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ORIGINAL PAPER

# *Thiobacillus* Bacteria-Enhanced Iron Biofortification of Soybean in a Calcareous Soil Enriched with Ferrous Sulfate, Mill Scale, and Pyrite

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## Abstract

The present study was conducted to investigate the possibility of using iron waste along with *Thiobacillus* bacteria to supply soybean iron requirement in a calcareous soil. In vitro, two strains of *Thiobacillus thiooxidans* (*T. thiooxidans*) and *Thiobacillus ferrooxidans* (*T. ferrooxidans*) have been investigated for their bioleaching potential from mill scale and pyrite in in the presence and absence of sulfur. In a greenhouse experiment, the effect of iron sources (control, ferrous sulfate, mill scale, and pyrite) and bacterial inoculation (*T. thiooxidans*, *T. ferrooxidans*, and simultaneous application of two bacteria) on iron uptake by soybeans was investigated. In laboratory experiment, the effect of *T. ferrooxidans* on iron bioleaching from the studied iron waste was greater than *T. thiooxidans*. *T. ferrooxidans* was more effective to enhance the iron dissolution from pyrite than mill scale. The application of sulfur increased the bioleaching efficiency. In the greenhouse experiment, inoculation with *T. thiooxidans* caused a significant increase in shoot iron concentration of soybean compared to control only in the application of pyrite, while *T. ferrooxidans* significantly increased iron uptake by soybean in the application of all iron sources as well as control treatment. The highest shoot iron concentration of soybean was obtained in simultaneous application of two bacteria species. While the addition of the mineral and waste iron components did not impact on iron uptake by soybeans, soil inoculation with *T. ferrooxidans* and simultaneous application of *T. ferrooxidans* and *T. thiooxidans* had a significant effect on iron biofortification in soybean.

**Keywords** Biofertilizer · Bioleaching · Iron waste · Waste management · Plant nutrition

## 1 Introduction

Anemia caused by iron deficiency is a major public health problem worldwide due to low quality diet, mainly lacking in animal source foods and low iron contents in foods from plant sources (García-Bañuelos et al. 2014). In other words, agricultural products are an important source of iron for humans. Although iron is generally abundant in soils, it mostly occurs in forms that are not readily available to plants. Especially phytoavailability of iron is severely restricted in calcareous soil because of high pH, low organic matter, and high carbonate contents. Therefore, the fortification of crops with iron to improve human nutrition is necessary. In this regard, biofortification is an appropriate method to fortification of agricultural products.

Biofortification is defined as a process to improve food crop's nutritional quality through agronomic practices, conventional plant breeding, or modern biotechnology (Garg et al. 2018). In agronomic biofortification, mineral fertilizers apply to increase the concentration of nutrients in crops that may have extra effects for increasing yield (Cakmak, 2008; Adu et al. 2018).

But as mentioned above, in calcareous soils, the application of iron mineral fertilizers is usually ineffective. To overcome this problem, some strategies are used that are different in terms of efficiency and cost-effectiveness such as foliar application (Aziz et al. 2019; Niyigaba et al. 2019; Singh

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et al. 2018), nitrogen supply (Aciksoz et al. 2011; Singh et al. 2018), application of organic compounds and cropping systems management (Chen et al. 2019), using ammonium or sulfur containing fertilizers (Granja and Covarrubias, 2018), application of elemental sulfur (Klikocka and Marks, 2018; Bouranis et al. 2018), and using synthetic Fe chelates (Lucena, 2006) and nano iron fertilizer (Rui et al. 2016; Askary et al. 2017; Yang et al. 2020).

In this regard, the use of waste containing iron compounds such as steel industry by-products and iron ores can be cost-effective. Abbaspour et al. (2005) applied mixtures of converter sludge with sulfuric acid, organic matter, and elemental sulfur in some calcareous soils. The result showed that sludge application, especially acidified sludge, increased DTPA (diethylenetriaminepentaacetic acid) extractable Fe in soils and iron uptake by maize. The positive effect of steel converter sludge application with elemental sulfur on iron availability in calcareous soils also has been reported by Mohammadi Torkashvand (2011) and Karimian et al. (2012). Marsolek and Hagstrom (1982) showed that the application of acidified iron rich residue obtained in the process of copper extraction from copper bearing ore was effective to reduce the iron deficiency chlorosis. They stated the acidic nature of this product as an important factor in its effectiveness.

Pyrites, as a mining waste, has long been used to supply Fe for plants and S to improve soil quality (Wallace and Wallace, 1992). Castelo-Branco et al. (1999) reported that pyrite application to the calcareous soils increased nutrient availability and plant yield. Ortas et al. (2015) reported that the decrease in soil pH due to oxidation of pyrite increased the availability of phosphorus and zinc in wheat and maize cultivation. Reducing soil pH with the application of high rate of pyrite was reported by Nesheim et al. (1997).

In recent years, microbial biofortification method, as a green technology through effective microorganisms, has been raised (Khan et al. 2019; Prasanna et al. 2016). Among the beneficial microorganisms, the *Thiobacillus* strains, which has been used to increase bioavailability of nutrients particularly P, Fe, and Zn, can be mentioned (Besharati, 2017; Akhtar et al. 2012).

It seems that the use of iron waste along with the application of microbial biofortification technology can be more effective in increasing the availability of iron from these inexpensive compounds. The aim of this study was to investigate the effect of *Thiobacillus* bacteria on the availability of iron from ferrous sulfate, pyrite, and mill scale in a calcareous soil.

Mill scale is a generic term for steel-making by-products formed on the outer surface of plates, rolls, sheets, or profiles during the hot-rolling process. The total iron content of mill scale is averagely about 70% (Ndlovu et al. 2017), consisting of the mixed elemental iron and iron oxide mainly FeO (wustite), but also contain $Fe_2O_3$ (hematite), $Fe_3O_4$ (magnetite), and other oxides.

## 2 Materials and Methods

Mill scale and pyrite were obtained from Kabakan steel mill of Mashhad and Sarcheshmeh copper mine of Kerman, respectively. Elemental composition of these two iron compounds was determined by XRF (X-ray fluorescence) method. Laboratory-grade ferrous sulfate was obtained from Merc Co. For use in laboratory and greenhouse experiments, mill scale and pyrite were sieved to pass through 125-µm mesh sieve after crushing.

### 2.1 Preparing Bacterial Cells

Mesophilic *Acidithiobacillus thiooxidans* PTCC No: 1692 (DSM 504) and *Acidithiobacillus ferrooxidans* PTCC No: 1646 (DSM 583) bacteria were obtained from the Persian Type Culture Collection (PTCC) center. The medium used for cultivation and reproduction of bacteria was offered by this center.

*Thiobacillus ferrooxidans* (*T. ferrooxidans*) has been grown in liquid medium containing the following: $K_2HPO_4$ (0.4 g), $MgSO_4.7H_2O$ (0.4 g), $(NH_4)_2SO_4$ (0.4 g), and $FeSO_4.7H_2O$ (33.3 g) per liter, and $H_2SO_4$ 0.1 N was used for adjusting the pH at 1.4. The culture medium of *Thiobacillus thiooxidans* (*T. thiooxidans*) contains the following: $K_2HPO_4$ (3 g), $MgCl_2.6H_2O$ (0.1 g), $NH_4Cl$ (0.1 g), $CaCl_2.2H_2O$ (0.14 g), and sulfur powder (10 g) per liter with adjusting pH to 4.2. The bacteria were inoculated on rotary shaker (150 rpm) at 30 °C for 10 days. After growing, the bacterial cells were separated by centrifugation at 5000 rpm and suspended in distilled sterilized water for using in laboratory and greenhouse experiments.

### 2.2 Laboratory Experiment

A batch-type bioleaching experiment was performed for the investigation of iron bioleaching potential of *T. thiooxidans* and *T. ferrooxidans* from pyrite and mill scale in the presence and absence of sulfur in a completely randomized factorial design with three replications. The experimental factors included two types of iron waste (pyrite and mill scale), three bacterial treatments (control, inoculation with *T. thiooxidans* and *T. ferrooxidans*), and sulfur at two levels (0 and 10 g $L^{-1}$). A total of 36 experimental units consisted of 100-mL glass Erlenmeyer flask, containing 50 mL of sterilized 9 K growth medium ($pH = 7.2$) and containing $(NH_4)_2SO_4$ (3 g), $MgSO_4.7H_2O$ (0.5 g), $K_2HPO_4$ (0.5 g), KCl (0.1 g), and Ca $(NO_3)_2$ (0.01 g) per liter that was prepared. For sulfur-containing treatments 10 g $L^{-1}$ sulfur powder and for iron source treatments 8.9 g $L^{-1}$ iron from pyrite or mill scale were supplied. Erlenmeyer flasks inoculated with approximately 2 mL volume of bacteria suspension containing about $10^7$ CFU $mL^{-1}$ of each species were incubated in a shaker incubator (180 rpm) at 30 °C for 15 days. McFarland's nephelometer method (McFarland, 1907) was used to estimate the number of cells per mL. Uniformly bacterial suspension was adjusted to 0.5 McFarland standard ($1.5\times10^8$ CFU $mL^{-1}$) and then diluted 1:10 (approximately $10^7$ CFU $mL^{-1}$). The pH and iron concentration was determined in the culture medium supernatant after centrifuging at 5000 rpm for 10 min by pH meter and atomic absorption spectrometry (PG990), respectively. For measuring the concentration of iron, the samples were acidified by the appropriate addition of nitric acid and 1% nitric acid was used for dilution.

### 2.3 Greenhouse Experiment

In the greenhouse experiment, the effect of soil inoculation with *T. thiooxidans* and *T. ferrooxidans* bacteria on iron uptake by soybean in a calcareous soil enriched with ferrous sulfate, mill scale, and pyrite was investigated in a completely randomized factorial design with three replications. The bacterial treatments included non-inoculated soil (*C*), inoculation with *T. thiooxidans* (*Tt*), inoculation with *T. ferrooxidans* (*Tf*), and simultaneous inoculation of *T. thiooxidans* and *T. ferrooxidans* (*Ttf*). Iron treatments included control (0 mg Fe) and 10 mg Fe per kg of soil from three kinds of iron sources (ferrous sulfate, pyrite, and mill scale). A loam agricultural soil with low available Fe (1.9 mg $kg^{-1}$) and 13% calcium carbonate equivalent (CCE) content was collected from 0 to 30 cm depth of the campus of Ferdowsi University of Mashhad, Razavi Khorasan province, Iran (36° 18′ 55.77″ N, 59° 31′ 34.11″ E). The soil was classified as Typic Haplocambid (Soil Survey Staff, 2010).

The electrical conductivity of saturated paste extract (ECe) and the pH of saturated paste of soil were measured 2.12 dS $m^{-1}$ and 7.65, respectively. Each kilogram of the soil consisted of 0.51 g total N, 3.5 g organic C, 7.0 mg available P, 151 mg available K, and 4.48 mg available Fe. Before applying the treatments, 40 mg $kg^{-1}$ of potassium sulfate, 80 mg $kg^{-1}$ of calcium phosphate, 12 mg $kg^{-1}$ of manganese sulfate, 20 mg $kg^{-1}$ of zinc sulfate, 120 mg $kg^{-1}$ of elemental sulfur powder, and 1% of composted cow manure were added to the soil. The bacterial suspension and iron treatments (10 mg Fe per kg of soil from ferrous sulfate, mill scale, and pyrite) were applied to the soil before planting. Bacterial treatments were inoculated with 30 ml of cell suspension for each pot (approximately $10^7$ CFU $mL^{-1}$). In simultaneous application of two bacteria, 15 ml per pot of each bacterial cell suspension (15 + 15) was added. Considering three replications, a total of 48 pots contained 3 kg of soil were prepared and maintained at filed capacity of moisture for a week. Due to the lack of soybean culture history in the studied soil, the soybean seeds (Katoul variety) were inoculated with native *Bradyrhizobium* bacteria. Ten pre-sprouted seeds were sown in each pot and then thin to 3 seedlings after germination. The pots were randomly arranged in the greenhouse under 30/22 °C temperature (day/night) and 14 h/10 h (light/dark) photoperiod and irrigated daily with deionized water to maintain the moisture at field capacity. The shoots and roots were separately collected from each pot after 60 days and rinsed with distilled water. Shoot and root dry weight was determined after drying at 65 °C. The oven-dried plant samples were crushed and passed through a 0.5-mm sieve. Dry ashing method at 500 °C was used for determination of Fe in plant material by atomic absorption spectrometry (PG990). After the plant harvesting and removing the roots, the soil of each pot air-dried, homogenized, and sieved to pass through a 2-mm mesh sieve. Soil pH in a 1:5 soil:water ratio and available Fe (Lindsay and Norvell, 1978) was determined in collected soil samples.

Analysis of variance was carried out using the MSTAT C software and significant difference between treatment means at the 5% level was determined using LSD test.

## 3 Results

The concentrations of chemical elements detected in mill scale and pyrite by XRF analysis are shown in Table 1. Approximately 99.5% of the mill scale contained iron oxides. Iron and sulfur content of pyrite was about 45.5% and 52%, respectively. The amounts of heavy metals in both compounds were negligible (Table 1).

### 3.1 Laboratory Experiment

The results showed that for both iron sources in non-inoculated treatments, the concentration of soluble iron was very low and the application of sulfur did not lead to significant increase in iron concentration (Table 2). In the application and non-application of sulfur, iron bioleaching from the studied compounds in inoculation with *T. ferrooxidans* (*Tf*) was much more than *T. thiooxidans* (*Tt*). Compared to *T. thiooxidans* in non-application of sulfur, *T. ferrooxidans* increased the concentration of soluble iron by 93.5 and 29.3 times in pyrite and mill scale treatments, respectively (Table 2). *T. ferrooxidans* was more effective in dissolving pyrite than mill scale. Inoculation with *T. ferrooxidans* in the application and non-application of sulfur increased the concentration of soluble iron by 2.29 and 5.47 times more in the pyrite treatment compared to the mill scale treatment.

**Table 1** LOI (loss on ignition) and chemical composition of mill scale and pyrite identified by XRF analysis

| | Compounds (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Si ($SiO_2$) | Al ($Al_2O_3$) | Na ($Na_2O$) | Mg (MgO) | K ($K_2O$) | Ti ($TiO_2$) | Mn (MnO) | Ca (CaO) | P ($P_2O_5$) | Fe ($Fe_2O_3$ + FeO) | S | LOI |
| Mill scale | nd | 0.01 (0.02) | nd | 0.06 (0.1) | nd | nd | 0.217 (0.28) | nd | 0.044 (0.1) | 70 (99.5) | nd | 0 |
| Pyrite | 0.26 (0.56) | 0.04 (0.08) | 0.067 (0.09) | nd | 0.008 (0.01) | 0.006 (0.01) | 0.008 (0.01) | 0.007 (0.01) | 0.009 (0.02) | 45.5 (Fe) | 52.2 | 0.52 |

Sulfur application had a positive effect on bioleaching efficiency and increased the percentage of Fe extraction from both studied iron compounds (Table 2). However, the addition of sulfur did not cause a remarkable increase in pyrite and mill scale dissolution in inoculated treatments with *T. thiooxidans*. Addition of sulfur had a great effect on increasing the iron bioleaching efficiency by *T. ferrooxidans* from pyrite as well as mill scale. The highest concentration of iron (6396.5 mg $L^{-1}$) was observed in the presence of sulfur and *T. ferrooxidans* in pyrite treatment. However, the effect of sulfur application on the increase of iron bioleaching from mill scale in the presence of *T. ferrooxidans* was higher than pyrite. Compared to non-sulfur treated samples, in the presence of sulfur, *T. ferrooxidans* inoculation increased the concentration of iron by about 2.67 and 6.36 times in pyrite and mill scale, respectively. In the presence of sulfur, the percentage of iron extraction from pyrite by *T. ferrooxidans* was about 71.8%, while in the same treatment for mill scale, this value was 31.3%.

The results showed that *Thiobacillus* bacteria inoculation led a significant reduction in pH and in the application of sulfur, pH decrease was more pronounced. So that in the application of sulfur in pyrite treatment, the solution of pH reduced from 6.22 to 1.35 and 1.75 by *T. thiooxidans* and *T. ferrooxidans*, respectively. This reduction for mill scale was from 6.91 to 1.32 and 1.78 in *T. thiooxidans* and *T. ferrooxidans* treatments, respectively (Table 2).

## 3.2 Green House Experiment

The results of greenhouse experiment showed that the application of ferrous sulfate, pyrite, and mill scale in inoculated and non-inoculated treatments had no significant effect on root dry weight and root nodulation of soybean. The effect of experimental treatments on shoot dry weight of soybean is shown in Fig. 1. Inoculation with bacteria and application of iron sources had no remarkable effect on the shoot biomass. The highest dry weight was related to ferrous sulfate treatment in the simultaneous application of two bacteria. This treatment also had the highest shoot iron concentration (Fig. 2). The simple main effect of inoculation with bacteria had a significant effect on shoot biomass of soybean. Simultaneous application of two bacteria species averagely increased the dry weight of the shoots by 12.7% compared to the non-inoculated treatments.

Iron concentration of soybean shoots was significantly affected by experimental treatments. Based on the results, in non-inoculated treatments, application of ferrous sulfate and mill scale did not have a significant effect on the concentration of Fe in shoots of soybean, and only application of pyrite (pyrite-*C*) significantly increased iron concentration by 13.6% compared to non-inoculated control treatment (control-*C*) (Fig. 2). However, iron concentration

**Table 2** The effect of iron compounds (pyrite and mill scale), bacterial treatments (*C* no inoculation, *Tt* inoculation with *T. thiooxidans*, *Tf* inoculation with *T. ferrooxidans*, *Ttf* inoculation with *T. thiooxidans* + *T. ferrooxidans*), and sulfur application (*S*- without elemental sulfur, *S* + using sulfur) on iron concentration and pH of the solution

| Iron compounds | Bacteria | Sulfur | Fe (mg/l) | Fe extraction (%) | pH |
|---|---|---|---|---|---|
| Pyrite | *C* | -S | 0.4 ± 0.2 | 0.005 | 6.37 ± 0.13 |
| | | + S | 1.0 ± 0.4 | 0.011 | 6.22 ± 0.03 |
| | *Tt* | -S | 25.7 ± 0.8 | 0.288 | 1.91 ± 0.01 |
| | | + S | 35.0 ± 4.2 | 0.393 | 1.36 ± 0.01 |
| | *Tf* | -S | 2400.0 ± 20.5 | 26.961 | 2.07 ± 0.04 |
| | | + S | 6396.5 ± 33.2 | 71.871 | 1.76 ± 0.02 |
| Mill scale | C | -S | 2.5 ± 0.9 | 0.028 | 6.96 ± 0.01 |
| | | + S | 5.2 ± 2.2 | 0.058 | 6.91 ± 0.08 |
| | *Tt* | -S | 15.0 ± 2.9 | 0.168 | 2.11 ± 0.04 |
| | | + S | 157.2 ± 9.2 | 1.767 | 1.33 ± 0.02 |
| | *Tf* | -S | 438.5 ± 11.4 | 4.926 | 2.51 ± 0.02 |
| | | + S | 2787.8 ± 24.4 | 31.323 | 1.79 ± 0.01 |

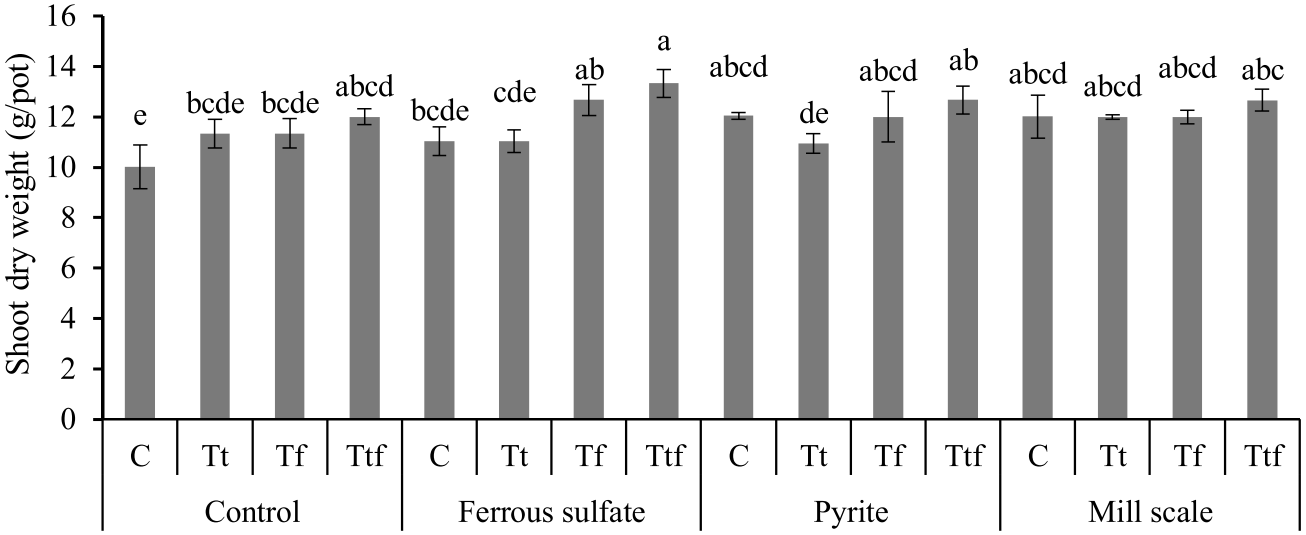

**Fig. 1** The effect of iron compounds (control, iron sulfate, pyrite, and mill scale) and bacterial treatments (C, no inoculation; Tt, inoculation with *T. thiooxidans*; Tf, inoculation with *T. ferrooxidans*; Ttf, inoculation with *T. thiooxidans* + *T. ferrooxidans*) on shoot dry weight of soybean. Different lowercase letters indicate significant differences between treatments ($P < 0.05$)

in this treatment was not significantly different from iron concentration in iron oxide and mill scale treatments.

According to Fig. 2, inoculation with *T. thiooxidans* had no significant impact on increasing iron concentration except in pyrite treatment. Compared to non-inoculated control treatment (control-*C*), inoculation with *T. thiooxidans* increased the shoot iron concentration in pyrite application by 25.6%. In all iron treatments (control, ferrous sulfate, pyrite, and mill scale), inoculation with *T. ferrooxidans* significantly increased iron concentration. The shoot iron concentration increased by 43.9% in control treatment inoculated by *T. ferrooxidans*. In control treatment, this bacterium was as effective in increasing iron as the pyrite treatment (Fig. 2).

In ferrous sulfate and mill scale treatments, the concentration of Fe was significantly influenced by inoculated with *T. ferrooxidans*, while shoot iron concentration was not affected by the addition of pyrite in the presence of this bacterium compared to the inoculated control treatment. In fact, the effect of *T. ferrooxidans* on increasing shoot iron concentration in using ferrous sulfate and mill scale was greater than pyrite (Fig. 2).

The results showed that simultaneous application of both studied bacteria had a positive impact on shoot iron concentration. Simultaneous inoculation increased the shoot iron concentration by 95.9%, 54.0%, and 89.2% compared to non-inoculated treatments and 28.2%, 23.3%, and 10.4% compared to inoculated treatments with *T. ferrooxidans* in

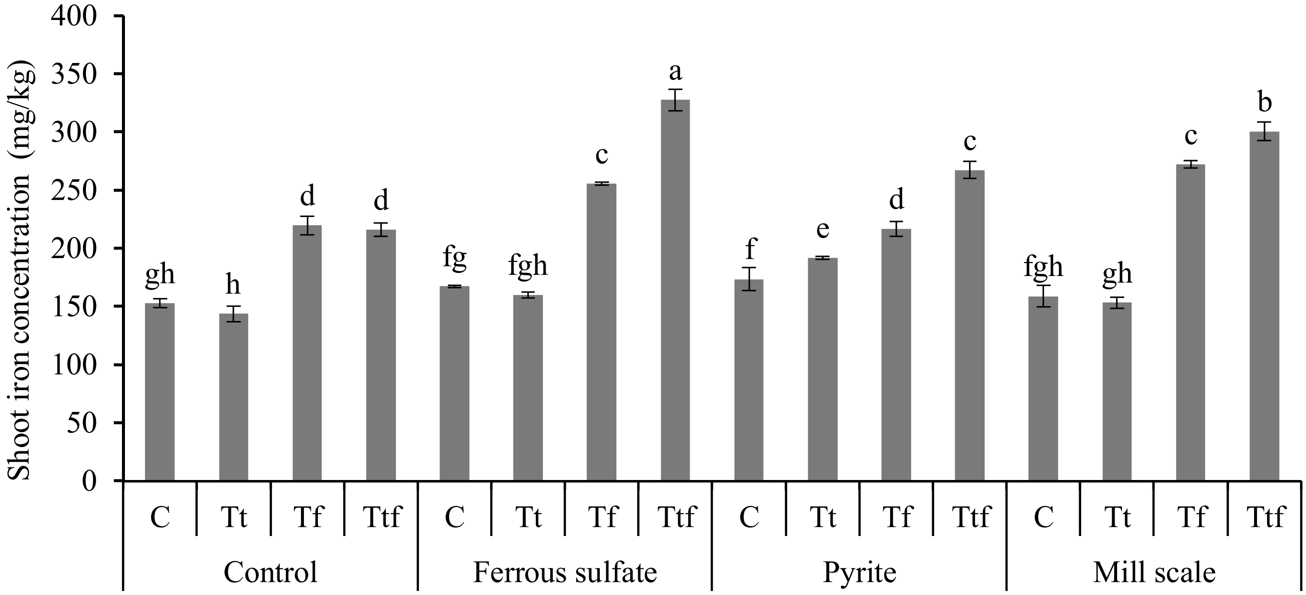

**Fig. 2** The effect of iron compounds (control, iron sulfate, pyrite, and mill scale) and bacterial treatments (C, no inoculation; Tt, inoculation with *T. thiooxidans*; Tf, inoculation with *T. ferrooxidans*; Ttf, inoculation with *T. thiooxidans* + *T. ferrooxidans*) on shoot iron concentration of soybean. Different lowercase letters indicate significant differences between treatments ($P < 0.05$)

application of ferrous sulfate, pyrite, and mill scale, respectively. The greatest effect of inoculation with *T. ferrooxidans* and *T. thiooxidans* was observed in using ferrous sulfate and then with a significant difference in mill scale application (Fig. 2).

Due to the fact that the experimental treatments did not exert a significant influence on the dry weight of plant shoots, a similar trend was observed for iron uptake by soybean and the shoots iron concentration (Figs. 2 and 3). Except that, in the application of pyrite, inoculation with *T. thiooxidans* did not have a significant impact on iron uptake compared to non-inoculated treatment (Fig. 3), while iron concentration showed a significant increase (Fig. 2).

The results showed that in the control treatment, bacterial inoculation did not cause a significant increase in soil available Fe (Fig. 4), while inoculation with *T. ferrooxidans* and co-inoculation with *T. thiooxidans* and *T. ferrooxidans*

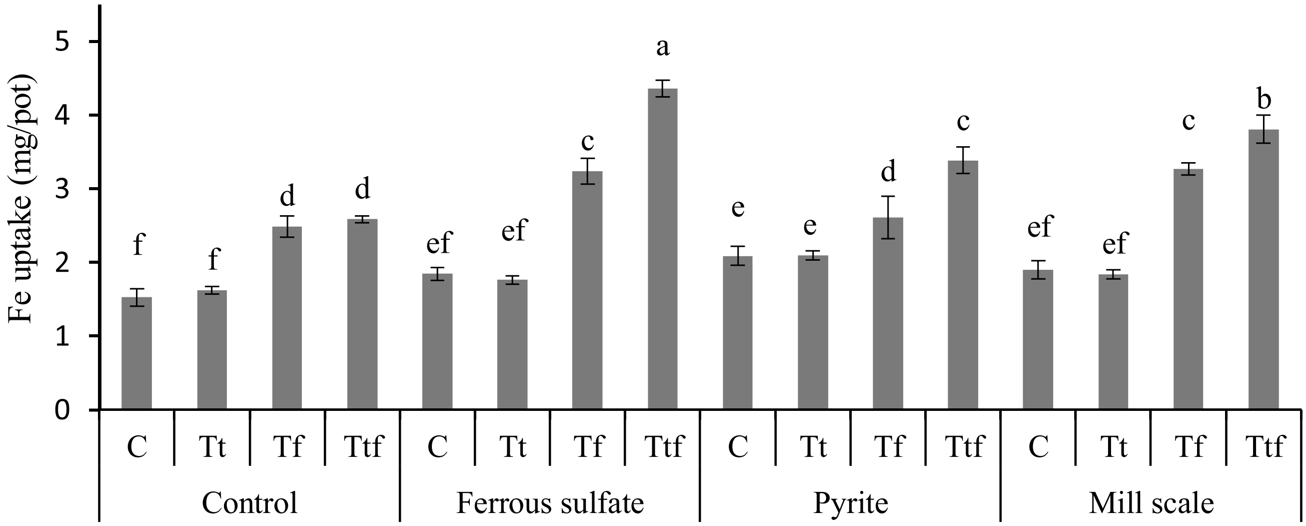

**Fig. 3** The effect of iron compounds (control, iron sulfate, pyrite, and mill scale) and bacterial treatments (C, no inoculation; Tt, inoculation with *T. thiooxidans*; Tf, inoculation with *T. ferrooxidans*; Ttf, inoculation with *T. thiooxidans* + *T. ferrooxidans*) on iron uptake by soybean. Different lowercase letters indicate significant differences between treatments ($P < 0.05$)

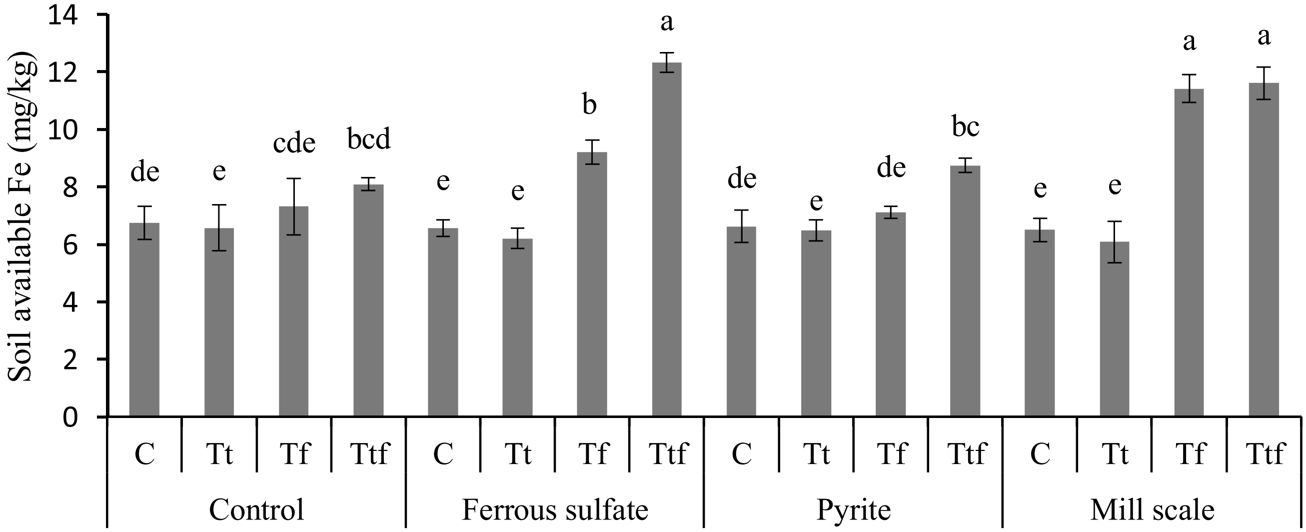

**Fig. 4** The effect of iron compounds (control, iron sulfate, pyrite, and mill scale) and bacterial treatments (C, no inoculation; Tt, inoculation with *T. thiooxidans*; Tf, inoculation with *T. ferrooxidans*; Ttf, inoculation with *T. thiooxidans* + *T. ferrooxidans*) on soil available Fe. Different lowercase letters indicate significant differences between treatments ($P < 0.05$)

significantly increased the uptake and concentration of iron in this treatment (Figs. 2 and 3).

In non-inoculated treatments, application of ferrous sulfate, pyrite, and mill scale exerted no significant effect on soil available iron compared to the control (Fig. 5). Inoculation with *T. thiooxidans* also did not increase the availability of soil iron in the application of these iron sources. Inoculation with *T. ferrooxidans* in control and pyrite treatments did not cause a significant increase in soil available iron (Fig. 4), while a significant increase in the concentration and uptake of iron was observed in these treatments (Figs. 2 and 3).

In application of ferrous sulfate and mill scale, inoculation with *T. ferrooxidans* increased the soil available iron by 36.3 and 69.2%, respectively (Fig. 4). The highest amount of soil available Fe was observed in simultaneous inoculation of two *Thiobacillus* species in ferrous

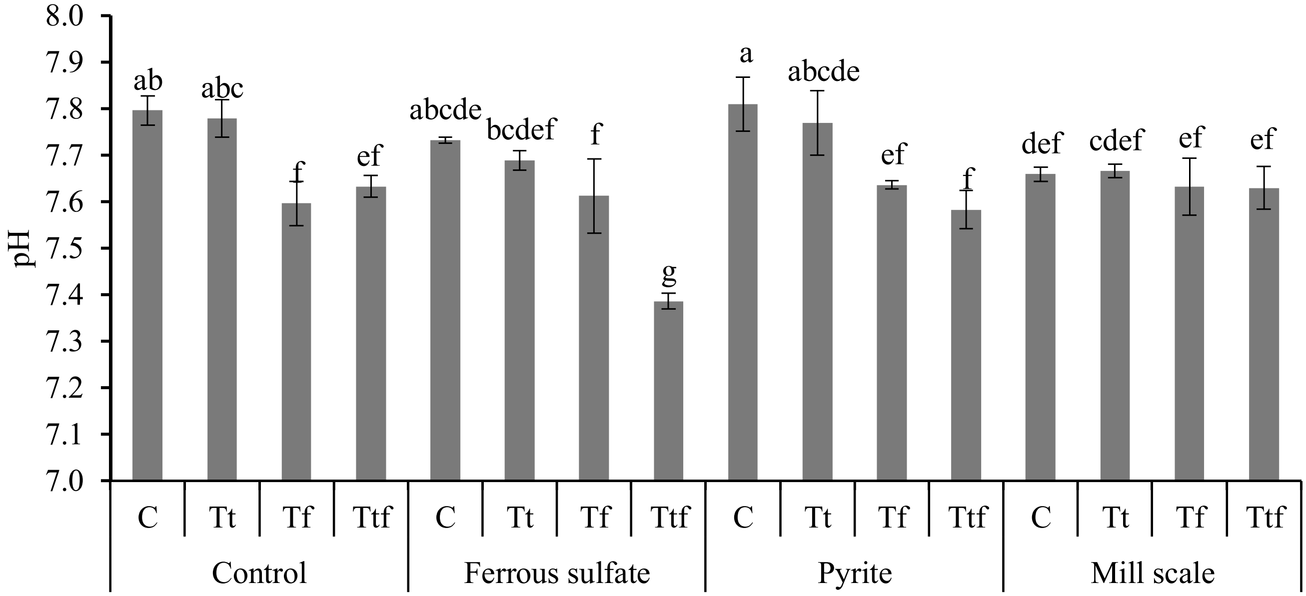

**Fig. 5** The effect of iron compounds (control, iron sulfate, pyrite, and mill scale) and bacterial treatments (C, no inoculation; Tt, inoculation with *T. thiooxidans*; Tf, inoculation with *T. ferrooxidans*; Ttf, inoculation with *T. thiooxidans* + *T. ferrooxidans*) on soil pH. Different lowercase letters indicate significant differences between treatments ($P < 0.05$)

sulfate treatment, which was not significantly different from mill scale treatments inoculated with *T. ferrooxidans* and co-inoculated with *T. thiooxidans* and *T. ferrooxidans* (Fig. 4), while the concentration and uptake of iron in these three treatments were significantly different (Figs. 2 and 3).

Although the changes in soil available Fe were not entirely consistent with changes in shoot iron concentration (Figs. 2 and 4), a significant positive linear relationship ($R^2 = 0.87$, $p < 0.01$) was observed between these two parameters (Fig. 6).

According to Fig. 5, *T. ferrooxidans* inoculation alone and mixed with *T. thiooxidans* decreased soil pH in all iron treatments. Soil inoculated with *T. thiooxidans* had no significant effect on soil pH in control, ferrous sulfate, and pyrite treatments. In application of mill scale in all bacterial treatments, soil pH significantly decreased compared to the non-inoculated control treatment. The lowest value of pH = 7.39 was observed in simultaneous application of both bacteria in using ferrous sulfate (Fig. 5). This treatment caused the highest concentration of iron in soybean shoots (Fig. 2). Although the experimental treatments had a significant influence on soil pH, the difference in pH between treatments was not remarkable and the maximum difference was 0.4 pH units. However, pH indicated a significant negative linear relationship with shoot iron concentration ($R^2 = 0.62$, $p < 0.01$) and soil available Fe ($R^2 = 0.49$, $p < 0.05$) (Figs. 7 and 8).

## 4 Discussion

### 4.1 Laboratory Experiment

The low concentration of iron in pyrite and mill scale treatments in non-inoculated bacterial treatments is due to the poor water solubility of these compounds. Inoculation with *T. thiooxidans* also had no considerable effect on iron bioleaching from pyrite and mill scale, while iron bioleaching efficiency in the application of *T. ferrooxidans* was remarkable. Bevilaqua et al. (2002) also reported that the oxidation of chalcopyrite by *T. thiooxidans* was negligible while *T. ferrooxidans* was quite effective. *T. ferrooxidans* is capable of deriving energy from the oxidation of ferrous ions. Therefore, oxidation of ferrous iron in pyrite and mill scale structure can lead to dissolution of these compounds and increase the concentration of soluble iron. Differences in chemical and physical characteristics of the studied iron compounds such as iron oxidation states, the presence of sulfur in mineral structure, particle size distribution, specific surface area, crystallization, purity grade, and interactions between bacteria and surface mineral can cause difference in their biological dissolution. The effect of *T. ferrooxidans* to improve the bioleaching efficiency of sulfide mineral especially pyrite has been reported in many studies (Jiang et al. 2007; Fowler et al. 1999; Rodríguez et al. 2003). While *T. thiooxidans* was unable to oxidize ferrous iron, both *T. thiooxidans* and *T. ferrooxidans* have a similar pathway for sulfur oxidation by utilizing molecular oxygen in aerobic condition. *T. ferrooxidans* can also use ferric iron to oxidize sulfur (Sugio et al. 1985; Espejo et al. 1988). The chemical

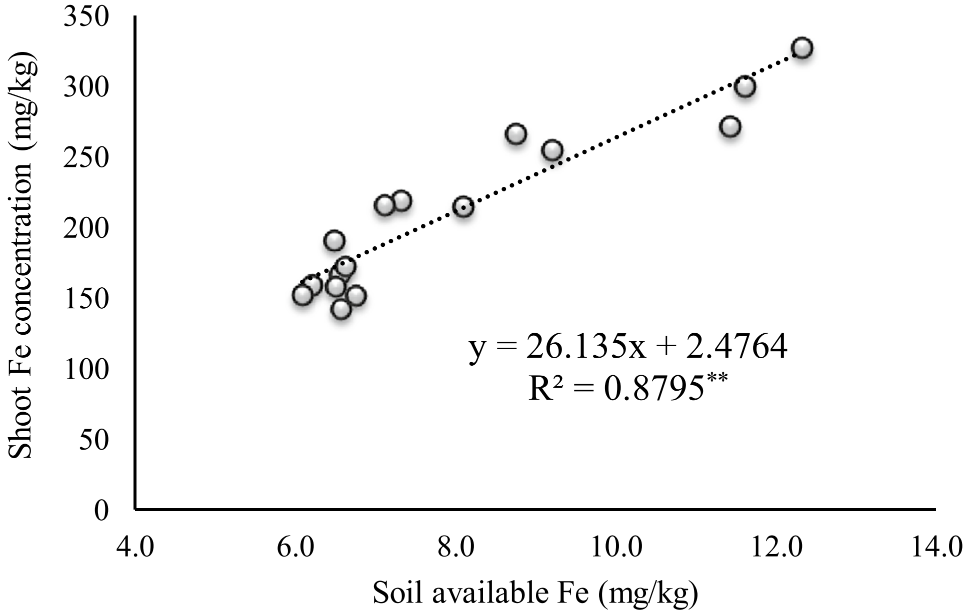

**Fig. 6** Relationship (linear regression) between shoot iron concentration of soybean and soil available Fe ($^{**}$significant at the 0.01 level)

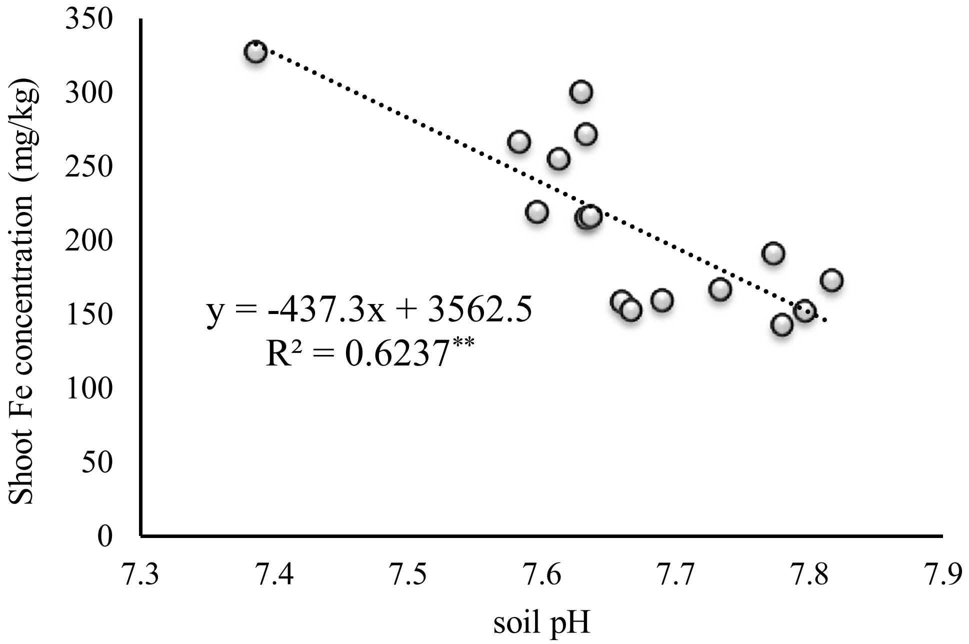

**Fig. 7** Relationship (linear regression) between shoot iron concentration of soybean and soil pH ($^{**}$significant at the 0.01 level)

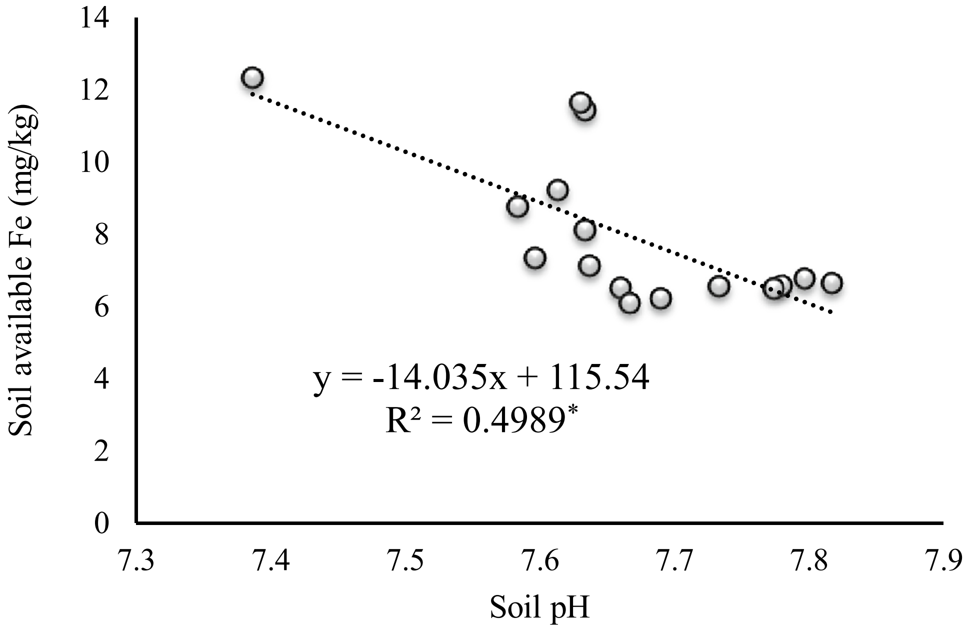

**Fig. 8** Relationship (linear regression) between soil available Fe and soil pH ($^{*}$significant at the 0.05 level)

and biological reaction during the bio-oxidation of pyrite is as follows (Jiang et al. 2007; Chandra and Gerson, 2010):

$$FeS_2 + 7/2O_2 + H_2O - - - - - - Fe^{2+} + 2SO_2^{2-} + 2H^+ \tag{1}$$

$$Fe^{2+} + 1/4O_2 + H - - bacteria - - - - - - Fe^{2+} + 1/2H_2O \tag{2}$$

$$FeS_2 + 14Fe^{3+} + 8H_2O - - - - - - 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \tag{3}$$

As shown in reactions 1, 2, and 3, the pyrite desolation is depending on the oxidation rate of ferrous ions and concentration of ferric ions. The oxidation of ferrous ions to ferric ions was catalyzed by *T. ferrooxidans* (Jiang et al. 2007). Biological regeneration of ferrous ions by *T. ferrooxidans* plays a key role in continued bio-oxidation of pyrite.

*T. ferrooxidans* are able to reduce ferric iron as an electron acceptor using elemental sulfur as electron donor. Presence of sulfur, oxidation state of iron, and ferric/ferrous ratio are important factors affecting the iron mineral oxidation by *T. ferrooxidans* (Jiang et al. 2007; Rodríguez et al. 2003).

Wu et al. (2019) showed that although adding elemental sulfur to the ferrous-containing medium caused the longer logarithmic phase of *T. ferrooxidans* growth, the final cell density was higher than the density of bacteria in medium without sulfur. Another important factor on bio-oxidation or biological dissolution of iron compounds is interactions between bacteria cells and surface minerals. Hosseini et al. (2005) attributed the difference in pyrite and chalcopyrite dissolution to the bacterial population bound to the mineral surface. In this regard, two different mechanisms, contact and non-contact, have been proposed. In non-contact or indirect mechanism, oxidation of soluble ferrous iron occurs by free cells or planktonic bacteria and involves the ferric-ferrous cycle. While in contact or direct mechanism, there is a physical connection between bacteria and mineral surface and redox reactions occur at mineral-bacteria interphase (Rohwerder et al. 2003). Rodríguez et al. (2003) stated cooperative bioleaching strategy that both free and attached to the mineral surface microorganisms is involved in the dissolution of pyrite. Bacterial attachment to minerals is a complex process. Interactions between bacteria and surfaces are controlled by many factors including mineral surface characteristics, surface properties of the cells via extracellular polymeric substances (EPS), and features of the culture medium such as, nutrients, pH, dissolved organic carbon, and type of bacteria. Extracellular polymeric substances (EPS) play a major role in bacterial attachment to the mineral surface (Sand and Gehrke 2006). The different substances in the bacterial culture medium affect the composition, functional groups, and the amount of EPS (Sharma et al. 2003; Devasia et al. 1993). Gehrke et al. (1998) reported that EPS from *T. ferrooxidans* mainly contain sugars in the presence of iron (II) sulfate while in the presence of sulfur, the major components were lipids. Therefore, it seems that addition of sulfur to the culture medium can affect the composition and amount of EPS and consequently the number of bacteria adhesions to mineral surfaces. In addition to the role of EPS in the electrostatic attachment of cell minerals, the importance of Fe (III) in EPS as a sulfide oxidizing agent at the cell-pyrite interface to enhance pyrite dissolution has been reported by Mitsunobu et al. (2016). Rapid attachment of both *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* bacteria to the pyrite surface was reported by Liu et al. (2011). Fowler et al. (1999) stated that the bacteria with increasing pH at the mineral surface enhance the solubility of pyrite.

Significant reduction in pH values were observed in inoculated treatments with *Thiobacillus* bacteria and the application of sulfur caused a further reduction. *Thiobacillus* bacteria can reduce pH in sulfur-containing minerals such as pyrite with oxidizing sulfur to sulfuric acid. Addition of elemental sulfur can lead to further reduction of pH. *T. ferrooxidans* also oxidize ferrous iron to ferric iron and chemical hydration of ferric iron produces additional hydrogen ions. Reduction of pH in mill scale treatment by *T. thiooxidans* can be the result of iron hydrolysis due to partial biochemical dissolution of this mineral. In *T. thiooxidans* inoculated treatments, a remarkable decrease in pH was observed. However, *T. thiooxidans* activity did not cause a significant increase in iron solubility, which indicates that the acidification of solution did not have a remarkable effect on dissolution of studied iron-containing minerals.

## 4.2 Green House Experiment

Some studies reported significant increase in plant growth after using different iron compounds (Tiwari et al. 1982; Purakayastha et al. 1998; Dubey and Mondal 1994; Bayat and Kaya 1998; Ortas et al. 2015; Tozsin, and Arol 2015). It seems that in this study, nodulation success by *Bradyrhizobium* and increasing soil fertility by adding sulfur and manure are the reasons for low impact of iron treatments on the shoot dry weight. Argaw et al. (2015) also reported that a non-significant effect of directly supplied of $FeSO_4$ (0 and 4 mg Fe $kg^{-1}$ soil) to the soil on nodulation and shoot biomass of soybean. They stated that genotype, high soil native N, and symbiotic effectiveness might be the cause of the ineffectiveness of iron application. Heitholt et al. (2003) also indicated that the application of $FeSO_4$ (0, 3, 10, 30, and 100 ppm Fe) had no effect on soybean biomass.

Without bacterial inoculation, the application of ferrous sulfate and mill scale had no impact on iron uptake by soybean compared to the control treatment. Probably, the use of pyrite as an energy source by chemoautotrophic bacteria such as soil native *Thiobacillus* species has been the reason for a significant increase of iron uptake and concentration only in pyrite treatment. Shenker and Chen (2005) stated that high soil pH, the presence of carbonates especially high calcium carbonate content, and high buffering capacity cause inefficiency or low efficiency of using iron mineral fertilizers such as ferrous sulfate on iron uptake by plants in calcareous soils. Although Mohammadi Torkashvand (2011) reported that the use of converter slag significantly increased shoot dry matter and Fe uptake by maize, the use of high amounts of converter slag (above 2%) seems to be the reason for increasing iron uptake by plant in this study. Wang and Cai (2006) showed that the application of steel slag at rates of 10 and 20 g $kg^{-1}$ increased corn dry matter yield and Fe uptake and extractable Fe in a calcareous soil. Wallace and Wallace (1992) reported that application of large amounts of pyrite can be used as useful fertilizers and soil amendments in crop production on alkaline calcareous soils by using ways to increase the oxidation rate. Joseph et al. (2014) reported that pyrite amendment alone and along with *T. ferrooxidans* improved yields and nutrient uptake in canola and wheat in a sulfur-deficient alkaline soil.

Based on the results, inoculation with *T. thiooxidans* only in the application of pyrite increased the concentration of iron in the plant. Oxidation of structural sulfur in this mineral is probably the reason for increasing its dissolution and consequently increasing the availability of iron, while *T. ferrooxidans* inoculation increased iron uptake by soybean in application of all studied iron sources as well as control treatment. In fact, without the use of any iron source, the effect of *T. ferrooxidans* on soybean iron uptake in the control treatment was significant. In this regard, *T. ferrooxidans* can be applied as biofertilizers to increase Fe uptake by plant in calcareous soils.

Low efficacy of *T. thiooxidans* compared to *T. ferrooxidans* was also observed in the laboratory experiment, and this bacterium did not have a remarkable effect on the solubility of pyrite and mill scale. In a soil bioleaching experiment, Ko et al. (2013) reported that the application of *T. ferrooxidans* generated lower soil pH and higher amount of $Fe^{3+}$ than *T. thiooxidans*.

The effect of *T. ferrooxidans* on increasing iron uptake in using ferrous sulfate and mill scale was greater than pyrite application. These results were inconsistent with the results of the laboratory section that *T. ferrooxidans* was more effective on pyrite dissolution than mill scale. The result is difficult to explain on the presented stage of experiments, although the impact of complex soil environment compared to controlled laboratory conditions may be the reason for the difference. Wallace and Wallace (1992) demonstrated that oxidation of pyrite in soil includes both chemical and biological processes and is affected by origin and crystallinity, particle size and purity of pyrite, and soil parameter such as pH, temperature, microbial activity, organic matter, and presence of phosphate. Pyrite oxidation processes under simulated calcareous soil conditions was studied by Lara et al. (2015). They reported that after the initial dissolution, meta stable siderite ($FeCO_3$)-like compound and subsequent jarosite and ferric oxyhydroxide were formed in pyrite surface depending on the surface acid condition reached in the systems. The formation of these secondary compounds was found to play a significant role in pyrite weathering.

Simultaneous soil inoculation with *T. ferrooxidans* and *T. thiooxidans* showed a synergistic effect on increasing iron uptake by soybean in all three studied iron sources. Liu et al. (2011) reported that the mixed culture of *T. ferrooxidans* and *T. thiooxidans* was more effective in pyrite solubilization than *T. ferrooxidans*. In addition, the effectiveness of simultaneous application of *T. ferrooxidans* and *T. thiooxidans* on metal bioleaching has been reported in some studies (Wang et al. 2009; Nguyen et al, 2015).

Soil pH is considered a major soil variable affecting iron availability. Due to the fact that calcium carbonate in calcareous soils causes a very high pH buffer capacity, the low pH changes in the experimental treatments were expected, although the addition of organic matter and sulfur to the studied soil before planting could be another reason for the low pH changes between the different treatments.

Simultaneous inoculation with *T. thiooxidans* and *T. ferrooxidans* had the greatest impact on soil available Fe as well as the shoot iron concentration and iron uptake by soybean. However, changes in soil iron alone were not sufficient to interpret changes in concentration and uptake of iron. This may be attributed to the rhizosphere effect. The effect of *Thiobacillus* bacteria on iron solubility may be limited to the rhizosphere area. Interactions between microbes and plant roots in rhizosphere can improve Fe availability by redox, complexation, and acidification processes (Colombo et al. 2014; Rengel 2015). Assuming that the effect of experimental treatments in the rhizosphere was greater than bulk soil, inoculation of seeds with bacteria may have similar results compared to soil inoculation, which should be considered. Bayat and Kaya (1998) stated that under field conditions, increasing wheat grain yield up to 25% due to application of pyrite may be as a result of decreased rhizosphere pH and as a consequence of increasing pyrite solubility. Awad et al. (2011) revealed that in soil inoculation with *Thiobacillus*, first the pH decreased and then increased over time and at the end of the growing period, the soil pH changes from 7.8 to 7.5. A decrease in soil pH from 7.2 to 7.0 has also been reported by Anandham et al. (2007).

Although in this study the effect of pyrite and mill scale application on the accumulation of heavy metals in plant and soil was not investigated, the effect of long-term use of these compounds on soil and plant contamination should be considered.

## 5 Conclusion

Microbial biofortification with application of effective microorganisms can be well used as a sustainable and economical way to address plant nutrient deficiencies. In the current study, the application of iron waste along with *Thiobacillus* bacteria exhibited effectiveness in increasing iron concentration in soybean shoots in a calcareous soil under greenhouse conditions. The results showed that *Thiobacillus ferrooxidans* was more effective than *Thiobacillus thiooxidans* on uptake of iron by soybean in the application of ferrous sulfate, mill scale, and pyrite. Simultaneous application of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* had a synergistic effect on the iron concentration of the plant. Based on the results, the use of iron waste along with *Thiobacillus* bacteria can be considered an agronomic biofortification method in plant iron nutrition in calcareous soils.

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## Declarations

**Conflict of Interest** The authors declare no competing interests.

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