Interaction between sulfur and selenium in agronomic biofortification of cowpea plants under field conditions

Vinícius Martins Silva[1], Lolita Wilson[2], Scott D. Young[2], Martin R. Broadley[2,3], Philip J. White[4], André Rodrigues dos Reis[1,5*]

[1] São Paulo State University (UNESP), Postal Code 14884-900, Jaboticabal, SP, Brazil

[2] School of Biosciences, University of Nottingham, Sutton Bonington, Leicestershire, LE12 5RD, UK

[3] Rothamsted Research, West Common, Harpenden AL5 2JQ, UK

[4] The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

[5] São Paulo State University (UNESP), Rua Domingos da Costa Lopes 780, Jd. Itaipu, Postal Code 17602-496, Tupã, SP, Brazil.

* Corresponding author: E-mail address: andre.reis@unesp.br

Abstract

Aims: Selenium (Se) as selenate shares similarities with sulfate in transport and assimilation by plants. Uptake and assimilation of Se might be affected by S and vice-versa, which could affect Se and S concentration in plant tissues, and metabolic pathways such as biosynthesis of sugars, amino acids, and storage proteins. This study aimed to evaluate Se and S combination on cowpea plants under field conditions.

Methods: The experimental design was a 4x4 interaction between four rates of Se (0, 10, 25, and 50 g ha^{-1}) and four rates of S (0, 15, 30, and 60 kg ha^{-1}) in two consecutive years of cowpea cultivation. Concentrations of Se, S, total sugars, sucrose, total free amino acids, and storage proteins in plant tissue were measured.

Results: The Se x S interaction did not affect cowpea yield or growth. Antagonistic effects of S on Se concentrations in leaves and seeds were observed mainly for the second crop season. Selenium did not decrease S concentrations in leaves and seeds of cowpea plants. The combination of 25 g Se ha^{-1} and 30 kg S ha^{-1} provided the greater concentrations of total sugars. Interaction between Se and S was associated with greater sucrose, amino acids, and storage proteins concentrations in cowpea seeds.

Conclusion: The Se and S interaction did not impair plant growth but application of S decreased Se content in cowpea. Further studies are needed to better understand the physiological roles of Se and S combination in producing primary metabolic compounds.

Keywords: *Vigna unguiculata* (L.) Walp; Selenate; Sulfate; storage proteins, amino acids, total sugars.

1.Introduction

Hidden hunger is defined as the inadequate consumption of vitamins and nutrients and still affects about 2 billion people around the world (Jiang et al., 2021). Although hidden hunger is a worldwide problem, it is more prominent in low and middle-income countries (FAO et al., 2019). The consumption of vegetables, cereals, and fruits of high nutritional quality can alleviate the problems of hidden hunger (Lenaertes & Demont, 2021). However, intensive plant-breeding strategies focus mostly on increasing yield, which might produce food with a lower concentration of minerals in the edible parts of crops (Schjoerring et al., 2018).

Selenium (Se) is an essential nutrient for human health and play a plethora of roles in the human body, such as its role in thyroid synthesis, its antioxidant properties (Ekuma et al., 2021), as well as due to its importance to immune synthesis (Rayman, 2000). Selenium is a beneficial element that can enhance antioxidant metabolism and the photosynthetic system of plants (Silva et al., 2020). However, available Se is scarce in most soils (Yang et al., 2019) and, thus, the edible parts of crops usually have low Se concentrations (Lanza & Reis, 2021). Among the elements related to hidden hunger, Se deficiency is the third most common nutrient deficiency in humans (Joy et al., 2015) and affects about 1 billion people around the world (Schiavon & Pilon-Smits, 2017).

Agronomic biofortification is a proven strategy to combat hidden hunger. The approach consists of enriching edible parts of crops with nutrients, such as Se, aiming to increase these nutrients in the human diet (White &Broadley, 2009; Reis et al., 2017). Studies of agronomic biofortification to increase Se concentration in plants have already been performed using a wide range of crops, including: cowpea, groundnut, soybean, wheat, rice, pear, strawberry, and others (White & Broadley, 2009; White, 2018; Lanza & Reis, 2021).

Agronomic biofortification of crops with Se has been extensively studied in the last decade. However, to fully understand Se uptake and assimilation by plants it is important to consider the relationships between Se and other elements. Selenium is absorbed and transported in plants by sulfur (S) transporters when applied as selenate (White, 2016; Natasha et al., 2018). Thus, the interaction between S and Se application can decrease Se uptake by plants (Liu et al., 2017; Deng et al., 2020). On the other hand, plants with a limited S supply might take up more Se due to an increase in S transporter activity (White, 2016). Sulfur is a macronutrient for plants, playing a role in protein, chlorophyll, and amino acid synthesis (Kopriva et al., 2016), thus its supply is essential for plant yield, quality, and growth (Chowdhury et al., 2020).

In addition to agronomic biofortification, Se might enhance other compounds in edible parts of plants. Some reports indicate that Se can increase total sugar concentration in fruits (Ren et al., 2021) and cereals (Lidon et al., 2018; Lara et al., 2019). Protein concentration can be affected by Se application, as previously observed in rice (Lidon et al., 2018). Since S is also important to proteins, amino acids and sugars synthesis (Kopriva et al., 2016; Dong et al., 2017; Najafi et al., 2020), the interaction between Se and S might affect not only just S and S concentration in plants, but also sugars, proteins, and amino acids on seeds.

Cowpea (*Vigna unguiculata* (L.) Walp) is resistant to drought and high temperatures (Carvalho et al., 2012). Cowpea seeds are one of the most important protein sources in low and middle-income countries (Manzeke et al., 2017). Cowpea protein concentration in seeds might surpass that of common beans (Teka et al., 2020). Previous reports demonstrate that, under proper Se application, cowpea can accumulate Se at safe concentrations in seeds (Silva et al., 2019). Selenium application also increases total sugar and sucrose content in cowpea leaves (Silva et al., 2020). Thus, the interaction of Se with

S in cowpea plants could be a useful line of investigation in trying to optimize Se biofortification in this crop.

Thus, this study aimed to evaluate the effect of Se and S interaction on cowpea on yield, Se and S accumulation in cowpea leaves and seeds, as well as the concentration of total sugars, sucrose, storage proteins, and amino acids in cowpea seeds.

2.Material and Methods

2.1. Experimental design and trial location

The experiment used a randomized complete block design, with four blocks and 16 treatments, totaling 64 plots. The plot comprised five rows of 2 m each, the rows were spaced by 0.45m. The treatments were a factorial scheme using four application rates of Se (0, 10, 25, and 50 g ha^{-1}) applied as sodium selenate (Sigma-Aldrich, St. Louis, Missouri, United States) and four application rates of S (0, 15, 30, and 60 g ha^{-1}) applied as ammonium sulfate. To balance the N supply in every plot, a stoichiometric correction was performed applying urea, to compensate for the N supplied as ammonium sulfate (Table 1).

The experiment was carried out in two consecutive years (2016 and 2017) at the 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 in the experimental area was classified as a Rhodic Haplusfox according to the Soil Survey Staff (2014). To determine the soil chemical properties, on September 10th, 2016, 20 soil subsamples were randomly collected from the top 20 cm depth of the experimental area. The subsamples were mixed together, homogenized, and evaluated according to Van Raij et al. (1997), revealing the following characteristics: pH (water) 5.5; P (resin) 34 mg kg^{-1}; S (calcium phosphate) 8 mg kg^{-1}; K (resin) 2.7 mmol$_c$ kg^{-1}; Ca (resin) 14 mmol$_c$ kg^{-1}; Mg (resin) 14 mmol$_c$ kg^{-1}; H+Al (SMP buffer) 26 mmol$_c$ kg^{-1}; cation exchange capacity 56.7 mmol$_c$ kg^{-1}; base saturation 54%; B (hot water) 0.19 mg kg^{-1}; Cu (DTPA) 2.7 mg kg^{-1}; Fe (DTPA) 19 mg kg^{-1}; Mn (DTPA) 12.4 mg kg^{-1}; Zn (DTPA) 6.1 mg kg^{-1}; organic matter 18 g kg^{-1}. Readily available Se was estimated at 3.6 µg kg^{-1} according to the methodology described by Silva et al. (2019). The daily mean temperature and rainfall during the experiment were recorded and are registered in supplementary Fig. S1.

2.2. Crop Husbandry and sampling

Since the experimental area presented a very compacted soil, before the first year of sowing, the soil was prepared by subsoiling, heavy disking, medium disking (twice), and leveling. The sowing was performed on October 18, 2016 (first year) and March 23, 2017 (second year) with a spacing of 0.45 m between rows and a sowing density of 11.2 seeds m^{-1}. Fertilization on the planting furrow consisted of 16.5 kg ha^{-1} K applied as KCl (33 kg ha^{-1}) and 8.7 kg ha^{-1} P applied as single superphosphate (110 kg ha^{-1}). The planting furrow fertilization was performed mechanically together with the sowing.

To avoid germination problems due to diseases and pests attack, 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 (*Bradyrhizobium* sp strain SEMIA 6462, product registration number SP 00581-10030-1, 2.0×109 colony-forming units g^{-1}, BIOMAX, São Joaquim da Barra city, Brazil), at an inoculation rate of 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 min. Emergence began on October 22, 2016, four days after sowing (DAS), in the first year, and March 28, 2017, five DAS, in the second year.

The treatments were applied at 39 DAS in the first and the second year. To ensure an accurate Se application in each plot, the total Se required for each treatment (four replicates) was weighed and diluted in 1 L of deionized water, generating a 1 L solution for each treatment. The stock solution was then subdivided into four portions of 250 mL each, the application was direct to soil, in each plot of five rows, resulting in 50 ml of stock solution being applied in each row. The different stock solutions, for each Se treatment, were stored in labeled bottles to avoid contamination. Ammonium sulfate and urea fertilization were performed by hand, in the line near the plant. The amount of ammonium sulfate and urea required was weighted and applied, for each 2m line, aiming to provide a proper application of the fertilizers.

During the experiment, pest control operations were performed as follows: in the first year, pest control measures were carried out at 16 DAS (abamectin, 0.50 L ha^{-1}), 27 DAS (bentazone, 0.8 L ha^{-1}; abamectin, 0.5 L ha^{-1} and imidacloprid, 0.4 L ha^{-1}), and at 37 DAS (haloxyfop-p-methyl, 0.3 L ha^{-1}). In the second year, pest control was carried out at 15 DAS (Clethodim + Alquilbenzene 0.40 L ha^{-1}, beta-cyfluthrin 0.15 L ha^{-1}, abamectin, 0.50 L ha^{-1}), 19 DAS (benthazon 1.2 L ha^{-1}), 20 DAS (deltamethrin 0.06 L ha^{-1}, beta-cyfluthrin+imidacloprid 0.87 L ha^{-1}) and 33 DAS (beta-cyfluthrin 0.15 L ha^{-1}, abamectin, 0.50 L ha^{-1}).

Leaf sampling was undertaken at 60 DAS in the first year and 58 DAS in the second year. The third trifoliate leaf (counting from the apex) was removed, dried in an oven at 40 °C to a constant mass, and ground in a Wiley mill with a 1 mm sieve. For each plot, ten trifoliate were collected randomly from 10 homogeneous plants. Harvest and plant height evaluations were performed at 81 DAS in the first year, and at 77 DAS in the second year. To harvest the experiment, two homogeneous rows were selected in each plot, from which all pods were collected manually; seeds were collected from the pods manually. The seeds were dried in an oven at 40 °C to a constant mass, and ground in a Wiley mill with a 1 mm sieve.

2.3. Selenium and sulfur analyses

For Se analysis, subsamples of ground leaf and seed samples equivalent to approximately 0.20 g dry weight (DW) were digested in 2 mL HNO_3 70%, 1 mL Milli-Q water, and 1 mL H_2O_2 prior to analysis by ICP-MS (Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany). The analysis was performed according to Thomas et al. (2016). The results were expressed in mg kg^{-1} on DW basis.

For S analysis, ground leaf and seed subsamples equivalent to approximately 0.25 g DW were digested in 3 mL HNO_3 and $HClO_4$ solution (2:1 v/v) prior to analysis by spectrophotometry according to Malavolta et al. (1997). The samples were evaluated in A spectrophotometer (SP-220, bioespectroTM), in absorbance, at 420 nm wavelength. The results were expressed in g kg^{-1}.

2.4. Determination of total sugars, sucrose, and free amino acids

Sucrose, total sugar, and free amino acids analysis were performed employing the same extraction method (Bielesk & Turner, 1996) and using ground seed subsamples equivalent to approximately 1.0 g DW for total sugar and sucrose, and 0.5 g DW for amino acids. The material was extracted in 5 mL MCW solution (60% methanol, 25% chloroform, and 15% water v/v). After extraction, the material was stored at 10 °C for phase separation.

From the hydrophilic phase, 10 μL were used to perform sucrose analysis according to Van Handel (1968). The observed results were expressed in mg g^{-1} DW and were quantified using a sucrose standard calibration curve. To perform total sugar analysis, 10 µL of the hydrophilic fraction was used according to the method described by Dubois et al. (1956). The results were expressed in mg g^{-1} DW and were quantified using a sucrose standard calibration curve. The free amino acid evaluation was performed according to Yemm et al. (1955) using 15 µL of the hydrophilic portion of the extract. Results were expressed in mg g^{-1} DW and quantified using a methionine standard curve. A spectrophotometer (SP-220, bioespectroTM) was used to perform all readings.

2.5. Determination of storage proteins

To perform albumin, globulin, prolamin and glutelin analysis, 0.25 g DW of seeds were extracted respectively in 5 mL deionized water for albumin analysis, 5 mL of NaCl 5% for globulin analysis, 2.5 mL ethanol 60% for prolamin analysis, and 5 mL NaOH 0.4% for glutelin analysis. The evaluation was performed according to Bradford (1976). The results were expressed in mg g^{-1} DW and quantified using a Bovine Serum Albumin standard curve. A spectrophotometer (SP-220, bioespectroTM) was used to perform all readings.

2.6. Statistical analysis

An Anderson-Darling test was performed on the obtained data to verify normality. The variance analysis (F test) was performed. When differences were observed among treatments, a Tukey test at 5% probability was used to compare the means. Analysis was performed in the R software (version 3.5.1).

3. Results

Cowpea yield in the first year and plant height in the first and second year were not affected by Se application, S application, or Se and S interaction (Fig. 1a, b, d). In the second year, an interaction was observed between Se and S for cowpea yield ($p < 0.05$; Table 2). In this case, under the Se application rate of 10 g ha^{-1}, the application of 30 kg ha^{-1} of S produced a yield 12% higher than under an application of 15 kg ha^{-1}, and 7% higher than the control, which presented a mean yield of 1815 kg ha^{-1}. Under a S application rate of 30 kg ha^{-1}, Se applied at the rate of 10 g ha^{-1} produced a cowpea yield 66 % higher than under an application rate of 25 g ha^{-1} and 33% higher than the control(Fig 1.c).

The concentrations of Se in leaves and seeds of cowpea in the first year were not affected by S application, or by an interaction between Se and S, but were affected by Se application rate ($p < 0.01$; Table 2). In both tissues, the increase in Se application rates produced a direct increase in Se concentration in leaves and seeds (Fig. 2a, b). In leaves, a Se concentration at an application rate of 50 g ha^{-1} (1.5 mg Se kg^{-1} DW) was 25 times greater than at 0 g Se ha^{-1} (0.06 mg Se kg^{-1} DW); in seeds, at 50 g ha^{-1} (1.24 mg Se kg^{-1} DW) the Se concentration was 26 times greater than 0 g Se ha^{-1} (0.047 mg Se kg^{-1} DW).

In the second year, an interaction between Se and S application was observed in leaves ($p < 0.01$; Table 2) and seeds ($p < 0.01$; Table 2). In leaves, under Se application rates of 25 and 50 g ha^{-1}, S applications decreased Se concentration in tissue. The more prominent observed decrease was under 50 g ha^{-1} of Se application, in which the S application of 30 kg ha^{-1} (0.6 mg Se kg^{-1} DW) produced a decrease of 79% in Se concentration, compared to 0 g S ha^{-1} (3.2 mg Se kg^{-1} DW). Under S application rates of 0, 15, and 60 kg ha^{-1}, increasing S rates directly decreased the effect of Se application on Se concentration in leaves. Under S application rate of 0 kg ha^{-1}, a 15 times increase between 0 g ha^{-1} (0.31 mg Se kg^{-1} DW) and 50 g ha^{-1} (3.2 mg Se kg^{-1} DW) of Se was observed, followed by 60 kg ha^{-1} (13 times increase 0 and 50 g ha^{-1} of Se), and less accentuated under 15 kg ha^{-1} of S (7 times increase between 0 and 50 g ha^{-1} of Se; Fig. 2c).

In the second year, in seeds, under a Se application of 50 g ha^{-1}, S application decreased Se concentration at all rates compared to 0 kg ha^{-1} of S (1.8 mg Se kg^{-1} DW). Under all Se application rates, there was an interaction with S application: the greater the S application rate, the smaller was the increase in Se concentration provided by Se applications (Fig. 2d).

The concentrations of S in leaves and seeds were affected only by S application rates, in both the first and second year ($p < 0.01$; Table 2). In the first year, S application produced an increase in S concentration in leaves at all application rates, with the application of 60 kg ha^{-1} (1.9 g S kg^{-1} DW) providing the greatest increase: 35% compared to 0 g ha^{-1} of S (1.4 g S kg^{-1} DW - Fig. 3a). In seeds in the first year, S application increased the S concentration, but without any differences among the 15, 30, and 60 kg ha $^{-1}$ of S application rates compared to 0 g ha $^{-1}$ of S (1.51 g S kg^{-1} DW - Fig. 3b). In the second year, on the other hand, S concentration in both leaves and seeds was higher under a S application rate of 30 kg ha $^{-1}$: 12%, and 15% higher in leaves and seeds, respectively, than under 0 kg ha^{-1}, in which S concentration was, respectively 1.31 and 1.34 g S kg^{-1} for leaves and seeds (Fig. 3c, d).

In both years, interactions between Se and S application rates were observed for the concentration of total sugar, sucrose, and free amino acids ($p < 0.01$; Table 3) in seeds. In the first year, the greatest total sugar concentration observed was under an application of 25 g ha^{-1} of Se and 30 kg ha^{-1} of S, 23% higher than the control (16.7 mg kg^{-1} DW). The smallest sugar concentration occurred under 0 g ha^{-1}of Se and 15 kg ha^{-1} of S: 11% lower than the control (Fig. 4a). Considering sucrose concentration in seeds in the first year, the highest concentration was observed under 10 g ha^{-1}of Se and 30 kg ha^{-1} of S, 42% greater than the control (7.2 mg kg^{-1} DW). The smallest concentration was observed under 25 g ha^{-1}of Se and 0 kg S but was not statistically different from the control (Fig. 4b).

In the second year, the highest total sugar concentration was observed under 25 g a^{-1}of Se and 0 or 30 kg ha^{-1} of S, 46% higher than control (11.9 mg kg^{-1} DW, Fig. 4c). Regarding sucrose concentration in seeds in the second year, the highest concentration was observed under 0 g ha^{-1}of Se and 60 kg ha^{-1} of S, 39% higher than control (5.7 mg kg^{-1} DW, Fig. 4d).

For free amino acids in the first year, the greatest concentration was observed under 50 g ha^{-1}of Se and 30 kg ha^{-1} of S, 26% larger than control (26.13 mg kg^{-1} DW), and the lowest under 25 g ha^{-1}of Se and 0 kg ha^{-1} of S, 25% lower than control (Fig. 4e). In the second year, the highest concentration of free amino acids was observed under 10 g ha^{-1}of Se and 0 kg ha^{-1} of S, 55% higher than the control, and the lowest under 25 g ha^{-1}of Se and 60 kg ha^{-1} of S, 28% lower than control (17.7 mg kg^{-1} DW, Fig. 4e).

For all storage proteins in seeds, an interaction between Se and S application rates was observed in both the first and second year (Table 3). For albumin, in the first year, the highest concentration, 27% higher than control (77.83 mg kg^{-1} DW), was observed under 0 g ha^{-1}of Se and 15 kg ha^{-1} of S, the lowest, 9% lower than control, was observed under 50 g ha^{-1}of Se and 60 kg ha^{-1} of S (Fig. 5a). Regarding globulin in the first year, the highest concentration was observed under 0 g ha^{-1}of Se and 30 kg ha^{-1} of S, 77% higher than the control (36.44 mg kg^{-1} DW), the control presented the lowest globulin concentration, with statistically similar results observed under 0 g ha^{-1}of Se and 15 kg ha^{-1} of S, and 50 g ha^{-1}of Se and 0 kg ha^{-1} of S (Fig. 5b). In the second year, the albumin concentration was highest under 25 g ha^{-1}of Se and 30 kg ha^{-1} of S, 28% higher than the control (83.43 mg kg^{-1} DW), while the lowest albumin concentration, 6% lower than the control, was observed under 50 g ha^{-1}of Se and 30 kg ha^{-1} of S, ith statistically similar results observed under 0 g ha^{-1}of Se and 30 kg ha^{-1} of S, (Fig. 5c). Globulin in the second year presented the highest concentration, 41% higher than the control (45.87 mg kg^{-1} DW), under 25 g ha^{-1}of Se and 30 kg ha^{-1} of S, with statistically similar results observed under 10 g ha^{-1}of Se and 0 kg ha^{-1} of S, the lowest concentration of albumin was observed under 25 g ha^{-1}of Se and 0 kg ha^{-1} of S, 21% lower than control (Fig. 5d).

The highest prolamin concentration in the first year was observed under 0 g ha^{-1}of Se and 15 kg ha^{-1} of S, 98% higher than control (0.68 mg kg^{-1} DW), with statistically similar results observed under 50 g ha^{-1}of Se and 0 or 30 kg ha^{-1} of S. The lowest prolamin concentration in the first year, 42% lower than the control, was observed under 25 g ha^{-1}of Se and 60 kg ha^{-1} of S (Fig. 6a). The highest glutelin concentration in the first year was presented under 0 g ha^{-1}of Se and 30 or 60 kg ha^{-1} of S, 25% higher than control (43.56 mg kg^{-1} DW), the lowest concentration of glutelin in the first year was presented under 25 g ha^{-1}of Se and 0 kg ha^{-1} of S, 40% lower than control (Fig. 6b). In the second year, prolamin concentration was greatest under 25 g ha^{-1}of Se and 15 kg ha^{-1} of S, 109% higher than control (0.56 mg kg^{-1} DW), and was smallest (16% lower than control) under 25 g ha^{-1} of Se and 0 kg ha^{-1} of S (Fig. 6c). The concentration of glutelin in the second year was highest, 29% higher control (37.97 mg kg^{-1} DW), under 25 g ha^{-1}of Se and 60 kg ha^{-1} of S, and lowest (30% lower than control) under 10 g ha^{-1}of Se and 0 kg ha^{-1} of S (Fig. 6d).

4. Discussion

In general, Se and S application did not affect cowpea yield and plant height (Fig. 1), thus, agronomic biofortification of cowpea plants with Se associated with S showed no impairment of seed yield, which is valuable information. Previous studies have also observed that, under the range of 2.5 to 60 g ha^{-1} as selenate or selenite, Se application had no effect on cowpea yield (Silva et al., 2019). Sulfur application can increase the yield of plants, as reported for soybean (Deng et al., 2020), faba beans (Barłóg et al., 2018) and rapeseed (Liu et al., 2017) when this nutrient is in deficient in the soil, which is not the case in the current study, since the available S concentration in soil of 7 mg dm^{-3} is considered between average and high (Ambrosano et al., 1997)

Direct application of Se, as sodium selenate, to soil was an efficient means to provide Se to the plant (Fig. 2). In Brazilian soil conditions, sodium selenate is a more suitable Se source than selenite for cowpea (Silva et al., 2019). Although in the first year no interaction was observed, in the second year a S and Se interaction led to a decrease in leaf and seed Se concentration with increasing S supply to cowpea. The selenate anion is taken up by root cells by sulfate transporters (Cabannes et al., 2012; White, 2016; Natasha et al., 2018). In Arabidopsis, it was observed that the sulfate transporter AtSULTR1;1 might contribute more to Se uptake in plants that lack S than in S-replete plants (White et al. 2004; El Kassis et al., 2007; White, 2016). An inhibitory effect of S on Se uptake has been reported in previous experiments: in rapeseed, under application rate of 60 kg ha^{-1} as sulfate (and elementary S), S decreased Se concentration in seeds (Liu et al., 2017) and, on two distinct soil types, S application at the rate of 100 mg kg^{-1} inhibited the Se uptake in soybean (Deng et al., 2020). The present study revealed the antagonist effect of S to Se uptake. In the second year, mainly at 50 g ha^{-1} of Se application, increasing S rates produced a substantial decrease in Se concentration in both leaves and seeds (Fig. 2c,d), probably due to the saturation of transporters with sulfate.

Although sulfate application produced a decrease in Se uptake, the contrary was not observed as Se application did not have any influence in S concentration in leaves and seeds (Fig. 3). Similar behavior has been reported in other studies examining Se and S interactions, as previously mentioned for rapeseed (Liu et al., 2017). This outcome is likely to reflect the relative Se and S concentrations in the soil and plant tissue. Thus, the increase in Se content in the soil solution and plant tissue is unlikely to affect the S content, which is more than 1000 times greater.

In the first year, the S concentration increased directly with S application, while in the second year, the highest S concentration was observed in seeds and leaves under a S application rate of 30 kg ha^{-1} of S, whereas the highest application (60 kg ha^{-1} of S) produced a decrease in S concentration in plant tissue, compared to the application rate of 30 kg S ha^{-1}. There are no official recommendations for S applications to cowpea, however, considering soil with similar characteristics to the current experiment, according to Ambrosano et al. (1997), for other *Fabaceae* plants the ideal S supply would be 30 kg ha^{-1}. Application rates of 60 kg ha^{-1} could be excessive (Fig. 3c and d). Thus the concentration of Se in tissues was only affected by S application rate in the second year, whereas in the first year S applied in soil was not excessive and did not impair Se uptake. In the second year, the excessive S availability appeared to be detrimental to Se uptake by roots. Is noteworthy that, in the first year more days of rain were observed right after Se and S application (Fig. S1). This can have enhanced selenate availability in soil, hindering its interaction with sulfate.

Sucrose concentration varied without a specific pattern (Fig. 4b and d), but the interaction between S and Se application provided an intriguing increase in total sugar concentration in cowpea seeds (Fig. 4a and c), even though at higher S application, the Se concentration decreased in leaves and seeds (Fig 2). Previous reports have indicated an effect of both S and Se on sugar content. Sulfur deficiency in *Arabidopsis thaliana* decreased sucrose, fructose, and maltose concentration (Dong et al., 2017), while the application of S as nanoparticles up until 1 mg mL^{-1} can increase total sugar concentration in lettuce (Najafi et al., 2020). On the other hand, Se application has been reported to increase total sugar concentration in apples (Ren et al., 2021), seeds of rice (Lidon et al., 2018), shoots of wheat plants (Lara et al., 2019), and leaves of cowpea (Silva et al., 2020). The combination of 25 g ha^{-1} of Se and 30 kg ha^{-1} of S provided the greatest total sugar concentration. This was most likely due to possible detrimental effects of higher application rates: high levels of Se could decrease photosynthetic rates (Lanza et al., 2021) and, consequently, sugar content (Silva et al., 2019) in cowpea. Whereas, in rice, the application of S has been reported to decrease the activity of sugar-related enzymes, as well as total sugar content (Das et al., 2018). Thus, the ideal combination of S and Se supply might play an important role in providing optimal sugar levels in plants.

In the second year, a decrease in the concentrations of free amino acids was observed under combined high levels of Se and general applications of S. There is a narrow range between Se beneficial effects and toxicity in plants (Silva et al., 2018; 2019). High levels of Se may increase the concentration of reactive oxygen species (ROS) within the cell, such as H_2O_2, leading to lipid peroxidation (Mostofa et al., 2017). It is possible that under the higher Se application rates, mostly 50 g ha^{-1}, a stressful condition might lead to impaired free amino acids synthesis. Cysteine is a precursor of glutathione (Gigolashvil & Kopriva, 2014) which is an important compound in the scavenging of ROS (Cummins et al., 2011). Considering this information, one possibility is that at high levels of Se, the cysteine available is being converted in glutathione to play its defensive role in the ROS scavenging, which might explain the low free amino acid content observed in the second year.

The role of S in methionine and cysteine synthesis (Panduragan et al., 2015), and the possible substitution of Se in these two amino acids (White, 2018), also suggests that these elements might affect protein concentration. And, although storage protein concentration in seeds was affected by interactions between Se and S application in both years, the results observed varied widely. Previous studies have reported the effect of S on storage protein content in Fabaceae: considering an arable depth of 20 cm, it has been observed that the equivalent of 100 kg ha^{-1} of S can increase the concentration of all four storage proteins in soybean seeds (Ibañez et al., 2020). On the other hand, Se application could provide very distinct results regarding storage proteins: while some reports have suggested that Se application can increase storage protein in rice (Reis et al., 2019), there are also studies indicating that Se can promote the degradation of globulin and albumin (Liu et al., 2011), indicating that the effect of Se on storage proteins is complex and not yet fully understood. Thus, to evaluate the interaction between these two elements, further investigations might be necessary to explain these widely variable results.

5. Conclusions

The interaction between Se and S application did not impair the growth or yield of cowpea. However high levels of S application can decrease Se accumulation in leaves and seeds of cowpea. Thus, the supply of S as ammonium sulfate should be performed carefully in crops cultivated for agronomic biofortification of seeds with Se. The wide variation in seed quality indicate that Se and S interaction in the plant could be complex, and further studies should be performed to investigate how Se and S can regulate the accumulation of sugars, free amino acids, and storage proteins in seeds.

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Declarations and statements

Findings

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

André Rodrigues dos Reis idealized the study and provided supervision to the project. Vinicius Martins Silva performed the field experiment, lab analysis and wrote the first manuscript draft. Lolita Wilson and Scott D, Young provided technical support and background on lab analysis and results interpretation. Martin R. Broadley and Philip J. White provided intellectual background and support in the project idealization. All authors read and commented previous versions of the manuscript to improve it. All authors read and approved the final manuscript.