

Can Improve Iron Biofortification Antioxidant Response, Yield and Nutritional Quality in Green Bean?

Juan P. Sida-Arreola¹, Esteban Sánchez^{1*}, Graciela D. Ávila-Quezada², Paul B. Zamudio-Flores¹, Carlos H. Acosta-Muñiz¹

¹Centro de Investigación en Alimentación y Desarrollo, Chihuahua, México

²Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua, Chihuahua, México

Email: *esteban@ciad.mx

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Abstract

The aim of this study was to evaluate the effect of iron biofortification on antioxidant response, yield and nutritional quality of green bean (*Phaseolus vulgaris* L.) under greenhouse conditions. Fe was applied using two forms (FeSO₄ and Fe-EDDHA) at four doses of application (0, 25, 50 and 100 µm) added under a hydroponic system, and were tested over a period of 40 days. The Fe content was assessed in seeds, as well as the activity of antioxidant enzymes, production of H₂O₂, yield and nutritional quality. The results being obtained indicated that the accumulation of Fe in bean seeds enhanced with the application of Fe-EDDHA at the dose of 25 µm. This demonstrated that low Fe application dose was enough to increase Fe levels in seeds of common bean. In addition, Fe-EDDHA application form at 50 µmol was the best treatment to improve crop yield. Respect to antioxidant system, chelated form of Fe (Fe-EDDHA) was more effective in the activation of antioxidant enzymes (CAT, SOD and GSH-PX), and a lower content of H₂O₂ in green bean seeds. Finally, to raise the Fe concentration in bean under biofortification program was a promising strategy in cropping systems in order to increase the ingestion of iron and antioxidant capacity in the general population and provided the benefits that this element offered in human health.

Keywords

Phaseolus vulgaris L., Antioxidant Activity, Biofortification, Iron, Nutritional Quality

*Corresponding author.

1. Introduction

Beans (*Phaseolus vulgaris* L.) are grown and consumed in nearly all the world. In many developing countries, 20% of the available proteins are provided by beans and represent also integral part of dietary protein for 50% of the world's population [1]. This crop is cultivated mainly in the American Continent and East Africa. Edible crops, such as beans, require certain concentrations of macro and micronutrients for optimal development and yield [2]. Biofortification is a prominent strategy that can increase levels of micronutrients in crops. Agronomic and genetic biofortification represent complementary agricultural approaches. It is considered that application of Fe fertilizers is a short-term solution and complement to plant breeding [3].

Today, there is a convincing evidence that especially foliar application of micronutrients fertilizers (such as selenium and zinc) are effective in improving their concentration in grain of *Phaseolus vulgaris* L. cultivars [4]-[6]. Respect to Fe biofortification, it appears that improving nitrogen nutritional status of plants promotes accumulation of Fe in grain. Therefore, the plant nitrogen status deserves special attention in biofortification of food crops with Fe. Studies under both field and greenhouse conditions have demonstrated that increasing N application (soil or foliar spray of urea) significantly improves shoot and grains Fe concentrations in gramineous species [3] [4] [7]. However, a role of Fe fertilizers and their application methods in improving Fe concentration in legumes is rarely studied.

Fe is an essential micronutrient [8] and cofactor of many antioxidant enzymes, and it can act as pro-oxidant [9]. It has been shown that many enzymes require Fe in order to function properly. In particular, Fe is present in the active site of enzymes on antioxidant pathway involved in the scavenging of reactive oxygen species (ROS) [10]. Production and activity of ROS have been reported frequently in several plants under different types of stresses [11]. Failure to quench ROS or subsequent propagation of chain reaction may cause oxidative stress and lead to injury. The generation of ROS such as superoxide anion radical (O_2^-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxide radical (OH) can damage many cellular components, including protein, membrane lipids and nucleic acids.

To face with increased levels of H_2O_2 , plants have evolved different enzymatic and nonenzymatic mechanisms [11]. These include free radical scavengers, such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (GPX), and the enzymes involved in the ascorbate-glutathione cycle [12]. The enzymatic processes basically involve dismutation of O_2 by SOD that generates another partially reduced oxygen species, like H_2O_2 [11]. H_2O_2 is generated in cells by the direct transfer of two electrons to the superoxide anion (O_2^-). This is mediated by enzymes such as glycolate and glucose oxidases, or by the dismutation of O_2 , which frequently occurs in chloroplasts and peroxisomes under conditions of high light stress. In the dark, or in non-photosynthetic tissues, H_2O_2 is produced mainly by leakage of the electron transport chain in mitochondria or microsomes [13] and during fatty acid oxidation [14].

Many researches have identified several beneficial effects of biofortification with micronutrients, which include antioxidant properties that can stimulate plant growth and protect plants against different types of abiotic stress [5] [6] [15]. It demonstrates that biofortification with micronutrients (such as selenium, zinc and iron) induces higher increases in enzymatic activities that detoxify H_2O_2 , as well as an increase in the levels of antioxidant compounds (ascorbate, glutathione, etc). Thus, this technology than promoting the application of micronutrients at low rates can be used to control the induction in bean plants of the antioxidant system, thereby improving crop yield, stress resistance and accumulation of antioxidant compounds in bean seeds [6] [15].

The aim of the present study was to analyze the effect of Fe biofortification, with different application rates and forms of Fe, on enzymatic activities of SOD, CAT and GSH-PX as bioindicators of the antioxidant system efficiency and improve fruit yield of bean plants (*Phaseolus vulgaris* L.).

2. Materials and Methods

2.1. Crop Handling

Seeds of green bean (*Phaseolus vulgaris* L., cv. Strike) were germinated and grown in a substrate mix (peat moss, vermiculite and perlite at a ratio 3:1:1) at an experimental greenhouse located in Delicias, Chihuahua, Mexico. Green beans seeds cv. Strike was acquired from the company Agrow Mexico in Mexico City. This variety of green beans is characterized as a kind of short cycle (60 days), erect bearing, consumed both green pods and dry grains, thrives in warm and cold climates, it is grown in not very saline soils is suitable to grow in

greenhouses. The temperature inside of the greenhouse was controlled at $25^{\circ}\text{C} \pm 4^{\circ}\text{C}$, relative humidity of 60% - 80%, and a photoperiod of 16/8 h (light/darkness). The plants were grown in individual pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height) of 8 L volume, filled with substrate mix. Throughout the growing cycle the bean plants received a growth nutrient solution composed of 6 mm NH_4NO_3 , 1.6 mm K_2HPO_4 , 2.4 mm K_2SO_4 , 4.0 mm $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.4 mm MgSO_4 , 5 μM Fe-EDDHA, 2 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.0 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 μM $(\text{NH}_4)_2\text{MoO}_7 \cdot 4\text{H}_2\text{O}$ and 0.5 μM H_3BO_3 (pH 5.5 - pH 6.0). For Fe biofortification, plants were subjected to different Fe treatments described in next experimental design section. These Fe treatments were applied in combination with the nutrient solution for 40 days (beginning 20 days after of seed germination). The application of the different treatments of Fe was through a hydroponic system for 40 days.

2.2. Experimental Designs and Treatments

In this study was used a completely randomized experimental design, with different forms of Fe at different concentrations. This were four treatments of Fe chelate, at 4 doses and 4 replicates per treatment: Fe-EDDHA (Fe-EDDHA) and FeSO_4 (FeSO_4) at doses of 0, 25, 50 and 100 μmol , respectively. Doses and application forms Fe were selected based on the study of Hermosillo-Cereceres *et al.* [6].

2.3. Plant Sampling

Plant materials were sampled at 60 days after germination, when the plants had a phenological phase of complete development and fruit maturity. The different organs of each bean plant were separated (leaf, petiole, stem, root, pod, seed) and washed three times with tap water and once with deionized water. These materials were blotted on filter paper. One part of plant material (plant fresh matter) was frozen using liquid nitrogen and stored at -30°C , this material was used for antioxidant enzyme assays (SOD, CAT, APX and GSH-PX) and H_2O_2 quantification. The rest of the plant material (plant dry matter) was dried at 65°C and used to determine the biomass, crop yield and Fe concentration in bean seed.

2.4. Biomass and Yield

Plant biomass was determined as the average dry weight of the entire plant and expressed as mg DW^{-1} . Yield was expressed as the mean weight of fruits per plant in grams of dry weight.

2.5. Iron Quantification in Bean Seeds

Fe concentration was determined by an Induced Plasma Optical Emission Spectrometer (Agilent Technologies 700 Series ICP-OES) with a complete dissolution of 0.5 g of sample with 6 ml of concentrated HNO_3 in an analytical microwave oven, START D (Milestone, Italy) at a temperature of 180°C during 38 minutes. The resulting solution was diluted to 50 ml with deionized water and the metal concentration determined by ICP-OES. Fe concentration was expressed in $\text{mg} \cdot \text{kg}^{-1}$ of dry weight [16].

2.6. Activity of Antioxidant Enzymes

2.6.1. SOD Assay

SOD (EC 1.15.1.1) activity was determined by its ability to inhibit the formation of nitro blue formazan from NBT according to two methods [17], with some modifications. Total soluble proteins were extracted from 0.5 g of frozen bean seeds ground in 5 ml of extraction buffer (50 mm of Hepes-HCl, at pH 7.6) with a mortal pestle. This homogenized was centrifuged at 11,500 rpm, for 20 min, at 4°C ; the supernatant liquid was separated by decantation and used for the enzymatic assay of SOD and total protein quantification. The supernatant was diluted at a proportion of 1:5 with the maceration buffer. For the SOD assay, it was used 0.1 ml de protein extract for each sample and mix with 5 ml enzymatic reaction buffer (50 mm Na_2CO_3 , pH 10.0), 13 mm methionine, 0.025% v/v, triton X-100, 63 μmol NBT, 1.3 μM riboflavin). The reaction was illuminated at PPFD of $380 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, for 15 min. Reactions without illumination were used as a control to correct for background absorbance. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. SOD specific activity was expressed as $\text{U min}^{-1} \cdot \text{g}^{-1}$ FW. One unit of SOD was defined as the amount required inhibiting the photo reduction of NBT by 50%.

2.6.2. CAT Assay

CAT (EC 1.11.1.6) activity was determined by spectrophotometrically following H_2O_2 consumption at 240 nm [18]. Total soluble proteins were extracted from 0.5 g of frozen bean seeds ground in 5 ml of extraction buffer (25 mM of HEPES-HCl, at pH 7.8) with a mortar and pestle. This homogenized was centrifuged at 11,500 rpm, for 20 min, at 4°C. The supernatant liquid was separated by decantation and then this extract was used for the enzymatic assay of CAT and total protein quantification. CAT activity was determined using the next reaction: 0.75 ml of $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer (25 mM, at pH 7), 0.75 ml of EDTA- Na_2 (0.8 mM), 1 ml of H_2O_2 (20 mM) and 0.5 ml of protein extract. The absorbance change was measured at a wavelength of 240 nm, for 3 min. This enzymatic assay was performed at 25°C. The results were expressed as specific activity ($\text{nmol H}_2\text{O}_2$ decomposed $\text{min}^{-1}\cdot\text{mg}^{-1}$ protein).

2.6.3. GSH-PX Assay

GSH-PX (EC 1.11.1.9) activity was measured using H_2O_2 as substrate [19]. Total soluble proteins were extracted from 0.5 g of frozen bean seeds ground in 5 ml of extraction buffer (25 mM of KNaH_2PO_4 , at pH 7) with a mortar and pestle. This homogenized was centrifuged at 11,500 rpm, for 20 min, at 4°C. The supernatant liquid was separated by decantation and used for the enzymatic assay of GSH-PX and total protein quantification. For the enzyme reaction, an aliquot of 0.2 ml of the protein extract was mixed with 0.4 ml GSH (0.1 mM) and 0.2 ml KNaH_2PO_4 (0.067 M). This mixture was preheating on water bath at 25°C, for 5 min. The above mentioned reagents without supernatant extract were used for the non-enzyme reaction. After preheating, 0.2 ml H_2O_2 (1.3 mM) was added to start the reaction. The reaction lasted 10 min and was stopped by adding 1 ml, 1% trichloroacetic acid and cooling at 4°C, for 30 min. Then the mixture was centrifuged for 10 min at 11904 g. 0.48 ml of the supernatant was placed into a cuvette and 2.2 ml of 0.32 M Na_2HPO_4 and 0.32 ml of 1.0 mM DNTB were added for color development. The absorbance at wavelength 412 nm was measured after 5 min. The enzyme activity was calculated as a decrease in GSH within the reaction time when compared with that in the non-enzyme reaction.

2.6.4. Protein Assay

Concentration of total soluble proteins extracted of bean tissue, which were used for the 3 enzyme assays (CAT, SOD and GSH-PX), was determined according to the manufacturer's protocol of Quick Start Bradford Protein Assay Kit (BioRad, USA). The standard protocol was performed with the next relation: 20 μl of sample and 1 ml of 1x Dye Reagent. The protein standard used for these protein assays was bovine serum albumin, provided in the kit (BSA Standard Set with 7 concentrations: 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 $\text{mg}\cdot\text{ml}^{-1}$).

2.6.5. H_2O_2 Quantification

The H_2O_2 content of seed samples was colorimetrically measured [20]. Seed samples were homogenized with 5 ml of cold acetone to extract the H_2O_2 . An aliquot (1 ml) of this extract was mixed with 0.5 ml of titanium tetrachloride 20% (v/v) and 2.5 ml of NH_4OH , centrifuged at 5000 g for 15 min and the supernatant was used to measure the absorbance at 415 nm (intensity of the yellow color). The H_2O_2 concentration was calculated according with Sánchez *et al.* [21], using a standard curve of H_2O_2 .

2.7. Statistical Analysis

Data were subjected to a simple ANOVA at 95% confidence, using SAS (SAS Institute Inc, Cary, NC). Means were compared by Tukey test ($P \leq 0.05$). The data shown are mean values \pm standard error (SE).

3. Results and Discussion

3.1. Effect of Fe Biofortification on Yield and Fe Accumulation in Bean Seed

Previous studies pointed out that Fe biofortification improves biomass and yield in legumes, such as soybean and beans [22]. It has also been found that when Fe is applied in the form of Fe-EDDHA is more effective in increasing biomass, that when this is applied in the form of FeSO_4 in these plants [23]. Our results coincide with those studies, showing an improvement in the amount of biomass according Fe levels were increased (Figure 1, $P \leq 0.05$) and yield increases notably when Fe-EDDHA form was applied (Figure 2, $P \leq 0.05$). In this study,

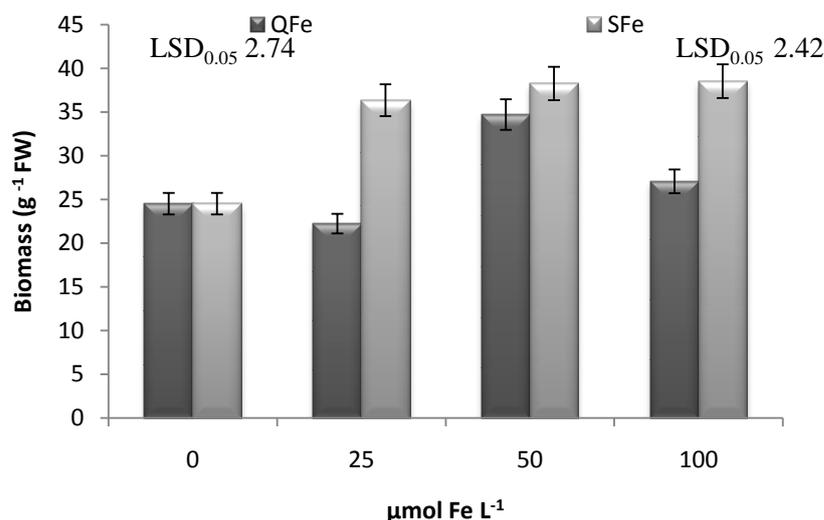


Figure 1. Biomass in plants of *P. vulgaris* L. cv strike subjected to different iron doses and forms. Data are means \pm SE ($n = 4$) ($P \leq 0.05$).

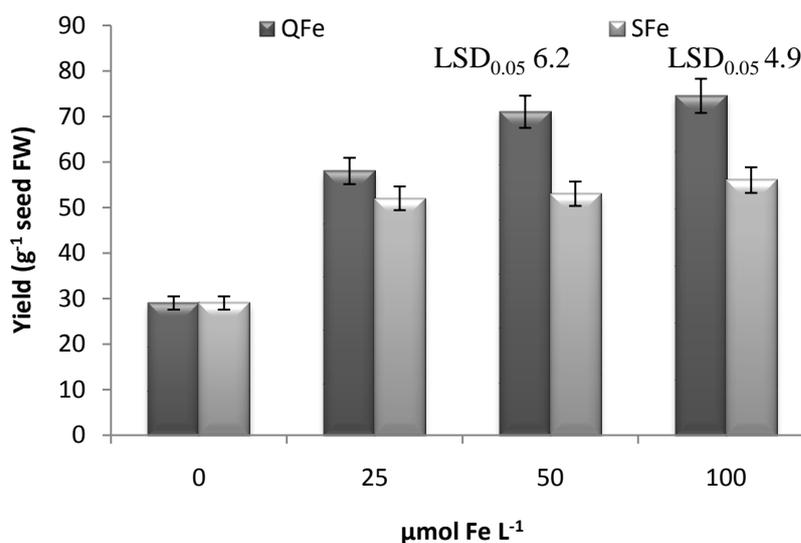


Figure 2. Yield in plants of *P. vulgaris* L. cv strike subjected to different iron doses and forms. Data are means \pm SE ($n = 4$) ($P \leq 0.05$).

there were significant differences in the Fe application form. Fe-EDDHA form, at 50 and 100 μmol doses, were significantly different with respect to the control, obtaining an increase in yield of 1.5 fold (Figure 2, $P \leq 0.05$).

Stress negatively affects growth and development in plants, generating ROS that damage macromolecules and cell structures. One of the most widely used indicators of stress is plant biomass [15]. Therefore, we studied this indicator of stress as a one way to define the effect of the different Fe treatments. In general, the results indicate that Fe-EDDHA is less toxic than FeSO_4 because the plants tolerated and responded positively to Fe-EDDHA that even enhanced growth up to rate of 25, 50, 100 μM . While this effect was not detected for FeSO_4 , the increases of biomass and crop yield were similar at rates of 25, 50 and 100 μM (Figure 1, $P \leq 0.05$).

Respect to Fe accumulation in seed, Fe biofortification induced the accumulation of this mineral in bean seed, with the two Fe forms applied (Figure 3, $P \leq 0.05$). However, our results indicate that biofortification with Fe-EDDHA was more effective in improving Fe accumulation bean seed. The dose of Fe applied to the lowest level in concentration (25 μM) produced a highly significant increase in the accumulation of Fe in bean seed, increasing 29% the seed levels of Fe, with respect to the control (Figure 3, $P \leq 0.05$). However, when Fe doses were applied at higher concentration (50 and 100 μM), we fail to increase the Fe accumulation in seeds (Figure 3,

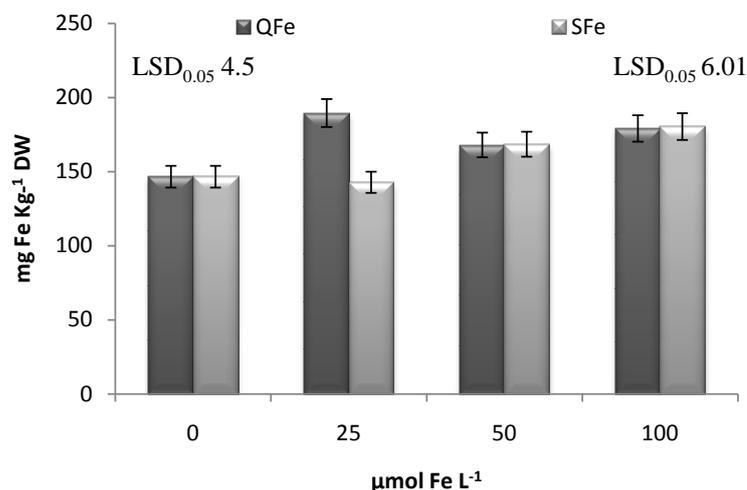


Figure 3. Fe concentration in bean seeds subjected to different concentrations and forms of Fe. Data are means \pm SE ($n = 4$) ($P \leq 0.05$).

$P \leq 0.05$). Therefore, we believe that these levels were toxic to these plants, so the plants did not accumulate more Fe in their seeds.

3.2. Effect of Fe Biofortification on Yield and Fe Accumulation in Bean Seed

Like other ROS, H_2O_2 accumulation is one of the causes of lipid peroxidation than attacks lipid membranes [24]. H_2O_2 is relatively “safe” in the absence of transition metals, being unreactive even at levels higher than biological system would ever generate. However, H_2O_2 is potentially quite reactive with molecules containing Fe^{2+} or other transition metals [25]. In previous studies of Se biofortification, it has been found that the content of H_2O_2 in seed can increase as increasing the doses of Se, which can be toxic levels and produce oxidative stress [15]. In this study, our results show a similar behavior with an increase in the H_2O_2 concentration as the Fe application rate increases (25, 50 and 100 μM of Fe). In addition, the H_2O_2 concentration detected is higher when the form of application used was $FeSO_4$ (Figure 4, $P \leq 0.05$). For these reasons, we assumed that $FeSO_4$ is more toxic than Fe-EDDHA in this bean crop, overall at a rate of 100 μM . In Figure 4, we can appreciate that these increases of H_2O_2 levels were notably high at 50 μM dose of Fe-EDDHA and 100 μM dose of $FeSO_4$, compared to the control. Fe-EDDHA at 50 and 100 μM doses had significant values of H_2O_2 in bean seed. Respect to $FeSO_4$ form, it was an increase of H_2O_2 concentration in more than two fold, compared to the control. These results indicate that studied plants were more tolerant to Fe-EDDHA than $FeSO_4$ improvement of stress tolerance to toxic levels of metals is often related to an increase in activity of antioxidant enzymes. Actually, early accumulation of H_2O_2 during metal exposition can signal an increase in antioxidant enzyme activities, which in turn protected plants from the oxidative stress caused by metals such as Fe. Thus, increased SOD, CAT and GSH-Px activities in bean seeds indicate that this specie of plant has the capacity to adapt to Fe toxicity by developing an antioxidant defense system.

CAT is a component of the plant antioxidant system, and controls ROS levels by removing the overproduction of H_2O_2 in their cells [26]. The effect of Fe application on CAT activity reported in this study, are in agreement with studies carried out in grasses plants. For example, it has been observed that doses of 50 and 100 μM of Fe applied to maize plants increased CAT activity in their seeds [27]. Our results are in line with this behavior, CAT activity shown an increase in bean seeds as the Fe doses were elevated, with both application forms of Fe ($FeSO_4$, Fe-EDDHA). However, $FeSO_4$ form produced a higher increase in the CAT activity of bean seed than Fe-EDDHA form, at the three doses used (Table 1, $P \leq 0.05$).

SOD is an important member of the cell protective antioxidant system. This enzyme catalyzes the dismutation of the superoxide anion into H_2O_2 plus molecular oxygen [28]. Similar to CAT, we found that SOD activity was increasing in bean seeds as higher Fe dose were used (25 and 50 μM), with the two forms of Fe used. These increases resulted highly significant with respect to controls. However, we did not detect an increase of SOD activity at the highest dose of Fe used (100 μM), for both compounds of Fe studied (Table 1, $P \leq 0.05$).

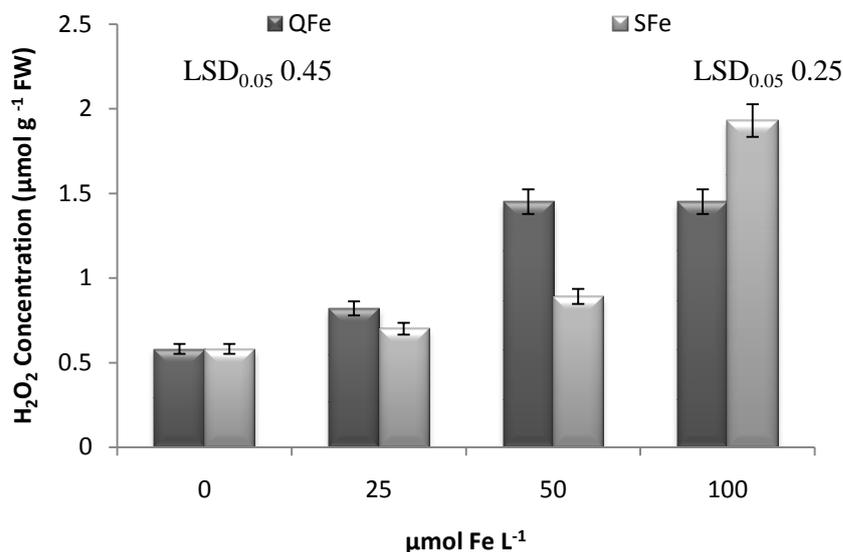


Figure 4. Hydrogen peroxide activity in seeds of *P. vulgaris* L. cv strike subjected to different iron doses and forms. Data are means \pm SE ($n = 4$) ($P \leq 0.05$).

Table 1. CAT, SOD and GSH-PX activities in bean seeds to different concentrations and forms of Fe.

Treatments	CAT ($\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \cdot \text{g}^{-1} \text{ FW}$)		SOD ($\text{U min}^{-1} \cdot \text{g}^{-1} \text{ FW}$)		GSH-Px ($\text{nmol GSH min}^{-1} \cdot \text{g}^{-1} \text{ FW}$)	
	Fe-EDDHA	FeSO ₄	Fe-EDDHA	FeSO ₄	Fe-EDDHA	FeSO ₄
Doses Fe (μmol)						
0	1.20 \pm 0.02 a	1.20 \pm 0.02 d	1.72 \pm 0.03 b	1.72 \pm 0.03 b	1.97 \pm 0.19 a	1.97 \pm 0.19 a
25	1.39 \pm 0.19 a	3.32 \pm 0.19 c	2.56 \pm 0.07 a	2.28 \pm 0.10 a	1.88 \pm 0.26 a	1.93 \pm 0.17 a
50	1.76 \pm 0.32 a	5.25 \pm 0.17 b	2.81 \pm 0.13 a	2.07 \pm 0.05 a	1.40 \pm 0.08 b	1.53 \pm 0.44 a
100	2.02 \pm 0.49 a	6.26 \pm 0.10 a	1.40 \pm 0.07 b	1.15 \pm 0.01 c	1.69 \pm 0.12 ab	1.41 \pm 0.23 a

CAT, catalase; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; FW, fresh weight. Data are means \pm SE. Means followed by different letters in each column are significant at $P < 0.05$ ($n = 4$).

GSH-PX is another enzyme responsible of the detoxification of H₂O₂ produced by SOD, through transformation of superoxide ion [29]. Compared to the increase in enzymatic activity detected for CAT and SOD, we did not detect such behavior with the activity of GSH-PX in bean seeds, cultivated through biofortification with two forms of Fe. Regarding the form of Fe that was applied as FeSO₄, we were unable to detect statically significant differences when we increased the dose of Fe at concentrations of 25, 50 and 100 μM (Table 1, $P \leq 0.05$). For the case of Fe-EDDHA form, we registered a small decrease in GPX activity at doses of 50 and 100 μM (Table 1, $P \leq 0.05$). In future projects, it would be necessary to confirm these results that focus on the study of another class of plant peroxidases, involved in this antioxidant system.

4. Conclusion

In conclusion, the increased SOD, CAT and GSH-PX activities in the bean seeds indicate that this specie has the capacity to adapt to Fe toxicity by developing an antioxidant defense system. In this study, the antioxidant system has significantly improved with respect to control, which can explain the beneficial effect of Fe found in plants subjected to diverse abiotic stress. In addition, we can indicate that the effect of Fe on this system depends largely on the form in which this trace element is applied. In bean seeds, application of FeSO₄ produces a higher increase in the activity of the antioxidant enzymes than Fe-EDDHA form at the doses studied. The biofortification with Fe increases significantly the accumulation of Fe in bean seeds, and we considered that the best treatment is the application of Fe-EDDHA at 25 μM dose, which increases in high percentage of the levels of Fe in bean seeds and crop yield of this cultivar. In general, these results will contribute to define the utility and application of Fe biofortification, which promote the application of micronutrients at low rates. This technology has a great potential to control the induction of the antioxidant system in bean plants, thereby improving crop yield,

stress resistance and accumulation of antioxidant compounds in bean seeds. Finally, to raise the Fe concentration in bean under biofortification program is a promising strategy in cropping systems in order to increase the ingestion of iron and antioxidant capacity in the general population and provide the benefits that this element offers in human health.

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