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RESEARCH ARTICLE

Biofortification of cowpea beans with iron: iron´s influence on mineral content and yield

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Abstract

Iron (Fe) deficiency is the most prevalent nutrient deficiency worldwide. Agronomic biofortification is an agricultural strategy for improving the micronutrient concentrations in staple food plants. At present, fertilization is a major vehicle for changing plant mineral contents and food quality. A greenhouse study was conducted to assess the effects of iron chelate and ferrous sulfate applications on the biofortification of Fe and its impacts on the mineral content and yield of cowpea beans. Four application rates of both forms were tested (0, 25, 50, and 100 µM L^{-1}) for 40 d. The amount and type of Fe application affected the mineral seed content, yield and yield components. Applying of Fe in the form of ferrous sulfate at 25 µM L^{-1} was found to be the optimal rate for biofortifying the cowpea bean plant, because it favored the seed yield and increased the bioavailable Fe content in the seeds over that of the control. The best iron chelate rate was 100 µM L^{-1}. Thus, it was considered feasible to implement an Fe fertilization program to improve the nutritional quality of cowpea bean crops by increasing the Fe concentrates in the seeds.

Keywords: *Vigna unguiculata*, iron chelate, minerals, ferrous sulfate

1. Introduction

Grain legumes (also known as pulses) occupy an important place in global food and nutrition, and they are an important constituent of the diets of a very large number of people. Legumes are rich not only in proteins but also in other nutrients such as starch, oil, vitamin, and minerals (Katoch, 2013). Most of the nutritional requirements of the rural population are still met by legumes, which constitute an important source of protein.

The cowpea bean (*Vigna unguiculata* [L.] Walp) is a dicotyledonous plant belonging to the order Fabales, family Fabaceae, Faboideae, Phaseoleae tribe, subtribe Phaseolinea*,* and genus *Vigna*. It is used in different parts of the world in traditional and cultural practices and, therefore, its cultivation is restricted to specialized geographical pockets in different agro-ecological regions, primarily by poor farming communities that derive their sustenance and livelihood from such crops (Carvalho *et al.*, 2012). As a result, the cowpea is commonly referred to as 'poor man's meat', particularly among the inhabitants of rural areas and urban slums in Tabasco, Mexico. The grains are processed into different types of food products such as tamalitos, bean tortillas, and soup. Green, immature cowpea pods are harvested and sold in local markets for consumption as a vegetable.

Iron (Fe) is an essential micronutrient for plants and for humans, and it is a constituent of a number of important macromolecules, including those involved in respiration, photosynthesis, DNA synthesis, and metabolism (Briat, 2011). Plants obtain Fe from the soil, where Fe exists in either the ferrous (Fe^{2+}) or ferric (Fe^{3+}) ionic state. Although Fe is the fourth most abundant element in the earth's crust, it is poorly bioavailable in soil because it binds rapidly to soil particles and forms insoluble complexes under aerobic conditions at a neutral or alkaline pH (Gómez-Galera *et al.*, 2010). There are estimates that some 3 billion people worldwide are afflicted by Fe deficiency; this deficiency is known to be particularly common in populations that depend on staple crops as the primary food and have little or no access to animal products (White and Broadley, 2005).

Agronomic biofortification is defined as the process of increasing the concentrations of essential elements in the edible portions of staple food plants through soil application, foliar application, by adding the elements to irrigation water (fertilization) or genetic improvement. This strategy was developed as a food-based method to address widespread deficiencies in Fe and Zn that remain prevalent to the greatest extent in low income countries (Sadeghzadeh and Rengel, 2013; Mao *et al.*, 2014). Micronutrient malnutrition, which is also known as "hidden hunger", is a major public health issue in most parts of the world and affects more than two billion people (Welch and Graham, 2004). More than 60% of the world's population is Fe deficient (Amarakoon *et al.*, 2012).

Research to develop biofortified foods is ongoing and has largely focused on increasing the Fe content of the world's most important staple food crops including maize [*Zea mays* (L.)] (Ortega-Blu and Molina-Roco, 2007), wheat [*Triticum sativum* (L.)] (Aciksoz *et al.*, 2011), common beans [*Phaseolus vulgaris* (L.)] (Blair, 2013), barley [*Hordeum vulgare* (L.)], and jerry oats [*Avena sativa* (L.)] (Bilski *et al.*, 2012) because of the high prevalence of deficiencies associated with this micronutrient in the developing world. Therefore, the aim of the experiment was to assess the effects of iron chelate and ferrous sulfate applications on Fe biofortification and to understand its impacts on the mineral content and yield of cowpea beans under greenhouse pot conditions.

2. Materials and Methods

2.1. Crop design and plant sampling

The pot experiment was conducted at the Academic Division of Agricultural Science of Juárez Autonomous University of Tabasco from October 2013 to January 2014. The black plastic pots used in the experiment were 30.0 cm in height, and 30.0 cm in diameter, and they were filled with an inert substrate (Tepetzil). Cowpea bean [*Vigna unguiculata* (L.) Walp 'De Castilla'] seeds were germinated and grown under greenhouse conditions (26-34 °C, 86-94% relative humidity) at a density of 44,444 plants per hectare. Throughout the growing cycle, each pot received 1 L of nutrition solution. During the first 20 d after sowing, the seedlings were kept in nutrient solution that was diluted to 20% of the original solution, which was prepared on the basis of a Hoagland and Arnon (1950) solution with 14 mM NO_3^-, 1 mM $H_2PO_4^-$, 2 mM SO_4^{2-}, 6 mM K^+, 4 mM Ca^{2+}, and 2 mM Mg^{2+}, and then modified to fit the Fe rates of the experiment. The solution was replaced every 3 d. The micronutrients in the nutrient solution were supplied with a commercial soluble trace elements mix (Tradecorp AZ). The pH value of the nutrient solution was maintained at 5.5 and the electrical conductivity was adjusted to 2.0 dS·m^{-1}. The irrigation water was classified as having a low salinity and a low sodium content (C1S1); E.C: 1.3 dS m^{-1}, pH: 7.0; cations (mM L^{-1}): Ca^{2+} = 4.6, Mg^{2+} = 1.3, K^{+} = 0.2, Na^{+} = 3.0 and anions (mM L^{-1}): HCO^{3-} = 4.6, Cl^{-} = 4.0, and SO_4^{2-} = 0.0. Four Fe rates (0, 25, 50, and 100 µM L^{-1}) were tested in a completely randomized experimental design with six replications. Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and iron chelate (Fe-EDDHA) were used as Fe sources, and they were added to the nutrient solution for 70 days beginning at 20 days after sowing.

2.2.Determining mineral nutrients

The seed material was thoroughly cleaned and ground in a Willey mill to pass through a 2-mm mesh and stored in airtight bags for further analysis. Following Wolf (1982), the N, P, K, Ca, Mg, Fe, Mn, Zn, and Cu were mineralized by wet digestion. For this process, 0.2 g of dry cowpea beans was ground and mineralized with $12H_2SO_4$ and 30% H_2O_2, with free P, at a temperature from 275 °C to 300 °C. Following the resulting mineralization, and after the addition of 20 mL of deionized H_2O, the mineral nutrients were determined as describe above.

The total N concentration and total crude protein content (N × 6.25) were determined by using the standard micro-Kjeldahl method (AOAC, 2002). The total P concentration was determined by using the colorimetric nitrovanadomolybdate method, and the total K concentration was analyzed by flame photometry (Alcántar and Sandoval, 1999). The total Mg and Ca concentrations were quantified by atomic absorption spectrophotometry, as were the micronutrients Fe, Cu, Mn, and Zn. All measurements were run in triplicate.

2.3. The physical characteristics of the seeds and their yields

The axial dimensions of length, width, and thickness for 25 whole healthy seeds were measured with a digital slide caliper with an accuracy of 0.01 mm (Mitutoyo). One hundred seeds from each treatment were weighed separately by using a digital electronic balance with an accuracy of 0.01 g (Sartorius 6124), and the total weight was recorded as the 100-seed weight. The plants were harvested 90 days after sowing, and the yield was expressed as the mean seed weight per plant. The collected cowpea beans were weighed on each plant at sampling. The seed yield was computed to have a 13% moisture content.

2.4. Statistical methods

The statistical significance for each treatment was assessed by using an orthogonal contrast test provided by the statistical package SAS 9.2 for Windows (SAS Institute, 2009).

3. Results and Discussion

3.1. Mineral content

Plants are the principal source of minerals for most of the Earth's population. In the present study, the highest crude protein and N contents were found in plants treated with 50 µM L^{-1} ferrous sulfate and 100 µM L^{-1} iron chelate with an increase of 5.0% and 3.6%, respectively, over the control (Figure 1A-1B). These values are frequently reported in the literature for cowpea bean seeds, and they range from 16.4% to 29.0% and 2.6% to 4.6%, respectively (Carvalho *et al.*, 2012). In comparison with the control, the P content increased by 12.9% and 23.3% in plants treated with 50 µM L^{-1} ferrous sulfate and 50 µM L^{-1} iron chelate (Figure 1C), respectively, whereas the K content declined by 10.07% and 12.9% with 25 and 100 µM L^{-1} ferrous sulfate, respectively, and 11.1%, 17.7%, and 10.3% with 25, 50, and 100 µM L^{-1} iron chelate, respectively (Figure 1D). However, the Ca and Mg contents increased by 28.5% and 8.8%, respectively over the control, with 25 µM·L-1 ferrous sulfate (Figure 1E), and 25.0% and 13.6%, respectively, with 50 µM L^{-1} iron chelate (Figure 1F). In this sense, low seed concentrations of N, P, K, Mg, Ca and S affect the levels of protein, fats, vitamins, antinutrientes, and other factors, in seeds (Welch and Graham, 2005).

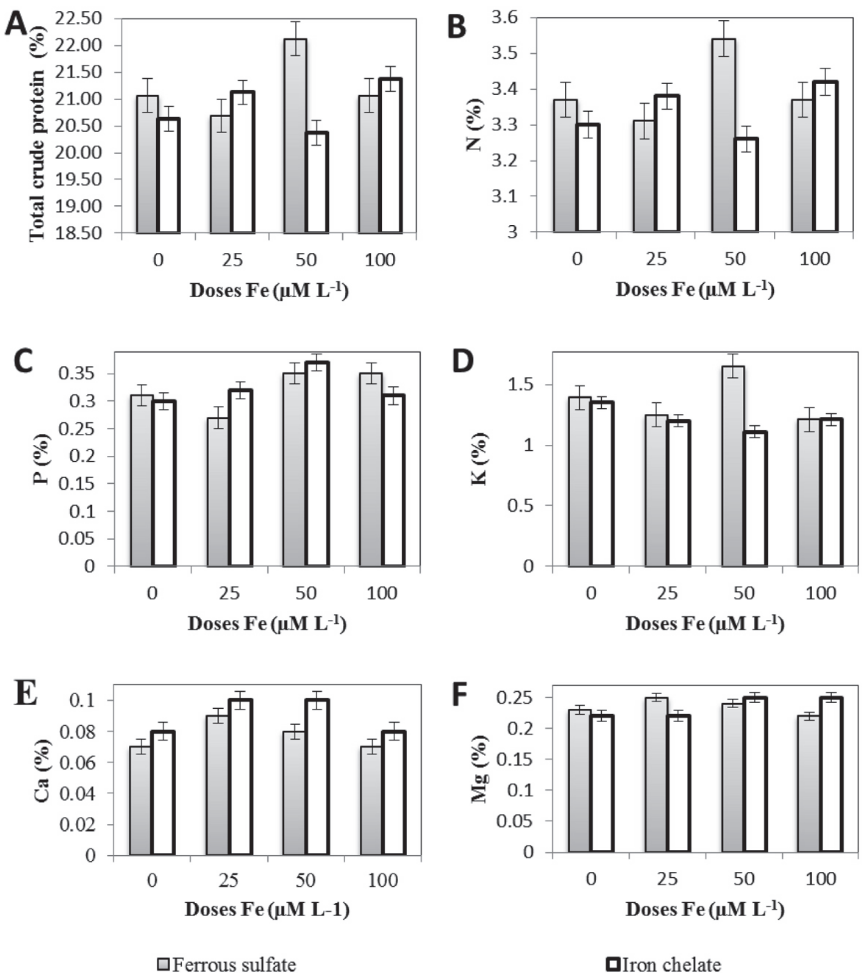

Figure 1. The total crude protein (A), nitrogen (B), phosphorus (C), potassium (D), calcium (E) and magnesium (F) contents in the seeds of the cowpea bean cultivar De Castilla in response to different application rates of ferrous sulfate and iron chelate. The data are ± the standard error (n = 4) and are significantly different from the initial values at $P < 0.001$ based on the orthogonal contrast test.

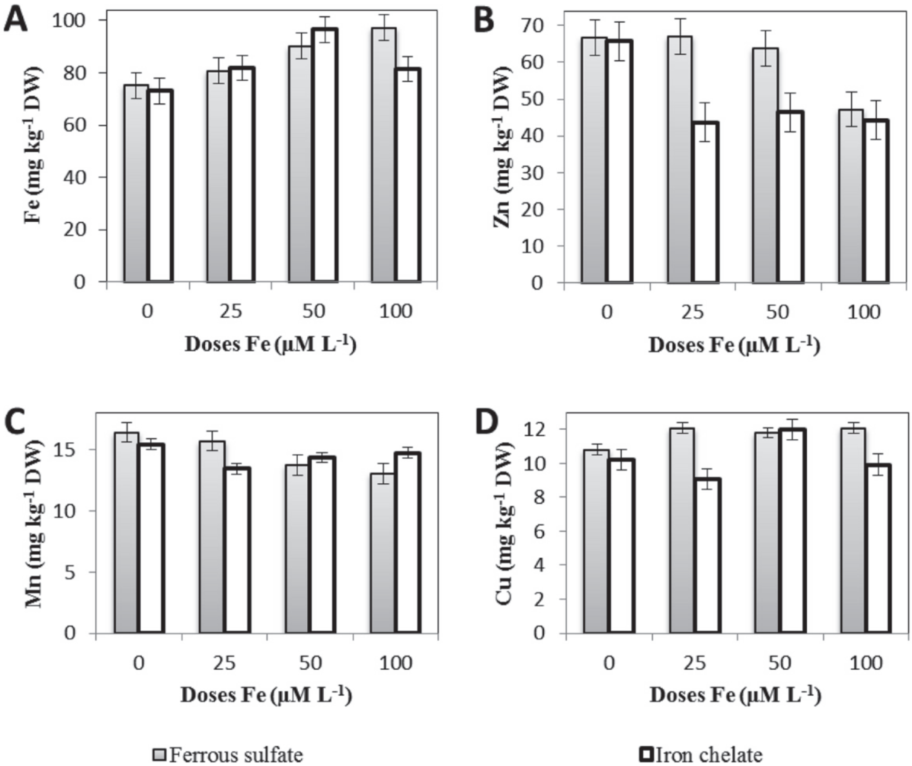

Figure 2. The iron (A), zinc (B), manganese (C) and copper (E) contents in the seed of cowpea bean cultivar De Castilla in response to different application rates of ferrous sulfate and iron chelate. The data are ± standard error (n = 4), and they are significantly different from the initial values at P < 0.001 based on the orthogonal contrast test.

In the present study, the concentration of cationic macronutrients in the seed tissue was less pronounced than it was in the results of a study by Espinosa-Moreno *et al*. (2013), who found that the seed N, P, K, Ca, and Mg contents were 3.5%, 0.35%, 1.52%, 0.28%, and 0.22%, respectively, with especially pronounced results for Ca and K. Because of its poor phloem mobility, Ca is known to be predominantly deposited in the vegetative tissues of maturing plants (Marschner, 2002).

Plants are the principal source of dietary Fe for most of Earth's population, and Fe deficiency can lead to major health problems. In our study, the Fe content had values varying from 73.1 to 97.2 ppm, and the highest Fe content was found in plants treated with 100 µM L^{-1} ferrous sulfate and 50 µM L^{-1} iron chelate, with an increase of 29.4% and 32.0%, respectively, over the control (Figure 2A). The results reported here demonstrated that agronomic biofortification through fertilization could significantly increase the Fe concentration in cowpea beans. A similar observation was reported previously by Aciksoz *et al*. (2011), Ortega-Blu and Molina-Roco *et al*. (2007), and Bilski *et al*. (2012). However, Aciksoz *et al*. (2011), noted that one major problem impairing the success of Fe soil or fertilization application is the rapid conversion of

Fe into unavailable forms when applied to calcareous soils and the poor mobility of Fe in the phloem, and thus soil Fe fertilization appears to be less effective than foliar Fe fertilization (Welch and Graham, 2005). The Zn content declined by 20.1% and 29.2% in plants treated with 50 and 100 µM L^{-1} ferrous sulfate, and 33.6%, 29.3%, and 32.5% with 25, 50, and 100 µM L^{-1} iron chelate, respectively, over the control (Figure 2B). In this sense, lower macronutrient concentrations (e.g., P and N) in seeds may sometimes be associated with a lower seed Zn concentration (Rengel, 2002). In comparison with the control, the Mn content declined by 20.7% and 12.9% with 100 µM L^{-1} ferrous sulfate and 25 µM L^{-1} iron chelate, respectively (Figure 2C). However, the B content increased by 4.2% with 50 µM L^{-1} ferrous sulfate and by 25.6%, 25.6%, and 18.1% with 25, 50, and 100 µM L^{-1} iron chelate, respectively, over the control (Figure 2D). The acquisition of metal ions, such as Fe, is important for plant survival. Iron has special importance because its ability to change redox states, making it an indispensable cofactor that is responsible for the function of electron transporter chains and catalytic processes (Briat, 2011). However, Fe over accumulation may lead to the overproduction of reactive oxygen species, resulting in cellular damage, necrosis, and, potentially, death. Therefore, the amount of Fe taken up by the roots must be strictly controlled (Ravet *et al.*, 2009). The re-partition of Fe between the various parts of a plant depends both on the physiological roles that are fulfilled by the metal ion and on the physiological function of the tissues. In this sense, the concentrations of free Fe^{2+} and Fe^{3+} in plant tissues are low because Fe cations are either incorporated into enzyme proteins or complexed with low-molecular-weight organic compounds (Briat, 2011).

Studies on wheat plants showed that raising the grain protein concentration increased the storage capacity for Fe and Zn (Cakmak *et al.*, 2010; Kutman *et al.*, 2010), supporting the idea that the grain capacity for accumulating Fe is largely influenced by the amount of grain protein (Gomez-Becerra *et al.*, 2010). These results and observations support the suggestion that the seed protein content helped to increase the seed Fe content of plants in this study.

3.2. The physical characteristics of the seeds and the yield

Trace elements are also important for the proper development of humans and plants, and they are normally required in small quantities. Fe is involved as a redox-active metal in photosynthesis, mitochondrial respiration, nitrogen assimilation, hormone biosynthesis, the production and scavenging of reactive oxygen species, osmoprotection, pathogen defense, and as a limiting factor for biomass production (Briat, 2011).

The ranges of the cowpea bean seed dimensions were 7.1 to 8.3 mm long, 5.3 to 5.9 mm wide, and 4.1 to 4.8 mm thick (Table 1), and the 100 seed weights varied from 12.0 to 15.0 g, which was consistent with measurements from the literature (Giami, 2005; Firouzi and Alizadeh, 2012). In the present study, the highest pods per plant, seeds per plant, and seed yield were found in plants treated with 25 µM L^{-1} ferrous sulfate and 100 µM L^{-1} iron chelate, with an increase of 80.0%, 48.7%, and 45.9%, respectively over the control, for ferrous sulfate and 13.6%, 19.3%, and 4.4%, respectively, for iron chelate over the control (Table 1). This finding exceeded the overall average yield of 13.0 g per plant obtained by local farmers by 283.8% and 206.2% (SIAP, 2013). Our results show a decline of 18.1 and 43.6% in the seed yield with 50 and 100 µM L^{-1} ferrous sulfate. This finding may be explained by the results obtained by Hemantaranjan and Garg (1988), who mentioned that increasing the Fe fertilization caused an initial increase in the *Triticum aes-*

tivum (L.) yield followed by a decline as the amounts of Fe fertilizer continued to increase.

The results presented here may have important implications for human nutrition. Iron deficiency is a growing public health problem in human populations that is associated with reduced dietary Fe intake (Cakmak *et al.*, 2010). There is an urgent need to improve the Fe concentrations in food crops to minimize Fe deficiency-related health problems in human populations. According to the results obtained in the present study, fertilizing with ferrous sulfate and iron chelate could improve the Fe concentration and seed yield significantly.

Table 1. The physical characteristics of seed and yield components for the cowpea bean cultivar De Castilla in response to different application rates of ferrous sulfate and iron chelate.

Treatment	Rates	Seed dimensions (mm)[a]			100 seed weight	Pods	Seeds	Seed yield
	($\mu M \cdot L^{-1}$)	Length	Width	Thickness	(g)[b]	per plant	per plant	(g per plant)
1. $FeSO_4$	0	7.7 ± 0.4	5.7 ± 0.2	4.8 ± 0.2	15.0 ± 1.9	20.0 ± 4.0	279.0 ± 88.0	34.2 ± 6.8
2. $FeSO_4$	25	8.2 ± 0.4	5.9 ± 0.1	4.6 ± 0.2	13.0 ± 2.6	36.0 ± 6.0	414.0 ± 114.0	49.9 ± 6.7
3. $FeSO_4$	50	7.1 ± 0.5	5.3 ± 0.6	4.1 ± 0.5	14.0 ± 4.2	20.0 ± 8.0	241.0 ± 158.0	28.0 ± 10.0
4. $FeSO_4$	100	7.7 ± 0.3	5.3 ± 0.6	4.3 ± 0.4	13.0 ± 2.3	13.0 ± 7.0	163.0 ± 63.0	19.3 ± 9.3
5. Fe-EDDHA	0	7.7 ± 0.4	5.7 ± 0.2	4.8 ± 0.2	15.0 ± 1.9	22.0 ± 4.0	280.0 ± 88.0	34.3 ± 6.8
6. Fe-EDDHA	25	7.6 ± 0.3	5.4 ± 0.1	4.3 ± 0.1	12.0 ± 4.0	25.0 ± 11.0	334.0 ± 177.0	35.8 ± 13.3
7. Fe-EDDHA	50	8.3 ± 0.6	5.7 ± 0.6	4.7 ± 0.4	12.0 ± 2.8	26.0 ± 7.0	332.0 ± 47.0	39.8 ± 5.1
8. Fe-EDDHA	100	8.1 ± 0.5	5.5 ± 0.5	4.5 ± 0.3	12.0 ± 1.2	31.0 ± 28.0	423.0 ± 326.0	47.3 ± 37.6
Treatment comparisons (P-values of linear orthogonal contrast)								
1 vs 2 + 3 + 4		0.333	0.652	0.194	0.144	0.885	0.441	0.518
2 vs 3 + 4		0.089	0.550	0.260	0.733	0.724	0.705	0.490
3 vs 4		0.610	0.724	0.364	0.767	0.588	0.493	0.561
5 vs 6 + 7 + 8		0.898	0.582	0.055	0.753	0.700	0.940	0.949
6 vs 7 + 8		**0.027**[C]	**0.049**	0.100	0.670	**0.034**	0.078	**0.029**
7 vs 8		0.161	0.929	0.629	0.882	0.458	0.560	0.503

4. Conclusions

Our experimental results suggest that the mineral seed contents and yields in cowpea plants are most affected by Fe fertilizer applications. Under the experimental conditions, ferrous sulfate at 25 $\mu M\ L^{-1}$ and iron chelate at 100 $\mu M\ L^{-1}$ could significantly improve the Fe concentration in cowpea beans and favored the seed yield over that of the control. In addition, the high ferrous sulfate rates caused a dramatic reduction in seed yield. Finally, it would be feasible to implement an Fe fertilization program in Tabasco to improve the nutritional quality of cowpea bean crops by increasing the Fe concentrates in the seeds.

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