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Impact of selenium fertilization on the activity of detoxifying enzymes of H_2O_2 in bean plants

Impacto de la fertilización de selenio sobre la actividad de las enzimas detoxificadoras de H_2O_2 en plantas de frijol

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Abstract. Selenium (Se) is an essential element for many organisms, although in high concentrations it may become toxic, leading to the generation of reactive oxygen species (ROS). In this study, bean plants received different application rates of Se (0, 10, 20, 40, 80, and 160 μM) in the form of selenite and selenate to evaluate the activity of the detoxifying enzymes of H_2O_2. The results indicated that the activity of these enzymes in bean plants depended on the chemical form of Se: selenite at rates of 20 μM or greater diminished biomass and yield, increasing the activity of superoxide dismutase (SOD). Even when catalase (CAT) activity also increased, it appeared that it was efficient at detoxifying H_2O_2 in the presence of Se, given also the rise in H_2O_2 production. Also, selenate diminished yield up to a rate of 160 μM and increased the activity of the enzyme GSH-Px, which reached its maximum activity at 160 μM, and thus proved less toxic than selenite.

Keywords: *Phaseolus vulgaris* L.; Green bean; Biofortification; Selenium.

Resumen. El selenio (Se) es un elemento esencial para muchos organismos. Sin embargo, en altas concentraciones puede llegar a ser tóxico, llevando a la generación de especies reactivas de oxígeno (ROS). En este estudio las plantas de frijol recibieron diferentes dosis de aplicación de Se (0, 10, 20, 40, 80 y 160 μM) en forma de selenito y selenato, con el objetivo de evaluar la actividad de las enzimas detoxificadoras de H_2O_2. Los resultados obtenidos indican que la actividad de estas enzimas en plantas de frijol es dependiente de la forma química de aplicación de Se: el selenito desde la dosis de 20 μM disminuyó la biomasa y el rendimiento, e incrementó la actividad de la superóxido dismutasa (SOD). Aun cuando también incrementó la actividad de la catalasa (CAT), parece que no fue eficiente en la detoxificación de H_2O_2 en presencia de Se, dado también el aumento en la producción de H_2O_2. Por otra parte, el selenato disminuyó el rendimiento hasta la dosis de 160 μM e incrementó la actividad de la enzima GSH-Px llegando a su máxima actividad en 160 μM, por lo que resultó menos tóxico que el selenito.

Palabras clave: *Phaseolus vulgaris* L.; Frijol ejotero; Biofortificación; Selenio.

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INTRODUCTION

Selenium (Se) is considered an essential micronutrient for human health (Terry et al., 2000; Zeng & Combs, 2008). However, this element has not yet been considered essential for plants (Terry et al., 2000). This is even though studies have shown the benefits of Se in plant metabolism, such as the elimination of free radicals. These and other oxygen radicals are highly oxidizing compounds for the cell, and are byproducts of redox biological reactions. As a defense mechanism, plants have an antioxidant system which involves various enzymes (Arora et al., 2002), mostly glutation peroxidase (GSH-Px) (Lu & Holmgren, 2009), which depends on Se for its activity. In addition, other nonenzymatic components also participate, such as glutation (GSH), which is involved both in the direct and indirect control of the concentrations of reactive oxygen species (ROS) (Foyer & Noctor, 2005). Recent studies on lettuce plants have shown that the activity of the enzyme GSH-Px depends exclusively on the Se content (Rios et al., 2008); thereafter, the Se deficiency is related to the low enzyme activity. In addition, these authors have indicated that Se is efficient for H_2O_2 detoxification on lettuce plants, reporting a greater activity of detoxifying enzymes such as the ascorbate peroxidase (APX). Studies in wheat indicate that Se boosted the activity of enzymes such as catalase (CAT) and peroxidase (POD) at low application rates (0.05 mM/Kg), whereas higher rates (0.15 mM/Kg) depressed their activity (Nowak et al., 2004).

Despite the benefits of Se in the metabolism of different plants are known, few works have evaluated the two inorganic available forms (i.e., selenate and selenite) in the same crop. In this context, the aim of the present work was to ascertain whether Se applied as sodium selenite or selenate exerted a positive effect on the oxide-reducing enzymes in bean plants.

MATERIALS AND METHODS

Crop handling and experimental design. *Phaseolus vulgaris* L., cv. Strike seeds were germinated and grown in a substrate mixture of peat, vermiculite, and perlite (3:1:1) in plastic pots (30 cm diameter) within a greenhouse. It was located in Delicias, Chihuahua (Mexico). Mean temperature inside the greenhouse was 25 ± 4 °C. Throughout the crop cycle, plants received a nutrient solution, prepared with distilled water, composed of: 6 mM NH_4NO_3, 1.6 mM K_2HPO_4, 2.4 mM K_2SO_4, 4.0 mM $CaCl_2 \cdot 2H_2O$, 1.4 mM $MgSO_4$, 5 μM Fe-EDDHA, 2 μM $MnSO_4 \cdot H_2O$, 1.0 μM $ZnSO_4 \cdot 7H_2O$, 0.25 μM $CuSO_4 \cdot 5H_2O$, 0.3 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.5 μM H_3BO_3 (Sánchez et al., 2004). The pH of the nutrient solution ranged from 5.5 to 6.0.

The Se sources used were sodium selenite and selenate (Na_2SeO_3 and Na_2SeO_4), at concentrations of 0, 10, 20, 40, 80, and 160 μM, added to the nutrient solution 20 days after sowing and during 40 days. We used a completely randomized experimental design with five treatments of selenite and other five of selenite. Each treatment was replicated four times. In all treatments, there was an additional treatment without Se application (control). Treatments containing Na_2SeO_3 or Na_2SeO_4 were analyzed separately.

Plant analysis. Plants were sampled 60 days after germination, when they were in the phenological phase of complete development and fruit maturity. Different organs separated from each bean plant (leaf, petiole, stem, root, pod, seed) were washed three times with tap water and once with deionized water.

One part of the plant material was used to quantify the total hydrogen peroxide (H_2O_2) and glutation contents as well as the enzyme activity (fresh plant material from the seed). The rest of the material was lyophilized and used to determine the Se concentration in the seed, the total biomass, and yield (plant dry matter).

Biomass and yield. Biomass was determined as the average dry weight of the whole plant. Yield was expressed as the mean weight of fruits per plant in grams of dry weight. When sampling each plant, bean fruits were weighed.

H_2O_2 concentration. The H_2O_2 concentration was measured colorimetrically following Mukherjee and Choudhuri (1983). One gram of seed was ground in 10 mL of cold acetone to do it. Thereafter, this material was centrifuged at 3500 rpm for 5 min. An aliquot of 1 mL of extract was mixed with 200 μL $TiCl_4$ at 20% dissolved in HCl 2 M, and 1 mL NH_4OH. Afterwards, the tubes were shaken and centrifuged at 3500 rpm for 5 min. The supernatant was discarded, and the sediment was washed three times with cold acetone. Next, 4 mL H_2SO_4 2 M were added and incubated for 15 min. Then, 3 mL H_2O_2 were filtered and measured spectrophotometrically at a wavelength of 415 nm against a pattern curve for H_2O_2.

Total Se content. For the determination of Se, 25 mg of the sample were digested with 2.5 mL of concentrated HNO_3 and 1 mL of H_2O_2 in a microwave oven. The resulting solution was diluted in 25 mL of deionized water and the Se concentration was determined by with the source of inductive coupling plasma mass spectrometry (ICP-MS) following Pedrero et al. (2006).

Activity of H_2O_2-detoxifying enzymes. The activity of GSH-Px (EC 1.11.1.9) was measured by the method of Flohé and Günzler (1984), using H_2O_2 as the substrate. The enzyme extract was made by grinding 0.5 g of fresh plant material in 5 mL of $KNaHPO_4$ buffer at pH 7.0, after centrifugation at 3000 rpm for 10 min. For the enzyme reaction, 0.2 mL of supernatant were added to the test, treatment tubes and

mixed with 0.4 mL of GSH 0.1 mM and 0.2 mL of $KNaHPO_4$, 0.067 M. The control tube was treated in the same way, replacing the enzyme extract with maceration buffer. After a preheating in a bath at 25 °C for 5 min, 0.2 mL of H_2O_2 1.3 mM were added to start the reaction. After 10 min at room temperature, the reaction was stopped by adding 1 mL of tricholoroacetic acid at 1% (v/v), and the mixture was placed in an ice bath for 30 min and centrifuged during 10 min at 3000 rpm. From the supernatant, 0.48 mL were removed and mixed with 2.2 mL of 0.32 M Na_2HPO_4 and 0.32 mL of 1 mM DNTB. The absorption was measured by spectrophotometry at 412 nm at a maximum time of 5 min.

The enzyme activity CAT (EC 1.11.1.6) was determined by the method of Rao et al. (1997). First, 0.5 g of dry plant material was ground in 5 mL of Hepes-HCI 25 buffer mM at pH 7.8, with PVPP at 10%; afterwards, this was centrifuged at 11500 rpm for 20 min. The following was mixed in test tubes: 0.75 mL of Na_2HPO_4-NaH_2PO_4 buffer 25 mM at pH 7, 0.75 mL de EDTA-Na_2 0.8 mM, 1 mL de H_2O_2, and 0.5 mL of enzyme extract. The absorbance change was measured at a wavelength of 240 nm for 3 min.

The superoxide dismutase (SOD; EC 1.15.1.1) activity was measured following Giannopolitis and Ries (1977) and Beyer and Fridovich (1987). First, 0.5 g de fresh material were ground in 5 mL Hepes-HCl 50 mM buffer at pH 7.6 and afterwards centrifuged at 11000 rpm for 10 min. The supernatant was diluted at a proportion of 1:5 with the maceration buffer. Next, the blank, control, and samples were prepared. The blank was prepared by the addition of 5 mL of reaction buffer consisting of: CO_3Na_2-CO_3HNa 50 mM buffer at pH 10.2, EDTA-Na 0.1 mM, L-methionine 12 mM, NTB 0.075 mM, and riboflavin 0.002 mM. In addition, 0.1 mL of maceration buffer were added to the tube. A control tube was prepared for each sample containing 0.1 mL of enzymatic extract and 5 mL of reaction buffer. In the tubes of the samples, 0.1 mL of enzyme extract and 5 mL of reaction buffer were placed. Also, 0.1 mL of enzyme extract and 5 mL of reaction buffer were placed in the test tubes, and the tubes were shaken and kept in darkness. Afterwards, readings were made at a wavelength of 560 nm. Then, the blank and the samples were illuminated for 15 min, with a light intensity of 380 μmol/(m^2 ·s). After 15 min, the samples were again measured by spectrophotometry at 560 nm, first the blanks and afterwards the control, and finally the samples.

Total glutation concentration. This was determined following the method of Gossett et al. (1994). The extraction was made by grinding 1 g of fresh plant material in 5 mL of metaphosphoric acid at 5% (v/v). This was centrifuged at 13500 rpm for 15 min. Next 50 μL of extract were added, as were 250 μL of Hepes-HCl buffer 50 mM at pH 7.6 (which contained 330 mM de betaine), and finally 150 μL of sulfasalicilic acid at 10% (p/v). Afterwards in a test tube, 150 μL of the above mixture were added, together with 700 μL of NADPH 0.3 mM, 0.1 mL of 5,5´dithiobis-(2- nitrobenzoic acid) (DNTB) 6 mM and 50 μL of reduced glutation (10 U/mL). The samples were read at 412 nm against a standard curve of glutation dissolved in metaphosphoric acid at 5%.

Statistical analysis. All data were analyzed using analysis of variance. After F tests were significant, means were compared with the LSD test at a 95% confidence level (SAS, 1987).

RESULTS

Our results showed that total biomass significantly differed only when applying selenite, with the greatest decline (a decrease of 45.7% with respect to the control) at the rate of 160 μM ($p \leq 0.05$). In terms of yield, our results indicated that favorable rates were 40 μM of selenite (2.8% over the control) and 20 μM of selenate (8.0% over the control). These rates, however, did not differ significantly with respect to the control ($p \leq 0.05$, Table 1).

Table 1. Biomass, yield, and H_2O_2 concentration in bean plants cv. Strike exposed to different application rates and forms of Se (selenate and selenite).
Tabla 1. Biomasa, rendimiento y concentración de H_2O_2 en plantas de frijol cv. Strike expuestas a diferentes dosis y formas de selenio (selenato y selenito).

Rate of Se (μM)	Total biomass (g d.w.)		Yield (g d.w.)		Concentration of H_2O_2 (μmol/g f.w.)	
	Selenate	Selenate	Selenate	Selenite	Selenate	Selenite
0	73.65 a	73.65 a	28.02 a	28.02 ab	4.27 c	4.27 b
10	68.72 a	73.11 a	25.83 a	27.47 ab	6.14 ab	4.38 b
20	71.37 a	66.43 b	30.28 a	23.59 bc	6.23 a	5.07 ab
40	62.62 a	69.24 ab	22.14 a	28.82 a	5.61 ab	5.64 ab
80	72.41 a	58.20 c	25.76 a	21.84 cd	5.36 b	5.78 ab
160	73.66 a	39.98 d	11.47 b	17.56 d	3.49 d	7.18 a
Significance	ns	*	*	*	*	*

Means with the same letters in a column are not significantly different (LSD, 0.05). d.w. (dry weight) and f.w. (fresh weight). The significance levels are given by *$p \leq 0.05$.

The greatest concentration of H_2O_2 appeared at the rate of 160 μM selenite, with an increase of 68% over the control ($p \leq 0.05$, Table 1).

For bean seed, this accumulation was favored by selenite application ($p \leq 0.05$), presenting the greatest accumulation at the rate of 160 μmol, which can be beneficial for the production of organic compounds of Se (Fig. 1).

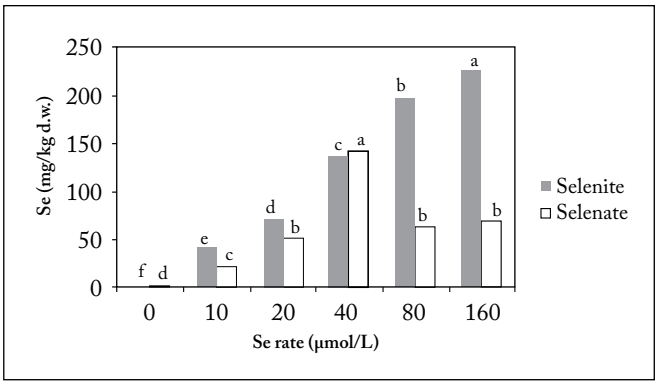

Fig. 1. Concentration of Se in bean cv. Strike under different application rates and forms of Se. Data are means ± standard error (n=4).
Fig. 1. Concentración de Se en la semilla de frijol cv. Strike bajo diferentes dosis y formas de Se. Los datos son medias ± error estándar (n=4).

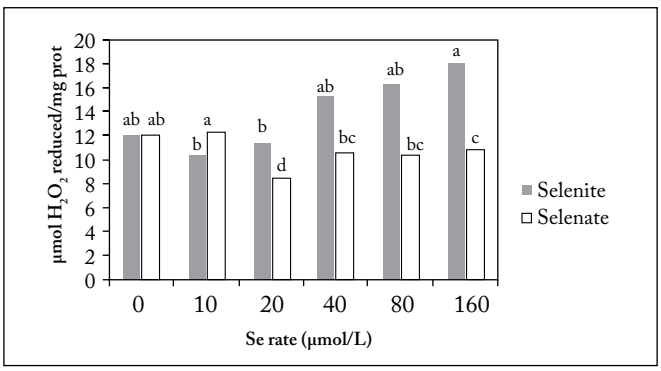

Fig. 3. Activity of the enzyme CAT in seeds of bean cv. Strike under different application rates and forms of Se. Data are means ± standard error (n=4).
Fig. 3. Actividad de la enzima CAT en semillas de frijol cv. Strike bajo diferentes dosis y formas de Se. Los datos son medias ± error estándar (n=4).

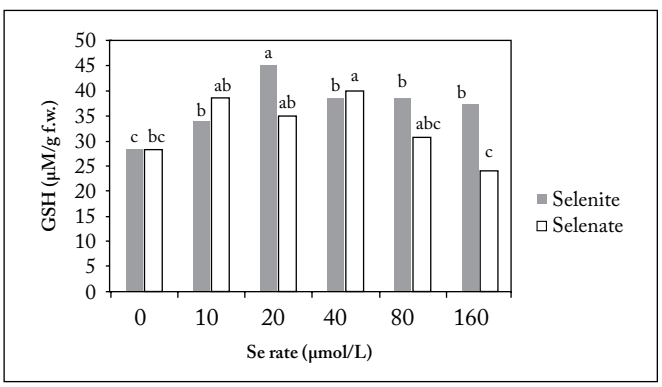

Fig. 5. Concentration of GSH in seeds of bean of cv. Strike under different application rates and forms of Se. Data are means ± standard error (n=4).
Fig. 5. Concentración de GSH en semillas de frijol cv. Strike bajo diferentes dosis y formas de Se. Los datos son medias ± error estándar (n=4).

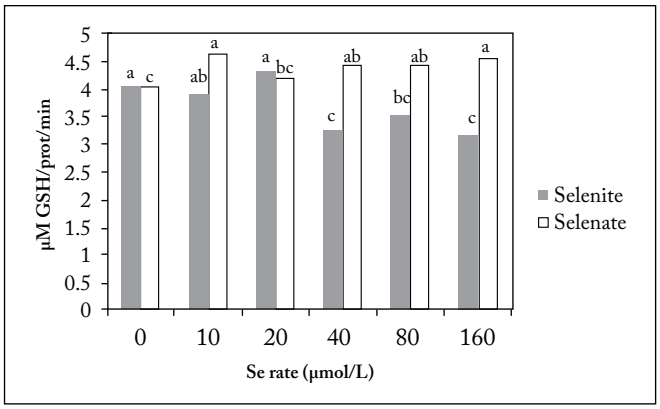

Fig. 2. Activity of the enzyme GSH-Px in seeds of bean cv. Strike under different application rates and forms of Se. Data are means ± standard error (n=4).
Fig. 2. Actividad de la enzima GSH-Px en semillas de frijol cv. Strike bajo diferentes dosis y formas de Se. Los datos son medias ± error estándar (n=4).

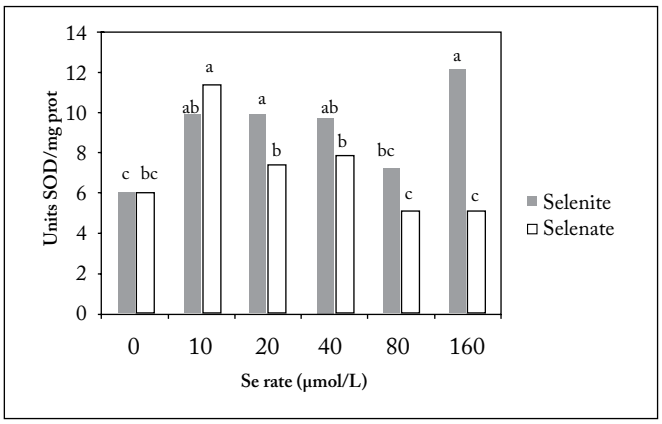

Fig. 4. Activity of the enzyme SOD in seeds of bean cv. Strike under different application rates and forms of Se. Data are means ± standard error (n=4).
Fig. 4. Actividad de la enzima SOD en semillas de frijol cv. Strike bajo diferentes dosis y formas de Se. Los datos son medias ± error estándar (n=4).

The GSH-Px presented the highest increase in the form of selenate, particularly at the rate of 10 µmol; increasing selenite applications diminished the activity of this enzyme ($p \leq 0.05$, Fig. 2).

The highest activity of CAT was found in plants treated with selenite at a rate of 160 µM ($p \leq 0.05$, Fig. 3).

SOD, the enzyme which produces H_2O_2 (Bowler et al., 1994), registered its highest activity with the application of selenate at a rate of 10 µmol, in agreement with the findings of Aggarwal et al. (2011) in bean. However, SOD activity increased in our results when selenite was applied, reaching the maximum value at the rate of 160 µM, surpassing the control by 100% activity ($p \leq 0.05$, Fig. 4).

Our results for total GSH indicated that Se in the form of selenite at rates of 20 μM presented the highest accumulation of this antioxidant compound ($p \leq 0.05$, Fig. 5).

DISCUSSION

The biomass and yield of a crop are reliable parameters to define the stress-tolerance index (Melchor et al., 2005). Se positively affects growth and stress tolerance in plants. However, according to its application rate and form (selenate or selenite) this micronutrient can also be toxic (Djanaguiraman et al., 2005; Ríos et al., 2009). Selenium is not considered an essential nutrient for plants, though it can be absorbed and accumulated in tissues. The sharp decrease in total biomass after Se application is attributed to its toxic effect, which is pro-oxidant at high rates provoking cell death in tissues, when levels of oxidative damage surpass the capacity of the antioxidant defense system (Yan & Spallholz, 1993). In the present work, the highest H_2O_2 concentration (Table 1) with rising rates of Se in the form of selenite coincided with the decline in crop yield. This is because of the phytotoxic effect of this form of Se at high concentrations. This is also important because one of the forms in which glutation decomposes H_2O_2 is through a reaction catalyzed by the glutation peroxidase (Szalai et al., 2009).

Hydrogen peroxide, a product of O_2 reduction, is potentially reactive oxygen but is not a free radical. However, when this compound is found in great quantities, it can damage plant cells, causing an oxidative stress. The influence of Se on the biomass of crops is related to the capacity of the plant to tolerate different quantities and sources of Se without these becoming phytotoxic.

High Se concentrations in plants can be toxic due to three main mechanisms, according to Spallholz and Hoffman (2002): (1) the generation of superoxide radicals, (2) the replacement of Se by sulfur in protein synthesis, and (3) the inhibition of methylation of organic compounds of Se. In the present study, the rates that favored yield (40 μM of selenite and 20 μM of selenate) presented high Se concentrations (138 and 51 mg/kg d.w., respectively; Fig. 1), indicating the high Se content that the plant can tolerate without presenting symptoms of toxicity, although differences were not significant. Most cultivated plants tolerate around 25 mg/kg of Se (Hasanuzzaman et al., 2010). Lettuce tolerates a maximum of 30 mg/kg of Se without showing toxicity (Ríos et al., 2009). Bean fruits contain a great quantity of proteins (Broughton et al., 2003). This might help plants to tolerate high Se concentrations without showing toxicity symptoms. This might be because plants transform inorganic forms of Se into organic compounds by the incorporation of this nutrient into non-specific forms of proteins (Terry et al., 2000).

Plants have an efficient system for eliminating ROS to protect against oxidative reactions. This system includes the detoxifying enzymes, notably GSH-Px, CAT, and SOD (Kong et al., 2005), in addition to compounds such as glutation. The results on the activity of the enzyme GSH-Px reflect the dependence of this enzymatic activity on the chemical form and rate of Se applied in this crop. In plants such as ryegrass and lettuce, the greatest enzymatic activity was found after applying selenate and selenite at a rate of 120 μM (Cartes et al., 2005; Ríos et al., 2009), relating this dependence more to the form than the rate of application. The lower activity of this enzyme at high selenite rates in our results (Fig. 3) coincides with the lower quantity of total biomass, lower dry matter production, greater accumulation of Se and maximum concentrations of H_2O_2. All this confirms the Se toxicity at high application rates in this crop, perhaps primarily due to the generation of superoxide radicals, as mentioned above.

The results found for the enzyme CAT indicate that, despite that selenite increases in activity, it is not an efficient enzyme in detoxifying H_2O_2 in this form. This is because that enzyme also presented the highest concentration of this compound. Other reports indicate that Se did not influence the enzymatic activity in plants such as lettuce (Ríos et al., 2009). However, in bean plants at 10 days of growth, the selenate rate of 20 μM spurred the activity of this enzyme, while at a rate of 60 μM slowed it (Aggarwal et al., 2011). These differences might be because the Se metabolism in plants differs according to the species, growth stage, and plant organ (Hasanuzzaman et al., 2010). This underscores the importance of knowing in detail the influence that Se exerts in this crop.

The results of the activity of the enzyme SOD showed high stress of the plant after applying selenite, coinciding with the lower total plant biomass and the greater H_2O_2 production (Fig. 4). These results agree with those of Dajanaguiraman et al. (2005), who reported a greater SOD activity in soybean after applying selenite at rates considered phytotoxic.

GSH acts as a component of the ascorbate-glutation cycle that participates in the elimination of excess H_2O_2 (Noctor & Foyer, 1998) in a reaction by which it is oxidized by glutation. The greater accumulation of GSH after applying selenite at a rate of 20 μM could account for the fact that Se in the form of selenate at high application rates presented lower fruit production due to the lower quantity of this antioxidant compound. Also, the high Se concentrations in the form of selenite did not lower the content of glutation in relation to the control; the same goes for the yield. Recent studies have demonstrated that Se not only can promote plant growth and development, but also increase their resistance to diverse types of stress. This is because it strengthens their antioxidant capacity by increasing the content of compounds such as glutation (Peng et al., 2002; Djanaguiraman et al., 2005; Rios et al., 2009). However, high Se concentrations seem to be toxic.

CONCLUSIONS

The results indicated that the activity of enzymes that detoxify H_2O_2 in bean plants cv. Strike depended on the chemical form of the applied Se. Selenite reduced biomass and yield from application rates of 20 μM, in addition to boosting SOD activity. Furthermore, these rates increased CAT activity, although it appeared that it was not efficient in detoxifying H_2O_2 in the presence of Se, given that it rather stimulated H_2O_2 production. Meanwhile, selenate lowered yield up to a rate of 160 μmol, and increased the activity of the enzyme GSH-Px, reaching its maximum activity at 160 μmol. Thus, selenate was less toxic than selenite.

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