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ORIGINAL PAPER

Can Bicarbonate Enhance the Performance of Carob Seedlings Grown in Nutrient Solutions with Different Fe Concentrations?

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Abstract

The aim of this work was to assess the effect of bicarbonate (Bic) ion on the nutritional status and performance of carob-tree seedlings, a species that normally grows in calcareous soil without exhibiting iron chlorosis symptoms. Seedlings were previously grown in nutrient solution with a small concentration of Fe (0.5–1 μM) to induce a moderate chlorosis. Afterwards, two experiments were established: in experiment 1, plants were grown for 21 days in the following treatments: Fe deficiency (Fe0), 0.5 μM Fe, 5 μM Fe, and 5 μM Fe plus calcium carbonate ($CaCO_3$). After assessing these results, a second experiment was conducted for 91 days, with the following treatments: Fe0, 1 μM Fe, 40 μM Fe and 40 μM Fe plus $CaCO_3$ and sodium bicarbonate ($NaHCO_3$). Chlorophyll of young leaves, biomass and mineral composition of leaves, stems and roots were assessed in both experiments. The ferric chelate reductase root activity (FC-R) and the genetic expression of calmodulin-regulated Ca^{2+}-ATPase pump (ACA gene) were evaluated in experiment 2. Fe-deficient plants exhibited reduced growth and enhanced macronutrients in leaves. Root micronutrient homeostasis changed as an adaptive mechanism in carob. The addition of bicarbonate did not aggravate Fe chlorosis, as leaf chlorophyll increased significantly. Root FC-R activity and ACA gene expression was not enhanced under Fe deficiency induced by bicarbonate (Fe40 + BicNa) which suggest a positive effect of bicarbonate in the metabolism of this crop. Nevertheless, small Fe concentrations (Fe1) induced a higher ACA gene expression thus indicating some stress response signalling.

Keywords Calmodulin-regulated Ca^{2+}-ATPase pump · *Ceratonia siliqua* L. · HCO_3^- · Iron chlorosis · Nutrients · Root FC-R

1 Introduction

There are several soil factors that promote iron (Fe) deficiency in field crops. Fe concentrations in alkaline soils (pH between 7.5 and 8.5) are approximately $10^{-10.4}$ M and insufficient for optimal plant growth, which require a Fe soluble range in the medium between 10^{-9} and 10^{-4} M (Guerinot and Yi 1994). In calcareous soils, the bicarbonate ion (HCO_3^-) aggravates the situation and is the most prevalent cause of Fe chlorosis in fruit tree crops. Soluble ferric (Fe^{3+}) and ferrous (Fe^{2+}) salts react rapidly with calcium carbonate to form Fe-hydroxides, which make Fe unavailable to plants (Bastani et al. 2018; Granja and Covarrubias 2018; Pestana et al. 2004). In nutrient solutions, HCO_3^- (added as calcium or sodium carbonate) induces Fe chlorosis in several horticultural crops like quince (Donnini et al. 2009), maize, sorghum and barley (Alhendawi et al. 2008), grapevine (Ollat et al. 2003) and citrus (Martínez-Cuenca et al. 2013; Pestana et al. 2005). In tomato, pea and cucumber, it was demonstrated that HCO_3^- could induce Fe deficiency by inhibiting the expression of root ferric reductase enzyme (FC-R), the Fe transporter (IRT) and the H^+-ATPase (HA) genes (Lucena et al. 2007). The bicarbonate ion (HCO_3^-) increases apoplast pH thus restricting Fe translocation and trapping it in the apoplast of maize root cells (Kosegarten and Koyro 2001), and an alkalinization of the xylem sap of intact maize seedlings due to the presence of HCO_3^- was observed by Wegner and Zimmermann (2004). For example, in the citrus genotype Forner-Alcaide 5, which is

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known to be tolerant to calcareous soils, the addition of 10 mM $NaHCO_3$ led to a reduction of Fe^{2+} uptake and shoot growth, as well as an enhanced expression of FRO2 gene (Martínez-Cuenca et al. 2013). In the grapevine rootstock 140 Rugeri grown in the presence of HCO_3^-, the lack of Fe in the solution led to a change of the root metabolism through an enhancement of root biomass and malic acid concentration, but interestingly, leaf chlorophyll was incremented (Covarrubias and Rombolà 2013). Mineral composition of plant tissues may be affected due to Fe deficiency, but in some cases, a balanced nutritional status is maintained (Granja and Covarrubias 2018; Covarrubias and Rombolà 2013).

Plants that preferentially grow in calcareous soil (calcicoles) can cope with nutritional constraints like less Fe availability and high soil Ca and HCO_3^-. These plants are more efficient on Fe uptake and use, and the toxic effect of bicarbonate on root growth is negligible (Marschner 2012). *Ceratonia siliqua* (L.) is a fruit tree species which grows in the Mediterranean ecosystem that is well adapted to limestone soils. Due to its particular features, this crop is efficient in using N and P (Correia and Martins-Loução 2005) which is also a particularity of calcicole species. Moreover, field-grown rootstock, grafted or ungrafted, do not develop symptoms of Fe chlorosis (Correia et al. 2014), indicating a conservative and optimized strategy regarding the use of Fe.

Fe deficiency normally enhanced the expression of several genes, such as the ferric reductase oxidase (FRO), HA and phosphoenolpyruvate carboxylase (PEPC), which encodes an increase of the activity of the corresponding enzymes, which may be regarded as an adaptive mechanism to Fe starvation (Michel et al. 2019; Martínez-Cuenca et al. 2013). Furthermore, other genes might also be involved. Several calmodulin-binding proteins may be important for understanding the molecular mechanisms involved in abiotic stress tolerance in plants (Virdi et al. 2015). In the susceptible *Poncirus trifoliata* rootstock, several genes related to cell wall modifications were overexpressed in Fe-deficient plants (Forner-Giner et al. 2010). One of these genes expresses a calmodulin-regulated Ca^{2+}-ATPase pump, present in the plasma membrane that regulates the concentration of Ca^{2+} in the cytosol. Ca^{2+}-ATPase pumps belong to either the P2A-ATPase or P2B-ATPase group, where the latter possesses a structural difference with an extended N-terminus which binds calmodulin serves as an auto inhibitory of pump activity (Bose et al. 2011).

Young carob plants are able to grow in calcareous soils without symptoms of iron deficiency and frequently, crystals of calcium oxalate are observed close to the primordia of reproductive buds in mature trees, suggesting an efficient mechanism to sequester the excess of calcium (unpublished results). Besides, in previous experiments, carob plants growing with low Fe availability (1 μM of Fe) did not show severe Fe chlorosis symptoms as expected (Pestana et al. 2012), and there was an increase in organic acid concentrations in root and shoots (Correia et al. 2014). However, the behaviour of carob seedlings under Fe shortage is not well-known and stress gene expressions have never been described.

We propose to conduct two experiments using carob seedlings previously grown under low Fe concentration in order to induce a moderate Fe stress during the initial stages of seedling development using either calcium carbonate (Bic) or calcium carbonate plus sodium bicarbonate (BicNa) as the sources of the bicarbonate ion. We expect to describe the physiological and morphological responses to Fe efficiency and understand which mechanisms are involved in the establishment and recovery of the seedlings under high HCO_3^- availability. Moreover, we want to assess if Fe availability can modulate the expression of the calmodulin-regulated Ca^{2+}-ATPase pump gene (ACA). Ultimately, we believe to provide new insights into the strategy of this crop in calcareous and alkaline soil conditions.

2 Materials and Methods

2.1 Seed Germination and Growth Conditions

For both experiments (1 and 2) conducted under glasshouse conditions, seeds were obtained from a commercial mixture and for germination, seeds were immersed in water heated to 80 °C for 1 h and afterwards transferred into water at room temperature and kept for 24 h. Then, seeds were sown in trays containing vermiculite which was disinfected by spraying with a solution of fosethyl-aluminium (2 g L^{-1}). The substrate was irrigated with distilled water to maintain adequate moisture content until germination.

2.2 Experiment 1

Carob seedlings with 1–2 pairs of leaves (plus the cotyledons) and 5 cm tall were transferred to 20-L containers with 1/4-strength Hoagland's nutrient solution. The solution was prepared using demineralized water with the following chemical composition (macronutrients in mM): 1.25 Ca $(NO_3)_2{\cdot}4H_2O$, 1.25 KNO_3, 0.25 KH_2PO_4, 0.5 $MgSO_4{\cdot}7H_2O$ and (micronutrients in μM): 11.5 H_3BO_3, 0.2 $ZnSO_4{\cdot}7H_2O$, 0.1 $CuSO_4{\cdot}5H_2O$, 9 $MnCl_2{\cdot}4H_2O$ and 0.02 $(NH_4)6Mo_7O_{27}{\cdot}H_2O$. Iron was added as Fe (III)-EDDHA (Basafer® from Compo, with 6% of Fe; 5.0% of Fe chelated by ortho-ortho EDDHA) at 0.5 μM, and plants were grown in these conditions for 60 days until the appearance of chlorosis (pre-treatment stage but without cotyledons, which were gradually senesced during this stage). Then, they were transferred into a newly made full-strength Hoagland's nutrient solution with the following chemical composition (macronutrients in mM): 5 Ca $(NO_3)_2{\cdot}4H_2O$, 5 KNO_3, 1 KH_2PO_4, 2 $MgSO_4{\cdot}7H_2O$ and

(micronutrients in μM): 46 H_3BO_3, 0.8 $ZnSO_4 \cdot 7H_2O$, 0.4 $CuSO_4 \cdot 5H_2O$, 0.9 $MnCl_2 \cdot 4H_2O$ and $(NH_4)6Mo_7O_{27} \cdot H_2O$. At this time, four treatments were imposed: Fe0 (0 μM Fe), Fe0.5 (0.5 μM Fe), Fe5 (5 μM Fe) and Fe5 plus 1 g L^{-1} $CaCO_3$ (Fe5 + Bic). For each treatment, 10 plants (replicates) were distributed by four 20-L containers, in a total of 40 plants under study. The initial pH of the nutrient solution was adjusted to 6.0 with NaOH (1 M) for Fe0, Fe0.5 and Fe5 (control) treatments. For Fe5 + Bic, the solution was not adjusted, and the pH value was approximately 7.0. The electrical conductivity (EC) was 2.0 ± 0.1 dS m^{-1} for all treatments. The nutrient solution was aerated throughout the experiment. The pH and EC values were monitored daily and concerning the Fe5 + Bic, the nutrient solution was stirred before readings. This experiment was concluded after apical leaves of Fe0 displayed severe chlorosis (yellowing of leaf mesophyll).

2.3 Experiment 2

In the second experiment, seedlings with four leaves, without cotyledons (due to short lifespan and senescence) and 10 cm tall were transplanted to 20-L containers and kept in full-strength Hoagland's solution, with 1.0 μM of Fe also for 60 days (pre-treatment stage). After this stage, the following treatments were imposed considering different Fe concentrations (in μM of Fe): 0 (Fe0), 1 (Fe1), 40 (Fe40) and 40 plus 1 g L^{-1} $CaCO_3$ plus 10 mM $NaHCO_3$ (Fe40 + BicNa). Iron was also added to the solutions as Fe (III)-EDDHA. As plants were taller and had more leaves, the Fe concentration was incremented from 5 to 40 μM in order to maintain green plants (Fe40, control plants). In the beginning of experiment 2, the pH of the solution was adjusted to 6.0 with NaOH (1 M) except for Fe40 + BicNa ($CaCO_3$ plus $NaHCO_3$) in order to simulate natural calcareous soil conditions. In this case, the pH ranged between 8.0 and 8.5 throughout the experimental period whereas in Fe0 and Fe1, the pH decreased 0.3 values while in Fe40 treatment, no variation was observed. The EC varied from 2.0 ± 0.1 dS m^{-1} for Fe0, Fe1 and Fe40 treatments while in Fe40 + BicNa, the EC ranged between 2.4 and 2.5 dS m^{-1}. Solutions were renewed once halfway through the experiment which was enough due to the slow-growing nature of this specie. Thus, we expect a slow rate of nutrients demand. Each treatment consisted of 10 plants per container in a total of 40 plants under study.

2.4 Plant Parameters

Leaf chlorosis was evaluated using the portable SPAD-502 meter (Minolta Corp., Japan) in fully expanded young leaves and in mature leaves. SPAD readings were taken in two different leaflets in all plants of each treatment in both experiments. SPAD readings were converted to total chlorophyll (μmol m^{-2}) using the calibration curve: Chlorophyll = 0.45 × $SPAD^2$ + 1.11 × SPAD + 27.3 (R^2 = 0.97; n = 35; P < 0.001) according to Correia et al. (2014). At the end of both experiments, stem, leaves and root dry weights (d.w.) were recorded as well as root length. Plants were harvested and separated into roots, stems and leaves and weighted for biomass assessment; root length determination and mineral composition of each plant organ were quantified at the end of the experiments (21 days and 91 days respectively for experiment 1 and 2).

2.5 Root Ferric Chelate Reductase Activity (FC-R)

The activity of root FC-R (EC 1.16.1.17) was measured by the formation of the Fe (II)-bathophenantrolinedisulfonate (BPDS) complex from Fe (III)-EDTA (Bienfait et al. 1983) at the end of the experiment 2. Measurements were performed with root tips excised with a razor blade from each plant. Each excised root tip (approximately with 2 cm and 0.013 ± 0.006 g of fresh weight—f.w.) was incubated in an Eppendorf tube in the dark with 900 μL of micronutrient-free 1/2 strength Hoagland's nutrient solution, containing 300 μM BPDS, 500 μM Fe (III)-EDTA and 5 mM MES, pH 6.0. After 2 h of incubation, sample absorbances were registered spectrophotometrically using a 535-nm wavelength. An extinction coefficient of 22.14 mM cm^{-1} was used. Blank controls without root tips were also used to correct for any unspecific Fe reduction. The FC-R activity was expressed in nmol Fe (II) min^{-1} g^{-1}.

2.6 Mineral Composition

Plant organs were separated into leaves (leaflets plus petiole), stems and roots. Plant material was washed in tap water, next in distilled water containing a non-ionic detergent, and then in 0.01 M HCl. Finally, three rinses were carried out in distilled water. The dry weight of each part was determined after drying at 60 °C for at least 48 h. Samples were ground and ashed at 450 °C and digested in HCl 1 M. Standardized procedures (A.O.A.C. 1990) were used to determine mineral composition. Nitrogen was analysed using the Kjeldahl method and concentrations of K, Ca, Mg, Zn, Cu, Mn and Fe were determined by atomic absorption spectrometry (AAS - M Series, Pye Unicam Ltd., Cambridge, UK). Phosphorus was determined colorimetrically using the molybdo-vanadate method with a 375-nm wavelength light source (spectrophotometer U2000, Hitachi Ltd., Tokyo, Japan).

2.7 Expression of mRNA of calmodulin-regulated Ca^{2+}-ATPase pump (ACA)

Plant RNA was extracted using the EZRNA Plant RNA Kit (OMEGA), following the manufacturer's guidelines, from approximately 300 mg (f.w.) of roots using three biological

replicates (100 mg per replicate) for each Fe treatment of experiment 2. Total RNA preparations were cleaned up with Turbo DNA-free Kit (Applied Biosystems) according to manufacturer instructions. Each RNA sample was quantified using the NanoDrop 2000c Spectrophotometer (Thermo Scientific) at 260 nm. The ratio of absorbance at 260 nm and 280 nm, used to assess the purity of RNA, in all samples was greater than 1.80.

Expression of ACA gene was based on the *Citrus medica* cDNA clone C31502B08, mRNA (accession number FC874904) and quantified by real-time reverse transcriptional polymerase chain reaction (RT-PCR) in an iCycler IQ (Biorad) using the iScript One-Step RT-PCR Kit with SYBR Green (Biorad) and included 200 nM of each of the ACA forward and reverse primers. ACA Fwd 5′-CAAATGGG GACGTTCAGTTT-3′ and ACA Rev 5′-AGTGCAAG AGCTCCCAGTGT-3′.

The amplifications were prepared in a total volume of 25 μL containing 12.5 μL of 2× SYBR Green master mix, 0.5 μL of each amplification primer, 0.5 μL of iScript reverse transcriptase and 1 μL of the RNA template. The thermo cycling parameters were reverse transcription for 10 min at 50 °C, denaturation for 5 min at 95 °C, followed by 40 cycles of 10 s denaturation at 95 °C, 30 s for annealing at 52 °C and 30 s at 72 °C for extension.

The efficiency of amplification of the ACA primers was determined by the iCycler software based in the amplifications of a series of dilutions made with three replicas for each dilution. Specificity of the amplifications was assessed by the melting curve analysis. Relative quantification of ACA expression was done according to the method of Pfaffl (2001), using 18S RNA amplification as a normalizing gene. Conditions for 18S RNA amplification were the same as used for ACA, except for the annealing temperature at 59° and the primers which were: 18SFwd 5′-GACTACGT CCCTGCCCTTTG-3′ and 18SRev 5′-TGATAAGG TTCAATGGACTTCTTC-3′. The efficiency of amplification of the 18S RNA was determined in a similar manner as for the ACA primers.

2.8 Statistical Analysis and Experimental Layout

Containers were distributed in a completely random layout. The effects of treatments were evaluated by one-way analysis of variance and the means compared using the Duncan multiple-range test (DMRT) at $P < 0.05$ (SPSS software version 17.0).

3 Results

The results obtained in experiment 1, and subsequently in experiment 2, were complementary. Plant material with different ages was used. Seedlings in experiment 1 were younger and thus submitted to a moderate stress imposition. In experiment 2, we simulate a severe stress using older plants for a longer time.

3.1 Experiment 1

At the beginning of this experiment (day 1), chlorophyll concentration for all treatments (150 < chlorophyll (μmol m^{-2}) < 325) indicated some degree of leaf chlorosis in young leaves (Fig. 1) just before treatment imposition. Subsequently, chlorosis was accentuated in both Fe0 and Fe0.5 treatments, and plants which grew with 5 μM of Fe (Fe5) without or with calcium carbonate (Fe5 + Bic) showed higher chlorophyll, respectively 205 and 575 μmol m^{-2} at day 21.

The initial measurement date (day 4) revealed symptoms of Fe chlorosis as in experiment 1. After 16 days of treatment imposition, plants of Fe0 and Fe1 treatments maintained a significant small chlorophyll concentration respectively, 65 and 92 μmol m^{-2}. By the end of the experiment, chlorophyll concentration in plants without or with low Fe concentration in the nutrient solution (Fe0 and Fe1) maintained the low values while in Fe40 and Fe40 + BicNa plants, chlorophyll increased to approximately 740 μmol m^{-2}.

Plants of Fe40 and Fe40 + BicNa treatments (in particular, Fe40) had also higher leaf number and dry weight and higher root length and root dry weight. This trend was consistently observed until the end of the experiment (91 days, Table 3). The R/S ratio was significantly higher in the Fe40 + BicNa.

As shown in Table 4, N, K and Mg concentrations were high in leaves of Fe0 and Fe1 treatments (no data is available for N in stems and roots).

In stems, P and Mg were also higher in deficient Fe treatments, and apparently also K. Calcium was significantly higher in roots of Fe40 + BicNa but not in stems or leaves. Regarding micronutrients, higher concentration of Fe was found in leaves of Fe40 + BicNa which converted to Fe content also showed higher leaf value (137 ± 74 μg per plant). In stems, the differences are not so clear but it seems that plants under Fe deficiency showed more Cu. The same is also true in roots, and Fe was significantly higher in Fe40 plants (with or without Bic and Na).

Plants grown without Fe (Fe0) incremented twofold the root activity of the FC-R enzyme (Fig. 3a) compared to the remaining plants, which present similar activity among them.

Using the Fe40 plants as reference (relative expression = 1), plants grown with 1 μM Fe (Fe1) showed an overexpression of ACA gene (Fig. 3b). However, in the presence of bicarbonate (Fe40 + BicNa), the expression level was not enhanced.

At the end of both experiments, severe symptoms of Fe deficiency, yellowing of mesophyll of young leaves, were

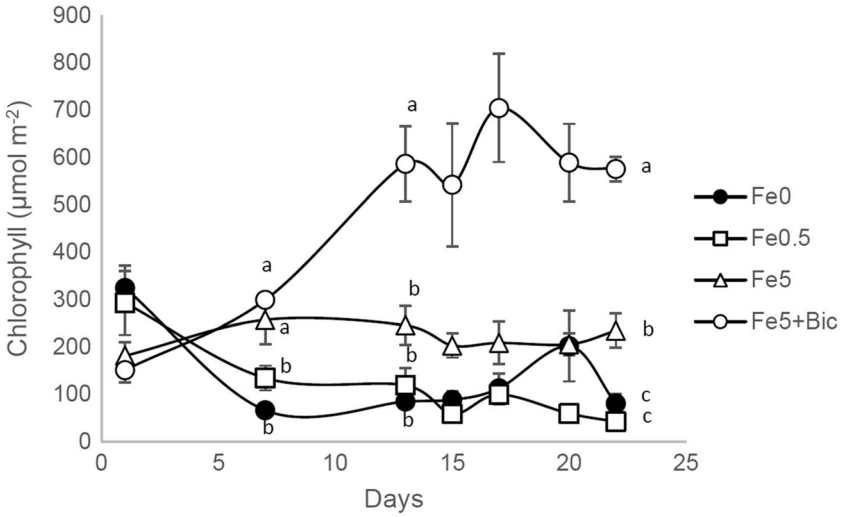

Fig. 1 Time course of total chlorophll (µmol m^{-2}) in young leaves for treatments of experiment 1. Values are means ± SE; $n = 5$. Means with the same letters are not signficantly different at $P < 0.05$ (Duncan test)

observed in plants grown without Fe in nutrient solution (Fig. 4). Plants growing with Fe5 showed moderate symptoms of Fe chlorosis (interveinal chlorosis of young leaves) while plants grown with Fe40 or with the addition of bicarbonate (Fe5 + Bic and Fe40 + BicNa) remained green during all experiments.

4 Discussion

Both experiments showed that seedlings that grew under limited Fe supply (0.5 or 1 µM of Fe) or without Fe (0 µM Fe) revealed clear Fe chlorosis symptoms in young leaves, which affected biomass accumulation and growth. The application of Fe just after treatment imposition, 40 µM in experiment 2, conducted to a rapid recovery of leaf chlorophyll and plant performance improvement, although the presence of bicarbonate led to an increase of R/S ratio. In fact, carob seedlings are able to recover from radiation and water stress effects, due to multiple strategies (maintenance of electron transfer rate or proline increment) as found by Osório et al. (2011). The slow growing adaptive strategy shown by this crop explains why in experiment 1 no differences were observed in R/S after 21 days.

Similar recovering results can be found in calcicole species. For example, a total re-greening of young leaves was observed in *Ulmus minor* after Fe resupply (Venturas et al. 2014) and partially disappeared in *Lupinus pilosus* (Ding et al. (2019).

The higher concentration of macronutrients (N, P, K and Mg) in leaves and stems of Fe-deficient treatments in comparison to Fe5 and Fe40 (with or without bicarbonate) was a response to Fe stress probably related to a dry weight reduction in stems and leaves and a concomitant "concentration effect." A similar response was reported in cucumber plants and in other crops (Tomasi et al. 2014 and references therein) explained by the depressed shoot dry matter production in Fe-deficient cucumber plants. In particular, P uptake was apparently reduced in plants grown in the presence of Bic (particularly visible in experiment 1), which is not entirely indicative of a calcicole behaviour (Neumann and Römheld 2012). It is possible that calcium phosphates produced in nutrient solution depressed P availability. K increase in leaves of Fe-deficient plants may be explained by the role of this cation in the regulation of osmotic adjustment to stress conditions. This response is probably not specific, since the same trend was registered in carob plants submitted to salinity stress (Correia et al. 2010). In roots, N followed a similar pattern as for aboveground tissues, but the depression of K accumulation in Fe-deficient plants, particularly in experiment 1, is not in accordance with the findings of Tomasi et al. (2014) for cucumber. More Ca was also found in leaves of Fe-deficient plants, particularly in experiment 2 (19.1 g kg^{-1} and

Table 1 Biomass parameters for each treatment at the end of experiment 1 (21 days). Means with different letter indicate significant differences at $P < 0.05$ (Duncan test). *R/S* root to shoot ratio

Treatments	Number of leaves	Stem d.w. (g)	Root length (cm)	Root d.w. (g)	Leaf d.w. (g)	R/S ratio
Fe0	5.8b	0.13b	28.2c	0.19a	0.44bc	0.33a
Fe0.5	5.7b	0.13b	34.2b	0.22a	0.36c	0.45a
Fe5	9.8a	0.20ab	46.7a	0.28a	0.64a	0.33a
Fe5 + Bic	9.3a	0.27a	40.8ab	0.26a	0.60ab	0.30a

Table 2 Mineral composition of roots, stems and leaves in treatments of experiment 1 (after 21 days of Fe treatments imposition). In each organ and nutrient, means with a different letter indicate significant differences at $P < 0.05$ between Fe concentrations (Duncan test)

	N	P	K	Mg	Ca	Cu	Zn	Mn	Fe
	g kg^{-1} d.w.					mg kg^{-1} d.w.			
Roots									
Fe0	44.1a	15.1a	26.2d	3.6b	16.9ab	214a	314a	850a	204b
Fe0.5	45.0a	15.1a	30.7c	4.2ab	14.9b	182a	254ab	1028a	212b
Fe5	34.7b	14.4a	40.4b	5.4a	24.0a	39b	194b	873a	345a
Fe5 + Bic	36.4b	5.2b	48.7a	4.7ab	16.5ab	33b	79c	1119a	252b
Stems									
Fe0	36.1a	10.0b	24.1b	3.2a	8.9b	4a	58a	33a	78a
Fe0.5	34.6a	13.0a	28.0a	2.8ab	6.5b	3a	60a	33a	91a
Fe5	25.3b	10.1b	26.0ab	2.3b	9.0b	7a	55a	31a	86a
Fe5 + Bic	24.1b	4.7c	25.1b	2.1b	12.6a	5a	34b	40a	71a
Leaves									
Fe0	36.4b	5.3b	31.5a	5.3a	23.0a	4a	60a	183a	57a
Fe0.5	38.8a	9.2a	29.8a	5.1a	23.3a	2a	65a	202a	58a
Fe5	28.6c	7.6a	21.1b	4.3b	21.8a	3a	49ab	210a	43a
Fe5 + Bic	28.2c	3.4b	20.8b	3.7b	16.5b	4a	31b	341a	48a

22.7 g kg^{-1} respectively for Fe0 and Fe1) as a probable result of Fe stress and tissue concentration; however, Ca accumulation was observed in roots (experiment 2) or stems (experiment 1) of plants, which grew in the presence of bicarbonate. It seems that under shortage of Fe, Ca is translocated from roots to leaves and concurrently, under excess (+Bic treatments), Ca is retained in roots (experiment 2). Uptake of Ca is proportional to Ca availability in the soil solution, and excess of Ca absorption may lead to the formation of crystalline Ca oxalate (Paiva 2019). Field-grown carob trees often show large amounts of those crystals which accumulate in the vicinity of the pre-emergent reproductive buds in lignified branches (unpublished results). Since the maintenance of low cytosolic Ca is imperative for the adaptation to Ca-rich soils (Lee 1998), the strategy of carob may include some sort of Ca-sequestering at root level.

Apparently, root micronutrient homeostasis was changed. Carob seems to increase Cu uptake when facing Fe deficiency, accumulating Cu in roots viewed in both experiments and stems in experiment 2. This response was reported by other authors (Cohu and Pilon 2007; Pestana et al. 2013), and it was attributed to the affinity of different enzymatic systems either for Fe or for Cu. In both experiments, it was noticed that Cu values in shoot tissues were low. Although no visual symptoms of Cu deficiency were observed in these experiments, this result may be due to formation of Cu (II) EDDHA chelates, an unavailable form for plants, which is known to occur

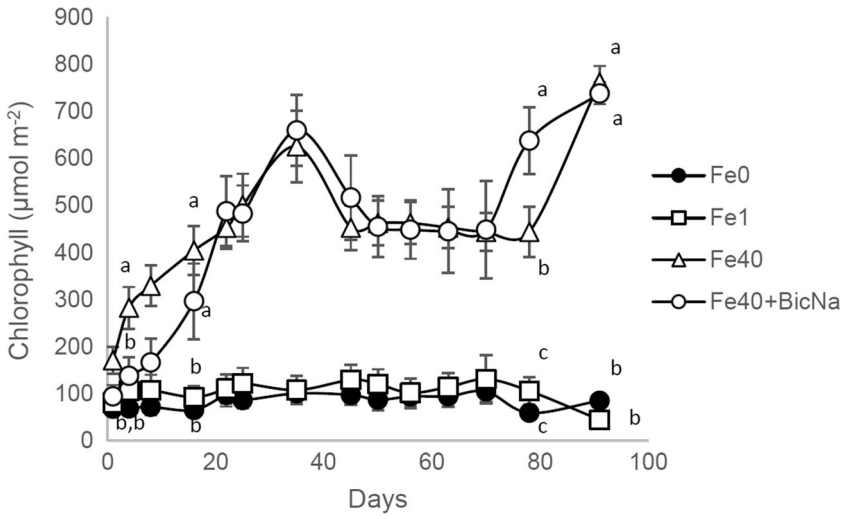

Fig. 2 Time course of total chlorophyll (µmol m^{-2}) in young leaves for treatments of experiment 2. Values are means ± SE; $n = 10$. Means with the same letters are not significantly different at $P < 0.05$ (Duncan test)

Table 3 Biomass parameters at the end of experiment 2 (91 days). Means with different letter indicate significant differences at $P < 0.05$ (Duncan test). *R/S* root to shoot ratio

Treatments	Number of leaves	Stem d.w. (g)	Root length (cm)	Root d.w. (g)	Leaf d.w. (g)	R/S ratio
Fe0	4.7b	0.15b	12.3b	0.04c	0.28c	0.09b
Fe1	4.0b	0.14b	17.0b	0.07bc	0.32c	0.15b
Fe40	11.0a	0.65a	54.0a	0.47ab	1.57a	0.21b
Fe40 + BicNa	9.0a	0.31b	41.7a	0.60a	0.97b	0.47a

when using commercial Fe chelates due to impurities of the isomers (Yunta et al. 2003).

Mn levels in leaves were in range of field conditions as reported by Correia et al. (2018) where leaf Mn in non-calcareous soils are relatively higher than in calcareous soils. Although the pattern of Mn was not conclusive, it was partially explained by the large variability within samples. In experiment 2, it was noticeable that the addition of bicarbonate (+Bic) lowered the Mn concentration in roots and leaves. Zn concentration seems to be depressed in the treatments with bicarbonate. In field studies, Fe concentrations in carob leaves may vary between 40 and 160 mg kg^{-1} d.w. in non-calcareous soils while in calcareous soils, the concentration is slightly lower varying between 35 and 100 mg kg^{-1} d.w. (Correia et al. 2018). The Fe concentrations were balanced out in leaves and stems in both experiments regardless of the amount of Fe in the nutrient solution. However, in the roots of Fe-sufficient plants, Fe5, and more expressively in Fe40 and Fe40 + BicNa, there was a threefold increase although no toxicity was observed probably because much of the Fe is in the root apoplast (Römheld and Nikolic 2007).

Reduced growth and leaf chlorosis increase is a common effect of bicarbonate; however, the physiological response to bicarbonate exposure is variable and depends on the plant species (Dąbrowska-Bronk et al. 2016). In the calcicole *Lupinus pilosus*, it was observed that plants grown with high pH and high calcium carbonate tend to reduce significantly the amount of Fe and Mn in roots and leaves and accumulate Ca in both of these organs (Ding et al. 2019). The addition of bicarbonate (+Bic) did not aggravate leaf Fe chlorosis, nor reduced growth nor inhibited Fe uptake from the solution. This was particularly visible in experiment 1 as chlorophyll values in young leaves of Fe5 + Bic plants increased, reaching values equal to those of Fe40 plants in experiment 2. Besides, symptoms of Fe chlorosis were no longer visible in Fe5 + Bic plants whereas for Fe5 plants, chlorophyll values remained low. Plants that received bicarbonate showed similar or even higher chlorophyll concentration than the control plants in both experiments, and the recovery was fast with no limiting

Table 4 Mineral composition of roots, stems and leaves in treatments of experiment 2 (after 91 days of Fe treatments imposition). In each organ and nutrient, means with a different letter indicate significant differences at $P < 0.05$ between Fe treatments (Duncan test). No data is available for N in roots and stems

	N	P	K	Mg	Ca	Cu	Zn	Mn	Fe
	g kg^{-1} d. w.					mg kg^{-1} d. w.			
Roots									
Fe0	–	10.5a	25.1a	4.3a	5.4b	162a	130a	319ab	409b
Fe1	–	11.6a	19.6a	3.6a	9.5b	89b	162a	396a	198b
Fe40	34.7[a]	10.3a	26.7 a	3.7a	11.7b	23c	124a	471a	1469a
Fe40 + BicNa	38.6[a]	5.0b	26.1a	5.4a	21.5a	12c	23b	113b	1251a
Stems									
Fe0	–	12.4a	22.2ab	5.3a	16.6a	16a	54a	26a	118a
Fe1	–	11.8a	25.1a	4.8a	18.1a	11ab	58a	21a	99a
Fe40	20.3[a]	4.9b	15.7bc	1.8b	8.4a	2c	22b	16a	92a
Fe40 + BicNa	20.5[a]	3.2b	11.3c	3.9ab	11.7a	6bc	34b	20a	149a
Leaves									
Fe0	38.1a	2.8ab	41.1a	5.9a	19.1ab	3a	36a	134ab	88a
Fe1	35.5a	3.5a	33.5a	6.2a	22.7a	3a	44a	201a	79a
Fe40	21.9b	3.1ab	14.9b	3.2b	13.0bc	2a	26ab	160a	74a
Fe40 + BicNa	21.1b	1.5b	19.4b	2.6b	10.3c	1a	14b	79b	153a

[a] $n = 2$

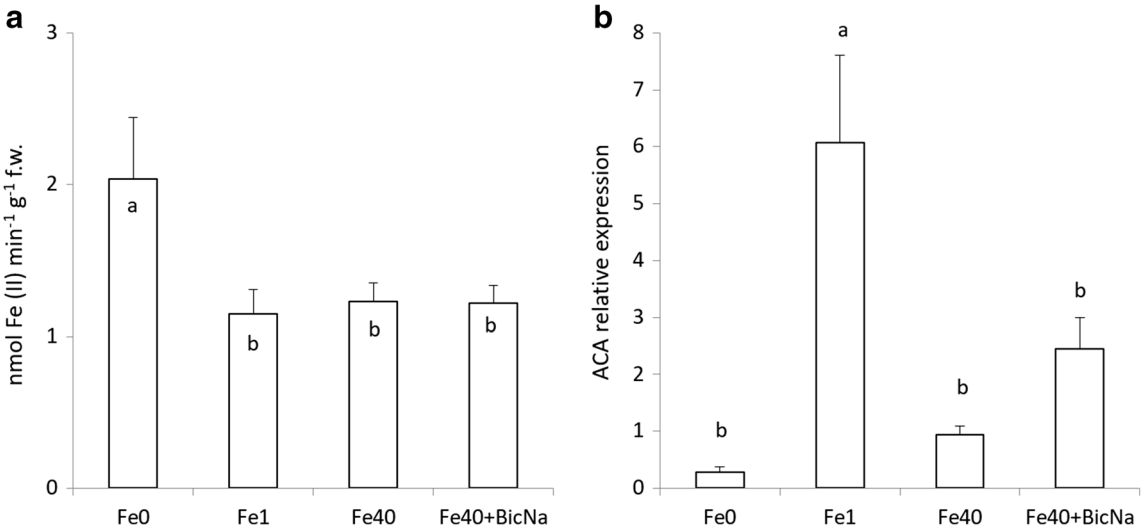

Fig. 3 Activity of root FC-R (**a**) and relative mRNA expression (**b**) of calmodulin-regulated Ca^{2+}-ATPase gene (ACA) for all treatments at the end of experiment 2. Columns with the same letter are not significantly different at $P < 0.05$ (Duncan test). Values are means ± SE. For FC-R activity, $2 < n < 3$ and for ACA relative expression, $4 < n < 9$

effect on plant observed. After being absorbed, HCO_3^- may be used in the synthesis of organic acids (Nikolic and Römheld 2007), incorporated into phosphoenolpyruvate and subsequently generating oxaloacetate and malate as part of the pH-stat mechanism (Rombolà et al. 2002). Recently, it was demonstrated that carob trees produce an important pool of organic acids in roots and shoots as a response to Fe deficiency (Correia et al. 2014). Succinic and malic acid

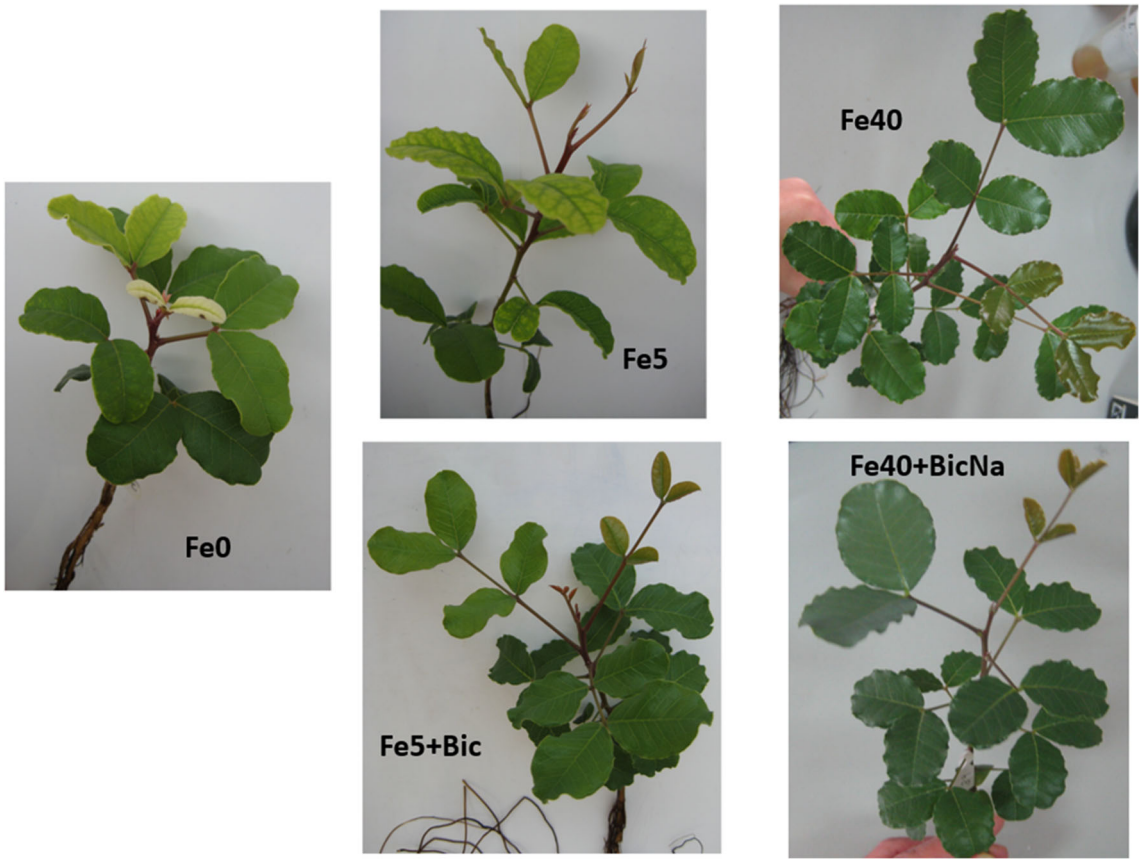

Fig. 4 Chlorotic carob plants of Fe0 treatment at the end of both experiments. Plants of Fe5 in experiment 1 exhibit moderate symptoms of iron deficiency. In experiment 2, for Fe40 plants, no symptoms were observed. Healthy plants were also obtained if bicarbonate (Fe5 + Bic) or bicarbonate plus 10 mM $NaHCO_3$ (Fe40 + BicNa) added in the nutrient solutions

concentrations were particularly enhanced in young chlorotic leaves, but there was a consistent translocation pattern of acids from roots to basal, middle and young leaves as a response to Fe deficiency.

Bicarbonate blocks the Fe-deficiency-induced responses in roots (Kim and Guerinot 2007), and it has also been suggested that ethylene may be involved in the negative effect of HCO_3^- in strategy I plants (García et al. 2013). Although there is some evidence that the increase in apoplast pH due to HCO_3^- induction may depress Fe^{3+} reduction (Mengel 1994), other studies indicate that the pH of the leaf apoplastic fluid is not affected by high HCO_3^- supply (Nikolic and Römheld 2002). Considering that the FC-R activity is pH dependent (Susin et al. 1996), the uptake of HCO_3^- at root level would inhibit the enzyme and thus depress the Fe^{3+} reduction. In carob, apparently, this was not the case since the activity of FC-R in Fe40 + BicNa treatments was similar to control plants in experiment 2, demonstrating the lack of inhibitory effect of Bic on FC-R activity. This is contrast with the findings of Donnini et al. (2012) which found that in *Parietaria diffusa* (calcicole shrub) the activity of this enzyme was enhanced as compared to control Fe-sufficient plants. In fact, only in total absence of Fe, roots of carob showed higher FC-R activity demonstrating a clear stress signalling occurred in these conditions. This response is normally described in the literature, and it was also reported for this species in a concurrent paper by Pestana et al. (2012).

The results point to the absence of a stress signalling induced by the addition of Ca and or Na bicarbonate. Moreover, it seems that carob seedlings used bicarbonate to overcome the stressed conditions imposed by a Fe-deficiency growing conditions just after germination, as occurred in experiment 2. In *Poncirus trifoliata*, a Citrus rootstock very susceptible to Fe deficiency, gene expression analysis showed that in Fe-stressed plants (0 μFe), several genes, including a calmodulin-regulated Ca^{2+}-ATPase pump (ACA), were overexpressed (Forner-Giner et al. 2010). In carob, no expression of ACA was registered either in Fe0 or in Fe40 + BicNa, but plants grown under Fe shortage (Fe1 treatment in experiment 2) did show overexpression of ACA gene, which may indicate some stress signalling in those plants confirmed by the lack of chlorophyll recover in that treatment. Carob root cell walls are thick and Ca is tightly bound to pectins of the root cell wall thus enhancing rigidity (Rhizopoulou 2004) which supports the pattern of Ca accumulation. These features may play an important role in the resistance to several types of stress, including nutritional stress.

5 Conclusions

Taken together (experiments 1 and 2), the results may support a bicarbonate use efficiency strategy of carob seedlings. Bicarbonate neither triggered growth inhibition nor aggravated Fe chlorosis in young leaves, which is the major finding of the present work and contrasts with most of the reported literature. Bicarbonate potentiated chlorophyll synthesis and, under these stress conditions, Ca metabolism is efficiently mobilized within plant organs. Moreover, Fe concentrations were not affected, due to bicarbonate addition in both experiments. Bicarbonate addition has positive effects on photosynthesis as referred by Wu and Xing (2012), and it has an important role in accelerating electron transfer to plastoquinone pools in PSII (for a review see Shevela et al. 2012). Presumably, bicarbonate potentiated the presence of Fe, the solution and electron transfer originating a fast build-up of chlorophyll in young leaves. Concurrently, it seems that bicarbonate does not inhibit root ferric chelate reductase activity or change the enzyme optimal environment. However, small Fe concentration (1 μM Fe in experiment 2) may conduct to overexpression of calmodulin-regulated Ca^{2+}-ATPase pump gene meaning some Fe-stress signalling mechanism at membrane level.

Author Contributions P.J. Correia wrote the manuscript. F. Gama and T. Saavedra executed the hydroponic experiments and were responsible for the acquisition, analysis, and interpretation of data. G. Nolasco and S. Dandlen supervised the gene expression study. A. de Varennes was responsible for mineral composition analysis. P.J. Correia and M. Pestana contributed to the experimental layout and discussion of the results.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

A.O.A.C (1990) Official methods of analysis of the Association of Official Analytical Chemists. Vol.1. K. Helrich (Ed.), Association of Official Analytical Chemists, 15th Ed. Arlington, Virginia

Alhendawi RA, Kirkby EA, Pilbeam DJ, Römheld V (2008) Effect of iron seed dressing and form of nitrogen-supply on growth and micronutrient concentration in shoots of sorghum grown in a calcareous sand culture. J Plant Nutr 31:1855–1865

Bastani S, Hajiboland R, Khatamian M, Saket-Oskoui M (2018) Nano iron (Fe) complex is an effective source of Fe for tobacco plants grown under low Fe supply. J Soil Sci Plant Nutr 18:524–541. https://doi.org/10.4067/S0718-95162018005001602

Bienfait HF, Bino RJ, van der Bliek AM, Duivenvoorden JF, Fontaine JM (1983) Characterization of ferric reducing activity in roots of Fe-deficient Phaseolus vulgaris. Physiol Plant 59:196–202. https://doi.org/10.1111/j.1399-3054.1983.tb00757.x

Bose J, Pottosin II, Shabala SS, Palmgren MG, Shabala S (2011) Calcium efflux systems in stress signaling and adaptation in plants. Front Plant Sci 2. https://doi.org/10.3389/fpls.2011.00085

Cohu CM, Pilon M (2007) Regulation of superoxide dismutase expression by copper availability. Physiol Plant 129:747–755

Correia PJ, Martins-Loução MA (2005) The use of macronutrients and water in marginal Mediterranean areas: the case of carob-tree. Field Crop Res 91:1–6

Correia PJ, Gama F, Pestana M, Martins-Loução MA (2010) Tolerance of young (Ceratonia siliqua L.) carob rootstock to NaCl. Agric Water Manag 97:910–916. https://doi.org/10.1016/j.agwat.2010.01.022

Correia PJ, Gama F, Saavedra T, Miguel MG, Paulo Da Silva J, Abadía A, De Varennes A, Pestana M (2014) Changes in the concentration of organic acids in roots and leaves of carob-tree under Fe deficiency. Funct Plant Biol 41:496. https://doi.org/10.1071/FP13204

Correia PJ, Saavedra T, Gama F, da Graça Miguel M, de Varennes A, Pestana M (2018) Biologically active compounds available in Ceratonia siliqua L. grown in contrasting soils under Mediterranean climate. Sci Hortic (Amsterdam). 235:228–234. https://doi.org/10.1016/j.scienta.2018.03.010

Covarrubias JI, Rombolà AD (2013) Physiological and biochemical responses of the iron chlorosis tolerant grapevine rootstock 140 Ruggeri to iron deficiency and bicarbonate. Plant Soil 370:305–315

Dąbrowska-Bronk J, Komar DN, Rusaczonek A, Kozłowska-Makulska A, Szechyńska-Hebda M, Karpiński S (2016) β-carbonic anhydrases and carbonic ions uptake positively influence Arabidopsis photosynthesis, oxidative stress tolerance and growth in light dependent manner. J Plant Physiol 203:44–54. https://doi.org/10.1016/j.jplph.2016.05.013

Ding W, Clode PL, Lambers H (2019) Is pH the key reason why some Lupinus species are sensitive to calcareous soil? Plant Soil 434:185–201. https://doi.org/10.1007/s11104-018-3763-x

Donnini S, Castagna A, Ranieri A, Zocchi G (2009) Differential responses in pear and quince genotypes induced by Fe deficiency and bicarbonate. J Plant Physiol 166:1181–1193

Donnini S, de Nisi P, Gabotti D, Tato L, Zocchi G (2012) Adaptive strategies of Parietaria diffusa (M.&K.) to calcareous habitat with limited iron availability. Plant Cell Environ 35:1171–1184. https://doi.org/10.1111/j.1365-3040.2012.02481.x

Forner-Giner MA, Llosá MJ, Carrasco JL, Perez-Amador MA, Navarro L, Ancillo G (2010) Differential gene expression analysis provides new insights into the molecular basis of iron deficiency stress response in the citrus rootstock Poncirus trifoliata (L.) Raf. J Exp Bot 61:483–490

García MJ, Romera FJ, Stacey MG, Stacey G, Villar E, Alcántara E, Pérez-Vicente R (2013) Shoot to root communication is necessary to control the expression of iron-acquisition genes in strategy I plants. Planta 237:65–75. https://doi.org/10.1007/s00425-012-1757-0

Granja F, Covarrubias JI (2018) Evaluation of acidifying nitrogen fertilizers in avocado trees with iron deficiency symptoms. J Soil Sci Plant Nutr 18:157–172. https://doi.org/10.4067/S0718-95162018005000702

Guerinot ML, Yi Y (1994) Iron: nutritious, noxious, and not readily available. Plant Physiol 104:815–820

Kim SA, Guerinot ML (2007) Mining iron: iron uptake and transport in plants. FEBS Lett 581:2273–2280. https://doi.org/10.1016/j.febslet.2007.04.043

Kosegarten H, Koyro HW (2001) Apoplastic accumulation of iron in the epidermis of maize (Zea mays) roots grown in calcareous soil. Physiol Plant 113:515–522. https://doi.org/10.1034/j.1399-3054.2001.1130410.x

Lee A (1998) How lipids interact with an intrinsic membrane protein: the case of the calcium pump. Biochim Biophys Acta - Rev Biomembr 1376:381–390. https://doi.org/10.1016/S0304-4157(98)00010-0

Lucena C, Romera FJ, Rojas CL, García MJ, Alcántara E, Pérez-Vicente R (2007) Bicarbonate blocks the expression of several genes involved in the physiological responses to Fe deficiency of strategy I plants. Funct Plant Biol 34:1002. https://doi.org/10.1071/FP07136

Marschner H (2012) Marschner's mineral nutrition of higher plants. 3rd edition. Academic press

Martínez-Cuenca M-R, Iglesias DJ, Forner-Giner MA, Primo-Millo E, Legaz F (2013) The effect of sodium bicarbonate on plant performance and iron acquisition system of FA-5 (Forner-Alcaide 5) citrus seedlings. Acta Physiol Plant 35:2833–2845

Mengel K (1994) Iron availability in plant tissues-iron chlorosis on calcareous soils. Plant Soil 165:275–283. https://doi.org/10.1007/BF00008070

Michel L, Beyá-Marshall V, Rombolà AD, Pastenes C, Covarrubias JI (2019) Evaluation of FE-heme applications or intercropping for preventing Iron deficiency in blueberry. J Soil Sci Plant Nutr 19:117–126. https://doi.org/10.1007/s42729-019-0017-9

Neumann G, Römheld V (2012) Marschner's mineral nutrition of higher plants, Marschner's mineral nutrition of higher plants. Academic Press. doi https://doi.org/10.1017/CBO9781107415324.004

Nikolic M, Römheld V (2002) Does high bicarbonate supply to roots change availability of iron in the leaf apoplast? Plant Soil 241:67–74. https://doi.org/10.1023/A:1016029024374

Nikolic M, Römheld V (2007) The dynamics of iron in the leaf apoplast. In: The Apoplast of higher plants: compartment of storage, transport and reactions. Springer Netherlands, Dordrecht, pp. 353–371. doi https://doi.org/10.1007/978-1-4020-5843-1_26

Ollat N, Laborde B, Neveux M, Diakou-Verdin P, Renaud C, Moing A (2003) Organic acid metabolism in roots of various grapevine (Vitis) rootstocks submitted to iron deficiency and bicarbonate nutrition. J Plant Nutr 26:2165–2176

Osório ML, Osório J, Vieira AC, Gonçalves S, Romano A (2011) Influence of enhanced temperature on photosynthesis, photooxidative damage, and antioxidant strategies in Ceratonia siliqua L. seedlings subjected to water deficit and rewatering. Photosynthetica 49:3–12. https://doi.org/10.1007/s11099-011-0001-7

Paiva EAS (2019) Are calcium oxalate crystals a dynamic calcium store in plants? New Phytol 223:1707–1711. https://doi.org/10.1111/nph.15912

Pestana M, De Varennes A, Abadía J, Faria EA (2005) Differential tolerance to iron deficiency of citrus rootstocks grown in nutrient solution. Sci Hortic (Amsterdam) 104:25–36. https://doi.org/10.1016/j.scienta.2004.07.007

Pestana M, Faria EA, de Varennes A (2004) Lime-induced iron chlorosis in fruit trees. In: Dris R., Jain S.M. (eds) Production practices and quality assessment of food crops. Springer Dordrecht, pp. 171–215. https://doi.org/10.1007/1-4020-2536-x_7

Pestana M, Gama F, Saavedra T, De Varennes A, Correia PJ (2012) The root ferric-chelate reductase of Ceratonia siliqua (L.) and Poncirus trifoliata (L.) Raf. responds differently to a low level of iron. Sci Hortic (Amsterdam) 135:65–67

Pestana M, Correia PJ, Saavedra T, Gama F, Dandlen S, Nolasco G, de Varennes A (2013) Root ferric chelate reductase is regulated by iron and copper in strawberry plants. J Plant Nutr 36:2035–2047. https://doi.org/10.1080/01904167.2013.816731

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29:e45

Rhizopoulou S (2004) Aspects of cell wall extensibility in Ceratonia siliqua L. Flora - Morphol Distrib Funct Ecol Plants 199:327–333. https://doi.org/10.1078/0367-2530-00160

Rombolà AD, Brüggemann W, López-Millán AF, Tagliavini M, Abadía J, Marangoni B, Moog PR (2002) Biochemical responses to iron deficiency in kiwifruit (Actinidia deliciosa). Tree Physiol 22:869–875. https://doi.org/10.1093/treephys/22.12.869

Römheld V, Nikolic M (2007) Iron. In: Barker AV, Pilbeam DJ (eds) Handbook of plant nutrition. CRC Press, Taylor and Francis Inc., New York, pp 329–350

Shevela D, Eaton-Rye JJ, Shen JR, Govindjee (2012) Photosystem II and the unique role of bicarbonate: a historical perspective, In: Biochimica et Biophysica Acta - Bioenergetics., 1817(8): pp. 1134–1151. https://doi.org/10.1016/j.bbabio.2012.04.003

Susin S, Abadía A, Gonzalez-Reyes JA, Lucena JJ, Abadia J (1996) The pH requirement for in vivo activity of the iron-deficiency-induced "turbo" ferric chelate reductase. A comparison of the iron-deficiency-induced iron reductase activities of intact plants and isolated plasma membrane fractions in sugar beet. Plant Physiol 110: 111–123. https://doi.org/10.1104/pp.110.1.111

Tomasi N, Mimmo T, Terzano R, Alfeld M, Janssens K, Zanin L, Pinton R, Varanini Z, Cesco S (2014) Nutrient accumulation in leaves of Fe-deficient cucumber plants treated with natural Fe complexes. Biol Fertil Soils 50:973–982. https://doi.org/10.1007/s00374-014-0919-6

Venturas M, Fernández V, Nadal P, Guzmán P, Lucena JJ, Gil L (2014) Root iron uptake efficiency of Ulmus laevis and U. minor and their distribution in soils of the Iberian Peninsula. Front Plant Sci 5(104). https://doi.org/10.3389/fpls.2014.00104

Virdi AS, Singh S, Singh P (2015) Abiotic stress responses in plants: roles of calmodulin-regulated proteins. Front Plant Sci 6. https://doi.org/10.3389/fpls.2015.00809

Wegner LH, Zimmermann U (2004) Bicarbonate-induced alkalinization of the xylem sap in intact maize seedlings as measured in situ with a novel xylem pH probe. Plant Physiol 136:3469–3477

Wu YY, Xing DK (2012) Effect of bicarbonate treatment on photosynthetic assimilation of inorganic carbon in two plant species of Moraceae. Photosynthetica 50:587–594. https://doi.org/10.1007/s11099-012-0065-z

Yunta F, Sierra MA, Gómez-Gallego M, Alcázar R, García-Marco S, Lucena JJ (2003) Methodology to screen new iron chelates: prediction of their behavior in nutrient solution and soil conditions. J Plant Nutr 26:1955–1968. https://doi.org/10.1081/PLN-120024256