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Foliar application of 3-hydroxy-4-pyridinone Fe-chelate [Fe(mpp)$_3$] induces responses at the root level amending iron deficiency chlorosis in soybean

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Abstract

Iron (Fe) deficiency chlorosis (IDC) affects the growth of several crops, especially when growing in alkaline soils. The application of synthetic Fe-chelates is one of the most commonly used strategies in IDC amendment, despite their associated negative environmental impacts. In a previous work, the Fe-chelate *tris*(3-hydroxy-1-(H)-2-methyl-4-pyridinonate) iron(III) [Fe(mpp)$_3$] has shown great potential for alleviating IDC in soybean (*Glycine max*) in the early stages of plant development under hydroponic conditions. Herein, its efficacy was verified under soil conditions in soybean grown from seed to full maturity. Chlorophyll levels, plant growth, root and shoot mineral accumulation (K, Mg, Ca, Na, P, Mn, Zn Ni, and Co) and *FERRITIN* expression were accessed at V5 phenological stage. Compared to a commonly used Fe chelate, FeEDDHA, supplementation with [Fe(mpp)$_3$] led to a 29% higher relative chlorophyll content, 32% higher root biomass, 36% higher trifoliate Fe concentration and a two-fold increase in leaf *FERRITIN* gene expression. [Fe(mpp)$_3$] supplementation also resulted in increased accumulation of P, K, Zn and Co. At full maturity, the remaining plants were harvested and [Fe(mpp)$_3$] application led to a 32% seed yield increase when compared to FeEDDHA. This is the first report on the use of [Fe(mpp)$_3$] under alkaline soil conditions for IDC correction, and we show that its foliar application has a longer-lasting effect than FeEDDHA, induces efficient root responses and promotes the uptake of other nutrients.

Keyords: iron fertilizers, ferritin, *Glycine max*, metal homeostasis, seed yield

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1-Introduction

Iron (Fe) is an essential mineral for plant growth that, despite being abundant in the soil, is predominantly present in the form of insoluble oxides and hydroxides unavailable for plant uptake (Wang and Peverly 1999, Souri 2016). Particularly, in calcareous alkaline soils total dissolved Fe is below the necessary concentration for maintaining the plant optimal nutritional status. Dicotyledonous plants utilize a reduction strategy for Fe uptake (strategy I), which consists in lowering the rhizosphere's pH through proton release, and favouring the reduction of Fe(III) to Fe(II) by a root membrane-bound ferric reductase enzyme (Morrissey and Guerinot 2009). However, the activity of this enzyme is inhibited under high pH conditions, leading to the impairment of Fe uptake (Abadía et al. 2011).

The expression of the *FERRITIN* gene, which encodes the main protein responsible for Fe storage (Jiang et al. 2006, Briat et al. 2010), has been shown to have a key role in plants' ability to adapt to Fe deficiency and the deleterious effects of reactive oxygen species (ROS) (DeLaat et al. 2014) intrinsically correlated to Fe metabolism (Santos et al. 2019). Additionally, the overexpression *FERRITIN* was also correlated with improved leaf iron content (Zang et al. 2017) and iron uptake and organ distribution (Yadav et al. 2017).

Since Fe is required for several physiological processes, such as photosynthesis, chlorophyll synthesis, respiration, nitrogen (N) fixation, enzyme activation and electron transfer (Prasad 2003), Fe deficiency may disrupt these processes at different rates and levels (Souri et al. 2018, Aslani and Souri 2018, Riaz and Guerinot 2021). Currently, 30% of the world's arable land corresponds to calcareous alkaline soils, which often lead to iron deficiency chlorosis (IDC), a condition that has a negative impact on agricultural production worldwide as it leads to severe yield losses in several crops (Álvarez-Fernández et al. 2003, Mohammadipour and Souri 2019, Zargar Shooshtari et al. 2020).

In terms of plant symptoms, IDC is generally characterized by yellowing of the younger leaves (interveinal chlorosis), reduced leaf area and stunted growth, but also results in high oxidative stress (Roriz et al. 2014, Santos et al. 2015, Santos et al. 2019). To prevent or treat IDC, the most common and efficient method is the application of synthetic fertilizers, usually prepared from polyamino-carboxylate ligands, such as EDTA and EDDHA (Martens and Westermaann 1991, Souri and Hatamian 2019). These ligands, especially EDDHA, are the most frequently used in agriculture (Hernández-Apaolaza and Lucena 2001) and are usually associated with high Fe uptake efficiency in plants (Rojas et al. 2008). However, commercially available formulations usually contain around 6% of Fe chelated, split into several isomers with different efficacy levels due to differences in solubility. They have also been shown to be highly recalcitrant chelating agents with a negative impact on the environment (Ylivainio 2010). These Fe-chelates are usually applied both directly to the soil or via foliar spraying. While soil application induces root growth and root nutrient accumulation (Schenkeveld et al. 2010), foliar spraying is generally more efficient to improve the plant nutritional status, leading to increased plant growth and chlorophyll content (Mohamadipoor et al. 2013, Carrasco-Gil et al. 2016). Also, foliar

application can reduce soil contamination, increase fertilization precision and induce Fe-deficiency mechanisms at the root level (Wei et al. 2012, Fuentes et al. 2018).

Hence, the characterization of new compounds with the ability to efficiently deliver Fe to plants at lower environmental cost is a very important strategy to support more sustainable agricultural practices (Bin et al. 2016, López-Rayo et al. 2019, Souri and Bakhtiarizade 2019). Ligands of the 3-hydroxy-4-pyridinone (3,4-HPO) class have multiple applications (Burgess and Rangel 2008, Rangel et al. 2009, Moniz et al. 2011, Ferreira et al. 2018) that stem from their high affinity to divalent and trivalent cations, and their distinct physicochemical properties (Leite et al. 2011, Moniz et al. 2013a, Moniz et al. 2013b). In recent studies, these ligands were shown to be highly effective in making Fe bioavailable for uptake in hydroponically-grown soybean plants (Santos et al. 2016a, Santos et al. 2020a).

Although 3,4-HPO ligands are optimized molecules to complex with Fe, their effect under soil conditions and when applied to chlorotic plants requires further studies to attest their efficacy for agricultural application. In this study, we hypothesize that the foliar application of $[Fe(mpp)_3]$ can be used in IDC amendment, being able to improve whole-plant responses to Fe deficiency when compared to FeEDDHA. Therefore, we analysed the efficacy of $[Fe(mpp)_3]$ on several plant growth and development parameters, yield, and chlorosis symptom development in soybean plants grown under calcareous soil conditions, from seed to full maturity. We also evaluated its mode of action via the analysis of mineral accumulation modulation and *FERRITIN* gene expression analysis.

2-Materials and methods

2.1-Synthesis and characterization of Fe-chelates

The ligand Hmpp and Fe-chelate $[Fe(mpp)_3]$ were prepared in our laboratory according to published procedures (Schlindwein et al. 2006, Queiros et al. 2011). Products were characterized by elemental analyses (EA; C, H, N), 1H and 13C NMR spectroscopy, and UV-vis spectroscopy. NMR spectra were recorded with a Bruker Avance III 400 spectrometer (400.15 MHz for 1H and 100.63 MHz for 13C) at Laboratório de Análise Estrutural, Centro de Materiais da Universidade do Porto (CEMUP; Portugal). Elemental analyses were performed at the analytical services of University of Santiago (Spain). The EA results are consistent with a tetrahydrate complex with the formula $[Fe(mpp)_3]\cdot 4H_2O$. EA for $C_{18}H_{18}N_3O_6Fe.4H_2O$, % calculated (% Found): C 43.22 (43.60) H 5.24 (5.23) N 8.40 (8.25).

2.2-Soil and plant growth conditions

An artificial soil was used, which consisted of 10% peat, 20% expanded clay and 70% fine quartz sand (V/V), according to the OECD standard protocol (OECD, 1984). This type of soil is frequently utilized to analyze mineral mobilization in soils and perform ecotoxicological evaluations (Lock and Janssen 2003, Martín-Fernández et al. 2017a, Pontoni et al. 2019). The properties of an artificial soil are stable, being an advantage for reproducible assays (Sydow et al. 2017, Pontoni et al. 2019). Also, as we did not test the application of the Fe-chelates in the soil, we aimed at utilizing a substrate mimicking the natural

soil morphology but with lower reactivity. Additionally, in natural soils, the clay percentage varies from 1 to 82% and organic carbon varies from 0 to 38% (Sydow et al. 2017), and the artificial soil used fits in this range of values (Table 1).

To adjust the soil pH to the desired level to guarantee soil alkalinity (i.e. pH > 7.5), 1 g of calcium oxide (CaO) was added per 1 kg of soil. The chemical characteristics of the soil are described in Table 1. During the assay, the pH was continuously monitored and plants were watered every two days with a nutrient solution composed of: 1.2 mM KNO_3; 0.8 mM $Ca(NO_3)_2$; 0.3 mM $MgSO_4.7H_2O$; 0.2 mM $NH_4H_2PO_4$; 25 µM $CaCl_2$; 25 µM H_3BO_3; 0.5 µM $MnSO_4$; 2 µM $ZnSO_4.H_2O$; 0.5 µM $CuSO_4.H_2O$; 0.5 µM MoO_3; 0.1 µM $NiSO_4$; 0.1 g L^{-1} CaO; 0.06 g $NaHCO_3$ (López-Rayo et al. 2016, Ferreira et al. 2019). Seeds of soybean (*G. max*) cultivar 'Williams 82' were germinated for seven days in the dark, at 25ºC. Two days prior to seedling transplantation, 2-L pots filled with soil (Table 1) were irrigated until reaching full soil water hold capacity and placed on flat individual dishes. Germinated seedlings were transferred to the pots, placed in a climate chamber (Aralab fitoclima 10000EHF) with 16 h day photoperiod providing 325 µmol $s^{-1}m^{-2}$ of photosynthetic photon flux density at plant level, supplied by a mixture of incandescent bulbs and fluorescent lights. Temperatures were set to 25ºC during the light period and to 20ºC during the dark period, whereas relative humidity was maintained at 75% throughout day and night.

2.3-Foliar fertilization

The experiment was based on a completely randomized design, using three treatments and twelve replications. Individual black pots were used for plant cultivation and each pot represented a replicate. The treatments consisted of water (control – without chelate application), FeEDDHA, or $[Fe(mpp)_3]$.

Foliar applications of Fe-chelates were performed two times during the vegetative growth phase, in a concentration of 5.5 mM for both chelates, specifically after plants reaching the V3 phenological stage (three trifoliate leaves fully expanded) and V4 phenological stage (four trifoliate leaves fully expanded). All trifoliate leaves of each individual plant were sprayed in the upper leaves surface with 3 mL of each treatment: water (control – without chelate application), 5.5 mM FeEDDHA, or 5.5 mM $[Fe(mpp)_3]$.

2.4-Plant growth analysis

The number of days after planting that were needed to reach the different development stages were registered individually for each plant (n = 12) from V1 (one fully expanded trifoliate leaf) to V5 (five fully expanded trifoliate leaves, before flowering). Leaf chlorosis was also assessed from V1 to V5 with Soil and Plant Analyzer Development (SPAD) readings, measured with a portable chlorophyll meter (Konica Minolta SPAD-502Plus) using the youngest trifoliate leaf of 12 independent biological replicates.

At V5, corresponding to 49-53 days after planting depending on the Fe treatment (Figure 1), the first destructive measurement was performed. Six plants from control and Fe-chelate treatments were

separated in roots and shoots. An equal, weighted sample from each tissue per plant was collected and frozen at -80ºC for further gene expression analyses. The rest of the samples was oven-dried at 70ºC until constant weight and dry weight was obtained.

The remaining six plants were allowed to reach full maturity and dry in situ. Pods were hand-harvested at R8 phenological stage, corresponding to reproductive stage 8 when 95% or more of pods have reached mature pod color. The number of seeds per plant and the average weight of 100 seeds were registered. Seed yield per plant was obtained from three groups of plants with two plants pooled in each group and calculated as described in (Soares et al. 2019) using the in situ dried seeds.

2.5-Mineral analysis

Dried root and shoot samples, collected at V5 phenological stage, of 6 plants per treatment were analysed for minerals (Fe, K, Mg, Ca, Na, P, Mn, Zn Ni, and Co) following the procedure described by Santos et al. (2020b).

2.6-*FERRITIN* gene expression analysis

Leaf tissue of six plants collected and frozen at V5 phenological stage was individually pulverized thoroughly with a mortar and pestle until a fine powder was obtained, and total RNA was extracted using Qiagen RNeasy Mini Kit (#74904) according to the manufacturer's instructions. RNA quality and quantity were checked by UV-spectrophotometry, using a nanophotometer. Single-stranded cDNA was then synthesized using First Strand cDNA Synthesis Kit (Fermentas UAB, #K1612), according to the manufacturer's instructions. *FERRITIN* primer sequences and the ones of the reference genes *18S rRNA* and *ACTIN* were taken from Santos et al. (2020a) (Table S1). qPCR reactions were performed on a CFX96 TouchTM Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) using iQTM SYBR Green Supermix (Bio-Rad Laboratories Inc.) with the following reaction conditions: 95°C denaturation for 10 min; and 40 cycles with 15 s at 95°C, 30 s at 56°C–58°C (depending on primers used), followed by melt curve stages to check that only single products were amplified. The comparative CT method (ΔΔCT; Livak and Schmittgen 2001) was used for the relative quantification of gene expression values of Fe-related genes using the geometric mean of the expression of the two stable reference genes as controls transcripts and the plants grown with no added Fe as the reference sample. Two technical replicates were analyzed and data were transferred to Excel files and plotted as histograms of normalized fold expression of target genes.

2.7-Statistical analysis

To test for significant differences between Fe treatments, data were analyzed as a completely randomized design using one-way ANOVA with Tukey's test, in GraphPad Prism 8 for macOS (GraphPad Software).

3-Results

3.1-Plant growth and chlorosis development

The foliar application of [Fe(mpp)$_3$] or FeEDDHA did not significantly influence plant development. Independently of the Fe-chelate treatment, plants took 40 ± 3 days from planting to V5 phenological stage (Fig. 1).

Leaf chlorosis symptoms development was assessed by the reading of SPAD values, which consistently decreased during plant development until the Fe-chelates application. At V3 phenological stage (moment of the first chelate application), all plants displayed chlorosis symptoms (Fig. 2). Those symptoms were alleviated only when plants were sprayed with [Fe(mpp)$_3$] as shown by a significant increase of 14% in SPAD values from V3 to V4 phenological stage and maintained in V5. Furthermore, when compared to control plants, the [Fe(mpp)$_3$]-treated ones had 30 and 33% higher SPAD values at V4 and V5, respectively. At V5 phenological stage, the SPAD values were also 29 % higher in plants treated with [Fe(mpp)$_3$] than in FeEDDHA-treated plants ($P < 0.05$) (Fig. 2). The visual development of chlorosis at V5 is represented in Figure 3A, showing that plants supplied with [Fe(mpp)$_3$] displayed no chlorosis symptoms at the time of sample collection.

No significant differences were found between treatments in terms of shoot dry weight (Fig. 3B). However, [Fe(mpp)$_3$] application significantly increased root growth when compared to the control plants (by 47%) and to the FeEDDHA treated plants (by 32%) (Fig. 3B).

3.2-Mineral accumulation

In the root tissues, while [Fe(mpp)$_3$] led to a significant increase of about two-fold in Fe concentration, FeEDDHA-treated plants had a similar amount of Fe than control plants (Fig. 4). On the other hand, Fe accumulation in the shoots significantly increased when using both types of Fe-chelates, but to a greater extent with the application of [Fe(mpp)$_3$] (Fig. 4). Indeed, [Fe(mpp)$_3$] ameliorated Fe concentrations in the shoots by seven-fold when compared to control plants (from 23 μg/g to 149 μg/g), whereas in FeEDDHA-treated plants (that had 63 μg/g of Fe) this represented a 2.7-fold increase.

The impact of foliar fertilization with the Fe-chelates on other minerals was also analysed (Table 2), both at the root and shoot level. The application of both Fe-chelates led to a significant increase in P concentration (44% with [Fe(mpp)$_3$] and 17% with FeEDDHA) when compared to the control, but only at the root level. Regarding K concentration, plants supplemented with [Fe(mpp)$_3$] and FeEDDHA accumulated 30 and 39% more K in roots,respectively, and 13 and 12% more K in the shoots when compared to the control. The application of [Fe(mpp)$_3$] generally maintained the Mg level at the control plants level, while FeEDDHA induced a significant increase both at the root (16%) and the shoot (12%) level. Similarly to Mg accumulation, [Fe(mpp)$_3$] did not induce any change in Na tissue concentration compared to control. However, FeEDDHA treatment led to a significant decrease of Na in the roots (13%) and increased Na concentration in the shoots (40%).

Regarding the micronutrient balance, [Fe(mpp)$_3$] treatment induced a significant decrease in Mn accumulation both at the root (42%) and at the shoot (40%) level compared to the control, while FeEDDHA supplementation only impacted the shoots, inducing a 38% decrease in Mn compared to the control (Table 2). Also, both [Fe(mpp)$_3$] and FeEDDHA induced a similar increase in total Zn content, accumulating a total of 30 and 31 µg, respectively, compared to the control (4 µg). However, while [Fe(mpp)$_3$] -treated plants displayed a balanced distribution of Zn between roots and shoots, FeEDDHA-treated plants accumulated 88% of their Zn pool in the root tissues (Table 2). Additionally, [Fe(mpp)$_3$] treatment also significantly increased the Co level in the shoots by 16% when compared to the control and 46% when compared to the FeEDDHA treatment.

To understand if the Fe-chelates would have an impact on heavy metal accumulation, the concentration of Cu, Al and Pb was also analysed, but their levels were below the detection limit (*data not shown*).

3.3-Leaf *FERRITIN* expression

The expression levels of leaf *FERRITIN* were significantly increased in plants treated with both Fe-chelates compared to the control plants (Fig. 5). But also, the application of [Fe(mpp)$_3$] induced a significantly higher increase in the expression of this gene (two-fold increase) than FeEDDHA treatment, which resulted in 1.25-fold increase compared to the control plants.

3.4-Seed yield

A significant increase in seed yield due to Fe-chelates treatment was observed (Fig. 6). Seed yield improvement was highest after [Fe(mpp)$_3$] treatment with an increase of 87% when compared to the control plants and of 32% when compared to FeEDDHA treated plants. FeEDDHA treatment also induced a significant increase in seed yield (41%) compared to the control plants, but this effect was less pronounced than that obtained for [Fe(mpp)$_3$].

Concerning the yield components, [Fe(mpp)$_3$] application resulted in a significant stimulation on the total number of seeds and in average seed mass (dry weight) compared to the control, whilst FeEDDHA treatment only had a significant positive impact on total number of seeds (*data not shown*).

4-Discussion

Since Fe has low mobility within plant tissues, the first deficiency symptoms are manifested in the younger trifoliate leaves (Marschner et al. 1996). Previous studies have determined that Fe fertilization should be performed during the vegetative stage for its efficient utilization (Schenkeveld et al. 2010, Martín-Fernández et al. 2017a). To understand the effect of Fe-chelates application on chlorosis treatment, SPAD values were measured in each consecutive phenological stage, from V1 to V5. Leaf SPAD values assessment is a good indicator of leaf chlorophyll content and mineral status, particularly in the case of Fe, N and K analysis (Ahmadi and Souri 2019, Aghaye Noroozio et al. 2019). Chlorophyll levels were lowest at the V3 leaf stage (between 35 to 40 days after planting depending on the Fe

treatment, Fig. 2), which is coherent with what was described in other studies with field-grown soybean plants under Fe deficiency (Nenova 2006, Bai et al. 2018). As shown after supplementation of other Fe-chelates compounds (Bin et al. 2016, Martín-Fernández et al. 2017b, Qiu et al. 2017, Ferreira et al. 2019), the pigment accumulation in leaves increased progressively, putatively reflecting the recovery of the photosynthetic apparatus, which was impaired due to Fe deficiency.

Furthermore, the SPAD results show that [Fe(mpp)$_3$] has a longer-lasting effect than FeEDDHA, since the SPAD values were maintained high from V3 to V4, while in plants treated with FeEDDHA there was a continuous decrease in SPAD values (Fig. 2). This short-term effect of FeEDDHA was previously reported (Cieschi and Lucena 2018, Cieschi et al. 2019). The fact that [Fe(mpp)$_3$]-treated plants maintain the chlorophyll levels for a longer time can be an indicator of its efficacy and stability, which are important requisites for the sustainable use of fertilizers in agricultural practices (Nadal et al. 2012), highlighting the potential of [Fe(mpp)$_3$]. Additionally, such efficacy allows for a single application of [Fe(mpp)$_3$] to induce an efficient response to Fe deficiency, while repeated applications are reported to be necessary for foliar spraying with other Fe fertilizers, such as FeEDTA or $FeSO_4$ (Fuentes et al. 2018).

Half of the samples were collected at V5, before the initiation of the reproductive stage, to understand Fe distribution within plant parts during the vegetative stage. The visual symptoms developed by plants were coherent with the SPAD values previously registered, where [Fe(mpp)$_3$]-treated plants were greener and without any sign of leaf interveinal chlorosis (Fig. 3A), as observed in our previous studies under hydroponic conditions (Santos et al. 2016a, Santos et al. 2020a).

Regarding the tissue dry weight, under calcareous conditions, although the Fe-chelates foliar application did not induce significant differences in shoot dry weight, plants treated with [Fe(mpp)$_3$] showed increased root growth (Fig. 3B). An increased root area increases the surface dedicated to nutrient absorption and is one of the main defence responses against nutrient limitation (Lemoine et al. 2013, Santos et al. 2016b). This result was also observed after the application of HBED/Fe^{3+} in soybean (Martín-Fernández et al. 2017a). However, the impact of fertilizing agents on plant biomass accumulation varies greatly and does not always correlate with the chelating agent fertilizing efficiency. For example, soil application of Fe-nanoparticles showed a positive effect in shoot biomass, but not at the root level (Cieschi et al. 2018). Azotochelin chelating agent (Ferreira et al. 2019) or the biodegradable chelating agent [S,S]-EDDS (López-Rayo et al. 2019) did not impact biomass accumulation despite increasing SPAD values.

Both foliar Fe-chelates were able to increase Fe concentration in the shoots; however, only [Fe(mpp)$_3$]-treated plants displayed increased Fe concentration in the roots (Fig. 4). As hypothesized in our previous studies (Santos et al. 2020a), this may be due to the hydrophilic nature of the interaction between [Fe(mpp)$_3$] and the plants' membranes, which allows for higher Fe solubility and mobility within the plant. Historically, the effectiveness of foliar fertilizers has been evaluated by their ability to deliver Fe to the leaves, which could then be translocated to the roots (Rodríguez-Lucena et al. 2010). Foliar spray

has been shown to be an effective method to deliver Fe to plants, as the nutrient is more available for direct utilization in chlorophyll synthesis and photosynthesis (Rodríguez-Lucena et al. 2010, Li et al. 2017, Fuentes et al. 2018). Our results evidence that the Fe delivered by [$Fe(mpp)_3$] was redistributed and that despite the low mobility of Fe in the phloem, it was translocated to the roots. The application of FeEDDHA led to a positive impact mainly in the organs where it was directly applied to (leaves), as also reported by others (El-Jendoubi et al. 2014). However, [$Fe(mpp)_3$] supplementation impacted the whole plant, leading to increased Fe concentration in different plant tissues as well as improved growth and development even under adverse calcareous conditions. Recent studies show that Fe translocation follows a root-to-shoot-to-root route, mediated by Fe concentration in the phloem, ensuring Fe transport to the whole plant (Valentinuzzi et al. 2020). The present results indicate that 3,4-HPO ligands might facilitate Fe long-distance transport within the plants, possibly due to their hydrophilic-lipophilic balance contributing to increased Fe solubility.

Furthermore, Fe-chelate applications generally maintained a good nutritional balance and the uptake was improved for some minerals, particularly with [$Fe(mpp)_3$] supplementation (Table 2). For example, P accumulation significantly increased in the root tissue after both chelates application, but [$Fe(mpp)_3$]-treated plants accumulated 24 % more P than the ones treated with FeEDDHA ($P < 0.0001$). Like Fe, P has low solubility in calcareous agricultural soils and P status was shown to affect Fe transport and accumulation in plants, particularly impacting the Fe storage organelles and *FERRITIN* gene transcription (Hirsch et al. 2006, Bournier et al. 2013). It is, therefore, important that [$Fe(mpp)_3$] is able to improve P status due to the reported direct link between Fe and P homeostasis (Xie et al. 2019).

Potassium accumulation was also significantly increased by both Fe-chelates in the roots and the shoots compared to the control. Fe and K share important regulatory mechanisms (Forieri et al. 2017), both impacted by the calcareous soil stress and improved by the Fe-chelates treatment.

Magnesium is generally more soluble under calcareous soil conditions (Jalali et al. 2020) and, here, [$Fe(mpp)_3$] addition had a low impact on this mineral accumulation (no significant changes when compared to the control), while FeEDDHA supplementation led to a significant decrease in root Mg concentration and increase in the shoots. Sodium also decreased in the roots of plants treated with FeEDDHA. One of the reasons behind these decreased concentrations can be cation competition (Gransee and Führs 2012), which with FeEDDHA appeared to be an issue.

In regards to the Mn accumulation pattern, it was interesting to note that Mn concentration significantly decreased in the root and shoot tissues under Fe fertilization. The negative interaction between Fe and Mn is well known (Engels et al. 2012), and several studies in different crops have shown that this might be due to the competition for common uptake and translocation mechanisms, not only at physiological but also at molecular and proteomic level (Korshunova et al. 1999, Vert et al. 2002, Eroglu et al. 2016, Ceballos-Laita et al. 2018).

Additionally, similarly to the pattern obtained with Fe accumulation, both chelates induced an increase in total Zn content. However, [$Fe(mpp)_3$]-treated plants displayed an even distribution of Zn between

shoots and roots, while FeEDDHA-treated plants accumulated most of the Zn pool in the roots. A cross-talk between Fe and Zn sensing and signalling has been demonstrated under different conditions since they share several molecular transporters (Shanmugam et al. 2012, Rai et al. 2015, Li et al. 2019). The application of Zn as EDDHA or EDDS chelate increased Zn nutrition in soybean plants (López-Rayo et al. 2016). In general, the Co level varied significantly between Fe treatments. It was reported in *Agrobacterium* studies that Co competes with Fe in certain proteins c and Co level is related with Fe homeostasis (Dokpikul et al. 2016).

We also investigated the expression of *FERRITIN*, as the protein is involved in Fe buffering and homeostasis (Briat et al. 2010). We have shown, under hydroponic conditions, that $[Fe(mpp)_3]$ supplementation is associated with increased *FERRITIN* expression (Santos et al. 2016a, Santos et al. 2020a) and here, under calcareous standard soil conditions, we observed that this pattern was maintained (Fig. 5). The overexpression of *FERRITIN* in yeast cells upregulated the iron uptake machinery and iron accumulation (de Llanos et al. 2016). In wheat plants, its overexpression enhanced the tolerance to oxidative and iron stresses, ultimately leading to increased leaf Fe content (Zang et al. 2017). Additionally, studies regarding banana plants have shown the role of FERRITIN in iron uptake and organ distribution (Yadav et al. 2017). These studies support that *FERRITIN* induction is an effective mechanism for Fe stress protection, attesting the efficiency of $[Fe(mpp)_3]$ (since it led to increased *FERRITIN* expression) in delivering sufficient amounts of Fe to the whole plant, even under alkaline conditions.

Finally, the plants grown to full maturity were analysed for seed yield (Fig. 6). Coherently to what was observed at V5, the application of $[Fe(mpp)_3]$ that induced an increase of 29% in SPAD values, of 32% in root dry weight and of two-fold in both shoot and root Fe accumulation when compared to FeEDDHA, also led to increased seed dry weight per plant. Although not as efficient in improving all of the previously mentioned parameters, FeEDDHA application also induced increased seed yield compared to the control plants, but this increase was 32% lower than $[Fe(mpp)_3]$. A study performed in cumin tested Fe fertilization by foliar spraying with different Fe sources and observed similar yield percentage increases with FeEDDHA application as the ones registered in the present study (Sabet and Mortazaeinezhad 2018). Also, in rice plants, the application of a nano-chelated iron fertilizer induced a 27% increase in yield (Fakharzadeh et al. 2020).

5-Conclusion

The responsiveness of plants to foliar treatment with $[Fe(mpp)_3]$, in terms of chlorosis development, plant growth and mineral accumulation, confirmed the great potential of the designed chelate when compared to the commercial control (FeEDDHA).

The improvement in IDC development was maintained through the vegetative phenological stages, attesting the long-term effect of this environmentally friendly chelate in the IDC amelioration of plants

grown in alkaline soils. Furthermore, through the mineral accumulation patterns and *FERRITIN* gene expression, we concluded that the mechanism of action of the [$Fe(mpp)_3$] chelate impacts the whole plant, eliciting plant resistance to Fe deficiency both at the root and at the shoot level.

We also registered a significant increase in P, K, Zn and Co accumulation and a significant negative correlation between Fe and Mn concentration in the leaves, thus showing that [$Fe(mpp)_3$] promoted the nutritional balance of different minerals.

Giving that most 3,4-HPO are highly versatile and non-toxic ligands, and considering the results obtained herein, it seems pertinent to better understand the ability of this ligand to deliver different mono- or divalent metal cations to plants for its use as a multi-nutrient fertilizer. New policies towards agricultural sustainability generally aim at lowering inputs for improved soil health and GHG emissions reduction. The demonstrated efficacy of this compound rmakes it advantageous with a low environmental cost as it could be used at a lower amount than the commonly used fertilizers. Thus, fertilizer formulations based on this compound could be suitable for precision agriculture applications, nano-encapsulation and other techniques that comply with more sustainable agricultural practices, contributing to climate change mitigation and sustaining plant productivity.

Author contributions

S.M.P.C., M.W.V. and M.R. conceived and designed the experiments; C.S.S., E.R., S.F., A. L. and T.M. performed the experimental work; C.S.S., E.R. and S.F. analysed the data; C.S.S. wrote the original draft of the manuscript; S.M.P.C, M.W.V. and M.R. reviewed and edited the manuscript; M.W.V. and M.R. obtained financial support for the project leading to this publication; all authors read and approved the final manuscript.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Fig. 1. The number of days after planting to reach phenological stages from V1 to V5 in soybean plants grown in soil under alkaline conditions to induce Fe deficiency. Plants were sprayed at V3 and V4 phenological stages (marked with an arrow 'FS') using water (control), 5.5 mM FeEDDHA or 5.5 mM [$Fe(mpp)_3$]. Symbols represent the means of 12 biological replicates. Vertical bars represent Tukey's Honestly Significant Difference HSD ($P < 0.05$) among treatments for each phenological stage.

Fig. 2. SPAD values of soybean plants grown from V1 to V5 phenological stages in soil under alkaline conditions to induce Fe deficiency. Plants were sprayed at V3 and V4 phenological stages (marked with an arrow 'FS') using water (control), 5.5 mM FeEDDHA or 5.5 mM [$Fe(mpp)_3$]. Symbols represent the means of 12 biological replicates. Vertical bars represent Tukey's Honestly Significant Difference HSD ($P < 0.05$) for the effect of SPAD values for each phenological stage.

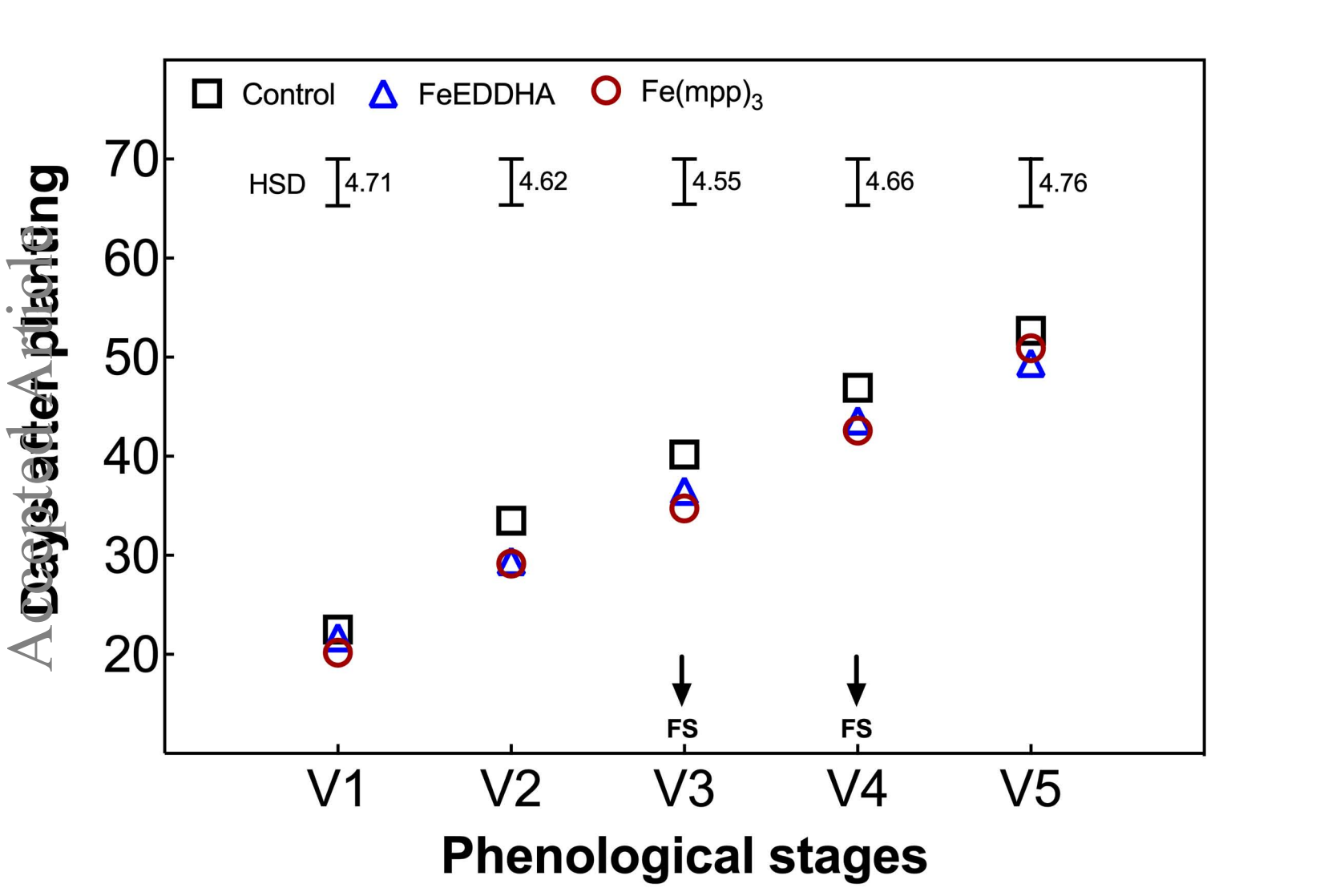
Fig. 3. Visual symptoms (A) and dry weight (B) of soybean plants grown in soil, under alkaline conditions to induce Fe deficiency, harvested at V5 phenological stage. Plants were sprayed at V3 and V4 phenological stages using water (control), 5.5 mM FeEDDHA or 5.5 mM [$Fe(mpp)_3$]. Bars represent the means of six biological replicates ± SE. Different letters represent significant differences ($P < 0.05$) by ANOVA with Tukey's test.

Fig. 4. Iron (Fe) concentration of shoots and roots of soybean plants grown in soil, under alkaline conditions to induce Fe deficiency, harvested at V5 phenological stage. Plants were sprayed at V3 and V4 phenological stages using water (control), 5.5 mM FeEDDHA or 5.5 mM [$Fe(mpp)_3$]. Bars represent

the mean of six biological replicates ± SE. Different letters represent significant differences ($P < 0.05$) by ANOVA with Tukey's test.

Fig. 5. Gene expression analysis of leaf *FERRITIN* in soybean plants grown in soil, under alkaline conditions, harvested at V5 phenological stage. Plants were sprayed at V3 and V4 phenological stages using water (control), 5.5 mM FeEDDHA or 5.5 mM [$Fe(mpp)_3$]. Bars represent the mean of six biological replicates ± SE relative to the housekeeping genes *18S rRNA* and *ACTIN*. Different letters represent significant differences ($P < 0.05$) by ANOVA with Tukey's test.

Fig. 6. Seed yield of soybean plants (grams of fresh weight per plant) grown in soil, under alkaline conditions, harvested at R8 phenological stage. Plants were sprayed at V3 and V4 phenological stages using water (control), 5.5 mM FeEDDHA or 5.5 mM [$Fe(mpp)_3$]. Bars represent the mean of three biological replicates ± SE. Different letters represent significant differences ($P < 0.05$) by ANOVA with Tukey's test.


Control
FeEDDHA
Fe(mpp)3
HSD
4.71
4.62
4.55
4.66
4.76
70
60
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Days after planting
FS
FS
V1
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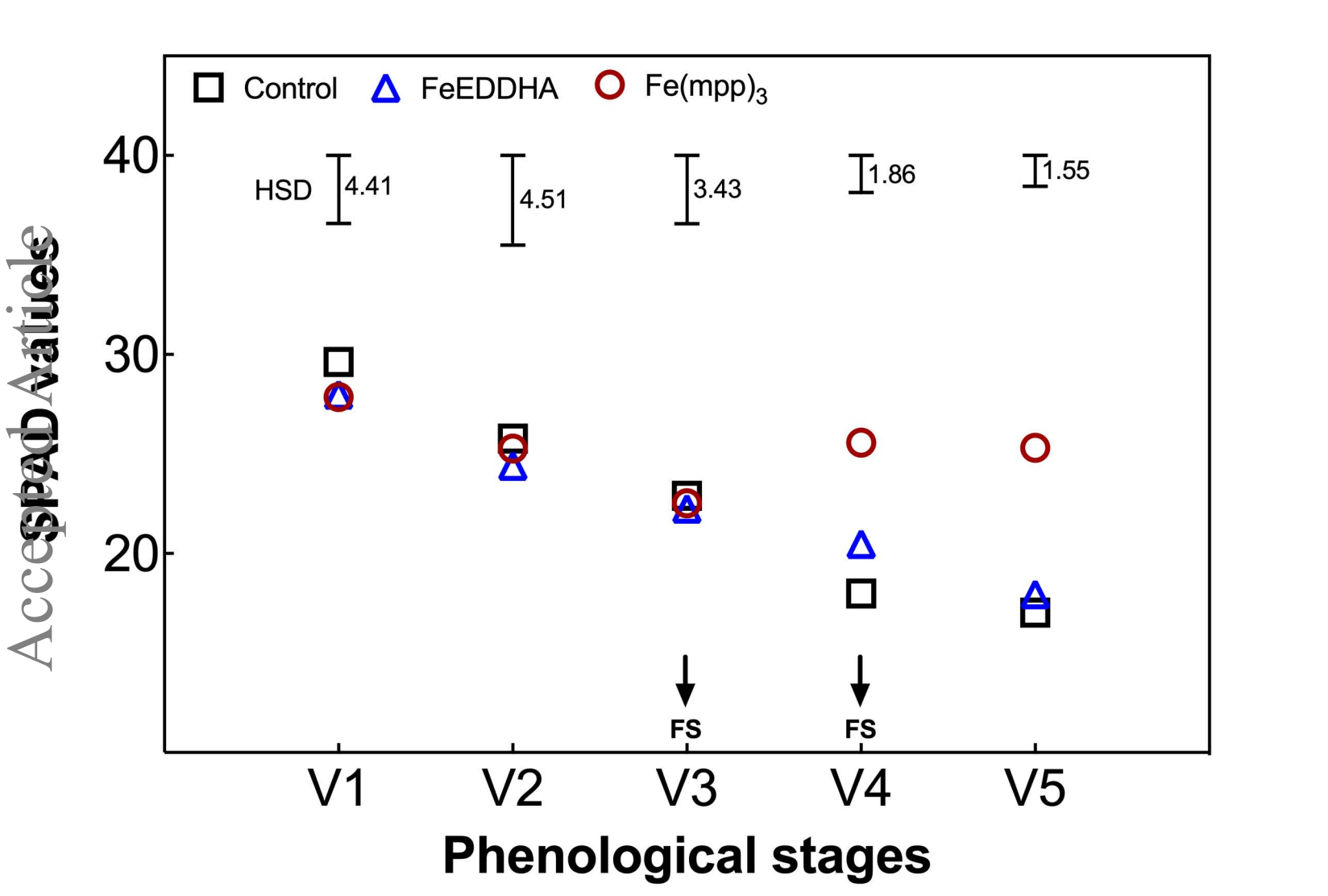
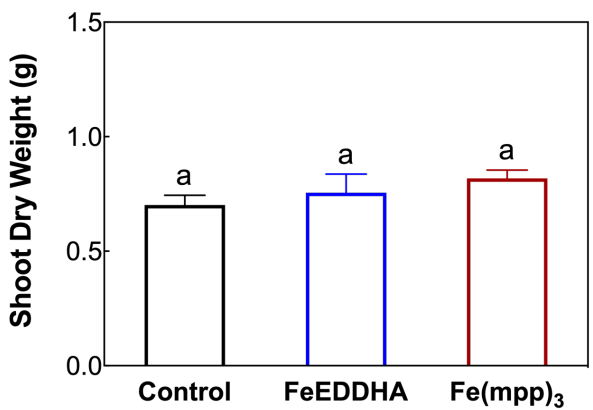
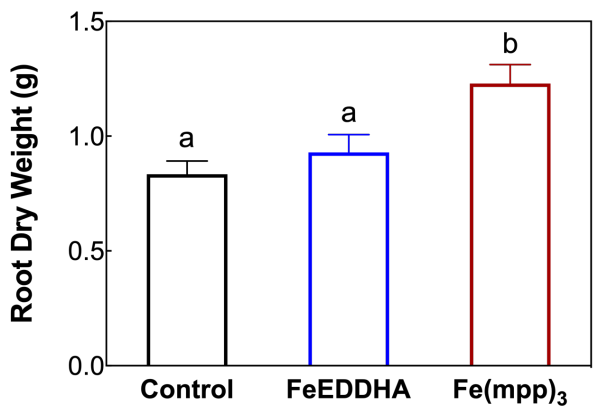
V4
V5
Phenological stages

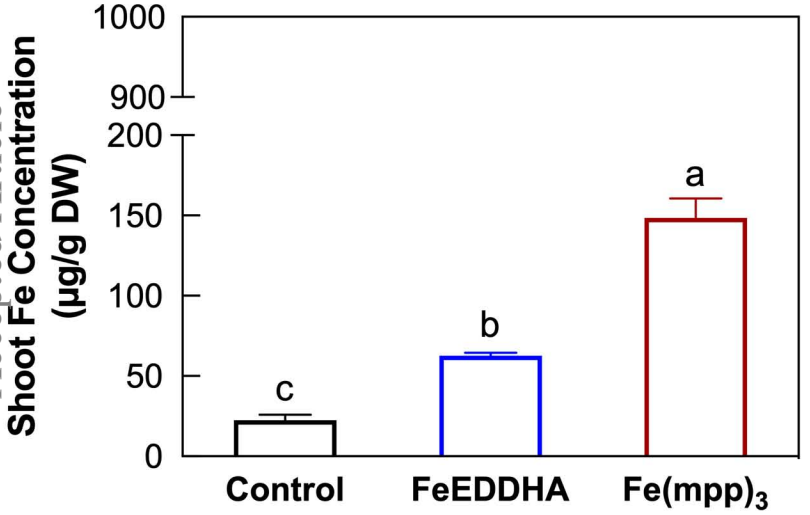


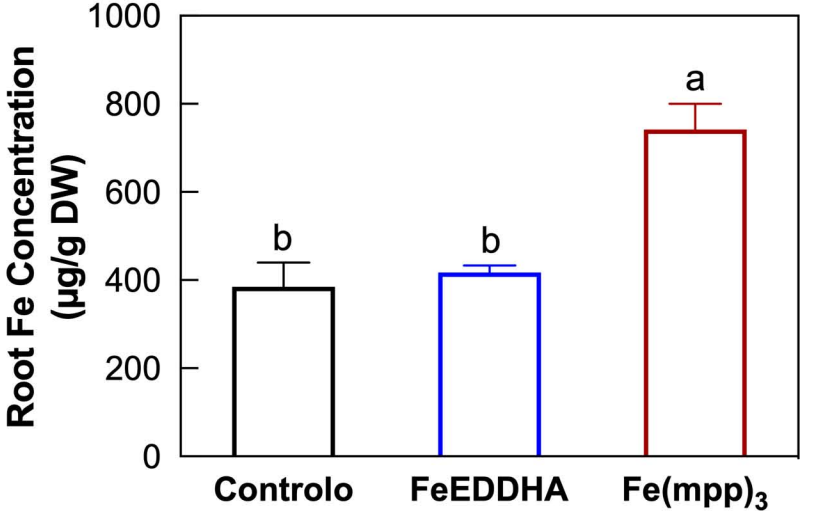
(A)

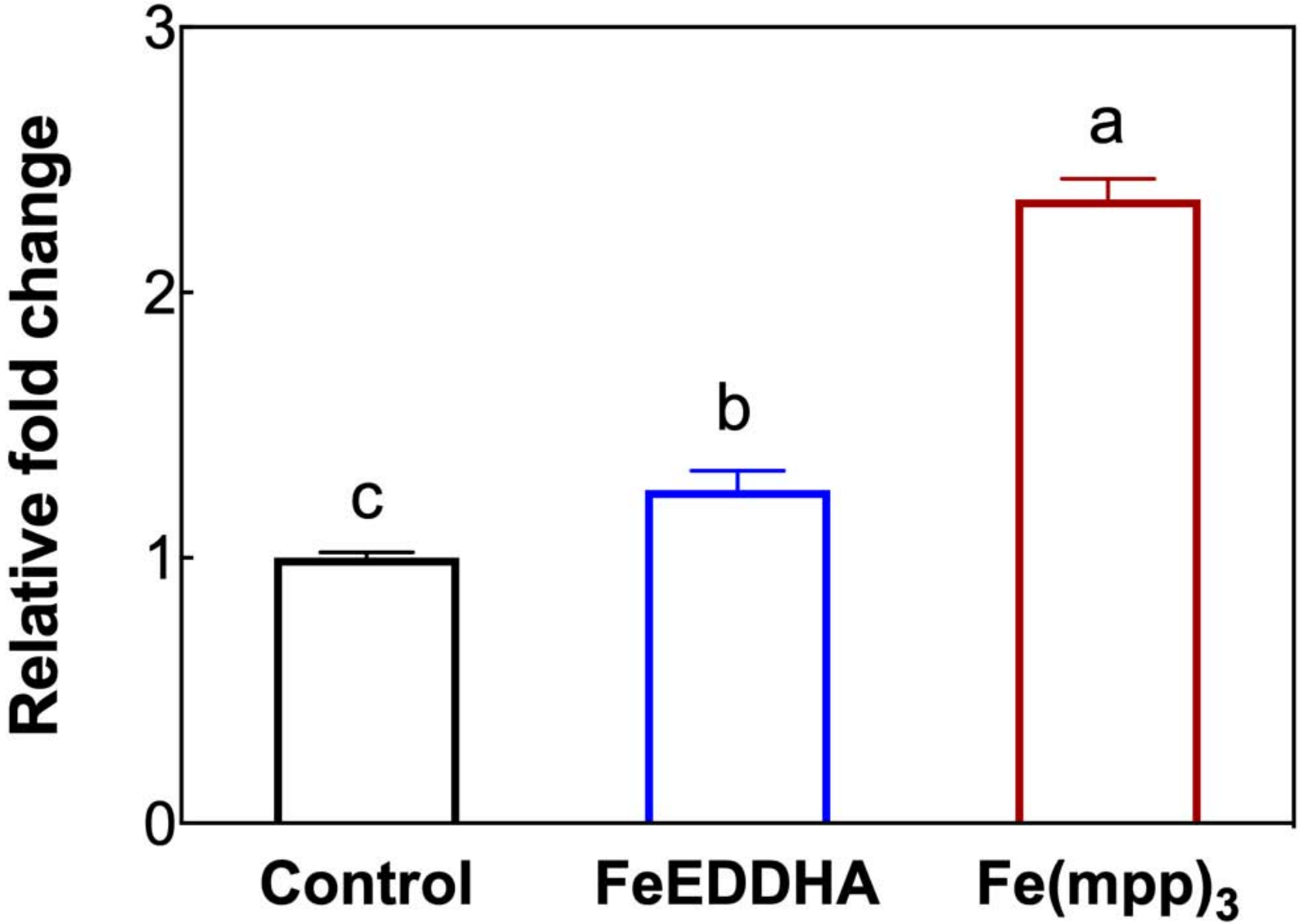
(B)











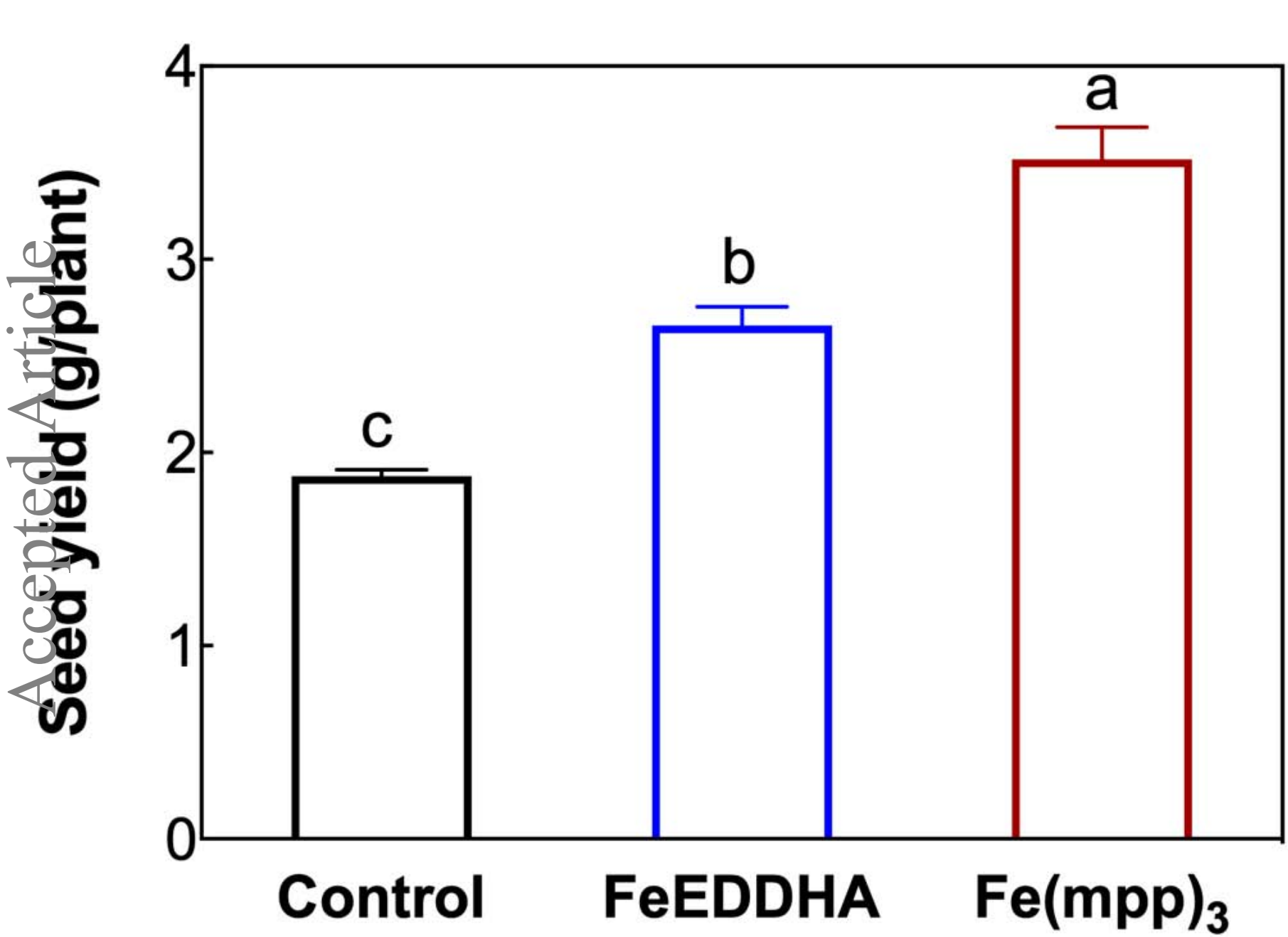


Table 1. Chemical characteristics of the soil utilized in the experiments

Parameters	
pH (H_2O)	8.4
pH (KCl)	7.5
EC (dS m^{-1})	0.078
OM (%)	0.54
N (g kg^{-1})	0.38
P (mg P_2O_5 kg^{-1})	10
K (mg K_2O kg^{-1})	18
Extractable micronutrients	
Cu (mg kg^{-1})	0.06
Zn (mg kg^{-1})	0.1
Fe (mg kg^{-1})	1.3
Mn (mg kg^{-1})	0.4

Table 2. Macro- and micromineral concentration in roots and shoots of soybean plants grown in standard artificial soil, under alkaline conditions, harvested at V5 phenological stage. Plants were treated by foliar spray with water (control) or 5.5 mM FeEDDHA or $Fe(mpp)_3$ at V3 and V4 phenological stages. Values represent means of six biological replicates ± SE. For each mineral (row), different letters, within a given plant organ, represent significant differences ($P < 0.05$) by ANOVA with Tukey's test (values in bold)

Mineral ($\mu g\ g^{-1}$)	Roots			*P*-value	Shoots			*P*-value
	Control	FeEDDHA	$Fe(mpp)_3$		Control	FeEDDHA	$Fe(mpp)_3$	
P	**1177 ± 36^{c}**	**1372 ± 134^{b}**	**1696 ± 18^{a}**	**< 0.0001**	1282 ± 47	1337 ± 76	1217 ± 70	0.0512
K	**14759 ± 2946^{b}**	**20477 ± 4871^{a}**	**19142 ± 1751^{a}**	**0.0274**	**9951 ± 556^{b}**	**11159 ± 637^{a}**	**10225± 423^{a}**	**0.0015**
Mg	**11111 ± 167^{b}**	**12922 ± 1272^{a}**	**11327 ± 92^{b}**	**0.0013**	**3775 ± 198^{b}**	**4220 ± 235^{a}**	**4056 ± 124^{ab}**	**0.0037**
Ca	7774 ± 812	7577 ± 527	8345 ± 655	0.1574	20461 ± 954	20251 ± 1062	21472 ± 106	0.0580
Na	**3313 ± 317^{ab}**	**2898 ± 812^{b}**	**3777 ± 401^{a}**	**0.0468**	**71.0 ± 7.3^{b}**	**99.1 ± 9.31^{a}**	**82.9 ± 10.0^{b}**	**0.0003**
Mn	**5.33 ± 1.11^{a}**	**4.54 ± 0.92^{ab}**	**3.11 ± 0.91^{b}**	**0.0047**	**14.8 ± 3.02^{a}**	**9.20 ± 2.11^{b}**	**8.90 ± 1.04^{b}**	**0.0004**
Zn	**4.61 ± 3.01^{c}**	**29.4 ± 10.1^{a}**	**14.1 ± 3.99^{b}**	**<0.0001**	**0.53 ± 0.13^{c}**	**4.12 ± 0.09^{b}**	**17.1 ± 0.055^{a}**	**< 0.0001**
Ni	2.27 ± 0.42	2.40 ± 0.33	2.50 ± 0.44	0.6163	0.45 ± 0.087	0.34 ± 0.19	0.36 ± 0.22	0.5258
Co	0.04 ± 0.02	0.12 ± 0.22	0.10 ± 0.25	0.0721	**0.58 ± 0.02^{b}**	**0.46 ± 0.07^{c}**	**0.67 ± 0.05^{a}**	**< 0.0001**