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RESEARCH ARTICLE

# Two Fe mining sub-products and three thiol compounds alleviate Fe deficiency in soybean (*Glycine max L.*) grown in a calcareous soil in greenhouse conditions

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**Abstract**

*Purpose* The aim of this work was to test the hypothesis that Fe mining sub-products and thiols can alleviate a moderate Fe-deficiency in the Strategy I species soybean (*Glycine max*) grown in a calcareous soil in greenhouse conditions.

*Methods* Combinations of three Fe sources [Fe(III)-EDDHA and two Fe mining sub-products, one containing Fe oxides and $FeS_2$ and the other Fe oxides], and three thiols (glutathione, dithiothreitol and thiophenol) were applied in solution to the soil, three times in a 55 day period, and different parameters related to Fe deficiency were measured. The thiol-mediated solubilization of Fe from the Fe mining sub-products was assessed by measuring in the solution total Fe and the reducible Fe pool using an Fe(II) chelator.

*Results* Application of Fe-EDDHA, the two Fe mining sub-products and the three thiols relieved the Fe deficiency symptoms to different extents, increased the Fe concentrations and contents throughout the plant and changed the redox state of leaves and roots, as judged from the changes in reduced and oxidized glutathione, ascorbate and antioxidant enzymes. When using Fe(III)-EDDHA, the addition of thiols led to a better leaf regreening. However, the addition of thiols did not cause further regreening in the case of the Fe mining sub-products, in spite of being able to solubilize Fe from them.

*Conclusion* Application of Fe-mining sub-products, thiols and the combination of Fe(III)-EDDHA and thiols could be used to alleviate moderate Fe deficiency in *G. max* grown in a calcareous soil.

**Keywords** Iron chlorosis · Iron fertilization · Iron nutrition · Iron solubilization · Thiols

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## Introduction

Iron (Fe) is very abundant in the Earth crust, but in aerated, high pH soils this metal occurs mainly in Fe(III) oxy-hydroxide forms that are not readily available for plants. Therefore, many crops growing in calcareous soils are affected by Fe deficiency and the produce yield and quality are reduced (Briat et al. 2015). To acquire Fe, dicotyledonous plants use a reduction strategy (Strategy I), that includes the

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elicitation of a root plasma membrane Fe(III) reductase enzyme (FCR) and a Fe(II) membrane transporter, as well as the secretion, depending on the plant species, of a number of substances, including protons, phenolic compounds and flavins (Abadía et al. 2011; Robe et al. 2021; Gheshlaghi et al. 2021). Graminaceous plants use a Fe chelation strategy (Strategy II), consisting in the root secretion of phytosiderophores (PS), which bind Fe in the rhizosphere, and subsequently the Fe(III)-PS complex is taken up by specific root transporters (Connorton et al. 2017).

Iron deficiency in crops is usually controlled by using synthetic Fe(III)-chelate fertilizers, which must be applied every year (El Jendoubi et al. 2011). Other common fertilization methodologies include foliar sprays with Fe compounds (Álvarez-Fernández et al. 2004; Rodríguez-Lucena et al. 2010a, b), although the transport of the added Fe from the foliage to other plant organs can be a limiting step (Rios et al. 2016). The application of Fe salts, alone or in combination with acidic substances and organic matter, is generally poorly effective in controlling Fe deficiency in crops growing in calcareous soils.

Sub-products from the Fe mining industry contain Fe in the form of different oxides and/or $FeS_2$ (Cornell and Schwertmann 2004), and this metal can be solubilized by leaching using inorganic (e.g., HCl and $SO_4H_2$) or organic acids such as oxalic acid (Blesa et al. 1987; Cornell and Schindler 1987; Panias et al. 1996; Ambikadevi and Lalithambika 2000). Compounds secreted by plants, including carboxylates, phenolics, flavins and flavonoids, can mobilize Fe from Fe oxides and oxi-hydroxides via reductive solubilization (Abadía et al. 2011; Sisó-Terraza et al. 2016; Robe et al. 2021; Gheshlaghi et al. 2021).

Thiols are produced in natural environments as a result of microbial deamination of amino acids (Kiene et al. 1990), microbial degradation of plant material (Sorensen 1988) and the reaction of dissolved sulfides with natural organic matter (Vairavamurthy and Mopper 1987). Thiols are known to be capable of reduce Fe(III) in oxides (Amirbahman et al. 1997) and clays (Morrison et al. 2013), and are used as electron shuttles by Fe(III)-reducing bacteria (Eitel and Taillefert 2017). Concentration is the main limiting factor of the electron shuttle promoting the bioavailability of Fe(III) (Yang et al. 2020).

Thiol compounds play many roles in the physiology of plants. The water-soluble antioxidant glutathione (GSH; L-g-glutamyl-L-cysteinyl-glycine) is abundant in plants and animals and has redox and regulatory functions, playing roles in ROS scavenging in the GSH-ascorbate cycle and as an electron donor for the enzyme glutathione peroxidase. Iron deficiency has been shown to cause increases in the root concentrations of GSH and ascorbate (Asc) in *Cucumis sativus* (Zaharieva et al. 1999) and *Beta vulgaris* (Zaharieva and Abadía 2003; Zaharieva et al. 2004). The synthesis of GSH in roots is regulated by nitric oxide (NO) (Matamoros et al. 1999), and it has been shown that thiol compounds play an essential role in Fe-deficiency tolerance and NO-mediated Fe-deficiency signalling in plants (Ramírez et al. 2008, 2013; Shanmugam et al. 2012; Kaya et al. 2020; Khan et al. 2021).

Iron oxides can be reductively solubilized by thiols such as GSH (Gheshlaghi et al. 2020, and references therein). It has been recently shown that when GSH was supplied to the roots of *Medicago scutellata* growing in a Fe-rich rock sand, GSH was capable of mobilize Fe from the substrate, increasing plant Fe concentrations and relieving Fe-deficiency symptoms (Gheshlaghi et al. 2020). Also, supplying GSH to the foliage of *M. scutellata* increased internal Fe availability and relieved Fe-deficiency symptoms (Gheshlaghi et al. 2019). The application of GSH to the soil to control Fe deficiency in plants has not been tested yet. However, the usefulness of this practice may be compromised by the fact that soil bacteria and fungi can consume and degrade exogenous GSH (Vergauwen et al. 2013), thus limiting to some extent the amount of GSH available for Fe reduction processes.

Soybean (*Glycine max*) is a species considered to be sensitive to Fe deficiency, and in alkaline and calcareous soils yield and seed quality are often reduced (Hansen et al. 2004; Merry et al. 2022). Soybean is a Strategy I species, and under Fe deficiency shows increases in the root FCR activity (Jolley et al. 1992), the capacity to acidify the medium (Zocchi and Cocucci 1990; Rahman et al. 2022), and the expression of the FCR -*GmFRO2*-, Fe(II) transporter -*GmIRT1*- and ATPase -*GmAHA2*- genes (Waters et al. 2018), as well as other genes in the roots (Moran Lauter et al. 2014; Santos et al. 2013, 2016). However, the decreases in the rhizospheric pH are less strong than in other plant species (Zocchi et al. 2007). Upon Fe deficiency, *G. max* secretes carboxylates (Zocchi 2006; Zocchi et al. 2007) and also

a still uncharacterized palette of phenolic compounds (Brown and Ambler 1973; Zocchi et al. 2007), but does not secrete flavins (Waters et al. 2018). *G. max* roots also contain flavonoids (d'Arcy-Lameta 1986; Graham 1991), compounds that have been found to be involved in Fe-deficiency responses in other species (Masaoka et al. 1993; Gheshlaghi et al. 2020). There are large differences between *G. max* genotypes regarding Fe uptake efficiency in calcareous soils (Lin et al. 1997; Jolley and Brown 1987; García-Mina et al. 2013; Vasconcelos and Grusak 2014; Waters et al. 2018; Raj et al. 2021). Within the plant, the concentrations of the natural chelators nicotianamine (NA) and citrate (Cit), which can bind Fe and participate in internal Fe transport, were highest in *G. max* among six species analyzed (Ariga et al. 2014). In *G. max*, the expression on NA synthase (NAS) increases under Fe deficiency (Atencio et al. 2021), and overexpression of barley NAS decreased chlorosis and increased Fe deposition in the seed (Nozoye et al. 2014; Nozoye 2018). In the *G. max* xylem, Fe was found to be transported by Cit (Tiffin 1970) and Cit-loading proteins are overexpressed in Fe-efficient cultivars compared to Fe-inefficient ones (Rogers et al. 2009; Qiu et al. 2017). Also, transgenic overexpression of *AtFRO2* resulted in an upregulation of Cit and malate (Mal) and increases in Fe concentrations (Vasconcelos et al. 2014).

The management of Fe deficiency in *G. max* usually involves the use of Fe-efficient cultivars and synthetic Fe(III) chelates, which often lead to an incomplete recovery (Wiersma 2005; Gamble et al. 2014). Application of Fe to the foliage can increase to some extent leaf Chl (Rodríguez-Lucena 2010a, b) but not always leads to yield increases (Goos and Johnson 2000; Merry et al. 2022). Many years ago, pyrite ($FeS_2$) was found to be useful for Fe deficiency management in soybean (Wallace et al. 1976, 1980), and it has been recently proposed that a pyrite-rich mine coal waste can ameliorate the nutrition of crops in calcareous soils, including *G. max* (Stander et al. 2022).

In this study, we tested the hypothesis that the application of Fe mining sub-products and thiols can increase the availability of Fe and alleviate a moderate Fe deficiency *in G. max* grown in a calcareous soil in greenhouse conditions. An Fe(III)-chelate, two different Fe mining sub-products (one containing Fe oxides and $FeS_2$ and the other Fe oxides) and three thiols, glutathione (GSH), dithiothreitol (DTT; *threo*-1,4-dimercapto-2,3-butanediol) and thiophenol (PhSH; benzenethiol) were applied to the soil, alone or in combination. Glutathione is a natural compound occurring in plants, whereas DTT and PhSH are synthetic low molecular mass reductants with sulfhydryl (-SH) groups. Parameters assessed were leaf chlorophyll levels, leaf and root biomass, plant height, leaf mineral composition, leaf and root GSH and oxidized GSH (GSSG), abscisic and gibberellic acids, Asc, Cit and Mal in leaves, and Asc peroxidase and GSH reductase (GR) in leaf extracts. Results indicate that the application of Fe sub-products and thiol compounds could be appropriate management practices to control Fe deficiency chlorosis in *G. max* grown in calcareous soils.

## Materials and methods

### Plant and soil materials and experimental design

Seeds of soybean (*Glycine max* L.; Katoul cultivar -also known as DPX 3589-, accession #1.2.3.11, from Aliabad-e Katul, Golestan Province, Iran) were obtained from the Seed and Plant Certification and Registration Institute, Karaj, Iran. Seeds were sterilized for 3 min in 1% (v/v) NaClO, washed for 20 min in sterile distilled water (removing any swollen seeds with broken seed coats), followed by three rinses in sterile distilled water.

The soil used was collected from the top layer (2–15 cm) in a non-agricultural land at the Ferdowsi University of Mashhad, Iran (Mashhad, Razavi Khorasan Province, Iran; 36° 18′ 3″ N, 59° 31′ 52″ E). The soil had a loamy texture (USDA classification system; 50% sand, 33% silt, and 17% clay). The soil characteristics and methods used are as follows: 19.3% $CaCO_3$ (Loeppert and Suarez 1996); pH 7.69 (in $H_2O$); 0.85 dS $m^{-1}$ electrical conductivity in saturated soil paste (Corwin and Rhoades 1982); and 12.21 $cmol_c$ $kg^{-1}$ soil cation exchange capacity; soil organic carbon content (Walkley-Black method; Nelson and Sommers 1996) 22 g $kg^{-1}$, and total Kjeldahl N (Bremner and Mulvaney 1982) 2 g $kg^{-1}$. Extractable P and K concentrations were 8.9 and 227 mg $g^{-1}$, respectively, and 2-{Bis[2 (bis(carboxymethyl) amino)ethyl]amino} acetic acid (DTPA)-extractable Fe was 3.5 mg Fe $kg^{-1}$. The soil was thoroughly

homogenized, air-dried, sieved (2 mm), and supplemented with urea, triple superphosphate and $KNO_3$, at rates of 80, 136.36 and 42.16 mg $kg^{-1}$ soil of N, $P_2O_5$ and $K_2O$, respectively. Each pot (17, 15.5 and 11 cm in height, and upper and lower diameters, respectively) was filled with 2.5 kg of soil. Seeds were sowed in the pots, the soil was moistened with Type I water, and after plant germination (at day 7) pots were irrigated to maximum water holding capacity with Type I water. After one week, seedlings were thinned to four per pot.

The experiment was carried out in a greenhouse at the Ferdowsi University of Mashhad. Day/night parameters were 25/18°C, 31/60% RH, and 16/8 h light/dark. The experiment was performed with a factorial, completely randomized design with three replications, using a combination of four Fe treatments and three thiol treatments. The Fe treatments used were implemented with zero Fe, Fe(III)-EDDHA (the Fe(III) chelate of ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid; thereafter called Fe-chelate) and two sub-products of the Fe mining industry: a mix of Fe oxides and a sulfur (S)-rich Fe ore. The Fe(III)-EDDHA was Helio Trace Fe(s), (Heliopotasse, Mulhouse, France; 6% EDDHA-chelated Fe, including 4.8% *o,o*EDDHA-chelated Fe). The two Fe mining subproducts used were an Iranian mine waste containing Fe oxides ($Fe_{oxi}$; 70.0% Fe, pH in water 4.07; obtained from a mine Company in Southern Iran) and an Fe ore ($Fe_{ore}$; 60.5% Fe, pH in water 4.48; obtained from Esfarayen Industrial Complex, North Khorasan, Iran), and before use they were passed through a Nº#100 sieve (150 µm mesh size). The thiol products used were reduced GSH, DTT and PhSH (Sigma-Aldrich, Taufkirchen, Germany; CAS numbers 70-18-8, 3483-12-3 and 108-98-5, respectively). Solutions were prepared at a final volume of 100 mL with no thiols, 5 mM GSH, 0.5 mM DTT or 5 mM PhSH in water. Then, 69.4 mg of Fe-EDDHA, 44.46 mg $Fe_{oxi}$ or 43.4 mg $Fe_{ore}$ were added, and the suspensions were shaken for 20 min in the dark at 15 °C. The mixtures were then slowly applied to each pot at sunset time at three different dates, 21, 28 and 35 days after sowing. Final Fe doses after the three applications were 5, 37.3 and 31.5 mg Fe $kg^{-1}$ soil for Fe(III)-EDDHA, $Fe_{oxi}$ and $Fe_{ore}$, respectively.

## Sampling, growth parameters and mineral analysis

Leaf chlorophyll was monitored 50 days after sowing with a SPAD-502 device (Minolta, Osaka, Japan). SPAD values shown are means of 24 measurements in young, fully developed leaves. Sixty days after sowing, plant height was measured ($n = 12$: all 4 plants per pot in each treatment). Plants were extracted from the soil, thoroughly washed with diluted soap, tap water and thrice with Type I water, and then divided in young (upper 3 trifoliates) and developed leaves (lower 4–5 trifoliates), stems and roots, and fresh weights (FW) were measured ($n = 9$: 3 plants in each of 3 pots in each treatment). Leaf midribs were not removed. Then, plant tissues were placed in an oven at 70 °C for 48 h to determine dry weight (DW). Dried tissues were ground to pass through a 1 mm stainless sieve and stored in plastic vials. For mineral analysis, tissues from three plants in a given treatment (two in the case of roots) in each pot were pooled. Nitric–perchloric acid digestion was carried out with standard procedures (AOAC 2000) as described in Gheshlaghi et al. (2019). The concentrations of Fe in the final solutions were determined by ICP-OES (Spectro Arcos, Spectro Analytical Instruments, Kleve, Germany), calibrating the device daily with certified standards and including standard solutions and blanks in each sample set. Six technical replications were made. Leaf 1,10-*o*-phenanthroline-extractable Fe (*extFe*) was determined using young leaves harvested 55 days after sowing (Katyal and Sharma 1984; Abadía et al. 1984). Washed leaves (1 g) were finely chopped using a Ti scissor, incubated for 24 h in 10 mL of 83 mM (1.5%) 1,10-*o*-phenanthroline in water, pH 3.0, and Fe in the filtered extracts was determined using AAS (PGI 990, PG Instruments Ltd., Lutterwoth, U.K.). To determine total S concentration, 1 g of dry tissue (pooled from 3 plants) was ashed at 600 °C in a muffle furnace, and dissolved in 10 mL of 3 M HCl. Samples were filtered through Whatman No. 42 paper, and S was determined by turbidimetry using $MgSO_4$ as a standard (Bardsley and Lancaster 1960). Three technical replications were made.

Fresh plant tissues (leaves and roots) from the remaining plant in each pot were immediately stored at -80 °C for biochemical analysis.

### Determination of leaf and root reduced glutathione and ascorbate peroxidase and glutathione reductase in leaf extracts

Reduced and oxidized GSH was measured spectrophotometrically in total foliage and root tissues with 2% metaphosphoric acid (w/v) as in Luwe et al. (1993). Since legumes contain hGSH and hGSSGh in varying amounts, the total GSH determined (tGSH) was the sum of GSH and hGSH, and the total GSSG determined (tGSSG) was the sum of GSSG and hGSSGh. Ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2) activities were measured in leaf extracts as in Nakano and Asada (1981) and Carlberg and Mannervik (1985), respectively. All these methods were described in detail in Gheshlaghi et al. (2019).

### Determination of ascorbate, gibberellic acid and abscisic acid in leaves

Ascorbate (Asc) was determined with α,α-dipyridyl (Masato 1980), as described in Gheshlaghi et al. (2019). Extraction and determination of gibberellic acid (GA3; thereafter called GA) was carried out according to Berríos et al. (2004) with some modifications. Fresh tissue from the total foliage (0.5 g) was ground in liquid $N_2$ and homogenized in 2 mL of pure methanol in a ball mill (MM 400, Retsch GmbH, Haan, Germany). The homogenate was centrifuged at 20,000 g at 4 °C for 15 min and the supernatant was collected. The sample was adjusted to pH 1–2 using 0.1 M HCl, transferred to a separatory funnel, and after adding 10 mL of ethyl acetate it was vigorously shaken for 2 min. Then, 10 mL phosphate buffer (pH 7.4) was added, shaking again for 3 min. The organic colored phase was discarded and the aqueous phase containing GA was collected. A 3 mL sample aliquot was mixed with 3 mL of 100% ethanol and 3 mL of 3.75 M HCl and stirred. After incubation at RT for 30 min, $A_{254}$ was measured spectrophotometrically (WPA S2000). Results were expressed as μg g $DW^{-1}$ using a GA calibration curve. Abscisic acid (ABA) was determined as in Kelen et al. (2004). Fresh leaf tissue (1.5 g FW from the total foliage) was ground with 60 mL of extraction solution (0.25 g of butylated hydroxytoluene and 0.44 g of Asc in 95% methanol) and the extract was maintained in the dark at 4 °C for 16 h. The extract was filtered (Whatman filter paper #42), the residue washed three times with extraction solution, and the pooled extract was concentrated using a freeze drier (FD-10 V, Pishtaz Engineering Co., Tehran, Iran) for 24 h and at -50 °C. Dried extracts were dissolved with a mixture of 0.5 M phosphate buffer (pH 8.5) and ethyl acetate (1:1; v:v). The ethyl acetate phase was filtered (Whatman No. 42 filter paper), dried at -50 °C and re-dissolved in 5 mL of 95% methanol. The mixture was vortexed and filtered (0.22 μm micro-sieve). A 20 μL extract aliquot was analyzed for ABA using a HPLC device (Waters Alliance e2695 XC with PDA detection) fitted with a $C_{18}$ column (250×4.6 mm i.d.). The mobile phase was 0.2% acetonitrile:100% methanol (50:50; v:v), and the flow rate was 0.8 mL $min^{-1}$. An ABA standard (Sigma-Aldrich) was used for quantification.

### X-ray diffraction analysis and scanning electron microscopy of $Fe_{oxi}$ and $Fe_{ore}$

The two Fe mining sub-products used (size ≤ 150 μm) and appropriate standards were subjected to X-ray diffraction analysis (XRD GNR Explorer device, G.N.R. Analytical Instruments Group, Theta/Theta XRD Explorer, Italy) with Match! software (Crystal Impact, Bonn, Germany). Analysis was made in the 2theta range of 20–80 °, with 6002 data points and alpha2 and background subtraction. The products were coated with Au for 60 s using a Sputter Coater (SCDOOS-Baltec, City, Switzerland). SEM observations were carried out on a Philips XL30 EM-EDS device with an accelerating voltage of 20 kV in the Central Laboratory of the Ferdowsi University. The products were dissolved in by HCl and $HClO_4$:HF 1:5 (Hlavay et al. 2004; Tighe et al. 2004), and total Fe determined by ICP-OES (Spectro Arcos, Spectro Analytical Instruments).

### Solubilization of $Fe_{oxi}$ and $Fe_{ore}$ by thiols

The solubilization of $Fe_{oxi}$ and $Fe_{ore}$ in the absence and presence of thiols was monitored as shown in Fig. S1. Fifty mg of each product were stirred at 2000 rpm and at 25 °C in a tube with 10 mL of assay medium (15 mM MES, pH 6.0, 300 μM BPDS) for different times (5, 10, 30, 60, 90, 110 and 120 min), and samples were centrifuged at 10,000 *x*g for 3 min. Total Fe in the supernatant (thereafter called *Total ICP-Fe*) was determined after digestion with HCl and

$HClO_4$:HF 1:5 (Hlavay et al. 2004; Tighe et al. 2004) using ICP-OES (Spectro Arcos, Spectro Analytical Instruments). The supernatant was supplemented with GSH, DTT or PhSH (to final concentrations of 5, 0.5 and 5 mM, respectively), and after 30 min samples were centrifuged again at 10,000 *x*g for 3 min. The supernatant was used to measure (i) absorbance in the range 400–700 nm (the complex Fe(II)-$BPDS_3$ has an absorption peak at 550 nm) using a spectrophotometer (UV2601, Rayleigh, Beiqing Road, Haidian District, Beijing, China), and (ii) Fe by ICP-OES (Spectro Arcos, Spectro Analytical Instruments) after acidification of the sample (3% $NO_3H$) (thereafter called *ICP-Fe*).

### Determination of leaf carboxylates

Citric (Cit) and malic (Mal) acids were determined enzymatically according to Mollering (1985, 1989), using specific kits (K-CITR 06/18 and K-LMAL-58 A/ K-LMAL-116 A 08/18; Megazyme, Wicklow, Ireland), and measuring $A_{340}$ spectrophotometrically. Results were expressed as mg Cit or Mal $g^{-1}$ FW.

### Data analysis

Data were analyzed by two-way ANOVA using SPSS 13.0 software. Significant differences among treatments were calculated using Duncan's multiple range test ($P < 0.01$). Significant differences between means for the thiol treatments in a given Fe treatment and for the Fe treatments in a given thiol treatment are indicated in the Figures using lower case and capital letters, respectively.

## Results

### Composition of the Fe sub-products used

The $Fe_{oxi}$ sub-product contained 82.2% FeO (wüstite), 2.3% $Fe_2O_3$ (hematite) and 7.9% $Fe_3O_4$ (magnetite) (Fig. S2), and the $Fe_{ore}$ one contained 36.5% $FeS_2$ (pyrite), 33.2% hematite, 14.1% magnetite, 4.9% wüstite and 4.9% $Fe_{0.4}Mg_{0.6}O$ (magnesiowüstite) (Fig. S3).

### Changes in leaf SPAD with Fe and thiol treatments

A picture of the plants at day 50 is shown in Fig. S4. The leaf chlorosis level in plants growing with no Fe in the absence of thiols was 2 (some chlorosis in the canopy; Merry et al. 2022). In the absence of thiols (grey bars, Fig. 1) all three Fe sources led to increases in the SPAD values in young leaves when compared to the control. In plants with no Fe added, the SPAD index increased with all thiols. In plants treated with Fe-chelate, the SPAD also increased with all thiols tested. However, in plants treated with $Fe_{oxi}$ and $Fe_{ore}$, the SPAD did not increase with thiols. When GSH was applied, all three Fe sources led to increases in SPAD when compared to the control. However, when DTT was applied, the Fe treatments did not lead to SPAD increases, and when PhSH was applied, the application of $Fe_{oxi}$ and $Fe_{ore}$, but not that of Fe-chelate, led to SPAD increases.

### Changes in biomass and plant height with Fe and thiol treatments

Changes observed in the shoot and root FW with Fe and thiol treatments are described in detail in the Supplementary materials file and Fig. S5.

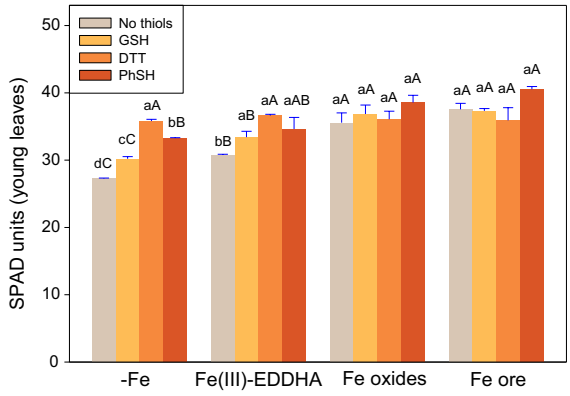


**Fig. 1** SPAD index in young leaves 50 days after sowing. Treatments were applied to the soil at days 21, 28 and 35, and consisted in 0, 5, 31.8 or 37.3 mg Fe $kg^{-1}$ soil (from Fe(III)-EDDHA, $Fe_{oxi}$ or $Fe_{ore}$, respectively), supplemented with no thiols, 5 mM GSH, 0.5 mM DTT or 5 mM PhSH. Values shown are means ± SE ($n = 24$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

When no thiols were added, the only shoot DW change was a decrease for $Fe_{oxi}$ (grey bars, Fig. 2A). In plants grown with no Fe, the DW was not changed when any of the thiols were applied. In plants treated with the Fe-chelate, the DW increased with GSH and PhSH. In plants treated with $Fe_{oxi}$ and $Fe_{ore}$, the DW decreased only with DTT and PhSH. In plants treated with GSH, DTT and PhSH, the the Fe-chelate led to increases in shoot DW when compared to the zero Fe control. Decreases in shoot DW were observed when any of the thiols were applied with $Fe_{oxi}$ and when DTT and PhSH were applied with $Fe_{ore}$.

When no thiols were added, all Fe sources led to increases in root DW (grey bars, Fig. 2B). In plants grown with no Fe, the DW increased with GSH and DTT. In plants treated with Fe-EDDHA, the DW decreased with GSH and DTT and increased with PhSH. In plants treated with $Fe_{oxi}$, the DW increased with DTT and PhSH and decreased slightly with GSH. In plants treated with $Fe_{ore}$, the root DW decreased with GSH and DTT and increased with PhSH. For any thiol treatment, all Fe sources led to increases in root DW.

When no thiols were added, all Fe sources led to large increases in plant height (grey bars, Fig. 2C). In the absence of Fe, height increased only with GSH and PhSH. In plants treated with any Fe source, height decreased with thiols. For any thiol treatment, supplementation with Fe-chelate led to increases in plant height. Other changes in height were decreases when GSH was applied with $Fe_{ore}$ and increases when DTT was applied with $Fe_{oxi}$ and $Fe_{ore}$.

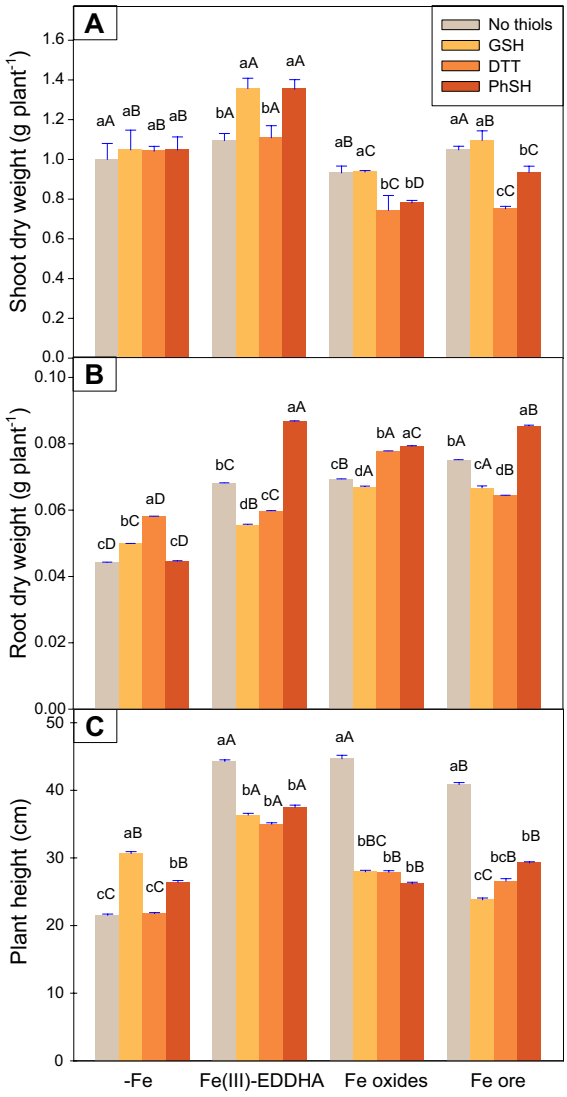


**Fig. 2** Plant biomass and height at the end of the experiment (at day 60). Treatments are as described in Fig. 1. Shoot DW (**A**), root DW (**B**) (in g plant$^{-1}$), and plant height (**C**; in cm). Values shown are means ± SE ($n = 9$ for DW and 12 for height). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

## Changes in Fe concentrations and contents in leaves, stems and roots

In the absence of thiols, the Fe-chelate and $Fe_{oxi}$ led to increases in the Fe concentrations in young leaves (grey bars, Fig. 3A) In plants grown with zero Fe, the Fe concentrations decreased with thiols. In plants treated with the three Fe sources, Fe concentrations increased with DTT, and decreased in the cases of the Fe-chelate and $Fe_{ore}$ with the other two thiols, and in the case of $Fe_{oxi}$ with PhSH. With any of the thiols, the three Fe sources led to increases in the Fe concentration when compared to the control, with the only exception of GSH with $Fe_{ore}$.

When no thiols were applied, all Fe sources led to increases in the Fe concentration in developed leaves when compared to the zero Fe control (grey bars, Fig. 3B). In plants grown with zero Fe, Fe concentrations increased with thiols. In plants treated with Fe-chelate, the Fe concentration increased with DTT and decreased with PhSH. In plants treated with $Fe_{oxi}$, leaf Fe concentrations increased with GSH and PhSH. In plants treated with $Fe_{ore}$, the Fe concentrations

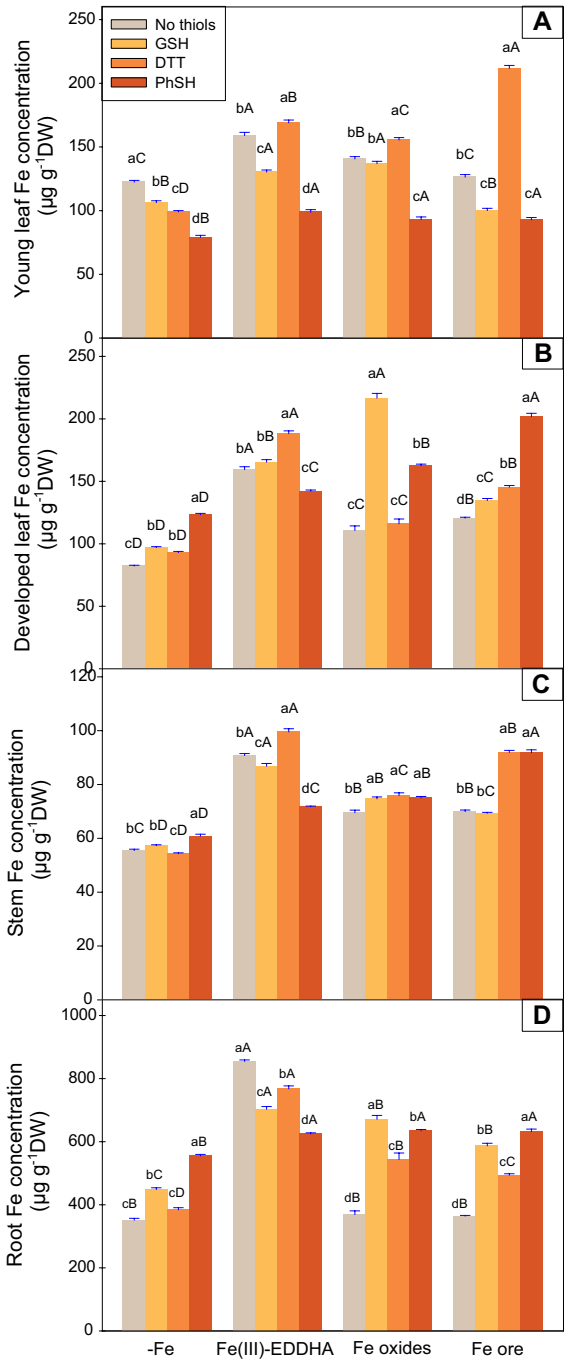


**Fig. 3** Iron concentrations at the end of the experiment (at day 60) in young leaves (**A**), developed leaves (**B**), stems (**C**) and roots (**D**) (in µg $g^{-1}$DW). Treatments are as described in Fig. 1. Values shown are means ± SE ($n = 6$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

increased with all thiols, with PhSH having the largest effect. For any thiol treatment, all Fe sources led to increases in Fe concentration when compared to the control.

When no thiols were used, all Fe sources led to increases in the Fe concentrations in stems (grey bars, Fig. 3C). In plants grown with no Fe, the Fe concentrations decreased slightly with DTT and increased with PhSH. In plants treated with the Fe-chelate, the Fe concentration increased with DTT and decreased with GSH and PhSH. In plants treated with $Fe_{oxi}$, the Fe concentration increased with all thiols, whereas in plants treated with $Fe_{ore}$ it increased with DTT and PhSH. For any thiol treatment, all Fe sources led to increases in the leaf Fe concentration when compared to the control.

In the absence of thiols, only the Fe-chelate led to increases in the Fe concentration in roots when compared to the zero Fe control (grey bars, Fig. 3D). In plants grown with no Fe, the Fe concentration only increased with GSH and PhSH. In plants treated with Fe-chelate, the Fe concentration decreased with all thiols. Conversely, in plants treated with $Fe_{oxi}$ and $Fe_{ore}$, the Fe concentration increased markedly with all thiols. When any of the thiols were applied, all Fe sources led to increases in the root Fe concentration when compared to the control.

When no thiols were applied, the only increase in leaf *extFe* (extractable Fe) was with $Fe_{oxi}$ (grey bars, Fig. S6). In plants grown with no Fe, the *extFe* increased only with GSH, whereas in plants treated with Fe-chelate it increased only with DTT and PhSH. In plants treated with $Fe_{oxi}$, the *extFe* decreased only with PhSH, and in those treated with $Fe_{ore}$ it increased with GSH and PhSH but decreased with DTT. When GSH was used, decreases and increases in *extFe* were found with the Fe-chelate and $Fe_{ore}$, respectively. When DTT was used, increases in *extFe* occurred with the Fe-chelate and $Fe_{oxi}$ and decreases with $Fe_{ore}$. When PhSH was used, increases in *extFe* were found with $Fe_{ore,}$ and decreases with $Fe_{oxi}$.

## Changes in Fe contents with Fe and thiol treatments

When no thiols were added, all Fe sources led to increases in the Fe contents in young leaves when compared to the control (grey bars, Fig. 4A). In plants grown with no Fe, the Fe contents decreased with all

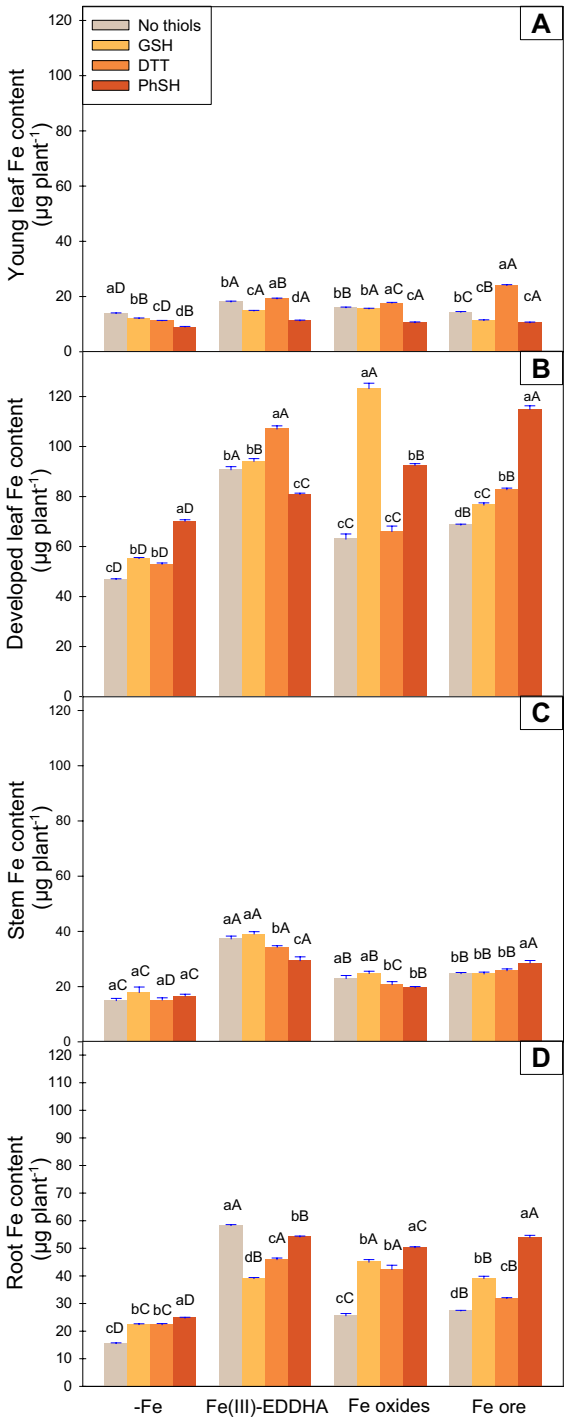


**Fig. 4** Iron contents at the end of the experiment (at day 60) in young (**A**) and developed (**B**) leaves, stems (**C**) and root (**D**), (in µg plant$^{-1}$). Treatments are as described in Fig. 1. Values shown are means ± SE ($n = 6$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

thiols. In plants treated with Fe-chelate, the Fe content increased with DTT and decreased with GSH and PhSH. In plants treated with $Fe_{oxi}$, the Fe content increased with DTT and decreased with PhSH. In plants treated with $Fe_{ore}$, the Fe content decreased with GSH and PhSH and increased with DTT. For any thiol treatment, all Fe treatments led to increases in the young leaf Fe content when compared to the control, with the only exception of $Fe_{ore}$ applied with GSH.

In the absence of thiols, all Fe sources led to increases in the Fe contents in developed leaves when compared to the control (grey bars, Fig. 4B). In plants grown with no Fe, the Fe contents increased when thiols were applied. In plants treated with the Fe-chelate, the Fe content showed increases and decreases with DTT and PhSH. In plants treated with $Fe_{oxi}$, the Fe contents increased with GSH and PhSH. In plants treated with the $Fe_{ore}$, the Fe contents increased with all thiols. With the three thiols increases in developed leaf Fe contents were found when using all three Fe sources.

When no thiols were used, all Fe sources led to increases in the Fe contents in stems when compared to the control (grey bars, Fig. 4C). In plants grown with no Fe, the Fe contents did not change with thiols. In plants treated with Fe-chelate and $Fe_{oxi}$, the Fe content decreased with DTT and PhSH, and in plants treated with $Fe_{ore}$ it increased only with PhSH. For any thiol treatment, all Fe sources led to increases in stem Fe contents when compared to the control.

When no thiols were added, all Fe sources led to increases in the Fe contents in roots when compared to the control (grey bars, Fig. 4D). In plants grown with no Fe, $Fe_{oxi}$ and $Fe_{ore}$, the Fe content increased with all thiols. In plants treated with the Fe-chelate, the Fe content decreased with all thiols. With any thiol treatment, all Fe treatments led to increases in the root Fe contents when compared to the control.

## Changes in S concentrations and contents with Fe and thiol treatments

Changes observed in the S concentrations and contents in leaves and roots with Fe and thiol treatments are described in detail in the Supplementary materials file and Fig. S7. Major changes observed included a

decrease in the root S concentrations with Fe-chelate, and a marked increase in S levels with GSH.

Changes in antioxidant compounds with Fe and thiol treatments

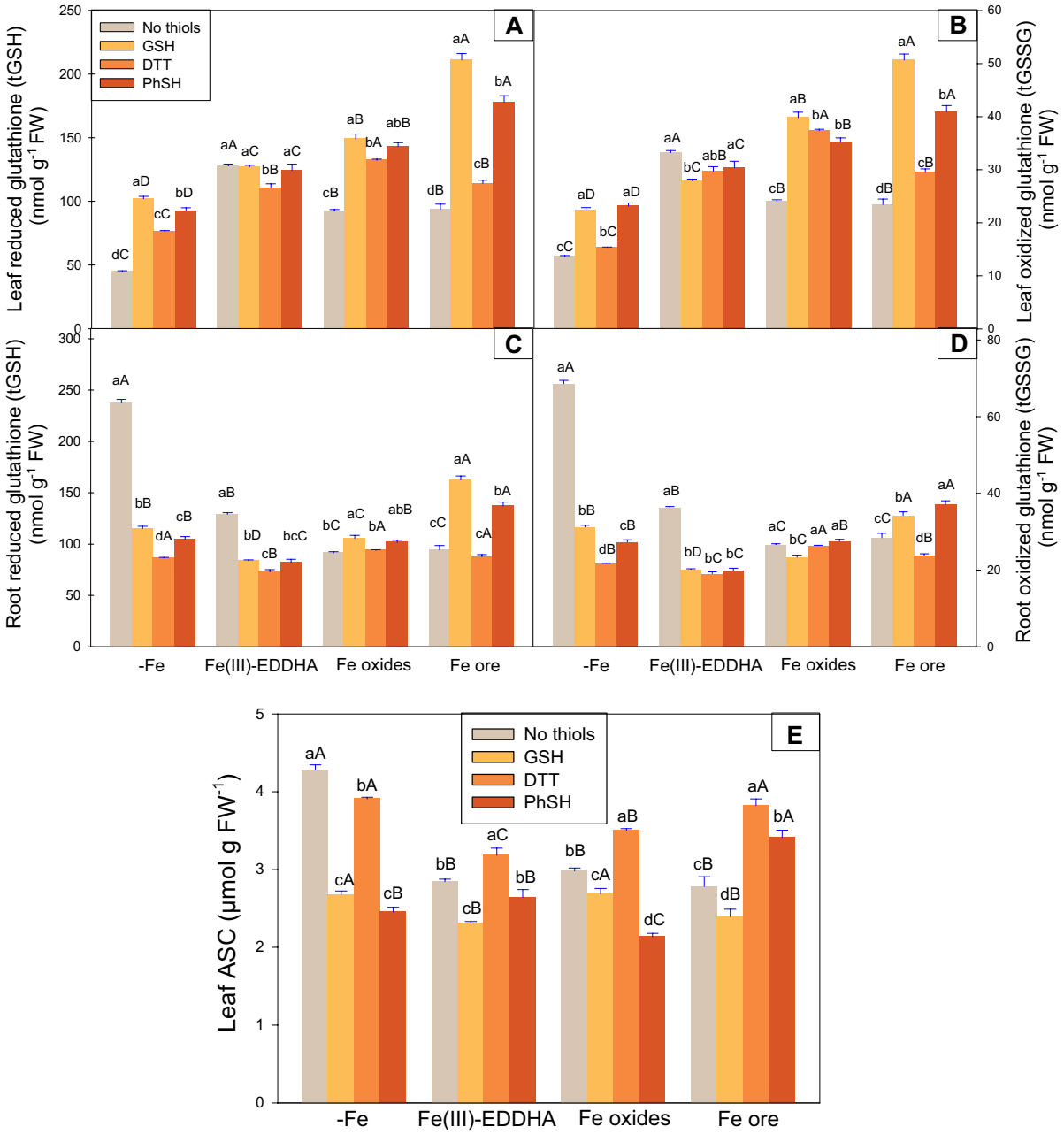


**Fig. 5** Antioxidant compounds at the end of the experiment (at day 60) (in nmol $g^{-1}$ FW). Treatments are as described in Fig. 1. Reduced glutathione (tGSH; in nmol $g^{-1}$ FW) in leaves (**A**) and roots (**C**), oxidized glutathione (tGSSG; in nmol $g^{-1}$ FW) in leaves (**B**) and roots (**D**) and ascorbate (Asc; µmol $g^{-1}$ FW) in leaves (**E**). Values shown are means ± SE ($n = 3$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

When no thiols were applied, all three Fe sources led to increases in the leaf tGSH concentrations when compared to the control (grey bars, Fig. 5A). In plants grown with no Fe, $Fe_{oxi}$ and $Fe_{ore}$, the tGSH concentrations increased with all thiols. In plants treated with Fe-chelate, the tGSH decreased with DTT. With any thiol treatment, all three Fe sources led to leaf tGSH increases.

In the absence of thiols, all three Fe sources led to increases in the leaf tGSSG concentrations when compared to the control (grey bars, Fig. 5B). In plants grown with no Fe, $Fe_{oxi}$ and $Fe_{ore}$, the tGSSG concentrations increased with all thiols, whereas in plants treated with the Fe-chelate they decreased with GSH. With any thiol treatment, all three Fe sources led to increases in leaf tGSSG when compared to the control.

When no thiols were added, all three Fe sources led to decreases in leaf Asc concentrations when compared to the control (grey bars, Fig. 5E). In plants grown with no Fe, the Asc concentrations decreased with DTT, and much more markedly with GSH and PhSH (Fig. 5E). In plants treated with Fe-chelate, the Asc concentrations decreased with GSH and increased with DTT. In plants treated with $Fe_{oxi}$, the Asc concentrations decreased with GSH and PhSH and increased with DTT. In plants treated with $Fe_{ore}$, the Asc concentrations decreased with GSH and increased with DTT and PhSH. When GSH was added, the Fe-chelate and $Fe_{ore}$ led to decreases in Asc concentrations when compared to the control. When DTT was added, the Fe-chelate and $Fe_{oxi}$ led to decreases in the Asc concentrations, and when PhSH was added, $Fe_{oxi}$ and $Fe_{ore}$ led to decreases and marked increases in leaf Asc concentrations, respectively.

When no thiols were added, all Fe sources led to marked decreases in the root tGSH concentrations when compared to the control (grey bars, Fig. 5C). In plants grown with no Fe and the Fe-chelate, tGSH decreased with all thiols. In plants treated with $Fe_{oxi}$, tGSH increased only with GSH, whereas in plants treated with $Fe_{ore}$ it increased with GSH and PhSH. When any of the thiols were applied, the Fe-chelate led to decreases in tGSH. Decreases in root tGSH were also found with GSH and $Fe_{oxi}$, and increases with GSH and PhSH and $Fe_{ore}$.

In the absence of thiols, all three Fe sources led to marked decreases in root tGSSG concentrations when compared to the control (grey bars, Fig. 5D). In plants grown with no Fe and the Fe-chelate, tGSSG decreased with all thiols. In plants treated with $Fe_{oxi}$, tGSSG decreased with GSH, whereas in plants treated with $Fe_{ore}$ it increased with GSH and PhSH and decreased with DTT. When any thiol was used, the Fe-chelate led to decreases in tGSSG when compared to the control. Other changes in root tGSSG included decreases when GSH was used with $Fe_{oxi}$, and increases when DTT was applied with $Fe_{oxi}$ and when PhSH was used with $Fe_{ore}$.

## Changes in antioxidant enzyme activities with Fe and thiol treatments

When no thiols were added, all Fe sources led to large increases in the GR activity in leaf extracts when compared to the control (grey bars, Fig. 6A). In plants grown with no Fe, the GR activity increased with the three thiols. In plants treated with Fe-chelate, the GR activity was not changed with any of the thiols. In plants treated with $Fe_{oxi}$ and $Fe_{ore}$, the GR activity increased with GSH and decreased with DTT. When GSH was used, $Fe_{oxi}$ and $Fe_{ore}$ led to minor increases in the GR activity, and when DTT was added, the Fe-chelate and $Fe_{oxi}$ led to increases in this parameter. Finally, when PhSH was added, $Fe_{oxi}$ and $Fe_{ore}$ led to small increases in the GR activity when compared to the control.

In the absence of thiols, all Fe sources led to increases in the APX activity in leaf extracts when compared to the controls (grey bars, Fig. 6B). In plants grown with no Fe, the APX activity increased with GSH and PhSH. In plants treated with Fe-chelate, the APX activity increased and decreased with GSH and DTT. In plants treated with $Fe_{oxi}$, the APX activity also decreased with DTT and PhSH. In plants treated with $Fe_{ore}$, the APX activity increased with GSH and decreased with DTT and PhSH. With GSH, the Fe-chelate and $Fe_{ore}$, but not $Fe_{oxi}$, led to increases in APX activity when compared to the control. When DTT was used, the Fe-chelate led to increases in APX activity, whereas $Fe_{oxi}$ and $Fe_{ore}$ led to decreases in this parameter. When PhSH was added, all three

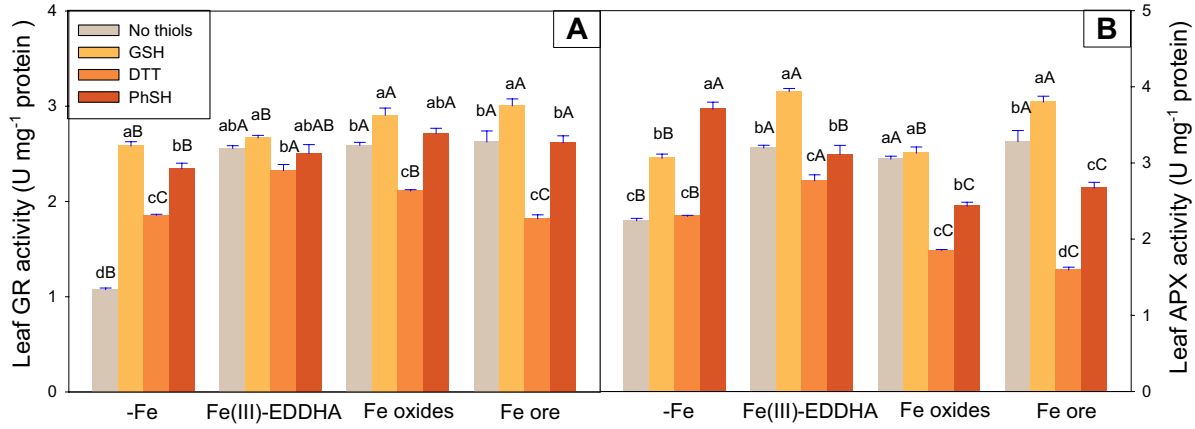


**Fig. 6** Antioxidant enzymes in leaves at the end of the experiment (at day 60). Treatments are as described in Fig. 1. Glutathione reductase (GR; A, in U $mg^{-1}$ protein) and ascorbate peroxidase activity (APX; B, in U $mg^{-1}$ protein). Values shown are means ± SE ($n = 3$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

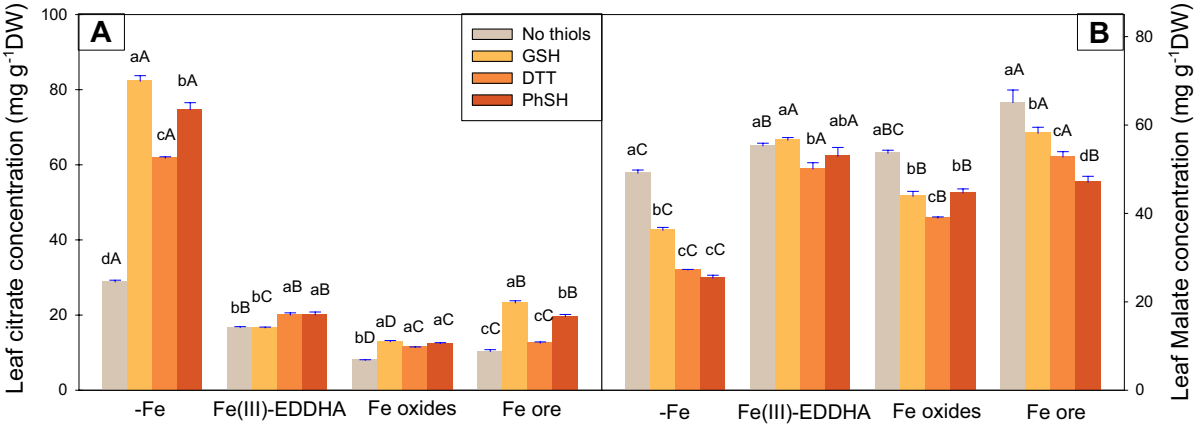


**Fig. 7** Leaf carboxylates at the end of the experiment (at day 60). Treatments are as described in Fig. 1. Leaf citrate (**A**) and malate (**B**) (in mg $g^{-1}$ DW). Values shown are means ± SE ($n = 3$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

Fe sources led to decreases in the leaf APX activity when compared to the control.

## Changes in carboxylates with Fe and thiol treatments

In plants grown in the absence of thiols, all Fe sources led to marked decreases in the leaf Cit concentration when compared to the control (grey bars, Fig. 7A). In plants grown with no Fe, the Cit concentration increased markedly with all thiols. In plants treated with the Fe-chelate, the Cit concentration increased with DTT and PhSH. In plants treated with $Fe_{oxi}$ the Cit concentration increased with the three thiols, whereas in plants treated with $Fe_{ore}$ it increased markedly with GSH and PhSH. With all thiols the three Fe sources led to marked decreases in leaf Cit when compared to the control.

When no thiols were added, all Fe products led to increases in leaf Mal concentrations (grey bars, Fig. 7B). In plants grown with no Fe, the Mal concentrations decreased when any of the thiols were added. In plants treated with Fe-chelate, the Mal

concentration decreased only with DTT. In plants treated with $Fe_{oxi}$ and $Fe_{ore}$, the Mal concentration also decreased with all thiols. With all the thiol treatments the three Fe sources led to increases in the leaf Mal concentrations when compared to the control.

## Changes in phytohormones with Fe and thiol treatments

When no thiols were added, all three Fe sources led to major increases in the leaf GA concentration when compared to the control (grey bars, Fig. 8A). In plants grown with no Fe, the GA concentrations increased markedly with GSH and PhSH. In plants treated with any of the three Fe sources the GA concentration decreased by half when any thiol was applied. When DTT was used, all three Fe sources led to major increases in the GA concentration when compared to the control. When GSH was added, the Fe-chelate and $Fe_{ore}$ led to increases and decreases in the GA concentration, respectively, and when PhSH was added the Fe-chelate and $Fe_{ore}$ led to increases in the leaf GA concentration.

In the absence of thiols all three Fe sources led to decreases in the leaf ABA concentrations when compared to the control (grey bars, Fig. 8B). In plants grown with no Fe, the ABA concentration decreased by half with GSH and PhSH and much less with DTT. In plants treated with any Fe source the ABA concentration did not change with thiols, with the exception of the $Fe_{ore}$ with PhSH, where there was a decrease. In plants treated with GSH, 2-fold increases in ABA concentrations were found with all Fe sources. When DTT was added, $Fe_{oxi}$ and $Fe_{ore}$ led to small decreases in the leaf ABA concentration, and when PhSH was added, all three Fe sources led to major increases in the leaf ABA concentration when compared to the control.

## Changes in DTPA-extractable soil Fe with Fe and thiol treatments

Changes observed in the DTPA-extractable soil Fe with Fe and thiol treatments are described in detail in the Supplementary materials file and Fig. S8. Some of the thiols increased markedly the DTPA-extractable soil Fe with some of the Fe sources.

## Solubilization of Fe oxides by thiols

Samples were homogenized in 15 mM MES, pH 6.0, supplemented with 300 µM BPDS, in the absence of thiols. After centrifugation to remove the bulk of the products, the *Total ICP-Fe* in the slightly opalescent solution was 8.9 and 175.6 mg Fe $L^{-1}$ in the $Fe_{oxi}$ and $Fe_{ore}$, respectively. When Fe was measured by ICP without a strong acid digestion (*ICP-Fe*), the Fe concentration in the solution was two orders of magnitude lower with $Fe_{oxi}$ than with $Fe_{ore}$ (0.1 and 9.3 mg

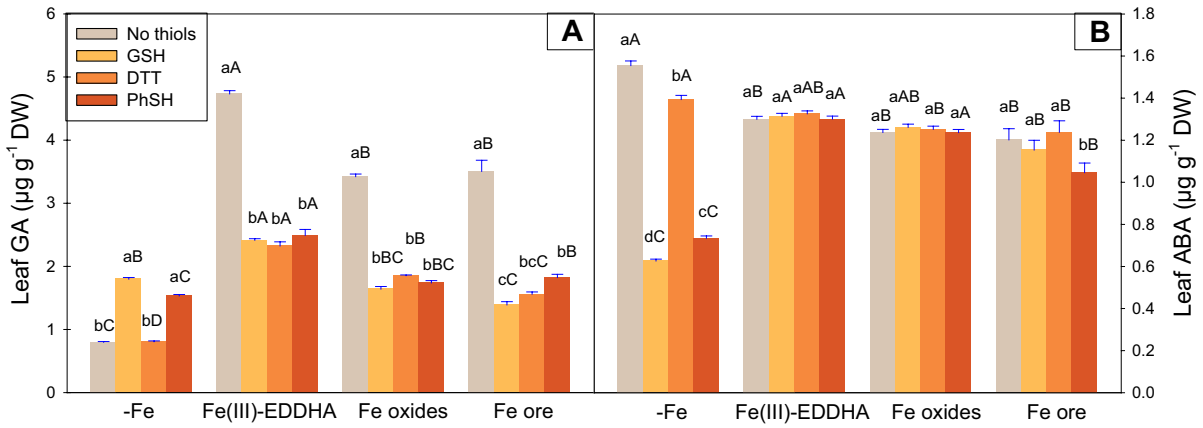


**Fig. 8** Gibberellic and abscisic acid in leaves at the end of the experiment (at day 60). Treatments are as described in Fig. 1. Gibbelleric acid (GA, in µg $g^{-1}$ DW) (**A**) and abscisic acid (ABA; in µg $g^{-1}$ DW) (**B**). Values shown are means ± SE ($n = 3$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

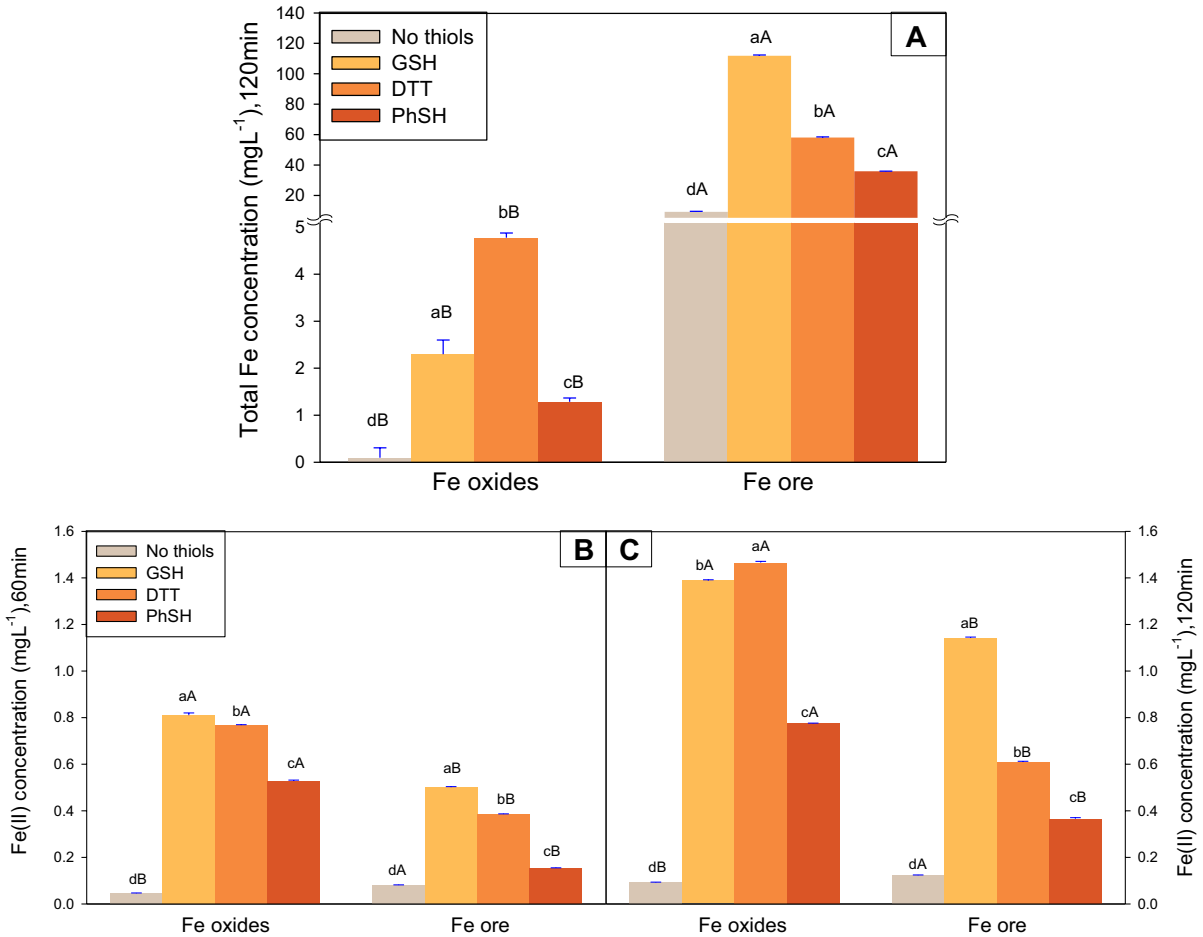


**Fig. 9** Iron measured by ICP-OES (without acid digestion; *ICP-Fe*) and Fe(II) in the solution after incubation of $Fe_{oxi}$ and $Fe_{ore}$ with BPDS (in mg $L^{-1}$). *Total ICP-Fe* (after acid digestion) in the solution was 8.9 and 175.6 mg Fe $L^{-1}$ with the $Fe_{oxi}$ and $Fe_{ore}$, respectively. *ICP-Fe* after 120 min (**A**), and Fe(II) measured spectrophotometrically after 60 (**B**) and 120 min (**C**). Fifty mg of product was stirred with 10 mL of buffer in 300 µM BPDS for different times. Values shown are means ± SE ($n = 3$). Letters above the columns indicate significant differences at $p \leq 0.01$ for the thiol treatments in a given Fe treatment (in lower case) and for the Fe treatments in a given thiol treatment (in capitals)

$L^{-1}$, respectively; grey bars, Fig. 9A). In the absence of thiols there was a measurable Fe(II) chelation by BPDS in both products, being larger at 120 than at 60 min (grey bars, Fig. 9B, C).

The addition of thiols led to large increases in the *ICP-Fe* in solution (measured after acidification), with the increases being higher for DTT in the case of $Fe_{oxi}$ and for GSH in the case of $Fe_{ore}$ (Fig. 9A). Thiols also led to large increases in the Fe(II) chelated by BPDS, increasing further with incubation time (Fig. 9B, C, and Fig. S9). The Fe(II) values were higher for $Fe_{oxi}$ than for $Fe_{ore}$ for all thiols at all incubation times. The highest and lowest Fe(II) values were for GSH and PhSH, respectively, both when using $Fe_{oxi}$ and $Fe_{ore}$.

In the absence of thiols, the *ICP-Fe* accounted for approximately 1% and 5% of the *Total ICP-Fe* (for $Fe_{oxi}$ and $Fe_{ore}$, respectively), whereas in the presence of thiols the *ICP-Fe* accounted for ca. 53% and 64% of the Total ICP-Fe (for $Fe_{oxi}$ and $Fe_{ore}$, respectively). The Fe chelated by BPDS constituted a larger fraction of the *ICP-Fe* in the case of $Fe_{oxi}$ (ca. 1%, 16%, 16% and 9% in the cases of the no thiol treatment, GSH, DTT and PhSH, respectively) than in the case of $Fe_{ore}$ (always < 1%).

## Discussion

### Effects of Fe deficiency in *G. max*

When *G. max* plants were grown with no Fe added, they showed symptoms of a moderate Fe-deficiency. Plants were small, young leaves were light green and root biomass was low. These moderate symptoms in plants grown in a 19% $CaCO_3$ soil indicate that the *G. max* cultivar used is an Fe-efficient (Fe-deficiency tolerant) one. Iron concentrations in developed leaves (82 μg $g^{-1}$ DW) were above the low Fe range for *G. max* (51 μg $g^{-1}$ DW; Benton Jones et al. 1991), and the Fe concentration in young leaves was even higher. Leaf chlorosis in the presence of relatively high leaf Fe concentrations (the "Fe-chlorosis paradox"), have been observed previously in *G.max* (Santos et al. 2015, 2019; Chen et al. 2020a, b). Leaves of Fe-deficient plants did not appear to show a strong oxidative stress, since the tGSH and tGSSG levels and the APX and GR activities were relatively low, in agreement with the moderate chlorosis observed. However, the root concentrations of tGSH and tGSSG were quite high, in line with the large decrease in root biomass. On the other hand, the leaf Asc, Cit and ABA levels were higher, and GA levels were lower, than those in the plants grown with the Fe-chelate.

When Fe deficiency is severe, oxidative stress in leaves and roots occurs in many plant species (M'sehli et al. 2009; López-Millán et al. 2013; Gheshlaghi et al. 2019; Rahman et al. 2022). However, when the Fe deficiency is moderate the oxidative stress is less intense, and for instance lower APX levels occur in Fe-efficient *G. max* cultivars compared to Fe-inefficient ones (Santos et al. 2019; Mira et al. 2021). Also, in *M. sativa* APX was lower in Fe-deficient plants than in Fe-sufficient ones (Rahman et al. 2021). Citrate concentrations usually increase throughout the plant with Fe deficiency (Abadía et al. 2002), and for instance in Fe-deficient *M. scutellata* the leaf Cit (and Asc) levels were higher than in Fe-sufficient controls (Gheshlaghi et al. 2019). However, in strongly Fe-deficient *G. max* leaves decreases in Cit and Mal were observed in a metabolomics study (Lima et al. 2014). On the other hand, the low and high leaf GA and ABA levels, respectively, confirm previous results in *G. max* (Chen et al. 2020a, b), and are likely associated with the decreases in plant size and biomass. In *Oryza sativa*, Fe deficiency has also been shown to decrease biologically active GA in leaves (Wang et al. 2017).

### Effects of applying Fe-EDDHA alone

The Fe-chelate at a 5 mg Fe $kg^{-1}$ soil dose was partially efficient in alleviating Fe-deficiency symptoms in *G. max*, leading to modest increases in SPAD and shoot biomass. A heatmap summarizing all changes found is included in Fig. 10. The increases were more marked in root biomass, and especially in plant height, Fe concentrations and contents in leaves, stems and roots, with little change in leaf *extFe*. These positive but limited effects are in line with previous results with Fe(III)-EDDHA in *G. max* (Wiersma 2005; García-Marco et al. 2006; Gamble et al. 2014). Oxidative stress parameters changed markedly with the Fe-chelate treatment. In leaves, the application of Fe-chelate led to marked increases in tGSH, tGSSG and GR, as well as decreases in Asc, suggesting the elicitation of a moderate oxidative stress, likely associated with an increase in the Fe being transported. Conversely, in the roots there were marked decreases in tGSH and tGSSG, indicating that the application of Fe-chelate relieved the oxidative stress in this compartment. A large increase in leaf GA, with decreases in leaf ABA, was also found, in agreement with a previous study (Chen et al. 2020a, b), also in line with the observed increases in plant size and shoot and root biomass. Leaf Cit decreased with no changes in Mal, suggesting that Cit could be being used for Fe transport (Rellán-Álvarez et al. 2011), in line with previous studies in *M. scutellata* (Gheshlaghi et al. 2019). The reason behind the decreases in leaf S concentrations and contents and root S concentrations with Fe-chelate is likely to be related to the shift in use of this element, which is also needed for the Fe-deficiency responses (Astolfi et al. 2021).

### Effects of applying $Fe_{oxi}$ and $Fe_{ore}$ alone

When stirred in buffer, the two Fe sub-products maintain a significant part of the Fe in solution/suspension (<1% and 6% of the Fe added for $Fe_{oxi}$ and $Fe_{ore}$, respectively) (Fig. 9A). Part of this Fe is likely in small particles that cannot be removed by

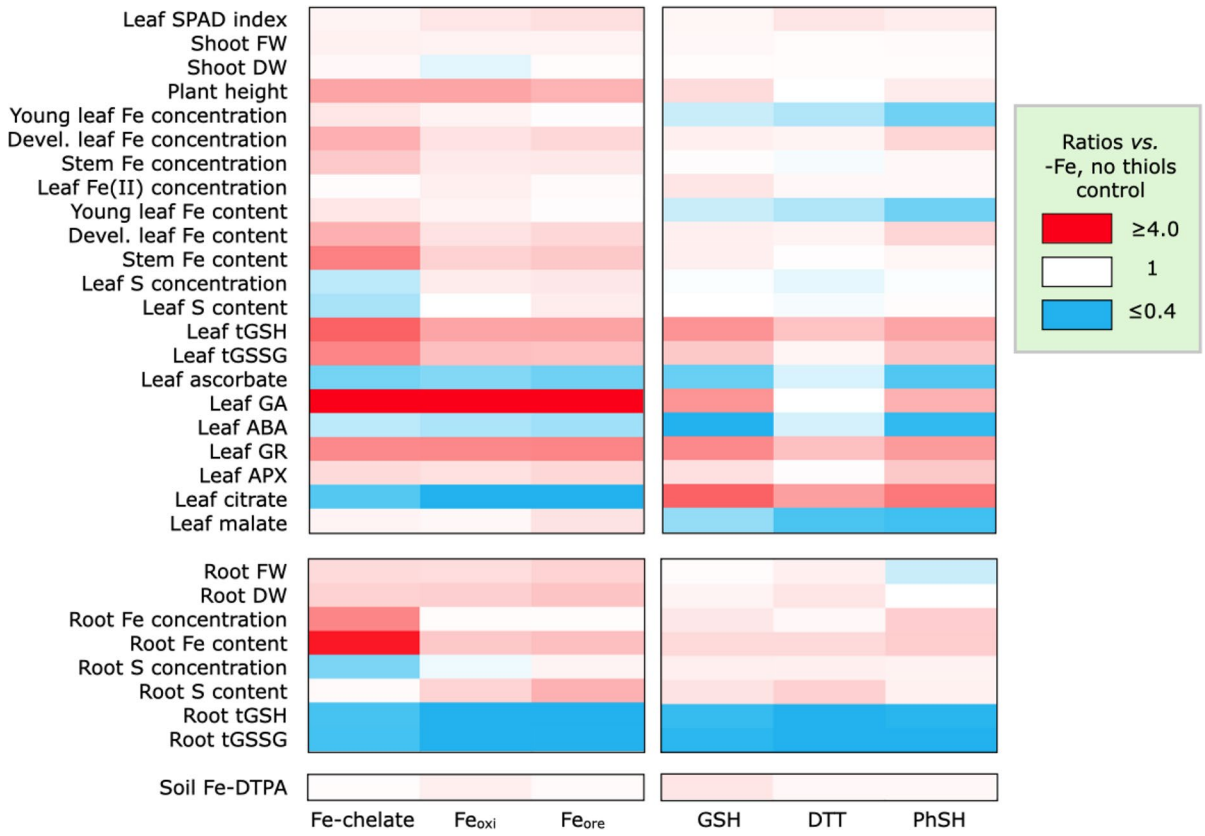


**Fig. 10** Heatmap summarizing the changes observed with the Fe-containing products or thiols were applied alone

centrifugation. A small fraction of this Fe in solution/suspension can be measured using ICP without acidification (1% and 5% of the Total ICP-Fe for $Fe_{ore}$ and $Fe_{oxi}$, respectively), and an even smaller part is converted, in a time-dependent manner, into chemical forms that are reduced in the presence of BPDS and can be measured as Fe(II)-$BPDS_3$ (Fig. 9B, C). These data indicate that $Fe_{oxi}$ and $Fe_{ore}$ are capable to deliver Fe in chemical forms potentially available for plants. However, in the case of the $Fe_{ore}$, part of the Fe put in solution was in non-reducible forms.

Indeed, when the two Fe sub-products were used alone, they alleviated Fe-deficiency symptoms in *G. max*, as judged by the increases in leaf SPAD, root biomass and plant height, with the leaf regreening being similar with both products and significantly better than that found with the Fe-chelate (see heatmap in Fig. 10). This indicates that the *G. max* cultivar used was an efficient one, capable of taking up Fe from $Fe_{oxi}$ and $Fe_{ore}$ using the natural Fe-acquisition toolbox in this plant species, which includes the secretion to the rhizosphere of protons, carboxylates and phenolics (Zocchi et al. 2007). Other changes in the measured parameters were in the same direction, but less intense, than those caused by the Fe-chelate, including increases in leaf Fe concentrations and contents, root Fe contents, and leaf tGSH and tGSSG. This suggests that $Fe_{oxi}$ and $Fe_{ore}$ also elicit a slight oxidative stress in leaves, but less intense than that observed with the Fe-chelate, likely because of a more controlled Fe supply and trafficking within the plant. In some parameters, such as the shoot biomass and root Fe concentrations, there were little changes when compared to the control. Conversely, the decreases in root tGSH and tGSSG, leaf ABA and leaf Cit were more marked than those observed with the Fe-chelate. Changes in the rest of parameters, including the large increase in leaf GA, were quite similar to those found with the Fe-chelate.

The positive results of Fe mining sub-products in alleviating Fe-deficiency are in line with previous studies in *G. max*, and indicate that this species can take up Fe from Fe chemical forms present in these materials using their natural acquisition mechanisms. The pioneering studies of Wallace et al. (1976, 1980) indicated that the application of $FeS_2$ could be useful for controlling Fe deficiency. Recently, the application of Fe-humic nanofertilizers containing the Fe oxi-hydroxide ferrihydrite have been shown to alleviate Fe deficiency in *G. max* (Cieschi et al. 2019). In *O. sativa*, the application of nanoparticles containing zero-valent Fe, but not those containing $Fe_2O_4$ and $Fe_2O_4$, were shown to cause some increases in leaf chlorophyll, in spite of the increases in plant Fe concentrations observed in all cases; these applications reduced the concentrations of GA and in some cases those of indole-3-acetic acid (Li et al. 2021). The possibility that $FeS_2$-rich mine coal wastes can ameliorate the nutrition of *G. max* and other crops in calcareous soils is currently being explored (Stander et al. 2022).

Effects of applying thiols alone

In the absence of Fe fertilization, the three thiols used (GSH, DTT and PhSH), alleviated Fe-deficiency symptoms in soybean, as judged by the leaf SPAD values, although growth parameters were not much changed, with the exception of increases in plant height with GSH and PhSH and root DW with DTT (see heatmap in Fig. 10). The Fe concentrations and contents in developed leaves and roots tended to increase whereas those in young leaves decreased, suggesting that the leaf regreening was associated to an Fe remobilization from pre-existing Fe pools as well as to a new Fe uptake. The application of thiols led to increases in leaf tGSH, tGSSG and antioxidant enzyme activities, and decreases in root tGSH and tGSSG and leaf Asc, again in line with an increase in Fe transport within the plant. Leaf hormone changes were not homogeneous, with GSH and PhSH causing large increases in GA and decreases in ABA, whereas DTT caused no major changes in leaf hormone concentrations. All thiols led to large increases in leaf Cit and decreases in leaf Mal, whereas the opposite was found with the Fe sources. The only thiol causing an increase in the soil DTPA-extractable Fe was GSH. The increases in root S concentrations and contents with the addition of thiols were expected.

The positive results of thiols in alleviating Fe-deficiency are in line with those observed in previous studies. In Fe-deficient Arabidopsis grown in hydroponics GSH increased the expression levels of Fe uptake- and transport-related genes and the Fe concentrations (Koen et al. 2012), and increased the leaf chlorophyll levels, but not the Fe concentrations, while preserving cell redox homeostasis (Ramírez et al. 2013). Later, it was shown that supplying GSH to the foliage of soil-grown *M. scutellata* relieved Fe-deficiency symptoms, and this was ascribed to the solubilization of pre-existing Fe pools within the plant via a reductive mechanism (Gheshlaghi et al. 2019). Also, when GSH was applied to the roots of *M. scutellata* growing in a Fe-rich rock sand, Fe was mobilized from the substrate via a reductive solubilization mechanism, increasing plant Fe concentrations and relieving Fe-deficiency symptoms (Gheshlaghi et al. 2020). Additional studies have focused on the use of NaHS, a donor of $H_2S$, which is rapidly incorporated into plant thiols such as GSH (Chen et al. 2011). The application of NaHS has been shown to promote chlorophyll synthesis in Fe-deficient *G. max* (Chen et al. 2020a, b), *Zea mays* (Chen et al. 2015) and *Fragaria x ananassa* (Kaya and Ashraf 2019).

Effects of applying Fe(III)-EDDHA together with thiols

The application of the Fe(III)-chelate supplemented with GSH and PhSH tended to decrease somewhat the Fe concentrations and contents in roots, stems and young leaves when compared to the Fe-chelate alone (see heatmap in Fig. S10). Furthermore, the application of Fe-chelate supplemented with thiols increased leaf SPAD, although plant height was decreased, and shoot biomass increased with GSH and PhSH when compared to the Fe-chelate alone. The addition of thiols caused decreases in the root tGSH and tGSSG, and normalized the leaf and root S concentrations and contents, which were low when the Fe-chelate was applied alone. When compared with the use of the Fe-chelate alone, thiols moderated the increases in leaf GA and the decreases in Cit. However, the hindering effect of the Fe-chelate on the Cit levels prevailed.

Effects of applying $Fe_{oxi}$ and $Fe_{ore}$ together with thiols

The application of $Fe_{oxi}$ and $Fe_{ore}$ supplemented with thiols increased the Fe concentrations and contents in roots, stems and developed leaves. However, the application of $Fe_{oxi}$ and $Fe_{ore}$ supplemented with thiols did not provide a further significant regreening, possibly because the effects of $Fe_{oxi}$ and $Fe_{ore}$ were already quite high when used alone. In all cases, when used in combination with Fe-containing products, the thiols caused marked relative decreases in plant height, and the application of GSH and PhSH decreased the Fe concentrations and contents in young leaves, as it occurs when the thiols were applied alone. On the other hand, the application of $Fe_{oxi}$ and $Fe_{ore}$ in combination with thiols decreased markedly the leaf GA (Fig. 8) and increased the leaf tGSH and tGSSG, when compared to the Fe sources used alone.

The thiol compounds are soluble in water at the concentrations used, and differ in mass (307.32, 154.25 and 110.17 g $mol^{-1}$ for GSH, DTT and PhSH), standard redox potential ($E^0$ -0.24, -0.33 and −1.71 for GSH, DTT and PhSH) and $pK_a$ of their -SH groups (9.65, 8.30 and 6.62 for GSH, DTT and PhSH). All of them are capable of reducing $Fe^{3+}$ to $Fe^{2+}$ ($E^0 + 0.77$) and compounds with -SH groups are known to be capable to form complexes with divalent metals (Singh and Kumar 2020) and enhance Fe leaching and bioavailability (Eitel and Taillefert 2017), but none of the chemical differences among the thiols included in the study seem to make a big difference in the ability to alleviate Fe deficiency in *G. max*.

## Concluding remarks

Results confirm the hypothesis that the application of Fe-mining sub-products, thiols and the combination of Fe(III)-EDDHA and thiols could be used to control a moderate Fe deficiency in *G. max* grown in a calcareous soil, with the different treatments relieving Fe deficiency symptoms to different extents. At the doses used, the two Fe-mining sub-products are more effective than the Fe-chelate, and this is likely due to their ability to release Fe forms that could be solubilized, and the metal subsequently taken up, by the Fe-acquisition toolbox existing in *G. max*.

Thiols are also effective in alleviating Fe deficiency symptoms in *G. max*, confirming for the first time that they are capable to facilitate Fe mobilization and uptake from the Fe oxy-hydroxide forms naturally occurring in a calcareous soil. This has been previously shown to occur in plants grown in nutrient solutions and in a rock sand substrate. This indicates that although the microbiome present in the calcareous soil is likely to consume thiols, at the doses used plants were still able to take advantage of the capacity of these compounds to mobilize Fe.

When applied in combination, the thiols only improved regreening in the case of Fe(III)-EDDHA, and this was associated to the mobilization in old leaves and stems of Fe forms elicited by the application of the Fe-chelate. In the case of the Fe mining sub-products the leaf Fe concentrations and contents also increased with the application of thiols, associated with a solubilization of Fe forms from the substrate, but leaves did not regreen further, probably due to the already marked effects of the Fe sub-products alone.

The doses used in this study are equivalent to approximately 175, 112 and 109 kg $ha^{-1}$ of Fe-chelate, $Fe_{oxi}$ and $Fe_{ore}$, respectively, and 387, 19 and 139 kg $ha^{-1}$ of GSH, DTT and PhSH, respectively. The positive results obtained suggest that the use of reasonable doses of Fe mining sub-products and GSH, which are cheap and easily available worldwide, can be an alternative for the alleviation of a moderate Fe deficiency chlorosis in *G. max*. New experiments should be envisaged to test the applicability of these management techniques in field conditions.

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