phosphate-buffered saline, 0.1% Tween-20, 1% BSA, (for monoclonal antibodies)] or TBS-TM blocking buffer [Tris-buffered saline, 0.1% Tween-20, 5% commercial dried skimmed milk, (for

polyclonal antibodies)]. Different antibodies were used, and the dilution ratios were: 1:6000 for a-CAT (Yamaguchi et al., 1984), 1:3000 for a-GOX (Nishimura et al., 1983), 1:3000 for a-PEX14 (Hayashi et al., 2000). All these antibodies were a kind gift from Dr Shoji Mano from the Department of Cell Biology, National Institute for Basic Biology, Okazaki, Japan. The incubation in primary antibody, diluted in blocking buffer, was carried out for 2 h at room temperature. After rinsing with TBS-TM or PBS-TB, nitrocellulose membranes were incubated at room temperature for 2 h with a 1:10000 diluted secondary antibodies [alkaline phosphatase-conjugated anti-rabbit (for polyclonals) or anti-mouse (for monoclonals) [IgG, Sigma, Milan, Italy]. After rinsing in TBS-T (Tris-buffered saline, 0.1% Tween-20) or PBS-T (phosphate-buffered saline, 0.1% Tween-20), filters were incubated in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (FAST BCIP/NBT, Sigma) for detection.

2.7. Native PAGE and SOD isoform visualization

Superoxide dismutase isoforms extracted from control and Fe-deficient plants 7 d after the start of the treatment as reported above, were separated by 12.5% native polyacrylamide gel electrophoresis (PAGE) at 100 V using the method described by Beauchamp and Fridovich (1971). Pre-treatment of the gels with 5 mM H₂O₂ and 3 mM KCN before SOD staining allowed us to characterize SOD isoforms as Cu/Zn-SOD, Fe-SOD or Mn-SOD because the isoenzymes were selectively inhibited by KCN or H₂O₂. In particular, Mn-SOD is resistant to both inhibitors, Fe-SOD is resistant to KCN and inhibited by H₂O₂, and both inhibitors inhibit Cu/Zn-SOD.

2.8. H₂O₂ content evaluation

H₂O₂ content in leaves from control and Fe-deficient plants 7 d after the start of the treatment, was measured following the method reported by Ranieri et al. (2001) based on the formation of the titanium-peroxide complex. The leaf samples were homogenized in cold 100% acetone (1:2; w/v), and centrifuged at 10000 g for 10 min; then 20% TiCl₄ in concentrated HCl was added to supernatant aliquots to give a final titanium concentration of 4%. After addition of NH₄OH (0.2 mL for each mL of the sample) to precipitate the titanium-peroxide complex, samples were centrifuged at 10000 g for 5 min, and the resulting pellet was washed five times in acetone and then resuspended in 2 N H₂SO₄. The absorbance of the solution was read at 415 nm against a blank containing H₂O instead of extracts.

2.9. Nitrate and ammonium analysis by capillary electrophoresis

Leaves from control (+Fe) and Fe-deficient (-Fe) plants 7 d after the start of the treatment were harvested 4 h after onset of light and immediately frozen in liquid nitrogen, and the plant material was stored at -80 °C. Nitrate and ammonium were extracted from the leaves with ultrapure water. Extracts were filtered through 0.2 µm filters and then analyzed by an Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, Santa Clara, CA, US). Ammonium was analyzed using a bare fused silica capillary with extended light path BF3 (i.d. = 50 µm, l = 56 cm, L = 64.5 cm). Sample injection was at 50 mbar for 5 s with +30 kV voltage and detection wavelength at 310/20 nm. Nitrate and nitrite were analyzed using a bare fused silica capillary with extended light path BF3 (i.d. = 50 µm, l = 72 cm, L = 80.5 cm). Sample injection was at 50 mbar for 4 s with -30 kV voltage and detection at 350/80 nm wavelength. Compounds were identified by using pure standards. The ion content was expressed as mg g⁻¹ FW.

2.10. Amino acid analysis

Amino acid (AA) analysis was performed on leaves of both Fe-deficient (-Fe) and control (+Fe) plants. Free AA were extracted from fresh tissues at 4 °C, first in 80% ethanol overnight, then in 60% ethanol for 1 h and finally in distilled water for 24 h. The supernatants of each sample were pooled, aliquoted and kept at -20 °C. Free amino acids were determined by HPLC as described before (Muller and Touraine, 1992).

2.11. Miscellaneous

RNA was purified from leaves from control and Fe-deficient plants 7 d after the start of the treatment and harvested 4 h after onset of light, according to Borlotti et al. (2012). Northern blot analysis procedures were carried out according to Borlotti et al. (2012). Gene sequences considered were Csa015274 (CsGS1), Csa0081189 (CsGS2), Csa021126 (CsNADH-GOGAT), Csa002676 (CsFd-GOGAT), Csa008224 (CsNR) according to Borlotti et al. (2012).

Metal contents were determined in dried leaf tissues after mineralization in HNO₃ by using a Microwave Digestion System (Multiwave ECO). Inductively coupled plasma-mass spectrometry (ICP/MS, aurora M90 BRUKER) was used to quantify the content of iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn) according to Vigani et al. (2018).

Total protein concentration was determined by using the dye-binding method of Bradford (1976), using BSA as a standard.

Statistical analyses were conducted with Past 3 software. Student's t-test was used to compare the means (three independent experiments \pm standard deviation) at the $P \leq 0.05$ level in all cases.

3. Results

3.1. Gas exchange parameters in cucumber leaves under Fe deficiency

After 7 d of Fe deficiency, cucumber leaves had a strong alteration in the CO₂ assimilation rate in response to increasing light or intercellular CO₂ concentration as evidenced by gas exchange parameters derived by the analysis of light curve and A/Ci response curves (Table 1; Fig. S1). The reduced ability in carbon assimilation displayed by Fe-deficient plants was evident both under low and saturating light conditions (Supplementary Fig. S1).

Table 1. Gas exchange parameters determined in leaves of cucumber plants subjected for 7 days to the absence (-Fe) or the presence of 50 μ M Fe (+Fe) in the nutrient solution. Parameters derived from the light curves determined at 21 and 2% pO₂ and A/Ci curve. For details see Materials and Methods section. Each value represents the means of three replicates (\pm standard deviation). In the last column, the significance of the difference between the means following Student's t-test is reported.

21% pO₂

Parameters	+Fe	-Fe	p	
A390 (μ mol CO ₂ m ⁻² s ⁻¹)	10.4 (0.2)	1.7 (0.7)		***
gs (mmol H ₂ O m ⁻² s ⁻¹)	233.8 (22.4)	110.5 (3.5)		**
Ci (μ mol CO ₂ mol ⁻¹ air)	283.5 (13.1)	367.0 (1.4)		**
Rd (μ mol CO ₂ m ⁻² s ⁻¹)	1.6 (0.7)	1.2 (0.2)		ns
Φ CO ₂ (μ mol CO ₂ μ mol photons ⁻¹)	0.05 (0.01)	0.008 (0.004)		**

V_{c,max} (mmol CO₂ m⁻²s⁻¹) 87 (7.0) 26 (4.1) ***
2% pO₂

Parameters	+Fe	-Fe	p
A390 (μmol CO ₂ m ⁻² s ⁻¹)	18.2 (1.5)	2.4 (0.2)	***
g _s (mmol H ₂ O m ⁻² s ⁻¹)	253.0 (15.6)	158.5 (14.9)	***
C _i (μmol CO ₂ mol ⁻¹ air)	247 (10.0)	354 (0.70)	**

ΦCO₂ (μmol CO₂ μmol photons) 0.07 (0.01) 0.012 (0.002) ns

A390: photosynthetic rate at ambient CO₂ and light-saturated conditions; g_s: stomatal conductance to water vapour at ambient CO₂ and light-saturated conditions; C_i: intercellular CO₂ concentration at ambient CO₂ and light-saturated conditions; R_d: dark respiration at ambient CO₂ and in the dark; ΦCO₂: quantum yield for CO₂ uptake; V_{c, max}: in vivo carboxylation efficiency of Rubisco enzyme.

***: P < 0.001; **: P < 0.01; ns: P > 0.05.

The quantum yield for CO₂ assimilation, ΦCO₂, was significantly reduced in Fe-deficient plants, indicating a reduction in the efficiency of the use of CO₂ per mole of absorbed photons (Table 1). A substantial reduction in CO₂ assimilation was also observed in saturating light conditions (-84% of A390 in comparison with the controls; Table 1). Likewise, as deduced by the gas exchange parameters, the reduction of CO₂ assimilation was attributable to a reduced stomatal conductance, but also to an alteration in the activity of the mesophyll considering the increase in the intercellular CO₂ concentration (C_i) (Table 1). On the other hand, V_{c, max}, that indicates the in vivo carboxylation activity of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), was strongly and significantly reduced in Fe-deprived leaves as compared to the controls (-87%).

Chlorophyll a fluorescence analysis (Table 2) underlined the altered photochemical efficiency of PSII (i.e. the significant reduction of F_v/F_m and ΦPSII) in Fe-deprived leaves (Table 2). The photochemical quenching coefficient, q_p, and the electron transport rate (ETR) in light conditions sharply decreased in Fe-deprived leaves (-68 and -73% as compared to their controls, respectively) while Fe-deficient leaves exhibited a remarkable increase in NPQ.

Table 2. Chlorophyll fluorescence parameters determined in leaves of cucumber plants subjected for 7 days to the absence of Fe (-Fe) or the presence of 50 μM Fe (+Fe) in the nutrient solution. Each value represents the means of three replicates (±standard deviation). In the last column, the significance of the difference between the means following Student's t-test is reported.

PARAMETERS	+Fe	-Fe	p
F ₀	196 (12)	330 (24)	***
F _m	993 (114)	550 (38)	***
F _v /F _m	0.81 (<0.01)	0.43 (0.09)	***
ETR (μmol electron m ⁻² s ⁻¹)	110.7 (26.4)	13.5 (1.7)	***
ΦPSII	0.46 (0.05)	0.05 (<0.01)	***
q _p	0.53 (0.13)	0.14 (0.03)	***
NPQ	0.41 (0.03)	2.06 (0.12)	***

F₀: minimal fluorescence determined in dark conditions; F_m: maximal fluorescence determined in dark conditions; F_v/F_m: potential quantum yield of PSII; ETR: electronic transport rate; ΦPSII:

photochemical PSII efficiency (in light conditions); qP photochemical quenching coefficient; NPQ: non-photochemical quenching. ***: $P < 0.001$.

3.2. Estimation of photorespiration impairment under Fe deficiency

Modelling of light response curves at both 21 and 2% pO₂ in control leaves suggest a slightly higher A390 asymptote at 2% as compared to the same analysis at 21% (pO₂) (Fig. S1B). Thus, stimulation of CO₂ assimilation rate by low pO₂ was expected, considering the elimination of the photorespiration process (Table 1). Even in Fe-deprived leaves, the absence of O₂ induced an increase in CO₂ assimilation rate.

The obtained results indicate that photorespiration contributes to the decrease of assimilated CO₂ by 43% and 29%, in controls and Fe-deprived leaves, respectively (Table 1). Such results suggested that the photorespiration process is still active under Fe deficiency, even though at a low rate.

3.3. Fe deficiency affects the biochemical activity of peroxisomes in leaves

In order to investigate the effect of Fe deficiency on photorespiration, we purified the peroxisome fraction from leaf tissues. The first reaction of photorespiration into peroxisome, i.e. the conversion of glycolate to glyoxylate, is carried out by the (enzyme) glycolate oxidase. During this reaction, H₂O₂ is typically generated as a by-product and then scavenged by CAT. Therefore, the determination of GOX and CAT activities was performed on the peroxisome purified fraction. As shown in Fig. 1A, Fe deficiency did not significantly affect the GOX activity. Accordingly, Western Blot analysis performed on the peroxisomal purified fraction LP-P1 did not reveal differences in the expression of glycolate oxidase protein under Fe deficiency condition (see Supplementary Material, Supplementary Fig. S2). However, CAT activity decreased by about 35% in the leaves of cucumber plants after 7d of growth under Fe deficiency as compared with the controls (Fig. 1B). Accordingly, the concentration of H₂O₂ in the leaves was higher (+40%) in the absence of Fe (Fig. 2A). Such H₂O₂ accumulation might be linked to the metal imbalance observed in Fe-deficient leaves (Fig. 2B) as well as to a different modulation of SOD activities (Fig. 2C). Fe-deficient leaf displayed a decrease of Fe (-60%) content along with an accumulation of Zn (+155%) and Cu (+55%) respect to the control (Fig. 2B). Accordingly, the in gel-activity of the Cu/Zn-SOD isoforms is higher as compared with the controls, whereas in the same leaves the activity of Fe-SOD was lower compared with the control condition (Fig. 2C). Although Mn content did not change, Mn-SOD in gel activity decreased in Fe-deficient leaves.

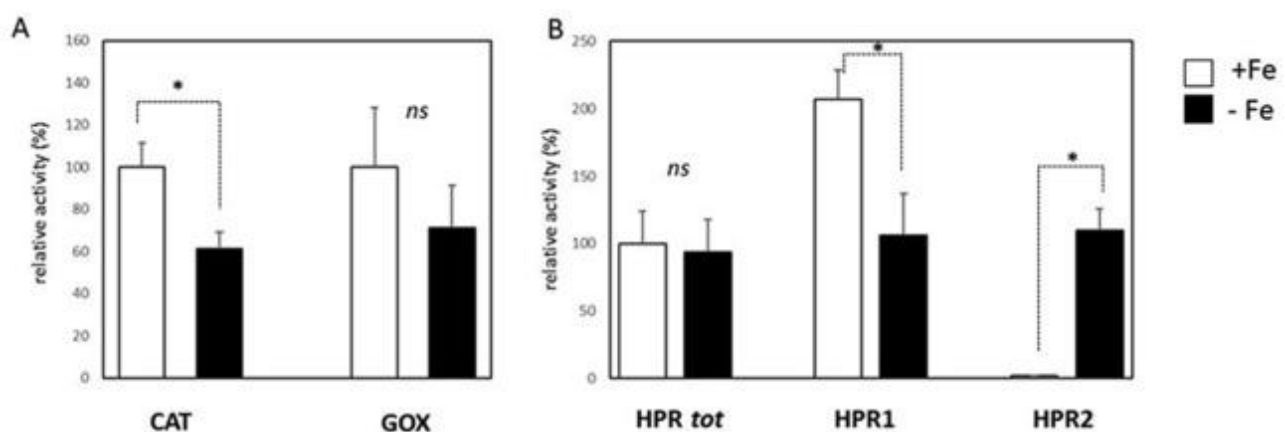


Fig. 1

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Fig. 1. Enzymatic activity of catalase (CAT), glycolate oxidase (GOX), total hydroxypyruvate reductase (HPR), HPR cytosolic (HPR2) and peroxisomal (HPR1) isoforms in leaves of cucumber plants grown in the presence (50 mM FeEDTA; white bar) or absence of Fe (black bar) for 7 days. Data are the means of three independent experiments (\pm standard deviation). The activities are expressed as percentage (%) of the relative activity of the Fe-deficient samples compared to control (+Fe) plants. Activity of control (+Fe) was 132.47, 12.23, 11.78 nmol NADH mg⁻¹ prot min⁻¹, for CAT, GOX and HPR (total activity) respectively. The asterisks indicate the significance following the Student's t-test for the comparison between -Fe and + Fe leaves. ns: $P > 0.05$; * $P < 0.05$.

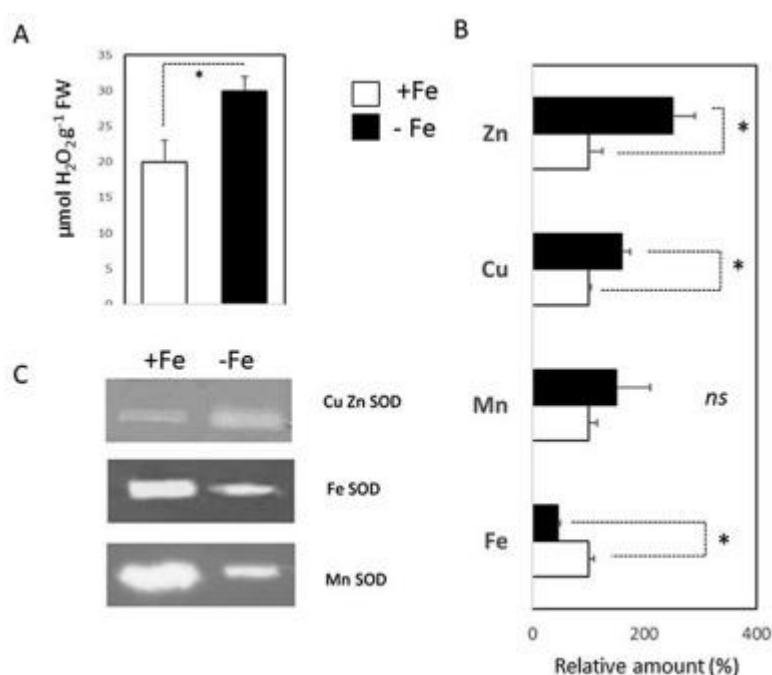


Fig. 2

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Fig. 2. Concentration of hydrogen peroxide (A); in-gel SOD isoforms activities assay (B), and metal content (Fe, Mn, Cu and Zn; C) in leaves of cucumber plants grown in the presence (50 mM FeEDTA; white bar) or absence of Fe (black bar) for 7 days. Metal contents are expressed as relative amount (%) of -Fe respect to + Fe leaf samples. The content of metals in +Fe was 148.32, 28.45, 18.97, 34.21 $\mu\text{g g}^{-1}$ DW for Fe, Mn, Cu and Zn respectively. Discrimination among isoforms was performed using KCN, H₂O₂ or both as inhibitors (see Materials and Methods); 12 μg protein per lane. Data are the mean (or the representative gel) of three independent experiments (\pm standard deviation). In Fig. 2A, the asterisks indicate the significance following the Student's t-test for the comparison between -Fe and + Fe leaves. ns: $P > 0.05$.

The lack of Fe during the plant growth did not affect the total HPR activity in the leaves of cucumber plants (Fig. 1B). Interestingly, by discriminating the activity of the two HPRs isoforms (cytosolic, HPR2 and peroxisomal, HPR1), in control leaves the total HPR activity was entirely attributable to the peroxisomal isoform (HPR1), while a very low cytosolic isoform (HPR2) activity was observed. On the contrary, in plants grown in Fe-deficient condition, the two isoforms of HPR had a similar activity (Fig. 1B), significantly lower than in the controls in peroxisome but significantly higher in

cytosol as compared with the controls. Finally, the activity of G3PDH was sharply increased in leaves of plants subjected to Fe deficiency (Supplementary Fig. S4).

3.4. Fe deficiency affects nitrogen (N) metabolism in cucumber leaves

An imbalance in the N metabolism was observed in plants subjected to Fe deficiency (Fig. 3). Although under Fe deficiency NR activity strongly decreased, GS activity was not affected, and the GOGAT (NADPH-dependent isoform) activity was induced (Fig. 3A). The expression of the relative genes partially mirrored what was observed at enzymatic level: under Fe deficiency the expression of CsNR gene decreased, while the expression of genes encoding for the GS isoforms was not affected (CsGS1) or induced (CsGS2). Furthermore, the expression of genes encoding for GOGAT isoforms did not change in Fe-deficient leaves compared with control plants (Supplementary Fig. S3). The alteration of nitrate reduction and ammonium organisation process led to the increase of NO_3^- content, as well as to unchanged level of NH_4^+ in Fe-deficient leaves with respect to control plants (Fig. 3B). Furthermore, the concentration of the amino acids in leaves changed under Fe deficiency conditions (Table 3). The content of arginine (Arg), asparagine (Asn) glutamine (Gln), and serine (Ser) accumulated in Fe-deficient leaves, while aspartate (Asp) and glutamate (Glu) did not change in their content in Fe-deficient leaves as compared to controls (Table 3). Besides, glycine (Gly) content decreased in Fe-deficient leaves (Table 3).

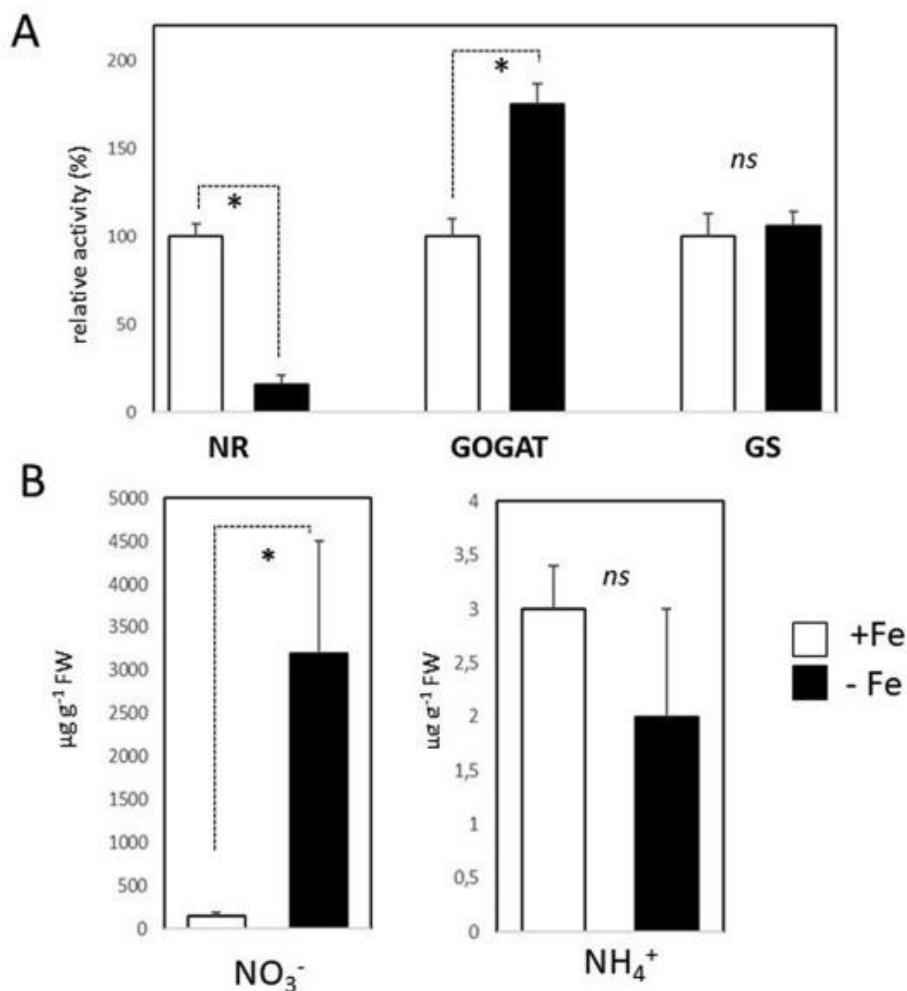


Fig. 3

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Fig. 3. N assimilation-related enzyme and nitrate and ammonium content in leaves of cucumber plants grown in the presence (+Fe, 50 mM FeEDTA; white bar) or absence (-Fe) of Fe (black bar) for 7 days. (A) Enzymatic activity of nitrate reductase (NR), glutamine oxoglutarate aminotransferase (GOGAT) glutamine synthase (GS). The activities are expressed as percentage (%) of the relative activity of the Fe-deficient samples compared to control (+Fe) plants. Activity of control (+Fe) was 11.21, 132.45, 225.67 nmol NADH mg⁻¹ prot min⁻¹, respectively for NR, GOGAT and GS, respectively. (B) Nitrate (NO₃⁻) and ammonium (NH₄⁺) content in leaves. Data are expressed as µg g⁻¹ FW. Data are the means of three independent experiments (±standard deviation). The asterisks indicate the significance following the Student's t-test for the comparison between -Fe and + Fe leaves. ns: P > 0.05; *: P < 0.05.

Table 3. Amino acid concentration (expressed as nmol mg⁻¹ FW) in leaves of cucumber plants subjected for 7 days to the absence of Fe (-Fe) or the presence of 50 µM Fe (+Fe) in the nutrient solution. Each value represents the means of three independent experiments (±standard deviation). In the last row, the significance of the difference between the means following Student's t-test is reported.

Treatment	Asp	Glu	Asn	Ser	Gln	Gly	Arg	
+Fe	0.88 (0.053)	2.65 (0.395)	0.08 (0.013)	0.08 (0.013)	0.29 (0.044)	0.44 (0.030)	0.13 (0.031)	0.15 (0.048)
-Fe	0.87 (0.156)	2.90 (1.710)	0.17 (0.058)	0.17 (0.058)	0.49 (0.258)	0.87 (0.615)	0.05 (0.035)	0.40 (0,099)
P	ns	ns	*	*	*	*	*	*

ASP: aspartic acid; GLU: glutamic acid; ASN: asparagine; SER: serine; GLN: glutamine; GLY: glycine; ARG: arginine. ns: P > 0.05; *: P < 0.05.

4. Discussion

It is well known that under Fe deficiency, leaves generally display low photosynthetic activity (Abadía et al., 1999; Hantzis et al., 2018), enhancing the risk for photo-oxidative damage, especially to PSII (Andersson and Aro, 2001).

Data obtained in this work confirm that Fe deficiency strongly impaired the photosynthetic machinery at different levels. The substantial decrease in CO₂ assimilation can be related to several changes occurring in Fe-deficient leaves: i) stomatal closure; ii) biochemical reduction of CO₂ assimilation, as evidenced by the decrease in carboxylation activity of Rubisco enzyme, iii) decrease in PSII photochemical efficiency (i.e. the reduction in Fv/Fm and ΦPSII) and a sharp decrease of ETR. The decrease in Fv/Fm, observed in Fe-deficient leaves, is attributable to the increase in the minimal as well as to the decrease in maximal chlorophyll fluorescence. An increase in F₀ [which represents the minimal fluorescence yield and occurs when all reaction centres are in an open state (Krause and Weiss, 1991)] under Fe deficiency might indicate losses of energy transfer from pigments to the reaction center, likely due to damage of the LHC associated with the PSII, as previously observed in conditions of Fe deficiency (Abadía et al., 1999). However, the analogous decrease of Fm yield reflects the increase of energy dissipation which may be related to denaturation of chlorophyll-binding proteins (Yamane et al., 1998), such as the antenna pigment-protein complex CP43-CP47 (Wang et al., 1999) and/or irreversible photo-damage to the RCII (Klughammer and Schreiber,

2008). However, NPQ increased in Fe-deficient leaves of cucumber plants, which is supportive for the activation of photo-protective mechanisms by these plants. These findings are in agreement with the previous investigations performed in other species, such as in pear (Morales et al., 2000a, b), tomato (Donnini et al., 2003), peach (Molassiotis et al., 2006), and pea (Jelali et al., 2011). Hantzis et al. (2018) reported that Fe-deficient *Arabidopsis* plants displayed lower values of NPQ as compared with control plants, suggesting that some plants displayed different modulation of photosynthetic process under low Fe availability. Later authors also demonstrated that a hierarchy for Fe utilization in photosynthetic tissue occurs in plants, indicating that different plant species/genotypes might display specific Fe-economy programs and, in turn, different acclimation strategies to avoid Fe deficiency (Vigani and Murgia, 2018).

In addition to photosynthetic-related parameters, this work offers new insights on the impact of Fe deficiency on the PR pathway. The amount of CO₂ assimilated is less affected by PR under Fe deficiency compared with control plants, indicating that such a pathway is slowed down when hardly any Fe is present. Accordingly, the *in vivo* carboxylation activity is strongly impaired while GOX activity did not change and CAT activities decreased under Fe deficiency. Such observations suggest that the modulation of both photosynthesis and photorespiration under low Fe availability allows plants to limit the losses of CO₂ assimilation by PR.

Along with CO₂ assimilation, the metabolic pathway of PR is interlinked to the N metabolism. The presence of the so-called photorespiratory N cycle allows the reincorporation of NH₄⁺, coming from Gly-Ser conversion, into Glu/Gln via GS-GOGAT cycle (Keys et al., 1978). Thanks to the ability of GOGAT to provide Glu also to Glutamate:Glyoxylate aminotransferase (GGAT) in the peroxisome, the NH₄⁺ recycled can be used for the synthesis of new Gly molecules. In this work, Fe deficiency affected peroxisome metabolism as well as photorespiratory N recycling by altering NO₃⁻ reduction, ammonia organication, and quali-quantitative amino acid composition in leaves. Such observations agree with the finding that under Fe deficiency, NR activity sharply decreased in leaves leading to foliar NO₃⁻ accumulation. On the other hand, the GS-GOGAT cycle was not impaired, and NH₄⁺ content did not show any changes under Fe deficiency, to indicate that NH₄⁺ content derived from AA recycling instead of from NO₃⁻ reduction. Furthermore, under Fe deficiency, the Gly content decreased in cucumber leaf along with an accumulation of Ser. Such findings suggest that the conversion of Gly into Ser is maintained at high rate under Fe deficiency. Such a reaction represents a crucial step to produce NH₄⁺ for photorespiratory N cycling (Peterhansel and Maurino, 2011). However, in addition to PR, two other pathways of Ser formation operate in plants and represent the branches of glycolysis diverging at the level of 3-phosphoglyceric acid. One branch (the glycerate-serine pathway) occurs in the cytosol and involves glycerate formation from 3-phosphoglycerate, while the other (the phosphorylated serine pathway) operates in plastids and forms phosphohydroxypyruvate as an intermediate (Igamberdiev and Kleczkowski, 2018). The differential modulation of hydroxypyruvate reductase (HPR) 1 and HPR2 activities occurred under Fe deficiency, which suggests that Ser synthesis from the non-phosphorylated pathways is unlikely to be induced under Fe deficiency. Hydroxypyruvate (Hpyr) can diffuse out of the peroxisome to the cytosol, where it is converted into glycerate by HPR2, a cytosolic paralog of peroxisomal HPR1 (Timm et al., 2008, Timm et al., 2012). Recent work reported that Hpyr in the cytosol might be involved in the alternative PR pathway involving glycerate kinase (GLYK) (Ushijima et al., 2017). This enzyme catalyzes the conversion of glycerate to 3-phosphoglycerate (3PGA) during the final step of photorespiration in the chloroplast (Boldt et al., 2005; Peterhansel and Maurino, 2011). However, a

cytosolic isoform of GLYK has recently been identified in the plant, which catalyzes the conversion of the cytosolic glycerate into 3PGA (Ushijima et al., 2017). The level of Gly, a photorespiratory intermediate, accumulated at significantly lower levels in GLYK overexpressing mutant (cytGLYK) plants than in the glyk mutant (Ushijima et al., 2017). Such findings may indicate that the low level of Gly observed in Fe-deficient leaves might be linked to the higher induction of the cytosolic photorespiratory pathway through the activation of HPR2. Therefore, the observed compensation HPR activity between peroxisomal and cytosolic isoforms along with the induction of G3PDH cytosolic activity suggest that Hpyr synthesized in the peroxisome is probably addressed to glycolysis throughout the cytosolic photorespiratory bypass (Fig. 4) (Ushijima et al., 2017).

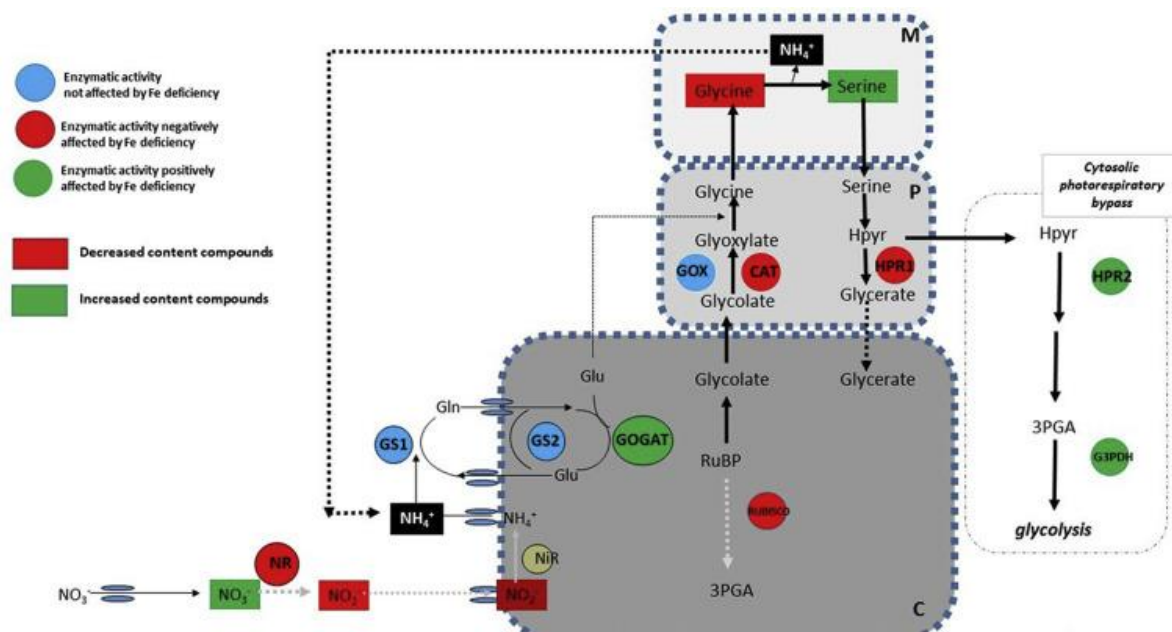


Fig. 4

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Fig. 4. Schematic representation of photorespiratory modulation and N recycling in Fe-deficient cucumber leaves. Fe deficiency determines i) a decrease (red circle) and an increase (green circle) of some enzymatic activities and ii) the accumulation (green rectangle) and a decreased content (red rectangle) of some compounds related to photorespiratory and N assimilation pathways. Proposed metabolic changes allow us to hypothesize that Fe deficiency leads to metabolic alterations aimed at sustaining N recycling and cytosolic photorespiratory bypass.

AbbreviationsC, chloroplast; P, peroxisome; M, mitochondrion; NR, nitrate reductase; GOGAT, glutamine oxoglutarate aminotransferase; glutamine synthase (GS); HPR, hydroxypruvate reductase; Hpyr; PGA and 3PGA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Overall, this work provides substantial evidence about the modulation of the PR in cucumber leaves under Fe deficiency. Our results suggest that plants subjected to Fe deficiency modulate PR-related processes, i.e. activating N recycling and cytosolic photorespiratory bypass, to sustain ammonia organication and glycolysis pathway respectively. A role of PR in the metabolic reprogramming occurring under Fe deficiency in cucumber leaves was highlighted, which adds a new piece to the puzzle of Fe metabolism in plants.

Contributions

GV, GZ and LG conceived the experimental work; FMC, ML, SD, LG performed the experimental analysis. GV drafted the manuscript, ML, LG, GZ revised critically the manuscript.

CRedit authorship contribution statement

Fabio M. Casiraghi: Investigation, Methodology, Formal analysis. Marco Landi: Investigation, Formal analysis, Writing - review & editing. Silvia Donnini: Investigation, Formal analysis. Andrea Borlotti: Investigation, Formal analysis. Graziano Zocchi: Writing - review & editing. Lucia Guidi: Methodology, Validation, Writing - review & editing. Gianpiero Vigani: Conceptualization, Resources, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

Supplementary Fig. S1

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Supplementary Fig. S1. Response curve of CO₂ assimilation in relation to intercellular CO₂ concentration (A/Ci curves) in leaves of cucumber control (round) and Fe deficient plants (square) after 7 days from the start of the experiment (A). A/Ci curves were determined at saturating light intensity, and each value represents the mean of three replicates. The response curve of the photosynthetic activity to increasing light intensity (light curves) under conditions of 21% O₂ (round symbol) and in the absence of O₂ (square symbol). Light curves were determined at 380 μmol mol⁻¹ CO₂, and each value represents the mean of three replicates.

Supplementary Fig. S2

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Supplementary Fig. S2. A) Western blot analysis of the different fraction of the mixed Percoll-Sucrose gradient on leaves of control plants (+Fe) and Fe deficient plants (-Fe). For each sample, 10 μg of protein was used. Antibody used was against catalase. CAT catalase; CE crude extract: S1 supernatant 1; I Percoll-Sucrose interface; B bottom of the centrifugation tubes. B) Western blot analysis of in leaves of control plants (+Fe) and Fe deficient plants (-Fe) after 7 days from the start of the experiment. For each sample, 12 μg of protein was used. Antibody used was against Glycolate oxidase. GOX catalase; LP-P1 Leaf peroxisome purified fraction

Supplementary Fig. S3

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Supplementary Fig. S3. Northern blot analysis of genes involved in N assimilation pathway in leaves of cucumber control (+Fe) and Fe deficient plants (-Fe) after 7 days from the start of the experiment.

Gene sequences considered were Csa015274 (CsGS1), Csa0081189 (CsGS2), Csa021126 (CsNADH-GOGAT), Csa002676 (CsFd-GOGAT), Csa008224 (CsNR) according to Borlotti et al. (2012).

Supplementary Fig. S4

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Supplementary Fig. S4. Activity of glyceraldehyde 3 phosphate dehydrogenase in leaves of cucumber control (+Fe) and Fe deficient plants (-Fe). The asterisks indicate the significance following the Student's t-test for the comparison between -Fe and + Fe leaves. ns: $P > 0.05$; * $P < 0.05$.

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