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Physiological, biochemical, and ultrastructural characterization of selenium toxicity in cowpea plants

Running title: Selenium toxicity responses in cowpea plants

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Highlights

- Foliar application higher than 50 g ha^{-1} caused leaf toxicity
- Selenium toxicity induced lipid peroxidation and caused lesions in the leaves
- Lesions were observed mainly near the trichomes
- μ-XRF revealed accumulation of selenium near the necrotic brown areas of the leaf lesions

Abstract

Selenium (Se) is considered a beneficial element for plants; however, in high concentrations, it causes negative effects on plant physiology and development. This study reports the first physiological, nutritional, and ultrastructural description of Se toxicity in cowpea growing under field conditions. Selenium was supplied as a foliar application of sodium selenite at varying concentrations (0, 50, 100, 200, 400, 800, 1200, and 1600 g ha^{-1}). An increased yield was observed with the application of 50 g ha^{-1} Se. Application of concentrations higher than 50 g ha^{-1} caused leaf toxicity. Increased lipid peroxidation and hydrogen peroxide concentration and reduced total sugars, sucrose, and carotenoid concentration were observed at highest doses tested (1200 and 1600 g ha^{-1}). Applications of more than 50 g ha^{-1} Se reduced the phloem diameter, caused chlorosis of the leaf blade with a coalescence of lesions, and caused pink salt deposits to appear. Lesions were observed mainly near the trichomes on the adaxial surface of the leaf blade. An analysis of the element distribution with microprobe X-ray fluorescence spectrometry (μ-XRF) revealed accumulation of Se, calcium (Ca), potassium (K), copper (Cu), and manganese (Mn) near the primary vein and in the necrotic brown areas of the leaf lesions. In contrast, Na was homogeneously distributed in the leaf tissue.

Keywords

Vigna unguiculata, antioxidant metabolism, photosynthetic pigments, toxicity, scanning electron microscopy

Introduction

Selenium (Se) is a micronutrient essential to metabolism in humans and animals. It is involved in thyroid function and the immune system and is also an antioxidant (Fairweather-Tait et al., 2011). In plants, Se is considered a beneficial trace element that can promote growth and help resist oxidative, biotic, and abiotic stress (White, 2016; Reis et al., 2017).

The concentrations of Se in agricultural food products depend on the availability of this element in the soil (Joy et al., 2015). Se concentrations tend to be very low (below 1 mg kg^{-1}) in plants growing in acidic and weathered conditions such as tropical soils (Pilon-Smits et al., 2009) and the Se deficiency is estimated to be a problem for more than 1 billion people (Combs, 2001; Schiavon & Pilon-Smits, 2017). Therefore, Se supplementation to plants might be an important strategy for increasing the daily intake of this element by humans and animals (White & Broadley, 2009; Chilimba et al., 2012; White, 2016; Reis et al., 2017).

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important legume due to its high nutritional value, adaptability, and versatility to different production conditions (Ehlers & Hall, 1997). Due to its resistance to water loss, it is suitable to hot arid regions (Hall, 2012). Cowpea also has a role as a dietary protein and mineral source, complementing cereals, tuberous vegetables, and root vegetables in diets in various developing countries in Africa, Latin America, and Asia (Phillips et al., 2003; Manzeke et al., 2017).

In spite of its nutritional importance, the range between deficient and toxic levels of Se is narrow (Schiavon & Pilon-Smits, 2017). Large Se concentrations may affect the development of plants, such as cowpea. The tolerated Se concentrations, as well as toxicity mechanisms, vary depending on the plant species (White et al., 2004).

The stress caused by high doses of Se in plants may change the concentrations of reactive species of oxygen (ROS) such as H_2O_2 and induce high lipid peroxidation rates in cell membranes (Mostofa et al., 2017). ROS are an indicative of stress in plants, indicating the damage to the plant cell but also act as signaling molecules in response to stressful compounds in the plant tissues (Wrzaczek et al., 2013; Shi et al., 2015; Luo et al., 2016). The abiotic stress in plants is evaluated also by the increase of antioxidative enzymes such as peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) in response to high levels of heavy metal exposure (Reis et al., 2017; Santos et al., 2017).

The most effective option for Se fertilization in crops is through foliar application, with the absorption and distribution being twice as efficient compared to soil application or seed treatment (Broadley et al., 2006; White & Broadley, 2009; Broadley et al., 2010; Chilimba et al., 2012; Ros et al., 2016). Foliar application places the solution directly in contact with the stomata and very close to the mesophyll. Thus, at high doses, a high risk exists of rapidly damaging the production of photoassimilates; high doses may also cause the accumulation of ROS, slow development, and decreased concentration of photosynthetic pigments (Molnárová & Fargašová, 2009).

Effects of large plant Se concentrations include a reduced number of chloroplasts in the mesophyll cells and the degradation of the cytoplasm, nucleus, and organelles in root meristems (Fatalieva, 1987; Ślusarczyk et al., 2015). However, information on the toxic effects of Se on plant ultrastructure, especially following foliar application, is still scarce.

No detailed information is available in the literature on the physiology and ultrastructure of plants grown under field conditions in response to the foliar application of sodium selenite. The present study presents a characterization of toxicity in cowpea based on the results obtained from antioxidant metabolism; from the analysis of histological, ultrastructural, and nutritional changes, and from the location of Se in different parts of the plant tissue analyzed through microprobe X-ray fluorescence spectrometry (μ-XRF).

Materials and methods

Experimental area

The experiments were conducted at the Teaching, Research and Extension Farm of São Paulo State University (UNESP) in the municipality of Selviria, Mato Grosso do Sul State, Brazil (20°20'43"S; 51°24'7"W, 355 m). The soil type is Distroferric Red Latosol with clayey texture (Demattê, 1980; EMBRAPA, 2013), with the following chemical characteristics: pH ($CaCl_2$ 0.01 M) 5.2; phosphorus (resin) 34 mg dm^{-3}; sulfur (calcium phosphate) 8 mg dm^{-3}; potassium (resin) 2.7 $mmol_c\ dm^{-3}$; calcium (resin) 14 $mmol_c\ dm^{-3}$; magnesium (resin) 14 $mmol_c\ dm^{-3}$; H+Al (SMP buffer) 26 $mmol_c\ dm^{-3}$; cation exchange capacity 56.7 $mmol_c\ dm^{-3}$; base saturation 54%; boron (hot water) 0.19 mg dm^{-3}; copper (DTPA) 2.7 mg dm^{-3}; iron (DTPA) 19 mg dm^{-3}; manganese (DTPA) 12.4 mg dm^{-3}; zinc (DTPA) 6.1 mg dm^{-3}; total Se 62 μg kg^{-1}, and organic matter 18 g dm^{-3}. These chemical characteristics were determined as described by Raij et al., (1997) after the collection of composite soil samples from 0–20 cm depth on September 10, 2016.

The experiment used a randomized complete block design, with four blocks and eight Se concentrations (0, 50, 100, 200, 400, 800, 1200, and 1600 g ha^{-1}), in four replicates. Sodium

selenite (Na_2SeO_3) was used as the Se source. Each experimental plot was composed of five 3-m-long rows in 0.45 m intervals, for a total parcel area of 6.75 m².

Experiment setup and procedure

The soil was prepared with subsoiling, heavy disking, medium disking (twice), and leveling. Sowing was carried out on October 18, 2016, with spacing of 0.45 m between rows and a sowing density of 11.2 seeds m^{-1}. Fertilization of the planting furrow consisted of 20 kg ha^{-1} K_2O applied as KCl (33 kg ha^{-1}) and and 20 kg ha^{-1} P_2O_5 applied as single superphosphate (110 kg ha^{-1}).

Cowpea seeds (*Vigna unguiculata* (L.) Walp.) of the BRS-Tumucumaque variety were treated with pyraclostrobin (25 g L^{-1} commercial product), thiophanate-methyl (225 g L^{-1} commercial product), and fipronil (250 g L^{-1} commercial product) at 2 mL product per kg of seeds. After the seeds were dried, they were inoculated with a premium peat inoculum for cowpea (strain SEMIA 6462, product registration number SP 00581-10030-1, 2.0 x 10^9 colony forming units g^{-1}, BIOMAX, São Joaquim da Barra city, Brazil), at 8 g kg^{-1} of seed. The inoculum was dissolved in a sugar solution (1 mL of water per gram of inoculant, 10% sugar) and gradually added and mixed with the seeds in a concrete mixer at a constant speed of 18 rpm for 5 minutes. Emergence began on October 22, 2016, four days after sowing (4 DAS).

At 40 days after emergence (DAE), the Se treatments were applied at 0, 50, 100, 200, 400, 800, 1200, and 1600 g ha^{-1} as sodium selenite diluted in water at 200 L ha^{-1}, by foliar application from a backpack sprayer calibrated for 28 mL of solution per row of cowpea. Additional treatments were carried out at 15 DAE (abamectin, 0.50 L ha^{-1} commercial product for mite control), 23 DAE (bentazone, 0.8 L ha^{-1} for weed control; abamectin, 0.5 L ha^{-1} and

imidacloprid, 0.4 L ha^{-1} for whitefly control), and at 33 DAE (haloxyfop-p-methyl, 0.3 L ha^{-1} for weed control).

Determination of antioxidant metabolism

Trifoliate leaves of the upper third of 10 plants were collected from each plot 48 hours after Se application and stored in liquid nitrogen (N). Leaf material was macerated in liquid N and stored in a freezer at -80ºC until it was processed for analyses of hydrogen peroxide concentration, lipid peroxidation, superoxide dismutase (SOD) activity, catalase (CAT) activity, ascorbate peroxidase (APX) activity, and glutathione reductase (GR) activity.

Lipid peroxidation was estimated based on the concentration of malondialdehyde (MDA) according to Heath and Packer (1968). Determination of the hydrogen peroxide concentration (H_2O_2) was carried out as described by Alexieva et al. (2001). For the determination of protein and the activity of superoxide dismutase (SOD, EC:1.15.1.1), catalase (CAT, EC:1.11.1.6), ascorbate peroxidase (APX, EC:1.11.1.11), and glutathione reductase (GR, EC:1.6.4.2), the material was weighed and prepared according to the methodology described by Gomes-Junior et al. (2007). The concentration of the total soluble protein was determined according to Bradford (1976). The SOD activity was quantified as described by Giannopolitis and Ries (1977). The CAT activity was determined by monitoring H_2O_2 degradation as described by Azevedo, Alas, Smith and Lea (1998). The APX activity was measured as described by Moldes et al. (2008). The GR was quantified as described by Gomes-Junior et al. (2006).

Determination of total sugar concentration and sucrose

The sugar concentration was determined from leaf samples extracted in 10 mL of MCW solution (60% methanol, 25% chloroform, and 15% water) and prepared for analyses as described

by Bieleski and Turner (1966). Total sugar was quantified according to the protocols in Dubois, Gilles, Hamilton, Rebers and Smith (1956), and sucrose was quantified according to Van Handel (1968).

Determination of the concentrations of photosynthetic pigments

Photosynthetic pigments (chlorophylls and carotenoids) were extracted and analyzed according to the method described by Lichtenthaler (1987) from plant tissue extracted in 80% acetone solution.

Nutrient analysis

Leaf samples were collected for nutrient analysis 16 days after the application of Se. The third trifoliate leaf (counting from the apex) was removed, dried in an oven at 40 ºC to a constant mass, and ground in a Wiley mill. The determinations of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), nickel (Ni), and selenium (Se) concentrations were conducted by inductively coupled plasma-mass spectrometry (ICP-MS) according to Thomas et al. (2016).

Stereomicroscopy, histological analysis, scanning electron microcopy, and μ-XRF

Morphometric measurements and photomicrographs of plant tissue were obtained with a computerized LAZ V4 (Leica Application Suite) image analysis system adapted to an APO S8-Leica stereomicroscope.

Leaf tissue destined for histological and ultrastructural analysis by scanning electron microscopy was fixed in FAA 70 (37% formaldehyde, acetic acid, and 70% ethanol at 1:1:18 –

v:v:v ratio) as described by Lavres-Junior et al. (2010). Histological analyses followed the methods described by Santos et al. (2017).

The location of Se and other elements in plant tissue was determined on excised trifoliate leaves using μ-XRF with an EDAX ORBIS PC analyzer. The distribution of elements in both healthy areas and tissue injured by the application of sodium selenite were characterized.

Yield components

The following parameters were analyzed to evaluate the effect of sodium selenite on yield components 37 days after application: plant height (PH), number of pods per plant (NPP), number of seeds per pod (NSP), pod length (PL), 100-seeds mass, and yield. The PH was estimated as the average height of four randomly selected plants in each plot. The NPP was calculated as the average of the total number of pods in four randomly selected plants per plot; ten pods from each of these four plants were selected to estimate PL and NSP. Harvesting was conducted by the selection of two homogeneous rows from each plot, from which all pods were manually harvested. The seeds were then weighed to obtain an estimate of yield, and 100 seeds were counted and weighed to determine the 100-seed mass. Yield and 100-seed mass were determined from seeds kept at 13% humidity.

Statistical analysis

The results were first submitted to Anderson-Darling normality tests; homogeneity was evaluated with Leven's test and, finally, with a variance analysis (F test). Differences between treatments were compared using a Tukey test at 5% probability or a polynomial regression, depending on the data set. Analyses were conducted using Minitab software, and graphs were prepared using SigmaPlot 12.5.

Results

Antioxidant metabolism response

The application of the sodium selenite had a significant effect on lipid peroxidation (MDA) (Figure 1a) and on hydrogen peroxide concentration (Figure 1b). Foliar application of Se up to 800 g ha^{-1} was associated with a reduction in lipid peroxidation relative to the control. A Se application of 1200 g ha^{-1} led to a 46% increase in the lipid peroxidation rates relative to the control. The highest rates of lipid peroxidation were recorded in leaves of plants that received 1600 g ha^{-1} Se, which had a rate of lipid peroxidation 3.5 times greater than the control.

An increase in hydrogen peroxide concentration was observed with the application of Se at 50 to 800 g ha^{-1}; however, these values were less than the H_2O_2 concentration recorded for the control treatment (Figure 1b). On the other hand, the application of 1600 g ha^{-1} increased the hydrogen peroxide concentration by 35% compared to the control treatment.

The application of Se affected the specific activity of SOD, CAT, APX, and GR in cowpea leaf tissue (Figure 2). The highest SOD activity (Figure 2a) was observed in plants that received 1200 g ha^{-1} Se. However, increased activity of this enzyme was also observed in the plants that received 100 and 800 g ha^{-1}. The SOD activity in the other treatments did not differ significantly from the control.

The CAT activity (Figure 2b) in leaves increased with increasing Se applications up to 100 g ha^{-1}. Selenium applications greater than 100 g ha^{-1} induced a marked decrease in CAT activity. No significant changes were observed in the APX activity in leaves (Figure 2c) with Se applications up to 800 g ha^{-1}; however, the application of 1200 g ha^{-1} Se led to an APX activity 3.6 times greater than the control.

The GR activity (Figure 2d) increased with increasing Se applications up to 100 and 200 g ha^{-1}. The greater GR activity was recorded in the plants that received 1200 g ha^{-1}, which had a GR activity 2.3 times greater than the control.

Photoassimilates and photosynthetic pigments

The application of sodium selenite affected the concentrations of sucrose, total sugars, total chlorophylls, and carotenoid concentration in cowpea leaves (Figure 3). Sucrose concentration in leaves (Figure 3a) increased with increasing Se application to a maximum in plants that received 400 g ha^{-1}, then decreased as Se applications were increased further. A similar result was obtained for total sugars (Figure 3b), which increased with increasing Se applications up to 400 g ha^{-1}.

Total chlorophyll concentration of leaves (Figure 3c) decreased with increasing Se applications up to 200 g ha^{-1}. However, the chlorophyll concentration increased in leaves of plants that received 400 and 800 g ha^{-1} and decreased again as more Se was applied. The highest concentrations of photosynthetic pigments were observed in the control. The concentration of carotenoids (Figure 3d) increased in leaves of plants that received 100 g ha^{-1} Se. The smallest concentrations of carotenoids were recorded in plants that received 400 and 1600 g ha^{-1} Se.

Yield components and nutritional analysis

Selenium application affected the 100-seed mass and overall yield of cowpea plants (Supplementary Material 1). No significant response to Se application was observed for PH, NPP, PL, or NSP (Supplementary Material 1a-d). The control plot showed the highest 100-seeds mass (Supplementary Material 1e), with this value decreasing with increasing Se application. The largest overall yield (Supplementary Material 1b) was obtained with plants that received 50 g

ha^{-1} Se, which yielded 1.6 times that of plants receiving no Se. For other treatments, the yield did not differ significantly from the control.

The concentrations of Se (Figures 4a, 4b) and Na (Figures 4c, 4d) in leaves and seeds were affected by the application of sodium selenite. The concentration of Se increased with increasing Se applications, whereas Na concentrations fitted a polynomial second-order regression model. The application of sodium selenite also affected leaf concentrations of P, Mg, K, and S, and the Ca concentration in the seeds (Supplementary Material 2). Plants that received 100 g ha^{-1} Se showed a 9.5% decreased in P concentration relative to the control (Supplementary Material 2a) and a 12% increase in Mg concentration in the leaves (Supplementary Material 2b). Plants that received 1200 g ha^{-1} showed similar K concentrations to the control; other concentrations led to a reduction of at least 24% in the K concentration (Supplementary Material 2c). A decrease was also observed in the S concentration in plants that received 50 and 200 g ha^{-1} (Supplementary Material 2d). An increased concentration of Ca was observed in seeds that received 800 g ha^{-1} applications (Supplementary Material 2e), corresponding to an increase of 38% relative to the control.

The application of sodium selenite also affected the concentrations of B, Cu, Fe, Mn, and Zn in the leaves and the Cu, Mn, and Ni in the seed (Supplementary Material 3). Plants that received 1200 g ha^{-1} Se had the largest concentrations of B (Supplementary Material 3a). The amounts for Cu recorded for the plants that received 50 and 100 g ha^{-1} were close to the control, whereas in other treatments, a decrease was observed in the Cu concentration (Supplementary Material 3b), with the largest concentration being observed in plants that received 200 g ha^{-1} Se. For Fe (Supplementary Material 3c), the largest concentrations were observed in plants that received 1200 g ha^{-1}, 1.45 times that of the control. Increased concentrations of Mn were recorded in plants that received sodium selenite (Supplementary Material 3d), with smaller values

being observed only for the 400 g ha^{-1} treatment, both in the leaves and seeds. Control plants showed the highest leaf concentrations for Zn (Supplementary Material 3e), with similar results obtained in the plants that received 100, 800, and 1200 g ha^{-1} Se. The Zn concentrations were lower than the control in all other treatments. Selenium application led to decreased amounts of Ni in the seeds (Supplementary Material 3f), with the most marked decrease having been observed in the highest doses of Se (1600 g ha^{-1}).

Ultrastructure and μ-XRF nutrient distribution analysis

The application of sodium selenite affected the diameter of the phloem and xylem and the thickness of the palisade parenchyma of cowpea leaves (Figure 5). The application of sodium selenite reduced the diameter of the phloem (Figure 5a). The largest phloem diameter was observed in the control treatment, with a reduction observed in plants treated with sodium selenite. Variation in the xylem diameter (Figure 5b) was also observed, with the smallest diameter being recorded for plants that received sodium selenite. The largest values for palisade parenchyma thickness (Figure 5e, Supplementary Material 4a) were observed in the control, and a significant reduction occurred in the thickness of the tissue of plants under applications of 50, 100 1200, and 1600 g ha^{-1} (Supplementary Material 4b-h).

The symptoms of toxicity of the cowpea leaves in response to application of Se is shown in Figure 6. Foliar applications of concentrations above 50 g ha^{-1} were sufficient to promote necrotic lesions in the adaxial surface of the leaves. Toxicity is characterized by chlorosis of the leaf blade, resulting in brown-colored borders. When concentrations were above 200 g ha^{-1} (Figures 6e-h), the lesions coalesced, and pink saline deposits were observed within them. At doses higher than 800 g ha^{-1} (Figures 6g-h), obstruction of the leaf conductive vessels was observed.

In scanning electron microscopy analyses (Figure 7), toxicity was observed mostly as lighter shade spots (Figures 7c-h), but closure of the stomata and other cells (Figures 7e-h) also occurred.

Figure 8 illustrates the distribution of the elements shown through μ-XRF in cowpea leaves in response to Se application at 1600 g ha^{-1} Se. High K-α values for Se (Figure 8b), S (Figure 8c), Ca (Figure 8d), K (Figure 8e), Co (Figure 8g), Cu (Figure 8h), and Mn (Figure 8i) were observed mainly in the leaf conductive vessels. On the other hand, Na (Figure 8d) seemed to be homogeneously distributed, with the green areas presenting higher readings for the element. The Na (Figure 8d) and Ca (Figure 8e) concentrations were highest in the cowpea leaves.

For the characterization of the foliar lesion caused by the application of 1600 g ha^{-1} of Se, the distribution of nutrients in the various regions of the plant tissue was analyzed using the line-scan technique (Figure 9). The red line in Figure 9a represents the scanned area. The highest K-α readings for Se, P, Ca, and K were observed in the region of the conductive vessels, followed by a higher count in the brown necrotic lesion area. The Se showed higher K-α readings than the P in the plant tissue (Figure 9b). Regions with higher Se values had low S values (Figure 9c). Sodium was the only element that showed homogeneous distribution in the leaf and was not similar to the K-α distribution of Se in the leaf (Figure 9d). Calcium, on the other hand, showed a high degree of similarity with the distribution of Se (Figure 9e). Potassium (Figure 9f) also showed similar distribution to Se in the conductive vessels and in the injured areas of the leaf.

Discussion

Antioxidant metabolism response

Although cowpea plants had a toxic reaction when exposed to more than 50 g ha^{-1} Se (Figures 6 and 7), lipid peroxidation and H_2O_2 concentration indicated stress in plants that received high concentrations (Figure 1). This is likely due to the increased enzymatic activities that are stimulated by plant stress; these activities are mainly observed in the increased activity of CAT (Figure 2b) and GR (Figure 2d) in plants exposed to low and intermediate doses of Se.

The increase in enzyme activity may have helped to maintain the MDA and H_2O_2 concentration at levels lower than in the control because CAT converts H_2O_2 into H_2O and O_2 (Igamberdiev & Lea, 2002) and GR catalyzes reduction of oxidized glutathione (GSSG) into reduced glutathione (GSH), an important compound in resistance to oxidative stress (Noctor et al., 2012). The reduction in hydrogen peroxide concentration in all treatments where the applied Se concentrations were lower than 1200 g ha^{-1} (Figure 1b) demonstrates the action of these enzymes. The decrease in lipid peroxidation follows a pattern similar to that of the peroxide concentration, given that the release of MDA occurs due to the destructive action of the reactive oxygen species (ROS) on cell membranes.

SOD showed a larger increase with very high concentrations of Se (1200 g ha^{-1}). This enzyme is responsible for the dismutation of O_2- into H_2O_2 (Fukai & Ushio-Fukai, 2011). The decrease in H_2O_2 concentrations caused by the activity of other enzymes (e.g., CAT and APX) may have provided an environment where there was no stimulus for SOD synthesis. Some studies have already shown that Se negatively impacts SOD activity in plants (Hartikainen et al., 2000; Xue & Hartikainen, 2000; Cheng et al., 2016; Reis et al., 2018).

Due to the very high doses of Se used in the present experiment, Se was observed to play a physiological role as a pro-oxidative agent, increasing the concentrations of H_2O_2, which may stimulate SOD activity in the 800 and 1200 g ha^{-1} treatments. The same is true for APX, an

enzyme that uses ascorbate as an electron donor to reduce H_2O_2 to H_2O (Caverzan et al., 2012) and showed higher activity in plants exposed to high Se concentrations (Figure 2c).

SOD, CAT, APX, and GR present peaks of activity at different Se concentrations, indicating that the element might be stimulating or suppressing different enzymes depending on its concentrations. According to Stewart et al. (1999), Se can catalyze the oxidation of thiol groups, promoting the release of superoxide ($O_2^{\bullet-}$), which may be one of the reasons the plants present stress symptoms.

Photoassimilates and photosynthetic pigments

Increases in sucrose and total sugar concentration up to the 400 and 800 g ha^{-1} treatments may be due to the activity of carbohydrate metabolism enzymes. Malik et al. (2011) observed that mung bean (*Phaseolus aureus* Roxb.) exposed to Se showed increased activity of amylases and invertases. Additionally, the stress generated by Se may itself have induced an increase in the accumulation of sugars in the leaf tissue.

Evidence that soluble sugars can be reduced has also been found. Ribeiro et al. (2016) observed lower starch degradation at nightfall in *Arabidopsis thaliana* under increased Se conditions, as well as a reduction in the concentration of saccharides and hexoses in the same period.

Among the observed variables, photosynthetic pigments were most sensitive to high doses of Se. A reduction in the leaf concentration of chlorophylls and carotenoids with the increased Se concentration has already been described for maize (Jiang et al., 2017).

Yield components and nutritional analysis

The increased yield observed in the plants with the application of 50 g ha^{-1} Se (Figure 4b), as well as the increase in foliar sucrose concentration (Figure 3), may be linked to the effect of Se in the mitigation of oxidative stress (Figure 1). At 50 g ha^{-1}, visual effects of Se toxicity were not observed (Figures 6 and 7).

The effect of Se on crop productivity is still controversial, but the beneficial effect of this element has previously been observed on the yield components of cowpea (Manaf, 2016) and other crops, such as mungbean (Kaur & Nayyar, 2015) and wheat (Yao et al., 2013), which agrees with the findings of the present study.

The leaf concentration of Se (Figure 4a) in plants that received the highest concentration treatments reached values close to 100 mg kg^{-1}, which is considered the upper limit tolerated by non-Se-accumulator plants (White, 2016). Regarding concentration of Se in seeds, an intake of up to 0.1 mg of Se per day is recommended for adults (Monsen, 2000), which is equivalent to a daily intake of more than 50 g of cowpea from the 50 g ha^{-1} treatment in our study (Figure 4b).

Absorption of selenite through the roots is partially mediated by phosphate transporters (Li, Mcgrath, & Zhao, 2008). However, under the conditions in the present study, Se was supplied in foliar applications, so the absorption of the selenite can occur passively (Gupta & Gupta, 2017), which explains the direct relation between the applied concentrations and the observed Se concentration. Selenite is rapidly converted to organoselenium compounds, such as selenocysteine (SeCys) and selenomethionine (SeMet) (White, 2016). The ease of assimilation of high amounts of selenite into sulfuric amino acids may have compromised the foliar concentration of S as observed in some treatments. The interaction between trace elements (such as Se) and micronutrients and other trace elements is a complex process, and sometimes, these elements may naturally present synergistic and antagonistic relations at the same time (Kabata-Pendias & Pendias, 2011).

Ultrastructure and μ-XRF nutrient distribution analysis

Selenium treatments showed direct effects on the diameter of the phloem (Figure 5a), compared to the xylem (Figure 5b), likely due to the application method (foliar), which favors the dispersion of the element through the phloem.

Visual observation of the effects of leaf tissue damage was possible (Figures 6, 7). No effect on the adaxial and abaxial epidermis thickness was observed (Figures 5c, 5d); however, a decrease in the palisade parenchyma thickness was observed. This indicates that the solutes were translocated through the first layer of the cells so that the toxic effect manifested in the palisade cells. Se can compromise the plant as a pro-oxidant (Hartikainen et al., 2000) or by inducing malformation of selenoproteins due to excess SeCys and SeMet compared to the sulfur forms of these amino acids (Gupta & Gupta, 2017).

The incidence of toxicity was observed mainly near the trichomes (Figure 7). Although the action of trichomes on water absorption is commonly associated with epiphytes, the effect of these structures on absorption has even been observed in forest trees (Eller et al., 2016) and Caatinga trees (Pina et al., 2017). No reports exist in the literature about the accumulation of Se in plant trichomes. However, trichomes might be regions of accumulation of Se and Na in foliar tissue, as already demonstrated for the accumulation of Mn in the trichomes in sunflower leaves (Blamey et al., 2015).

To our knowledge, no study characterizing necrotic lesions caused by toxicity of sodium selenite has been previously published. Therefore, this is the first study to characterize the toxicity of sodium selenite to plants. The distributions of the elements in the parts of the plant tissue, as shown by X-ray spectrometry (Figure 8), are indicated by the highest K-α readings of Se, S, Ca, K, Co, Cu, and Mn near the conductive vessels. This is probably due to the time of

sampling (48 hours after application). Symptoms of toxicity were observed 48 hours after the foliar application of sodium selenite. Therefore, the higher readings of these nutrients in the conductive vessels was likely due to insufficient time for the migration of the elements to other parts of the leaf tissue. In addition, damage to trichomes, cells, and stomata, as observed in Figure 7, may have contributed to compromising the natural pattern of the elements in the plant tissue.

In addition to the conductive vessels, high K-α counts of Se and other micronutrients mentioned above were observed in the necrotic brown region on the leaf (Figure 9a). The antagonistic relation between Se and S is apparent in Figure 9c. The similarity between the deposition of Se and Ca suggests that Ca has an important role in the attempt to relieve stress in the damaged area. The accumulation of Ca in damaged areas has already been observed in toxicity experiments with other elements, such as Mn (Blamey et al., 2015; Santos et al., 2017). Calcium accumulation may be a response to prevent cell degradation due to the role of the element in inhibiting enzyme activity during pectin polygalacturonase degradation (Hawkesford et al., 2012). However, the study in question does not directly show this relation with Ca, so future research should be developed to better investigate the role of Ca in Se toxicity in plants.

Conclusions

The application of Se at concentrations higher than 50 g ha^{-1} induces a reduction in phloem diameter and palisade parenchyma thickness. Toxicity symptoms are characterized by brown necrotic lesions next to the trichomes. The decrease in lipid peroxidation and in H_2O_2 concentration observed in selenium treatments with concentrations under 1200 g ha^{-1} is a response of the plant to stress.

The highest concentrations of Se, K, Ca, Cu, and Mn are found in conductive vessels, whereas Na is more homogeneously distributed in the plant tissue.

Further studies on the effects of Se on agronomic and structural characteristics of cowpea should be conducted to better understand the effects of Se on this plant.

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Conflict of interest

The authors have no conflicts of interest to declare.

References

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E. (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell and Environment* 24, 1337-1344.

Azevedo, R.A., Alas, R.M., Smith, R.J., Lea, P.J. (1998) Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. *Physiologia Plantarum* 104, 280-292.

Bieleski, R.L., Turner, N.A. (1966) Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. *Analytical Biochemistry* 17, 278-293.

Blamey, P., Hernandez-Soriano, M., Cheng, M., Tang, C., Paterson, D., Lombi, E., Kopittke, P.M. (2015) Synchrotron-based techniques shed light on mechanisms of plant sensitivity and tolerance to high manganese in the root environment. *Plant Physiology* 169, 2006-2020.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-259.

Broadley, M.R., White, P.J., Bryson, R.J., Meacham, M.C., Bowen, H.C., Johnson. S.E., Hawkesford, M.J., McGrath, S.P., Zhao, F.J., Breward, N., Harriman, M., Tucker, M. (2006) Biofortification of U.K. food crops with selenium (Se). *Proceedings of the Nutrition Society* 65, 169-181.

Broadley, M.R., Alcock, J., Alford, J., Cartwright, P., Fairweather-Tait, S.J., Foot, I., Hart, D.J., Hurst, R., Knott, P., McGrath, S.P., Meacham, M.C., Norman, K., Mowat, H., Scott, P., Stroud, J.L., Tovey, M., Tucker, M., White, P.J., Young, S.D., Zhao, F.J. (2010) Selenium biofortification of high-yielding winter wheat (*Triticum aestivum* L.) by liquid or granular Se fertilisation. *Plant and Soil* 332, 5-18.

Caverzan, A., Passaia, G., Rosa, S.B., Ribeiro, C.W., Lazzarotto, F., Margis-Pinheiro, M. (2012) Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology* 35, 1011-1019.

Cheng, B., Lian, H.-F., Liu, Y.-Y., Yu, X.-H., Sun, Y.-L., Sun, X.-D., Liu, S.Q. (2016) Effects of selenium and sulfur on antioxidants and physiological parameters of garlic plants during senescence. *Journal of Integrative Agriculture* 15, 566-572.

Chilimba, A.D.C., Young, S.D., Black, C.R., Meacham, M.C., Lammel, J., Broadley, M.R. (2012) Agronomic biofortification of maize with selenium (Se) in Malawi. *Field Crops Research* 125, 118-128.

Combs, G.F. (2001) Selenium in global food systems. *British Journal of Nutrition* 85: 517-547.

Dematt̂e, J. (1980) *Levantamento Detalhado dos Solos do Campus Experimental de Ilha Solteira*. Escola Superior de Agricultura Luiz de Queiroz, Piracicaba.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28, 350-356.

Ehlers, J.D., Hall, A.E. (1997) Cowpea (*Vigna unguiculata* L. Walp.). *Field Crops Research* 53, 187-204.

Eller, C.B., Lima, A.L., Oliveira, R.S. (2016) Cloud forest trees with higher foliar water uptake capacity and anisohydric behavior are more vulnerable to drought and climate change. *New Phytologist* 211, 489-501.

EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) (2013) *Sistema Brasileiro de Classificação de Solos*. Embrapa Solos, Rio-de-Janeiro.

Fairweather-Tait, S.J., Bao, Y., Broadley, M.R., Collings, R., Ford, D., Hesketh, J.E., Hurst, R. (2011) Selenium in human health and disease. *Antioxidants & Redox Signaling* 14, 1337-1383.

Fatalieva, S.M. (1987) Ultrastructure of mesophyll cells grown on different levels of selenium of two pea genotypes. In *Genetic Aspects of Plant Mineral Nutrition. Developments in Plant*

and Soil Sciences (eds W. H. Gabelman & B. C. Loughman), pp. 471-476. Springer, Dordrecht.

Freeman, J.L., Marcus, M.A., Fakra, S.C., Devonshire, J., Mcgrath, S.P., Quinn, C.F., Pilon-Smits, E. A. H. (2012) Selenium hyperaccumulator plants *Stanleya pinnata* and *Astragalus bisulcatus* are colonized by Se-resistant, Se-excluding wasp and beetle seed herbivores. *PLoS One* 7, e50516.

Fukai, T., Ushio-Fukai, M. (2011) Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxidants & Redox Signaling* 15, 1583-1606.

Giannopolitis, C.N., Ries, S.K. (1977) Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiology* 59, 309-314.

Gomes-Junior, R.A., Gratão, P.L., Gaziola, S.A., Mazzafera, P., Lea, P.J., Azevedo, R.A. (2007) Selenium-induced oxidative stress in coffee cell suspension cultures. *Functional Plant Biology* 34, 449-456.

Gomes-Junior, R.A., Moldes, C.A., Delite, F.S., Pompeu, G.B., Gratão, P.L., Mazzafera, P., . . . Azevedo, R.A. (2006) Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. *Chemosphere* 65, 1330-1337.

Gupta, M., Gupta, S. (2017) An overview of selenium uptake, metabolism, and toxicity in plants. *Frontiers in Plant Science* 7, 2074.

Hall, A.E. (2012) Phenotyping cowpeas for adaptation to drought. *Frontiers in Physiology* 3, 155.

Hartikainen, H., Xue, T., Piironen, V. (2000) Selenium as an anti-oxidant and pro-oxidant in ryegrass. *Plant and Soil* 225, 193-200.

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Møller, I.S., White, P. (2012) Functions of macronutrients. In *Marschner's Mineral Nutrition of Higher Plants* (ed P. Marschner), pp. 135-189. Academic Press, San Diego.

Heath, R.L., Packer, L. (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoiichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125, 2141-2145.

Igamberdiev, A.U., Lea, P.J. (2002) The role of peroxisomes in the integration of metabolism and evolutionary diversity of photosynthetic organisms. *Phytochemistry* 60, 651-674.

Jiang, C., Zu, C., Lu, D., Zheng, Q., Shen, J., Wang, H., Li, D. (2017) Effect of exogenous selenium supply on photosynthesis, Na+ accumulation and antioxidative capacity of maize (*Zea mays* L.) under salinity stress. *Scientific Reports* 7, 42039.

Joy, E.J.M., Broadley, M.R., Young, S.D., Black, C.R., Chilimba, A.D.C., Ander, E.L., Watts, M.J. (2015) Soil type influences crop mineral composition in Malawi. *Science of the Total Environment* 505, 587-595.

Kabata-Pendias, A. & Pendias, H. (2011) *Trace Elements in Soils and Plants*. CRC Press, Boca Raton.

Kaur, S., Nayyar, H. (2015) Selenium fertilization to salt-stressed mungbean (*Vigna radiata* L. Wilczek) plants reduces sodium uptake, improves reproductive function, pod set and seed yield. *Scientia Horticulturae* 197, 304-317.

Lavres Junior, J., Reis, A.R., Rossi, M.L., Cabral, C.P., Nogueira, N.D.L., Malavolta, E. (2010) Changes in the ultrastructure of soybean cultivars in response to manganese supply in solution culture. *Scientia Agricola* 67, 287-294.

Li, H.-F., Mcgrath, S.P., Zhao, F.-J. (2008) Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. *New Phytologist* 178, 92-102.

Lichtenthaler, H.K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology* 148, 350-382.

Luo, Z.B., He, J., Polle, A., Rennenberg, H. (2016). Heavy metal accumulation and signal transduction in herbaceous and woody plants: Paving the way for enhancing phytoremediation efficiency. *Biotechnology Advances* 34, 1131-1148.

Malik, J.A., Kumar, S., Thakur, P., Sharma, S., Kaur, N., Kaur, R., . . . Nayyar, H. (2011) Promotion of growth in mungbean (*Phaseolus aureus* roxb.) by selenium is associated with stimulation of carbohydrate metabolism. *Biological Trace Element Research* 143, 530-539.

Manaf, H.H. (2016) Beneficial effects of exogenous selenium, glycine betaine and seaweed extract on salt stressed cowpea plant. *Annals of Agricultural Sciences* 61, 41-48.

Manzeke, M.G., Mtambanengwe, F., Nezomba, H., Watts, M.J., Broadley, M.R., Mapfumo, P. (2017) Zinc fertilization increases productivity and grain nutritional quality of cowpea (*Vigna unguiculata* [L.] Walp.) under integrated soil fertility management. *Field Crops Research* 213, 231-244.

Moldes, C.A., Medici, L.O., Abrahão, O.S., Tsai, S.M., Azevedo, R.A. (2008) Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. *Acta Physiologiae Plantarum* 30, 469-479.

Molnárová, M., Fargašová, A. (2009) Se(IV) phytotoxicity for monocotyledonae cereals (*Hordeum vulgare* L., *Triticum aestivum* L.) and dicotyledonae crops (*Sinapis alba* L., *Brassica napus* L.). *Journal of Hazardous Materials* 172, 854-861.

Monsen, E.R. (2000) Dietary reference intakes for the antioxidant nutrients: vitamin C, vitamin E, selenium, and carotenoids. *Journal of the American Dietetic Association* 100, 637-640.

Mostofa, M.G., Hossain, M.A., Siddiqui, M.N., Fujita, M., Tran, L.S.P. (2017). Phenotypical, physiological and biochemical analyses provide insight into selenium-induced phytotoxicity in rice plants. *Chemosphere* 178, 212-223.

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y.I., Neukermans, J., Marquez-Garcia, B., Foyer, C.H. (2012) Glutathione in plants: an integrated overview. *Plant, Cell & Environment* 35, 454-484.

Phillips, R.D., Mcwatters, K.H., Chinnan, M.S., Hung, Y.-C., Beuchat, L.R., Sefa-Dedeh, S., Saalia, F.K. (2003) Utilization of cowpeas for human food. *Field Crops Research* 82, 193-213.

Pilon-Smits, E.A.H., Quinn, C.F., Tapken, W., Malagoli, M., Schiavon, M. (2009) Physiological functions of beneficial elements. *Current Opinion in Plant Biology* 12, 267-274.

Pina, A.L.C.B., Zandavalli, R.B., Oliveira, R.S., Martins, F.R., Soares, A.A. (2017) Dew absorption by the leaf trichomes of *Combretum leprosum* in the Brazilian semiarid region. *Functional Plant Biology* 43, 851-861.

Raij, B.V., De Andrade, J.C., Cantarella, H., Quaggio, J.A. (1997) *Análise Química Para Avaliação da Fertilidade de Solos Tropicais*. Instituto Agronômico de Campinas, Campinas.

Reis, A.R.D., El-Ramady, H., Santos, E.F., Gratão, P.L., Schomburg, L. (2017) Overview of selenium deficiency and toxicity worldwide: affected areas, selenium-related health issues, and case studies. In *Selenium in Plants: Molecular, Physiological, Ecological and Evolutionary Aspects* (eds E. A. H. Pilon-Smits, L. H. E. Winkel & Z.-Q. Lin), pp. 209-230. Springer International Publishing, Cham.

Reis, A.R., Barcelos, J.P.Q., Osório, C.R.W.S., Santos, E.F., Lisboa, L.A.M., Santini, J.M.K., Santos, M.J.D., Furlani Junior, E., Campos, M., Figueiredo, P.A.M., Lavres, J., Gratão,

P.L. (2017). A glimpse into the physiological, biochemical and nutritional status of soybean plants under Ni-stress conditions. *Environmental and Experimental Botany* 144, 78-87.

Reis, H.P.G., Barcelos, J.P.Q., Furlani Junior, E., Santos, E.F., Silva, V.M., Moraes, M.F., Putti, F.F., Reis, A.R. (2018). Agronomic biofortification of upland rice with selenium and nitrogen and its relation to grain quality. *Journal of Cereal Science* 79, 508-515.

Ribeiro, D.M., Silva Júnior, D.D., Cardoso, F.B., Martins, A.O., Silva, W.A., Nascimento, V.L., Araújo, W.L. (2016) Growth inhibition by selenium is associated with changes in primary metabolism and nutrient levels inArabidopsis thaliana. *Plant, Cell & Environment* 39, 2235-2246.

Ros, G.H., Van Rotterdam, A.M.D., Bussink, D.W., Bindraban, P.S. (2016) Selenium fertilization strategies for bio-fortification of food: an agro-ecosystem approach. *Plant and Soil* 404, 99-112.

Santos, E.F., Kondo Santini, J.M., Paixão, A.P., Júnior, E.F., Lavres, J., Campos, M., Reis, A.R.D. (2017) Physiological highlights of manganese toxicity symptoms in soybean plants: Mn toxicity responses. *Plant Physiology and Biochemistry* 113, 6-19.

Schiavon, M., Pilon-Smits, E.A.H. (2017) The fascinating facets of plant selenium accumulation - biochemistry, physiology, evolution and ecology. *New Phytologist* 213, 1582-1596.

Shi, G.W., Li, H., Liu, T.X., Polle, A., Peng, C.H., Luo. Z.B. (2016). Exogenous abscisic acid alleviates zinc uptake and accumulation in Populus × canescens exposed to excess zinc. *Plant, Cell and Environment* 38, 207-223.

Ślusarczyk, J., Wierzbicka, M., Suchocki, P., Kuraś, M. (2015) Ultrastructural changes in onion (*Allium cepa* L.) root tip meristem cells treated with Selol and sodium selenate (IV).

Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics 68, 306-316.

Stewart, M.S., Spallholz, J.E., Neldner, K.H., Pence, B.C. (1999) Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis. *Free Radical Biology and Medicine* 26, 42-48.

Thomas, C.L., Alcock, T.D., Graham, N.S., Hayden, R., Matterson, S., Wilson, L., Young, S.D., Dupuy, L.X., White, P.J., Hammond, J.P., Danku, J.M.C., Salt, D.E., Sweeney, A., Bancroft, I., Broadley, M.R. (2016) Root morphology and seed and leaf ionomic traits in a *Brassica napus* L. diversity panel show wide phenotypic variation and are characteristic of crop habit. *BMC Plant Biology* 16, 214.

Van Handel, E. (1968) Direct microdetermination of sucrose. *Analytical Biochemistry* 22, 280-283.

White, P.J., Bowen, H.C., Parmaguru, P., Fritz, M., Spracklen, W.P., Spiby, R.E., Meacham, M.C., Mead, A., Harriman, M., Trueman, L.J., Smith, B.M., Thomas, B., Broadley, M.R. (2004) Interactions between selenium and sulphur nutrition in *Arabidopsis thaliana*. *Journal of Experimental Botany* 55: 1927-1937.

White, P.J., Broadley, M.R. (2009) Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist* 182: 49–84.

White, P.J. (2016) Selenium accumulation by plants. *Annals of Botany* 117, 217-235.

Wrzaczek, M., Brosché, M., Kangasjärvi, J. (2013). ROS signaling loops-production, perception, regulation. *Current Opinion in Plant Biology* 16, 575–582.

Xue, T., Hartikainen, H. (2000) Association of antioxidative enzymes with the synergistic effect of selenium and UV irradiation in enhancing plant growth. *Agricultural and Food Science in Finland* 9, 177-186.

Yao, X., Jianzhou, C., Xueli, H., Binbin, L., Jingmin, L., Zhaowei, Y. (2013) Effects of selenium on agronomical characters of winter wheat exposed to enhanced ultraviolet-B. *Ecotoxicology and Environmental Safety* 92, 320-326.

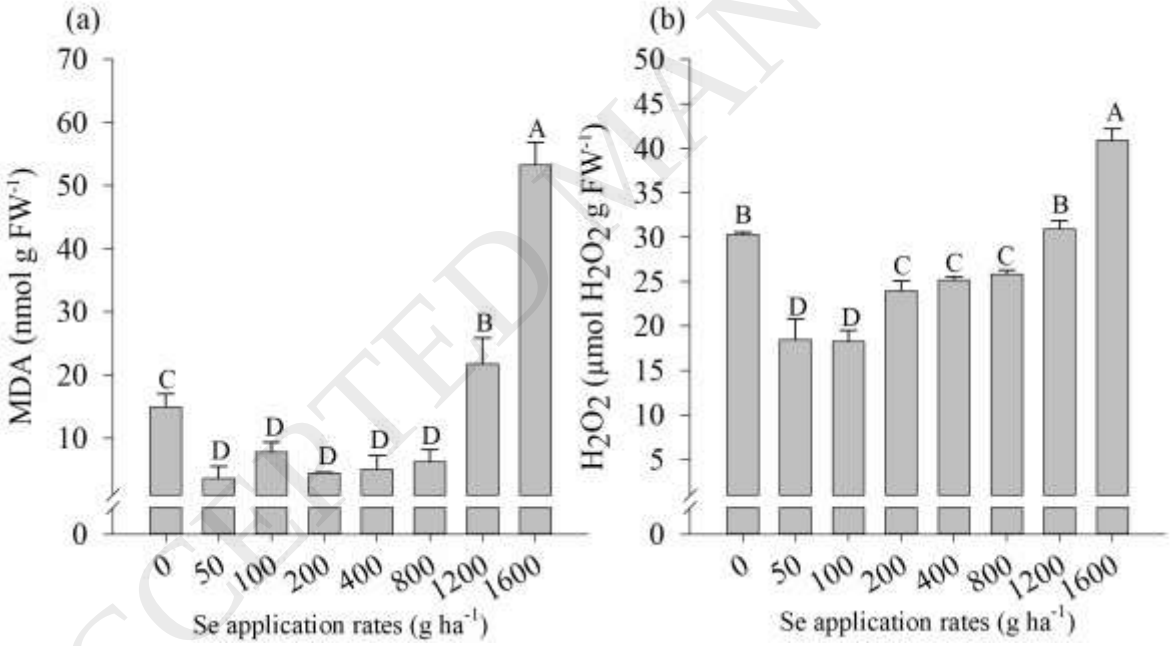


Figure 1. Estimates of lipid peroxidation assayed (a) by MDA concentration and (b) hydrogen peroxide concentration in the third trifoliate leaves of cowpea plants sprayed with sodium selenite at various application rates. Error bars indicate the standard error of the mean (n=4 replicates); identical letters indicate that no significant difference exists between the means according to the Tukey test ($p \leq 0.05$). CV(%)=16.60 (a) and 3.06 (b).

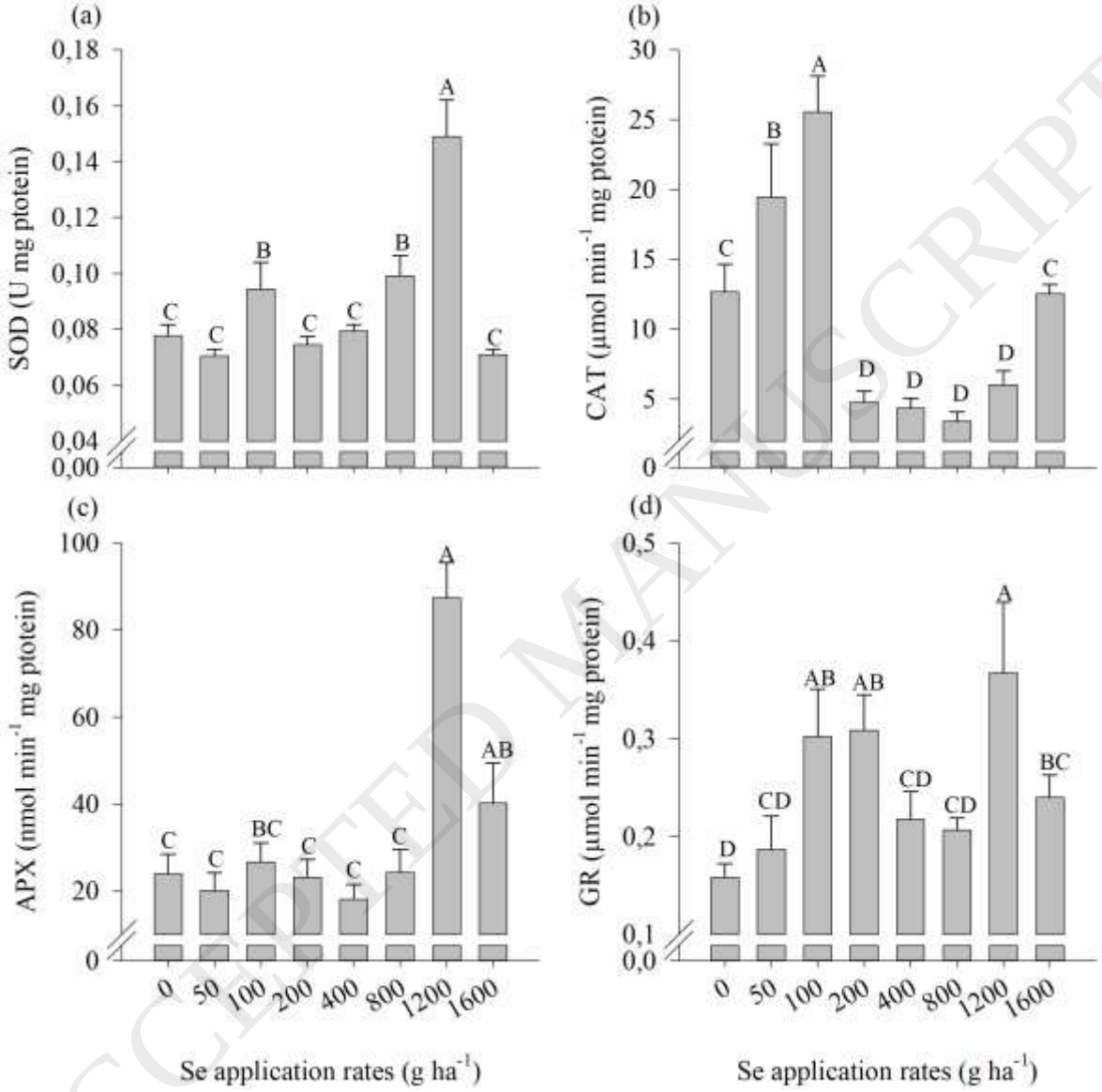


Figure 2. Specific activity of (a) superoxide dismutase, (b) catalase, (c) ascorbate peroxidase, and (d) glutathione reductase in the third trifoliate leaves of cowpea plants sprayed with sodium selenite at various application rates. Error bars indicate the standard error of the mean (n=4 replicates); identical letters indicate that no significant difference exists between the means according to the Tukey test ($p \leq 0.05$). CV(%)=6.22 (a), 17.39 (b), 17.76 (c), and 13.65 (d).

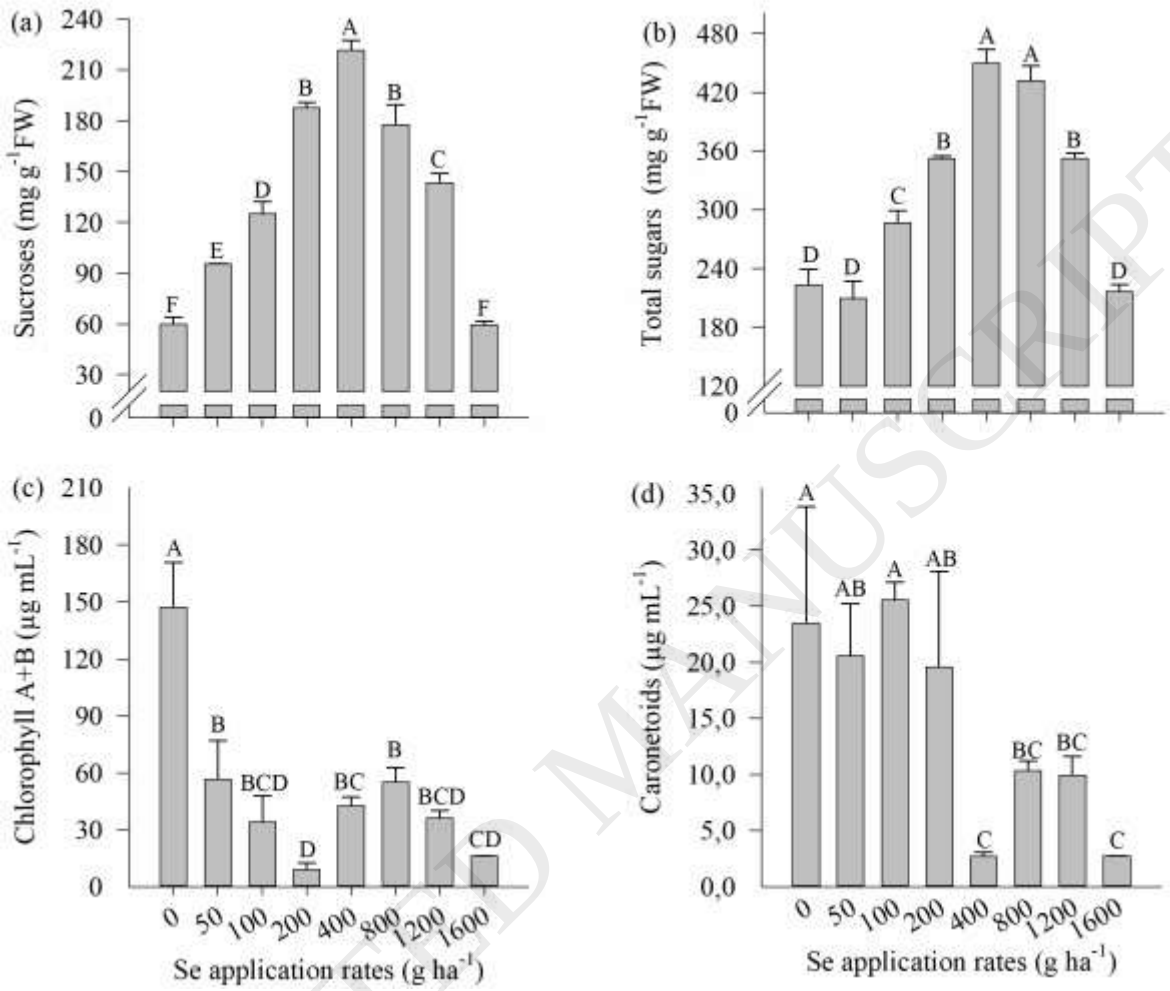


Figure 3. Concentrations of (a) sucrose, (b) total sugar, (c) chlorophylls A+B and (d) carotenoids in the third trifoliate leaves of cowpea plants sprayed with sodium selenite at various application rates. Error bars indicate the standard error of the mean (n=4 replicates); identical letters indicate that no significant difference exists between the means according to the Tukey test ($p \leq 0.05$). CV(%)=4.68 (a), 4.20 (b), 23.68 (c), and 36,70 (d).

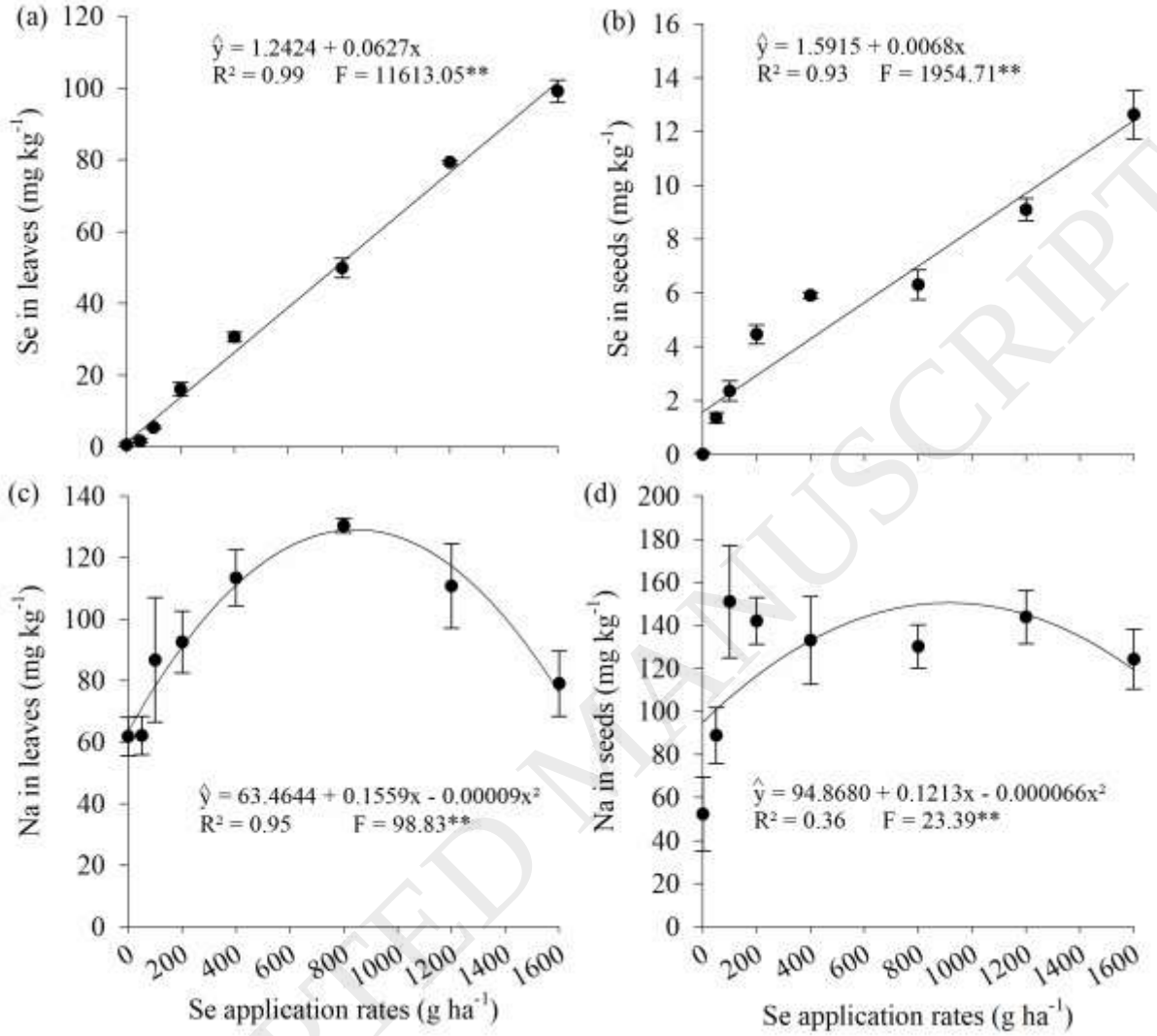


Figure 4. Selenium concentrations in (a) leaves and (b) seeds and sodium concentrations in (c) leaves and (d) seeds of the third trifoliate leaves of cowpea plants sprayed with sodium selenite at various application rates. Error bars indicate the standard error of the mean (n=4 replicates).

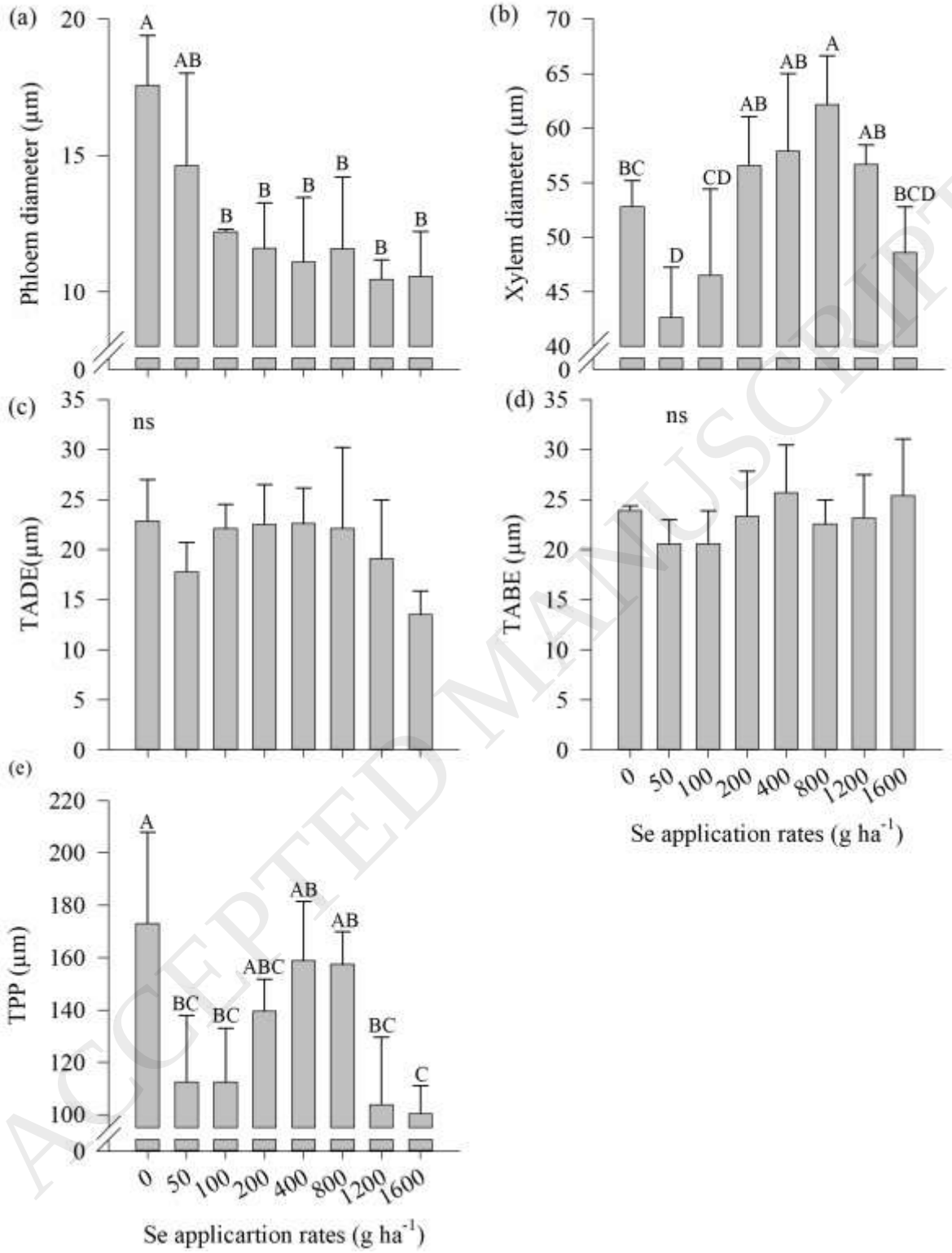


Figure 5. Diameters of (a) phloem and (b) xylem and (c) the thicknesses of the adaxial epidermis, (d) abaxial epidermis, and (e) palisade parenchyma the third trifoliate leaves of

cowpea plants sprayed with sodium selenite at various application rates. Error bars indicate the standard error of the mean (n=4 replicates); identical letters indicate that no significant difference exists between the means according to the Tukey test. ($p \leq 0.05$), ns = not significant. CV(%)=16.41 (a), 7.43 (b), 17.57 (c), 23.24 (d) and 17.69 (e).

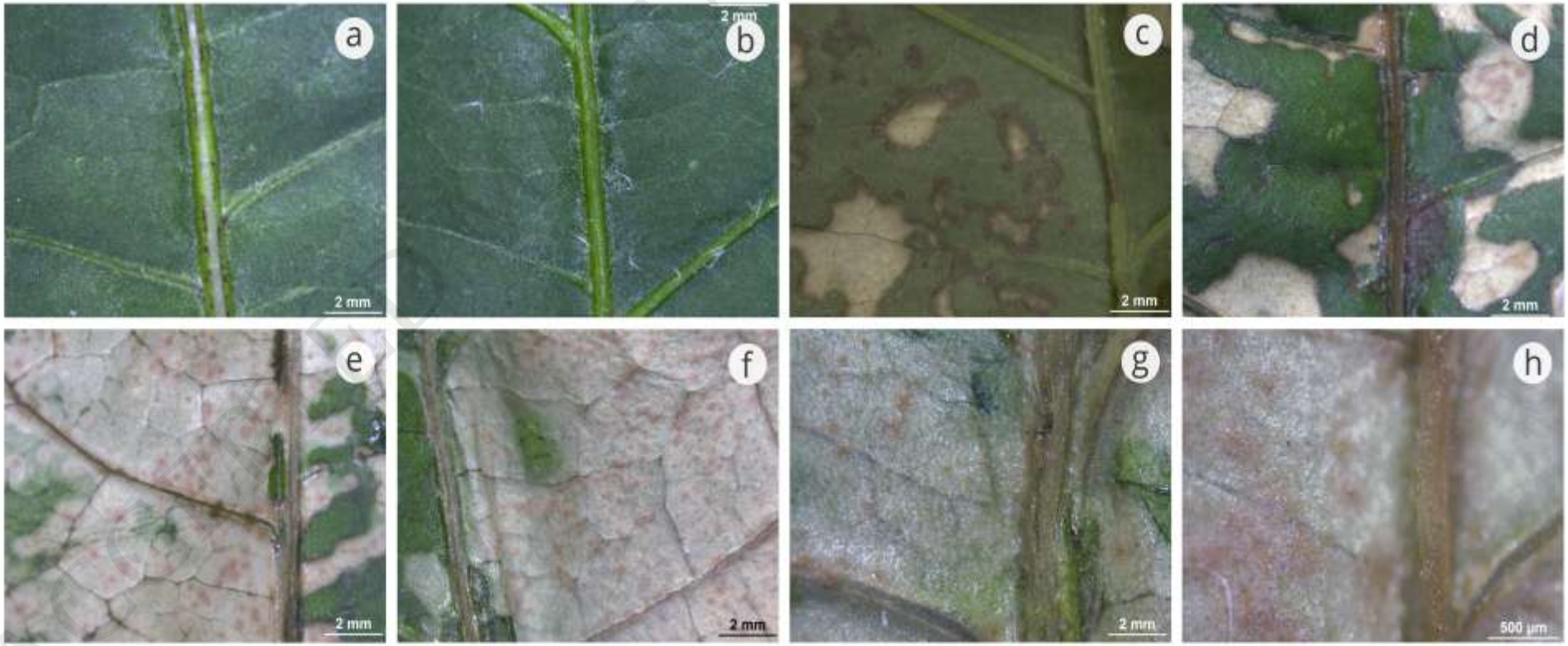


Figure 6. Stereomicroscope images of the adaxial surface of the leaf blade of cowpea plants sprayed with sodium selenite at rates of (a) 0, (b) 50, (c) 100, (d) 200, (e) 400, (f) 800, (g) 1200, and (h) 1600 g ha^{-1} Se.

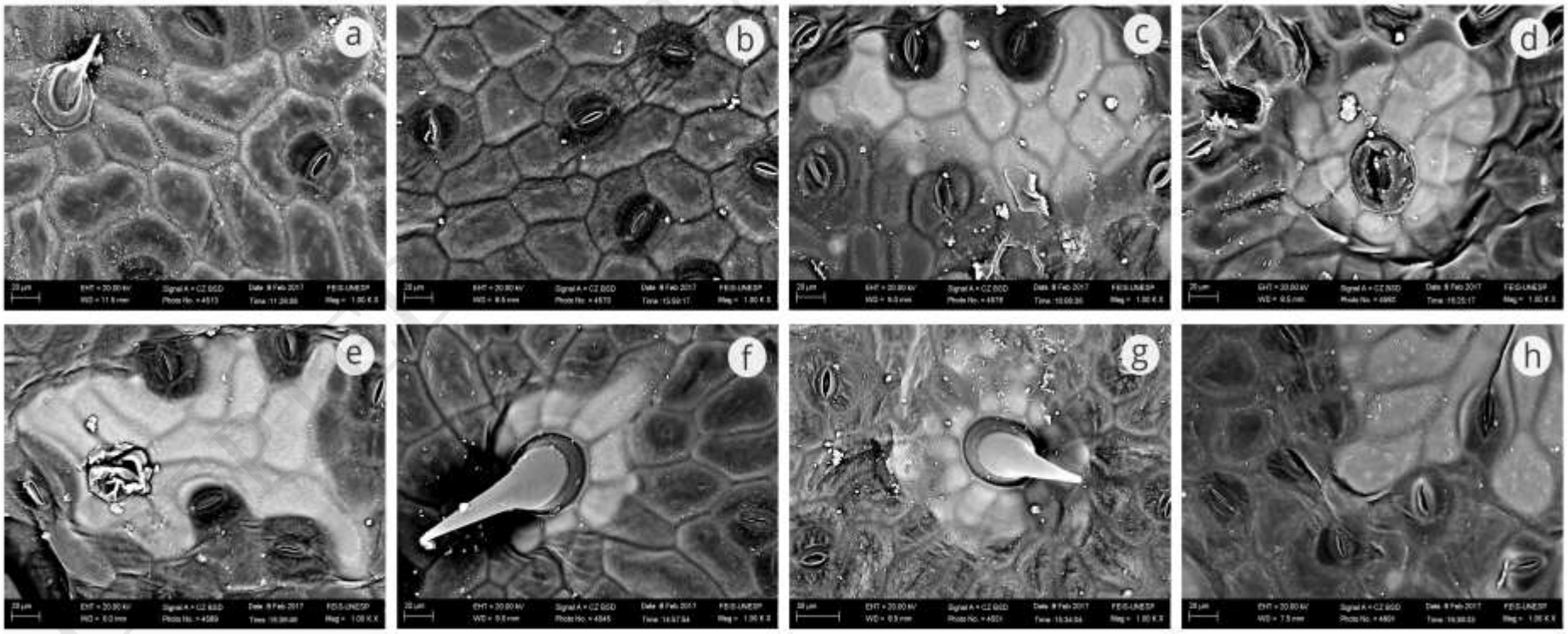


Figure 7. Scanning electron microscopy of the adaxial surface of the leaf blade of cowpea plants sprayed with sodium selenite at rates of (a) 0, (b) 50, (c) 100, (d) 200, (e) 400, (f) 800, (g) 1200, and (h) 1600 g ha^{-1} Se.

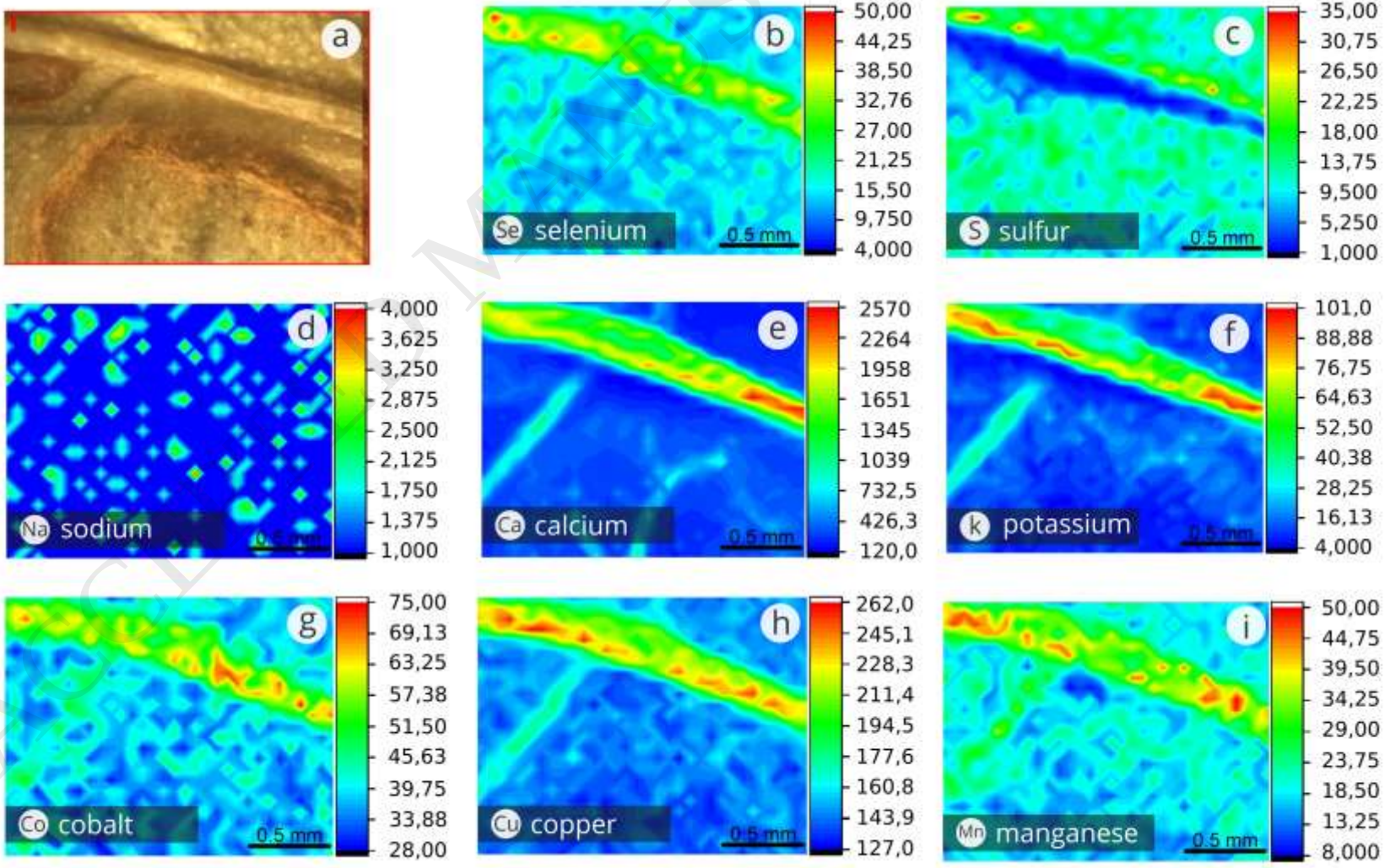
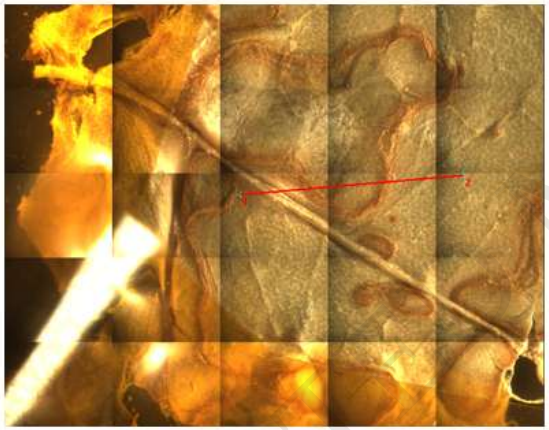
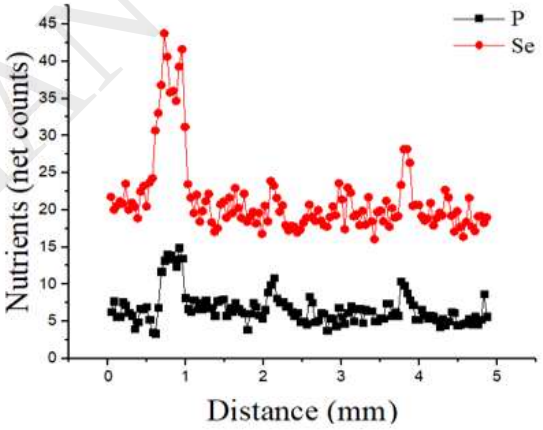
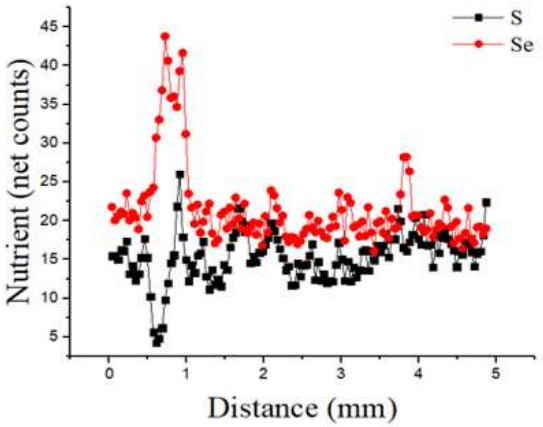
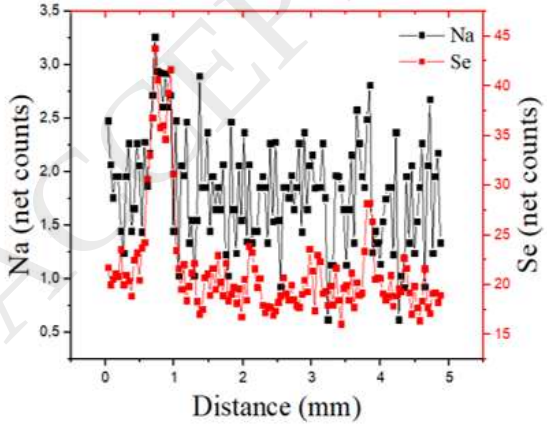


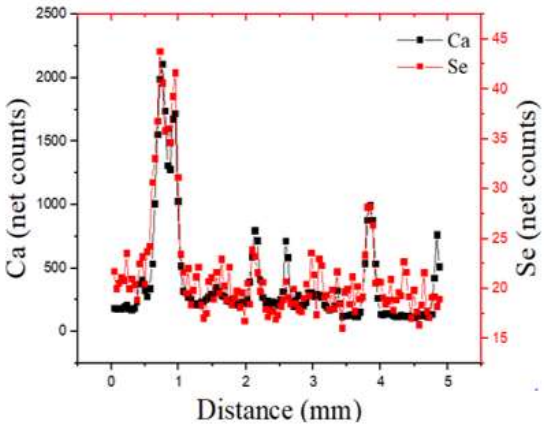
Figure 8. Distribution maps of elements in leaves of cowpea plants sprayed with Se at 1600 g ha^{-1} Se observed using μ-XRF.











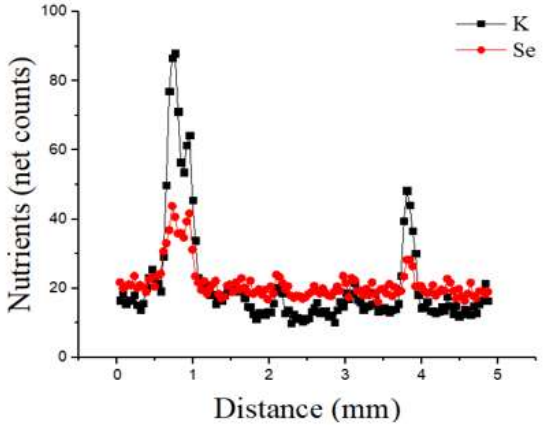


Figure 9. Distribution of elements in leaves of cowpea plants sprayed with Se at 1600 g ha^{-1} Se observed using μ-XRF.