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BASIC AREAS - Article

Physiological and toxic effects of selenium on seed germination of cowpea seedlings

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ABSTRACT: Selenium (Se) is considered a beneficial chemical element for plants, but in high concentrations it may present symptoms of toxicity. The present study aimed to evaluate 11 concentrations of Se (0; 0.1; 0.5; 1; 5; 10; 20; 40; 80; 400; 800 mg.L^{-1}) to determine the low and high (toxicity) critical levels to seed germination of cowpea (*Vigna unguiculata*). In addition, alterations in the rate of photosynthetic pigments, lipid peroxidation and sugars during the initial growth development of seedlings were analysed. Seeds exposed to 800 mg.L^{-1} of Se showed a decrease of 20% of seed germination index compared to the control treatment. The decrease in seedling growth reflected in the increase of total sugars and sucrose concentration in both the shoot and root in response to exposure to Se concentration. There was a decrease in the concentration of leaf chlorophyll, carotenoids and pheophytin from seedlings exposed to high Se concentration. The rate of lipid peroxidation and the hydrogen peroxide concentration in the shoot was reduced up to the concentration of 1 mg.L^{-1} with subsequent increase in response to Se concentration applied. In the roots, the lipid peroxidation rate increased at concentrations higher than 80 mg.L^{-1}. The highest oxidation rate of the cellular lipid membrane in response to Se occurred in the shoot, due to oxidation reactions in the chloroplast. Degradation of photosynthetic pigments and accumulation of total sugars and sucrose can be considered efficient biomarkers to indicate the toxicity of Se in cowpea seedlings and probably in other crops.

Key words: soluble sugars, oxidative metabolism, phytotoxicity, *Vigna unguiculata*.

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INTRODUCTION

Selenium (Se) concentration in the soil is the product of weathering of rocks and/or discharge of anthropogenic sources (Wadgaonkar et al. 2018). Anthropogenic sources and mining operations may increase elemental pollutants in some sensitive areas, making them a risk of environmental contamination (Winkel et al. 2015; Jones et al. 2017). The excessive use of fertilizers containing Se can also be a major contributor to releasing it into agrosystems (Reis et al. 2017; Silva et al. 2018). Selenium concentration in plants and animals is directly correlated with its concentration in soil (White 2016). Toxic concentrations of Se appears to be a potential risk to plants, as well as to human and animal health (El-Ramady et al. 2014; Reis et al. 2017; Reis et al. 2018).

Humans and animals feed on plants, which take up Se from soil or nutrient solutions in hydroponic crops (Joy et al. 2015; Dinh et al. 2017; Li et al. 2017). Several countries, such as India, China and the United States have soils with a high concentration of Se, above 10 $mg \cdot kg^{-1}$, which are categorized as seleniferous soils (Fordyce 2013; Mostofa et al. 2017). Thus, it is extremely relevant to understand the physiological and biochemical mechanisms of how seeds and plants behave in contaminated environments or in the presence of high concentrations of Se in soil (Schiavon and Pilon-Smits 2017).

Cowpea (*Vigna unguiculata* L. 'Walp.') belongs to the Fabaceae Family. This genus is composed of four subspecies, among which *unguiculata* is the most cultivated in the world (Chen et al. 2017). Cowpea seeds are rich in protein and carbohydrate, having on average 20 to 25% of proteins and 45 to 55% carbohydrates (Sreerama et al. 2012). For the low-income population, therefore, it is an alternative for protein consumption, especially in continents such as Africa and Latin America (Almeida et al. 2010; Silva et al. 2018).

Application of Se at low concentration shows a positive effect on germination and physiological quality of seeds in several crops, such as cabbage, alfalfa, radish, sorghum, wheat (Carlson et al. 1989), barley, white mustard, oilseed rape (Molnárová and Fargałová 2009), and rice (Khaliq et al. 2015, Moulick et al. 2016).

High concentrations of Se can interfere with germination by acting on inactivating hydrolytic carbohydrate enzymes and may lead to the death of the embryo (Sreekala and Lalitha 1998; Khaliq et al. 2015). Some studies show that seed germination is inhibited with high Se supply in bitter melon (Chen and Sung 2001; Hartikainen et al. 2000), brown mustard (Prins et al. 2011), and *Arabidopsis thaliana* (EL Mehdawi et al. 2011) plants.

Application of Se at low concentration increases the antioxidant properties of higher plants, reflecting the reduction of reactive oxygen species (Moulick et al. 2016; Silva et al. 2018). However, high concentrations of Se may be toxic to plants due to high generation of reactive oxygen species, such as hydrogen peroxide and induce high lipid peroxidation rates in cell membranes (Hartikainen et al. 2000; Cartes et al. 2005; Mostofa et al. 2017; Reis et al. 2018).

This study aimed to evaluate the critical levels of Se toxicity during seed germination and early development of cowpea seedlings. The photosynthetic pigments (chlorophylls, carotenoids and pheophytins), production of malondialdehyde and hydrogen peroxide, total sugars and sucrose were measured in cowpea seedlings to establish biomarkers tools to evaluate the toxicity of Se in germinated seedlings.

MATERIAL AND METHODS

Samples and experiment design

Seeds of cowpea cultivar 'BRS Xique-Xique' used in this study were collected from the germoplasm bank of Sao Paulo State University. Before installing the germination test, a stock solution of sodium selenate was prepared with dilutions equivalent to 0.1, 0.5, 1, 5, 10, 20, 40, 80, 400, and 800 $mg \cdot L^{-1}$ of Se. Deionized water was used as control treatment, being considered as concentration 0 $mg \cdot L^{-1}$ of Se.

A completely randomized experimental design was made up of 11 treatments and 4 replications. In order to evaluate the effect of Se concentrations on the physiological quality of the cowpea seeds, a germination test was carried out. Then, length and weight measurement of the shoot and the root and quantification of the pigments, oxidative stress and sugars were analysed.

Germination test

In order to perform the germination test of cowpea seeds exposed to Se concentrations, 25 seeds with 4 replicates were used. The seeds were stored in rolls of germitest paper, previously moistened to 2.5 times their own mass, with respective treatments concentrations. Rolls of germitest papers →

were kept in incubators at 25 °C under a 12-hour light and a 12-hour dark period. The count of seed germination was made at 8 days after sowing. The results were expressed as percentage of normal seedlings.

Length and fresh seedling biomass

After the germination count, the shoot and root length of 10 seedlings of each replicate were measured with the aid of a ruler and the results were expressed in cm·seedling^{-1}. Then, the shoot was detached from the root and the same 10 plants were weighed using an analytical balance and the results were expressed as mg·seedling^{-1} (Nakagawa 1994).

Hydrogen peroxide concentration

The concentration of hydrogen peroxide (H_2O_2) was determined by reaction with potassium iodide (KI), according to Alexieva et al. (2001). One g of the plant material from each replicate was weighed and grinded with liquid nitrogen, to which 4 mL of 0.1% trichloroacetic acid (TCA) was added. The material was homogenized using a mortar and pestle and centrifuged at 10,000 rpm for 15 minutes at 4 °C in a refrigerated centrifuge. For the reaction, 200 µL of supernatant, 200 µL of potassium phosphate buffer pH 7.5 (100 mmol·L^{-1}) and 800 µL of KI solution (1 mol·L^{-1}) were added. The samples were kept on ice for 1 h. After warmed up to room temperature, absorbance readings were taken at 390 nm. The hydrogen peroxide concentration in the shoot and root tissue was calculated based on a standard curve and the results expressed in nmol·g^{-1} FW (fresh weight).

Lipid peroxidation

The evaluation of lipid peroxidation was determined by the production of metabolites reactive to 2-thiobarbituric acid (TBA), mainly malondialdehyde (MDA), according to Heath and Packer (1968). The extraction was carried out by homogenizing 1 g of plant material from each replicate in 4 mL of 0.1% (p/v) trichloroacetic acid (TCA) + 20% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 10,000 rpm for 5 min in a refrigerated centrifuge at 4 °C. To 250 µL of the supernatant was added 1 mL of 20% of TCA + 0.5% of TBA.

The samples were kept in dry bath at 95 °C for 30 min, and then transferred to ice, where it remained for another 10 min. Thereafter, the material was again centrifuged at 10,000 rpm for 10 min. Samples were read at two wavelengths, one at 535 nm and one at 600 nm. The results were expressed as nmol MDA·g^{-1} FW.

Analysis of photosynthetic pigments

Determinations of the photosynthetic pigments (chlorophyll, carotenoids and pheophytin) were based on the description of Lichtenthaler and Wellburn (1983), using acetone 80% as solvent. Extraction was carried out with 0.5 g of fresh matter from each replicate in 5 mL acetone 80%. The concentrations of chlorophyll a, chlorophyll b, total chlorophyll, pheophytin a, pheophytin b, total pheophytin and carotenoids were expressed in µg·mL^{-1}.

Determination of total sugars and sucrose

For extraction and determination of total sugars and sucrose, 1 g of plant sample was extracted in 10 mL of MCW solution (60% methanol, 25% chloroform and 15% water). After 48 h in the refrigerator, an aliquot of 4 mL of the supernatant was removed, and 1 mL of chloroform + 1.5 mL of deionized water were added in another tube. After the separation phase, total sugars and sucrose were determined in the aqueous phase, according to the method described by Bieleski and Turner (1966).

The total sugars were determined according to Dubois et al. (1956). For total sugars quantification, 12.5 µL of the supernatant, 0.5 mL of 5% phenol and 2 mL of sulphuric acid were used. Total sugars concentrations were determined based on the standard sucrose curve and the results were expressed as mg·g^{-1} FW.

The sucrose concentration was performed using the methodology described by Van Handel (1967). From the aqueous phase of the supernatant, a 50 µL aliquot of the shoot and 100 µL of the root were removed, and 0.5 mL of 30% potassium hydroxide and 2 mL of sulphuric acid were added. The tubes were homogenized in a vortex mixer and taken to the oven at 100 °C for 10 min. After cooling the tubes, readings were taken at the wavelength of 490 nm. The sucrose concentrations were based on the standard sucrose curve and the results expressed in mg.g^{-1} FW.

Statistical analysis

In all considered datasets, normality of the data was analysed using the Anderson-Darling test and homoscedasticity of the data was verified with the variance equation test (or Levenn's test). The data was submitted for analysis of variance, with levels of significance at 0.05 of probability, by the F test. Where significant, the means were submitted to the Tukey test at 0.05 probability level using the statistical program Minitab and Sigmaplot.

RESULTS

Effect of Se on germination and early growth development of seedlings

Germination of cowpea seeds was lower than the control at 800 mg·L^{-1} (Fig. 1a), while the other Se concentrations did not affect germination. The concentration of 800 mg·L^{-1} negatively affected the germinative power in the order of 20% in comparison to the control treatment.

The shoot and root growth were lower in seedlings exposed to Se concentrations in the solution of 40 and 800 mg·L^{-1} compared to the control (Fig. 1b-c). Root growth started to decrease by the concentration of 1 mg·L^{-1}. A drastic decrease was observed in shoot and root growth in the concentrations of 400 and 800 mg·L^{-1} in the order of 74 and 82% (shoot) and 87 and 94% (root) in comparison to the control treatment, respectively.

Figure 2 adds descriptive information to the effect of increasing Se concentrations on early development of cowpea seedlings. It can be observed that, in the concentrations of 40 to 80 mg·L^{-1}, it is possible to notice decrease in the root system, absence of lateral roots and signs of lipid peroxidation, as well as decrease in shoot and delay of leaf initiation.

Oxidative metabolism response to Se concentration

The concentration of carotenoid, leaf and root lipid peroxidation rates, shoot and root H_2O_2 concentration (Fig. 3) were affected by Se concentrations. The concentration of carotenoids decreased with increasing Se concentrations from 0.5 to 800 mg·L^{-1} (Fig. 3a), showing decreases of 25, 47, 79, 59, 70, 80, 77, 87, and 89%, respectively.

The lipid peroxidation rates in the shoot was lower than in control treatment at concentrations from 0.5 to 10 mg·L^{-1}, while concentrations of 400 and 800 mg·L^{-1} were 34% higher on average (Fig. 3b). The lipid peroxidation →

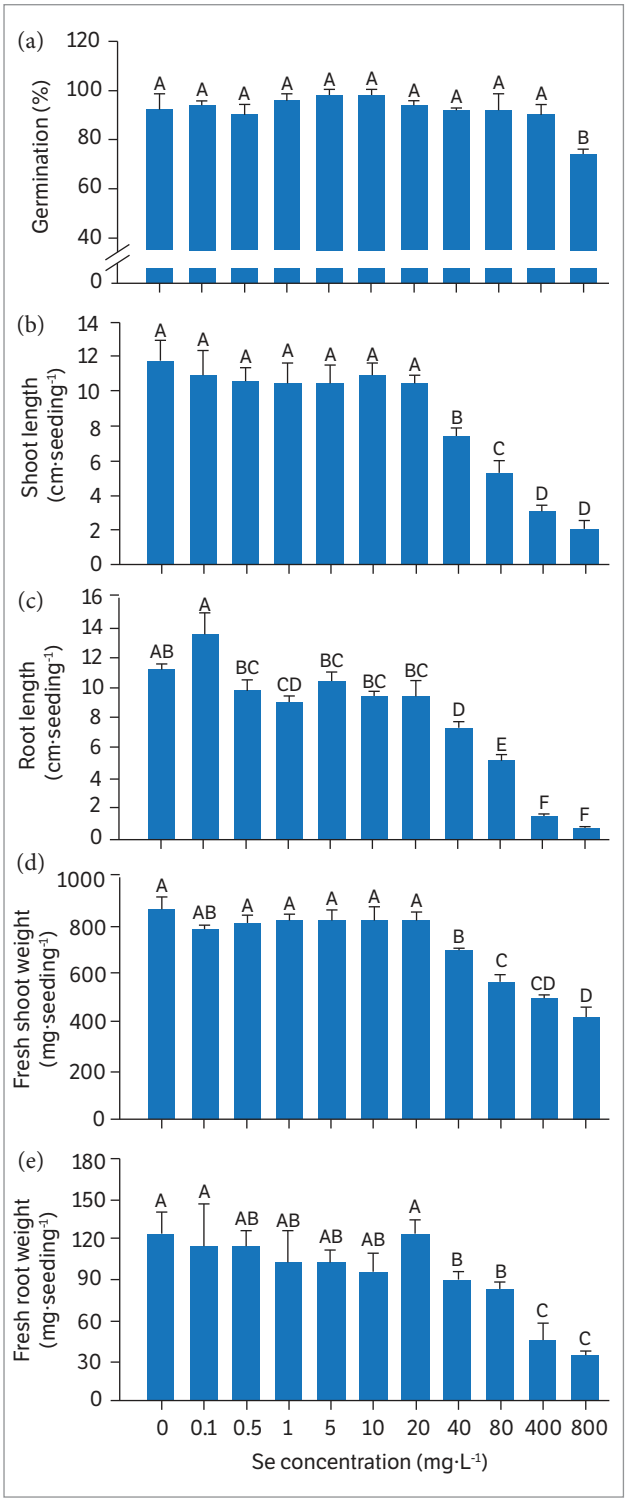

Figure 1. Percentage of seed germination (a); length of the shoot (b) and root (c); fresh mass of shoot (d) and root (e) of cowpea seedlings in response to Se application rates. Vertical bars represent the standard deviation (n = 4).

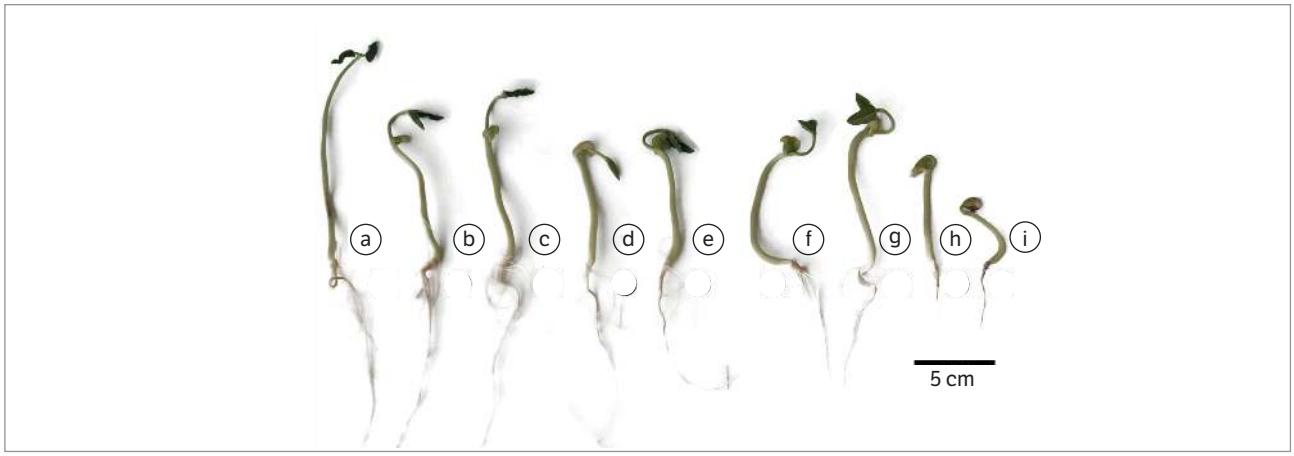

Figure 2. Germination of cowpea in response to Se application rates, where; (a) 0 mg·L^{-1}; (b) 0.1 mg·L^{-1}; (c) 0.5 mg·L^{-1}; (d) 1 mg·L^{-1}; (e) 5 mg·L^{-1}; (f) 10 mg·L^{-1}; (g) 20 mg·L^{-1};(h) 40 mg·L^{-1}; (i) 80 mg·L^{-1}.

rates from the root also followed the trend of reduction followed by increase (Fig. 3c), but the concentrations that had lower rates than the control were those from 0.1 to 40 mg·L^{-1}, and those with higher rates were from 80 to 800 mg·L^{-1}, showing an increase of 17, 10, and 11%, respectively. The concentration of 80 mg·L^{-1} was toxic to the root and not to the shoot in comparison to the control treatment.

The H_2O_2 concentration in the shoot in the concentrations of 1, 5, 40, and 80 mg·L^{-1} was lower than the control (Fig. 3d). The H_2O_2 concentration in the root in concentrations of 0.1 from 5, and 40 mg·L^{-1} was lower than the control (Fig. 3e). On the other hand, the concentrations of 400 and 800 mg·L^{-1} increased the H_2O_2 concentration in the leaf and root compared to the control, the latter being decreased by the concentration of 80 mg·L^{-1}. The percentage increases were respectively 25 and 10% (in the shoot); and 37, 125, and 42% (in the root).

Photosynthetic pigments and sugars

The concentration of chlorophyll a, chlorophyll b and total chlorophyll was affected by Se concentrations, showing lower values in comparison to the control from the concentration of 0.1 mg·L^{-1} (Fig. 4a,b,e). The highest differences in pigment content occurred at the concentrations from 5 to 800 mg·L^{-1}, which had average decreases of 81, 58, 74, 83, 78, 91 and 92%, respectively, when compared to control treatment.

Total pheophytin at concentrations of 0.1 and 0.5 mg·L^{-1} of Se did not differ from the control (Fig. 4c). However, high Se concentration promoted a deleterious effect on the seedlings. The most drastic deleterious effects comprised the same concentrations as observed in chlorophyll (5 to 800 mg·L^{-1}) (Fig. 4a,b,e), showing decreases of 80, 62, 77, 83, 80, 91 and 92%, respectively, in relation to the control. The concentration of pheophytin a, pheophytin b and total pheophytin also responded negatively in response to Se concentrations (Figure 4d,e), but they differed from the control from the concentration of 0.5 mg·L^{-1}, maintaining the most critical concentrations in the same range already observed (5 to 800 mg·L^{-1}), showing average decreases of 83, 64, 77, 82, 80, 91, and 91%, in comparison with control treatment.

Total sugars in shoots were lower than in control at Se concentrations of 0.1, 0.5 and 20 mg·L^{-1} (Fig. 5a), while concentrations of 40 and 800 mg·L^{-1} provided an increase in relation to the control treatment, presenting increments of 23, 49, 72, and 98% respectively. On the other hand, in the root, all concentrations were higher than control (Fig. 5b), except for the concentration of 1 mg·L^{-1} that did not differ from the control, achieving increments of 32, 53, 56, 21, 100, 97, 108, 201, and 125%, respectively.

Sucrose concentration in shoots was lower in the concentrations of 0.1, 0.5, and 1 mg.L^{-1} than in the control treatment (Fig. 5c). The increase of sucrose occurred in the concentration of 20 mg·L^{-1}, which did not differ from the control; the increases were 8, 23, 33, 33 and 45%, respectively, in relation to the control. Similarly to total sugars in roots, all concentrations of sucrose in the root were higher than in the control treatment (Fig.5d), except for the concentration of 80 mg·L^{-1}, which did not differ from it, achieving increases of 20, 53, 58, 46, 34, 36, 39, 87, 164, and 85%, respectively, in relation to the control.

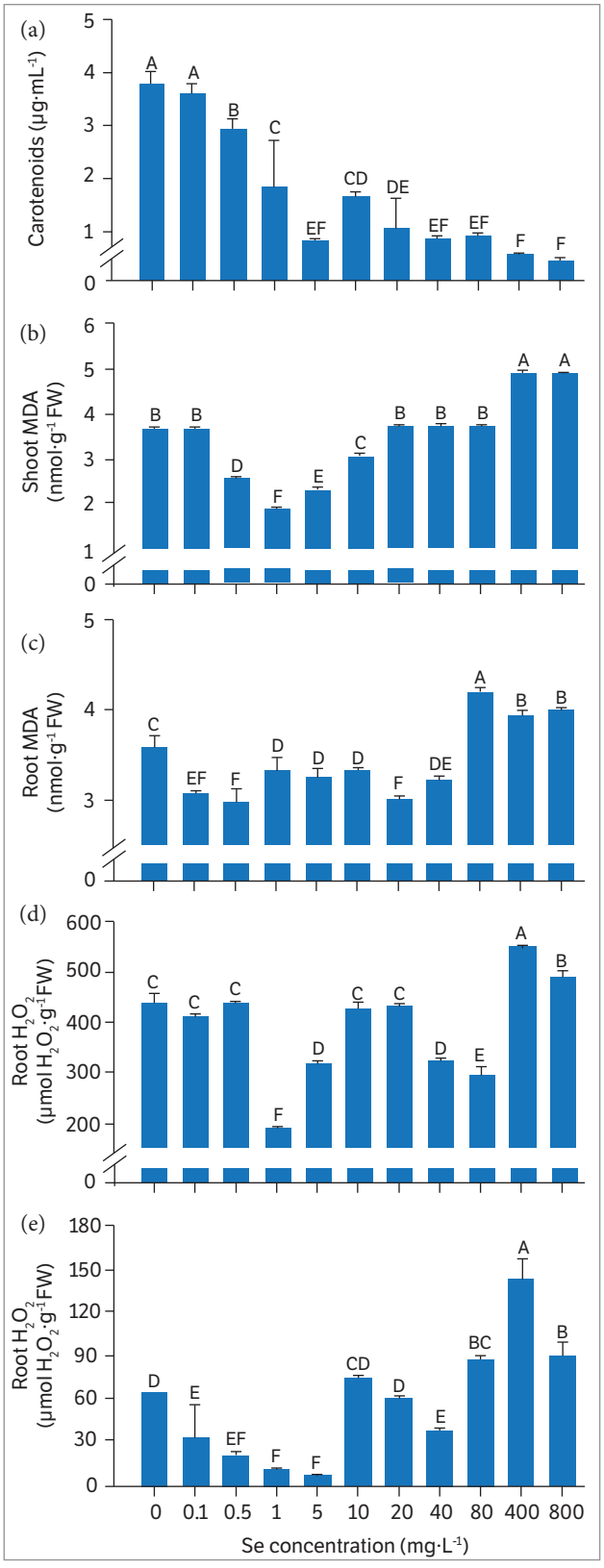

Figure 3. (a) Concentration of carotenoids, malondialdehyde in shoot (b) and root (c); concentration of hydrogen peroxide in shoot (d) and root (e) of cowpea seedlings in response to Se application rates. Vertical bars represent the standard deviation (n = 4).

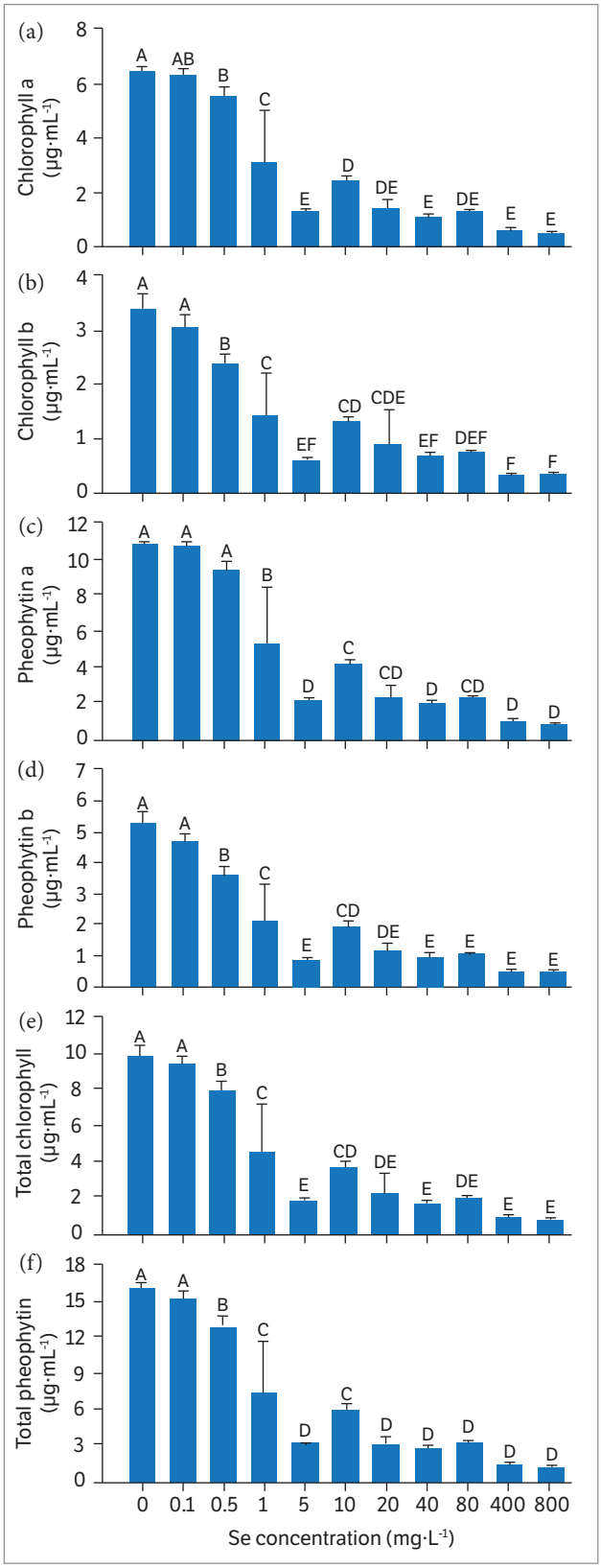

Figure 4. (a) Photosynthetic pigments chlorophyll a, chlorophyll b (b), pheophytin a (c), pheophytin b (d), total chlorophyll (e) and total pheophytin (f) of shoots of cowpea in response to Se application rates. Vertical bars represent the standard deviation (n = 4).

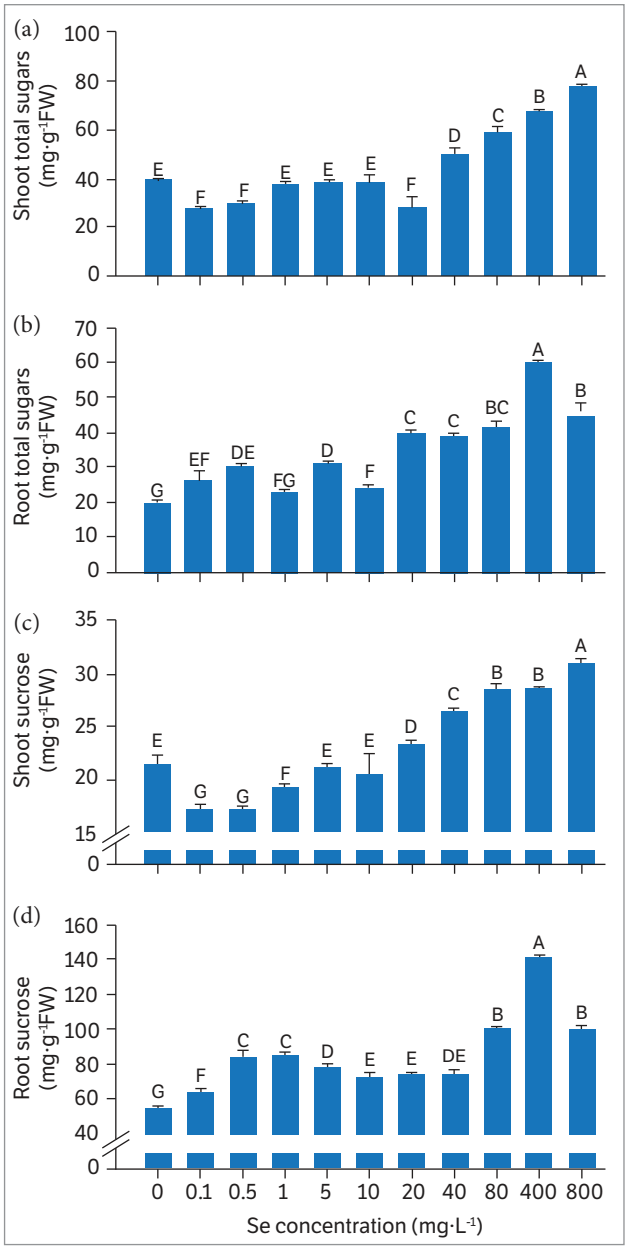

Figure 5. Concentration of total sugars in shoot (a) and root (b); sucrose in shoot (c) and root (d) of cowpea seedlings in response to Se application rates. Vertical bars represent the standard deviation (n = 4).

DISCUSSION

Effect of Se on germination and early growth development of seedlings

Recent studies also showed inhibition of germination when seeds were submitted to increasing Se concentrations (Du et al. 2019). Prins et al. (2011) studied the effect of selenate on the reproductive functions of accumulating and hyperaccumulating species of Se. These authors observed that the germination rate decreased with increasing concentrations of selenate in brown mustard. Mehdawi et al. (2011) observed reduction and inhibition of the germination of *A. thaliana* species sown in soil close to Se hyperaccumulator species due to their apparent ability of concentrate Se, in which the concentration of 10 mg·kg^{-1} of Se inhibited seed germination by 50%. Other studies did not observe an inhibitory effect on germination with the application of selenite and selenate (Carlson et al. 1989; Molnárová and Fargałová 2009), but these results are probably due to the fact that the researchers used smaller Se concentrations than this study.

The inhibitory effect observed in the germination of cowpea seeds when exposed to higher concentrations of Se may be related to inhibition of the activity of enzymes that hydrolyze the metabolites required for embryo development (Sreekala and Lalitha 1998; Khaliq et al. 2015). In this study, it was inferred that these concentrations of Se were more damaging to the root growth when compared to the shoot. The lower root and shoot growth response to selenate and selenite were also verified in other studies (Carlson et al. 1989; Molnárová and Fargałová 2009).

Cowpea seedling length, the shoot and root fresh weight decreased from the concentration of 40 mg·L^{-1} in comparison to the control treatment (Fig. 1d,e). The concentrations of 400 and 800 mg·L^{-1} registered decreases of 43 and 51% (shoot) and 65 and 75% (root), respectively. Application of 800 mg·L^{-1} of Se in rolls of germitest paper decreased 20% of cowpea seed germination (Fig. 1). Application of 20 mg·L^{-1} decreased the biomass of shoot and roots of cowpea seedlings (Figure 2).

It is inferred that the lower growth of cowpea seedlings at the concentrations from 40 to 800 mg·L^{-1} is reflected in a smaller increase of biomass weight. Prins et al. (2011) reported that concentrations of Se higher than 500 mg·kg^{-1} DW in the floral organ impaired plant growth and reproduction, causing reduction in biomass production, pollen germination and seed production. Mehdawi et al. (2011) also found exorbitant decreases in the production of *A. thaliana* biomass growing close to Se hyperaccumulating plants.

It is important to point out that some studies had an opposite effect to those found in this study, such as increased germination, growth and weight of shoot and root with the application of selenite (Chen and Sung 2001; Moulick et al. 2016; Khaliq et al. 2015). In these studies with a positive effect in the physiological quality, the seeds underwent an →

osmotic pre-treatment with Se, in order to later apply the germination test, and in the present study the seeds germinated and developed under Se solution, which may be related with the differential response observed.

Oxidative metabolism response to Se concentration

Carotenoids participate in the light absorption process and act to protect the chlorophyll destruction and degradation of the plasma membrane through the elimination of the reactive oxygen species (Asada 2006; Molnárová and Fargałová 2009; Gratão et al. 2015). The degradation observed in the carotenoids results in the decrease of plant resistance to reactive oxygen species, which favours the increase of lipid peroxidation rates and H_2O_2 concentration (Fig. 3).

High Se concentrations increased the lipid peroxidation rates and H_2O_2 concentration in seedling tissues (Fig. 3), indicating a high peroxidation rate of the lipid bilayer of leaf and root cell membranes. Previous results regarding the effect of high concentrations of selenite and selenate also revealed similar behaviour for lipid peroxidation of cell membranes (Hartikainen et al. 2000, Cartes et al. 2005). In this study, it is possible to observe the beneficial and toxic effects of Se, since it acted as an antioxidant at low concentrations, while high concentrations were toxic to the seedlings. However, Hawrylak-Nowak et al. (2013) observed that lipid peroxidation rates in lettuce leaves treated with selenite and selenite in hydroponics increased under higher Se concentrations, while a small reduction in the lipid peroxidation occurred in the roots.

Photosynthetic pigments and sugars

Exposure of cowpea seed to high concentration of Se showed a drastically decrease in chlorophyll and pheophytin concentrations (Fig. 4). Changes in chlorophyll a, chlorophyll b and total chlorophyll concentrations were similar to changes in pheophytin a, pheophytin b and total pheophytin concentrations. Pheophytins are the first electron carriers in photosystem II (Bodnar et al. 2016). Thus, the amount of these pigments is directly related to the number of reaction centres of photosystem II, so the pheophytin restriction reduces the functional activity of the photosynthetic reaction centres (Silva et al., 2018).

Although the amount of pigments was generally affected from the concentration of 0.5 $mg \cdot L^{-1}$ of Se. Seedling growth and weight generally declined only from the concentration of 40 $mg \cdot L^{-1}$, which is probably due to the fact that cotyledons provide the nutrients, carbon and energy required for early seedling growth, maintaining the supply until the leaves established the photosynthetic process (Lapaz et al. 2017). Similar results were found with hydroponics in selenite-selenate-enriched medium, where the chlorophyll concentration, growth and weight decreased in response to the increase in Se concentrations (Akbulut and Çakir 2010; Mostofa et al. 2017).

Total sugars and sucrose concentration in cowpea seedlings increased in response to Se concentration exposure (Fig. 5). Higher concentration of total sugars and sucrose in shoots may be associated with the lower growth of the cowpea seedlings generated by the stress caused by the Se concentrations. This happens because concentrations of 20 $mg \cdot L^{-1}$ caused lower seedling growth and higher accumulation of sugars in the shoot of seedlings. Similar results were obtained by Silva et al. (2018) working with leaf Se fertilization at high concentrations in cowpea plants under field conditions.

In general, there was an increase in the accumulation of sugars in the shoot and roots of cowpea seedling in response to high Se concentration exposure. This effect is associated with lower seedling growth, and may also be associated with increased activity of amylases and invertases (Malik et al. 2011) and delay in the disappearance of R-galactosides after germination (Vidal-Valverde et al. 2002).

CONCLUSION

The increase of total sugars and sucrose levels in shoot and root and the drastic decrease of photosynthetic pigments are related to the lower seedling growth development in response to high Se exposure. These responses can be considered efficient biomarkers to indicate the phytotoxicity of Se in cowpea seedlings and probably in other crops.

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AUTHORS' CONTRIBUTION

Conceptualization, Reis A. R.; Methodology, Reis A. R., Lapaz A. M. and Yoshida C. H. P.; Investigation, Lapaz A. M., Yoshida C. H. P., Heinrichs R. and Campos M.; Writing – Original Draft, Lapaz A. M.; Writing – Review and Editing, Reis, A. R.; Resources, Heinrichs R. and Reis A. R.; Supervision, Reis A. R.

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