Expression analysis and biochemical characterization of beans plants biofortificated with zinc

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Abstract The present work was carried out in greenhouse conditions at the Centro de Investigación en Alimentación y Desarrollo AC in Delicias, Chihuahua, México. Four different concentrations (0, 25, 50 and 100 μM L⁻¹) of Zn chelate and sulfate were used to study the antioxidant system of Phaseolus vulgaris L. Three genes related with antioxidant activity [superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT)] were selected for expression study. Results showed that when Zn chelate at 50 and 100 μM L⁻¹ were applied SOD was repressed and GSH-Px expression was low at 0, 25 and 100 μM L⁻¹ while with sulfate form SOD expression was low and GSH-Px expression was strong in all treatment. CAT was highly expressed in all forms and treatments. For a biochemical study the same enzymes were spectrophotometrically measured. SOD activity shows differences in both forms of Zn, chelate form was different at 25, 50 and 100 μM L⁻¹ with less activity at 100 μM L⁻¹ and sulfate treatment shows differences in all concentrations used. GSH-Px activity shows significant differences with sulfate form at 25, 50 μM L⁻¹ where at 50 μM the activity was higher and low at 100 μM L⁻¹, CAT does not exhibit significant differences but with chelate treatment at 50–100 μM L⁻¹ the activity was higher compared to sulfate. Finally, to raise the Zn concentration in bean under biofortification program is a promising
strategy in cropping systems in order to increase the ingestion of zinc and antioxidant capacity in the general population and provided the benefits that this element offered in human health.

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1. Introduction

Zn is necessary for the activity of more than 100 enzymes involved in several metabolic routes and consequently participates in various biochemical and immunological functions. Zn deficiency affects physical development, immune system, reproductive function and neuro development. For children these deficiencies are manifested as little gain in weight and height, diarrhea, anorexia and neurological problems. Additionally, the deficiency of this element is associated with increases in mortality (Hotz and Brown, 2004).

Nowadays, an innovative approach to solve the problems of micronutrients malnutrition is called “biofortification” (Bouis, 2003). Biofortification has been defined as a process where the concentration of bioavailable essential elements in the edible parts of the crop plants increases through crop management (fertilization).

To ensure the effectiveness of Zn biofortification, the concentration should be established not only to prevent deficiencies of this element in the fruits, but also to maintain the GSH-Px, SOD and CAT enzymes at a high level, and deficiencies of this element in the fruits, but also to maintain concentration should be established not only to prevent management (fertilization).

Few studies have focused on gene expression of the enzymes mentioned in biofortification processes. Comprehension of the responses of gene expression of antioxidant enzymes is important for future understanding of the molecular factors that control the antioxidant defense in the bean plants, for this reason the objective of this research is to analyze the temporal expression and enzyme activity of SOD, GSH-Px and CAT in bean plants (Phaseolus vulgaris L.) biofortified with Zn-sulfate and Zn-chelate.

2. Materials and methods

The present work was carried out in greenhouse conditions at the Centro de Investigación en Alimentación y Desarrollo AC in Delicias, Chihuahua, México. Seeds of bean (Phaseolus vulgaris L. cv. Strike) were germinated in a chamber at 28 °C for 48 h. After this period, the plants were grown in a greenhouse. For 10 days after transplantation and prior to application of the experimental treatments, the plants received a complete Hoagland nutrient solution suited by Sanchez et al. (2004); the experimental treatments, the plants received a complete Hoagland nutrient solution suited by Sanchez et al. (2004); the nutrient solution was renewed every three days.

Subsequently, 10 days after germination the following zine treatments were applied (for 50 days) in the forms of sulfate and chelate: T1 = 0 μM, T2 = 25 μM, T3 = 50 μM, T4 = 100 μM.

The intermediate leaves were collected after 60 days, after complete crop cycle, and were used to perform biochemical and molecular analyses. The fruits were also harvested for yield determination.

2.1. Molecular analyses

The total RNA was extracted using the RNA purification kit GenElute™ RNA/DNA/Protein Plus.

The first cDNA strand was synthesized using the SuperScript III System (Invitrogen Life Technologies, USA). A polymerase chain reaction (PCR) was carried out in three steps of one cycle each at 94 °C for 5 s, 55 °C for 30 s and 68–72 °C for 1 min.

Differential expression of genes encoding proteins widely related to oxidation mechanisms in plants (SOD, GSH-Px, and CAT) were analyzed using RT-PCR. The primers were designed using the Primer Express 2.00 (Applied Biosystems software), based on the sequences obtained from the database of the National Center for Biotechnology Information (NCBI) (Table 1). RT-PCR was performed in a BioRad standard cycle programmer system.

The amplification consisted of one cycle for denaturation at 94 °C for 2 min, 40 cycles of annealing at specific temperature for each gene (Table 1) for 30 s, 58 °C for 30 s and extension at 72 °C for 1 min followed by a cycle of final extension at 72 °C for 2 min. The results were visualized in agarose gel 1%.

2.2. Biochemical analyses

2.2.1. Biomass and bean yield (P. vulgaris)

Plants biomass was determined as the average dry weight of whole plants and expressed as mg.DW −1. Yield was expressed as dry weight of fruits per plant (in grams).

2.2.2. GSH-Px assay

The activity of GSH-Px (EC 1.11.1.9) was determined spectrophotometrically as described by Rao et al. (1997). Activity was measured by following the decrease in absorbance at 340 nm and expressed as μmoles of NADPH oxidized (mg prot) −1 (min) −1.

2.2.3. CAT assay

Total catalase (CAT; EC 1.11.1.6) activity was determined in a spectro-photocolorimeter as described by Sánchez et al. (2000). The activity was determined by monitoring the degradation of H2O2 at 240 nm during 1 min against a sample without plant extract. The enzyme activity was expressed in μmoles of H2O2 (mg prot) −1 (min) −1.

2.2.4. SOD assay

Extraction and quantification of the SOD (EC 1.15.1.1) was performed as established by Sánchez et al. (2004). The activity of this enzyme was measured by optical density at 560 nm, based on the inhibition of the photochemical reduction of Nitro Blue Tetrazolium (NBT). The enzyme activity was expressed in units of SOD (mg prot) −1 (min) −1.
2.2.5. Statistical analyses

The experimental design consisted of random distribution of the different treatments and their repetitions. Each treatment had four repetitions with four plants treated by repetition. All data were subjected to analysis of variance. LSD test was used at 95% (SAS, 2001) for the difference between means.

3. Results and discussion

In bean plants the biomass increases with significant differences at 0 (control) and 25 \( \mu\)M L\(^{-1}\) when Zn chelate was used (Table 2), and in the control with respect to the others treated with Zn sulfate (Table 2).

With respect to yield the results were similar to those of the biomass where the higher values were at 50 and 100 \( \mu\)M L\(^{-1}\) and when Zn chelated was employed, significant differences were found (Table 2). Zn sulfate form induces significant differences between the higher concentrations (50 and 100 \( \mu\)M L\(^{-1}\)) and the lowers one (0 and 25 \( \mu\)M L\(^{-1}\)). The lower yields were obtained in the control (0 \( \mu\)M L\(^{-1}\)) with both forms of Zn used (Table 2).

The increase in biomass and yield when the crop was fertilized with this nutrient indicate that biofortification is an appropriate and necessary method for bean plants, this is also reflected in low yields and biomass obtained in control (no nutrient). These results are similar to those obtained by Weldua et al. (2013) when bean plants were fertilized with Zn in the maturation phase (plants per pot) and yield and biomass significantly increased.

To study the temporal expression of SOD in leaves of beans with different concentrations of Zn sulfate and chelate, the transcript related to rRNA 18S was analyzed through RT-PCR (Fig. 1).

Table 1  Primers design for RT-PCR.

| Primer          | Accession number (Gen Bank) | Size (pb) | Tm (°C) | Annealing temperature (°C) | Sequence (5'-3')         |
|-----------------|----------------------------|-----------|---------|----------------------------|--------------------------|
| SOD ARNm (for)  | JQ043347.1                 | 20        | 66.8    | 65                         | 5' CCTAAAGGGAACCTCGCTG3' |
| SOD ARNm (rev)  | JQ043348.1                 | 20        | 63.3    | 65.4                       | 5' AGCTCGTAACCACACTAGGC3'|
| CAT ARNm (for)  | EU884307.1                 | 20        | 64.3    | 64.5                       | 5'TCACAGAGGCACAGACTGG3' |
| CAT ARNm I (rev)| EU884307.1                 | 20        | 66.9    |                            | 5'TGTGGTGAGACCTTTGGGA3' |

Table 2  Total biomass production (g plant\(^{-1}\)) and yield (g plant\(^{-1}\)) in bean plants cv. Strike in response to different dose of Zn sulfate and chelate. Significant level ± SE. P < 0.05 (n = 4).

| Dose of Zn (\( \mu\)mol L\(^{-1}\)) | Biomass (g ps\(^{-1}\)) | Yield (g s\(^{-1}\)) |
|-----------------------------------|-------------------------|----------------------|
|                                   | Zn EDDHA                | ZnSO\(_4\) | Zn EDDHA | ZnSO\(_4\) |
| 0                                 | 23\(^a\)                | 21\(^b\)     | 40\(^d\) | 39\(^c\) |
| 25                                | 28\(^b\)                | 30\(^a\)     | 62\(^a\) | 75\(^b\) |
| 50                                | 34\(^a\)                | 32\(^a\)     | 2.33\(^b\) | 87\(^a\) |
| 100                               | 31\(^a\)                | 27\(^a\)     | 2.20\(^b\) | 93\(^a\) |

Fig. 1  RT-PCR analysis of temporal expression of: A) Superoxide dismutase (SOD); B) Glutathione peroxidase (GSH-Px); C) Catalase (CAT), in beans plants. (1) Zn chelate 0 \( \mu\)M L\(^{-1}\), (2) Zn chelate 25 \( \mu\)M L\(^{-1}\), (3) Zn chelate 50 \( \mu\)M L\(^{-1}\), (4) Zn chelate 100 \( \mu\)M L\(^{-1}\), (5) Zn sulfate 0 \( \mu\)M L\(^{-1}\), (6) Zn sulfate 25 \( \mu\)M L\(^{-1}\), (7) Zn sulfate 50 \( \mu\)M L\(^{-1}\), (8) Zn sulfate 100 \( \mu\)M L\(^{-1}\) (9) rRNA 18 S.

The results indicate no expression when Zn chelate was used in concentrations of 50 and 100 \( \mu\)M L\(^{-1}\), this shows the absence of the superoxide radical which works on the enzyme associated with SOD activity or an activity inhibiting. In the other treatments the expression was low showing that the doses used did not cause the appearance of the superoxide radical, harmful element for plants. The expression of the gene in the control is probably because like other proteins induced they have a temporal expression profile in many hosts mostly transitory which favors cellular energy savings (Buchanan et al., 2000).
When SOD activity was analyzed by spectrophotocolorimetry, significant differences were found for all treatments with Zn sulfate and when Zn chelate was used, differences were found between control (0 μM L⁻¹) and 25 μM L⁻¹ as shown in Table 3. In both forms of Zn used, the higher activity of the enzyme was found at 25 μM L⁻¹.

The enzymatic activity of SOD was higher at 25 μM L⁻¹ followed by 50 μM L⁻¹ for both forms of Zn used (chelate and sulfate) with significant differences between both concentrations. Enzyme activity decreased at the higher concentrations and in the control (without Zn) (Table 3).

A high SOD activity may indicate the presence of superoxide radical so the enzyme is active to diminish the negative effects of this radical in bean plants, this situation can be observed at the lower concentration of Zn (25 μM L⁻¹) meaning that plants still have deficiency of this element when stress is created. The lower yields obtained at this concentration with both forms of Zn used also support the idea of a deficiency in this element (Table 2).

With the higher concentration (100 μM L⁻¹) the lowest value was obtained for enzyme activity, mainly in the form of sulfate indicating an enough concentration for the evaluations made. Also at this high concentration total biomass production and good yields were obtained per plant (Table 2).

Hacisalihoglu et al. (2003) reported that under stress by Zn deficiency, SOD Cu/Zn activity decreases due to Zn participation in both gene expression and protein synthesis. Cakmak (2000) found that Zn can induce stress and at this point the activity of some enzymes (SOD, CAT) is inhibited. Results from this research do not match with those authors because SOD shows more activity at the lower concentration of Zn indicating a stress probably due to deficiency of the mineral element.

The expression of GSH-Px in bean leaves is shown in Fig. 1B.

This gene was strongly expressed at 50 μM L⁻¹ of Zn chelate and in all concentrations of Zn sulfate, as shown in Fig. 1B. The presence of GSH-Px gene in all concentrations used may be because of its biological importance, since it allows adjustment of cellular redox environment under normal conditions and after the stress appearance.

It is important to highlight the role of GSH-Px in protecting the photosynthesis process that could lead to oxidative damage (Milla et al., 2003) and the reason of why the gene could be expressed in all treatments and forms of Zn employed because the study was conducted on leaves that are the primary organ of this process.

The results of GSH-Px activity as enzyme were higher at 0 μM L⁻¹ (control) when Zn sulfate was used with the lower activity at a higher concentration (100 μM L⁻¹) (Table 3). No significant differences were found with Zn chelate.

The activity of GSH-Px in all treatments and forms of Zn used is relatively high compared with SOD, this evidence proves one more time the biological importance of this enzyme in plants.

The GSH-Px accumulates in response to increases in ROS, or to compensate declines in the defense capacity of other antioxidants, as we can see at 0 μM L⁻¹ where no Zn was used and the enzyme activity is high because of the stress create.

CAT expression in bean leaves were analyzed too (Fig. 1C). CAT expression in all treatments and forms of Zn used was high. This could be explained by the ability of metal to increase the activity of the reactive oxygen scavenging enzymes (CATs, Ascorbate peroxidase, Guaiacol peroxidase) (Tewari et al., 2005).

The fact that many stresses activate or allow the CAT genes to be expressed could explain the expression of CATs in the study we address because plants are always exposed to some stress, the conditions are never ideal. Moreover in the family of CAT genes that exists (CAT 1, CAT 2 and CAT 3) the CAT 2 are activated in photosynthetic tissues (leaves) so in this case it could be this gene.

CAT total activity analyzed in the spectrophotocolorimeter showed no significant difference in any of the treatments or forms of Zn used as shown in Table 3.

CATs remove H₂O₂ produced during photorespiration in peroxisomes. Increasing this element (H₂O₂) in shoots and roots of plants after applying Zn concentrations between 500 and 2000 μM Zn²⁺ suggests that some of the enzymes remove the peroxide work efficiently (Kishor and VeePrakash, 2009). In our experiment Zn concentrations used were below 500 μM that may be explained why were no significant differences and the concentrations should not induce oxidative stress because of the metal.

Although significant differences weren’t found lower enzyme activities were observed with Zn sulfate in all concentrations used indicating less oxidative stress.

Generally in this research the lower activity of the studied enzymes were observed with Zn sulfate indicating lower oxidative stress and ROS formation.

This work provides an insight into molecular and biochemical level associated with different concentrations and forms of Zn used in bean plants in a biofortification process and how different quantities and forms influence in different ways on genes and enzymes related to antioxidant defense system in plants. The gene expression studies complemented with biochemical indicators are tools that help support a program of Zn biofortification of beans.
4. Conclusion

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This work provides an insight into molecular and biochemical level associated with different concentrations and forms of Zn used in bean plants in a biofortification process and how different quantities and forms influence in different ways on genes and enzymes related to antioxidant defense system in plants.

The lower expression or activity of SOD and GSH-Px genes and enzymes were found in the concentration of 100 μM L⁻¹ followed by 50 μM L⁻¹ with Zn sulfate been in relation to the highest yields and biomass amount so these concentrations can be used for biofortification of beans.

The gene expression studies complemented with biochemical indicators are tools that help support a program of Zn biofortification of beans.

References

Boius, H.E., 2003. Micronutrient fortification of plants through plant breeding: can it improve nutrition in man at low cost? Proc. Nutr. Soc. 62, 403–411.

Buchanan, B.B., Gruissem, W., Jones, R.L., 2000. Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, USA.

Cakmak, I., 2000. Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytol. 146 (2), 185–200.

Hacisalihoglu, G., Hart, J.J., Wang, Y.H., Cakmak, I., Kochian, L.V., 2003. Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. Plant Physiol. 131, 595–602.

Hafeez, B., Khanif, Y.M., Saleem, M., 2013. Role of zinc in plant nutrition: a review. Am. J. Exp. Agric. 3 (2), 374–391.

Hotz, C.H., Brown, K.H., 2004. Assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr. Bull. 25 (1), 121–123.

Kishor, P.M., VeePrakash, V., 2009. Antioxidant modulation in response to zinc induced oxidative stress at different pH in Glycine max L. cv. Merrill. Am. Eurasian J. Agric. Environ. Sci. 6 (4), 485–493.

Milla, M.A., Maurer, A., Rodriguez, A.H., Perry, J.G., 2003. Glutatione peroxidase genes in Arabidopsis are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. Plant J. 36, 602–615.

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., Watkins, C.B., 1997. Influence of salicylic acid on H₂O₂ production, oxidative stress, and H₂O₂ metabolising enzymes. Plant Physiol. 115, 137–149.

Sanchez, E., Soto, J.M., Garcia, P.C., Lopez-Lefebre, L.R., Rivero, R. M., Ruiz, J.M., Romero, L., 2000. Phenolic compounds and oxidative metabolism in green bean plants under nitrogen toxicity. Aust. J. Plant Physiol. 27, 973–978.

Sanchez, E., Rivero, R.M., Ruiz, J.M., Romero, L., 2004. Changes in biomass, enzymatic activity and protein concentration in roots and leaves of green bean plants (Phaseolus vulgaris L. cv. Strike) under high NH₄NO₃ application rates. Sci. Horit. 99, 237–248.

Statistical Analysis System (SAS) Institute, 2001. SAS user’s guide. Statistics. Version 8. SAS Inst., Cary, NC, USA. Quality, and elemental removal. J. Environ. Qual. 19, 749–756.

Tewari, R., Kumar, K.P., Sharma, P.H., 2005. Signs of oxidative stress in the chlorotic leaves of iron starved plants. Plant Sci. 169, 1037–1045.

Weldua, Y., Haileb, M., Habtegebrielb, K., 2012. Effect of zinc and phosphorus fertilizers application on yield and yield components of faba bean (Vicia faba L.) grown in calcareous cambisol of semi-arid northern Ethiopia. J. Soil Sci. Environ. Manage. 3 (12), 320–326.