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ORIGINAL ARTICLE

Mechanistic assessment of tolerance to iron deficiency mediated by *Trichoderma harzianum* in soybean roots

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Abstract

Aims: Iron (Fe) deficiency in soil is a continuing problem for soybean (*Glycine max* L.) production, partly as a result of continuing climate change. This study elucidates how *Trichoderma harzianum* strain T22 (TH) mitigates growth retardation associated with Fe-deficiency in a highly sensitive soybean cultivar.

Methods and Results: Soil TH supplementation led to mycelial colonization and the presence of *UAOX1* gene in roots that caused substantial improvement in chlorophyll score, photosynthetic efficiency and morphological parameters, indicating a positive influence on soybean health. Although rhizosphere acidification was found to be a common feature of Fe-deficient soybean, the upregulation of Fe-reductase activity (*GmFRO2*) and total phenol secretion were two of the mechanisms that substantially increased the Fe availability by TH. Heat-killed TH applied to soil caused no improvement in photosynthetic attributes and Fe-reductase activity, confirming the active role of TH in mitigating Fe-deficiency. Consistent increases in tissue Fe content and increased Fe-transporter (*GmIRT1*, *GmNRAMP2a*, *GmNRAMP2b* and *GmNRAMP7*) mRNA levels in roots following TH supplementation were observed only under Fe-deprivation. Root cell death, electrolyte leakage, superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) substantially declined due to TH in Fe-deprived plants. Further, the elevation of citrate and malate concentration along with the expression of citrate synthase (*GmCs*) and malate synthase (*GmMs*) caused by TH suggest improved chelation of Fe in Fe-deficient plants. Results also suggest that TH has a role in triggering antioxidant defence by increasing the activity of glutathione reductase (GR) along with elevated S-metabolites (glutathione and methionine) to stabilize redox status under Fe-deficiency.

Conclusions: TH increases the availability and mobilization of Fe by inducing Fe-uptake pathways, which appears to help provide resistance to oxidative stress associated with Fe-shortage in soybean.

Significance and Impact of the Study: These findings indicate that while Fe deficiency does not affect the rate or degree of TH hyphal association in soybean roots, the beneficial effects of TH alone may be Fe deficiency-dependent.

Journal of Applied Microbiology

KEYWORDS

antioxidant enzymes, beneficial fungus, Fe-efficiency, mineral deficiency, mycelial colonization

INTRODUCTION

Iron (Fe) deficiency is common in soil, producing chlorosis, inefficient photosynthesis, growth retardation and yield loss in plants (Kabir et al., 2015; Robe et al., 2020; Waters et al., 2018). This problem arises most frequently in highly alkaline soils (Alcantara et al., 2002) or, less frequently, in soil with a high cation exchange capacity (Alcantara et al., 2002; Kabir et al., 2015). Calcium carbonate deposits are frequent in soils with a high pH (Zou et al., 2020). Both chlorosis in young leaves and leaf/root stunting are early signs of Fe shortage (Kabir et al., 2012; López-Millán et al. 2000). Fe-deficiency can result in significant production loss in affected fields, including. 20%–30% yield reductions in soybean fields (Assefa et al., 2020).

Plants have two distinct strategies for Fe uptake associated with a series of mechanisms, broadly known as strategies I and II. Reduction-based Fe uptake is a set of mechanisms in dicots and non-grass monocots that is referred to as strategy I (Curie & Briat, 2003; Kabir et al., 2012; Santi & Schmidt, 2009; Waters et al., 2018). This process consists of a series of reactions that allow plants to adjust to varying Fe levels. On the other hand, strategy II plants possess chelation-based mechanisms and are mostly found in graminaceous (grass) species wherein plants release phytosiderophores (PS) into the rhizosphere in order to mobilize inorganic Fe-III compounds by forming Fe-PS complexes (Li et al., 2019). One of the hallmarks of plants that use strategy I is a ferric chelate reductase used to convert ferric Fe (Fe-III) to ferrous Fe (Fe-II) (Kabir et al., 2015). Plants can induce strategy I responses, but this largely depends on the genotype and cultivar's sensitivity or tolerance. Bicarbonate is capable of inducing Fe chlorosis by inhibiting the expression of the ferric reductase, Fe transporter and H^+-ATPase genes, most likely by altering the expression of Fe efficiency reactions or FER-like transcription factors (Lucena et al., 2007). Phenolic compounds released through root exudates have chelating, reducing and radical scavenging action in response to Fe shortage (Curie & Mari, 2017; Rodríguez-Celma & Schmidt, 2013). The acidification of the rhizosphere by secreting H^+ is another key mechanism plants use to solubilize soil Fe (Kabir et al., 2012). Until coumarin-derived phenolics were discovered in Arabidopsis in high pH environments, this trait was not considered part of the Fe uptake mechanism (Fourcroy et al., 2014; Rodríguez-Celma et al., 2013). However, the passage of solubilized Fe through the root system is aided by metal transporters. The protein encoded by *IRT1* (a Fe-regulated transporter) is the common Fe transporter found in several plant species (Kabir et al., 2012; Vert et al., 2001). *ZIP1* (a zinc-regulated transporter gene) also encodes functional Zn or Fe transporters that may be involved in divalent metal ion absorption in plant cells (Li et al., 2013). The protein NRAMP1 has a wide variety of metal substrates and is induced by Fe deprivation, suggesting that it may be involved in Fe homeostasis (Briat et al., 2015). Plants also secrete organic acids (e.g. citrate, malate) to help with Fe mobilization in the rhizosphere (Correia et al., 2014).

Abiotic stress tolerance in plants is influenced by their interactions with microbes such as mycorrhizal fungi, endophytic fungi and plant-growth-promoting rhizobacteria, in addition to their innate resistance (Ali et al., 2014; Egamberdieva et al., 2016). Several *Trichoderma* fungi have been shown to be antagonistic towards a variety of agricultural diseases (Li et al., 2018). *Trichoderma* species (common soil fungi) can also increase host plant tolerance to a variety of abiotic stimuli through their involvement in root growth promotion, nutritional absorption maintenance and the activation of defensive mechanisms to prevent oxidative damage (Hermosa et al., 2012). Furthermore, *T. harzianum* has been shown to solubilize a variety of plant nutrients (Li et al., 2015) and to increase the availability of P and Fe to plants, resulting in a considerable increase in plant biomass (Yedidia et al., 2001). Colonization by *T. harzianum* also promotes the activity of antioxidant enzymes, thereby limiting the abundance of reactive oxygen species (ROS) in plants under abiotic stresses (Ahmad et al., 2015; Mastouri et al., 2012). However, no efforts have yet been made to elucidate the mechanisms underpinning the involvement of *T. harzianum* in Fe-deficiency tolerance in soybean.

Soybean (*Glycine max* L.) is an important crop plant used for food, feed and a variety of non-food industrial products (Chaudhary et al., 2015). Fe deficiency is a major factor constraining soybean yields. In the US, where soybean cultivation has been expanded to areas of high pH calcareous soils, Fe-deficiency chlorosis (IDC) has been estimated to account for an approximate yield deficit of 340,000 Mg, or $820 million in revenue per year (Hansen et al., 2004). Investigations of the genetic basis of IDC tolerance in sorghum have uncovered QTL that are associated with IDC tolerance, which can be considered when choosing the cultivar of sorghum to plant in calcareous areas (Assefa et al., 2020). Keeping this in mind, we expect to find useful findings that would be crucial in elucidating the real-life interactions of beneficial microbes, such as *T. harzianum* to promote the use of microbial biofertilizer for improved Fe-efficiency in Fe-starved

soybean. In this study, we investigated the role of *T. harzianum* in the induction of the adaptive features and regulatory mechanisms that mitigate Fe-deficiency symptoms in soybean plants, using physiological and molecular methods. Our focus was on the strategy I response, Fe-uptake and chelation processes that plants possess under differential Fe availability in the soil.

MATERIALS AND METHODS

Plant cultivation and fungal treatment

Soybean seeds of a highly sensitive cultivar (BARI soybean-5) to Fe-deficiency were germinated on a tray for 3 days at room temperature. The seedlings were then grown in 1 kg of soil in the absence (Fe-sufficient) or presence (indirect Fe-deficient) of 1500 mg $CaCO_3$ and were cultivated following a randomized complete block design (RCBD) in a growth room having 10 h of light and 14 h of darkness (550–560 μmol s^{-1} per μA) at 25°C. *Trichoderma harzianum* T22 spores (1 g kg^{-1}) were applied in a granular form (Dragonfli) by mixing with the soil before seed planting. The average pH of the soil at the start of the experiment was 6.0 and 7.6 for Fe-sufficient (control) and Fe-deficient soil, respectively. In a separate experiment, heat inactivation of TH was performed by heating the TH granule in an electric oven at 90°C for 1 h. The plants were cultivated having three plants/replicate several times in the same growth conditions up to 4 weeks to ensure the colonization and establishment of exogenous TH with plants and to confirm the reproducibility of the results.

Microscopic and molecular detection of *T. harzianum* T22 in roots

Fungal colonization was visualized in roots cut into 1 cm segments (Zhang et al., 2012). Root samples were first immersed in 10% (w/v) KOH for 2 h at 90°C before being stained with trypan blue. Root pieces were mounted on plates using polyvinyl alcohol and glycerol and viewed under a digital microscope at 20× magnification.

The molecular detection of TH in soybean roots was performed as previously described (Horn et al., 2016). Briefly, DNA was extracted from soybean roots using Wizard Genomic DNA Purification Kits (Promega Corporation) per the manufacturer's protocol. DNA was then quantified using a NanoDrop ND-1000 Spectrophotometer (Wilmington) and used for PCR amplification with primers designed to amplify a fragment of the *UAOX1* gene of TH. The *UAOX1* forward (5′-GTC GGT AGC TGA AAG GGG AT-3′) and reverse (5′-ATG TAG AGG CCG GAA ACA CC-3′) primer combinations (Horn et al., 2016) resulted in a 486-bp fragment from a chromosomal location just upstream of the *UAOX1* coding region. The PCR product was then visualized in a Gel Documentation System (Thermo Fisher Scientific).

Measurement of morphological and photosynthetic attributes

A digital calliper was used to measure the length of the longest root and shoot of each plant. The root and shoot were also dried for 3 days at 80°C in an electric oven to access dry weights. We further measured the chlorophyll score and photosynthetic attributes in intact leaves to see whether TH is able to restore chlorophyll synthesis and photosynthesis in Fe-starved soybean. A SPAD (soil plant analysis development) meter (Minolta) was used for estimating the chlorophyll content of fully expanded young leaves. Photosystem II (PSII) activity, including Fv/Fm (quantum efficiency of photosystem II), Pi_ABS (photosynthesis performance index), ET2o/RC (electron transport flux further than QA DIo/RC) and Mo (approximated initial slope in ms^{-1} of the fluorescent transient), were recorded using FluorPen FP 100 on fully expanded young leaves after keeping them in the dark for 1 h (Photon Systems Instruments).

Determination of ferric chelate reductase activity, rhizosphere acidification and total phenolics in roots

A Ferrozine assay was used to determine ferric chelate reductase (FCR) activity in roots (Kabir et al., 2015). The root surface was first cleaned with 0.2 mM $CaSO_4$ and Milli-Q water. One millilitre of the assay mixture (100 mM Fe(III) EDTA, 0.10 mmol MES-NaOH (pH 5.5), 300 mM ferrozine) was then added to the root sample. Samples and blank tubes (without the assay solution) were kept at 25°C for 20 min in the dark. The absorbance was read at 562 nm. FCR activity was calculated based on the molar extinction coefficient of ferrozine.

Rhizosphere pH was measured at day 0 after transferring the 3 day-old young seedlings to the soil pot. Rhizosphere pH was also measured on day 21. Rhizosphere pH was measured from a soil suspension. One gram of soil attached to the root was stirred with 10 ml water and incubated at room temperature for half an hour before measuring pH using a digital pH meter. Total phenolic concentrations in the roots were measured as previously described (Kogure et al., 2004). In short, the root extract was mixed with a solution of 80% Folin–Ciocalteu reagent containing 20% Na_2CO_3. The optical density of the solution was read at 765 nm. Sample

concentrations were measured using a gallic acid calibration curve and expressed as mg g^{-1} extract (GAE).

Estimation of cell death and electrolyte leakage

Cell death (%) was calculated using an Evans blue viability assay (Zhao et al., 2005). Entire fresh roots and shoots were placed in a tube with 2 ml of Evans blue mixture and left for 15 min. Afterwards, 1 ml of 80% ethanol was added and the mixture was kept for 10 min at room temperature. The tubes containing solutions were incubated for 15 min at 50°C in a water bath before being centrifuged for 10 min at 12,000 rpm. The supernatant was then transferred to another tube before measuring the absorbance at 600 nm. Finally, cell death (%) was calculated based on the fresh weight of the root and shoot.

A conductivity meter was used for evaluating the electrolyte leakage that results from the loss of cell membrane integrity in both root and shoot tissues, as in the previously described technique (Lutts et al., 1996), with some modifications. Root and shoot surface components were washed three times with deionized water. The fresh specimens were then moved in a deionized water beaker (20 ml) and held at 25 °C for 2 h. The electrical conductivity (EC1) of the solution was then computed. The samples were then heated in a water bath at 95°C for 20 min before cooling at 25°C. After that, the final EC (EC2) was calculated as follows: EC = (EC1/EC2) 100 (%).

In vivo microscopic visualization of ROS species

Superoxide ($O_2^{\bullet-}$) was visualized in root tissue using the specific probe dihydroethidium (DHE). Soybean root tips were incubated in a PBS solution (pH 6.8) for 30 min containing 10 M DHE. The root tips were then washed with fresh buffer. The fluorescence of the $O_2^{\bullet-}$ specific probe DHE was visualized with a fluorescent microscope (CLS-01-00076, Logos Biosystem Inc) using 488 and 520 nm excitation and emission, respectively (Sandalio et al., 2008; Singh et al., 2021).

Hydrogen peroxide (H_2O_2) was visualized under oxidative stress conditions using 2′,7′-dichlorofluorescein diacetate (DCF-DA) as a qualitative marker for H2O2 detection. We prepared 50 mM DCF-DA in 50 mM phosphate buffer saline (PBS, pH 7.2). Three times with deionized water, soybean root tips were washed, and excess water was removed with tissue paper (Kimteck). The root tips were inoculated with DCF-DA probe in the dark for 30 min at room temperature, washed with distilled water and the reaction of H_2O_2 with fluorescent DCF-DA molecules was monitored using a fluorescence microscope (CLS-01-00076, Logos Biosystem Inc) with excitation at 480 nm and emission at 530 nm (Sandalio et al., 2008; Singh et al., 2021).

Analysis of citrate and malate levels by high-performance liquid chromatography

The high-performance liquid chromatography (HPLC) analysis was carried out in the same way as before, with a few changes (Guo et al., 2011; Kabir et al., 2015). Root samples were homogenized in methanol before centrifuging at 12,000 rpm for 10 min. The clear phase was then filtered through 0.22 micro Minisart® Syringe Filters (Sartorius). The sample was derivatized by adding 40 µl BODIPY-aminozide, 2 µl 3 M (N′-ethylcarbodiimide hydrochloride), and 15 µl 20% pyridine. Before vortexing, the mixture was diluted to 100 L of water-acetonitrile (2:8, v/v) solution. After that, the samples were heated to 40C for 30 min prior to injection. A 20 µl extract was injected into an HPLC system (Binary Gradient HPLC System, Waters Corp) equipped with a C18 reverse phase-HPLC column (particle size: 5 mm, pore size: 300 A, pH range, 1.5–10; dimension). In the mobile phase, buffer A (water and 0.1% TFA) and buffer B (80% acetonitrile and 0.1% TFA) were utilized at gradients of 1–24 min 100% A, 25–34 min 100% B, and 35–60 min 100% A. Each metabolite's retention time was then measured using a dual absorbance detector (Waters Corporation) at 215 and 245 nm.

Determination of elemental concentrations in root and shoot

Collected roots and shoots were washed with deionized water and immersed in 0.1 mM $CaSO_4$ for 5 min before performing a final wash with deionized water. Roots and shoots were then dried at 70°C for 72 h. Equal amounts of tissue for each plant were weighed and digested using a $HNO_3/HClO_4$, 3:1 (v/v) solution. Elemental concentrations (Fe, Z, S, Ca) in the digestion were measured through inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7700). Standard solutions of each element (Roth) were used to create standardized curves.

Gene expression analysis by real-time PCR

Total RNA was isolated from plant tissues using RNeasy® plant mini kits (QIAGEN). A total of 0.1 g of ground plant tissue was mixed with an RNA extraction buffer containing 2 M DDT and 1% (v/v) β-ME. The mixture was vortexed and centrifuged at ≥13,000 rpm for 2 min. Total RNA was

obtained following multiple filtering and washing steps. A micro-drop UV/Vis spectrophotometer (UVISDrop-99) was used for measuring the RNA concentration of the samples. First-strand cDNA was synthesized using cDNA synthesis kits (Bio-Rad). Gene expression analysis by real-time PCR (qPCR) was performed with the CFX96 Real-Time system (Bio-Rad). The gene-specific primers for selected genes responsible for Fe-uptake and mobilization were designed with NCBI primer blast (Table S1) analysis. The qPCR reactions were 95°C for 3 min, 40 amplification cycles of 5 s at 95°C, 30 s annealing at 58°C, and a final extension of 5 min at 60°C. Expression levels of target genes were determined using the $dd^{-\Delta Ct}$ method (Livak & Schmittgen, 2001), where *actin* mRNA was used for normalization.

Antioxidant enzyme activities

Antioxidant enzyme activities in plant tissue were measured following the protocols of Haque et al. (2021). One hundred milligrams of tissue was homogenized with 0.5 ml of 100 mM potassium phosphate (KP-buffer, pH 7.0) and vortexed. The mixture was then centrifuged at 10,000 rpm for 15 min, and the supernatant was collected for enzymatic analysis. Superoxide dismutase (SOD) activity was determined by adding 100 µl of extract to 1 ml of EDTA (0.1 mM), $NaHCO_3$ (50 mM, pH 9.8) and epinephrine (0.6 mM). The formation of adrenochrome from this chemical reaction was monitored at 475 nm. To determine ascorbate peroxidase (APX) activity, 100 µl of the sample extract was combined with 1 ml of EDTA (0.1 mM), KP-buffer (50 mM, pH 7.0), hydrogen peroxide (0.1 mM), and ascorbic acid (0.5 mM). The mixture was then read at 290 nm and the activity was based on the extinction coefficient 2.8 mM^{-1} cm^{-1}. Catalase (CAT) activity was measured using a mixture containing 1 ml of KP-buffer containing 6% hydrogen peroxide which was added to 100 µl of sample extract. Absorbance through the mixture was read at 240 nm (extinction co-efficient 0.036 mM^{-1} cm^{-1}) considering 30s as the initial and 60s as the endpoint. For GR (glutathione reductase) activity, 100 µl of plant extract was combined with 1 ml of 0.2 M KP-buffer (pH 7.0) that contained 1 mM EDTA, 20 mM GSSG (oxidized glutathione) and 0.2 mM NADPH. The reaction was triggered with GSSG and reduced in absorbance at 340 nm because of NADPH oxidation. Glutathione reductase (GR) activity was then calculated using the extinction coefficient of 6.12 mM^{-1} cm^{-1} (Halliwell & Foyer, 1978).

Statistical analyses

All measurements were carried out using three biological replicates. Analysis of variance (ANOVA) was used to determine significant differences between treatment groups ($\alpha = p \leq 0.05$) followed by Duncan's Multiple Range Test (DMRT) in both root and shoot separately, where applicable. Statistical analysis was conducted using SPSS Statistics 20.0 (IBM). GraphPad Prism 6.0 was used for graphical presentation.

RESULTS

TH colonization, plant phenotype and chlorophyll

TH colonization of soybean roots was confirmed using both microscopic and molecular techniques. Microscopic visualization showed the colonization of mycelium in soybean roots only when inoculated with TH (Figure 1a). Further, DNA from the roots plants inoculated with TH showed a single PCR band of 486 bp, indicating *UAOX1* gene amplification with *UAOX1*-specific primers, but no band was observed in uninoculated controls, consistent with the microscopic evidence (Figure 1b). After 4 weeks of cultivation, the soybean plants showed distinct variations in morphology and leaf chlorophyll status across the different combinations of Fe deficiency and TH supplementation. Fe deficiency caused stunted growth along with leaf chlorosis. Significant levels of chlorosis were not detected visually or by SPAD analysis in Fe-starved plants that were inoculated with TH (Figure 1c,d). However, chlorophyll levels in the young leaves were significantly decreased due to Fe deficiency in contrast to Fe-sufficient controls in the absence of TH (Figure 1c). The addition of TH in the Fe-deficient soil caused a significant increase in the chlorophyll score compared to Fe-deprived plants. Although Fe-deficient plants showed root stunting and foliage disruption, control Fe-sufficient, Fe-sufficient plus TH and Fe-deficient plus TH all exhibited similar vigorous plant phenotypes (Figure 1e).

Photosynthetic and morphological parameters

Handheld chlorophyll fluorescence analysis showed that Fv/Fm, Pi_ABS and ET2o/RC were significantly decreased under Fe deficiency relative to the young leaves of Fe-sufficient plants (Figure 2a–c). All photosynthesis parameters significantly increased with TH colonization in Fe-deficient plants. Plants cultivated with TH in Fe-sufficient soil showed similar Fv/Fm, Pi_ABS and ET2o/RC to that of controls (Figure 2a–c). The Mo value significantly increased following Fe deficiency compared to controls, but the addition of TH on Fe-deficient plants created a substantial increase in Mo. The leaves of control plants

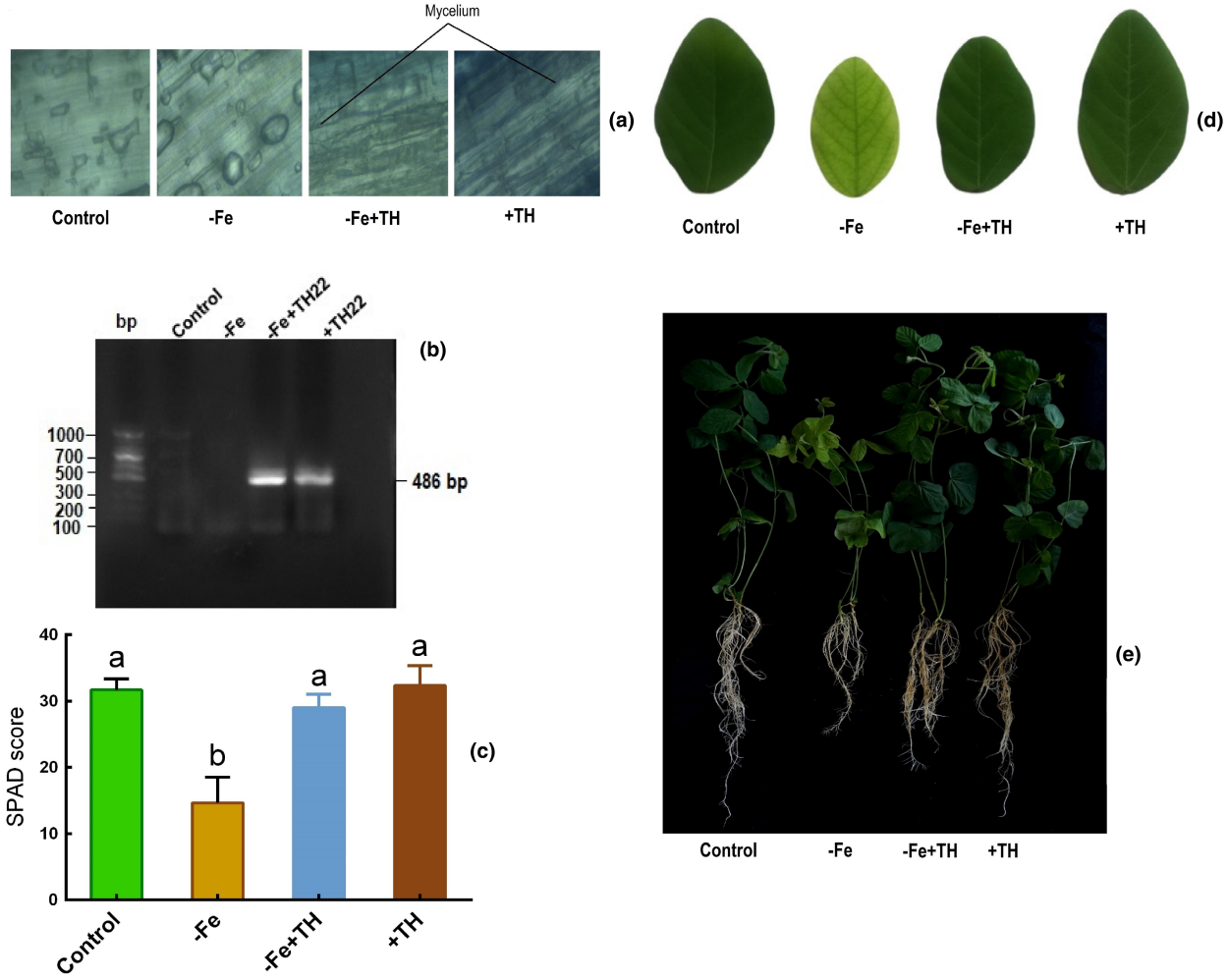

FIGURE 1 Microscopic visualization of TH mycelium (a), molecular detection of the presence of the TH *UAOX1* gene (b), SPAD score (c), leaf chlorosis (d) and plant phenotype (e) in response to different growth conditions of Fe and TH in soybean. Data in (c) represent mean ± SD of three independent biological samples. Different letters indicate significant difference at $p < 0.05$ level, where applicable.

cultivated with TH showed a similar Mo value to that of TH-lacking controls (Figure 2d). The root length (cm), root dry weight (g), shoot height (cm) and shoot dry weight (g) significantly declined in response to Fe deficiency in comparison with controls (Figure 2e–h). However, the cultivation of Fe-deficient plants with TH gave rise to a significant increase in these morphological parameters compared to Fe-deprived plants (Figure 2e–h).

Changes in strategy I response and stress indicators

Fe deficiency caused a significant drop in FCR activity in roots relative to controls. However, the inoculation of TH into Fe-deficient soils yielded a significant increase in FCR activity relative to Fe-starved plants (Figure 3a). The plants cultivated with TH in Fe-sufficient soil showed similar FCR activity in roots compared to TH-treated plants grown in Fe-deficient soils (Figure 3a). We further estimated the rhizospheric acidification at 0 and 28 days after seedling transplantation to observe the extrusion of protons from soybean roots in response to Fe deficiency and TH treatment. Interestingly, plants treated with or without TH in controls conditions showed no significant variations in rhizospheric acidification (Figure 3b). However, Fe-deficient plants with or without TH in the soil showed a significant decrease in the rhizospheric acidification at the end of the cultivation period compared to the initial state (Figure 3b). Moreover, the total phenol concentration in the roots showed no significant variations among the three treatments (control, −Fe and +TH). However, the presence of TH in the soil caused a significant rise in total phenol in Fe-deficient plants (Figure 3c). Furthermore, cell death (%) and electrolyte leakage in the root and shoot of soybean significantly increased with Fe-shortage compared to Fe-sufficient controls (Figure 3d–e). However, the presence of TH in the soil promulgated

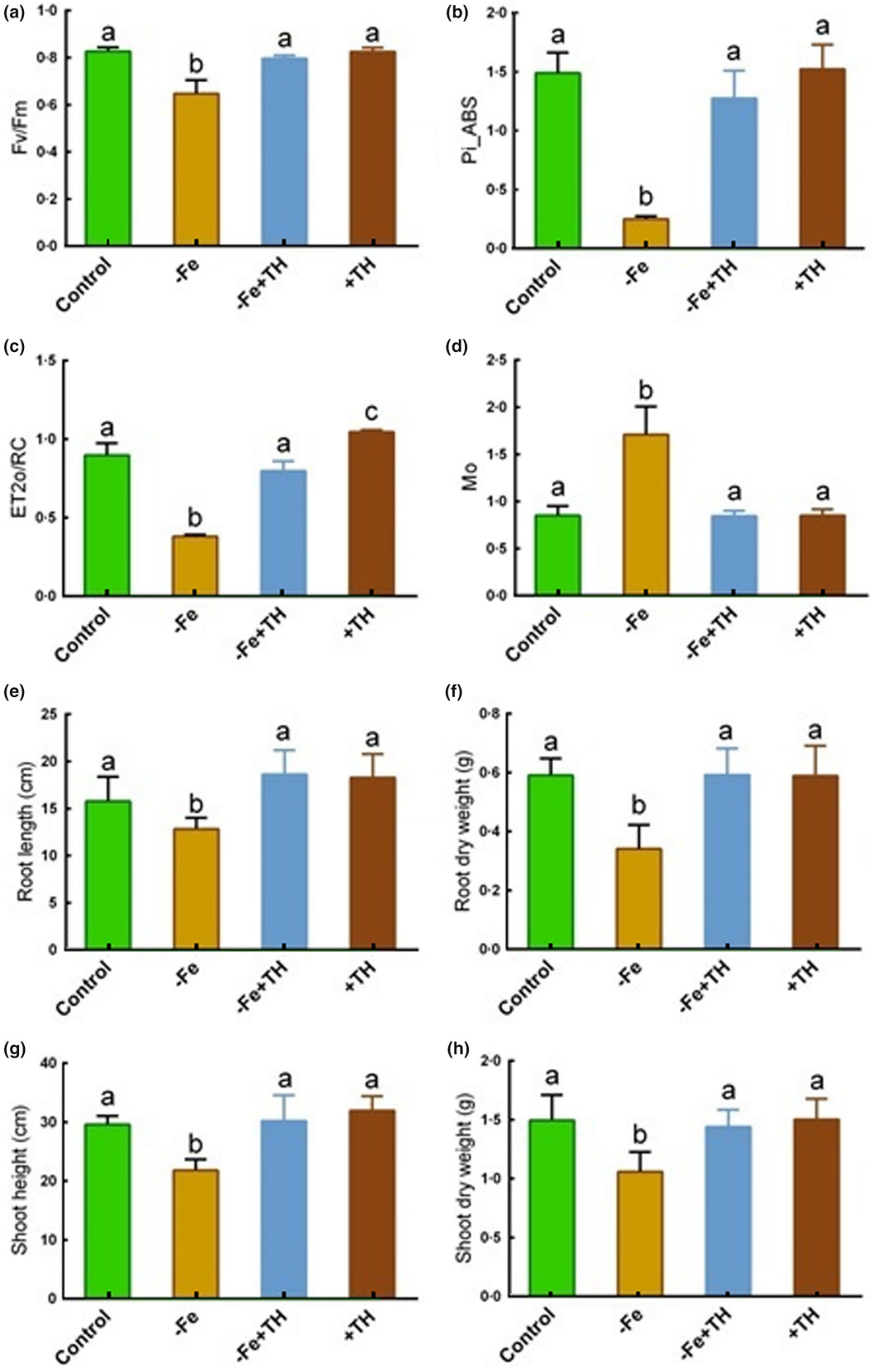

FIGURE 2 Fv/Fm (a), Pi_ABS (b), ET2o/RC (c), Mo (d), root length (e), root dry weight (f), shoot height (g) and shoot dry weight (h) in soybean cultivated in different growth conditions of Fe and TH. Data represent means ± SD of three independent biological samples. Different letters indicate significant differences at a $p < 0.05$ level.

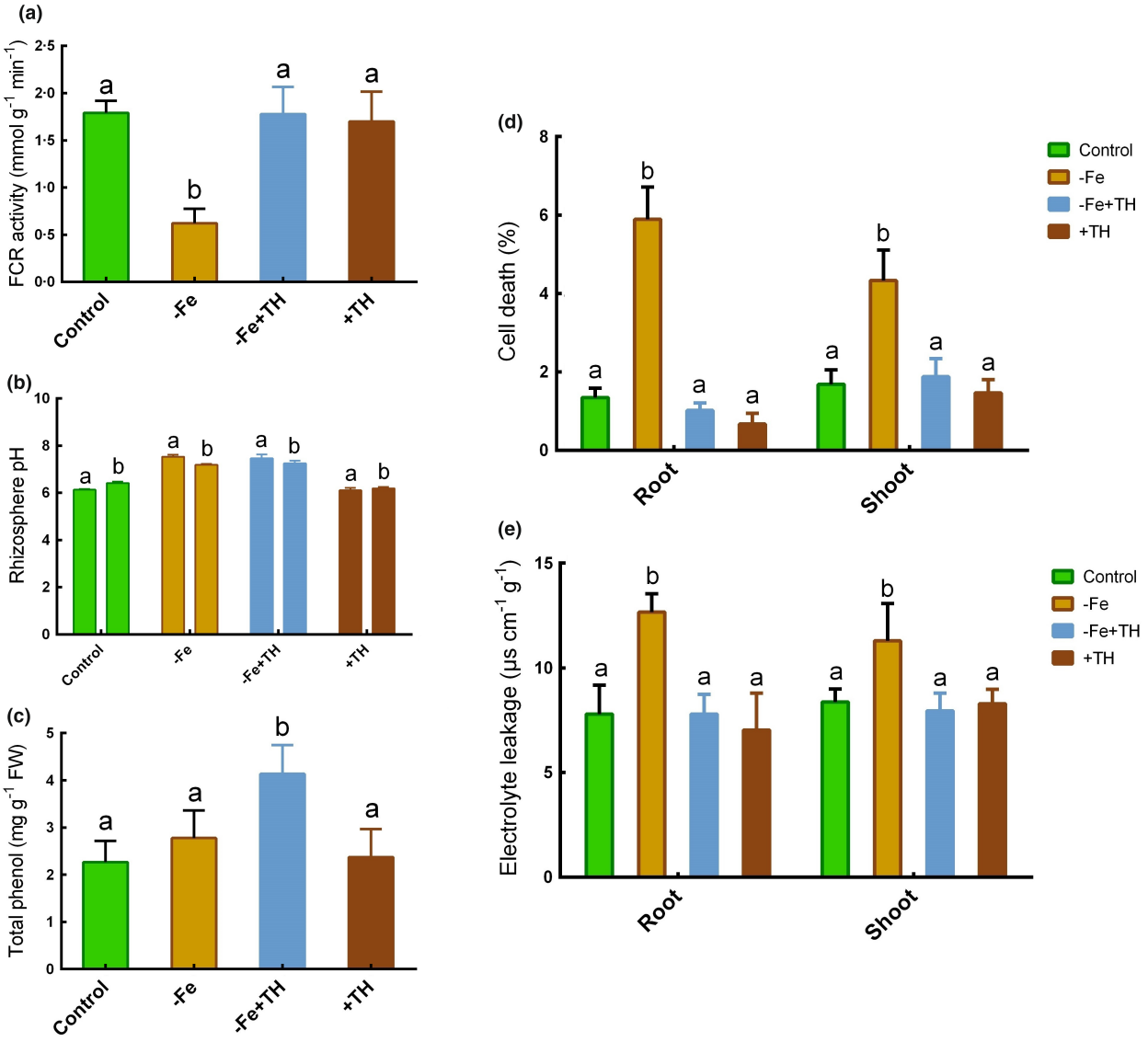

FIGURE 3 FCR activity (a), rhizosphere pH at day 0 (initial) and day 28 (end) after transferring the 3d-old seedling to the soil pot (b), total phenol concentration (c), cell death (d) and electrolyte leakage (e) in soybean cultivated in different growth conditions of Fe and TH. Data represent means ± SD of three independent biological samples. Different letters indicate significant differences at a $p < 0.05$ level.

a significant decrease in these two stress indicators in the root and shoot of Fe-deficient plants. The level of cell death (%) and electrolyte leakage in plants cultivated with TH in Fe-sufficient soils showed a similar status of these stress indicators to that of Fe-sufficient controls that were not inoculated with TH (Figure 3d–e).

Changes in ROS generation

Our qualitative analysis demonstrated that Fe deficiency led to soybean roots with a substantial accumulation of ROS ($O_2^{\cdot-}$ and H_2O_2), indicated as intense DHE and DCF-DA fluorescence, indicating the presence of $O_2^{\cdot-}$ and H_2O_2, respectively. However, the plants treated with TH under Fe deficiency showed a substantial decrease in DHE and DCF-DA fluorescence in roots compared to Fe-depleted plants. Similar fluorescence intensity was also observed for Fe-sufficient plants with or without TH (Figure 4a,b).

Changes in organic acids and S-metabolites in roots

Although Fe deficiency caused no significant changes in the concentration of citrate and malate in the roots of soybean, the addition of TH on Fe-deficient plants gave rise to a significant increase in these two organic acids (Table 1), although the malate enhancement was only significant on Fe-deficient soils. In addition, the concentrations of glutathione and methionine significantly declined in roots of Fe-deficient soybean in comparison with controls, but

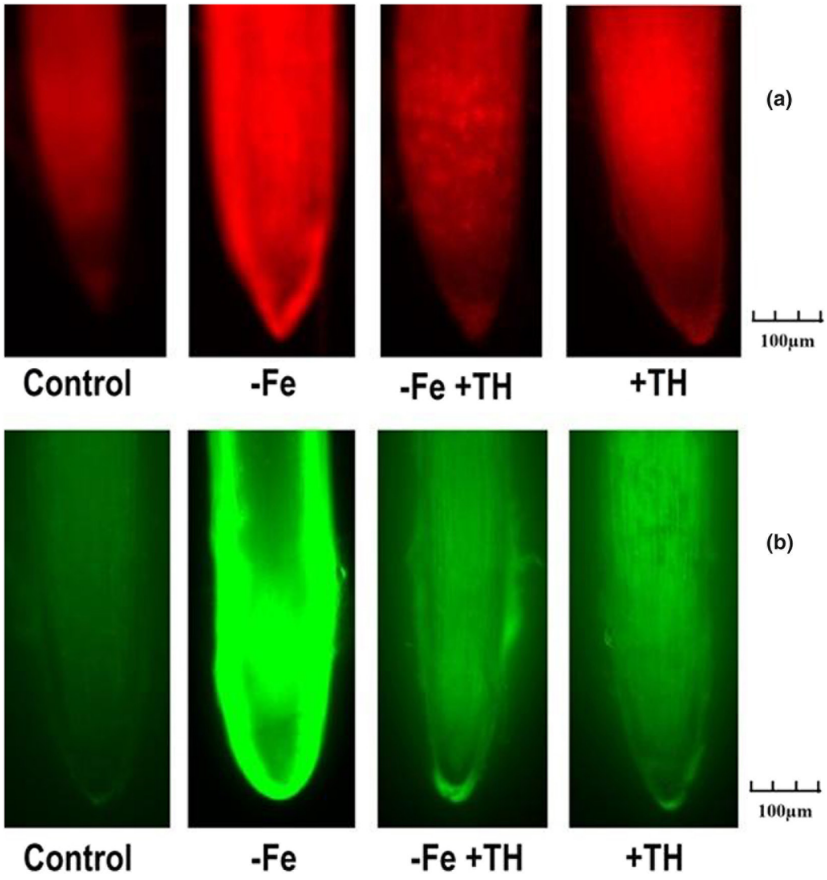

FIGURE 4 Fluorescence visualization of O_2^- (a) and H_2O_2 (b) in roots of soybean cultivated in different growth conditions of Fe and TH by DHE and DCF-DA staining, respectively. Scale bar = 100 μm.

TH reversed this decline (Table 1). The concentration of cysteine showed no significant changes in the roots of soybean among the different treatments of Fe and TH (Table 1).

Effect of heat inactivation on TH effects

To confirm the active role of TH in mitigating Fe deficiency, we killed TH spores by heating prior to application in the soil. We observed that plants showed distinct chlorosis in the young leaf due to Fe deficiency, which was fully restored following TH application (Figure 1). However, heat-inactivated TH did not prevent chlorosis on soybean grown in Fe-deficient soils (Figure 5a). Similarly, soybean grown in soil with heat-activated TH showed significant decreases in Fv/Fm, SPAD score and FCR activity just as seen in Fe-sufficient plants that did not receive TH (Figure 5b–d).

Changes in elemental concentrations in root and shoot

Following Fe deficiency, the Fe concentrations in roots and shoots of soybean significantly decreased (Figure 6a). With the addition of TH to Fe-deficient soils, the plants showed a significant increase in Fe concentration in both root and shoot relative to Fe-deficient plants. Plants cultivated with TH in Fe-sufficient soils exhibit similar Fe status to that of controls and Fe-deficient plants grown with TH (Figure 6a). On the other hand, Zn concentrations in roots and shoots were significantly increased because of Fe deficiency, with the increase greater in the root than the shoot. However, plants cultivated with TH and Fe-deficient soils showed similar Zn status in root and shoot to that of control Fe-sufficient plants cultivated with or without TH (Figure 6b). Finally, soybean showed no significant variations in S and Cd status in either root or shoot due to the different treatments of Fe and TH (Figure 6c,d).

Changes in mRNA transcripts linked to Fe-deficiency responses

We performed real-time PCR analysis on a series of genes associated with Fe uptake and mobilization in roots of soybean subjected to different Fe and TH treatments. The mRNA levels of *GmFRO2* and *GmIRT1* in roots were significantly decreased in response to Fe deficiency relative to Fe-sufficient controls. However, the plants showed a significant increase of *GmFRO2* and *GmIRT* mRNAs when subjected to TH supplementation in soil with or without Fe deficiency (Figure 7a,c).

Journal of Applied Microbiology

TABLE 1 Organic acid and S-metabolite concentrations (μM) in roots of soybean cultivated in different combinations of Fe and TH

Treatments	Citrate	Malate	Glutathione	Methionine	Cysteine
Control	1.37 ± 0.16^{a}	1.03 ± 0.15^{a}	1.79 ± 0.18^{a}	1.31 ± 0.08^{a}	2.87 ± 0.23^{a}
−Fe	1.52 ± 0.34^{a}	1.08 ± 0.10^{a}	1.12 ± 0.12^{b}	0.84 ± 0.16^{b}	2.76 ± 0.25^{a}
−Fe + TH	3.60 ± 0.47^{b}	2.63 ± 0.28^{b}	1.49 ± 0.12^{a}	1.64 ± 0.21^{a}	2.62 ± 0.29^{a}
+TH	3.89 ± 0.34^{b}	0.84 ± 0.56^{a}	1.56 ± 0.13^{a}	1.48 ± 0.12^{a}	2.59 ± 0.36^{a}

Note: Different letters indicate significant differences at a $p < 0.05$ level.

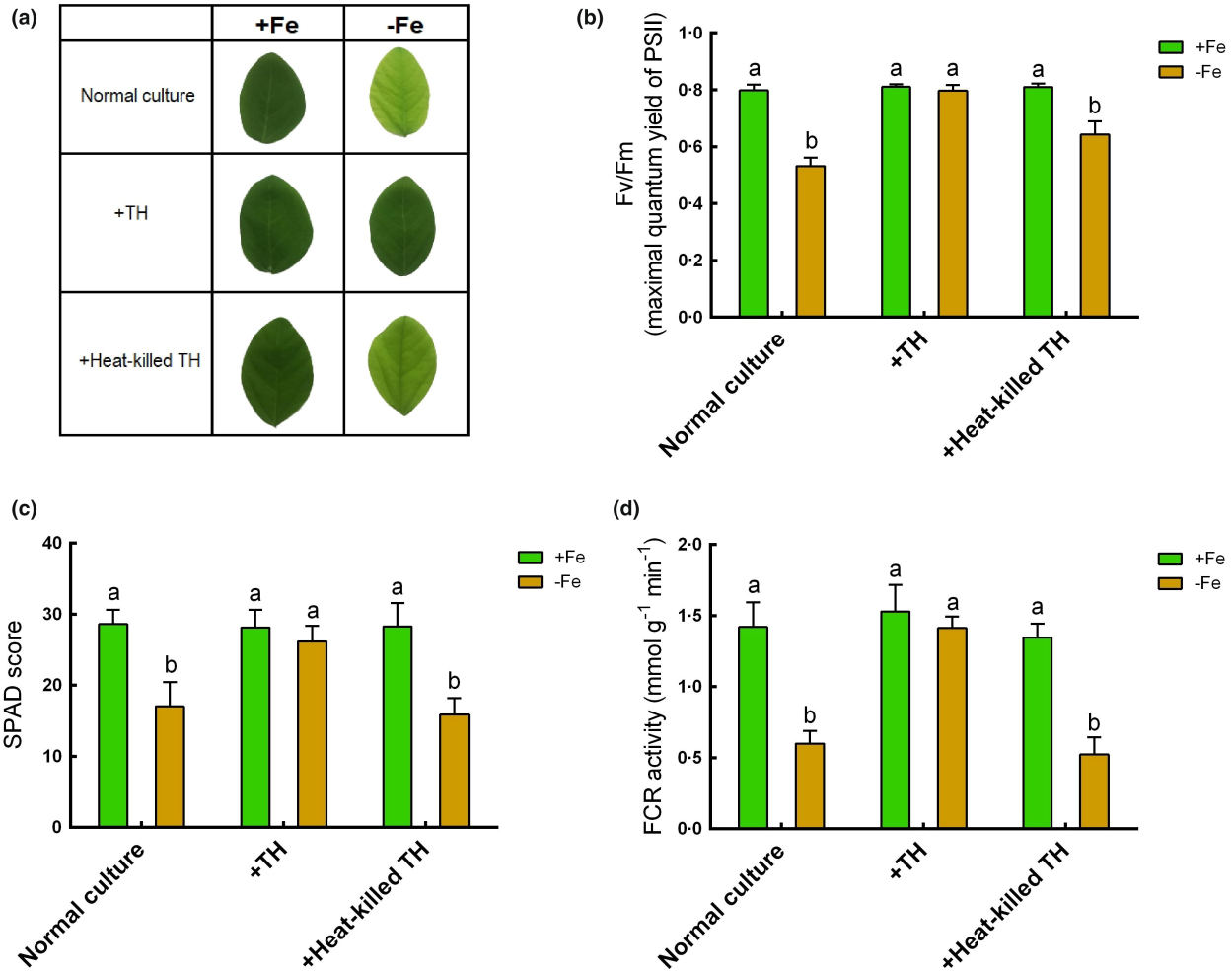

FIGURE 5 Leaf chlorosis (a), Fv/Fm (b), leaf SPAD score (c) and FCR activity in roots of soybean in the presence or absence of added Fe, active TH or inactive TH. Data represent means ± SD of three independent biological samples. Different letters indicate significant differences at a $p < 0.05$ level, where applicable.

Further, the amount of *GmATPase4* mRNA significantly increased under Fe deficiency in the absence or presence of TH compared to non-treated controls. Plants treated with TH on Fe-sufficient soils exhibit similar mRNA levels for *GmATPase4* in roots to that of controls (Figure 7b). The abundance of mRNAs for three Fe-transporter genes (*GmNRAMP2a*, *GmNRAMP2b* and *GmNRAMP7*) was significantly decreased in response to Fe deficiency relative to Fe-sufficient controls. However, these genes appear to be significantly induced following TH supplementation under Fe-shortage compared to Fe-deprived plants. Plant treated with TH with or without Fe deficiency showed a similar abundance pattern of *GmNRAMP2a*, *GmNRAMP2b* and *GmNRAMP7* in roots of soybean similar to that of untreated controls (Figure 7d–f). In addition, we did not see any significant changes in *GmZIP1* mRNA levels in the roots of soybean following Fe deficiency or TH supplementation (Figure 7g). Further, the abundance of mRNAs for *GmCs* and *GmMs*, genes responsible for citrate and malate synthesis, respectively, showed no significant changes associated with Fe deficiency. However, the mRNA levels for these genes were significantly increased

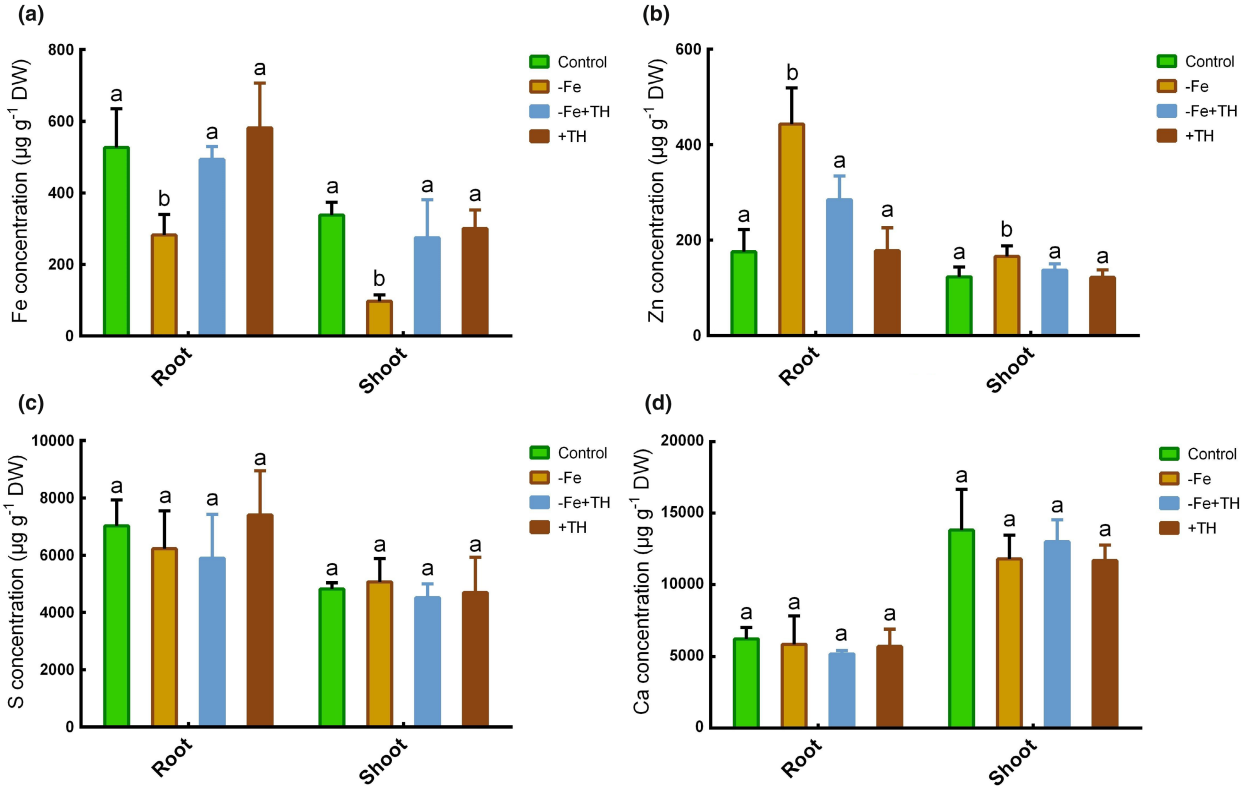

FIGURE 6 ICP-MS analysis of Fe (a), Zn (b), S (c) and ca (d) concentrations in root and shoot of soybean cultivated in different growth conditions of Fe and TH. Data represent means ± SD of three independent biological samples. Different letters indicate significant differences at a $p < 0.05$ level.

by TH inoculation under Fe-shortage compared to control plants. The plants grown with TH in Fe-sufficient soils showed a similar mRNA level of these genes to that of control or Fe-deficient plants (Figure 7h,i).

Changes in antioxidant enzymes

The activities of CAT and APX showed no substantial changes in response to the different treatments of Fe and TH in soybean (Figure 8a,b). However, the SOD (superoxide dismutase) activity in the roots was significantly increased under Fe deficiency in the absence or presence of TH in the soil compared to plants cultivated with sufficient Fe with or without TH (Figure 8c). Further, Fe deficiency caused a significant decrease in GR activity in the roots of soybean relative to controls. However, the addition of TH under control or Fe-deficient conditions showed a significant increase in GR activity in roots compared to Fe-deficient plants (Figure 8d).

DISCUSSION

Crops are exposed to several abiotic stresses on a constant basis. Although genetics has been used to increase plant tolerance against abiotic stress, these are technology-intensive solutions that have not been adopted in many nations around the world. Microbiome engineering may be an economical and environmentally beneficial technique for avoiding the shortcomings of other strategies. The adverse effects of Fe deficiency in calcareous soil are well known (Santos et al., 2015; Waters et al., 2018), and were further confirmed by the decreased biomass, severe chlorosis, photosynthetic disturbance, ion imbalance and oxidative stress detected in our analyses (Assefa et al., 2020; Kabir et al., 2012). In this study, we investigated a series of physiological and molecular traits associated with strategy I responses and other adaptations underlying Fe-deficiency symptoms in soybean. This study demonstrates the beneficial impact that TH can play in mitigating Fe-deficiency symptoms and responses in soybean.

Colonization by TH does not negatively impact plant physiology in Fe-sufficient soils

In this study, the addition of TH in the soil and its colonization in the root substantially improved chlorophyll

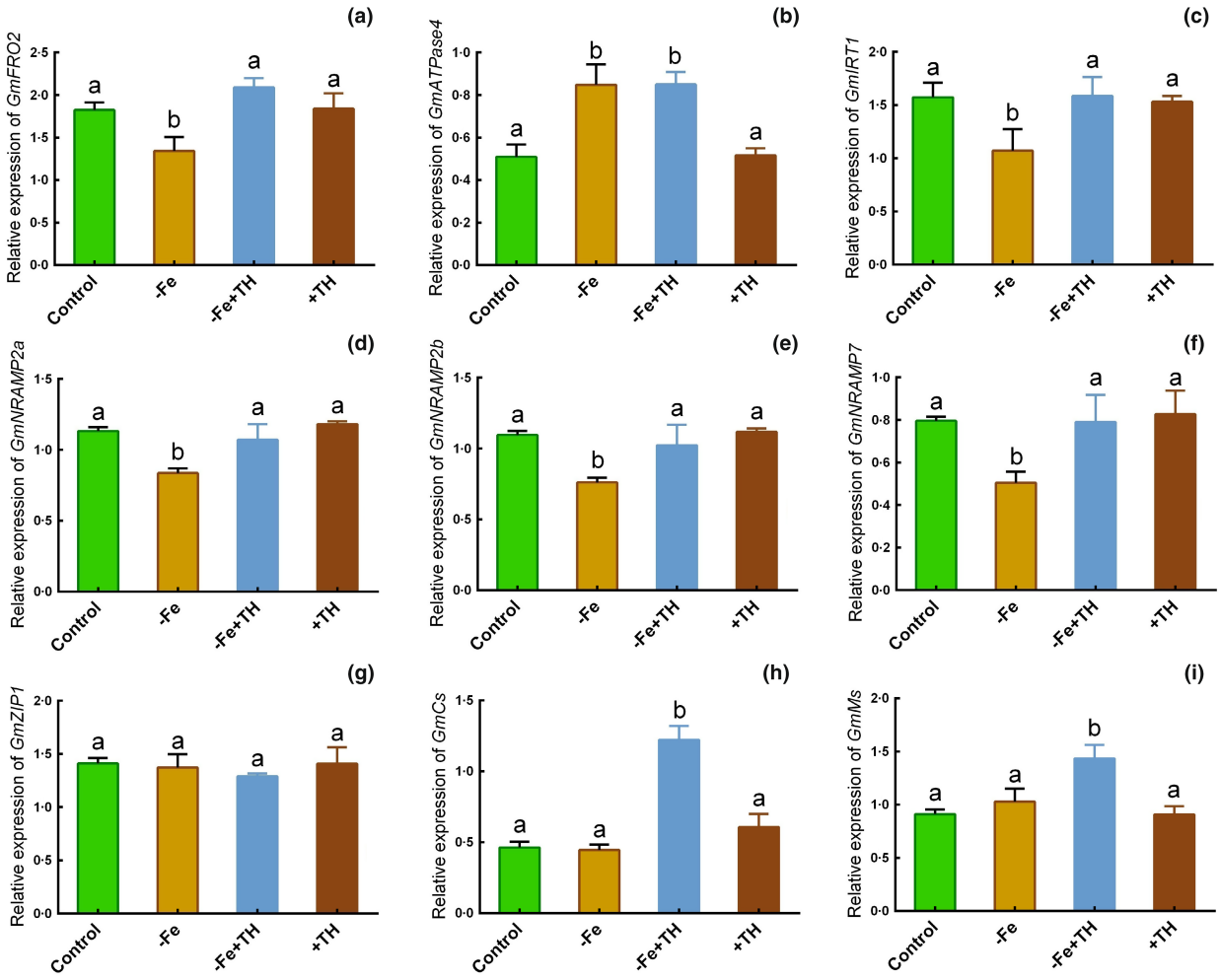

FIGURE 7 Real-time PCR analysis of *GmFRO2* (a), *GmATPase4* (b), *GmIRT1* (c), *GmNRAMP2a* (d), *GmNRAMP2b* (e), *GmNRAMP7* (f), *GmZIP1* (g), *GmCs* (h) and *GmMs* (i) in roots of soybean cultivated in different growth conditions of Fe and TH. Data represent means ± SD of three independent biological samples. Different letters indicate significant differences at a $p < 0.05$ level.

synthesis, photosynthetic attributes and morphological parameters, but only in the presence of Fe deficiency, suggesting that TH is not helpful for this process in many soil types or environments. On the other hand, TH did not negatively influence any of the investigated parameters of Fe-sufficient soils, suggesting that TH addition will either be a positive factor (under Fe deficiency) or a neutral contributor, in strong contrast to such microbial amendments as arbuscular mycorrhizal fungi (AMF) that are growth promoters in poor soils but growth inhibitors in rich soils (Klironomos, 2003). Further, it has been demonstrated that different mycorrhizal species respond differently depending on the plant species with which they are associated (Ortas & Ustuner, 2014). Interestingly, some fungicide and/or herbicide treatments have no effect on mycorrhizal growth (Huey et al., 2020). Hence, the synergistic interactions of plants and fungi are highly variable but can be critical for sustainable agriculture because they allow plants to grow and develop naturally rather than through the use of agrochemicals.

Co-evolution of TH with its plant hosts

Mycorrhizal fungi colonize intercellular spaces in roots or develop within cells (Bonfante & Genre, 2010). The earliest stage of the plant-TH interaction is for the fungus to colonize the host roots. To facilitate colonization, *Trichoderma* must deal with plant defence responses during the early stages of the relationship by suppressing innate immunity on a large scale, as demonstrated previously for Arabidopsis roots (Jacobs et al., 2011). It has been reported that TH facilitates the access of AMF to the roots of plants that are not routinely colonized by AMF (Poveda et al., 2019). Further, increased productivity has been reported in several plant species as a result of the combined application of TH and AMF, although these beneficial effects are also dependent on the fungal strains used (Baum et al., 2015; Martínez et al., 2004). Thus, the interaction/combination effect of TH and AMF in mitigating Fe-deficiency symptoms in soybean is worth investigating. We observed that TH vigorously colonized the soybean

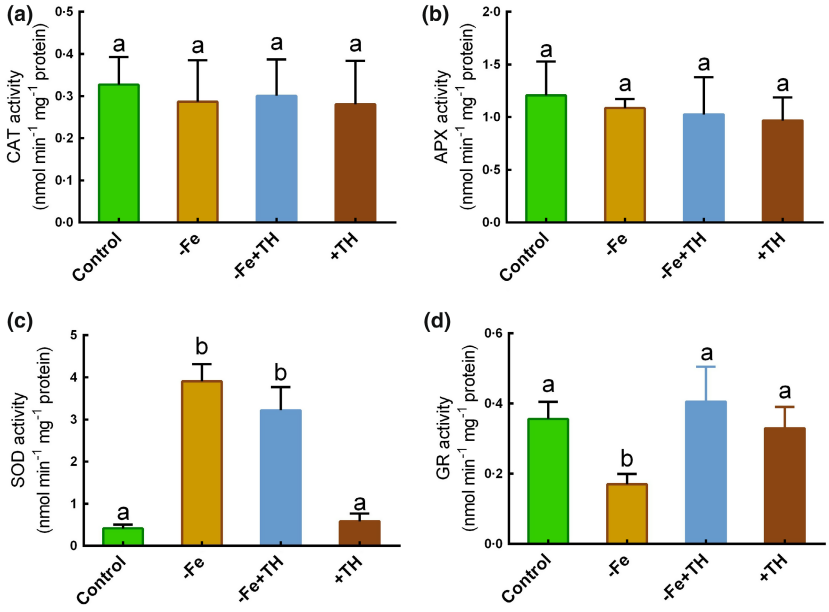

FIGURE 8 Changes in the activities of CAT (a), APX (b), SOD (c) and GR (d) in roots of soybean cultivated in different growth conditions of Fe and TH. Data represent means ± SD of three independent biological samples. Different letters indicate significant differences at a $p < 0.05$ level.

roots within a short period, as evident from the microscopy and the molecular detection of TH *UAOX1* gene presence. In earlier studies, *Trichoderma* colonization was seen to occur in Arabidopsis roots as early as 24 h after infection, resulting in significant alterations in the levels of several plant transcripts linked to biotic and abiotic stress resistance (Brotman et al., 2013). The presence of TH DNA was also detected in roots of tomato and Arabidopsis as early as 2 weeks after Fe deficiency was introduced (Martínez-Medina et al., 2017). In another study, colonization of TH in the roots of tomato was observed 10 days after fungal inoculation when the plants were 4 weeks old (Poveda et al., 2019). These studies suggest that there is no effect of Fe deficiency on the rate or degree of TH hyphal association in soybean root, but that the beneficial effects of TH alone may be Fe deficiency-dependent, while associated effects of other co-evolved beneficial microbes may show a more general TH enhancement.

Fe-deficiency effects on soybean and their reversal by TH addition

In this present work, Fe deficiency in soybean was associated with lower chlorophyll score, decreased photosynthetic efficiency and less plant biomass, as previously reported in earlier studies of other soybean cultivars (Santos et al., 2015). Plants require Fe at a concentration of 10^{-9} to 10^{-4} moll^{-1} at the intracellular level for chlorophyll biosynthesis and optimal growth (Kim & Guerinot, 2007). In this study, the addition of TH to Fe-deficient soil substantially improved chlorophyll synthesis, photosynthetic attributes and morphological parameters, suggesting that TH does have a role in enhancing Fe uptake in soybean. Under metal stress, AMF also has been seen to exhibit a protective role in restoring PSII activity in chloroplasts, thus improving water usage efficiency in *Robinia pseudoacacia* (Yang et al., 2015). Fe deficiency alters the shape of the thylakoid membrane, lowering PS-II effectiveness in plants and interfering with the Fe-dependent chlorophyll synthesis process (Allen et al., 2008; Petrou et al., 2014). Under Fe deprivation, leaf Fv/Fm ratio, which is regarded as a good diagnostic predictor of PS-II damage (Murchie & Lawson, 2013), increased dramatically in TH-treated soybean plants. Due to TH inoculation under Fe deficiency in soybean, Pi_ABS, which is connected to the performance index for energy conservation from photons absorbed by PS-II antenna, was also consistently increased. Various findings show that alterations in these OJIP parameters caused by Fe shortage are linked to damage to the reaction centre or other parts of the energy transfer route in the PS-II system (Kabir et al., 2020; Prity et al., 2020). We conclude that TH can help the host plant acquire sufficient Fe in the PS-II reaction centre, thus improving the photosynthetic efficiency of the soybean plants, as evidenced by the higher Fv/Fm ratio and Pi_ABS under Fe deficit in the presence of TH.

We not only found considerable improvement in morpho-physiological features and induction of regulatory mechanisms linked to Fe uptake but also demonstrated a significant increase in the Fe concentrations in roots and shoots of soybean plants cultivated under the dual conditions of Fe deficiency and TH supplementation. In a previous study in soybean, some Fe absorption genes (*IRT1*, *FRO2*) were regulated similarly by Fe deficiency in soybean, but many other genes related to Fe-acquisition were not, implying that Fe-shortage increases the expression of Fe uptake genes but has other consequences as well (Waters et al., 2018). Similarly, our studies of the expression patterns

of a series of Fe-transporters responsible for Fe-uptake in dicot plants indicated that many were not induced by the absence or presence of Fe deficiency and TH in the soil (data presented in Figure S1), but substantially higher mRNA levels for *GmIRT1*, *GmNRAMP2a*, *GmNRAM2b* and *GmNRAMP7* were observed in roots of Fe-deficient soybean infected with TH. It is unclear if the upregulation of Fe-uptake genes is because plants directly sense TH at the roots, or if the upregulation is due to a TH-originated or local root signal that is enhanced by TH under low Fe concentration in plant tissues. Thus, it appears that TH colonization would be beneficial even from the early stages of soybean growth and development under Fe deficiency.

The results of the current research present the opportunity to begin to build a mechanistic (systems) network of plant-TH contributions to reducing Fe-deficiency symptoms in soybean. To confirm that this was an active contribution, we investigated heat-inactivated TH. Heat-killed TH did not reverse any Fe-deficiency symptoms, indicating that it was not just the added biomass of the TH that was responsible for the positive effects. Studying the efficiency of microbes by SynCom technology and in gnotobiotic systems is relatively new in plant stress research (Harbort, Hashimoto, Inoue, Schulze-Lefert, 2020). Our initial studies suggest additional system building and testing that can be used to further elaborate the system networks for Fe deficiency-soybean-TH interactions.

Induction of strategy I mechanisms

Numerous studies have shown that induction of strategy I responses for Fe uptake confers tolerance to Fe shortage in Arabidopsis and other dicot plants (García et al., 2010; Kabir et al., 2012). In this study, TH addition to the soil was shown to be involved in strategy I responses of the soybean through the induction of increased FCR activity. The FCR enzyme is known to carry out a rate-limiting step in the absorption of Fe (Morrissey & Guerinot, 2009), making its activity critical to increasing Fe uptake under low Fe conditions. Both FCR activity and the transcription of its candidate gene (*GmFRO2*) were significantly lower under Fe-deficient conditions in comparison to the control, though both were found to be increased when TH was present in the roots of soybean under these same conditions. Despite the fact that FCR is generally increased in Fe-deficient plants, Parafield, a Fe-deficient pea cultivar, showed a significant reduction in FCR (Kabir et al., 2012), which is similar to what we have seen in this soybean genotype. Kabir et al. (2012) also demonstrated that the induction of FCR in Fe-deficiency tolerant pea was considerably higher at day 5 after the deficiency was imposed. As a result, the soybean genotype employed in this study may be more vulnerable to Fe deficit, making the fact that TH was able to restore Fe in response to Fe deficiency on such a sensitive plant genotype all the more remarkable. It is known that Fe becomes more available by reducing Fe^{3+} to the more soluble Fe^{2+} prior to Fe uptake in strategy I plants (Li et al., 2004; Waters et al., 2002). Our results support the hypothesis that TH may play a role in the FCR-mediated process that converts Fe^{3+} to Fe^{2+}, thereby making Fe^{2+} more available to Fe-deficient soybean. In Arabidopsis, coumarin-type exudates mobilize Fe(III) by chelation and also contribute to the reduction of Fe^{3+} (Tsai et al., 2018). It has been demonstrated that coumarins (phenolic substances) generated from plants influence the composition of Arabidopsis root microbial communities in Fe-deficient Arabidopsis (Perkowska et al., 2021). Also, bacterial root microbiota, which is activated by coumarins released by plants, plays a critical role in plant adaptation to Fe-deficient soils (Harbort, Hashimoto, Inoue, Niu, et al., 2020). Another study demonstrated that *Trichoderma* fungi release volatile compounds that activate the Fe acquisition machinery in the roots of Arabidopsis and tomato (Martínez-Medina et al., 2017). Thus, it is possible that TH, either directly or indirectly through its influence on other microbial communities, stimulates the production of coumarin or airborne signals in soybean roots in order to cope with Fe-depleted conditions. This is consistent with the substantial increase of total phenol concentrations observed in the roots of Fe-deficient soybean colonized by TH. As an adaptive response to Fe deficiency, phenolic production in the roots of dicots has been shown to increase apoplastic Fe reutilization to conserve Fe within the shoot (Jin et al., 2007). This was also previously hypothesized to be a key mechanism for surviving chlorosis in soybean (Longnecker & Welch, 1990). The exact mechanism by which apoplastic Fe is repurposed is unknown, however, but a variety of root exudates are thought to be involved (Canarini et al., 2019). By the induced expression of the phenylpropanoid pathway, Fe-deficient Arabidopsis enhances the production of phenolics of the coumarin class (Rodríguez-Celma et al., 2013; Schmidt et al., 2014). Interestingly this is one of few adaptive responses observed in this study where TH addition is not contributing to preserving an aspect of normal plant function under Fe stress, but rather actively stimulating excess phenol production, suggesting that this may play a key mechanistic role.

Fe-deficient soybean plants showed a substantial increase in rhizosphere acidification over the course of the experiment, and the end-point expression of the *GmATPase4* gene was high regardless of TH colonization. Proton extrusion can be mediated by this *ATPase* gene across the plasma membrane of roots, a process induced

by Fe deficiency and thought to aid in the mobilization of Fe in plants (Martínez-Cuenca et al., 2015; Santi & Schmidt, 2009). The extrusion of H^+ to lower the pH of the rhizosphere is a known adaptive response of Fe-deficient plants (Kabir et al., 2012; Morrissey & Guerinot, 2009), and is further confirmed in this study. This response may serve to increase Fe-uptake by releasing oxide-bound Fe into the soil water solution for plant uptake. However, the induction of *GmATPase4* or rhizosphere acidification was observed in Fe-deficient conditions regardless of the presence of TH in the soil. From these results, we suggest that the proton pumping observed in soybean may not be related to the presence of TH in the soil per se, but rather a homeostatic plant response to the high pH conditions initially present in Fe-deficient soils.

Organic acids have been shown to have the ability to help mobilize Fe (Nworie et al., 2017) and can be triggered in plants by mycorrhizae and/or soil bacteria (Adeleke et al., 2012; Andrino et al., 2021). To gain more insight into the Fe mobilization process, we further investigated the concentrations of key organic acids related to Fe absorption in soybean. Due to chelating properties, organic acids form complexes with some soil minerals, resulting in the acquisition of otherwise inaccessible nutrients for plant growth and development (Morgan et al., 2005). The increased concentration of citrate and malate accompanied by the upregulation of *GmCs* and *GmMs* genes in roots, by TH under Fe deficiency, indicates that TH can play a critical role in the elevation of these key organics acids to mobilize. However, it is unknown whether the increased citrate and malate accumulation in the roots occurred as a result of the plant's response to TH or whether TH was capable of releasing these acids to mobilize Fe. Mycorrhizal fungi, particularly AMF, have been shown to release significant amounts of organic acids in response to phosphorus deficiency in plants (Andrino et al., 2021; Li et al., 2021). Further research is required to determine how TH promotes the production of organic acids in plants that aid in the acquisition of Fe.

Induction of antioxidant systems

Under abiotic stressors such as Fe deficiency, ROS can act as key signalling molecules to initiate and regulate adaptive mechanisms (Mittler et al., 2004; Sun et al., 2016), though they can also cause oxidative damage to proteins, DNA and lipids of both the plant and any associated microbes (Apel & Hirt, 2004). The regulation of ROS by the fine-tuning of ROS-scavenging enzyme activities and metabolites is one of the tolerance mechanisms that plants often possess to cope with stress (Kabir et al., 2016). Although our assessment was largely qualitative, we observed that there were noticeably higher amounts of ROS observed in the shoots of Fe-deficient soybean than was observed in the presence of TH. This qualitative assessment can also be validated by measuring the activity of ROS-scavenging enzymes that regulate ROS concentrations. ROS-scavenging enzymes were found to show variable responses to Fe deficiency. While CAT and APX

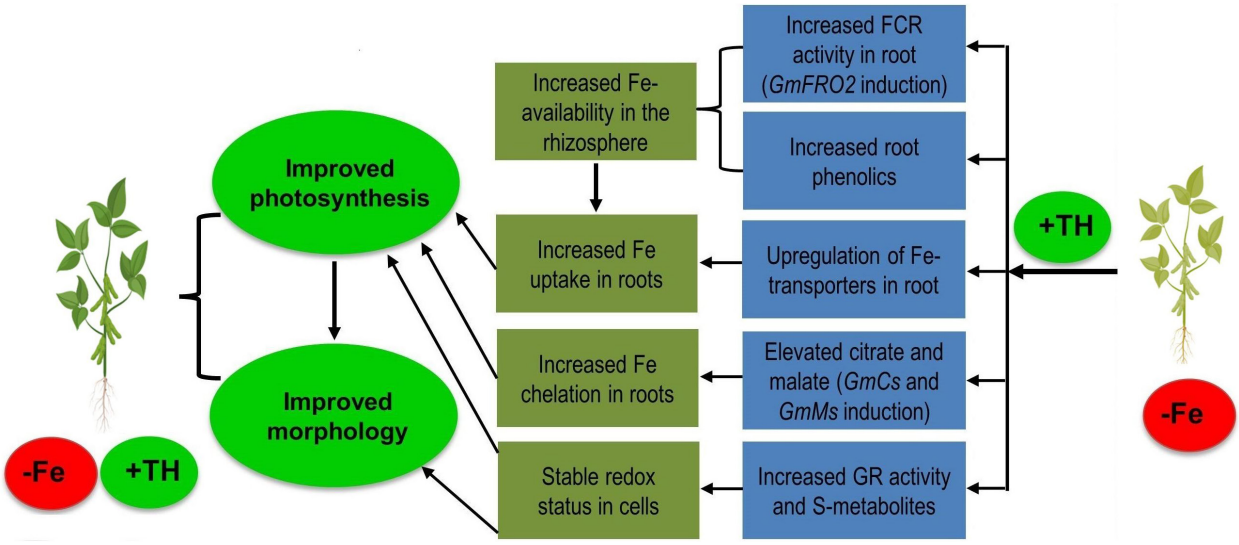

FIGURE 9 Simple systems diagram of TH-mediated mitigation of Fe-deficiency in soybean. The addition of TH increases FCR activity and phenolics content in the rhizosphere, resulting in increased Fe availability. TH also promotes the expression of Fe transporters, which increases Fe uptake in roots. Additionally, TH can result in an increase in organic acids (citrate and malate), which increases Fe-chelation in roots. TH is associated with an increase in GR activity and S-metabolites, both of which contribute to the redox stability of cells. The addition of TH enhances iron uptake and photosynthesis, as well as antioxidant defence, thereby improving the morphological parameters of Fe-deficient soybean plants.

activity exhibited no differences across the treatment combinations, SOD activity was highly upregulated relative to controls in Fe-deficient plants regardless of TH addition. GR activity was downregulated relative to the controls only in Fe-deficient plants without TH colonization. GR is a powerful antioxidant that scavenges H_2O_2 and $O_2^{\cdot-}$ (Gill et al., 2013). GR is also required to produce GSH, an S-metabolite that further protects cells from oxidative damage (Shankar et al., 2016). Several studies also demonstrated the role of TH in inducing antioxidant defence in response to salinity and drought in plants (Ahmad et al., 2015; Pandey et al., 2016). The importance of antioxidant process was further supported by our observation of elevated S-metabolites (glutathione and methionine) in Fe-deficient roots following TH supplementation. TH allows for higher S-metabolites in Fe-deficient soybean plants, presumably by regulating glutathione cycles to mitigate ROS damage. In a similar study, alfalfa plants were ineffective at inducing GR activity under Fe deficiency, but the addition of AMF aided its induction and hence may have a role in scavenging ROS in Fe-deficient conditions (Rahman et al., 2020). Also, higher SOD, CAT and APX activity in mycorrhizae-infected plants may be linked to improved plant growth and nutrient uptake (Alguacil et al., 2003). However, different fungal species colonize different host plants, which can lead to variable antioxidant enzyme activity under abiotic stress (Evelin et al., 2009). It is notable that, in this study, TH colonization led to a reduction in ROS generation and an increased capacity for the protection of cellular structures from oxidative stress. A detailed pathway by which fungi detoxify ROS is still to be proven, it is known that fungi can induce salicylic and jasmonic acid production in plants, which causes defence genes to be more highly expressed to combat oxidative stress (Corradi et al., 2009; Lanfranco et al., 2005). Further studies are needed to determine whether TH employs a similar jasmonate and or salicylate induction process for reducing oxidative harm in plants suffering from Fe shortage or other abiotic stresses.

In summary, TH was shown to improve photosynthetic efficiency, and morphological parameters, associated with its beneficial effects on Fe-deficient soybean. The elevation of Fe-reductase activity (*GmFRO2*) was one of the main mechanisms that significantly boosted TH-induced Fe availability. Further, cellular stress indicators and ROS under Fe deficiency were significantly reduced due to TH. The substantial increase of citrate and malate revealed that TH caused more Fe chelation in Fe-deficient plants. The Fe levels in tissues and the expression of Fe-transporters in roots (*GmIRT1*, *GmNRAMP2a*, *GmNRAMP2b* and *GmNRAMP7*) both were induced considerably by TH under Fe deficiency. Results also suggest that TH may have a role in inducing antioxidant defence by increasing GR enzymatic activity and S-metabolites (glutathione and methionine) to stabilize the redox state in Fe-deficient cells (Figure 9). Using TH for Fe-deficiency mitigation is predicted to help reduce the need for synthetic fertilizers and other chemicals, encouraging sustainable and environmentally friendly agriculture.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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