This is the author's final version of the contribution published as:

Slatni T, Vigani G, Ben Salah I, Dell'Orto M, Gouia H, Zocchi G, Abdelly C.

Metabolic changes of iron uptake in N$_2$-fixing common bean nodules during iron deficiency

The publisher's version is available at:

http://www.sciencedirect.com/science/article/pii/S0168945211001269?via%3Dihub#!

When citing, please refer to the published version.

Link to this full text:

https://ac.els-cdn.com/S0168945211001269/1-s2.0-S0168945211001269-
main.pdf?_tid=8f575e4a-f9e2-11e7-9729-
00000aacb35e&acdnat=1516013892_13429a03a4a22aad45c5a89461591966

Abstract
Abstract
Iron is an important nutrient in N2-fixing legume nodules. The demand for this micronutrient increases during the symbiosis establishment, where the metal is utilized for the synthesis of various iron-containing proteins in both the plant and the bacteroid. Unfortunately, in spite of its importance, iron is poorly available to plant uptake since its solubility is very low when in its oxidized form Fe(III). In the present study, the effect of iron deficiency on the activity of some proteins involved in Strategy I response, such as Fe-chelate reductase (FC-R), H+-ATPase, and phospho*enol*pyruvate carboxylase (PEPC) and the protein level of iron regulated transporter (IRT1) and H+-ATPase proteins has been investigated in both roots and nodules of a tolerant (Flamingo) and a susceptible (Coco blanc) cultivar of common bean plants. The main results of this study show that the symbiotic tolerance of Flamingo can be ascribed to a greater increase in the FC-R and H+-ATPase activities in both roots and nodules, leading to a more efficient Fe supply to nodulating tissues. The strong increase in PEPC activity and organic acid content, in the Flamingo root nodules, suggests that under iron deficiency nodules can modify their metabolism in order to sustain those activities necessary to acquire Fe directly from the soil solution.
Highlights
► Nodules can improve FC-R and H+-ATPase activities. ► The nodules contribute with roots to Fe solubilization. ► A strong increase in the PEPC activity in nodules. ► A important increase in organic acid content in nodules. ► The main results prove that Fe uptake directly by nodules.

1. Introduction

Iron is an essential element required for many functions in plants, including heme and chlorophyll biosynthesis, photosynthesis and respiration [1,2], and as a component of Fe–S cluster containing enzymes [3]. Its ability to gain and loose an electron confers to the ion important properties in the redox reactions. This microelement is also vital for the establishment and function of symbiotic nodules involved in nitrogen fixation in legumes [4,5]. Therefore, the bacteroids, which differentiate from bacteria during the symbiosis, need to be supplied with Fe in functioning nodules. Iron metabolism is of particular importance in nodules since it is a constituent of key proteins such as nitrogenase and leghemoglobin [4]. In addition, it is necessary to synthesize their cytochromes of the respiratory chain and other redox components organized as a branched system [6]. Although abundant in the environment, Fe is often a limiting nutrient for plant growth and nodules establishment due to the low solubility of its oxidized form at near neutral soil pH [7]. Thus, plants have evolved efficient Fe acquisition mechanisms. In particular Strategy I plants (dicotyledonous and non-graminaceous plants), which include common bean, take up Fe from the rhizosphere by reducing ferric into ferrous forms through the action of plasma membrane-bound Fe(III)-chelate reductase (FC-R) [8,9]. Iron reduction is then followed by the uptake of Fe2+ into root cells by a metal ion transporter (IRT1) [3,10,11]. Furthermore, roots of Strategy I plants release more protons under Fe deficiency, thereby lowering the rhizosphere pH and increasing Fe solubility. The importance of rhizosphere acidification in the responses of Strategy I plants to Fe deficiency has

been recognized for many years [12,13]. This response has been associated with the activity of a plasma membrane-localized H+-ATPase [12]. A recent work using a reverse genetic approach has shown that root ferric reductase is encoded by the *FRO2* gene [3,8,14], and that IRT1, a ZIP family member, is the main Fe transporter responsible for root Fe uptake from the soil solution [15]. IRT1 and FRO2 play a key role in root Fe uptake in response to Fe deficiency [3,8] and are considered among the principal regulatory targets that control Fe uptake and homeostasis in plants. Their expression is rapidly induced by Fe deficiency in roots [16] as well as drastically turned off, via transcriptional and post-transcriptional modifications, in Fe resupplied plants [17].

At the level of symbiotic nodules, a ferric-chelate reductase activity associated with the peribacteroid membrane (PBM), which effectively excludes the bacteroid from the host cytosol and which controls the movement of metabolites between the two symbiotic partners [18], has been detected and its involvement in the Fe uptake into symbiosomes has been assumed [19]. Thus, Fe transport into the bacteroid must occur across the PBM. In fact, it has been shown that iron is transported in purified soybean symbiosomes [19] and that citrate appears to play an important role in the process. In particular, the ferrous form was taken up by symbiosomes more efficiently than the ferric one indicating that the transport from the plant host cell to the microsymbiont *in vivo* may occur mainly as Fe2+.

The nodules also possess an H+-ATPase activity localized on the PBM which pumps protons outside bringing to a generation of a transmembrane electrical potential [20]. The H+-pumping ATPase was one of the first enzymatic activities to be detected on the PBM [21] and it plays a critical role in the regulation of nutrient exchange between legume and bacteroids [22]. The essential exchange between the two partners of symbiosis is the flux of carbon skeletons from the plant into the bacteroid and the product of nitrogen fixation from the bacteroid to the plant. There is a general agreement that dicarboxylates, especially malate, are the main source of carbon supplied to the bacteroid and that ammonium is the form in which fixed nitrogen is supplied to the plant [23]. Phospho*enol*pyruvate carboxylase (PEPC) has an important role in providing carbon skeletons to sustain amino acid synthesis. This enzyme catalyses the fixation of bicarbonate to phospho*enol*pyruvate to produce oxaloacetate and Pi and consequently keep the glycolysis rate sufficiently high in the plant [24] thus sustaining the demand for ATP and NAD(P)H and contributing to the cytosolic pH-stat. PEPC plays a very important role in N2-fixing root nodules, providing both C for N assimilation into amino acids and malate as an energy source for bacteroid nitrogenase [25]. Both the protein and the mRNA of PEPC are significantly more abundant in legume nodules as compared to roots and the PEPC protein represents at least 1% of nodule soluble protein [25].

In order to characterize the changes induced by Fe deficiency on physiological and biochemical behaviour of nodules and to verify whether nodules are able to take up Fe directly from soil solution, an attempt has been made to measure the activities of FC-R, H+-ATPase and PEPC in N2-fixing common bean roots and nodules subjected to Fe deficiency.

2. Materials and methods

2.1. Plant material and growth conditions

Two common bean cultivars, Coco blanc, sensitive, and Flamingo, tolerant to Fe deficiency [26], were inoculated with *Rhizobium tropici* CIAT 899 originating from International Center of Tropical Agriculture, Colombia and maintained in the "Laboratory interactions legumes Micro-organisms" (CBBC, Hammam-Lif, Tunisia) and grown individually in the glasshouse under natural light in 1 L pots filled with the following N-free nutrient solution [27]: KH2PO4 (1.60 mM), MgSO4 (1.50 mM), K2SO4 (1.50 mM), CaSO4 (3.50 mM), H3BO3 (4 μM), MnSO4 (4 μM), ZnSO4 (1 μM), CuSO4 (1 μM), CoCl2 (0.12 μM), Na6Mo7O24 (0.12 μM) added with 5 μM Fe as K–Fe–EDTA and 1 mM urea as starter N needed for growth during 2 weeks. The nutrient solution was aerated with a flow of 400 mL min−1 of filtered air via a compressor and "spaghetti tube" distribution system. After 2 weeks of pre-treatment (appearance of nodules), initial harvest was performed and plants were separated in two plots, the first one received no iron (deficient plants), and the second one received 45 μM Fe(III) EDTA (control plants). After 3 weeks (time necessary to reach the maximum

of N2-fixation [28]) of treatment, leaves, roots and nodules were harvested separately. Samples of fresh material were dried at 70 °C for 72 h and ground to fine powder using a grinder with agate pots. Mineral concentration was determined by atomic absorption spectrophotometer (Varian Spectr AAS 220). Quantification of active Fe was performed according to Köseoglu and Açikgöz [29]. Total nitrogen was determined by the Kjeldahl method. Quantities of N fixed were calculated from the differences between whole-plant N content at the final and initial harvest.

2.2. Preparation of microsomal and soluble fraction

Roots and nodules of common bean plants were excised, rinsed in distilled water and homogenized in a mortar at 2–4 °C in a buffer containing 50 mM Tris–HCl pH7.5 containing 10 mg mL^{-1} leupeptin, 10% glycerol, 20% PVPP and 10 mM MgCl2, 1 mM EDTA, 14 mM β-mercaptoethanol, 1 mM PMSF. After filtration through four layers of gauze, the homogenate was centrifuged at 13,000 g for 15 min and the supernatant was again centrifuged at 80,000 g for 30 min to obtain a microsomal fraction (pellet) and a soluble fraction (supernatant). The microsomal pellet was re-suspended in the same buffer and stored in small aliquots in liquid N2 until testing. [30].

2.3. Enzyme assays

H+-ATPase activity was determined spectrophotometrically by coupling ATP hydrolysis to NADH oxidation according to Dell'Orto et al. [31]. The assay was performed at room temperature in a final volume of 2 mL with the following composition: 250 mM sucrose, 50 mM KCl, 25 mM Mops-BTP (pH 6.5), 1 mM ATP, 0.25 mM NADH, 1 mM PEP, 15 μg mL^{-1} lactate dehydrogenase (EC 1.1.1.27) (Boehringer Mannheim), 30 μg mL^{-1} pyruvate kinase (EC 2.7.1.40) (Boehringer Mannheim), 0.015% (w/v) Lubrol and 10–20 μg mircosomal fraction protein. The reaction was started by the addition of 1 mM MgSO4. The oxidation of NADH was followed at 340 nm for 5 min period.

The NADH-dependent FC-R activity was assayed in a medium containing 250 mM sucrose, 15 mM MOPS-BTP (pH 6.0), 0.25 mM K3Fe(CN)6, 0.25 mM NADH, and 0.01% Lubrol (Sigma–Aldrich). Reaction was started by the addition of 20–50 μL of microsomal fraction preparation. NADH oxidation was followed at 340 nm and the absorbance changes monitored over a 5 min.

Phospho*enol*pyruvate carboxylase (PEPC) (EC 4.1.1.31) was determined as reported by De Nisi and Zocchi [30]. Reaction was started by adding aliquots of protein extracts and the enzymatic assay was performed at 25 °C in 1.5 mL final volume. Oxidation of NADH was followed spectrophotometrically at 340 nm.

2.4. Organic acid assays

Organic acids, such as malate and citrate, were extracted from root or nodules grown in the presence or absence of Fe as reported by Rabotti et al. [32]. Roots and nodules, of each treatment, were rinsed in distilled water, homogenized in the presence of 5 mL of 10% (v/v) perchloric acid and centrifuged for 15 min at 10,000 g. Supernatant pH was brought to 7.5 with 0.5 M K2CO3 to neutralize the acidity and to precipitate the perchlorate. The extract was clarified with another centrifugation at 15,000 g for 15 min. Citric and malic acid contents were determined enzymatically, according to Rabotti et al. [32]. The recovery of both organic acids was more than 90% as determined by the use of an internal standard.

2.5. Protein gel blot analysis

Soluble or microsomal protein extracted from roots and nodules of plants grown in the presence and in the absence of Fe was loaded on a discontinuous SDS-polyacrylamide gel [3.75% (w/v) acrylamide stacking gel, and typically 10% (w/v) acrylamide separating gel], according to the method of Laemmli [33]. After SDS-PAGE, proteins were electrophoretically transferred to PVDF membrane filters (Sigma) using a semi-dry blotting system with a buffer containing 10 mM 3-cyclo-hexylamino-1-propane sulphonic acid (pH 11 with NaOH) and 10% (v/v) methanol for 1.5 h at room temperature at a current intensity of 0.8 mA cm^{-2}. Polyclonal antibodies raised against an IRT1 (a kind gift from Dr. E. Connolly), H+-ATPase (a kind gift from Dr. R. Serrano) and PEPC (a kind gift from Dr. J. Vidal) isoform were used. Antisera were diluted 1:1000 in TBS-T buffer (20 mM Tris–HCl (pH 7.5), 200 mM NaCl, 0.05% (w/v) Tween 20) and incubation was carried out overnight at

4 °C. After rinsing with TBS-T, PVDF membranes were incubated at room temperature for 2 h with a 1:25,000 diluted secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma). After rinsing in TBS-T the filters were incubated in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (FAST BCIP/NBT, Sigma).

Protein concentration was determined according to Bradford [34] by using the BioRad reagent and BSA as a standard.

2.6. Statistical analysis

A two-way analysis of variance (ANOVA), with the cultivars and treatments as factors, was performed for the data set using the SPSS 10.0 program. Means were compared using the Duncan's test at $P < 0.05$ level when significant differences were found [35].

3. Results

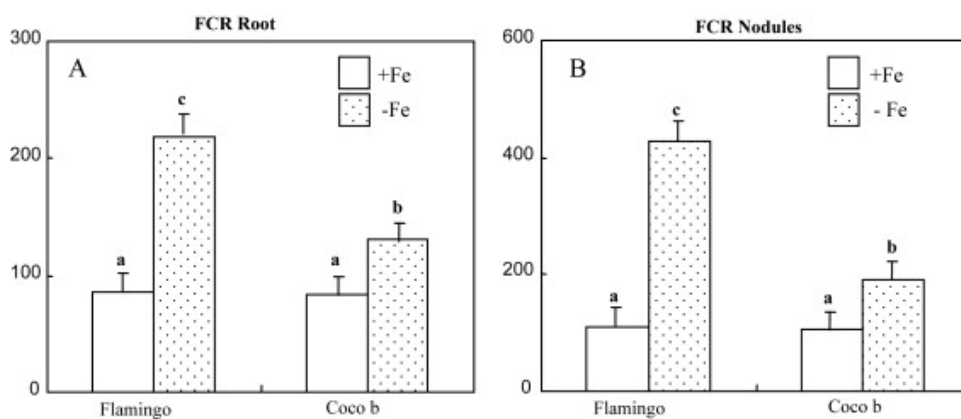3.1. H+-ATPase, FC-R and PEPC activities in roots and nodules under Fe

Fig. 1 shows the H+-ATPase activity in the microsomal fraction extracted from roots and nodules of both cultivars grown in the presence and in the absence of Fe. The H+-ATPase activity was higher in Fe-deficient than in Fe-sufficient plants for both cultivars (Fig. 1). However, Flamingo shows a higher H+-ATPase activity than Coco blanc. At the root level (Fig. 1A), this activity increased 3.8 and 2.1 times in Fe-deficient Flamingo and Coco blanc plants, respectively, compared with the control plants. Accordingly, the H+-ATPase activity was 2.6 and 1.4 times higher in nodules of Fe-deficient Flamingo and Coco blanc plants, respectively compared with the controls (Fig. 1B). Additionally, under Fe deficiency the H+-ATPase activity in roots was twice higher than that observed in nodules in both cultivars.
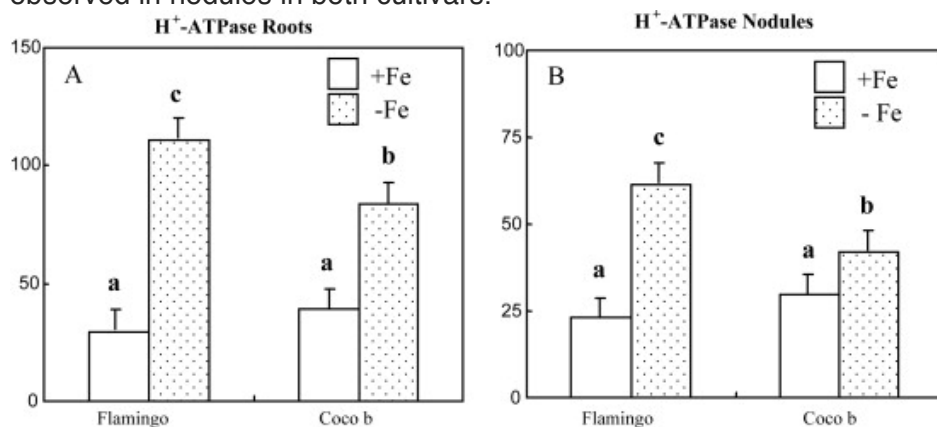
[Download full-size image]

Fig. 1. H+-ATPase activity (nmol NADH min−1 mg−1 Protein) determined in microsomal fraction of roots (A) and nodules (B) of common bean plants grown in the presence or in the absence of Fe. Data are the means ± standard error of 4 replicates. Mean values followed by the same letter are not significantly different at $P \leq 0.05$.

The FC-R activity measured in the same microsomal fractions was found to be higher under Fe deficiency than in control condition. In roots it was 2.6 and 1.6 times higher in Fe-deficient Flamingo and Coco blanc respectively, compared with the control (Fig. 2A). As well, as, the FC-R activity was 4 and 1.8 times higher in Flamingo and Coco blanc Fe-deficient nodules, respectively (Fig. 2B), compared to control. An intraspecific difference was found for FC-R activity, since under Fe deficiency Flamingo, which is the tolerant cultivar, exhibits a higher value than the sensitive cultivar Coco blanc (2.3 and 1.9 times, in nodules and roots respectively). In addition, contrarily to what observed for H+-ATPase, under Fe deficiency, the FC-R activity in nodules is around twice higher than in roots.

Fig. 2. FC-R activity (nmol NADH min−1 mg−1 Protein) determined in microsomal fraction of roots (A) and nodules (B) of common bean plants grown in the presence or absence of Fe. Data are the means ± standard error of 4 replicates. Mean values followed by the same letter are not significantly different at $P \leq 0.05$.

PEPC, activity decreases in the roots of both cultivars subjected to Fe deficiency (Fig. 3A) reaching a lower value in the nodules of Coco blanc, while it increases significantly in nodules of the tolerant variety (Fig. 3B). Moreover, the PEPC activity was 8.5 and 6.4 times higher in Flamingo and Coco blanc nodules subjected to Fe deficiency with respect to the roots, respectively.
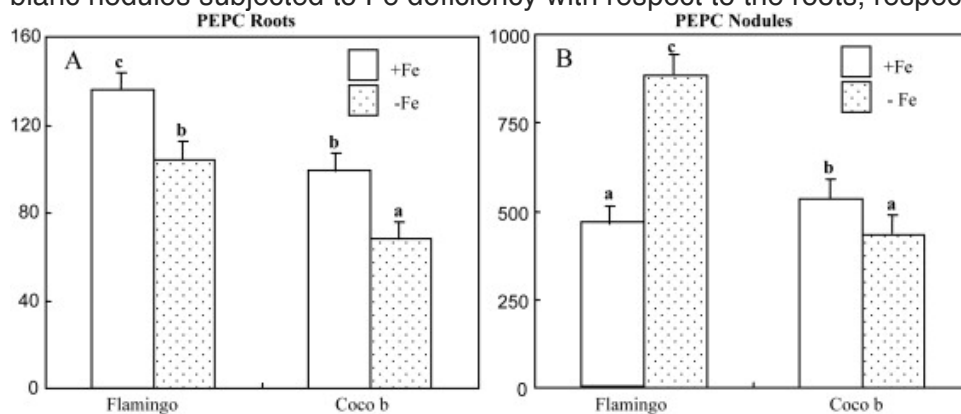
Fig. 3. PEPC activity (nmol NADH min−1 mg−1 Protein) determined in microsomal fraction of roots (A) and nodules (B) of common bean plants grown in the presence or absence of Fe. Data are the means ± standard error of 4 replicates. Mean values followed by the same letter are not significantly different at $P \leq 0.05$.

3.2. Protein levels of H+-ATPase, IRT1 and PEPC

Protein levels of H+-ATPase, IRT1 and PEPC was determined by Western blot analysis (Fig. 4). Although the antibody against the H+-ATPase detected weak bands, probably due to its low effectiveness on the microsomal fraction, a slight increase in the H+-ATPase protein is more evident in Fe-deficient nodules of both cultivars, while at the root level this difference is less evident. In any case, even if the increase is weak it might in part explain the enhanced H+-ATPase activity determined in Fe-deficient nodules.
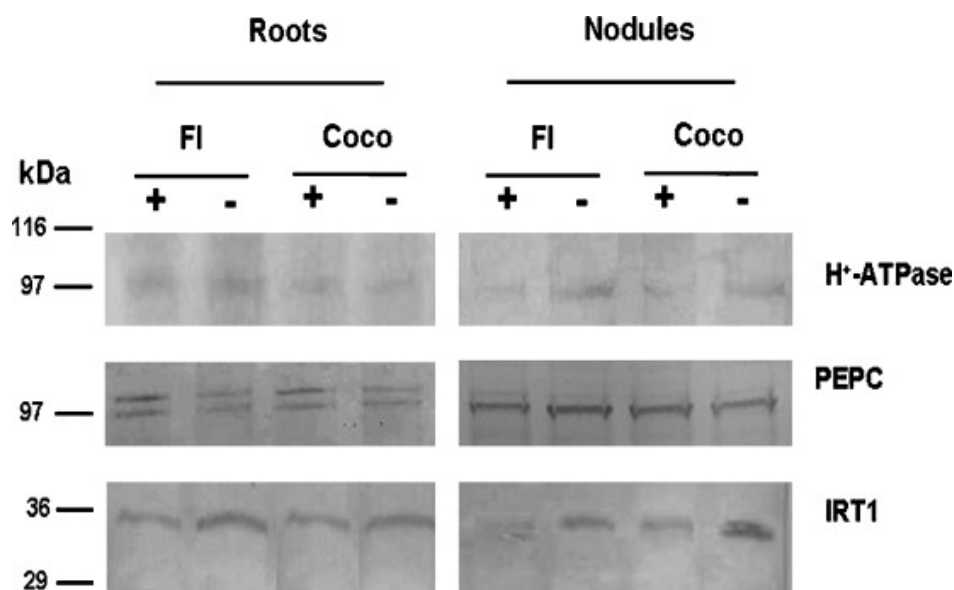
Fig. 4. Western blot determination using SDS-PAGE and immunoblotting analysis of IRT1 and H+-ATPase extracted from microsomal fraction and PEPC extracted from soluble fraction of roots and nodules of common bean plants grown in the presence and in the absence of Fe. 12.5 µg of roots and nodules proteins were loaded in each lane for IRT1 and H+-ATPase. 12.5 µg and 10 µg proteins of respectively roots and nodules were leaded in each lane for PEPC. The determination was performed at least 3 times with different extracts with similar results.

The IRT1 antiserum recognized a clear band, at the expected height, in the microsomal fraction of both root and nodules. At the root level, the IRT1 protein increased in –Fe Flamingo while in –Fe Coco a lower increase was observed with respect to the relative control conditions. At the nodule level IRT1 greatly accumulate in both –Fe Flamingo and –Fe Coco with respect to the relative control conditions (Fig. 4). Concerning the amount of PEPC protein, this decreased in the soluble fraction of Fe-deficient roots of both cultivars in agreement with the enzymatic activity. On the contrary, in nodules from Flamingo samples a very low increase in the protein amount can be appreciated in the –Fe condition, while in nodules of the sensitive cultivar (Coco blanc) the PEPC protein slightly decreased in –Fe condition.

3.3. Mineral composition

The mineral content of different plant organs is summarized in Table 1 and shows that the Fe content was several times lower in both roots and leaves of plants subjected to Fe deficiency compared to control plants, mainly in the sensitive genotype. Accordingly, the Fe content decreased in nodules of the two cultivars grown under Fe deficiency by about 29% in Flamingo and 56% in Coco blanc, respect to the control.

Table 1. Minerals status of leaves, roots and nodules of plant grown in the presence (+Fe) or in the absence (–Fe) of Fe. Data are the means ± standard error of 4 replicates. Mean values followed by the same letter are not significantly different at $P \le 0.05$.

	Divalent ion (mg g^{-1} DW)	Fe	Zn	Cu	Mg	Ca
Flamingo						
Leaves	+Fe	0.185 ± 0.07c	0.047 ± 0.002a	0.015 ± 0.001a	8.88 ± 0.35i	8.4 ± 0.42g
	–Fe	0.074 ± 0.02b	0.048 ± 0.003a	0.018 ± 0.001a	6.72 ± 0.27f	7.1 ± 0.15e
Roots	+Fe	0.814 ± 0.21i	0.085 ± 0.004c	0.039 ± 0.002bc	4.3 ± 0.21c	5.3 ± 0.32d
	–Fe	0.266 ± 0.08e	0.130 ± 0.001d	0.040 ± 0.003bc	3.24 ± 0.11b	3.5 ± 0.11a
Nodules	+Fe	0.576 ± 0.13g	0.075 ± 0.002b	0.075 ± 0.004d	6.96 ± 0.23f	6.7 ± 0.34d
	–Fe	0.411 ± 0.15f	0.084 ± 0.003c	0.086 ± 0.003e	7.5 ± 0.37g	5.2 ± 0.25d

Coco blanc						
Leaves	+Fe	0.172 ± 0.08c	0.041 ± 0.002a	0.017 ± 0.002a	8.2 ± 0.41h	7.8 ± 0.37f
	−Fe	0.045 ± 0.03a	0.054 ± 0.003a	0.023 ± 0.003ab	6.3 ± 0.23e	6.6 ± 0.25e
Roots	+Fe	0.781 ± 0.21h	0.092 ± 0.005c	0.045 ± 0.003c	4.05 ± 0.18c	4.1 ± 0.21b
	−Fe	0.214 ± 0.07d	0.269 ± 0.004e	0.100 ± 0.005f	2.6 ± 0.11a	3.2 ± 0.12a
Nodules	+Fe	0.516 ± 0.12g	0.075 ± 0.003b	0.032 ± 0.003b	5.3 ± 0.21d	5.5 ± 0.23d
	−Fe	0.225 ± 0.11d	0.321 ± 0.001f	0.079 ± 0.002de	3.8 ± 0.23bc	4.7 ± 0.25c

On the contrary, the amount of Zn strongly increased in Fe-deficient Coco blanc cultivar compared to the control, particularly in roots and nodules; in Flamingo, only the roots showed a considerably increase in Zn content under Fe deficiency, while its content slightly increased in nodules and did not show significant increase in leaves. A similar trend has been observed for the Cu contents. In fact, in the sensitive cultivar, Fe deficiency induced an enhanced amount of the element in all tissues, while in Fe-deficient Flamingo plants Cu content slightly increased in nodules, while it did not show any significant difference in roots and leaves, compared to the control. Concerning Ca and Mg, these divalent cations behave in the same manner, showing a decrease in all the tissues of both cultivars under Fe deficiency, except for Flamingo nodules which show a significant increase in Mg content.

3.4. Organic acids

The determination of some organic acids involved in carbon supply to bacteroids (Fig. 5) shows an increase in their content especially in nodules of Flamingo cultivar subjected to Fe deficiency: the content was about 4 and 2.7 times higher in Fe-deficient nodules than in Fe-sufficient ones for citrate and malate, respectively. In Coco blanc (sensitive cultivar) the citrate content slightly increased (about +23%) and the malate content decreased (about −19%) in nodules subjected to Fe deficiency. In roots the citrate content increases only in Flamingo by about 33.5% in plants grown under Fe-deficiency, while in Coco blanc it decreases by about 36.5%. The malate content decreases in roots subjected to Fe deficiency by about 49% and 30% in Flamingo and Coco blanc respectively.
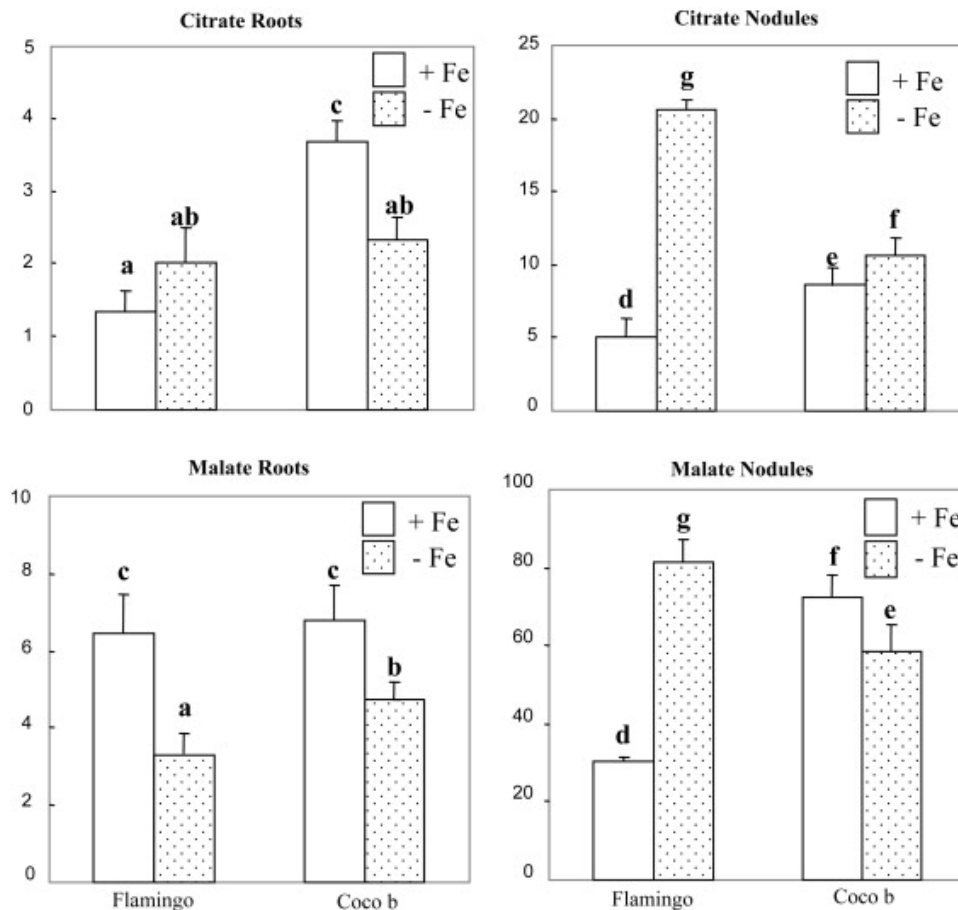
Fig. 5. Effect of iron deficiency on citrate (A) and malate (B) content (μg g−1 FW) in roots and nodules grown in the presence or in the absence of Fe. Data are the means ± standard error of 4 replicates. Mean values followed by the same letter are not significantly different at $P \le 0.05$.

4. Discussion

The results obtained in this work show that the adaptive responses of Strategy I plants involved in Fe uptake were increased under Fe deficiency in the two common bean cultivars considered. In a preliminary time-course screening the acidification and reduction activities were determined after 1, 2 and 3 weeks of –Fe treatment (data not shown). The choice to determine these activities after a 3-week treatment period, in which the difference between the two cultivars is still high, also corresponds to the maximum N2-fixation, In this work FC-R and H+-ATPase activities were increased under Fe deficiency in the roots of both cultivars, but Flamingo presents higher values. Similar results were observed in other plants belonging to leguminosae: for instance, pea [35] and soybean [36], suggesting that the induction of FC-R and H+-ATPase is correlated to the tolerance to Fe deficiency. It is also noteworthy that the activity of FC-R under Fe deficiency was higher in nodules than in roots (about two fold), suggesting that under this condition nodules play an important role in Fe^{3+} reduction and Fe uptake and consequently in Fe supply to the bacteroids. Several studies on Fe acquisition in legumes or in rhizobia are directed to answer two essential questions: (1) how do the plant hosts acquire enough Fe for the symbiosis? and (2) how do rhizobia acquire Fe as symbionts? [8,37]. In the literature there is no information about the possibility of nodules to acquire Fe directly from the soil solution and in order to verify this hypothesis, a preliminary investigation using "*in vivo*" techniques was performed in common bean [38]. Results showed that both Fe(III)-chelate reductase and acidification of the external medium were increased in roots and especially in nodules when grown in a Fe-deficient medium [38]. Indeed, FC-R activity appears particularly localized around nodules subjected to Fe deficiency suggesting that this organ might play an important role in the total Fe^{3+} reduction, especially in the

tolerant cultivar, Flamingo [38]. With the additional data obtained in this work we might hypothesize that when common bean plants grow with low Fe availability, nodules acquire the capacity to take up Fe not only from the plant cytosol, but directly from the nutrient solution.

Previous results showed that the nodules were less affected by Fe deficiency compared with the plant tissues, suggesting that these symbiotic organs are protected against an excessive decrease of their Fe concentration [26], as well as those results show that the performance of the tolerant cultivar is related to a better Fe allocation to nodules and to a significantly better Fe-use-efficiency [26]. In this current study Fe content in nodules (Table 1) is less affected than in other plant tissues and suggests that nodules maintain the integrity of their mechanism to allocate more Fe to bacteroids in order to keep the nitrogenase activity high. Western blot analysis shows the presence of IRT1 protein in roots and in nodules of both cultivars. The amount of this protein is increased under Fe deficiency also in nodules suggesting that, similarly to what occurs in roots, IRT1 may be involved in Fe uptake in nodules.

It is known that the apoplast of roots might contain some Fe atoms [39]. The cell wall, the major component of root apoplast, contains highly negatively charged sites that can serve as a sink for most cationic mineral nutrients, such as Fe. Under Fe deficiency conditions Fe desorption from the cell wall could be achieved by acidification of the apoplast (H+-ATPase), and the subsequent reduction of Fe^{3+} (FC-R) allows its uptake by IRT1.

Interestingly, in Coco blanc nodules, Fe deficiency induced a lower increase in H+-ATPase and FC-R activities (limiting the availability of apoplastic Fe) with respect to Flamingo, although a comparable IRT1 protein accumulation is observed. Since IRT1 is a specific transporter of Fe^{2+} but can also transport other bivalent cations, such as Zn^{2+}, Mn^{2+} and Cd^{2+} under Fe deficiency [40], the accumulation of Zn in nodules of the sensitive cultivar might be the result of a lower apoplastic Fe availability (Table 1). Nevertheless, Zn accumulation in nodules could be due to the presence of other specific transporters (i.e. ZIP family carriers).

Several metabolic changes occur under Fe deficiency (i.e. increase in PEPC activity, organic acid accumulation), since this nutritional stress affects mainly the carbon and the energy metabolism [41]. This is true not only for the plant but also for the bacteroids [42]. In fact, bacteroids fixing nitrogen are dependent on the host plant for organic compounds and energy: the PEPC catalyses the fixation of bicarbonate to phospho*enol*pyruvate to produce oxalacetate that, along with malate and citrate, provides the carbon skeletons for the amino acid synthesis and the production of ATP and reducing equivalents necessary for the nitrogenase activity [42]. In the same context, nodule immunolabeling and *in situ* mRNA hybridization studies show that PEPC is present in both infected and uninfected cells of the N2-fixing zone [43]. The dependence of nodule function on PEPC activity has been shown through the effects of antisense expression of PEPC in nodules [44]. Inhibition of nodule PEPC *via* antisense resulted in a decrease of nodule mRNA and enzyme activity, lower nitrogenase activity and decreased N accumulation and plant growth [44].

Our results showed that the PEPC activity increased only in Flamingo nodules, while in Coco nodules and in roots of both cultivars, similarly to what found in soybean [36], its activity slightly decreased (Fig. 3). Interestingly, the organic acid accumulation in the different samples follows the same trend of PEPC activity (Fig. 5). Several studies show that, under Fe deficiency, the increased PEPC activity results in a greater production of organic acids, mainly malate and citrate [24,36,45,46]. Malate is addressed to mitochondria to replenish the Krebs cycle sustaining the increased demand for ATP, [41], while citrate is considered the main chelating agent for Fe translocation. The importance of organic acid accumulation has been assessed also in nodules [19]. In fact, the increased synthesis of ATP is important to sustain the nitrogenase activity and the H+-ATPase activity necessary to acidify the peribacteroid space (PBS) to take up Fe [19]. Furthermore, it is well documented that citrate is involved in the chelation and transport of Fe^{3+} from the plant cytosol to the peribacteroid space (PBS) via the peribacteroid membrane (PBM) [19]. Our results suggest that Fe deficiency induces these metabolic changes only in Flamingo nodules which showed the highest activation of FC-R and the highest Fe content. How can we explain these results? The PEPC regulation is quite complex and is the result of different factors

acting on the enzyme [30]. The high amount of Fe in Flamingo nodules allows the nitrogenase to work more efficiently thus requesting a stronger effort in the production of carbon skeletons to assimilate the NH4+ produced. The activity of PEPC is one of the main links between carbon and nitrogen metabolism, by producing organic acids which are substrate of nitrogen assimilation. The increased content of citrate and malate in −Fe Flamingo nodules could be a direct consequence of PEPC activation; nevertheless these organic acids could be transported from the roots to the nodules as a consequence of an increased request of carbon skeleton both for amino acid synthesis and for the production of energy, explaining their lower content in roots. In fact, the increase of citrate content in −Fe Flamingo nodules suggests that the transport of Fe(III)–citrate into the nodules could provide, at the same time, not only Fe but also carbon skeleton to the bacteroids. Nevertheless, concerning the different response of the two bean cultivars to Fe deficiency, we assumed that it may be related to a different tolerance to this stress. The possibility of a different supply of carbon skeleton from the leaves might also explain the observed variability. The chlorosis score (data not shown) shows only a small difference between the two cultivars at the beginning of the experiments, but a marked difference occurred after 3 weeks of exposure to Fe deficiency. However, this difference never reached values for which it is possible to ascribe the decrease in the response at both root and nodule levels to a heavy loss of the photosynthetic activity. Hence, the susceptible cultivar might be more chlorotic than the tolerant one because of its lower capacity to respond to Fe deficiency and to take up iron. Recently [38], we found that Fe use efficiency for chlorophyll biosynthesis (FeUE/Chl), defined as the ratio of chlorophyll concentration (mg g FW−1) to leaf Fe concentration (mg Fe g DW−1), was 3 times higher in Flamingo than in Coco blanc, confirming the sensitivity of the latter cultivar. The cause/effect relationship is difficult to establish, but our findings strongly suggest that the adaptive responses of the two cultivars are in agreement with their different susceptibility to Fe deficiency.

In conclusion, the present work suggests that, under Fe deficiency, the nodules differentially act depending on the genotype of host plant. Under Fe deficiency, in Coco plants the Fe content of nodules drastically decreased (Table 1) as well as the relative N2 fixation [26], probably due to their insufficient activation of Fe uptake activities both in roots and in nodules. By the contrast, in −Fe Flamingo plants the Fe content of nodules only slightly decreased (Table 1) as well as the relative N2 fixation [26]. This might be due to the activation of Fe uptake activities in roots and even more in nodules; in fact, the strong increase in the PEPC activity and organic acid content, suggests that, under Fe deficiency, the tolerant genotype may induce modifications of nodule metabolism in order to sustain both N2 fixation and Fe uptake directly from the soil solution.

References

[1]

J.F. Briat, C. Curie, F. Gaymard

Iron utilization and metabolism in plants

Curr. Opin. Plant Biol., 10 (2007), pp. 276-282

ArticlePDF (752KB)View Record in Scopus

[2]

G. Vigani, D. Maffi, G. Zocchi

Iron availability affects the function of mitochondria in Fe-deficient cucumber roots

New Phytol., 182 (2009), pp. 127-136

CrossRefView Record in Scopus

[3]

J. Jeong, M.L. Guerinot

Homing in on iron homeostasis in plants

Trends Plant Sci., 15 (2009), pp. 280-285

ArticlePDF (217KB)View Record in Scopus

[4]

C. Tang, S.J. Zheng, Y.F. Qiao, G.H. Wang, X.Z. Han

Interactions between high pH and iron supply on nodulation and iron nutrition of *Lupinus albus* L. genotypes differing in sensitivity to iron deficiency

Plant Soil, 279 (2006), pp. 153-162

CrossRefView Record in Scopus

[5]

V. Rotaru, T.R. Sinclair

Interactive influence of phosphorus and iron on nitrogen fixation by soybean

Environ. Exp. Bot., 66 (2009), pp. 94-99

ArticlePDF (680KB)View Record in Scopus

[6]

C.A. Appleby

Leghemoglobin and rhizobium respiration

Annu. Rev. Plant Physiol., 35 (1984), pp. 443-478

CrossRef

[7]

N. Grotz, M.L. Guerinot

Molecular aspects of Cu, Fe and Zn homeostasis in plants

Biochim. Biophys. Acta, 1763 (2006), pp. 595-608

ArticlePDF (304KB)View Record in Scopus

[8]

N.J. Robinson, C.M. Procter, E.L. Connolly, M.L. Guerinot

A ferricchelate reductase for iron uptake from soils

Nature, 397 (1999), pp. 694-697

CrossRefView Record in Scopus

[9]

J. Jeong, E.L. Connolly

Iron uptake mechanisms in plants: functions of the FRO family of ferric reductases

Plant Sci., 176 (2009), pp. 709-714

ArticlePDF (189KB)CrossRefView Record in Scopus

[10]

H. Ding, L. Duan, J. Li, H. Yan, M. Zhao, F. Zhang, W.X. Li

Cloning and functional analysis of the peanut iron transporter AhIRT1 during iron deficiency stress and intercropping with maize

J. Plant Physiol., 167 (2010), pp. 996-1002

ArticlePDF (942KB)View Record in Scopus

[11]

B.M. Waters, R.P. Sankaran

Moving micronutrients from thesoil to the seeds: Genes and physiological processes from a biofortificationperspective

Plant Science, 180 (2011), pp. 562-574

ArticlePDF (346KB)View Record in Scopus

[12]

S. Santi, S. Cesco, Z. Varanini, R. Pinton

Two plasma membrane H+-ATPase genes are differentially expressed in iron-deficient cucumber plants

Plant Physiol. Biochem., 43 (2005), pp. 287-292

ArticlePDF (565KB)View Record in Scopus

[13]

S. Santi, W. Schmidt

Laser microdissection-assisted analysis of the functional fate of iron deficiency-induced

root hairs in cucumber
J. Exp. Bot., 59 (2008), pp. 697-704
CrossRefView Record in Scopus
[14]
I. Mukherjee, N.H. Campbell, J.S. Ash, E.L. Connolly
Expression profiling of the Arabidopsis ferric chelate reductase (FRO) gene family reveals differential regulation by iron and copper
Planta, 223 (2006), pp. 1178-1190
CrossRefView Record in Scopus
[15]
P.B. Hall, M.L. Guerinot
The role of ZIP family members in iron transport
L.L. Barton, J. Abadia (Eds.), Iron Nutrition in Plants and Rhizospheric Microorganisms, Springer, Dordrecht, The Netherlands (2006), pp. 311-326
CrossRefView Record in Scopus
[16]
G. Vert, J.F. Briat, C. Curie
Arabidopsis IRT2 gene encodes a root-periphery iron transporter
Plant J., 26 (2001), pp. 181-189
CrossRefView Record in Scopus
[17]
E.L. Connolly, J.P. Fett, M.L. Guerinot
Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation
Plant Cell, 14 (2002), pp. 1347-1357
CrossRefView Record in Scopus
[18]
L.F. Whitehead, D.A. Day
The peribacteroid membrane
Plant Physiol., 100 (1997), pp. 30-44
CrossRefView Record in Scopus
[19]
K. Le Vier, D. Day, M.L. Guerinot
Iron uptake by symbiosomes from soybean root nodules
Plant Physiol., 111 (1996), pp. 893-900
CrossRefView Record in Scopus
[20]
M.K. Udvardi, D.A. Day
Electrogenic ATPase activity on the peribacteroid membrane of soybean (*Glycine max* L.) root nodules
Plant Physiol., 90 (1989), pp. 982-987
CrossRefView Record in Scopus
[21]
D.P.S. Verma, V. Kazazian, V. Zogbi, A.K. Bal
Isolation and characterization of the membrane envelope enclosing the bacteroids in soybean root nodules
J. Cell Biol., 78 (1978), pp. 919-936
CrossRefView Record in Scopus
[22]
E. Fedorova, R. Thomson, L.F. Whitehead, O. Maudoux, M.K. Udvardi, D.A. Day
Localization of H+-ATPases in soybean root nodules
Planta, 209 (1999), pp. 25-32

CrossRefView Record in Scopus

[23]

D.A. Day, L. Copeland

Carbon metabolism and compartmentation in nitrogen fixing legumes nodules

Plant Physiol. Biochem., 29 (1991), pp. 185-201

View Record in Scopus

[24]

J. Abadia, A.F. López-Millán, A. Rombolà, A. Abadia

Organic acids and Fe deficiency: a review

Plant Soil, 241 (2002), pp. 75-86

CrossRefView Record in Scopus

[25]

C.P. Vance

Amide biosynthesis in root nodules of temperate legumes

E.W. Triplett (Ed.), Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process, Horizon Scientific Press, Wymondham, UK (2000), pp. 589-608

[26]

T. Slatni, A. Krouma, S. Aydi, C. Chaiffi, H. Gouia, C. Abdelly

Growth, nitrogen fixation and ammonium assimilation in common bean (*Phaseolus vulgaris* L) subjected to iron deficiency

Plant Soil, 312 (2008), pp. 49-57

CrossRefView Record in Scopus

[27]

E.J. Hewitt

Sand and Water Culture Methods Used in the Study of Plant Nutrition

(2nd ed), Commonwealth Bureau of Horticulture Technical Communications (1966)

pp. 22

[28]

T. Slatni, A. Krouma, S. Aydi, C. Chaiffi, H. Gouia, C. Abdelly

Symbiotic nitrogen fixation and leghemoglobin accumulation in common bean (*Phaseolus vulgaris* L) subjected to iron deficiency

Biologia Tunisie, 4 (2006), pp. 127-128

View Record in Scopus

[29]

A.T. Köseoglu, V. Açikgöz

Determination of iron chlorosis with extractable iron analysis in peach leaves

J. Plant Nutr., 18 (1995), pp. 153-161

CrossRefView Record in Scopus

[30]

P. De Nisi, G. Zocchi

Phospho*enol*pyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization

J. Exp. Bot., 352 (2000), pp. 1903-1909

View Record in Scopus

[31]

M. Dell'Orto, S. Santi, P. De Nisi, S. Cesco, Z. Varanini, G. Zocchi, R. Pinton

Development of Fe-deficiency responses in cucumber roots: involvement of plasma membrane H+- ATPase activity

J. Exp. Bot., 51 (2000), pp. 695-701

CrossRefView Record in Scopus

[32]

G. Rabotti, P. De Nisi, G. Zocchi

Metabolic implications in the biochemical responses to iron deficiency in cucumber roots

Plant Physiol., 107 (1995), pp. 1195-1199

CrossRefView Record in Scopus

[33]

U.K. Laemmli

Cleavage of structural proteins during the assembly of the head of bacteriologe T4

Nature, 227 (1970), pp. 680-685

CrossRefView Record in Scopus

[34]

M.M. Bradford

A rapid and sensitive method for the quantization of micrograms quantities of protein utilizing the principle of protein-dye binding

Anal. Biochem., 72 (1976), pp. 248-254

ArticlePDF (403KB)View Record in Scopus

[35]

N. Jelali, W. M'sehli, M. Dell'Orto, C. Abdelly, M. Gharsalli, G. Zocchi

Changes of metabolic responses to direct and induced Fe deficiency of two *Pisum sativum* cultivars

Environ. Exp. Bot., 68 (2010), pp. 238-246

ArticlePDF (1MB)View Record in Scopus

[36]

G. Zocchi, P. De Nisi, M. Dell'Orto, L. Espen, P. Marino Gallina

Iron deficiency differently affects metabolic responses in soybean roots

J. Exp. Bot., 58 (2007), pp. 993-1000

CrossRefView Record in Scopus

[37]

M.L. Guerinot

Iron uptake and metabolism in the rhizobia/legume symbioses

Plant Soil, 130 (1991), pp. 199-209

CrossRefView Record in Scopus

[38]

T. Slatni, A. Krouma, H. Gouia, C. Abdelly

Importance of ferric-chelate reductase activity and acidification capacity in root nodules of N2-fixing Common bean (*Phaseolus vulgaris* L.) subjected to iron deficiency

Symbiosis, 47 (2009), pp. 35-42

CrossRefView Record in Scopus

[39]

C.W. Jin, G.Y. You, Y.F. He, C. Tang, P. Wu, S.J. Zheng

Iron deficiency-induced secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover

Plant Physiol., 144 (2007), pp. 278-285

CrossRefView Record in Scopus

[40]

C.K. Cohen, D.F. Garvin, L.V. Kochian

Kinetic properties of a micronutrient transporter from *Pisum sativum* indicates a primary function in Fe uptake from the soil

Planta, 218 (2004), pp. 784-792

View Record in Scopus

[41]

G. Zocchi

Metabolic changes in iron-stressed dicotyledonous plants
L.L. Barton, J. Abadía (Eds.), Iron Nutrition in Plants and Rhizospheric Microorganisms, Springer, Netherland (2006), pp. 359-370
CrossRefView Record in Scopus

[42]
J. Prell, P. Poole
Metabolic changes of rhizobia in legume nodules
Trends Microbiol., 14 (2006), pp. 161-168
ArticlePDF (403KB)View Record in Scopus

[43]
C.P. Vance
Carbon and nitrogen metabolism in legumes nodules
M.J. Dilworth, *et al.* (Eds.), Nitrogen-Fixing Leguminous Symbioses (2008), pp. 293-320
View Record in Scopus

[44]
J. Schulze, L.F. Shi, J. Blumenthal, D.A. Samac, J.S. Gantt, C.P. Vance
Inhibition of alfalfa root nodule phospho*enol*pyruvate carboxylase through an antisense strategy impacts nitrogen fixation and plant growth
Phytochemistry, 49 (1998), pp. 341-346
ArticlePDF (490KB)View Record in Scopus

[45]
A.F. López-Millán, F. Morales, Y. Gogorcena, A.A. Abadía, J. Abadía
Metabolic responses in iron deficient tomato plants
J. Plant Physiol., 166 (2009), pp. 375-384
ArticlePDF (256KB)View Record in Scopus

[46]
S. Jiménez, N. Ollat, C. Deborde, M. Maucourt, R. Rellán-Álvarez, M.Á. Moreno, Y. Gogorcena
Metabolic response in roots of Prunus rootstocks submitted to iron chlorosis
J. Plant Physiol., 168 (2011), pp. 415-423
ArticlePDF (592KB)View Record in Scopus