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Testing a Bovine Blood-Derived Compound as Iron Supply on *Cucumis sativus* L.

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Abstract: A new powder formulation obtained from bovine blood (Fe-heme) was tested on cucumber (*Cucumis sativus* L.) to investigate its effectiveness as iron supply in comparison with two synthetic iron-chelates fertilizers: ethylenediaminetetraacetic acid (EDTA/Fe³⁺) and ethylenediamine-N'N'-bis(2-hydroxyphenyl acetic acid) (o,oEDDHA/Fe³⁺). Green stressed cucumber plants were evaluated in their recovery (SPAD index and weight variations) and to test the iron reduction capacity of the roots at pH 7.5 and 6.0 using each iron treatment as iron supply. The blood-derived product showed similar effects on decreasing iron-deficiency symptoms: SPAD increments and the weights of plants were similar. Noteworthy, the average of Fe³⁺ reduction capacity in roots was higher for EDTA/Fe³⁺, while it was similar for o,oEDDHA/Fe³⁺, and Fe-heme at pH 7.5. Fe-heme showed a complex behavior due to aggregation and low solubility at pH 6 and showed an unexpectedly high contribution of root exudates to iron reduction.

Keywords: iron deficiency; blood-derived fertilizers; biochemical responses; Fe-chelate reductase; root exudates

1. Introduction

Iron chlorosis is a nutritional disorder often occurring in plants cultivated on calcareous and/or alkaline soils [1]. The iron chlorosis prevention/cure with synthetic iron chelates is a widespread agronomical practice in vineyards and fruit trees [2,3]. Nevertheless, such an approach implies environmental and health risks coupled to high costs, and therefore, their use is economically justified only for high-value crops [3]. In this context, some key aspects should be considered, such as minimizing environmental impacts and the reutilization of organic materials to reduce the inputs for production (byproducts from agro-industries, e.g., beet vinasse, lignosulfonates, animal blood-derived fertilizers, etc.) [4–7]. Natural materials containing iron as a cofactor, such as plants and animals byproducts, have been used as iron sources for plants [3,8]. A blood meal is a natural iron source containing 20–30 g Fe kg⁻¹ in the form of ferrous iron (Fe²⁺) in the heme group of the hemoglobin molecule. However, in solution, Fe²⁺ is rapidly oxidized in air to Fe³⁺ [9], which is the main form of iron in blood-meal formulations [10]. A blood meal is a byproduct of industrial slaughterhouses used as fertilizer in organic farming, not only as a natural iron source but also as a slow-release nitrogen fertilizer [11].

The synthetic ethylenediaminetetraacetic acid (EDTA/Fe³⁺) and ethylenediamine-N,N'-bis(2-hydroxyphenyl acetic acid) (o,oEDDHA/Fe³⁺) present high stability constants (10²⁵ and 10³⁵, respectively) and are stable over a wide range of pH: the latter is stable until pH 10, while the former has poor stability at alkaline pH [12]. Fe-heme compounds are extremely stable compounds: the insertion and the depletion of Fe ions are both enzyme-catalyzed reactions; into mitochondria, ferrochelatase (EC 4.99.1.1) inserts Fe²⁺ into the porphyrin ring, while it is removed into the endoplasmic reticulum by heme oxygenase (EC 1.14.99.3) [9]. Those reactions cannot occur outside living cells. However, the loss of the entire Fe-heme prosthetic group (in the form of hemin) has been measured, and it is higher at pH around 5.5 and above 8, thus determining a rapid denaturation of the protein [9].

In this work, a new iron (Fe)-heme containing fertilizer was tested in iron-deficient cucumber (*Cucumis sativus* L.) plants, whose iron nutrition mechanisms have been deeply investigated [13–16]. Therefore, this species was chosen as a model to evaluate the impact of these fertilizers on plant physiology (leaf chlorophyll and plant growth) in iron-deficient conditions and determine if plants take up the iron through the typical enzymatic-induced reduction mechanism. According to Mori [17], the incorporation of iron from hemoglobin into the root cells may follow a similar mechanism as the uptake of iron in animal cells (endocytosis). Recent works by López-Rayó et al. [18,19] demonstrated that Fe-heme fertilizers could represent an alternative to synthetic iron chelates since they improve iron nutrition in grapevine plants grown in calcareous soils conditions [18]. In particular, Fe-heme induced an increased iron reduction capacity in the roots and a lower exudation of oxalic acid, associated with the accumulation of oxalate compounds in roots [19].

Under iron depletion, Strategy I plants exhibit several physiological adaptation responses in roots to increase iron uptake [20]. These responses have been profusely described elsewhere [21,22] and include, among others, the activation of the plasma membrane-bound Ferric-chelate reductase (EC 1.16.1.7) (FCR) to reduce Fe³⁺ to Fe²⁺ on the root surface [20], which represents an essential step for iron uptake in the Strategy I plants [23]. Moreover, a complex mixture of substances (i.e., carbohydrates, organic acids, amino acids, phenolics, phytosiderophores, and enzymes) is released by roots and is involved in the modulation of iron availability [24]. The same authors highlighted that phenolics and organic acids are involved in iron solubilization and mobilization of iron through reduction and complexation processes. More in detail, Pavlovic et al. [25] reported increased concentrations of organic acids (mainly malic and citric) and phenolic compounds (riboflavin, catechin, epicatechin, and gallic acid) in cucumber root tips in response to iron deficiency. Zamboni et al. [26] showed that iron-deficient tomatoes modulate the expression of iron uptake, translocation, and homeostasis, together with the modulation of a specific branch of phenolic biosynthesis. This class of compounds present in root exudates is relevant but presumably underestimated in many iron-deficiency studies. Recently, López-Rayó et al. [19] evaluated the contribution of roots exudates to the total iron reduction to be about 30–50% in iron-deficient grapevines using different iron sources and pH conditions.

The present work focuses on the effects of Fe-heme, dispensed on chlorotic cucumber plants, on the re-greening effect, and root iron-reduction and compare them with two synthetic chelates. This research ascribes to the studies on the efficiency of Fe-heme fertilizers and their consequences on the rhizosphere [18,19], such as organic acid exudation [19].

2. Materials and Methods

2.1. Reagents and Products

All chemicals were of analytical grade. The chelating agents, o,oEDDHA, 94.49% (LGC Standards), Na₂H₂-EDTA 99% (Titriplex III), were commercially available. The titrimetric purity of o,oEDDHA was previously calculated as described elsewhere [27]. A dark reddish powder formulation obtained by centrifugation of whole bovine blood of food-grade and later hemolyzed and subsequently digested with an alkaline protease (Fe-heme) was kindly provided by FarproAgro, Modena, Italy. Chemical and

reactivity assessment of Fe-heme product was profusely studied elsewhere [10,18] together with the iron complexation rate.

The o,oEDDHA/Fe³⁺, and EDTA/Fe³⁺ chelate solutions were prepared following the protocol described in Nadal et al. [13]. Briefly, chelating agents were initially dissolved in sufficient NaOH (1:3 molar ratio), then an amount of Fe(NO₃)₉·H₂O (Merck) stock solution, calculated to be 5% above the molar amount of ligand, was slowly added on both chelating agent solutions. During the chelation process, pH was kept between 6.0 and 8.0, and it was finally adjusted to 7.0 by adding a diluted solution of either HNO₃ or NaOH, as needed. The resulting solutions of o,oEDDHA/Fe³⁺ and EDTA/Fe³⁺ were left to stand overnight to allow Fe excess precipitation as iron hydr(oxides). Then, they were filtered through 0.45 µm cellulose membrane filters and diluted by adding type I water.

2.2. Recovery Assay

Cucumber seeds (*Cucumis sativus* L., cv Ashley) were germinated in standard seed germination papers moistened with a macronutrient solution [1.0 mM Ca(NO₃)₂, 0.9 mM KNO₃, 0.3 mM MgSO₄, 0.1 mM KH₂PO₄] in diffuse light in a growth chamber for 5 days as reported by Nadal et al. [28]. Twenty-four plants (six for each treatment) were inserted into darkened pots for hydroponic cultures (constantly aerated) with a nutrient solution at a concentration of 5 µM Fe in the N,N'-bis(2-hydroxybenzyl)-ethylenediamine-N,N'-diacetic acid (HBED/Fe³⁺) form [29], where they remained for 8 days before the imposition of the different Fe-treatments. The nutrient solution composition was as follows: macronutrients Ca(NO₃)₂ 1 mM, KNO₃ 0.9 mM, MgSO₄ 0.3 mM, KH₂PO₄ 0.1 mM; micronutrients NaCl 35 µM, H₃BO₃ 10 µM, Na₂MoO₄ 0.05 µM, MnSO₄ 1.0 µM, CuSO₄ 1.0 µM, ZnSO₄ 1.0 µM, NiCl₂ 1.0 µM, CoSO₄ 1.0 µM, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 0.1 mM, KOH 0.05 mM [13]. The initial pH of the nutrient solution was set to 7.5, to simulate calcareous soil conditions.

Plants were grown in the above-described nutrient solution in a growth chamber (model CCK, Dycometal, Barcelona, Spain) provided with fluorescent and sodium vapor lamps, wherein the temperature was 25–28 °C with 40–70% relative humidity.

Plants were divided into 4 treatments of 6 plants each: (1) –Fe, (2) o,oEDDHA/Fe³⁺, (3) EDTA/Fe³⁺, and (4) a new powder formulation derived from bovine-blood (Fe-heme) [10,19]. The Fe concentration for the 3 treatments was 10 µM, which already proved to permit to observe differences among treatments in the case of synthetic Fe chelates [13]. Other micronutrients were provided: manganese (MnSO₄) 1.0 µM, copper (CuSO₄) 0.5 µM, zinc (ZnSO₄) 0.5 µM, cobalt (CoSO₄) 0.1 µM, and nickel (NiCl₂) 0.1 µM [13].

The treatments were applied only once, and the effects of the treatments were observed for two weeks: as mentioned before, plants remained for 8 days in iron deficiency conditions, then the recovery effects induced by the treatments were evaluated during the following 7 days; the day of the application of the treatments corresponds to 0 DAT (days after treatments). Then plants were cut into roots, leaf, and shoot, weighed and measured separately for fresh weight (FW) determination and then dried in a forced-air oven at 65 °C for 3 days for the subsequent dry weight (DW) determination. Chlorophyll Index (Soil Plant Analysis Development (SPAD) MINOLTA 502, Osaka, Japan) was measured twice before applying the treatments and then daily on each leaf of the cucumber plants. Leaves were divided into two groups: mature leaves, corresponding to the fully expanded leaves already present on the plants at the beginning of the experiment, i.e., 8 days before the imposition of the treatments (so at –8 DAT, Days After Treatment); on the contrary, young leaves were fully expanded leaves appearing during the experiment. Those are usually more sensitive to iron deficiency [30]; therefore, the subdivision was aimed at evaluating the effects of the different treatments on the two types of leaves. The recovery after the treatments was estimated by the SPAD index variation (ΔSPAD), where ΔSPAD is the difference between the SPAD value measured during the experiment and the first SPAD value measured at that node level, taken as a reference: –8 DAT for nodes 1, 2 and 3; at –2 DAT for nodes 4 and 5; 1 DAT for node 6 in EDDHA/Fe³⁺ treated plants and 4 DAT for node 6 in Fe-heme treated plants.

To evaluate the effects of the treatments during vegetative growth, Δ SPAD data were averaged over the leaves of the same node, where node 1 corresponds to the oldest leaves and node 6 to the youngest.

2.3. Assay on Fe-Reduction Capacity

Cucumber seeds were germinated as reported in the previous paragraph, and then uniform seedlings were selected. Bunches of two individual plants were wrapped together with polyurethane foam and placed in a 12-L polypropylene bucket (12 pairs of plants per bucket) containing the nutrient solution with 0.1 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and 2.4 g CaCO_3 for pH buffering at 7.5 to simulate calcareous soil conditions.

Plants were grown for 14 days in the above-described continuously aerated nutrient solution in a Dycometal type CCK growth chamber. Deionized water was added every 2 days to keep the volume constant, and the nutrient solution was renewed weekly.

The concentration of iron added (5 μM) was found by Lucena and Chaney [29] to be the most adequate to produce green cucumber plants, but with a high FCR activity (stressed plants) in an experiment with similar experimental conditions. The Ferric-chelate reduction activity measurement was firstly made at pH = 7.5, where the Fe-heme formulation is highly soluble [10], but where enzymatic FCR activity is quite low [31,32]. Then, it was repeated on similar plants at the optimal pH = 6, at which most of the FCR activity studies have been conducted [29], but where coagulation and aggregation processes for the used Fe-heme product were observed and reported elsewhere [10].

The assay solution contained macronutrient and sodium bathophenanthrolinedisulfonate (Na_2BPDS) (300 μM) as a specific chelating agent for Fe^{2+} complexation. The experiment was initiated within 2 h after the daylight period. A bunch of 2 plants was transplanted into a 200-mL assay solution, where they set for 2 h without Fe sources. Then, 5 mL of the treatment solution (o,oEDDHA/ Fe^{3+} , EDTA/ Fe^{3+} , or Fe-heme) were added (time 0) so that the final concentration was 100 μM Fe. Aliquots of 3 mL were collected after 0, 10, 20, 60, and 120 min for spectrophotometric measurements. Six pairs for each treatment were arranged. Besides that, two replicate blanks per product, consisting of solutions without plants, were included to correct reduction rates for slow photoreduction of the different Fe-sources [29]. The $(\text{BPDS})_3/\text{Fe}^{2+}$ concentration was calculated as in Lucena and Chaney [29] by determination of absorbance at 535 nm (maximum absorbance of $(\text{BPDS})_3/\text{Fe}^{2+}$), at 480 nm (near the maximum absorbance of o,oEDDHA/ Fe^{3+}) and at 370 and 400 nm (maximum absorbancies of Fe-heme) [10] to consider the contribution of the applied treatments on the total absorbance. The absorbancies were measured by a Jasco V-650 Uv-vis spectrophotometer. The concentration of each chelate was calculated by solving the two-equation system (e.g., for Fe-heme):

$$A_{535} = a_{\text{FeBPDS}535} \times [\text{Fe}(\text{BPDS})_3] + a_{\text{BB}535} \times [\text{Fe-heme}]$$

$$A_{400} = a_{\text{FeBPDS}400} \times [\text{Fe}(\text{BPDS})_3] + a_{\text{BB}400} \times [\text{Fe-heme}]$$

where A_{535} and A_{400} are the absorbancies measured for each sample at 535 and 400 nm, respectively; $a_{\text{FeBPDS}535}$, $a_{\text{FeBPDS}400}$, $a_{\text{BB}535}$, and $a_{\text{BB}400}$ are the molar absorptivity in the experimental conditions (see Table 1).

Table 1. Absorptivity coefficients at 370 nm (a_{370}), 400 nm (a_{400}), 480 nm (a_{480}), and 535 (a_{535}) of Fe chelates used to assess Fe-reduction activity.

Iron Source	Molar Absorptivity Coefficients/L mol ⁻¹ cm ⁻¹			
	a_{370}	a_{400}	a_{480}	a_{535}
$(\text{BPDS})_3/\text{Fe}^{2+}$	1.79×10^3	4.11×10^3	1.73×10^4	2.26×10^4
o,oEDDHA/ Fe^{3+}	1.19×10^3	2.25×10^3	5.05×10^3	3.33×10^3
EDTA/ Fe^{3+}	8.73×10^2	3.98×10^2	2.05×10^2	1.88×10^2
Fe-heme	4.35×10^4	1.04×10^4	4.41×10^4	1.33×10^4

The fresh weight of the roots was determined at the end of the experiment. The slope of the plots of produced Fe^{2+} ($\mu\text{mol g}^{-1}$ fresh root) against time (h) was used as the Fe^{3+} reduction rate for each pair of plants at 20, 60, and 120 min (0.33, 1, and 2 h, respectively).

The contribution to iron reduction by root exudates was determined in a parallel experiment. Fe sources were added after removing cucumber plants since FCR is produced by roots only once iron is added. Similarly, the experiment was initiated within 2 h after the daylight period. Three pairs of plants for each treatment were transplanted into a 200-mL assay solution and left for 2 h without iron sources to collect exudates from cucumber roots. The duration of root exudates collection was chosen based on a previous protocol used by Lopez-Rayó et al. [19] to collect root exudates in grapevine for a similar experiment on Fe reduction capacity of roots. BPDS and 5 mL of the iron sources (o,oEDDHA/ Fe^{3+} , EDTA/ Fe^{3+} , or Fe-heme, 100 μM) were added after removing the plants from the nutrient solution (time 0). Aliquots of 3 mL were collected after 0, 10, 20, 60, and 120 min for spectrophotometric measurements, in analogy with the FCR assay. It was repeated at both pH 7.5 and pH 6. Iron reduction capacity by roots was calculated by subtracting the value obtained from the iron reduction capacity by exudates to the total iron reduction.

2.4. Statistical Analysis

Statistical analyses were performed with SPSS statistical software (version 19.0; SPSS Inc., Chicago, IL, USA). Data were statistically handled by a one-way analysis of variance (ANOVA). Statistical comparison of means was carried out to reveal the differences between chelates using Duncan Multiple Range Test ($\alpha = 0.05$). Data on Fe-reduction were expressed as the mean reduction rates, including the standard error corresponding to six plants replicates per treatment.

3. Results

3.1. Recovery Assays

To assess the effectiveness of the different iron sources in recovering from the early chlorotic symptoms on leaves, discrimination between old and young, i.e., developed after the first day of SPAD measure corresponding to -8 DAT (Day After Treatment) leaves, was performed.

SPAD data showed a similar trend for young and old leaves, as depicted in Figure 1A,B: the application of iron fertilizers induced a rapid increase of the SPAD index significantly higher from $-Fe$ plants in young leaves since 1 DAT (Figure 1A). Similar effects were found for old leaves (Figure 1B) even if, in this case, no significant differences were found between the Fe-heme product and $-Fe$ treatment. Table S1, in the Supplementary materials, lists the standard errors associated with SPAD indexes.

The recovery from chlorosis after the treatment application was assessed by the SPAD index variation (ΔSPAD) for each node during the assay (Figure 2). Positive SPAD increments from leaves treated with EDTA/ Fe^{3+} and o,oEDDHA/ Fe^{3+} were detected for node 1 (Figure 2A) and 2 (Figure 2B) only at the end of the experiment. Positive SPAD increments were also detected for all iron sources on higher nodes (Figure 2C–E).

Regarding vegetative growth, only plants treated with o,oEDDHA/ Fe^{3+} , and Fe-heme reached up to node 6 level (Figure 2F), indicating the positive effect of Fe-heme supply on both re-greening and recovery of vegetative growth. Table S2, in the Supplementary Materials, lists the standard errors associated with ΔSPAD values.

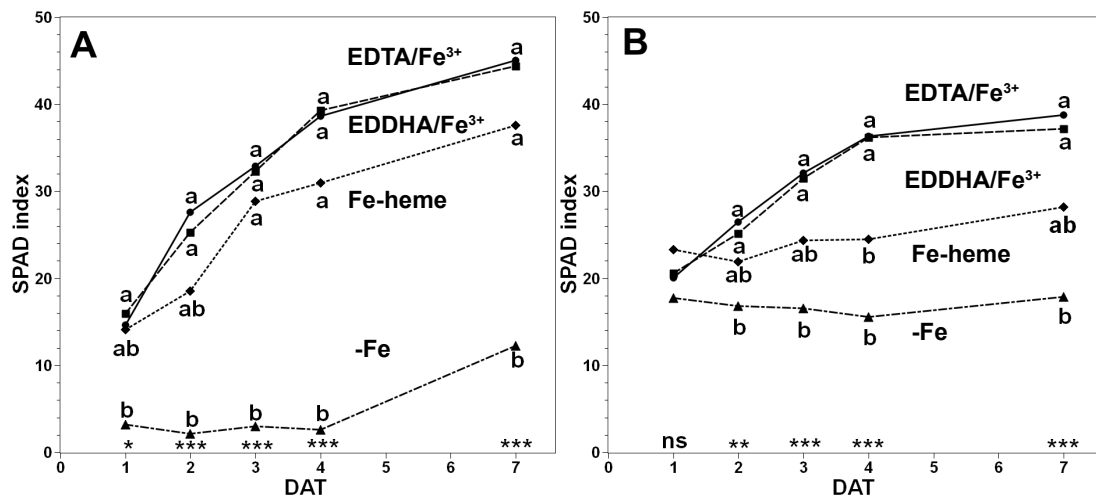


Figure 1. SPAD readings from (A) young cucumber leaves (leaves developed after the first measure at DAT –8) and (B) mature cucumber leaves for the tested treatments (dashed and dotted line and triangles: –Fe; dashed line and squares: o,EDDHA/Fe³⁺, continuous line, and circles: EDTA/Fe³⁺, dotted line, and diamonds: Fe-heme). Different letters indicate significant differences among treatments in a post-hoc Duncan test (*: $p < 0.1$; **: $p < 0.05$; ***: $p < 0.001$; ns, non-significant difference); $n = 6$.

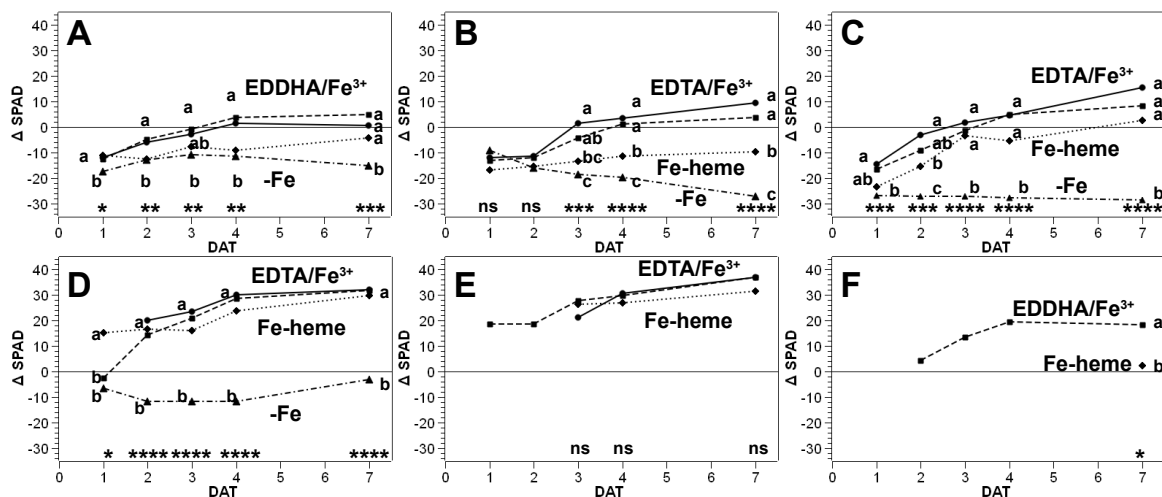


Figure 2. SPAD increments along plant axis for node 1 (A), node 2 (B), node 3 (C), node 4 (D), node 5 (E) and node 6 (F) for the tested treatments (dashed and dotted line and triangles: –Fe; dashed line and squares: o,EDDHA/Fe³⁺, continuous line and circles: EDTA/Fe³⁺, dotted line and diamonds: Fe-heme). Different letters indicate significant differences among treatments in a post-hoc Duncan test. (*: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.005$; ****: $p < 0.001$; ns, non-significant difference).

Growth data on cucumber plants showed a uniform behavior (Table 2): the –Fe treated plants showed the lowest values for all the determined parameters, being statistically different from o,EDDHA/Fe³⁺ treated plants for all values except for stem dry weight (DW), in which the highest value was measured in Fe-heme treated plants. For the other parameters, Fe-heme was found to be not significantly different from synthetic chelates and –Fe plants.

Table 2. Growth parameters of different plant organs at the end of the recovery experiment (FW: fresh weight; DW: dry weight). Data are mean \pm standard error of six replicates. Different letters indicate significant differences among treatments in a post-hoc Duncan test. (*: $p < 0.1$; **: $p < 0.05$; ***: $p < 0.005$; ****: $p < 0.001$); $n = 6$.

Treatments	Total	Root	FW/g Stem	Leaves	Single Leaf	N° Leaves
–Fe	2.9 \pm 0.9 ^b	1.0 \pm 0.1 ^b	0.63 \pm 0.09 ^b	1.3 \pm 0.7 ^b	0.38 \pm 0.19 ^b	3.3 \pm 0.5 ^b
o,oEDDHA/Fe ³⁺	7.1 \pm 3.2 ^a	2.9 \pm 1.3 ^a	0.94 \pm 0.39 ^a	3.2 \pm 1.6 ^a	0.61 \pm 0.18 ^a	5.2 \pm 1.5 ^a
EDTA/Fe ³⁺	5.9 \pm 2.5 ^{ab}	2.4 \pm 1.2 ^{ab}	0.76 \pm 0.18 ^{ab}	2.7 \pm 1.6 ^a	0.56 \pm 0.21 ^{ab}	4.7 \pm 1.4 ^{ab}
Fe-heme	4.5 \pm 2.6 ^{ab}	1.5 \pm 1.3 ^b	0.77 \pm 0.36 ^{ab}	2.2 \pm 1.1 ^{ab}	0.46 \pm 0.17 ^{ab}	4.8 \pm 1.0 ^a
Significance	**	****	*	**	***	**

Treatments	Total	Root	Stem	Leaves	-
–Fe	0.31 \pm 0.10 ^b	0.058 \pm 0.021 ^b	0.045 \pm 0.011 ^b	0.21 \pm 0.07 ^b	-
o,oEDDHA/Fe ³⁺	0.59 \pm 0.22 ^a	0.104 \pm 0.042 ^a	0.069 \pm 0.027 ^{ab}	0.42 \pm 0.18 ^a	-
EDTA/Fe ³⁺	0.48 \pm 0.18 ^{ab}	0.085 \pm 0.024 ^{ab}	0.057 \pm 0.012 ^{ab}	0.34 \pm 0.16 ^{ab}	-
Fe-heme	0.45 \pm 0.14 ^{ab}	0.064 \pm 0.032 ^b	0.081 \pm 0.013 ^a	0.31 \pm 0.10 ^{ab}	-
Significance	**	**	***	**	-

3.2. Fe Reduction by Roots and Root Exudates

At pH = 6 (Table 3 and Figure 3A,B), EDTA/Fe³⁺ followed a linear dependence of the total reduced Fe³⁺ with time, as showed in Figure 3A and by the correlation values in Table 3 ($R^2 = 0.974$ after 2 h). Moreover, plants treated with EDTA/Fe³⁺ showed the highest total iron reduced amount (0.560 $\mu\text{molFe g}^{-1} \text{h}^{-1}$) that was statistically different from the other 2 iron sources at time > 1 h.

o,oEDDHA/Fe³⁺ and Fe-heme showed a more complex behavior (see Figure 3A): the amount of total reduced Fe³⁺ is no longer linear with time (after 2 h, $R^2 = 0.667$ for o,oEDDHA/Fe³⁺, and $R^2 = 0.502$ for Fe-heme, see Table 3): almost all Fe³⁺ is reduced in the first 60 min for o,oEDDHA/Fe³⁺ and the first 10 min for Fe-heme, after those times the total reducing capacity reaching a plateau value.

Regarding iron reduction from root exudates, a non-linear trend with time was observed for all the iron sources (shown in Figure 3B) with a plateau region reached after 10–20 min. Moreover, the amount of reduced Fe³⁺ was not statistically different between the synthetic chelates and Fe-heme (Figure 3B), ranging from 0.053 (EDTA/Fe³⁺) and 0.0017 $\mu\text{molFe g}^{-1} \text{h}^{-1}$ (Fe-heme) (Table 3). However, the contribution of this kind of reduction to the total root Fe-reducing capacity seemed to be source dependent: it was found to be minimal for the synthetic chelates (in particular in the case of EDTA/Fe³⁺, where it accounted for the 15.8–4.8%) while higher for Fe-heme (37.1–32.5%) (see Table 3). Moreover, it showed a decreased contribution only in o,oEDDHA/Fe³⁺ treated plants (from 42.4% after 20 min, to 18.4% after 2 h; see Table 3) while remaining almost constant for the other 2 iron sources.

At pH = 7.5, all iron sources induced lower values of reduced Fe³⁺ if compared to pH = 6 (Table 3, Figure 3C,D): EDTA/Fe³⁺ was confirmed to induce the highest total Fe-reducing capacity with an almost linear trend (0.328 $\mu\text{molFe g}^{-1} \text{h}^{-1}$ and $R^2 = 0.962$, after 2 h). Interestingly, o,oEDDHA/Fe³⁺ and Fe-heme showed similar total Fe³⁺ reduction capacities (Figure 3C,D). At this pH value, the influence of Fe-source on the exudates reduction was found after 1 h (Figure 3D): EDTA/Fe³⁺ showed the highest values, while the other 2 iron sources remained similar. In general, the contribution of root exudates to total Fe reduction was found to be higher than those observed at pH = 6, in particular in the case of Fe-heme for which it represented more than 56% of total reducing capacity after 2 h (Table 3). Moreover, at this pH, the effects of root exudates on the total Fe³⁺ reduction represented the main contribution to iron reduction up to 20 min for all the 3 iron sources (ranging from 83% in Fe-heme and 60% in o,oEDDHA/Fe³⁺ treated plants), but then decreased with time, accounting for 30–35% in chelates treated plants.

Table 3. Total Fe reduction, Fe reduction by root exudates, and % of Fe reduction capacity by roots (calculated by subtracting the value obtained from the Fe reduction capacity by exudates to the total Fe reduction) determined in cucumber roots at pH = 6.0 and pH = 7.5.

Time (h)	Fe Source	Total Fe-Reduction ($\mu\text{mol}_{\text{Fe}} \text{g}^{-1} \text{h}^{-1}$) Slope \pm Error [R ²]	Fe Reduction by Root Exudates ($\mu\text{mol}_{\text{Fe}} \text{g}^{-1} \text{h}^{-1}$) Slope \pm Error [R ²]	% Fe Reduction by Roots
pH = 6.0				
1/3	EDTA/Fe ³⁺	0.595 \pm 0.024 [0.892]	0.094 \pm 0.025 [0.289]	84.2 \pm 4.8
	<i>o,o</i> EDDHA/Fe ³⁺	0.203 \pm 0.032 [0.642]	0.086 \pm 0.026 [0.422]	57.6 \pm 19.5
	Fe-heme	0.143 \pm 0.038 [0.243]	0.053 \pm 0.041 [0.437]	62.9 \pm 38.5
1	EDTA/Fe ³⁺	0.611 \pm 0.011 [0.968]	0.047 \pm 0.008 [0.420]	92.3 \pm 1.5
	<i>o,o</i> EDDHA/Fe ³⁺	0.239 \pm 0.032 [0.609]	0.043 \pm 0.008 [0.565]	82.0 \pm 5.8
	Fe-heme	0.077 \pm 0.012 [0.408]	0.025 \pm 0.018 [0.454]	67.5 \pm 28.4
2	EDTA/Fe ³⁺	0.560 \pm 0.008 [0.974]	0.027 \pm 0.004 [0.437]	95.2 \pm 0.8
	<i>o,o</i> EDDHA/Fe ³⁺	0.185 \pm 0.018 [0.667]	0.034 \pm 0.004 [0.680]	81.6 \pm 4.0
	Fe-heme	0.049 \pm 0.006 [0.502]	0.017 \pm 0.008 [0.702]	65.3 \pm 20.6
pH = 7.5				
1/3	EDTA/Fe ³⁺	0.225 \pm 0.031 [0.636]	0.138 \pm 0.039 [0.257]	38.7 \pm 25.8
	<i>o,o</i> EDDHA/Fe ³⁺	0.110 \pm 0.025 [0.500]	0.065 \pm 0.017 [0.506]	40.9 \pm 28.9
	Fe-heme	0.076 \pm 0.032 [0.140]	0.063 \pm 0.027 [0.204]	17.1 \pm 70.4
1	EDTA/Fe ³⁺	0.277 \pm 0.009 [0.919]	0.089 \pm 0.040 [0.318]	67.9 \pm 15.5
	<i>o,o</i> EDDHA/Fe ³⁺	0.083 \pm 0.015 [0.515]	0.030 \pm 0.007 [0.482]	63.9 \pm 15.0
	Fe-heme	0.051 \pm 0.013 [0.369]	0.041 \pm 0.013 [0.481]	19.6 \pm 46.0
2	EDTA/Fe ³⁺	0.328 \pm 0.006 [0.962]	0.085 \pm 0.021 [0.395]	74.1 \pm 6.9
	<i>o,o</i> EDDHA/Fe ³⁺	0.067 \pm 0.009 [0.581]	0.020 \pm 0.004 [0.500]	70.2 \pm 10.0
	Fe-heme	0.060 \pm 0.005 [0.766]	0.034 \pm 0.007 [0.631]	43.3 \pm 16.4

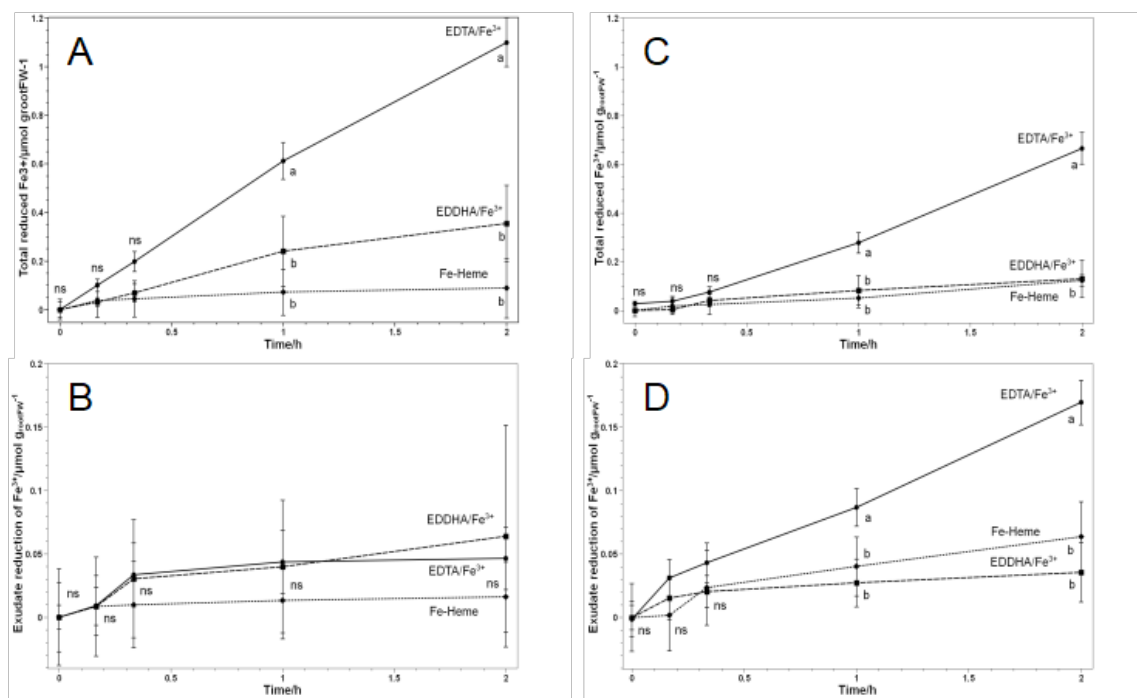


Figure 3. Trend of Fe³⁺ reduction observed at pH = 6.0 and pH = 7.5 in Fe-deficient cucumber plants. (A) Total Fe³⁺ reduction at pH = 6.0, *n* = 6; (B) Fe³⁺ reduction by root exudates at pH = 6.0, *n* = 3; (C) Total Fe³⁺ reduction at pH = 7.5, *n* = 6; (D) Fe³⁺ reduction by root exudates at pH = 7.5, *n* = 3. Different letters indicate significant differences among treatments in a post-hoc Duncan test (*p* < 0.05); ns: non-significant difference. Standard errors were used as error bars.

4. Discussion

The effectiveness of a Fe-heme formulation in comparison with synthetic chelates was evaluated in a recovery assay by measuring the SPAD index on leaves, also considering their position (node) in the plant. Figure 1 showed that the Fe-heme formulation was more effective on younger leaves than on control leaves. More in detail, Fe-heme was as effective as synthetic chelates and promoted leaf growth until node 6 as o,oEDDHA/Fe³⁺ (Figure 2). López-Rayó et al. [19] in a similar experiment on the grapevine, conducted under controlled conditions using the same Fe-heme formulation at the concentration of 10 µM Fe, reported a significant difference between o,oEDDHA/Fe³⁺, and Fe-heme treated plants about the SPAD index measured on the youngest expanded leaf, explaining this finding with the highest chemical stability of Fe-heme complex compared to Fe-EDDHA [19]. Plants' organs fresh and dry weights (Table 2) were higher in plants treated with the two synthetic iron chelates (in particular o,oEDDHA/Fe³⁺); however, it is worth noting Fe-heme formulation was not significantly different from iron chelates. More in details, plants treated with Fe-heme were statistically different from o,oEDDHA/Fe³⁺ treated plants only in root FW and DW (Table 2), and showed the highest stem dry weight. These findings agree with previous work on grapevine [18], which highlighted that reducing the dose of Fe-heme formulations could be more effective in promoting root growth. The Fe reducing capacity assay was performed in two different experiments: the first operating at pH = 6.0, which ensured the highest FCR activity [29] and the second at pH = 7.5, which represented a compromise between FCR activity and optimal solubility of Fe-heme formulations, previously demonstrated to be around pH 8.2 [10].

The Fe-heme formulation showed an overall iron reduction rate similar to that of o,oEDDHA/Fe³⁺ (particularly at pH = 7.5, Table 3 and Figure 3C,D), but lower compared to EDTA/Fe³⁺. This result has been previously described by Lucena and Chaney [29], reporting that the best substrates for the root reduction activity in Fe-stressed cucumber roots are the weaker iron chelates. Therefore, the lowest stability of EDTA/Fe³⁺ complex compared to o,oEDDHA/Fe³⁺ [12], could explain the highest reduction rate of EDTA/Fe³⁺ compared to o,oEDDHA/Fe³⁺ (Table 3, Figure 3C,D), which has been previously described also by García-Marco et al. [33] and Hernández-Apaolaza et al. [34] on analogous experiments on stressed cucumber plants. In particular, the activity values calculated using o,oEDDHA/Fe³⁺ and EDTA/Fe³⁺ as iron substrates of FCR well agree with those reported by Nadal et al. [13] for o,oEDDHA/Fe³⁺ and by Lucena and Chaney [29] for EDTA/Fe³⁺ in similar cucumber plants. The high stability of Fe-heme derivatives could, in part, explain the lower roots Fe reduction. In fact, the release of ferric/ferrous ion from Fe-heme compounds is an enzyme-mediated process by Heme Oxygenase since the heme prosthetic group acts as an extremely stable coordination agent towards iron. However, the similarities with o,oEDDHA/Fe³⁺ treated plants may suggest that different mechanisms of Fe intake could be involved in the case of Fe-heme products, i.e., endocytosis [17]. Moreover, iron reduction data suggested that Fe-heme reduction could be less pH-dependent than synthetic iron chelates (Table 3), further supporting that other mechanisms different from enzymatic iron reduction could be involved in the rhizosphere level. However, it should be recalled that Fe-heme formulations showed a tendency to aggregate at mildly acidic pH [10], thus lowering iron availability at pH = 6.

One of the main goals of the present study was to discriminate the contribution of root exudates inside the overall iron reduction capability of roots. The characterization of root exudates was not among the purposes of this study. However, Zhao et al. [35] reported an extensive analysis of cucumber's root exudates (subjected to copper accumulation stress) through gas chromatography and NMR spectroscopy. Exudates were found to be a complex mixture of several metabolites belonging to five main biochemical groups: amino acids (among them Serine was reported to play a major role in the Cu-chelating process), organic acids (i.e., citric acid), fatty acids (i.e., pelargonic acid, an indicator of membrane damage), sugars (which showed a down-regulation due to citric acid exudation), and phenolic compounds (i.e., salicylic and benzoic acids which showed antioxidant and antifungal activities). Among these compounds, organic acids, phenolics, and flavins compounds are

the most effective reducing and complexing agents, increasing iron mobility and availability [24,36]. In particular, Pavlovic et al. [25] reported increased concentrations of organic acids (mainly malic and citric) and phenolic compounds (riboflavin, catechin, epicatechin, and gallic acid) in cucumber root tips in response to iron deficiency. At the same time, Bityutskii et al. [37] described an increased exudation of organic acids such as citric, fumaric, and gluconic in Fe deficient cucumber plants. A recent paper by Gattullo et al. [38] reported a 10-fold phenols (mainly in the form of rutin) and 3-fold organic acids (mainly in the form of citric acid) exudation in Fe-deficient cucumber plants grown on calcareous soils. The same authors reported a 3-fold increased exudation of amino acids in similar conditions: a possible contribution of amino acids to improve iron mobilization by Fe-heme formulation has been previously hypothesized by Lopez-Rayó et al. [19].

The present data suggest that the contribution by root exudates to the overall root iron reduction decreased with time, but increased with pH. The pH-dependency is not surprising since FCR-activity is known to be higher at pH = 6 than at pH = 7.5 [39]; moreover, Donnini et al. [40] found an increased phenolics release at pH = 8 in *Parietaria diffusa*. Recently, López-Rayó et al. [19] evaluated the root iron reduction induced by Fe-heme treated grapevine plants in buffered and non-buffered conditions at pH = 6, using EDTA/Fe³⁺ as an iron source for the iron-reducing capacity assay. Their data showed that the contribution of exudates to the overall iron reduction ranged from 15 to 45%, in agreement with the present results. Finally, the 'exudates' contribution to iron reduction seemed to be iron source dependent only at pH = 7.5 and in particular when using the Fe-heme formulation, suggesting a possible stimulation of phenols exudation by roots by this iron source [19]. Fourcroy et al. [41] showed that iron deficiency upregulates the expression of genes involved in the synthesis and secretion of phenolic compounds belonging to the coumarins family in Arabidopsis roots, improving plant iron nutrition. In the same species, Sisò-Terraza et al. [42] found that the secretion of phenolics was more intense at pH = 7.5 than at 5.5 and attributed to fraxetin (a catechol coumarin) the main role in mobilizing iron from Fe³⁺-oxides in soils.

It is important to note that the highest iron reduction rate observed on plants treated with EDTA/Fe³⁺ does not correspond to the best physiological performances in the recovery experiment (SPAD index, FW and DW of plant organs; see Figures 1 and 2 and Table 2). A similar observation was reported by Vasconcelos and Grusak [43] on soybeans, while Garcia-Marco et al. [33] found a direct correlation between FCR activity and iron chelates efficacy in soybeans. Therefore, this parameter may not be appropriate in predicting the efficacy of a natural iron complex, since other physiological mechanisms of interaction between the product and roots should be considered.

5. Conclusions

The effectiveness towards Fe-chlorosis symptoms on stressed cucumber plants of a new Fe-heme formulation was evaluated, focusing on the physiological aspects involved in Fe intake by roots (root and root exudates contribution to total Fe reduction capacity). This animal blood-derived product, whose use may be of interest for organic cultivations, confirmed to be as effective as synthetic iron chelates (EDTA/Fe³⁺ and *o,o*EDDHA/Fe³⁺) to recover from iron deficiency in a mild basic environment. As a matter of fact, in these pH conditions, Fe remains bound to porphyrin organic compounds along with a broad pH interval as hemin at acid pH's or hematin at basic pH's, preventing Fe release and precipitation as amorphous Fe (hydr)oxide. Other common synthetic Fe fertilizers such as EDTA/Fe³⁺ chelate present limited stability in calcareous soil and are not recommended at pH higher than 6.5, for which Fe-heme formulations could represent an interesting alternative.

In conclusion, the positive effects of Fe-heme in alleviating Fe-deficiency symptoms (in particular in promoting vegetative growth and the re-greening effect on young leaves), together with its reduced environmental impact and lower cost for farmers, may contribute to its use as an alternative to synthetic Fe-fertilizers. With this aim, specific field experiments using similar Fe-heme formulations [44] could help to gain more insight into the biochemical and physiological mechanisms of iron supply from this class of fertilizers.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/10/1480/s1>. Table S1: SPAD values and associated standard errors from young cucumber leaves (leaves developed after the first measure at DAT -8) and mature cucumber leaves for the tested treatments. Different letters indicate significant differences among treatments in a post-hoc Duncan test; n = 6, Table S2: SPAD increments and standard errors along plant axis for nodes 1 to 6 for the tested treatments. Different letters indicate significant differences among treatments in a post-hoc Duncan test (ns: non-significant difference).

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