UNIVERSIDADE ESTADUAL PAULISTA (UNESP)
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CÂMPUS DE JABOTICABAL

INTEGRATIVE EFFECTS OF SELENIUM AND SULFUR ON NUTRITIONAL AND PHYSIOLOGICAL RESPONSES OF COWPEA (*Vigna unguiculata* (L.) Walp) GENOTYPES

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DADOS CURRICULARES DO AUTOR

VINICIUS MARTINS SILVA – Nascido em 31 de maio de 1991, Natural de Ilha Solteira, São Paulo. Em 2009, ingressou no curso de agronomia na Faculdade de Engenharia de Ilha Solteira (UNESP), foi bolsista de iniciação cientifica da Fundação de Amparo à Pesquisa do Estado de São Paulo (11/00412-1) sob orientação do professor Dr. Salatier Buzetti. Graduou-se em 2014 e após breve período trabalhando no setor sucroalcooleiro, em 2016 iniciou mestrado em Agronomia (Sistemas de Produção) na Faculdade de Engenharia de Ilha Solteira (UNESP), sob orientação do Prof. Dr. André Rodrigues dos Reis e Coorientação do Prof; Dr. Marco Eustáquio de Sá. Foi bolsista de mestrado pela Fundação de Amparo à Pesquisa do Estado de São Paulo (16/19773-8) e bolsista BEPE (17/21950-8) como pesquisador visitante na University of Nottingham (Inglaterra) sob supervisão do Prof. Dr. Martin Broadley. Em 2019 defendeu o Mestrado e iniciou o doutorado em Agronomia (Produção Vegetal) na Faculdade de Ciências Agrárias e Veterinárias (UNESP), sob orientação do Prof. Dr. André Rodrigues dos Reis e Coorientação do Prof. Dr. Arthur Bernardes Cecílio Filho e da Profª. Drª. Priscíla Lupíno Gratão. Foi bolsista de doutorado pela Fundação de Amparo à Pesquisa do Estado de São Paulo (18/18936-6) e bolsista BEPE (20/12218-4) como pesquisador visitante na Cornell University (Estados Unidos) sob supervisão da Profª. Drª. Li Li. Foi laureado como melhor aluno da 38ª turma do curso de agronomia da Faculdade de Engenharia de Ilha Solteira. Junto ao seu orientador André Rodrigues dos Reis, foi laureado (3º lugar) com o Prêmio Péter Murányi 2020 – Alimentação, pelo trabalho realizada com Biofortificação de Feijão-Caupi. Atuando nas áreas de nutrição vegetal, biofortificação e fisiologia de plantas, publicou mais de 30 artigos em periódicos revisados por pares, é revisor dos periódicos *Scientific Reports, Gesunde Pflanzen* e *The Brazilian Journal of Biosystems Engineering*. De acordo com o banco de dados da Web of Science, sua produção científica soma 384 citações e resulta em índice H 11 (19/08/2023).

“Somewhere, something incredible is waiting to be known.”

- Sharon Begley, Jornalist
August 15, 1977, Newsweek magazine

DEDICO

A minha parceira, companheira, a mulher que escolhi para estar ao meu lado durante toda a minha vida, Líria Nakamiti Novaes. Que me apoia em cada decisão, me motiva a ser cada vez melhor e mais forte, para poder apoiá-la também.

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INTEGRATIVE EFFECTS OF SELENIUM AND SULFUR ON NUTRITIONAL AND PHYSIOLOGICAL RESPONSES OF COWPEA (*Vigna unguiculata* (L.) *Walp*) GENOTYPES

RESUMO – Selênio (Se) e enxofre (S) são elementos similares que afetam a absorção e assimilação um do outro pelas plantas, principalmente na forma de selenato e sulfato, pois compartilham transportadores nas plantas. Essa tese objetivou investigar mais a fundo a interação entre Se e S no metabolismo de plantas, estudando o feijão-caupi (*Vigna unguiculata*), uma importante fonte de proteína e nutrientes em países em desenvolvimento. Na revisão de literatura (capítulo 1), foi observado que a interação entre Se e S pode afetar produtividade das culturas, concentração de metabólitos e que o S pode atenuar a toxicidade de plantas a exposição ao Se. Muitos trabalhos mencionam a importância dos transportadores de sulfato (SULTRs) na absorção e assimilação de Se e S, mas apenas alguns estudos de fato estudam o efeito desses elementos combinado na expressão desses genes. No capítulo dois, o efeito da aplicação de selenito e selenato no sistema antioxidante e na concentração de pigmentos e açúcares foi avaliado em experimento de feijão-caupi cultivado em condições de campo. A aplicação de doses baixas de selenato (2,5 e 5 g ha^{-1}) resultou em notável aumentos na concentração de pigmentos e açúcares, bem como estimulou o sistema antioxidante. Nos capítulos três e quatro, 29 genótipos de feijão-caupi sob aplicação de Se foram avaliados para observação da partição do Se pelos tecidos (cap 3) e o seu efeito na biossíntese de açucares e ureídeos, bem como a produtividade (cap 4). E os resultados observados demonstram que BRS-Guariba é um bom candidato de acordo com sua resposta para aplicação de Se. No capítulo 5, um experimento foi realizado visando observar a interação entre selenato e sulfato em condições de campo. Os resultados reportados demonstram que a interação entre S e Se no campo pode gerar aumentos de proteínas, amino ácidos e açúcares, sem comprometer a produtividade. Enfim, no capítulo 6, foi observado o efeito da interação entre Se e S na expressão gênica, nodulação e síntese de flavonoides no genótipo guariba. A interação entre Se e S resultou no aumento da expressão de genes transportadores de sulfato (SULTRs), enquanto a nodulação e síntese de flavonoides foram afetadas negativamente pela aplicação combinadas desses elementos.

Palavras-chave: *Vigna unguiculata*, variação genotípica, selenato, sulfato, nodulação, flavonoides, SULTRs

INTEGRATIVE EFFECTS OF SELENIUM AND SULFUR ON NUTRITIONAL AND PHYSIOLOGICAL RESPONSES OF COWPEA (*Vigna unguiculata* (L.) *Walp*) GENOTYPES

ABSTRACT – Selenium (Se) and Sulfur (S) are similar elements that affect each other uptake and assimilation in plants, mainly Selenate and Sulfate since both share transporters in plants. In this thesis, it was aimed to further investigate S and Se interaction in plant metabolism, studying cowpea (*Vigna unguiculata*), an important source of protein and nutrients in developing countries. In the literature review (Chapter 1), it was observed that S and Se affect crop yield, and the concentration of metabolites, in addition was observed that S application can increase plant's tolerance to Se toxicity. Many studies mention sulfate transporters' importance in Se and S uptake and assimilation, but just a few studies focus on transporters under the application of both elements. In chapter two, the effect of selenite and selenate in pigment concentration, antioxidant system, and sugar content were evaluated in cowpea plants under field conditions. The application of low rates of selenate (2.5 and 5 g Se ha^{-1}) enhances pigments, sugar concentration, and antioxidant activity in plants. In chapters three and four, 29 cowpea genotypes under Se application were studied to observe this element partitioning (Ch3) and its effect on yield, and on sugars and ureides biosynthesis (Ch4). Considering the observed results, BRS-Guariba is a good genotype regarding response to Se. In chapter 5, a field experiment was carried out to observe sulfate and selenate interaction. The reported results demonstrate that Se and S interaction in the field might lead to increased sugars, proteins, and amino acids concentration in seeds without impairing crop yield. Finally, in chapter 6 the effect of Se and S interaction on gene expression, root nodulation, and flavonoid synthesis was reported in cowpea genotype BRS Guariba. The interaction between Se and S resulted in the up-regulation of SULTRs genes, while nodulation and flavonoid biosynthesis were negatively affected by Se and S combined application.

Keywords: *Vigna unguiculata*, genotypic variation, selenate, sulfate, nodulation, flavonoid, SULTRs

CHAPTER 1 – INTRODUCTION: INTEGRATIVE EFFECTS OF SELENIUM AND SULFUR ON NUTRITIONAL AND PHYSIOLOGICAL RESPONSES OF COWPEA (*Vigna unguiculata* (L.) *Walp*) GENOTYPES

ABSTRACT

Selenium (Se) is a nutrient for animals and a beneficial element for plants, and sulfur (S) is a macronutrient for all organisms. Both elements share a plethora of similarities and, mainly as sulfate and selenate, are absorbed and assimilated in plants through the same pathways. Those similarities lead to interactions between Se and S that turn into plant responses in the uptake and assimilation of these elements and these compounds as well as physiological and growth alterations. This review aimed to compile studies about Se and S interaction and how the elements affect plant metabolism, yield, and elemental assimilation and uptake. Since sulfate and selenate are absorbed by sulfate transporters (SULTRs) in plant roots, most works focus on the observations regarding sulfate and selenate in plants, however, some papers reported the effect of S on selenite. In general S application decreases Se uptake by roots which is detrimental when the focus is Se biofortification but might be beneficial to avoid Se toxicity. Selenium on the other hand usually increases S uptake, mostly likely by enhancing SULTRs expression in plant roots. A minority of reports observed S enhancing Se uptake, and Se hindering S uptake, indicating that complex interaction between Se and S could be affected by plant species, Se accumulation pattern and soil type. Due to the effect of Se and S interaction in plant metabolism, some studies reported the interaction of those elements affecting crop quality, such as increase in sugar content and decrease in erucic acid in plants seeds. Although many studies mention sulfate transporters' influence on Se and S uptake and assimilation, a minority of reports focus on investigating those genes expression under Se and S interaction. Further investigation of Se and S interaction should consider environmental and plants-specific aspects to better understand the complex behavior of Se and S within crops. The effect of Se and S interaction in sulfate transporters should be further investigated.

Keywords: selenate, sulfate SULTR, elemental interaction, nutrient uptake, nutrient assimilation

1. INTRODUCTION: INTEGRATIVE EFFECTS OF SELENIUM AND SULFUR ON NUTRITIONAL AND PHYSIOLOGICAL RESPONSES OF COWPEA (*Vigna unguiculata* (L.) *Walp*) GENOTYPES

Sulfur (S) is a nutrient for all organisms (Kopriva et al., 2019), is part of essential amino acids, thus plays a role in protein formation in every living being (Nakai et al., 2020). In plants, sulfur is a macronutrient, and is considered the fourth most required for plant nutrition after N, P and K (Li et al., 2020), S is also related to several metabolic processes in plants such as sugar and lipids synthesis, as well as respiration (Liang et al., 2022).

Selenium (Se) is a nutrient for animals and humans (Ekuma et al., 2022). Due to the Se-containing aminoacid Selenocysteine (SeCys) being incorporated in proteins Se plays many roles in human health (Rayman, 2000). The element is important in central nervous system maintenance (Steinbrenner & Sies, 2013),Se also plays a role in the thyroid gland function (Derumeaux et al., 2003). And considering Se effect as an antioxidant, the element protects sperm cells from oxidative damage, thus being important to male fertility (Chen et al., 2013), as well as protecting DNA from oxidative damage (Giray & Hincal 2002). While in plants Se is a beneficial element that can enhance antioxidant enzymes activity (Silva et al., 2020; Lanza & Reis 2021), pathogens resistance (Schiavon & Pilon-Smits, 2017) and nodulation in Fabaceae (Cunha et al., 2022). Selenium can also increase the concentration of pigments in plant leaves (Silva et al., 2020) as well as enhance the sugar accumulation in grains and the N metabolism (Silva et al., 2023).

Se and S share chemical similarities, especially sulfate and selenate (Cabannes et al., 2011; Natasha et al., 2018), since both Se and S are in the same group in the periodic table (group 16), thus, these elements share similarities in chemical and physical properties (Dauphas, 2013; Wang and Becker, 2013). These similarities lead to S and Se interaction in soil (Song et al., 2022), plants (Silva et al., 2022), and other organisms (Gilbert et al., 2022). And those interactions generate responses on plants that affects the elements uptake and accumulation (Silva et al., 2022; Guimarães et al., 2022) as well as metabolism such as senescence and antioxidative system (Cheng et al., 2016). The effect of Se and S interaction also affects the availability of those

elements in soil solution and how they are reduced by microorganisms (Zher & Oremland; 1987; Dowdle & Orelamd, 1998).

Cowpea (*Vigna unguiculata* [L.] *Walp*) is a Fabaceae plant with high protein concentration in its grains and high tolerance to drought, high temperature and low fertility. Thus it is a crucial crop to provide protein for low income regions and countries. Cowpea also has a very wide genetic variability and previous studies demonstrate it has a high response to Se application considering biofortification potential as well as the physiological responses of Se to mitigate oxidative stress.

The aim of this thesis was to evaluate the effects of Se and its interactions with S in cowpea metabolism, S and Se uptake, nodulation as well as genotypic response of cowpea to Se application as sodium selenate..

2. INTERACTION BETWEEN SULFUR AND SELENIUM ON PLANT METABOLISM AND CROP YIELD

2.1 Selenium and sulfur on soil and its interaction on plant uptake

Sulfur is the fourteenth most abundant element in the earth's crust (Liang et al., 2022), in cultivated soil, the average S concentration is 0.7 g kg^{-1}, varying between 0.03 and 10 g kg^{-1} around the world (Alloway, 2003). On the other hand, Se is a very rare element in the earth's crust, present in most soils between 0.01 and 2 mg kg^{-1} (average of 0.40 mg kg^{-1}), but in some rare cases, some seleniferous soil might present Se concentration up to 1200 mg kg^{-1} (Dhillon & Dhillon, 2003).

The speciation of S in soils varies as inorganic form S exists in different oxidation states such as sulfate (SO_4^{2-}), sulfite (SO_3^{2-}), sulfide (S^{2-}), and nonvalent S^0, while organic S is bound to organic matter or animals and plant residues and can represent around 60% of total S in the soil (Liang et al., 2022). Organic S is not available for plants uptake and must be mineralized (Lucheta & Lambais, 2012), while SO_4^{2-} is the main S source available for plants uptake (Narayan et al., 2022). The distinct pools of S in soil are in a dynamic change all the time: organic S is constantly under mineralization to be converted in inorganic S such as SO_4^{2-}, while the immobilization of S occurs under high levels of SO_4^{2-}, which is bound to carbon skeletons forming sulfate esters (C-O-S). This dynamic is dependent of C:S ratio in soil, under C:S ratio < 200, Sulfur mineralization is promoted, if C:S ratio is between

200 and 400, the organic and inorganic S are in balance and no net changes occurs, and if C:S ratio > 400 Sulfur immobilization is promoted (Whilhem, 2009; Lucheta & Lambais, 2012). The mineralization of organic S occurs mainly by biologic process in which microorganisms use C-bonded to S compounds as C sources to get energy, and S is released as a by-product of C oxidation into CO_2, in natural aerobic conditions of most soils the decomposed organic matter generates SO_4^{2-} (Liang et al., 2022).

The speciation of Se in soil is comprised mainly by selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), since selenite is strongly adsorbed by soil particles such as Fe and Mg oxides (Sharma et al., 2015), in soil solution Se is mainly found as selenate (Silva et al., 2019; Araujo et al., 2020). Is noteworthy that Selenite (SeO_3^{-2}) interacts with HPO_3^{2-} in soil instead of SO_4^{2-} (Nakamura & Sekine, 2008). Other forms such as elemental Se (Se^0) and selenide (Se^{-2}) are observed in less amounts or unavailable to plants uptake: Se^0 is bound to organic matter, and Se^{2-} is found in precipitates or organic bound to volatile components (Zhang & Moore, 1996), and Se association with organic matter limits its mobility in soil and decreases Se availability to plants (Tolu et al., 2022). Selenium availability in soil solution is enhanced by microorganisms' activity in organic matter mineralization (Eswayah et al., 2016), and by soil minerals dissolution (Shaheen et al., 2017). Plants roots secretion of organic acids also can dissolve Se fixed by soil and can prevent it from being adsorbed in soil colloids again in oxides (Dinh et al., 2017). Selenate can be reduced to Se^0 in and further to Se^{2-} by microorganisms (Eswayah et al., 2016).

Before the plant's uptake, Se and S already undergo a series of interactions in the soil solution. As aforementioned, in natural conditions, on most soils, Se predominant forms are Selenate and Selenite (Wang et al., 2022), however selenite adsorption in soils is usually higher than selenate (Silva et al., 2019; Araujo et al., 2020), thus selenate is usually the main source of Se in soils. While most S in the soil is present in organic matter, Sulfate-SO_4 is the dominant form of S free in soil solution, ready for plant uptake (Narayan et al., 2022). As sulfate and selenate, S and Se compete for positive charges in soil colloids (Araujo et al., 2020). Thus, Se and S compete for adsorption sites in the soil as SeO_4^{2-} and SO_4^{2-}, and sulfate presence may affect Se adsorption and vice-versa, as observed in table 1, which comprises Se and S interaction effect in soil. Araujo et al (2020), observed that SeO_4 availability in tropical

soil was affected by SO_4 presence: studying the effect of sulfate (applied as phosphogypsum) on Se adsorption in distinct soil depths, it was observed that Se adsorption increased at higher Soil depths, and decreased under higher sulfate concentration in soil. The presence of SO_4 in soil solution can contribute to reduction of selenate to Se^0 (Olegario et al., 2010), which reduces Se avaibility. On the other hand, the adsorption capacity of iron oxide is higher to SO_4^{2-} than SeO_4^2 (Ryden et al., 1987), thus, by competition sulfate can increase selenate availability in soil solution, since sulfate is more strongly adsorbed by iron oxide than selenate.

Table 1. Selenium and Sulfur behavior on soil and the outcome of its interaction in the element's availability.

Soil Type	S application	Se application	Outcome	Reference
Not described	0, 25, 50, 75 and 100 mg/kg S	No additional Se	Exogenous S application increased Solube Se in soil and decreased Se bound to organic matter particles	Jiang et al., 2022
gray brown luvisol classified as a sandy clay	0 and 50 mg/kg as sulfate	0, 0.1, 1.0 and 5.0 Se as selenite or selenate	Sulfate addition in soils with selenate further increased extractable Se	Kikkert et al., 2013
Not described	20 mg/kg S as potassium sulfate	20 µg/kg as sodium selenate	Sulfate addition increased Selenate recovery in the soil by 12 to 17%	Stround et al., 2010
Yellow-brown soil and calcareous alluvial soil	0 or 100 mg kg S as elemental S	0 or 2 mg/kg Se as sodium selenate	additional S application decrease avaiable Se by 12.6% in Yellow brown soil and 14.4% in calcareous alluviual soil	Deng et al., 2021

This phenomenon was previously observed: Se partitioning in soil solution of a grey-brown luvisoil is enhanced by sulfate ions presence due to competition for same adsorption sites (Kikkert et al., 2013). In an experiment in oxidic clayey soil, the application of sulfate provided an increase in selenate desorption from soil particles (Santos et al., 2021). Jiang et al. (2022) studying the effect of S and P application in Pak Choi, observed an increase in Solube-Se and a decrease in Se bound to organic matter under exogenous S application, although the experiment was performed with both P and S application, both S and P interaction and only S application led to increase in soluble Se availability. The Selenate recovery is increased under potassium sulfate by 12 to 17% (Stroud et al., 2010), according to the authors, in this case sulfate might be suppressing microbial reduction of selenate into unavailable Se forms. This is supported by classical experiments with the sulfate reducing bacteria *Desulfovibrio desulfuricans* subsp. *aestuarii*, which is capable of reducing selenate to selenide, but the selenate reduction is reduced under the presence of 1 mM sulfate (Zehr & Oremland, 1987). In rice experiment studying Se and S interaction in two soil with different textures (Clay and Sandy clay loam), it was reported a stronger interaction between both selenate and sulfate in the sandy clay soil (Cardoso et al., 2022). The authors observed a lower adsorption of Se in the sandy clay loam texture soil, consequently in this soil, Se was more available in the solution, leading to more interaction with sulfate.

On the other hand, in experiment with two types of soil (calcareous alluvial soil and yellow-brown soil), the application of elemental S decreased the soluble portion of Se in both soils, 12.6% in yellow-brown, and 14.4% in calcareous alluvial (Deng et al., 2011), however is noteworthy that elemental S was used in those experiments instead of selenate.

The knowledge regarding S and Se interaction in soil is still emerging and further investigations are necessary to better understand how these elements influence each other’s availability to plants.

2.2 Selenate and sulfate uptake and metabolism on plants

Is noteworthy that S and Se uptake, translocation, distribution and assimilation depend of plant species, developmental stage, S and Se form and physiological circumstances (Gupta & Gupta, 2017). However, both Selenate and Sulfate share the same uptake and assimilation pathway in plants.

Selenate and sulfate uptake and transport is mediated by sulfate transporters (SULTRs) present in plant cells (White, 2018 Takahashi, 2019). There is a plethora of SULTRs observed in plants, especially in *Arabidopsis thaliana*. Transporters such as SULTR1 and SULTR2;1 observed in *A. thaliana* are responsible for both selenate and sulfate transport, and SULTR1;2 is probably the main selenate transporter (El Kassis et al., 2007). And transporters such as SULT3;1 were observed to lead sulfate into the chloroplast of *A. thaliana* (Cao et al., 2013). The genes *AtSULTR1;1* and *AtSULTR1;2* encode high affinity sulfate transporters in root hairs, cortical and epidermal cells of *A. thaliana* roots, both responsive to sulfate while only *AtSULTR1;1* is upregulated by selenate in the medium (Yoshimoto et al., 2002). The genes *AtSULTR4;1* and *AtSULTR4;2* encodes transportes localized predominantly in the pericycle and xylem parenchyma cells of roots and hypocotyls of *A. thaliana*, in roots SULTR4;1 was accumulated constantly while SULTR4;2 is more common under S starvation, while in shoots both transporters where more common under low S (Kataoka et al., 2004)

Sulfate and selenate share pathways in plant metabolism. The uptake by occurs due to SULTR1;1 and SULTR1;2 activity, then it the molecules could be loaded into the vacuole for accumulation, on into the chloroplast in which most steps of assimilation and metabolism occurs (Gigolashiv & Kopriva, 2014). Sulfate and selenate are assimilated and converted into methionine (Met) or cysteine (Cys), and Se-methionine (Se-Met) or Se-cysteine (Se-Cys), or other compounds such as glutathione, this process occurs mainly in the chloroplast (Gupta & Gupta, 2017). In the first step sulfate/selenate is converted into sulfite/selenite by a reaction catalyzed by two enzymes: ATP sulfurylase (APS) and APS reductase (APR). In the first step, APS catalyzes the replacement of two phosphate groups of ATP molecule by a sulfuryl/selenyl group, generating a adenosine phosphosulfate/phosphoselenate (APS/APSe), then the APR catalyzes the reaction in which glutathione donate two electrons to APS/APSe to convert it into sulfite/selenite (Sors et al., 2005). In

sequence, the enzyme sulfite reductase catalyzes the conversion of sulfite/selenite into sulfide/selenide by acquiring six electrons from ferredoxin (Gupta & Gupta, 2017). The sulfide/selenide is converted into Cys/Se-Cys after coupling with O-acetyl serine (OAS) in a reaction catalyzed by cysteine synthase (Kopriva et al., 2006; Gupta & Gupta, 2017). Cysteine/Se-Cys is the first stable compound from sulfate/selenate assimilation (Kopriva, 2006; White, 2018). Cysteine can be a precursor for other S containing compounds as Met and gluthathione (Nikiforova et al., 2004), while Se-Cys can be converted into Se-Met (While, 2016). Both molecules are assimilated in the aforementioned pathway, thus the interaction between sulfate and selenate in plants is even more complex than in soil, as will be further discussed in the next section.

2.3 Selenium and sulfur interaction effect on plants

Due to the aforementioned mechanisms of uptake and assimilation, S and Se application and concentration in plant tissue affects each other assimilation pathways, which lead to several distinct behaviors that vary according to plant species, supply of S and Se, soil texture and tolerance to Se in tissue. A comprehensive compilation of plants response to Se and S interaction is compiled in table 2.

Table 2. Selenium and Sulfur effect in plant metabolism and Se and S concentration.

Crop	S application	Se application	Outcome	Observations	Reference
Pak Choi (*Brassica rapa* L. *ssp. chinensis*)	0, 25, 50, 75 and 100 mg/kg S	No addition Se	75 mg/kg S increase shoot Se concentration	Interaction between S and P further increase Se concentration	Jiang et al., 2022
Pak Choi (*Brassica rapa* L. *ssp. chinensis*)	-	0, 0.5, 2, 20, 30 mg Se/Plant)	Se increased S relative concentration in pak choi tissue		Abdalla et al., 2022 a
Soybean (*Glycine max* L. Merr)	0 or 100 mg kg S as elemental S	0 or 2 mg/kg Se as sodium selenate	addition S application decrease Se concentration in soybean Grains	No S effect on Se speciation	Deng et al., 2021
Cowpea (*Vigna unguiculata* L.Walp)	0, 15, 30 and 60 kg/ha as sodium sulfate	0, 10, 25 and 50 g/ha as sodium selenate	Se concentration in grains and leaves decreased under S application	No Se effect on S concentration in leaves and seeds, Se and S combination might enhance total sugar concentration in seeds.	Silva et al., 2022
Rapeseed (*Brassica napus*)	0 and 60 kg/ha as elemental S and magnesium sulfate	0 and 60 g/ha as selenite and selenate	Both S sources decreased Se grain concentration in both Se treatments did not affect Se distribution in seeds.	Se and S combination improves rapeseed quality by increasing oleic acid and decreasing erucic acid concentration in seeds	Liu et al., 2017
Rapeseed (*Brassica napus*)	29,05 µM, 0.5 mM, 1 mM, 2mM and 4mM	0, 10 and 100 µM as sodium selenite	Sulfate application decreased Se concentration in roots and shoots	Sulfate application decreased Se translocation	Ren et al., 2023
Nannochloropsis oceanica	0, 1, 2, 3, 4, 5, 6.48 and 28 mM	0 and 30 µM	Se accumulation increased 8 fold under 2mM S application or less		Guimarães et al., 2022
Strawberry (*Fragaria* x *ananassa* Duch).	0 and 60 mg/dm3	0, 0.3, 0.6, 1.2, and 2.4 mg/dm3	S impaired Se concentration in fruits and shoot; Se enhanced S uptake	Se application enhanced strawberry yield	Santiago et al., 2018
Lettuce (*Lactuca sativa* L. Asteraceae)	1 and 2 mM	0, 1.3 and 3.8 µM	High levels of S increased Se accumulation. Under high levels of S, Se	Enhanced amino acids accumulation and decrease nitrate concentration and yield	Abdalla et al., 2022b

			application increase S accumulation		
Broccoli (*Brassica oleracea* var. *italica*)	1 and 5 mM	0, 50, 100 and 150 µM	High Se decreases S in leaves; high S might decrease Se in leaves, but increase it in florets.	100 µM Se and 4 mM S enhances sulforaphane yield	Mao et al., 2020
Wheat (*Triticum aestivum* L.)	-	0, 1, 5, 10 and 20 µM	Se application increases S concentration in 7 wheat lines	Se application enhances SULTR1;1, SULTR1;3 and SULTR4;1, gene expression	Boldrin et al., 2016
Rice (*Oryza sativa* L.)	0.1 and 0.5 mM (nutrient solution); 0; 45 and 90 m/dm3 (soil)	0 and 20 µM (nutrient solution); 0; 0.5; 1.0; 2.0 and 4.0 m/dm3 (soil)	S enhances Se concentration in shoots, but might decrease it in grains	Se application enhances gene expression of OsSULTR1;1 and OsSULTR1;2. Se and S combination enhances CAT and APX activity	Cardoso et al., 2022
Tartary buckwheat (*Fagopyrum tataricum* Gaertn)	126 µM	126 µM	Se and S combined application provided the highest concentration of Se in roots, leaves, huscas and seeds		Golob et al., 2016
Brassica juncea L. Czern	200 µM SO4 or SO4 starvation in nutrient solution	0, 200 µM	SO4 starvation led to increased Se concentration in roots and shoots	Se applications impair S uptake in S replete plants, but improves S uptake under S starvation	Shiavon et al., 2012

Regarding Se concentration in tissue, plants are classified as (1) Se non-accumulators, that do not tolerate Se concentration in tissues higher than 100 mg kg^{-1}; (2) Se accumulators, that tolerate more than 100 mg Se kg^{-1} in its tissue; and finally (3) Se hyper-accumulators, these species are able to accumulate 1000 to 15000 mg Se kg^{-1} in their tissue (White, 2016). Many of Se accumulator plants are from *Brassicaceae* family, which also presents a unique S metabolism and accumulation (Wiley & Wilkins, 2006) due to the biosynthesis of glucosinolates (Bednarek, 2012). However, Se accumulator tends to present a higher [Se]/[S] ratio than non-accumulator plants (White, 2007).

Pak Choi experiment studying the effect of P and S on Se uptake, the application of 75 mg kg^{-1} of S (and no P application) increased shoot Se concentration, mostly due to S effect on enhancing Se bioavailability on soil (Jiang et al., 2022). On the other hand, the authors did not observe root Se concentration increase under S application. Is noteworthy that P and S combination further increased Se concentration in both roots and shoots. In another Pak Choi experiment, although no S application was performed, the Se application increased S relative content in plant tissues (Abdalla et al., 2020a).

In Chinese mustard (*Brassica juncea*) cultivated in nutrient solution with 200 µM sulfate or sulfate starvation combined with 0 or 200 µM selenate, plants under S starvation presented higher Se concentration in roots and shoots (Schiavion et al., 2012). The authors also reported that Se impaired S uptake in S-replete plants, and enhances S uptake in S-starving plants.

In a soybean experiment with two types of soil (calcareous alluvial soil and yellow-brown soil), additional S application provided a decrease in gain Se concentration of 55% and 38% in both soil types, respectively (Deng et al., 2021). However, the authors did not observe S effect on Se speciation in soybean grains.

While in wheat under S starvation, Se concentration was 7 fold higher in grains compared to grains with proper S supply, the low S plants also presented higher Se in vegetative tissue and better Se mobility along the plant (Shinmachi et al., 2010). In an experiment evaluating seven lines of wheat under application of 0, 1, 5 10 and 20 µM sodium selenate, all lines presented S concentration increase when Se was applied

(Boldrin et al., 2016). The authors suggests that Se application mimics S starvation in plants, thus, enhancing S assimilation and absorption mechanisms, which increases the nutrient uptake.

In rapeseed, the application of 60 kg ha^{-1} of S as elemental sulfur or magnesium sulfate in interaction with the application of 60 g ha^{-1} of Se as selenate and selenite, it was observed that elemental S and sulfate decreased Se concentration in rapeseed grains under selenite and selenate application (Liu et al., 2017). In addition, S did not affect Se distribution in seeds.

The negative effect of sulfate on Se concentration was also observed in cowpea: in a field experiment studying 4 sulfate and selenate application rates interaction, sulfate led to a decrease in Se concentration in cowpea seeds and leaves, while Se application didn't impair S uptake in leaves and seeds (Silva et al., 2022).
In strawberry cultivated in greenhouse conditions on pot experiments, under application of five rates of Se as sodium selenate, 60 mg dm-3 of S applied as gypsum led to decreased Se concentration in shoots and fruits under all Se application rates. On the other hand, the Se application enhanced S uptake (Santiago et al., 2018). In the algae *Nannochloropsis oceanica,* under Se and S application, under lower concentrations of S applied (less than 2mM) Se accumulation increased by eightfold in the algae FW (Guimarães et al., 2022).

Although in most cases Se concentration is reduced under S supply, some studies also reported beneficial effect of Sulfate in Se uptake and accumulation by plants. In an experiment with Tartary and common buckwheat, under application of 0 or 126 µM of selenate or sulfate, in tartary buckwheat the concentration of Se was higher in roots, leaves, seeds, and husks when both elements were applied in conjunction (Golob et al., 2016). The authors did not observed the same behavior for common buckwheat, since in this case both Se and Se + S application provided the same result of Se concentration in all tissues evaluated.

In rice, a study comprised of two experiments was performed, a seedling experiment in nutrient solution with application of 0 and 20 µM Se, and 0.1 and 0.5 mM S; and a long term experiment in two types of soil, with application of 0; 0.5; 1.0; 2.0 and 4.0 mg dm^{-3} Se and 0; 45; and 90 mg dm^{-3} S (Cardoso et al., 2022). The authors observe in the seedling experiment a decrease in Se concentration in roots and shoots

under S application, while S and Se combination increase S concentration in shoots. In the soil experiment, under high levels of Se, S enhances Se concentration in shoots, however in rice grains, S enhances Se concentration in sandy clay loam soil but impairs the element concentration in plants grown in clay soil texture soil.

In lettuce under two levels of S (1 and 2 mM as potassium sulfate) and three of Se (0, 1.3 and 3.8 µM as sodium selenate), S provided an increase in Se accumulation under the highest Se and S applications combined. However, the highest S and Se level also decreased lettuce head yield (Abdalla et al., 2022b). Those authors also observed that under high levels of S, Se application enhances S concentration.

Most experiments demonstrate that Se supply enhances S uptake, in some cases the contrary is observed. In broccoli, the application of 150 µM Se reduced leaf S concentration in plants under low S supply (1 mM), however under 4 mM S, Se application did not affect leaf S concentration. On the other hand, Se impaired S concentration in florets in broccoli plants under 4 mM S application, but not under 1 mM S (Mao et al., 2020). The same authors observed that 4 mM S application decreases leaf Se concentration in broccoli plants under 100 and 150 µM Se, but increases floret Se concentration in plants under 50 µM Se.

Although the major effect observed is between Se as selenate and S as sulfate, other forms of Se are also affected by S applicartion. In rapessed (*Brassica napus*) under 0, 10 and 100 µM selenite and 29,05 µM, 0.5, 1, 2 and 4 µM sulfate, Se concentration was higher in shoots and roots under 29,05 µM selenate in both selenite application, translocation of Se was also impaired under increasing S supply (Ren et al., 2023). Selenite might not share root transporters with sulfate, however both sulfate and selenite share the same assimilation pathway within the plant.

The interaction between Se and S might affect other plant aspects in addition to Se and S accumulation. In lettuce under combined high levels of S (2 mM) and Se (3.8 µM), an increase was observed in some aminoacids accumulation, such as aspartate, asparagine; glutamic acid and glutamines (Abdalla et al., 2022b). In addition to that, these authors also reported decreased levels of nitrate and lower yield under combined high levels of Se and S. In strawberries, the combined application of Se and S increased fruit yield (Santiago et al., 2018). Sulforaphane, a bioactive produced by

Brassicaceae is enhanced by combined Se and S application in broccoli, specifically under 100 µM Se and 4 mM S (Mao et al., 2020).

Sulfur also might regulate selenium tolerance, since in chinese mustard, S starving plants, when compared to S replete plants, presented a decrease in the expression of *Selenium binding protein 1* (SBP1), a gene related to *Arabdopsis thaliana* tolerance to Se exposure (Shiavon et al., 2021). Another evidence was observed in rice seedlings in hydroponic experiment (Cardoso et al., 2022), in which 20 µM Se decreased root and shoot DW, but the combined application of Se and S resulted in shoot and root DW similar to treatments without Se application. The same authors also observed an increase in CAT and APX activity as well as GSH concentration in shoots.

Selenium and sulfur interaction could also alter other compounds concentration in plants. Selenate application increases rapeseed quality by increasing oleic acid and reducing erucic acid content in its seeds, and the combination with selenate further increases oleic acid and reduces erucic acid (Liu et al., 2017). In cowpea, the combination of 25 g Se ha^{-1} and 30 kg S ha^{-1} increases total sugars concentration in seeds, enhancing the quality of the material (Silva et al., 2022).

Although the interaction between Se and S is complex and may vary due to plant species, tissue and environmental conditions. In general, the effect of S changing Se uptake and assimilation by plants is more common than the effect of Se changing S uptake. And regarding S effect on Se, is more common to observe S impairing Se uptake and assimilation than the contrary. While for Se effect on S (when observed), more studies report Se enhancing S uptake than impairing it.

2.4 Selenium and sulfur interaction effect on sulfate transporters

Although many papers mention the effect of sulfate transporters in selenate and sulfate uptake and assimilation by plants in its discussion, a minority of works effectively study the effect of S and Se interaction in gene expression, as observed in the compilation presented in table 3. In wheat lines, the expression of SULTR1;1, SULTR1;3 and SULTR4;1, genes related to Se and S uptake were enhanced in roots under 10 µM selenate application, those genes are related to sulfate transport and uptake (Boldrin et al., 2016). This upregulation of genes under Se application is

important since it can enhance S uptake by plants. The upregulation of S transporters genes was also reported in rice, in which the application of 20 µM Se increased the expression of two genes in plant roots, *OsSULTR1;1* and *OsSULTR1;2* (Cardoso et al., 2022). Intriguingly those authors reported a decrease in *OsSULTR1;1* expression under Se + S application.

Table 3.Sulfate transporters expression due to interaction of Se and S on plants.

Crop	Genes	S application	Se applicaiton	Outcome	Observations	Reference
Brassica Juncea	BjSULTr2;1	200 µM SO4 or SO4 starvation in nutrient solution	0, 200 µM	Gene expression decrease by Se after 6h and by Se + S after 24 h of exposure		Shiavon et al., 2012
Rice	OsSULTR1;1 OsSULTR1;2	0.1 and 0.5 mM (nutrient solution); 0; 45 and 90 m/dm3 (soil)	0 and 20 µM (nutrient solution); 0; 0.5; 1.0; 2.0 and 4.0 m/dm3 (soil)	20 µM Se increased the expression of both genes	Se + S combined decreased OsSULTR1;1 expression	Cardoso et al., 2022
Rapeseed	BnSULTR2; BnSULTR4;1 BnSULTR4;2	29,05 µM, 0.5 mM, 1 mM, 2mM and 4mM	0, 10 and 100 µM as sodium selenite	Overexpression of SULTR2;2 under S application and underexpression under Se+S. Overexpression of SULTR4;1 under Se+S. Undexprression of SULTR4;2 under S application and overexpression under Se application		Ren et al., 2023

The application of Se in Chinese mustard decreased expression of *BjSULTr2;1*, a low sulfate transported related gene only expressed in roots, after 6 h of exposure and the combined application of Se and S decrease this gene expression after 24h of exposure to the treatments (Schiavon et al., 2012).

In rapeseed under sulfate and selenite application, the expression of many Sulfate transporters was observed, the most preeminent results regarding genes of the groups *BnSULTR2;2,* which are involved in long distant Se and S distribution, while *BnSULTR4;1*, and *BnSULTR4;2,* that plays a role in tonoplast efflux and movement between vacuoles and cytoplasm of sulfate and selenate (Ren et al., 2023). The authors reported an upregulation of *BnSULTR2;2* expression under when S was applied, and a decrease in its expression under Se and S combination, while SULTR4;1 expression was increased under Se+S application, and SULTR4;2 expression decreased under S application, but increased under Se application.

2.5 Conclusion

In general, the effect of Se and S in most cases lead to a decrease in Se concentration due to S application and to increase in S concentration due to Se application, but the opposite effects sometimes are observed. Thus, plant species, soil type and application method should be taken into consideration to explain the elements behavior. Although the main interaction regarding Se and S is due to selenate and sulfate interaction, other sources of those elements also play roles in the interaction, such as gypsum and selenite.

Selenium and sulfur effect on plant metabolism might lead to a some interesting responses, such as: (1) increased tolerance of plants to excessive Se due to S application; (2) reduction of erucic acid and increase in oleic acid in *brassicaceae*; (3) increase in total sugar concentration in seeds of *fabaceae*; (4) increase in amino acids concentration in vegetable leaves; and (5) yield enhancement.

Sulfate transporters play a important role in Se and S uptake by plants, and more experiments with Se and S interaction studying the expression of transporters should be performed.

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CHAPTER 2 –SELENATE AND SELENITE AFFECT PHOTOSYNTHETIC PIGMENTS AND ROS SCAVENGING THROUGH DISTINCT MECHANISMS IN COWPEA (*Vigna unguiculata* (L.) walp) PLANTS

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ABSTRACT

Selenium (Se) is a beneficial element to higher plants. Application of Se at low concentrations enhances the antioxidant metabolism reducing the reactive oxygen species (ROS) generated by plant membrane cells. This study aimed to evaluate how the application of Se in the forms sodium selenate and sodium selenite regulates ROS scavenging in field-grown cowpea plants. Seven Se application rates (0; 2.5; 5; 10; 20; 40 and 60 g ha^{-1}) of each of the two Se forms were applied to plants via the soil. Photosynthetic pigments concentration, gas exchange parameters, lipid peroxidation by malondialdehyde (MDA) concentration, hydrogen peroxide concentration, activity of catalase (CAT, EC:1.11.1.6), glutathione reductase (GR, EC:1.6.4.2), ascorbate peroxidase (APX, EC:1.11.1.11) and Se concentration in leaves and grains were evaluated. In general, Se application led to a decrease in chlorophyll a concentration whilst leading to an increase in chlorophyll b, conserving total chlorophyll amount. Application of 2.5 g ha^{-1} of Se as selenate provided a notable increase in total chlorophyll and total carotenoids compared to the other application rates. Selenate and selenite application decreased lipid peroxidation. However, each Se source acted in a different pathway to combat ROS. While selenate have more potential to increase activity of APX and GR, selenite showed a higher potential do increase CAT activity. The negative correlation between CAT and GR is indicative that both pathways might be activated under distinct circumstances. The more prominent activity of CAT under high rates of selenite resulted in a negative correlation of this enzyme with chlorophyll a and carotenoids. Both selenate and selenite application increased sucrose and total sugars concentration in leaves of cowpea plants. Overall, these results indicate that application of Se in cowpea under field conditions stimulates distinct pathways to ROS scavenging system. This could prove beneficial to mitigate oxidative stress during plant development.
Keywords: Antioxidant metabolism; photosynthesis; oxidative stress; sodium selenite; sodium selenate.

Abbreviations: Selenium (Se); reactive species of oxygen (ROS); malondialdehyde (MDA); catalase (CAT, EC:1.11.1.6); glutathione reductase (GR, EC:1.6.4.2); ascorbate peroxidase (APX, EC:1.11.1.11); glutathione (GSH); ascorbic acid (AsA); glutathione peroxidase (GSH-Px, EC:1.11.1.9); arsenic (As); cadmium (Cd); days after sowing (DAS); chlorophyll a (Chl a); chlorophyll b (Chl b); pheophytin a (Pheo a); pheophytin b (Pheo b);

1. INTRODUCTION

Selenium (Se) is an essential element in human and animal nutrition. It acts as a constituent of selenoproteins, which confer a key role in antioxidant metabolism, as well as thyroid hormone and immune system functioning (Rayman, 2012). Selenium in global food systems originates in the soil, (Combs Junior, 2001), where it is generally regarded as a trace-element, present at very low concentrations worldwide (Reis et al., 2017). For example, in some Brazilian regions, soil Se concentrations may vary between 0.06 and 0.6 mg kg^{-1} (Silva Junior et al., 2017). In China, the variation in soil Se is likely to fall between 0.2 and 0.6 mg kg^{-1} (Song et al., 2020) and in Malawi, soil Se may vary between 0.12 and 0.3 mg kg^{-1} (Chilimba et al., 2012). Soil containing Se at concentrations below 0.6 mg kg^{-1} can be considered deficient (Gupta & Gupta, 2000). This can lead to reduced nutritional quality of edible foods and hence human and animal Se undernutrition (Silva et al., 2019). For instance, in Africa, the risk of Se deficiency in humans is ranked third among mineral nutrients (Joy et al., 2014) and it was estimated that up to 1 billion people worldwide may suffer from and Se deficiency (Combs Junior, 2001).

In plants, Se is considered a beneficial element, potentially promoting growth and helping to alleviate oxidative stress (Silva et al., 2019; Schiavon & Pilon-Smits, 2017). Selenium has previously been shown to enhance the antioxidant defense system in chloroplasts in tomato under salt stress (Diao et al., 2011). In addition, hydroponically cultivated garlic plants exposed to 60 mM of NaCl showed an increase in chlorophyll and carotenoid concentration under Se application of 8 mg L^{-1} (Astaneh et al., 2018). Therefore, leaf photosynthesis and pigment concentrations may also be affected by exogenous Se concentrations. Selenium application has also been shown to increase sugar concentrations in plants. Application of Se as nanoparticles at 20 and 40 ppm increased total sugar concentration in shoots of different groundnut genotypes (Hebat-

Allah et al., 2019). Application of 0.75 ppm of Se as sodium selenate was also shown to increase sugar concentration in mungbean seedlings (Malik et al., 2011).

Further evidence as to the role of Se in alleviating oxidative stress in plants has been shown through its potential to reduce lipid peroxidation in cucumber (Jóźwiak & Politycka, 2019). Over-production of reactive oxygen species (ROS) by higher plants can increase the lipid peroxidation of cell membranes (Silva et al., 2018). Increased concentrations of hydrogen peroxide (H_2O_2), oxygen radicals (O_2^-) and OH radicals are indicative of oxidative stress in plants. However, in stressful conditions, H_2O_2 might act signaling molecules in plant tissues (Farooq et al., 2019). The estimation of lipid peroxidation by malondialdehyde (MDA) concentration, hydrogen peroxide and the activity of antioxidant enzymes such as catalase (CAT, EC:1.11.1.6); glutathione reductase (GR, EC:1.6.4.2) and ascorbate peroxidase (APX, EC:1.11.1.11) form further useful markers to evaluate the combat of oxidative stress in plant metabolism (Santos et al., 2017). The peroxidation of unsaturated fatty acids in phospholipids membranes caused by ROS increases the MDA concentration (Farooq et al., 2019). The enzymes CAT, APX and GR are part of the scavenging system of ROS within the cell, mitigating the oxidative stress-induced damage. Catalase catalyzes the conversion of H_2O_2 into H_2O (Nazmir & Gosh, 2018), APX uses ascorbate as an electron donor for the reduction of H_2O_2 into H_2O and O_2^- (Foyer & Noctor, 2011) and GR maintains a reducing intracellular status by converting glutathione disulfide (GSSG) into the reduced form of glutathione (GSH) (Deponte, 2013). In a previous study, application of sodium selenite to soil at the rate of 790 µg of Se per pot containing six rice seedlings (~60 days old), increased protein, GSH, and ascorbic acid (AsA) concentration, and also the activity of glutathione peroxidase (GSH-Px, EC:1.11.1.9) (Yin et al. 2019). In a further study, soil application of 25 g Se ha^{-1} as sodium selenate increase CAT and SOD activity, and decreased MDA concentration in rice leaves (Reis et al., 2018). Thus, there is building evidence for the role of Se in alleviating oxidative stress in plants.

Several research programs on genetic and agronomic biofortification are currently focused on increasing Se concentrations in edible crop tissue (Reis et al., 2018). Selenium can be applied to plants in the form of both sodium selenite and sodium selenite. Since selenite is more adsorbed into soil particles, application of Se as selenate via soil

is more efficient in providing Se for accumulation in plant grains (Silva et al., 2019). However, since only limited work has been performed on analyzing the effect of soil application of Se fertilizers on antioxidant system functioning and photosynthetic pigment concentrations, it is unclear which form is more efficient for this use (Yin et al., 2019). Thus, further investigation in this area is needed.

Cowpea (*Vigna unguiculata* (L.) Walp.) originated from West Africa (Nwokolo & Ilechukwu, 1996). Cowpea grains have very important nutritional value, complementing the human diet in developing countries (Silva et al., 2019). Cowpea is considered drought tolerant and suitable for growth in arid regions as well as on low fertility soils and in high temperatures (Carvalho et al., 2019). According to our former study, cowpea shows great potential to accumulate safe concentrations of Se in its grains under field conditions (Silva et al., 2019). Our hypothesis is that Se application provides physiological changes that may not be observed in cowpea growth and yield due its rusticity. In this study, we aimed to evaluate the effect of sodium selenate and sodium selenite application on photosynthesis and antioxidant metabolism of field-grown cowpea plants. If Se application has a positive effect on antioxidant metabolism, it may help to combat ROS stress in plant cells, thus contributing to an increase in productivity.

2. MATERIALS AND METHODS

2.1 Experimental Design

The experiment was carried out at the São Paulo State University (UNESP) Farm, Mato Grosso do Sul State, Brazil. The experimental area soil was classified as Rhodic Haplusfox according to the Soil Survey Staff (2014). A total of 20 soil subsamples from the top 20 cm depth were collected and homogenized to evaluate chemical soil properties according to Raij et al. (1997). The observed chemical properties were: pH: 5.5; P: 34 mg kg^{-1}, S: 8 mg kg^{-1}, B 0.19 mg kg^{-1}, Cu 2.7 mg kg^{-1}, Fe: 19 mg kg^{-1}, Mn: 12.4 mg kg^{-1}, Zn: 6.1 mg kg^{-1}; K: 2.7 $mmol_c$ kg^{-1}, Ca: 14 $mmol_c$ kg^{-1}, Mg: 14 $mmol_c$ kg^{-1}, H + Al: 26 $mmol_c$ kg^{-1}, cation exchange capacity: 56.7 $mmol_c$ kg^{-1}, V%: 54 and organic matter: 18 g kg^{-1}. An analysis was performed to quantify readily available soil Se concentrations in the soil according to Silva et al. (2019). The observed readily available Se was 3.6 µg kg^{-1}. Daily

rainfall, mean, maximum and minimum temperatures recorded during the experiment can be found in Supplementary material 1.

Seeds were sown on October 18th 2016, using the seed density of 11 seeds m^{-1}. In this study, cowpea (*Vigna unguiculata* (L.) Walp.) variety BRS Tumucumaque was used because is the most cultivated cowpea variety in Brazil. The fertilization with P and K was performed in planting furrows, at the application rates of 20 kg ha^{-1} of P_2O_5 (110 kg ha^{-1} of single superphosphate) and 20 kg ha^{-1} of K_2O (33 kg ha^{-1} of KCl).

Each experimental plot consisted of five rows of 5 m, with 0.45 m spacing between rows. The experiment was designed in a complete randomized block in a factorial scheme (7 × 2), in which four replicates of seven Se rates (0, 2.5, 5.0, 10, 20, 40, and 60 g ha^{-1}) and two Se sources (sodium selenate and sodium selenite) were applied via soil application.

A peat inoculum specific for cowpea (2.0 × 10^9 colony-forming units g^{-1}, strain SEMIA 6462), was used to perform inoculation. The rate of inoculation was at 8 g kg^{-1} of seed. Prior to inoculation, seeds were treated, with the thiophanate-methyl (225 g L^{-1}), fipronil (250 g L^{-1}), and pyraclostrobin (25 g L^{-1}) at the rate of 2 mL product kg^{-1} of seeds. Emergence began at 4 days after sowing (DAS) on October 22nd, 2016.

In order to accurately perform the Se application, a stock solution was made for each treatment, comprised of the total Se required for all four replicates. Sodium selenate and sodium selenite were weighed and diluted in 2 L of deionized water. For Se application in each plot, the stock solution was subdivided into four portions of 500 mL each, considering that each plot was comprised of five rows. For each row, 100 mL of solution was applied to the planting furrow, close to the base of the plants. The operation was performed using a small malleable polyethylene bottle with a pierced cap. Each treatment was applied with a specific bottle to avoid contamination. Applications of Se were performed 37 DAS. Pest control comprised application of abamectin (0.5 L ha^{-1}) at 16 and 27 DAS, bentazone (0.8 L ha^{-1}) and imidacloprid (0.4 L ha^{-1}) at 27 DAS, and haloxyfop-p-methyl (0.3 L ha^{-1}) at 37 DAS.

2.2 *Photosynthetic pigment concentration analysis*

For chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl a+b), carotenoids, and pheophytin a (Pheo a) and pheophytin b (Pheo b) analysis, ten trifoliate leaves of the upper third of plants were collected, in each plot at 46 DAS. The leaf tissue was macerated in 80% acetone, an aliquot of 200 µL of the extract was then collected and added to 1.8 mL of 80% acetone. A spectrophotometer (SP-220, bioespectro^{TM}) was used to take measurements at the following wavelengths (absorbance): 470, 647, 653, 663 and 665nm. The pigment concentrations were estimated according to Lichtenthaler (1987). The results were expressed in µg mL^{-1}.

2.3 *Digestion Gas exchange parameters*

Determination of gas exchange was carried out using a portable gas exchange device (Infra Red Gas Analyzer –IRGA, brand ADC BioScientific Ltd, model LC-Pro) according to Santos et al. (2017). The following parameters were determined: net photosynthetic rate (µmol CO_2 m^{-2} s^{-1}), stomatal conductance (mol H_2O m^{-2} s^{-1}), internal CO_2 concentration in the sub stomatal chamber (µmol CO_2 mol^{-1}) and transpiration rate (mmol H_2O m^{-2} s^{-1}). For measurements, the standard conditions used were: 1000 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), provided by LED lamps, 380 ppm of CO_2, and a chamber temperature of 28 °C, according to Jiang & Xu (2001).

2.4 Lipid peroxidation and peroxide analysis

Sampling for evaluation of MDA, hydrogen peroxide, catalase (CAT, EC:1.11.1.6), ascorbate peroxidase (APX, EC:1.11.1.11), glutathione reductase (GR, EC:1.6.4.2) and proteins, was performed 46 DAS, nine days after the treatment application. Ten trifoliate leaves of the upper third of plants were randomly collected from each plot. The material was collected, snap-frozen in liquid nitrogen and stored at -80 ºC.

Lipid peroxidation was evaluated according to Heath & Packer (1968). For extraction, leaf material was macerated in 4 mL of 0.1% (w/v) trichloroacetic acid solution, then centrifuged at 4,000 rpm for 15 min at 4°C. To 200 µL of supernatant was then added 1.5 mL of 0.5% thiobarbituric acid (TBA). The mixture was agitated vigorously and incubated at 95°C for 30 min. The material was kept accommodated on ice inside a closed

polystyrene box for 10 minutes and centrifuged at 4,000 rpm for 1 min. The absorbance was then recorded at 535 nm, and a non-specific absorbance at 600 nm using a spectrophotometer (SP-220, bioespectro^{TM}). To calculate the content of MDA, the molar extinction coefficient 155 mM^{-1} cm^{-1} was used, and the results were expressed in nmol MDA g^{-1} fresh weight (FW).

The hydrogen peroxide concentration was determined according to Alexieva et al. (2001). For extraction, leaf material macerated in with 4 mL of 0.1% (w/v) trichloroacetic acid solution and centrifuged at 10,000 rpm for 15 min at 4 °C. To 200 µL of supernatant was added then added 200 µL of 0.1 M potassium phosphate buffer (pH 7.5) and 800 µL of 0.1 M KI solution. The microtubes rest for 1 hour in the dark accommodated on ice inside a closed polystyrene box. The absorbance was then recorded at 390 nm using a spectrophotometer (SP-220, bioespectro^{TM}). Results were calculated based on a calibration curve and expressed in µmol H_2O_2 g^{-1} FW.

2.5 Protein concentration and enzymes activity analysis

To evaluate the concentration of proteins, catalase (CAT, EC:1.11.1.6), ascorbate peroxidase (APX, EC:1.11.1.11) and glutathione reductase (GR, EC:1.6.4.2), sampling was performed 46 DAS, nine days after the treatment application. Ten trifoliate leaves of the upper third of plants were collected randomly from each plot. The material was snap-frozen in liquid nitrogen and stored at -80 ºC.

The extraction for enzymes and protein analysis was performed using 5 mL of 0.1 M potassium phosphate buffer at pH 7.5 containing 3 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid. The mixture was centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatant was then kept at −80 °C. For protein analysis, absorbance was recorded at 595 nm using a spectrophotometer (SP-220, bioespectro^{TM}). Protein determination was performed according to Bradford (1976), using a calibration curve of bovine serum albumin. The protein content was expressed as mg g^{-1} FW.

The determination of ascorbate peroxidase (APX, EC:1.11.1.11) activity was performed according to Gratão et al. (2008). First a solution containing 600 µL of 80 mM potassium phosphate buffer (pH 7.0) + 100 µL of 5 mM ascorbic acid, and 100 µL of 1 mM EDTA was made. The solution was kept in water bath at 30°C for 10 min. Then, 100

µL of 0.1 mM H_2O_2 and 100 µL of protein extract was added. The extract was rapidly shaken and then immediately analyzed using a spectrophotometer (SP-220, bioespectro^{TM}) at 290 nm for 1 minute. The results were expressed as nmoL min^{-1} mg $protein^{-1}$.

Catalase (CAT, EC:1.11.1.6) activity was determined using the methodology of Azevedo et al. (1998). In Eppendorf tubes, 1 mL of 0.1 M potassium phosphate buffer (pH 7.5), and 2.5 µL H_2O_2 30% was added, followed by 25 µL of protein extract. Immediately after the addition of the protein extract, the tubes were quickly mixed by vortexing. Enzyme activity was determined by monitoring the absorbance recording at 240 nm over a 1 minute interval using a spectrophotometer (SP-220, bioespectro^{TM}). The results were expressed as µmoL min^{-1} mg $protein^{-1}$.

For glutathione reductase (GR, EC:1.6.4.2) activity, determination was performed according to Gomes-Junior et al. (2006). First 1 mL of 0.1 M potassium phosphate buffer at pH 7.5 was mixed with 500 µL of 0.1M 5'5-dithiobis (2-nitrobenzoic acid) (DTNB). The mixture was incubated in a water bath at 30 ºC for 15 min. Then, 100 µL of 1 mM of oxidized glutathione (GSSG), 100 µL of 0.1 mM NADPH and 50 µL of protein extract was added. The mixture was rapidly shaken and then immediately analyzed using a spectrophotometer (SP-220, bioespectro^{TM}) at 412 nm for 1 minute. The results were expressed as µmoL min^{-1} mg $protein^{-1}$.

2.6 Sucrose and total sugar analysis

To perform sucrose and total sugars analysis, ten trifoliate leaves of the upper third of plants were randomly collected from each plot at 46 DAS. The material was collected and stored in liquid nitrogen. For extraction, 1 g of fresh weight was ground in liquid nitrogen and incubated for 24 hours in 10 mL MCW solution (60% methanol, 25% chloroform, and 15% water v/v) at 4°C in a refrigerator. The material was then centrifuged at 10,000 rpm for 10 minutes at 4ºC. After 24 hours, an aliquot of 4 mL was transferred to a clean tube, to which 1 mL of chloroform and 1.5 mL of deionized water was added for phase separation. The aqueous phase was taken, to which sucrose and total sugar analyses were performed.

Sucrose analysis was carried out according to Van Handel (1968). In 100 µL of supernatant, 500 µL of 30 % KOH (w/v) and 2 mL of 100 % H_2SO_4 was added. The mixture was heated in an oven at 100°C for 10 minutes. The tubes were then allowed to rest for 15 minutes for cooling, after which the absorbance was recorded at 490 nm using a spectrophotometer (SP-220, bioespectroTM). Total sugars analysis was carried out according to Dubois et al. (1956). To 100 µL of supernatant, 500 µL of 5 % phenol (w/v) and 2 mL of 100 % H_2SO_4 was added. The tubes were allowed to rest for 10 minutes for cooling, and then the absorbance was recorded at 490 nm using a spectrophotometer (SP-220, bioespectroTM). Results for sucrose and total sugars analysis were calculated based on a calibration curve using 1 mg mL^{-1} sucrose, and then expressed in terms of mg sucrose g^{-1} FW and mg total sugar g^{-1} FW, respectively.

2.7 Mineral analysis

For mineral analysis, the third trifoliate leaves were collected 63 DAS, the grains were collected 81 DAS. The material was dried in an oven at 40 °C for 72 h until a constant dry mass was achieved, and later ground in a Wiley mill (Marconi, MA 340, Piracicaba, Brazil) using a 1 mm sieve. The samples were digested and analyzed by ICP-MS following Thomas et al. (2016). The results were expressed in mg kg^{-1}.

2.8 Statistical analysis

Anderson-Darling normality tests and Leven's homogeneity tests were used to analyze the dataset. Variance and covariance analyses were performed, and then, to compare differences among treatment means, Tukey tests at 5% were used. To perform all the tests, softwares Agroestat and Minitab were used, and SigmaPlot 12.5 software was used to prepare the graphs.

Using the software R (R Development Core Team 2015), heatmaps were made using Pearson correlation coefficients ($p < 0.05$) to evaluate the correlation among the cowpea parameters under the treatments included in the experiments. The pack 'corrplot' was accessed to generate the heatmaps using the functions 'corr' and 'cor.mtest' to create coefficient matrices and p-values, respectively.

3. RESULTS

3.1 Photosynthetic pigments

An interaction between Se source and application rate was observed for chlorophyll a ($p<0.01$), chlorophyll b, total chlorophyll ($p<0.01$) and carotenoids concentration ($p<0.01$; Table S2). Selenate application led to an increase in chlorophyll a concentration at Se rates of 2.5 and 5 g ha^{-1}. Compared to control, a decrease in chlorophyll a was observed at 10, 20 and 60 g ha^{-1}. For selenite treatments decreases in chlorophyll a, compared to control, were observed at rates of 10, 20, 40 , 60 g Se ha^{-1}. Chlorophyll a concentrations were higher under selenate than selenite application at Se concentrations of 2.5, 5 and 60 g ha^{-1} (Fig 1a). As for chlorophyll b concentrations, under selenate application, increase in concentrations compared to control were observed at 2.5, 5, 40 and 60 g Se ha^{-1}. For selenite, chlorophyll b was found in higher concentrations compared to control under application rates of 10, 20 and 40 g Se ha^{-1}. Chlorophyll b concentrations were highest under selenate than selenite application at 5 g Se ha^{-1}, and higher under selenite than selenate application at 10 and 20 g Se ha^{-1} (Fig 1b).

Selenium application at the rates 2.5 and 5 g Se ha^{-1} as sodium selenate provided an increase in total chlorophyll concentration compared with untreated plants. Considering selenite application, a difference was only observed at a rate of 60 g Se ha^{-1}, corresponding to a decrease (Fig 1c). At application rates of 2.5, 5, 40 and 60 g Se ha^{-1}, total chlorophyll was higher under selenate application than under selenite application. However, selenite induced a higher total chlorophyll concentration compared to selenate at rate of 20 g Se ha^{-1} (Fig 1c).

Total carotenoids concentration under selenate application increased at 2.5 and 5 g Se ha^{-1} and decreased at 10 and 20 g Se ha^{-1} compared to untreated plants. Selenite application at a rate of 60 g Se ha^{-1} decreased total carotenoids compared to untreated plants (Fig 1e). Carotenoid concentration was higher under selenate application at rates of 2.5, 5 and 60 g Se ha^{-1} and higher at selenite application at rate 20 g Se ha^{-1}, each compared to the other Se source (Fig 1d).

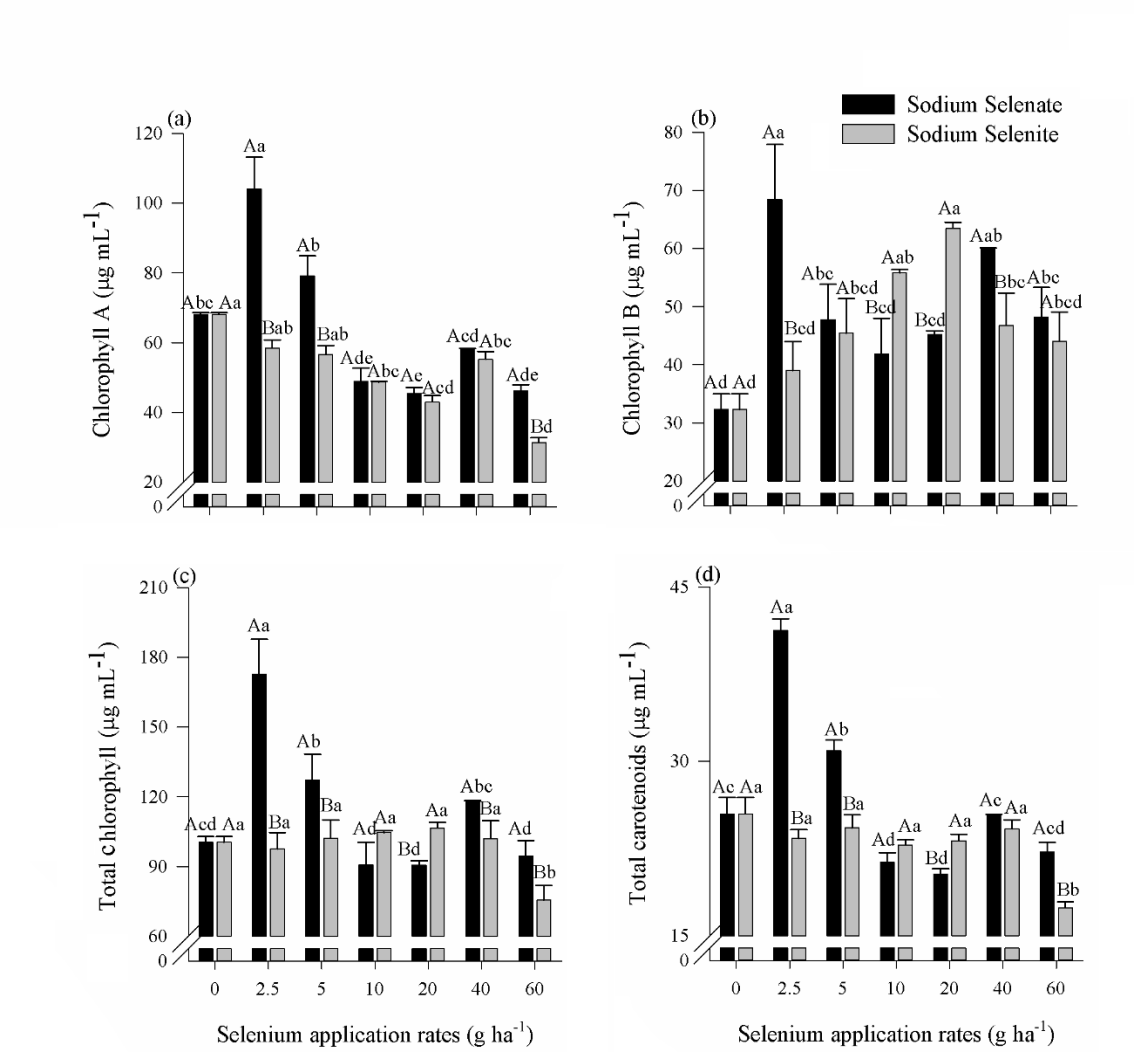

Figure 1. Effects of sodium selenate and sodium selenite application on chlorophyll a (a), chlorophyll b (b), total chlorophyll (c) and total carotenoids concentration in cowpea leaves. The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate difference between means according to a Tukey test ($p \leq 0.05$). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se application rates. CV (%) = 9.92 (a); 13.34 (b); 9.27 (c) and 6.77 (d).

3.2 Gas Exchange parameters

Interactions were observed between Se source and application rate for net photosynthetic rate ($p<0.01$) stomatal conductance ($p<0.01$) and transpiration rate ($p<0.01$; Table S3). Internal CO_2 concentration was not affected by Se source or the interaction between factors, but was affected by the Se application rate ($p=0.023$; Table S3).

Selenate application increased net photosynthetic rate at an application rate of 20 g Se ha^{-1} compared to untreated plants. Selenite application provided higher net photosynthetic rate than selenate at an application rate of 2.5 g Se ha^{-1}, although the inverse was observed at application rates of 5 and 60 g Se ha^{-1} (Fig 2a). Considering stomatal conductance in comparison to control plants, selenate application rates provided increase at rate of 5 g Se ha^{-1}, stomatal conductance was increased by selenite application at 10, 20 and 40 g Se ha^{-1} (Fig 2b).

Internal CO_2 concentration was affected only by Se application rates. The highest CO_2 concentration was observed at 20 g Se ha^{-1}, the lowest as 60 g Se ha^{-1}. Both results were statistically different from each other, but not when compared to control treatment (Fig 2c). Selenate application decreased the transpiration rate at 60 g Se ha^{-1} compared to untreated plants. Selenite increased the transpiration rate at Se applications of 2.5, 10 and 40 g Se ha^{-1}, compared to control. The transpiration rate was also higher under selenite than selenate at rates of 2.5, 40 and 60 g Se ha^{-1} (Fig 2d).

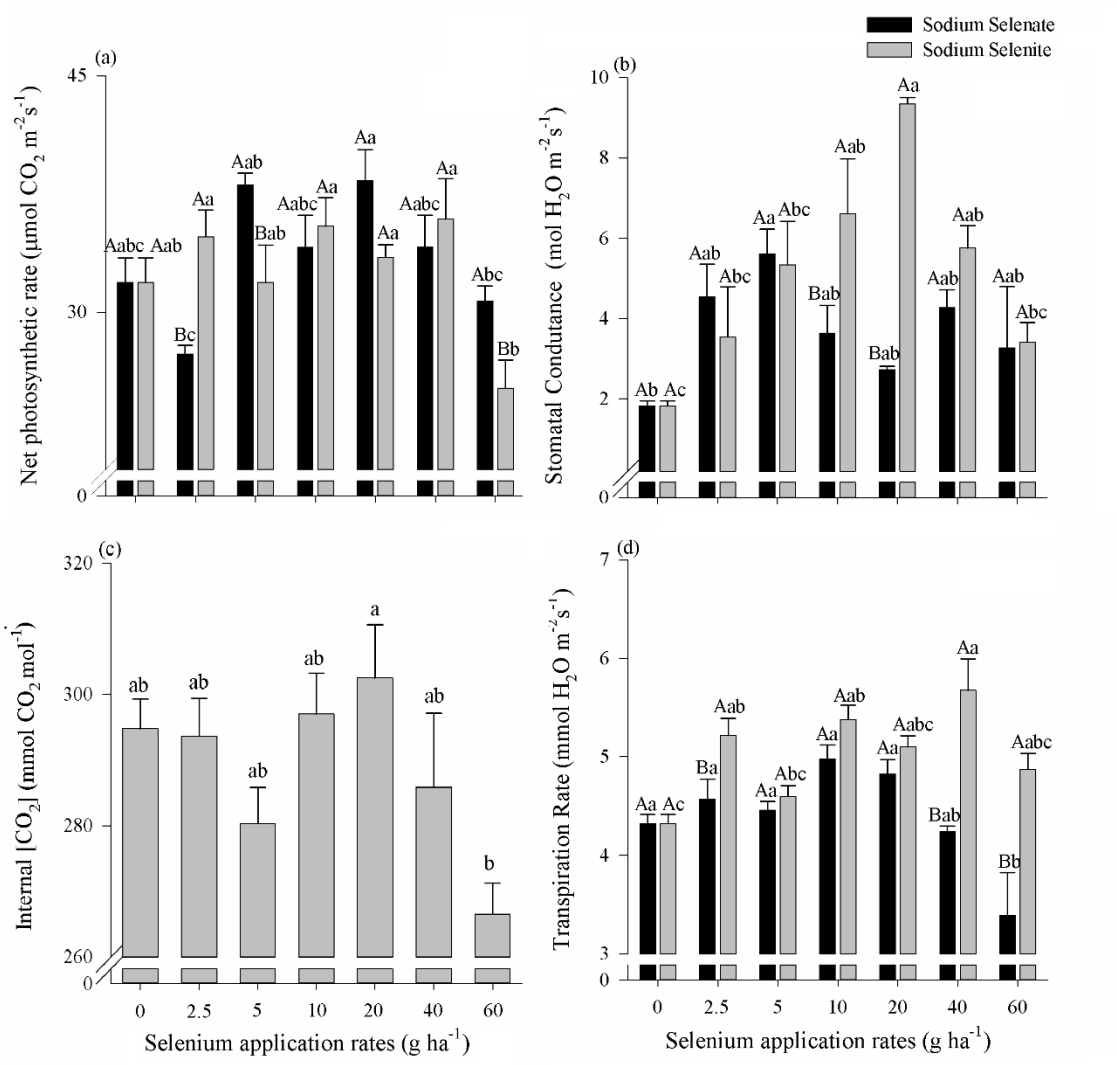

Figure 2. Effects of sodium selenate and sodium selenite application on net photosynthetic rate (a), stomatal conductance (b), internal CO_2 concentration (c) and transpiration rate (d) in cowpea leaves. The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate difference between means according to a Tukey test ($p \leq 0.05$). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se application rates. CV (%) = 10.47 (a); 37.38 (b); 7.13 (c); and 8.32 (d)

3.3 Oxidative stress and antioxidante metabolism

Interactions between Se source and application rate were observed for lipid peroxidation assayed as MDA ($p<0.01$), H_2O_2 concentration ($p<0.01$), activity of APX ($p<0.027$), CAT ($p<0.01$) and GR ($p<0.01$) as well as protein concentration ($p<0.01$; Table S4; S5). Application rates of 2.5, 5, 10, 20 and 60 g Se ha^{-1} as sodium selenate led to a decrease in lipid peroxidation of leaf cell membranes. Selenite application decreased lipid peroxidation at rates of 40 and 60 g Se ha^{-1}(Fig 3a). Lipid peroxidation rates were lower under selenate than selenite at application rates of 2.5 and 5 g Se ha^{-1}. Conversely, selenite presented lower lipid peroxidation than selenate at Se rates of 40 and 60 g Se ha^{-1}.

Cowpea plants treated with 2.5, 20, 40 and 60 g Se ha^{-1} as sodium selenate, and selenite showed elevated H_2O_2 concentrations compared to untreated plants. Application of 10 g Se ha^{-1} as selenite decreased H_2O_2 compared to control (Fig 3b). Hydrogen peroxide concentration in cowpea leaves treated with 20, 40 and 60 g Se ha^{-1} was lower under application in the form of selenate than selenite, and selenite provided lower H_2O_2 than selenate at 10 g Se ha^{-1} (Fig 3b).

Ascorbate peroxidase activity was higher under application of Se at rates of 10, 20 and 60 g Se ha^{-1} in selenate form, compared to control. Under selenite application, the rate of 20 g Se ha^{-1} led to an increase in APX activity compared to untreated plants. Selenium application at rates of 10 and 60 g ha^{-1} as selenate induced higher APX activity than when applied as selenite (Fig 4a).

Selenium application at rates of 20, 40 and 60 g ha^{-1} as selenate increased CAT activity. An increase in CAT activity was observed under all application rates when applied as selenite. (Fig 4b). Application of Se as selenite also led to higher CAT activity than when applied as selenate at application rates of 2.5, 5, 10 and 60 g Se ha^{-1} (Fig 4b)

Glutathione reductase activity was increased under selenate application at rates 2.5, 5 and 40, and decreased at 60 g Se ha^{-1}, compared to untreated plants. Selenite application decreased GR activity in all application rates (Fig 4c). Glutathione reductase activity was higher under selenate than selenite under application rates of 2.5, 5, 10, 20 and 40 g Se ha^{-1}. However, higher GR activity was recorded under selenite than selenate application at 60 g Se ha^{-1} (Fig 4c).

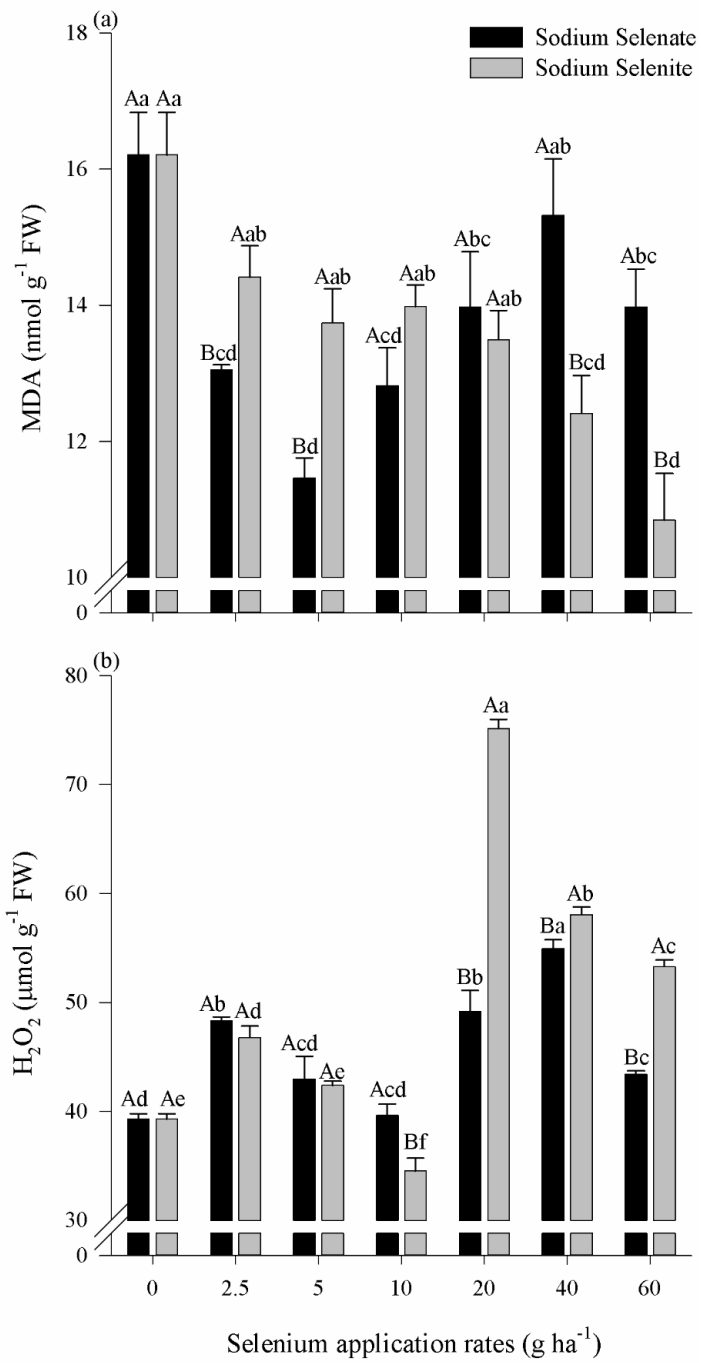

Figure 3. Effects of sodium selenate and sodium selenite application on lipid peroxidation essayed by MDA concentration (a) and hydrogen peroxide concentration (b) in cowpea leaves. The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate difference between means according to a Tukey test ($p \leq 0.05$). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se application rates. CV (%) = 5.97 (a) and 3.81 (b).

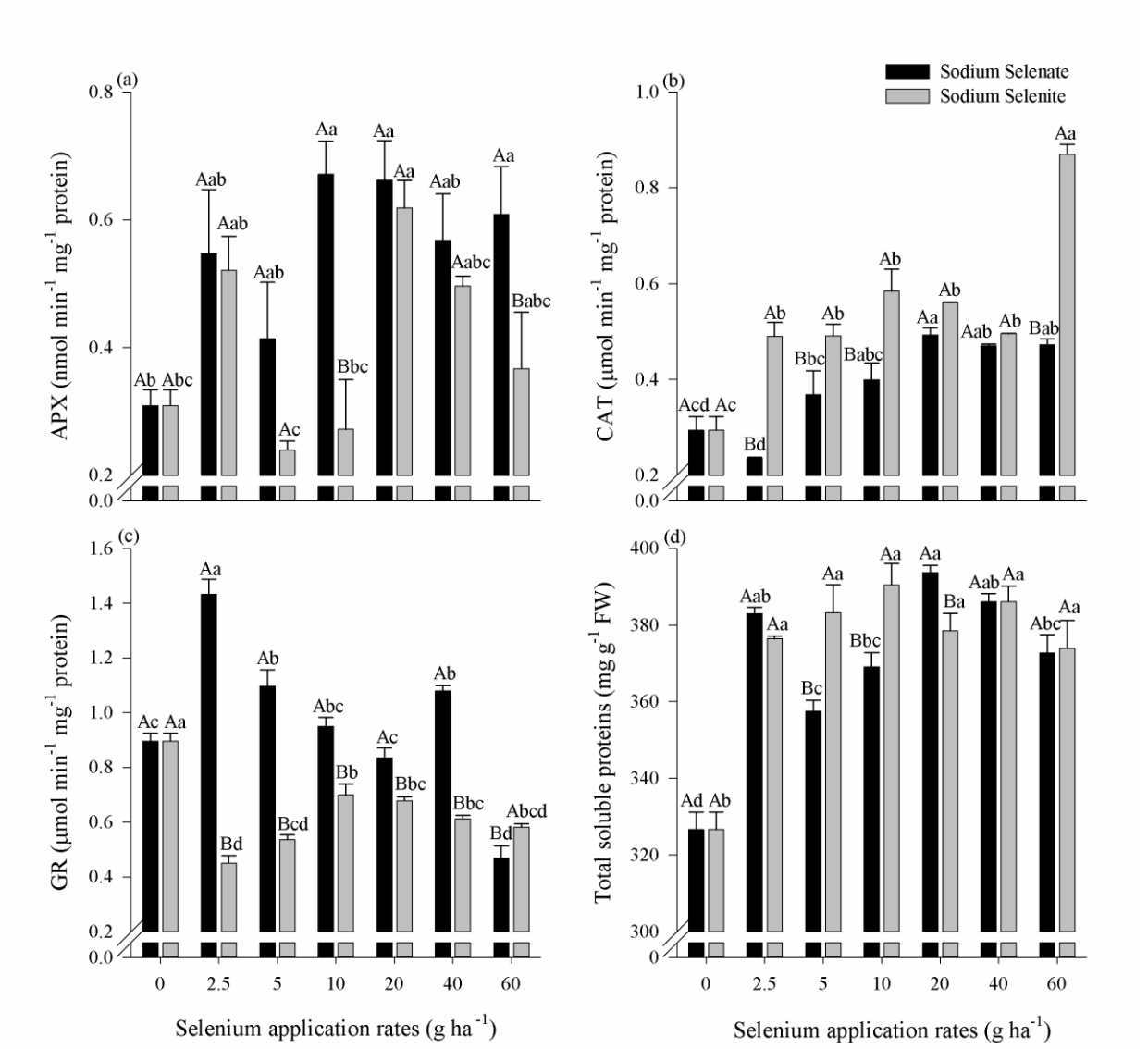

Figure 4. Effects of sodium selenate and sodium selenite application on enzymatic activity of APX (a), CAT (b) GR (c) and total soluble proteins (d) in cowpea leaves. The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate difference between means according to a Tukey test ($p \leq 0.05$). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se application rates. CV (%) = 26.30 (a); 10.55 (b); 8.40 (c) and 2.24 (d).

3.4 Sucrose and total sugars concentration

Interactions between Se source and application rate were observed for leaf sucrose concentration ($p<0.01$) and total sugar concentration ($p<0.01$; Table S5).

Application of sodium selenate at rates 2.5, 5, 10, 40 and 60 g ha^{-1} led to an increase in leaf sucrose concentration compared to untreated plants. The largest increase was recorded at 118 % under 2.5 g Se ha^{-1}. Under sodium selenite treatment, all application rates increased leaf sucrose concentration compared to untreated plants. The largest increase of 108 % was observed at 2.5 g Se ha^{-1}. Selenate application led to an elevated sucrose concentration in leaves compared to selenite application at the rates of 2.5 and 5 g Se ha^{-1}, and selenite led to elevated sucrose in leaves than selenate at rates of 20 and 40 g Se ha^{-1} (Fig 5a)

Selenium application as both selenate and selenite increased the total sugars concentration in comparison to untreated plants at all rates. The highest increase in total sugars under selenate application was 177% recorded at 2.5 g Se ha^{-1}. Under selenite application, the highest total sugar concentration was recorded at 40 g Se ha^{-1}, 157% higher than the concentration observed in untreated plants. Similarly, Se application as selenate led to greater leaf total sugar concentrations than selenite at 2.5 g Se ha^{-1}, and selenite application provided greater leaf total sugar concentrations than selenate at 40 g Se ha^{-1-} (Fig 5b).

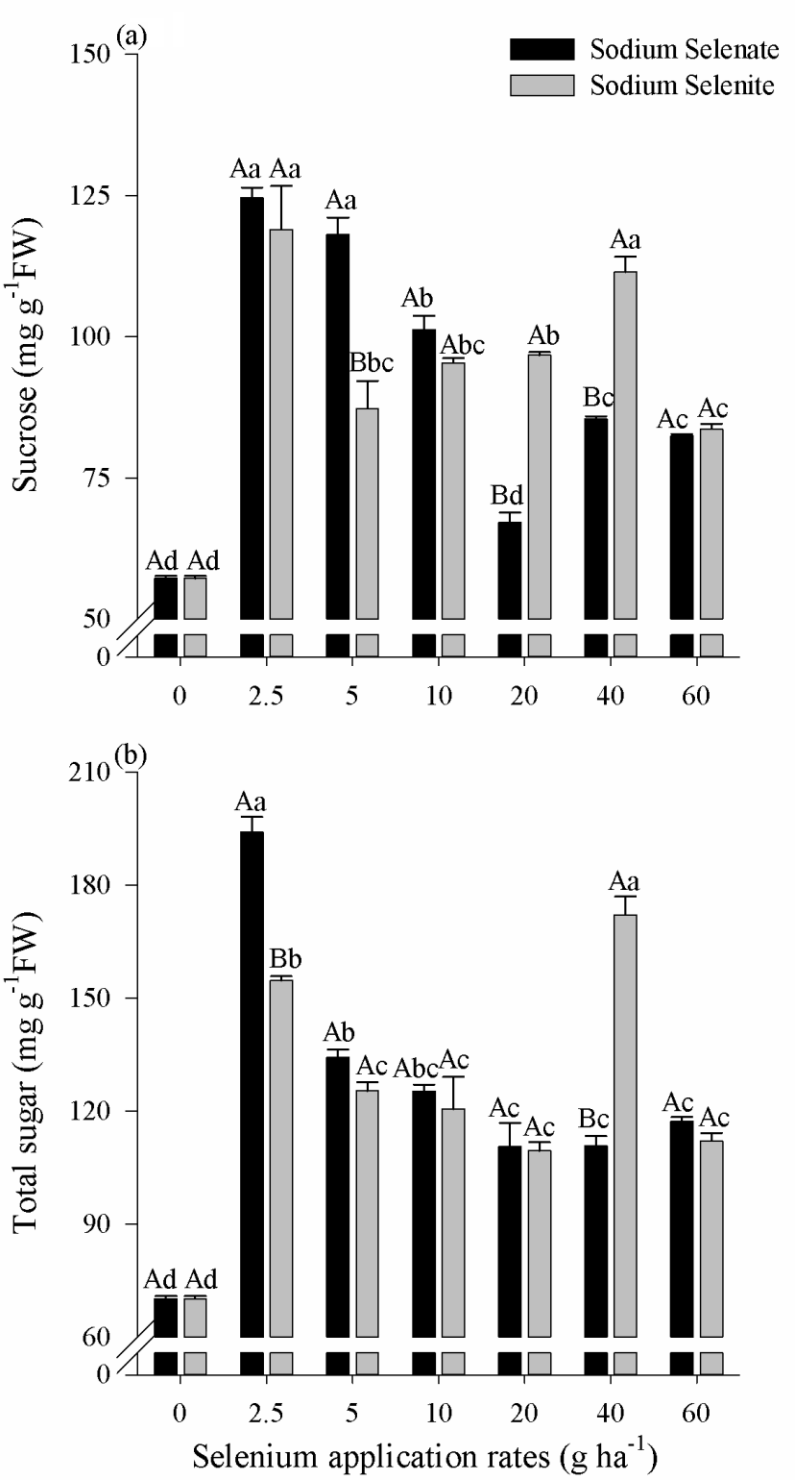

Figure 5. Effects of sodium selenate and sodium selenite on sucrose (a) and total sugar concentration (b) in cowpea leaves. The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate difference between means according to a Tukey test ($p \leq 0.05$). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se application rates. CV (%) = 6.26 (a) and 5.96 (b).

3.5 Selenium concentration in plant tissue

Interactions were observed between Se source and application rate for leaf ($p=0.046$) and grain ($p<0.01$) Se concentrations (Table S6). Leaf Se concentration increased from 0.06 mg kg^{-1} in untreated plants up to 0.52 mg kg^{-1} under application of 40 g Se ha^{-1} as selenate. However, no significant increase in leaf Se was observed under Se application as selenite. Similar trends were observed in cowpea grains; under selenate application, grain Se concentration increased from 0.05 mg kg^{-1} in untreated plants to 0.84 mg kg^{-1} at an application rate of 40 g Se ha^{-1}, whilst limited differences were observed under selenite application apart from at an application rate of 60 g Se ha^{-1}, in which grain Se concentration reached 0.32 mg ka^{-1} (Table S1).

3.6 Heatmap and correlation among parameters

A positive correlation was observed between leaf Se concentration and grain Se concentration (Fig 6). A negative correlation was observed between CAT and chlorophyll a, carotenoids, MDA and GR activity, whilst a positive correlation was observed between CAT and soluble proteins, H_2O_2 and transpiration rate (Fig 6). Positive correlations were also observed between Se concentration in leaves and grains with APX, and negative correlations were observed between Se concentration in leaves and grains with transpiration rate (Fig 6). Total chlorophyll, chlorophyll a, total carotenoids and pheophytin were also positively correlated (Fig 6).

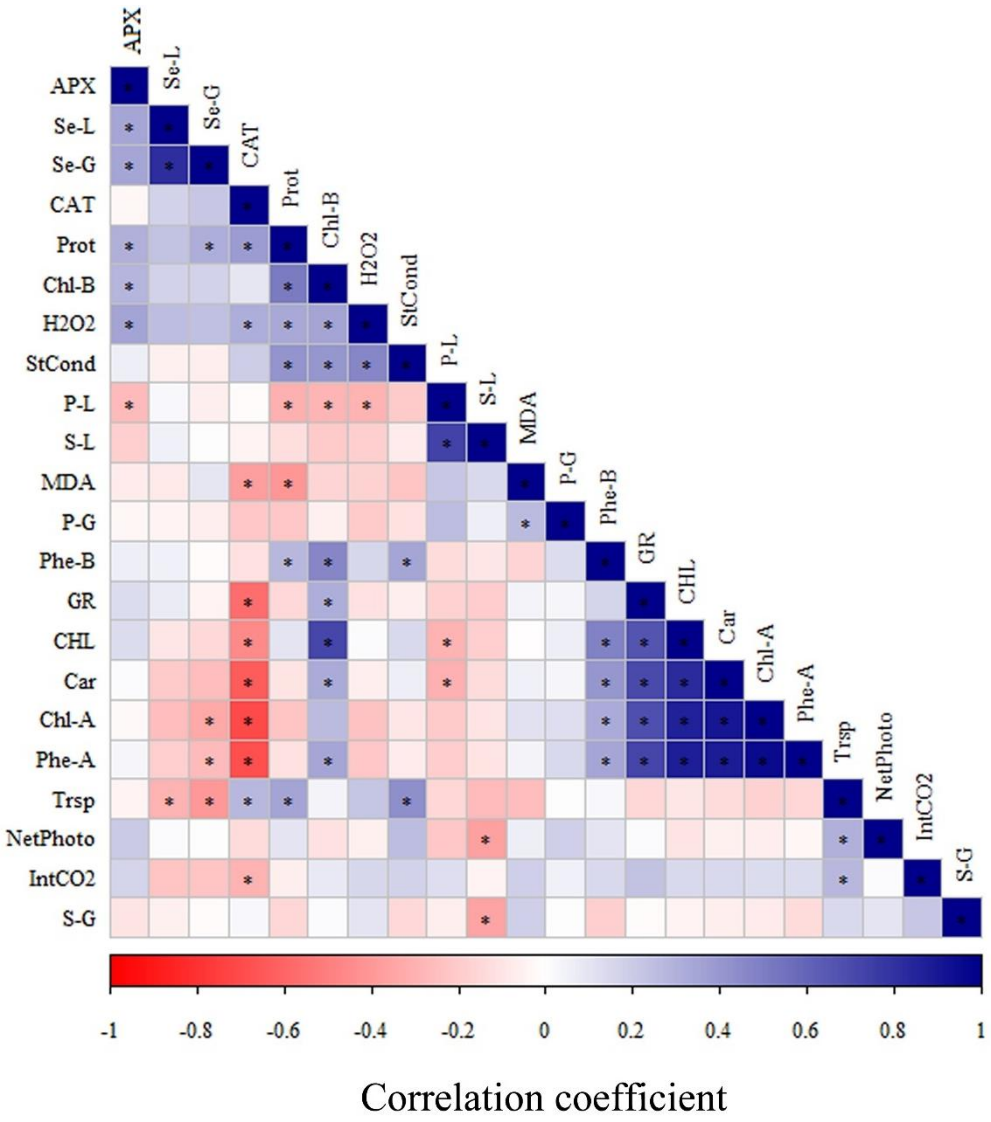

Figure 6. Heatmap showing the Pearson correlation among the physiological and biochemical parameters from cowpea plants in response to Se application rates as selenite or selenite source. * indicates significant relationship ($p \leq 0.05$).
Abbreviations: chlorophyll a (Chl-A), chlorophyll b (Chl-B), total chlorophyll (CHL), total carotenoids (Car), pheophytin A (Phe-A), pheophytin B (Phe-B), internal CO_2 concentration ($IntCO_2$), transpiration rate (Trsp), stomatal conductance (StCond), net photosynthetic rate (NetPhoto), leaf Se concentration (Se-L), grain Se concentration (Se-G), leaf P concentration (P-L), grain P concentration (P-G), leaf S concentration (S-L), grain S concentration (S-G).

4. DISCUSSON

The effect of Se application on chlorophyll and carotenoids concentration was previously observed in hydroponic cultivated garlic (Astaneh et al., 2018). Photosynthetic pigment concentration is very sensitive to variation in the concentration of ROS and can be used as an indicator of cellular metabolic state (Chutipaijit et al., 2011). In this study, Se applied at a rate of 2.5 g ha^{-1} in the form of sodium selenate provided a notable increase in concentrations of pigments, as observed for chlorophyll a and b, total chlorophyll and carotenoids concentration (Fig 1). Handa et al. (2019) also reported an increase in total chlorophyll and total carotenoids followed by a decrease in these pigments as plants were exposed to higher Se concentrations. In pumpkin plants, a foliar spray of 1.5 mg Se L^{-1} as sodium selenate facilitates respiration and electron donation in the respiratory chain, which leads to accelerated chlorophyll biosynthesis (Germ et al., 2005). This might explain the increase in pigments under 2.5 g Se ha^{-1} when applied as selenate. Interestingly, a decrease in chlorophyll a concentration was observed under most of the other Se application rates, whilst a general increase in chlorophyll b concentration was observed. The concentration of total chlorophyll in most treatments remained constant and not lower than control. These results indicate that decreases in chlorophyll a is compensated by the increase in chlorophyll b concentration (Fig 1a,b,c).

Pigment concentration alteration might be related to changes in photosynthetic rate and gas exchange parameters (Handa et al., 2019). However, no correlation was observed between these two measures in this study (Fig 6). Selenium application rates and sources promoted a variable effect on photosynthetic rates. Only selenate application rates affected the internal CO_2 concentration, whilst stomatal conductance and transpiration rate were most affected by selenite application rates (Fig 2). Higher stomatal conductance under selenite application at rates above 5 g ha^{-1} (Fig 2b) might be associated with Se availability in the soil. Selenate in soil is more readily available to plants than selenite due to lower soil adsorption properties in the former (Silva et al., 2019). However, selenite absorbed by plant roots is converted into organic compounds faster than selenate (White, 2016). This may explain the results generated by this study, as at low Se application rates, selenite would not be efficiently taken up by plants, but at higher rates, more may be taken up and converted to organic forms more quickly than

selenate, enabling a more efficient increase in stomatal conductance. Under application rates higher than 20 g ha^{-1}, selenite and selenate provided equal but limited increases in stomatal conductance, indicating an optimum Se application rate for this benefit to be achieved.

Selenium can enhance photosynthetic apparatus and pigment concentration. These results are usually associated with Se applied under induced stress conditions (Silva et al., 2018). In maize plants under salt stress in the presence of 100 mM NaCl, the addition of 1 µM Se as selenite in Hoagland solution increased plant chlorophyll and carotenoid concentration, net photosynthetic rate and stomatal conductance (Jiang et al., 2017). An increase in net photosynthetic rate, transpiration rate, stomatal conductance and internal CO_2 concentration was also observed in *Brassica juncea* plants under application of 4 µM kg^{-1} of Se as selenate via soil application before sowing (Handa et al., 2019). Selenium might act repairing chloroplasts damaged by environmental stress (Feng et al., 2013) and protecting enzymes chloroplast enzymes (Pennanen et al., 2002). Thus, the lack of induced stress in this study might be the cause of no clear responses in chlorophyll and photosynthesis to selenate and salenite application.

Lipid peroxidation is a good indicator of ROS activity damaging cells, since the oxidative stress causes the peroxidation of unsaturated fatty acids, whilst increasing MDA concentration (Farooq et al., 2019). Selenium application could decrease lipid peroxidation by enhancing the activity of enzymes to combat ROS (Reis et al., 2018; Yin et al., 2019). In this study, selenate application promoted lower lipid peroxidation rates in comparison both to plants subject to selenite application and untreated plants (Fig 3a). Application of 2.5 and 5 g Se ha^{-1} decreased the lipid peroxidation as measured by MDA concentration, followed by an increase showing the highest rates at 40 g Se ha^{-1}. Selenite application tended to gradually decrease MDA with increasing Se rates (Fig 3a). Higher leaf Se concentration was observed in plants treated with selenate than those treated with selenite (Table S1). This might explain the more prominent alteration in MDA under selenate application. In *Brassica chinensis* grown in a hydroponic system, lower MDA concentrations were observed in response to selenate than selenite at rates 5 and 20 mM (Yu et al., 2018). However, application of selenite provided lower lipid peroxidation than application of selenate in tomato plants under application of 1 mM of Se (Alves et al.

2019). The effect of Se rates and sources on lipid peroxidation rates may vary according to plant species, and further work is required in order to underpin the mechanism driving this.

Under increased concentrations of MDA, the increase in H_2O_2 indicates that this molecule might be acting in oxidative stress promotion (Feng et al., 2013), but under lower concentrations of MDA, increases in H_2O_2 could indicate signaling (Farooq et al., 2019). Hydrogen peroxide concentrations in foliar tissues increased under most Se application rates compared to untreated plants in this study (Fig 3b). Selenium application at high concentrations can promote oxidative stress by consuming compounds that act in ROS scavenging, such as thiols ferredoxins and NADPH (Feng et al., 2013; Yu et al., 2018) which might lead to the increase in H_2O_2 concentration observed here. However, since H_2O_2 can also act as a signaling molecule in plant tissue (Farooq et al., 2019) and might act in plant development processes, such as leaf development (Lu et al., 2014), it is also possible that the high H_2O_2 concentrations observed at high Se application levels are a result of H_2O_2 acting as a signaling agent in the cowpea leaf tissues. In these study conditions, selenite is highly adsorbed to soil and selenate is more readily available to plants (Silva et al., 2019). However, as mentioned above, once taken up, selenite is more easily converted in amino acids and selenoproteins (White, 2016). That could explain why the effects of selenite application were only observed at the higher Se application rates.

Regarding APX, while in most application rates no difference was observed between selenite and selenate application, at 10 and 60 g ha^{-1} selenate provided a higher APX activity. In previous studies soaking wheat seedling in 5 mg L^{-1} selenate solution, was observed an increase in Ascorbic acid production (Akladious, 2012), which is substrate for APX activity. The observed changes in CAT and GR activity under the treatments in this study suggest that each Se source might act distinctly on each enzyme. Selenite application led to a generally higher CAT activity than selenate application (Fig 4b). In tomato plants cultivated in a pot experiment, the application of 1 mM of Se as selenite also provided higher CAT activity than selenate at the same application rate (Alves et al., 2019). In addition, in *Brassica chinensis* cultivated in a hydroponic system at a Se application rate of 10 mM, CAT activity was higher under selenite than selenate exposure (Yu et al., 2018). The application of selenate led to greater GR activity than the

application of selenite (Fig 4c). Inorganic Se can increase glutathione (GSH) concentration in order to synthetize organic-Se (Feng et al., 2013). Under higher levels of GSH, higher levels of GR are expected (Deponte, 2013). Since Se from selenate tends to be more slowly converted to organic-Se than selenite, this might explain the observed differences in GR activity between the two forms of Se.

A negative correlation between CAT and GR was observed (Fig 4a, Fig 6), indicating that the activity of one enzyme might be suppressing the other and vice-versa. Greater CAT activity was generally observed under selenite application than selenate. Previous results in cowpea also indicate that CAT presents higher activity than GR under selenite application of 100 g Se ha^{-1} or lower (Silva et al., 2018). The inorganic pool of Se provided by selenate might be inducing antioxidant activity by the ascorbate-glutathione antioxidant pathway (Asada, 2006). While the rapidly organic-Se provided by selenite might be inducing enzymatic activity of CAT, which also acts to decrease lipid peroxidation and may help to explain the results observed here.

Total sugars and sucrose concentration increased substantially in leaves of cowpea plants under almost all selenite or selenate application rates (Fig 5). Beneficial effects of Se on sugar concentration were also observed in previous studies. In shoots of groundnut, application of 20 and 40 ppm of Se nanoparticles increased total soluble sugars (Hebat-Allah et al., 2019). These authors also observed a decrease in MDA in one groundnut groundnut genotype (“Giza 6”) in addition to the increase in sugar concentration, consistent with some of the results presented here. The application of 0.75 ppm of Se as sodium selenate increased the activity of α-amylase and β-amylase in shoots of mungbean seedlings (Malik et al., 2011), indicating that Se might promote starch degradation by enhancing enzyme activity. The increase in soluble sugar concentration with a decrease in MDA under Se application might indicate that sugar accumulation could be an osmotic regulator, acting to maintain optimal cellular metabolism and protect cellular structures from ROS (Hebat-Allah et al., 2019). In this study, the results suggest that sucrose and total sugars concentration play an important role when combined with antioxidant enzymes to regulate the oxidative stress in cowpea plants controlled by selenate or selenite application (Fig 7).

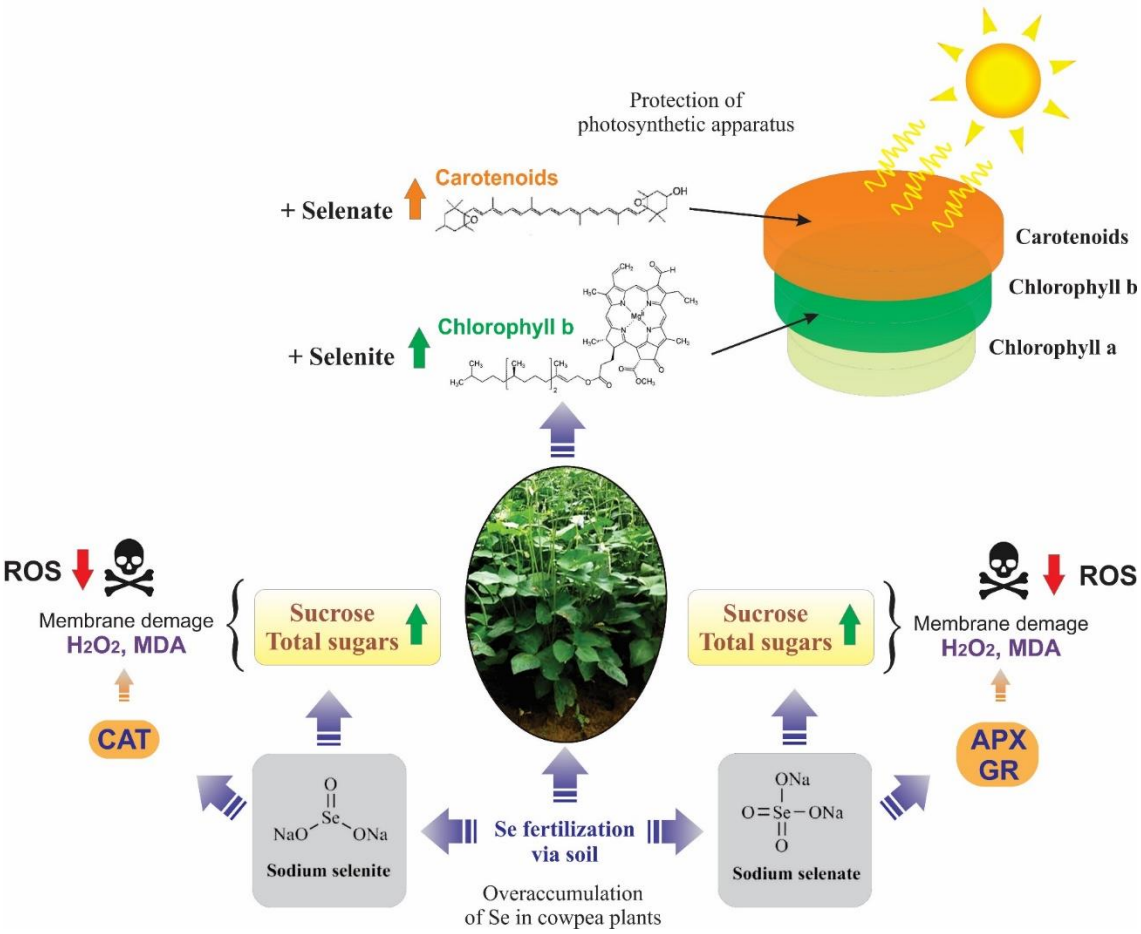

Figure 7. Proposed mechanism of general effects of sodium selenite and sodium selenate application on ROS scavenging system in cowpea plants.

Selenium application at high concentrations can drastically increase oxidative stress in plants (Silva et al., 2018). However, Se application up to a concentration of 100 g ha^{-1} can promote plant growth and antioxidant metabolism depending on plant species (Ding et al., 2017; Jiang et al., 2017). This study demonstrates that Se application might be beneficial to plants under field conditions by ameliorating antioxidant metabolism, enhancing the photosynthetic responses and increasing total sugars and sucrose concentration of cowpea plants. This is proposed to occur via to a Se-induced increase in activity of CAT, APX and GR, thus mitigating lipid peroxidation. It is noteworthy that previously published results do not indicate decreased cowpea grain yield and plant growth under Se application (Silva et al., 2019). Thus, Se application might prove beneficial in alleviating oxidative stress, without impacting on productivity.

5. CONCLUSIONS

Selenium application at 2.5 g ha^{-1} as sodium selenate provided an increase in total chlorophyll and carotenoids concentration. Selenium effects on gas exchange parameters are variable, depending on the form and Se rate applied. Further studies are necessary to better understand the relationship between Se and the photosynthetic system in cowpea under field conditions. In general, the effect of Se application differed between source. Selenium applied as sodium selenate showed a higher potential to increase GR activity, acting in the ascorbate-glutathione cycle, while sodium selenite application provided a higher potential to increase CAT activity. However, both selenate and selenite decreased lipid peroxidation levels, indicating that Se application is generally beneficial to the anti-oxidative system in field-grown cowpea plants. Despite the decrease of MDA concentration, the observed increases in H_2O_2 at high Se application rates may be related to signaling for oxidative stress combat instead of acting as a stressor agent. Both selenate and selenite application also increased leaf sucrose and total sugars concentration in cowpea leaves which may be an indicator of ROS scavenging. The combination of sugar metabolism and activation of antioxidative enzymes may form the biochemical response to enhance ROS scavenging induced by selenate and selenite application in cowpea plants under field-grown conditions, even in the absence of induced stress. Thus, the physiological response of Se to cowpea could be crucial to mitigate possible impairment provided by field stress.

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7. SUPPLEMENTARY TABLES

Table S1. Leaf and grain Se concentration in response to sodium selenate or sodium selenite application via soil. Leaf and grain material harvested 63 and 81 DAS, respectively.

Se application rates (g ha^{-1})	Leaf Se concentration		Grain Se concentration	
	Selenate	Selenite	Selenate	Selenite
	mg kg^{-1}			
0	0.06 Ad	0.06 Aa	0.05 Ac	0.05 Ab
2.5	0.12 Acd	0.09 Aa	0.09 Ac	0.09 Ab
5	0.27 Abcd	0.10 Ba	0.13 Ac	0.10 Ab
10	0.33 Aabc	0.08 Ba	0.24 Abc	0.08 Bb
20	0.29 Aabcd	0.20 Aa	0.44 Ab	0.19 Bab
40	0.52 Aa	0.25 Ba	0.84 Aa	0.23 Bab
60	0.39 Aab	0.29 Aa	0.67 Aa	0.32 Ba
CV(%)	----------48.02----------		----------37.30----------	

Different letters indicate difference between means according to a Tukey test ($p \leq 0.05$). Uppercase letters correspond to Se sources, and lowercase letters correspond to Se application rates.

Table S2. F ratio and P value of analysis of covariance (ANCOVA) for chlorophyll a, b, total chlorophyll and total carotenoids concentration. Sources of variation: Se sources, Se application rates and the variation between the factors.

Chlorophyll a		
Factor	F ratio	Pr (<F)
Se source (A)	67.53**	0.0001
Application rate (B)	56.14**	0.0001
A X B	17.25**	0.0001
Chlorophyll b		
Factor	F ratio	Pr (<F)
Se source (A)	2.01NS	0.1646
Application rate (B)	11.55**	0.0001
A X B	12.66**	0.0001
Total chlorophyll		
Factor	F ratio	Pr (<F)
Se source (A)	23.90**	0.0001
Application rate (B)	21.26**	0.0001
A X B	19.85**	0.0001
Total carotenoids		
Factor	F ratio	Pr (<F)
Se source (A)	69.24**	0.0001
Application rate (B)	50.42**	0.0001
A X B	35.52**	0.0001
Pheophytin a		
Factor	F ratio	Pr (<F)
Se source (A)	150.69**	0.0001
Application rate (B)	90.61**	0.0001
A X B	25.71**	0.0001
Pheophytin b		
Factor	F ratio	Pr (<F)
Se source (A)	0.07NS	0.7943
Application rate (B)	4.22**	0.0023
A X B	2.36*	0.0485

F: F ratio. P: value. ** significant at 0.01 of probability. * significant at 0.05 of probability. ^{NS} not significant.

Table S3. F ratio and P value of analysis of covariance (ANCOVA) for Net photosynthetic rate, Stomatal conductance, Internal CO_2 concentration and transpiration rate. Sources of variation: Se sources, Se application rates and the variation between the factors.

Net photosynthetic rate		
Factor	F ratio	Pr (<F)
Se source (A)	0.87NS	0.3557
Application rate (B)	5.59**	0.0003
A X B	4.11**	0.0027
Stomatal conductance		
Factor	F ratio	Pr (<F)
Se source (A)	10.44**	0.0025
Application rate (B)	6.19**	0.0001
A X B	5.14**	0.0006
Internal CO2 concentration		
Factor	F ratio	Pr (<F)
Se source (A)	0.09NS	0.7618
Application rate (B)	2.80*	0.0230
A X B	0.79NS	0.5869
Transpiration rate		
Factor	F ratio	Pr (<F)
Se source (A)	35.78**	0.0001
Application rate (B)	7.78**	0.0001
A X B	4.75**	0.0010

F: F ratio. P: value. ** significant at 0.01 of probability. * significant at 0.05 of probability. ^{NS} not significant.

Table S4. F ratio and P value of analysis of covariance (ANCOVA) for lipid peroxidation essayed as MDA, H_2O_2 concentration and enzymes activity. Sources of variation: Se sources, Se application rates and the variation between the factors.

MDA concentration		
Factor	F ratio	Pr (<F)
Se source (A)	1.25NS	0.2709
Application rate (B)	18.45**	0.0001
A X B	13.16**	0.0001
H2O2 concentration		
Factor	F ratio	Pr (<F)
Se source (A)	87.46**	0.0001
Application rate (B)	200.19**	0.0001
A X B	67.31**	0.0001
APX activity		
Factor	F ratio	Pr (<F)
Se source (A)	17.01**	0.0002
Application rate (B)	7.24**	0.0001
A X B	2.71*	0.0268
CAT activity		
Factor	F ratio	Pr (<F)
Se source (A)	130.71**	0.0001
Application rate (B)	48.78**	0.0001
A X B	16.43**	0.0001
GR activity		
Factor	F ratio	Pr (<F)
Se source (A)	336.12**	0.0001
Application rate (B)	32.19**	0.0001
A X B	61.71**	0.0001

F: F ratio. P: value. ** significant at 0.01 of probability. * significant at 0.05 of probability. ^{NS} not significant.

Table S5. F ratio and P value of analysis of covariance (ANCOVA) for lipid peroxidation essayed as MDA, H_2O_2 concentration and enzymes activity. Sources of variation: Se sources, Se application rates and the variation between the factors.

Protein concentration		
Factor	F ratio	Pr (<F)
Se source (A)	2.98NS	0.0973
Application rate (B)	49.58**	0.0001
A X B	6.24**	0.0001
Sucrose concentration		
Factor	F ratio	Pr (<F)
Se source (A)	1.98NS	0.1819
Application rate (B)	99.58**	0.0001
A X B	25.58**	0.0001
Total Sugars concentration		
Factor	F ratio	Pr (<F)
Se source (A)	0.02NS	0.8847
Application rate (B)	149.52**	0.0001
A X B	33.64**	0.0001

F: F ratio. P: value. ** significant at 0.01 of probability. * significant at 0.05 of probability. ^{NS} not significant.

8. SUPPLEMENTARY FIGURES

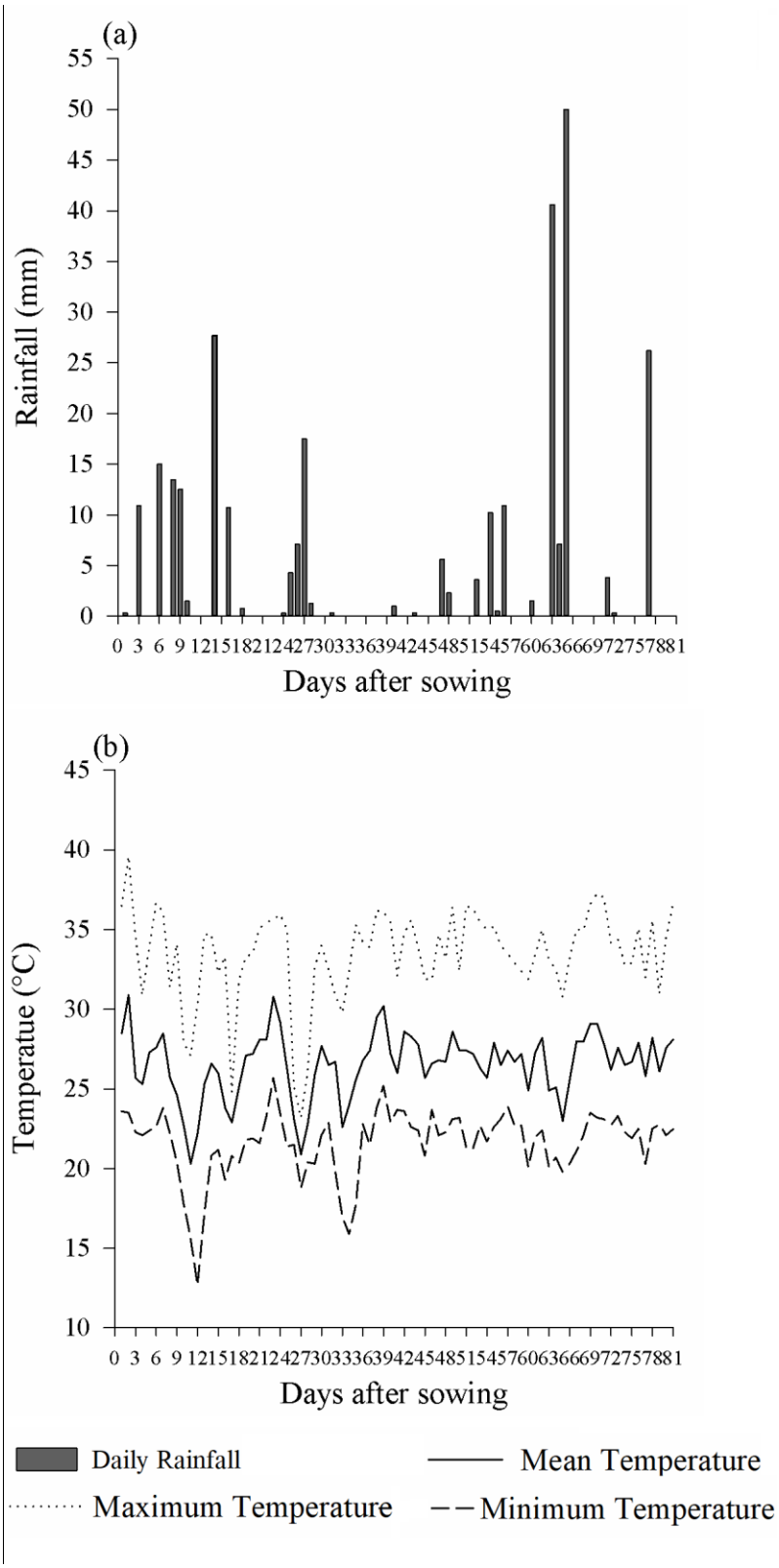

Figure S1. Rainfall (a) and temperature (c) of cowpea crop experiment field.

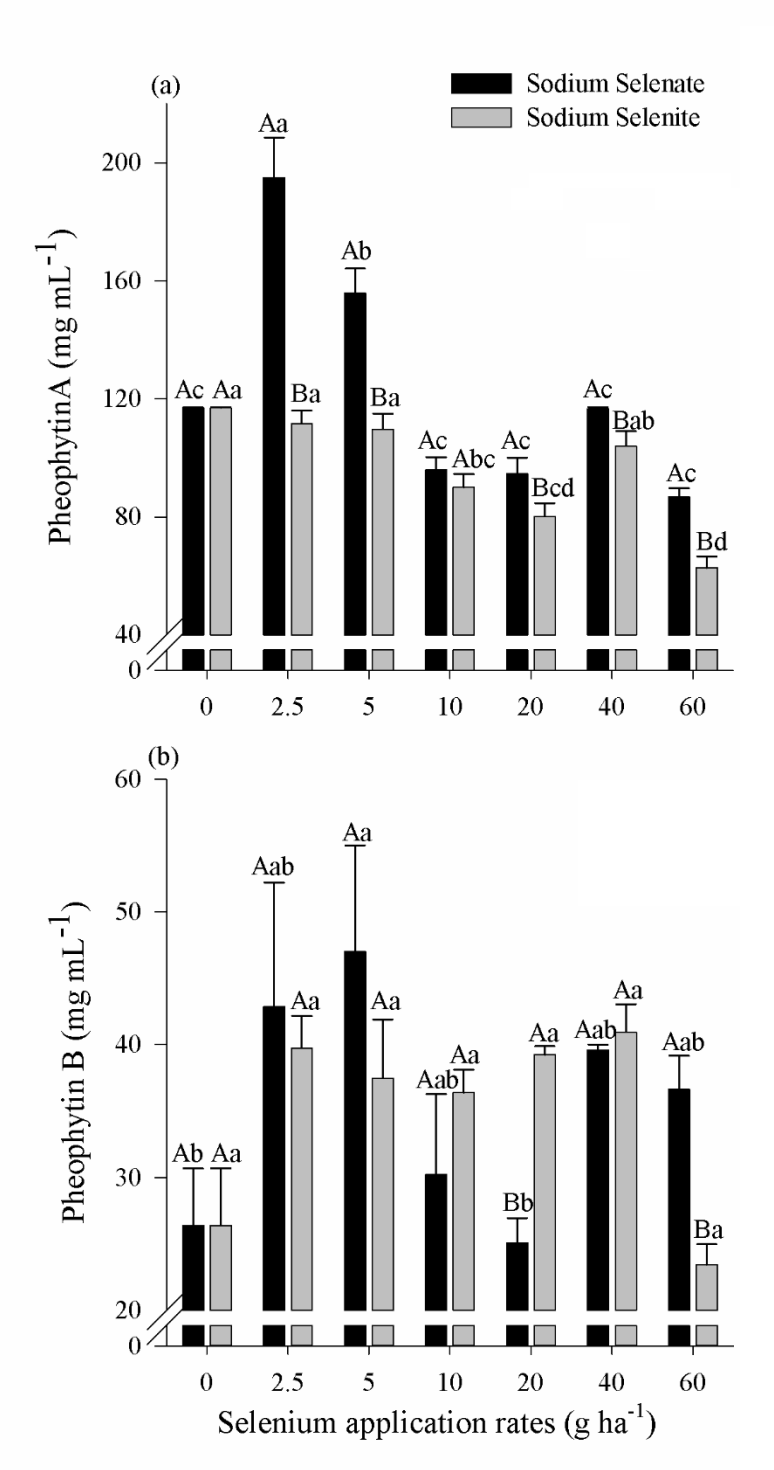

Figure S2. Effect of sodium selenate and sodium selenite application on pheophytin a (a) and pheophytin b (b) in cowpea leaves. The standard error of the mean (n = 4) is indicated by error. CV (%) = 7.40 (a) and 24.26 (b).

CHAPTER 3 – APPLICATION OF SODIUM SELENATE TO COWPEA (*Vigna unguiculata* L.) INCREASES SHOOT AND GRAIN SE PARTITIONING WITH STRONG GENOTYPIC INTERACTIONS

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ABSTRACT

Backgorund: Cowpea is a crop widely used in developing countries due its rusticity. Besides its rich genotypic variability, most breeding programs do not explore its potential to improve elements uptake. Selenium (Se) is a scarce element in most soils, resulting in its deficiency being common in human diets. This study aimed to evaluate the interaction between biofortification with Se and genotypic variation in cowpea, on the concentrations of Se in roots, leaves + stem and grains.

Methods: Twenty-nine cowpea genotypes were grown in a greenhouse in the absence (control) and presence of Se (12.5 µg Se kg^{-1} soil) as sodium selenate, in fully randomized scheme. The plants were cultivated until grains harvest. The following variables were determined: roots dry weight (g), leaves + stems dry weight (g), grains dry weight (g), Se concentration (mg kg^{-1}) in roots, leaves + stems and grains, and Se partitioning to shoots and grains.

Results: Selenium application increased the Se concentration in roots, leaves + stems and grains in all genotypes. At least twofold variation in grain Se concentration was observed among genotypes. Selenium application did not impair biomass accumulation, including grain dry weight. Genotype “BRS Guariba” had the largest Se concentration in grains and leaves + stems. Genotype MNC04-795F-158 had the largest partitioning of Se to shoots and grain, due to elevated dry weights of leaves + stems and grain, and high Se concentrations in these tissues.

Conclusion: This information might be valuable in future breeding programs to select for genotypes with better abilities to accumulate Se in grain to reduce widespread human Se undernutrition.

Keywords: *Vigna unguiculata* (L.) Walp, sodium selenite, selenium partitioning, grain quality, biofortification, pulses

1. INTRODUCTION

Currently, about 820 million people worldwide are estimated to consume insufficient food to meet their dietary energy requirements (FAO, 2019). Further, more than half of the world's population are likely to consume insufficient quantities of one or more micronutrients (White & Broadley, 2009). This is, in part, a consequence of plant breeding aimed at increasing crop yield, which can reduce tissue nutrient concentrations through growth dilution (White et al., 2009)]. Crop-breeding programs need to expand their focus from yield alone, to also increasing nutrient concentrations in edible plant tissue to combat human micronutrient malnutrition.

Selenium (Se) is an essential element for human and animal nutrition, serving in antioxidant processes, and contributing to proper immune system and thyroid functioning (Fairweather-Tait et al., 2011). It is also considered a chemo-preventative agent, since appropriate Se nutrition is able to reduce the risk of cancer, and may delay or reduce the prevalence of its recurrence (Serrano-Sandoval et al., 2019). In plant nutrition, Se is considered a beneficial element, acting in the biotic and abiotic stress response as an antioxidant promoter (Pilon-Smits et al., 2009; Reis et al., 2017; Reis et al., 2018; Silva et al., 2018; Whit, 2016).

Despite its nutritional importance, Se is a trace-element, usually present in low concentrations in the soil (Lazo-Vezes et al., 2015) and in most foods. Plant-based diets often result in Se undernutrition in both humans and animals, leading to Se deficiency symptoms (Rayman, 2012). It is estimated that more than 1 billion people suffer from Se deficiency (Schiavon & Pilon-Smits, 2017). Among human micronutrient deficiency risks, Se ranks as the third most prevalent, with particularly high deficiency risks in Africa (Ngigi et al., 2019).

Cowpea (*Vigna unguiculata* (L.) Walp.) is a legume (Fabaceae) believed to have originated in West Africa (Nwokolo & Ilechukwu, 1996). It is rich in protein (Timko et al., 2007) and well adapted to low fertility soils, high temperatures and drought conditions (Carvalho et al., 2019). These traits make cowpea a good food source to complement other edible plants, and it is currently used to provide protein to diets in African, Asian and Latin American countries (Timko et al., 2007; Manzeke et al., 2019). The use of improved cowpea genotypes with higher yields, might reduce poverty and lead to

increase household income (Manda et al., 2019). An additional benefit of cowpea in the human diet is the potential for its sprouts to help reduce colorectal-cancer (Teixeira-Guedes et al., 2019).

Breeding programs for cowpea aim to develop improved lines with high grain yield potential, resistance to biotic stresses, tolerance to abiotic factors, adaptation to major production, and traits preferred by consumers and producers (Rocha et al., 2017). Recently, breeders have focused on cowpea genotypes with the potential to accumulate micronutrients in edible tissue, aiming to benefit human health by combating undernutrition (Oliveira et al., 2017).

There is considerable genotypic variation in seed Se concentration in many grain crops (Timko et al., 2007). In rice, roots of cultivars that accumulate more Se appear to release more organic acids, which increases Se availability in rhizosphere (Zhang et al., 2019a)]. However, some rice genotypes accumulate more Se in roots and leaves, with limited translocation of Se to grain (Zhang et al., 2019b), thus impacting on their effectiveness in improving human Se nutrition. These studies exemplify the importance of selecting genotypes that not only acquire Se effectively but also translocate Se to edible portions, which are independent processes.

The effect of Se applications on seed Se concentration in crops is well-established (Ros et al., 2006). For example, in rice, a Se application rate of 25 g ha^{-1} increased grain Se concentration from 0.03 to 0.32 mg kg^{-1} and also increased albumin and glutelin in seeds (Reis et al., 2018). In wheat, Se application at a rate of 10 g ha^{-1} resulted in substantial increases in the concentrations of Se and selenomethionine in both grain and bread (Broadley et al., 2010) and Se application at a rate of 21 g ha^{-1} increased both Se concentration in grain and grain yield (Lara et al., 2019). In cowpea, a Se application rate of 10 g ha^{-1} provided sufficient Se to increase daily human Se intake by up to 14 µg d^{-1}, based on current dietary trends, without impairing cowpea yield and biomass (Silva et al., 2019).

Since little is known about genotypic variation in Se accumulation in cowpea, this study aimed to evaluate variation in the ability of 29 cowpea genotypes to accumulate Se in roots, shoots and grains, as well as the interactions between genotypic effects and Se application rates.

2. MATERIALS AND METHODS

2.1 Experimental Design

The experiment was conducted in a greenhouse at São Paulo State University (UNESP) in the municipality of Tupã, São Paulo State, Brazil. Twenty-nine cowpea genotypes (Table 1) were cultivated in the absence (control) and presence of additional Se (12.5 µg Se kg^{-1} soil). Cowpea genotypes were received from Brazilian Agricultural Research Corporation (EMBRAPA). The experiment had a completely randomized design, with three replicates per genotype per Se treatment, totaling 174 pots.

The soil was collected from the experimental farm at São Paulo State University and sieved to fill pots to the volume of 5 dm^3 in November 2016. Soil chemical characteristics were as follows: pH ($CaCl_2$ 0.01 M) 4.6; phosphorus (resin), sulfur (calcium phosphate), boron (hot water), copper (DTPA), iron (DTPA), manganese (DTPA) and zinc (DTPA): 6, 3, 0.07, 0.5, 11, 12 and 0.2 mg dm^{-3} respectively; potassium (resin), calcium (resin), magnesium (resin), H+Al (SMP buffer) and cation exchange capacity: 0.9, 5, 3, 16 and 24.9 mmolc dm^{-3} respectively; base saturation: 36%. These chemical characteristics were determined as described by Raij et al. (1997). Total and exchangeable Se concentration in unamended soil were 45 µg kg^{-1} and 3.2 µg kg^{-1}, respectively. To determine soil Se concentrations, 4 g of air-dried soil was weighted and added into a 50 mL centrifuge tube, in which was added 20 mL of 0.01M KNO_3. A rotary shaker was used to homogenize the solution for 2 hours, then a centrifugation was performed for 30 min at 1650xg. After centrifugation, into a < 0.22 µm syringe filter 9 mL of the supernatant was pipetted. The supernatant was filtered into a tube containing 0.1 M KH_2PO_4 and 10% tetramethylammonium hydroxide (TMAH) for determination of total and exchangeable Se, respectively. The determination was carried out using an inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany), as described by Silva et al (2019).

Table 1. Identity and characteristics of cowpea genotypes used in this study based on grain color, plant architecture, maturation cycle and geographical recommendation in Brazil.

ID	Genotype	Plant architecture	Grain color	Maturation Cycle*	Geographical recomendation**
1	BR 17 - Gurguéia	Prostrate	Brown	Early médium	A**
2	BR 3-Tracuateua	Prostrate	White	Early	“São Paulo” State
3	BRS Aracê	Semi-	Green	Early médium	B**
4	BRS Cauamé	Semi-eret	White	Early	A
5	BRS Guariba	Semi-erect	White	Early	A
6	BRS Itaim	Erect	White	Early	B
7	BRS Juruá	Semi-	Green	Early medium	B
8	BRS Marataoã	Semi-	Brown	Early medium	A
9	BRS Milênio	Prostrate	White	Early medium	“Pará” State
10	BRS Novaera	Semi-eret	White	Early	A
11	BRS Pajeú	Semi-	Brown	Early Medium	North and Northeast regions
12	BRS Potengi	Semi-erect	White	Early	North and Northeast regions
13	BRS Rouxinol	Semi-	Brown	Early Medium	“Bahia” State
14	BRS Tumucumaque	Semi-eretc	White	Early	A
15	BRS Urubuquara	Semi-	White	Early Medium	“Para” State
16	BRS Xiquexique	Semi-	White	Early Medium	A
17	California CB-5	Erect	White	Early	California State
18	Inhuma	Semi-	Brown	Early Medium	A
19	MNC01-631F-20-5	Semi-	Brown	Early Medium	North and Northeast regions
20	MNC04-769F-62	Semi-erect	Brown	Early	A
21	MNC04-782F-108	Semi-	Brown	Early Medium	A
22	MNC04-792F-143	Semi-erect	Brown	Early	A
23	MNC04-792F-146	Semi-erect	Brown	Early	A
24	MNC04-795F-158	Semi-	Brown	Early Medium	A
25	Patativa	Prostrate	Brown	Early Medium	“Ceará” State
26	Paulistinha	Semi-	Brown	Early Medium	“Piauí” and “Ceará” states
27	Pingo de Ouro-1-2	Semi-	Brown	Early Medium	A
28	Pingo de Ouro-2	Semi-	Brown	Early Medium	Northeast Region
29	Pretinho	Semi-	Black	Medium	A

*“Early” maturation cycle last for 60 to 65 days; “Early Medium” maturation cycle lasts for 70 to 75 days, and “Medium” maturation cycle lasts for 75 to 80 days. ** “A” for genotypes recommended for North and Northeast regions, and “Mato Grosso” and “Mato Grosso do Sul” states; “B” For genotypes recommended for “Pará”, “Roraima”, “Mato Grosso”, “Piauí”, “Tocantins”, “Maranhão”, “Bahia” and Sergipe States

Prior to sowing cowpea seeds, each pot received 1.25 g of lime, 0.17 g KCl, and 0.46 g single superphosphate. The soil was incubated 30 days prior sowing the seeds. For inoculation, a peat inoculum specific for cowpea was used (2.0 x 10^9 colony forming units g^{-1}, strain SEMIA 6462, 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 then added and mixed with the seeds. Sowing was performed on December 29 2016. Emergence began on January 5 2017, 7 days after sowing (DAS). An application of urea (0.10 g pot^{-1}), was made 27 DAS. The pots were irrigated by a custom computerized irrigation system. Each pot was irrigated five times daily for one minute each time, at a flow rate of 100 mL min^{-1} via a low-density polyethylene (LDPE) pipes. Data regarding temperature and potential evapotranspiration were presented in figure A.1.

2.2 *Procedure and sampling*

According to general recommendations for cowpea cultivation, 29 days after emergence (DAE), each pot received 20 mL of a solution containing urea (0.22 g pot^{-1}), KCl (0.17 g pot^{-1}), and single superphosphate (0.45 g pot^{-1}). The same fertiliser applications were made 36 DAE and 61 DAE. Selenium treatments were applied to the relevant pots 44 DAE at a rate of 12.5 µg kg^{-1} soil per pot, using sodium selenate. The application rate and salt were selected according to previous studies of the group regarding Se application rates and sources in cowpea (Silva et al., 2019).

Harvest was performed across a series of days for each genotype according to the pod's maturity. The material was separated into grains, leaves + stems and roots. Root material was washed with deionized water. Root, leaves + stems, and seeds of cowpea plants were dried in an oven at 40 °C for 72 hours to a constant mass to measure the dry weight (DW) of grains, leaves + stems and roots. After determining its dry weight, sample material was ground and homogenised in a Wiley mill for chemical analysis.

2.3 *Digestion and nutritional analysis of roots, leaves + stems and grains*

Subsamples (~0.20 g) of dried, milled root, leaves + stems and grain materials were weighed (exact weights recorded) and digested in digestion tubes of a perfluoroalkoxy (PFA) liner material containing 2 mL 70 % Trace Analysis Grade HNO_3, 1 mL Milli-Q water, and 1 mL H_2O_2. Prior to elemental analysis, the digestates were diluted 1-in-10 using milli-Q water. The digestion of plant material and analysis by ICP-MS were performed according

to the methods of Thomas et al (2016). Data processing was undertaken using Qtegra™ software (Thermo Fisher Scientific).

2.4 Selenium translocation estimative

Selenium partitioning was calculated for each genotype following Abichequer & Bohnen (1998) using the following equations:

$$Se\ partitioning\ to\ shoot\ (\%) = \frac{(B \times E) + (C \times F)}{(A \times D) + (B \times E) + (C \times F)} \quad \text{(Equation 1)}$$

$$Se\ partitioning\ to\ grains\ (\%) = \frac{(C \times F)}{(A \times D) + (B \times E) + (C \times F)} \quad \text{(Equation 2)}$$

where:

A – Root dry weight (kg)

B – Leaves + stems dry weight (kg)

C – Grains dry weight (kg)

D – Root Se concentration (µg kg^{-1})

E – Leaves + stems Se concentration (µg kg^{-1})

F – Grains Se concentration (µg kg^{-1})

2.5 Statistical analysis

The normality of the data was determined using the Anderson-Darling normality tests; Leven's test was used to determine homogeneity, then a variance analysis (F test) was performed. Mean differences between treatments were compared using a Scott-Knott test at 5% probability. Analyses were performed using R (version 3.5.1; source: https://cran.r-project.org/bin/windows/base/old/). A decision matrix was performed for variables that presented interaction between Se application and genotypes, to helps do pinpoint which is the best genotype for each characteristic.

3 RESULTS

The effect of Se on the dry weights of roots, leaves + stems and grain of the 29 cowpea genotypes is reported in Fig. 1. Regardless of the Se application, genotypes 1, 2, 4, 5, 6, 7, 8, 13, 20, 21 and 29 had larger root dry weight than the other genotypes (Fig. 1a, Table A.2; $p \leq 0.05$). The leaf + stem dry weights and grain dry weights were not affected by Se application (Fig. 1b-c; Table A.1). However, regardless of Se application, genotypes

4, 6, 8, 12, 17, 19, 21, 23, 24, 25 and 26 had larger leaf + stem dry weights than other genotypes, and genotypes 4, 6, 10, 12, 15, 17, 19, 23, 24, 25, and 26 had larger grain dry weights than other genotypes (Fig. 1c; Table A.2).

An interaction was observed between Se application and genotype in root dry weight ($P<0.05$; Table A.1). Selenium application resulted in an increase in root dry weight in genotypes 8 and 19, and a decrease in root dry weight in genotype 4; root dry weight was not affected by Se application in the other genotypes (Fig. 1a, Table A.2).

The application of Se increased Se concentration in grain, leaves + stems and roots of most genotypes (Fig. 2, 3 and 4, respectively). However, an interaction between Se application and cowpea genotypes was observed for Se concentrations in grain, leaves + stems and roots ($P<0.0001$; Table A.1). There was no difference in Se concentration in any tissue among genotypes grown without Se application. However, wide genotypic variation was observed in Se concentrations of grain, leaves + stems and roots when Se was applied (Fig. 2, 3 and 4, respectively).

Selenium application increased grain Se concentration in all 29 genotypes (Fig. 2). The increase in grain Se concentration when Se was applied ranged from 549 µg Se kg^{-1} dry weight in genotype 16 to 2462 µg Se kg^{-1} dry weight in genotype 5 (Fig. 2; Table A.3). A significant interaction was observed between Se application and genotype on Se concentration in grain ($P<0.0001$; Table A.1).

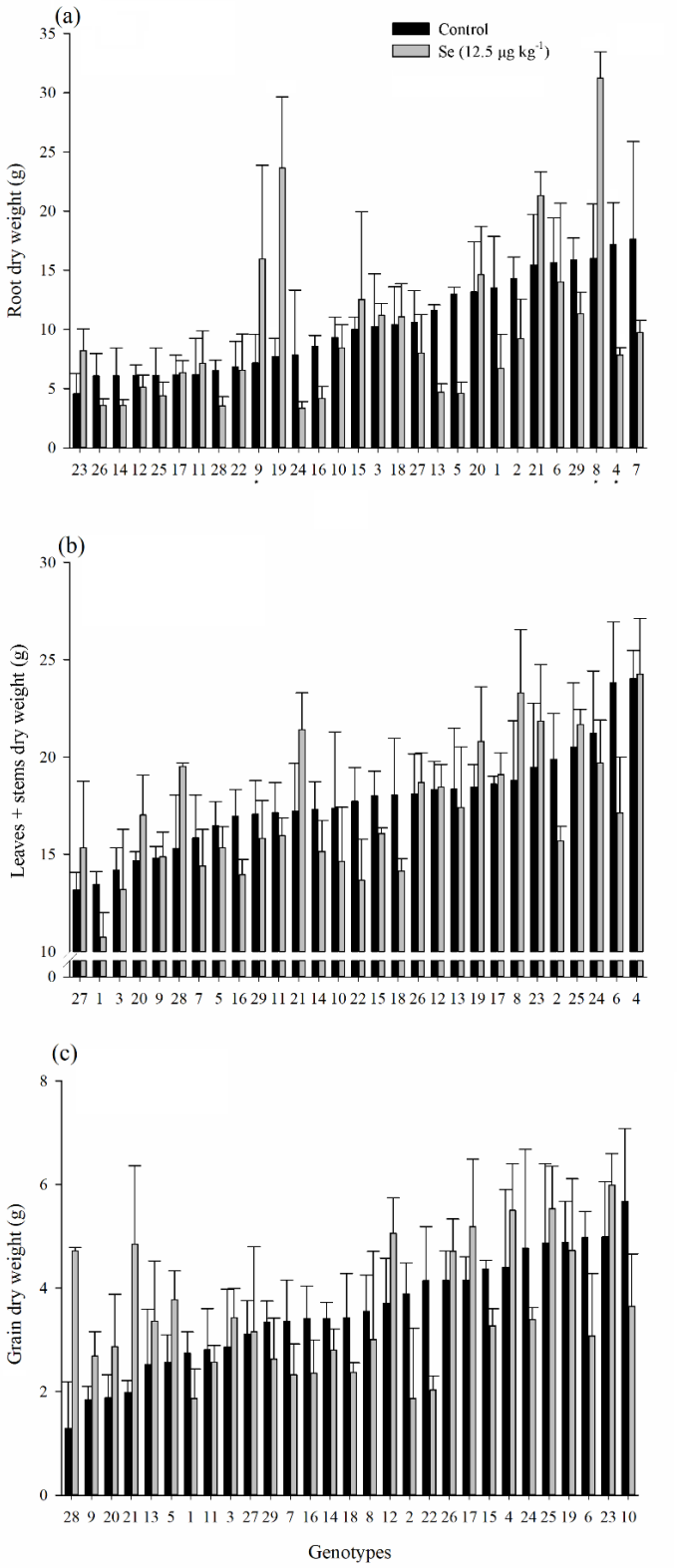

Fig. 1. Root (a), leaves + stems (b) and grain (c) dry weight of cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 55.28 (a), 21.20 (b) and 44.00 (c). '*' Below numbers indicates difference between means of the same genotype under absence or presence of Se application according to the Scott-Knott test ($p \leq 0.05$).

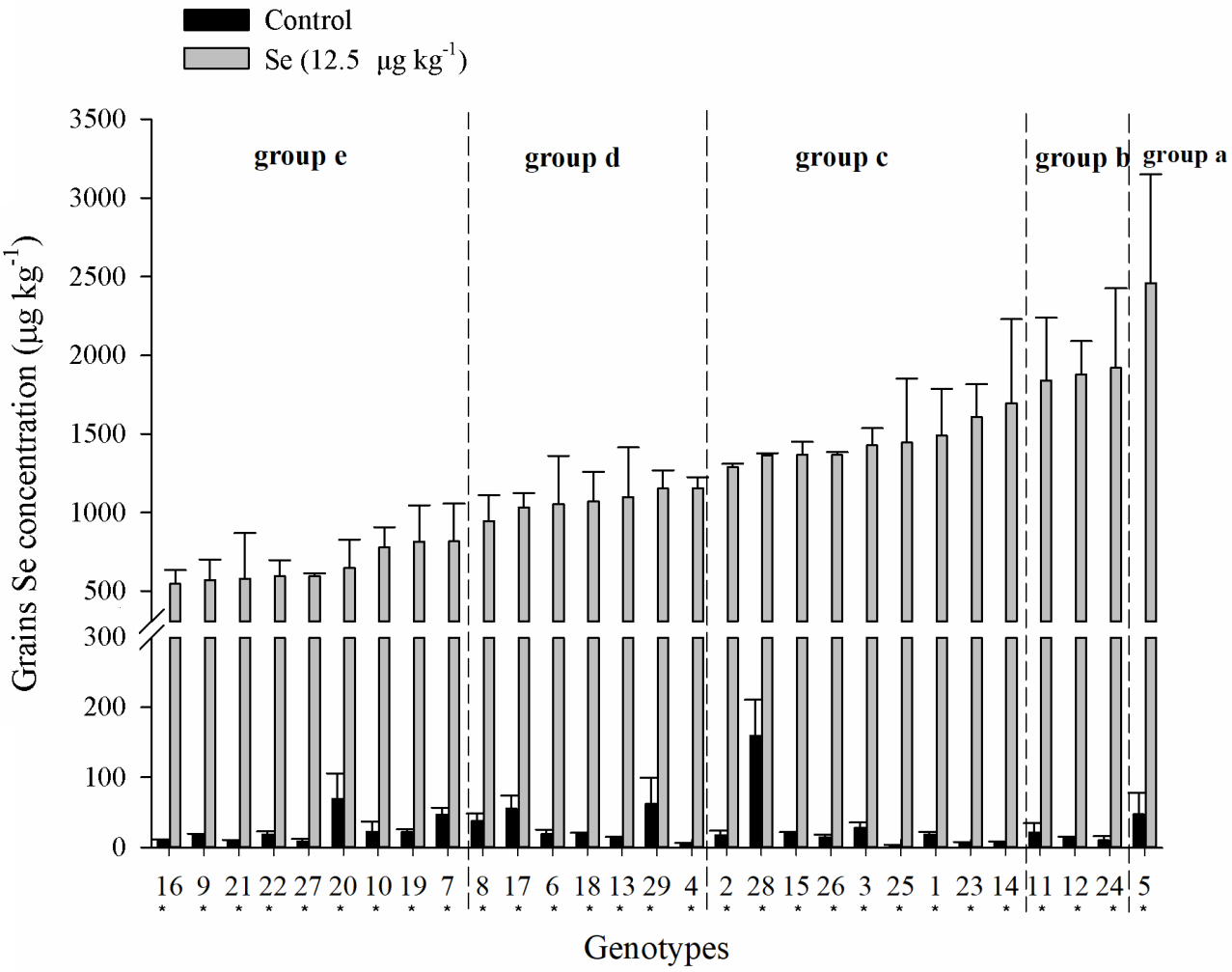

Fig. 2. Grain Se concentration in cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 53.93. '*' Below numbers indicates difference between means of the same genotype under absence or presence of Se application according to the Scott-Knott test, different letter groups indicate difference among means of distinct genotypes under Se application ($p \leq 0.05$).

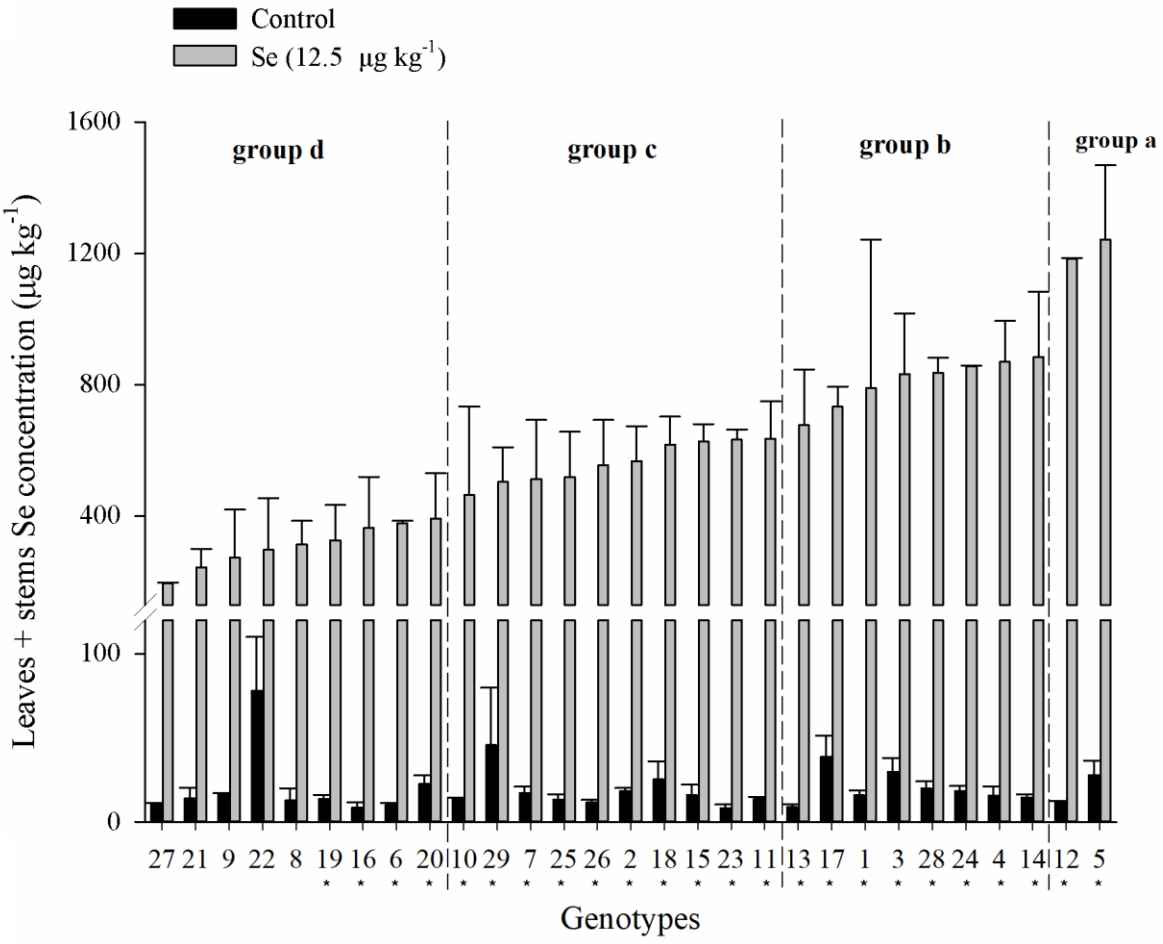

Fig. 3. Leaves + stems Se concentration in cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 60.84. '*' Below numbers indicates difference between means of the same genotype under absence or presence of Se application according to Scott-Knott test, different groups indicate difference among means of distinct genotypes under Se application ($p \leq 0.05$).

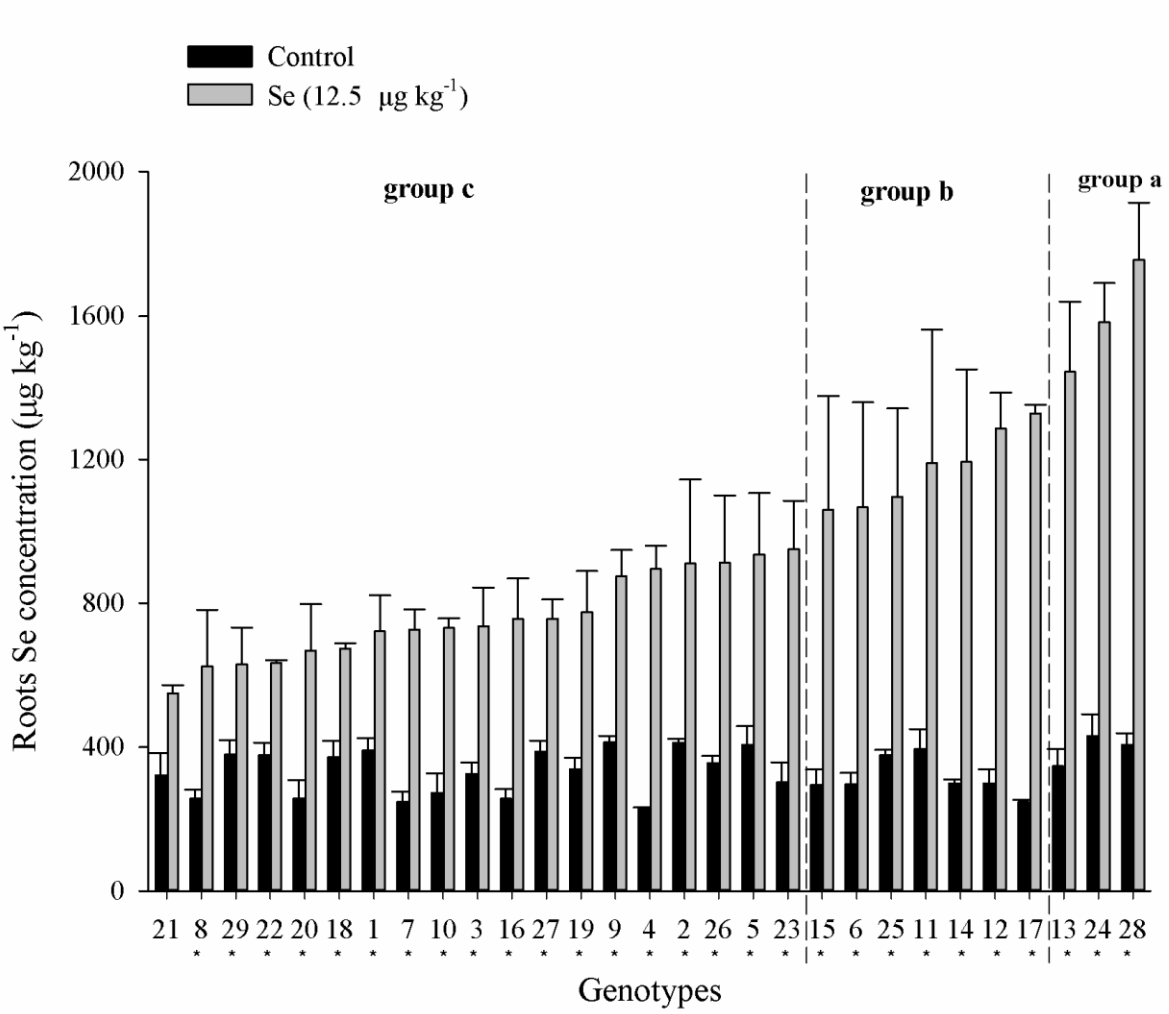

Fig. 4. Root Se concentration in cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 32.37. '*' Below numbers indicates difference between means of the same genotype under absence or presence of Se application according to the Scott-Knott test, different groups indicate difference among means of distinct genotypes under Se application ($p \leq 0.05$).

An increase in Se concentration in leaves + stems in response to Se application was observed in most genotypes, except genotypes 8, 9, 21, 22 and 27 (Fig. 3). Genotype 19 showed the smallest increase in the Se concentration of leaves + stems (313 µg kg^{-1}) and genotype 5 the largest increase in the Se concentration of leaves + stems (1218 µg kg^{-1}) following Se application (Fig. 3; Table A.3). There was a significant interaction between Se application and genotype on the Se concentration in leaves + stems ($P<0.01$; Table A.1).

Selenium application did not affect root Se concentration in genotypes 1, 18, 21, 22 and 29, but increased the root Se concentration of the other 25 genotypes (Fig. 4; $p \leq 0.05$). The largest root Se concentration after Se application was 1755 µg kg^{-1} dry weight in genotype 28 (Fig. 4; Table A.3).

Regarding Se concentrations in grains, leaves + stems and roots from Se-biofortified genotypes, five, four and three distinct groups were observed according to Scott-Knott test, respectively (Fig. 2, 3, 4, respectively, Table A.3). For grain Se concentration, genotype 5 had the largest mean Se concentration of 2462 µg kg^{-1} dry weight when Se was applied (Fig. 2; Table A.3). For leaves + stems, two genotypes, 5 and 12, had the largest mean Se concentrations, which were equal to or greater than 1183 µg kg^{-1} dry weight when Se was applied (Fig. 3; Table A.3). For roots, genotypes 13, 24 and 28, had the largest mean Se concentrations, which were equal or greater than 1444 µg kg^{-1} when Se was applied (Fig. 4; Tables A.3).

Selenium partitioning to the shoot was evaluated by comparing Se accumulation in leaves + stems (shoot) plus grains with Se accumulation in the whole plant. The partitioning of Se to the shoot was affected by genotype and by Se application ($P<0.0001$; Table A.1). There was also an interaction between Se application and genotype on the partitioning of Se to the shoot ($P<0.0001$; Table A.1). The application of Se influenced Se partitioning to the shoot in all genotypes except genotypes 9 and 22, according to the Scott-Knott test (Table A.4; $p \leq 0.05$). Genotypes were divided into three groups according to the Scott-Knott test, for their partitioning of Se to the shoot (Fig. 5). For group a, Se partitioning to the shoot was between 64% (genotype 15) and 91% (genotype 5) following Se application; for group b, Se partitioning to the shoot was between 47% (genotype 20) and 61% (genotype 18) following Se application; and for group 3, Se partitioning to the shoot ranged from 28% (genotype 9) to 37% (genotype 19) following Se application (Fig. 5, Table A.4). Considering all genotypes, the mean Se partitioning to shoot was 61.5% following Se application, but only 14.2% in the absence of Se application (Fig. 5, Table A.4).

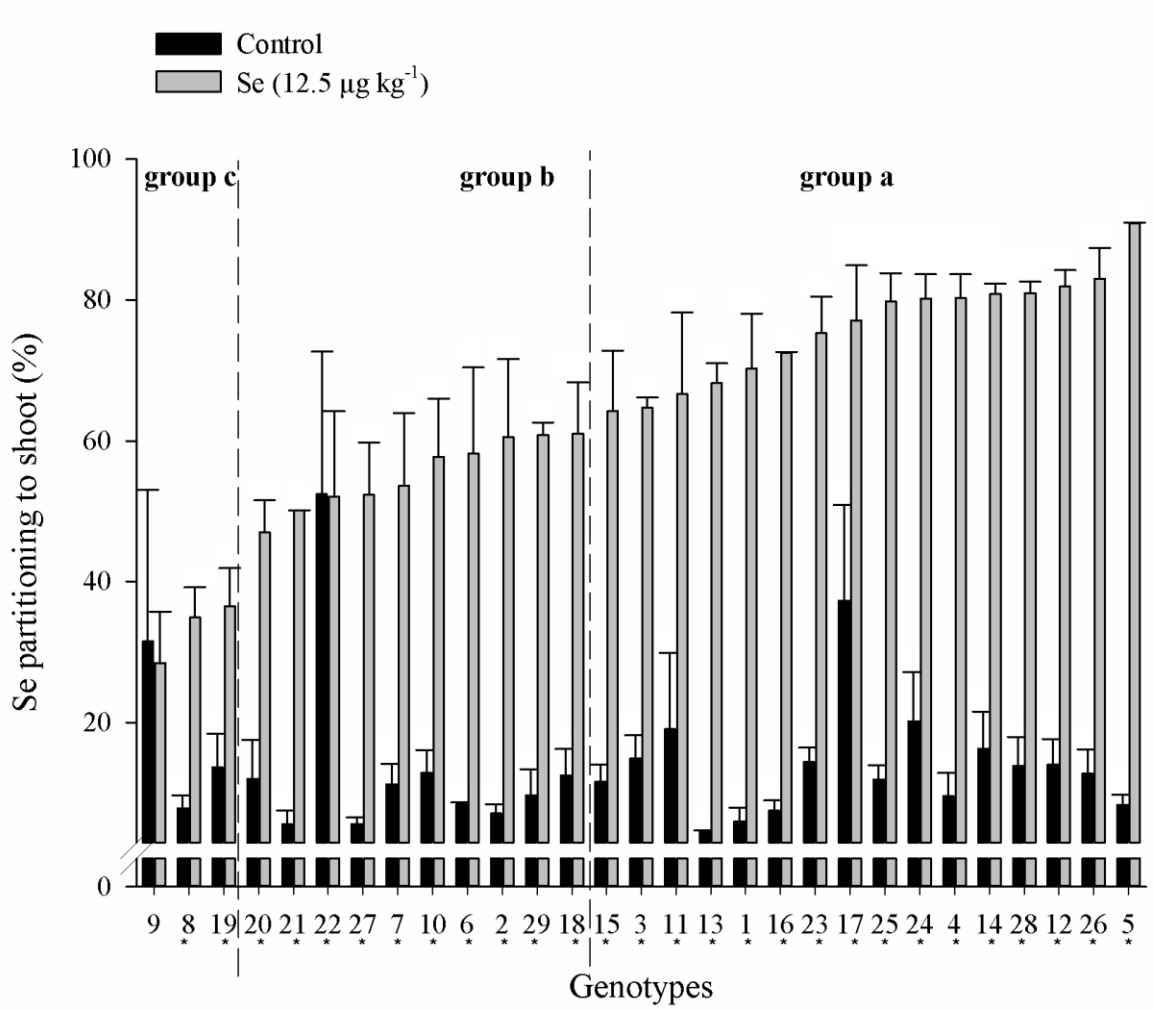

Fig. 5. Selenium partitioning to shoot in cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 30.06. '*' Below numbers indicates difference between means of the same genotype under absence or presence of Se application according to the Scott-Knott test, different groups indicate difference among means of distinct genotypes under Se application ($p \leq 0.05$).

Selenium partitioning to grains was also evaluated. The mean Se partitioning to grains was 20.0% when Se was applied to plants, but only 2.3% when no Se was applied (Table A.5). Genotypes were divided into two groups for their partitioning of Se to grain, regardless of whether Se was applied or not, according to the Scott-Knott test (Fig. 6, Table A.3). Group a was represented by genotypes in which Se partitioning was

between 11.8%, (genotype 15) and 17.8% (genotype 26), and group b was represented by genotypes in which Se partitioning was between 5.3% (genotype 27) and 11.5% (genotype 3; Fig. 6, Table A.5). Although effects of Se application and genotype were observed for Se partitioning to grains, there was no interaction between these factors (Table A.1; Table A.5).

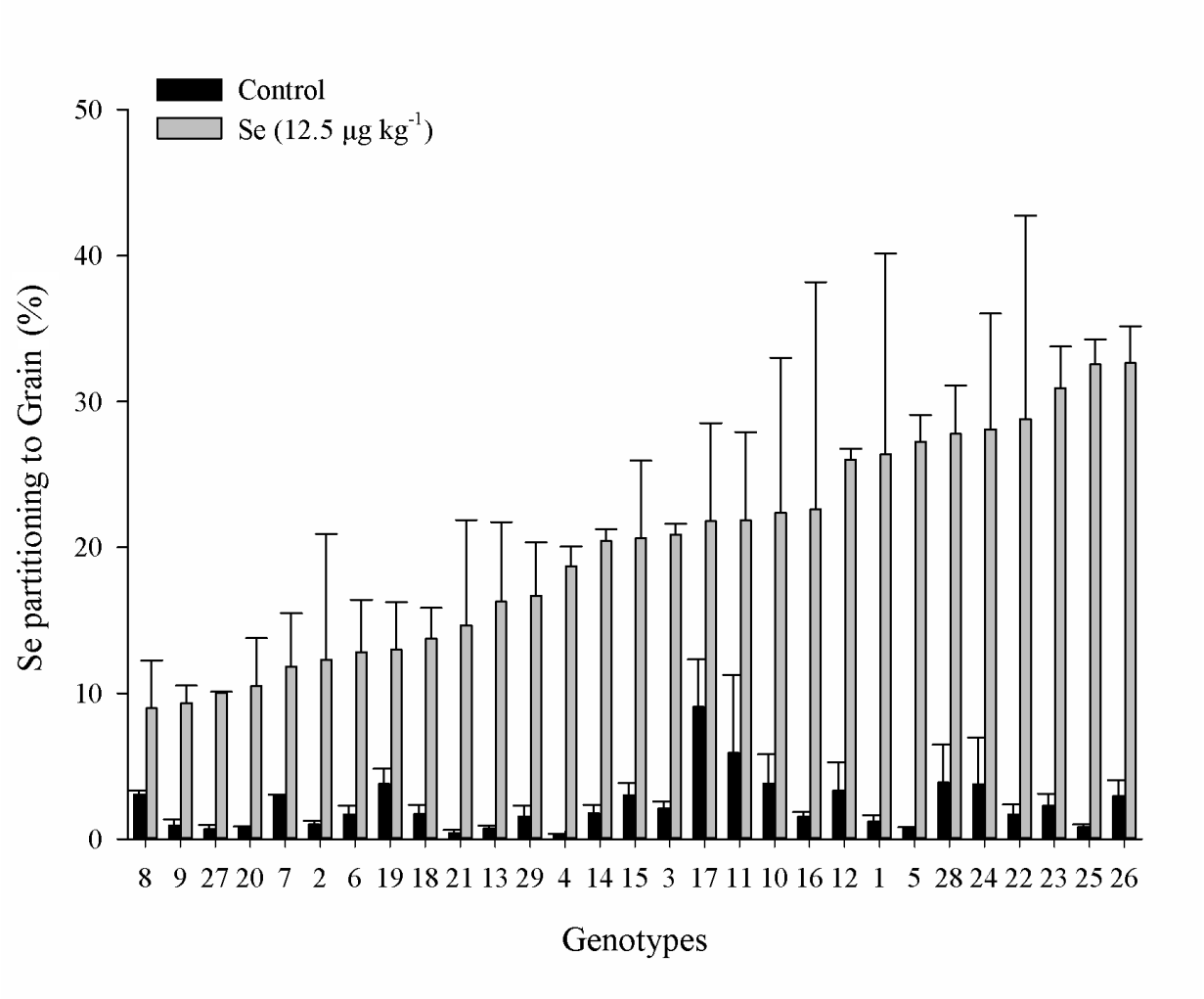

Fig. 6. Selenium partitioning to grains in cowpea genotypes with and without application of Se. Error bars indicates the standard error of mean (n=3). CV (%) = 72.10.

4 DISCUSSION

Selenium application had little effect on plant growth in this study. Selenium application had no effect on leaf + stem and grain dry weights in any of the genotypes studied (Fig. 1b-c). However, the root dry weight of genotype 4 (BRS Cauamé) was reduced by Se application (Fig. 1a), suggesting a potential impairment in root development following Se application in some genetic backgrounds. Genotypes 8 (BRS Marataoa) and 19 (MNC01-631F-20-5) exhibited larger root dry weights following Se application (Fig. 1a).

The range of tissue Se concentrations is relatively narrow between those causing beneficial and toxic effects in plants, varying from 100 µg kg^{-1} for beneficial effects, to 1500 µg kg^{-1} to cause toxic effects (Pilon-Smits et al., 2009; Silva et al., 2018; White, 2016). Increased growth or yield of plants following Se application is occasionally observed (Pilon-Smits et al., 2009; White, 2016) and, for example, the seedling biomass of nine out of 26 genotypes of lentil (Thavarajah et al., 2017) and the yield of wheat (Lara et al., 2019) have been increased by the application of Se. By contrast, application of excess Se impairs plant growth under field, glasshouse and laboratory conditions (Silva et al., 2018; White, 2016; Pezzarossa et al., 2009). Thus, the observation that a substantial increase in Se concentrations in cowpea tissues can be achieved without a reduction in cowpea yield at the Se application rates used in this study is valuable agronomic information.

The Se concentrations in control plants was considered the typical Se concentration range for the cowpea genotypes used in this study. In grain from control plants, the typical Se concentration varied from 4.31 µg kg^{-1} in genotype 25 (Patativa) to 159.91 µg kg^{-1} in genotype 28 (Pingo de Ouro 2; Fig. 2). The reported Se concentrations in seeds and grain of crops grown without Se fertiliser applications varies widely (White, 2016). For example, Thavarajah et al (2017), studying Se concentration in grains of 191 lentil accessions grown without the application of Se fertiliser, observed that grain Se concentrations of most of the accessions was within the range of 250 µg kg^{-1} to 750 µg kg^{-1} of Se, although some accessions reached concentrations of more than 2000 µg kg^{-1} . However, no Se was detected in mature seeds of common bean genotypes when no Se fertiliser was applied (Figueiredo et al., 2017). The relatively low

Se concentrations observed here in grain of cowpea observed in the present study (Fig. 4) might be due to the small amounts of Se acquired and the small percentage of the Se acquired being partitioned to grain (Fig. 6). In the control plants, Se partitioning to grain was only about 2% (Fig. 6, Table A.5) and most of the Se in plants grown without Se fertiliser application tended to be accumulated in shoots (leaves + steam) (Fig. 5, Table A.4). The typical Se concentration in leaves + stems varied from 8.42 µg kg^{-1} in genotype 23 (MNC04-792F-146) to 78.16 µg kg^{-1} in genotype 22 (MNC04-792F-143; Fig. 3). The typical Se concentration in roots varied from 230 µg kg^{-1} in genotype 4 (Cauamé) to 430 µg kg^{-1} in genotype 24 (MNC04-795F-158; Fig. 4). Similar results, indicating higher Se concentrations in roots than in above ground tissues in plants that did not receive Se fertiliser were observed in a study comparing two genotypes of *Prunus* rootstock, in which Se concentrations in roots varied from 260 µg kg^{-1} to 330 µg kg^{-1} dry weight, whilst in twigs and leaves, Se concentration varied from 10 µg kg^{-1} to 130 µg kg^{-1} dry weight and from 100 µg kg^{-1} to 120 µg kg^{-1} dry weight, respectively (Pezzarossa et al., 2009).

The Se concentrations in tissues of cowpea plants after the application of Se fertiliser varied widely between genotypes (Fig. 2, 3 and 4). The application of Se fertiliser increased Se concentration in grains, leaves + stems and roots of all the genotypes evaluated (Fig. 2, 3 and 4). Grain Se concentration in plants treated with Se ranged from 549 µg kg^{-1} dry weight (genotype 16; BRS Xique-Xique) to 2462 µg kg^{-1} dry weight (genotype 5; BRS Guariba). In all the genotypes evaluated, control plants had larger Se concentrations in roots than in grains, whereas, when Se fertiliser was applied, genotypes in groups a, b and c had larger Se concentrations in grain (Fig. 4) than in roots (Fig. 2), the only exception being genotype 28 (Pingo de ouro 2), which had the largest Se concentration in roots (Fig. 4). Selenium partitioning to grains also increased substantially, from 2.3% in control plants to 20% in plants receiving Se fertiliser, on average (Table A.5). This is indicative of a high Se translocation efficiency from roots to grains when Se availability is high, particularly in the genotypes from groups a, b and c for grain Se concentration (Fig. 2). Root Se concentrations in plants receiving Se fertiliser ranged from 550 µg kg^{-1}dry weight (genotype 21; MNC04-782F-108) to 1755 µg kg^{-1} dry weight (genotype 28; Ping de ouro 2).

The greater increase of Se concentration in grain than in roots of cowpea genotypes when Se fertiliser is applied might be related to factors that facilitate sodium selenate translocation from roots to grains. Sodium selenate is a readily available source of Se for plants because it does not form insoluble salts in the soil solution and is converted to organic Se compounds slowly (Natasha et al. 2018). Selenate is transferred from root to shoot tissue via the xylem and then to reproductive organs via the phloem (White, 2016; 2018; Winkel et al., 2015). It has been observed, in tomato, that selenate concentrations in the xylem are 7 to 13 times larger than in external media, indicating the ability of plants to transport selenate readily across roots to the xylem (Asher et al., 1977).

In the present study, the total Se in the natural soil (45 μg kg^{-1}) was larger than the Se fertiliser added as a treatment (12.5 μg kg^{-1}), although it is still a very small soil Se concentration, since soils with less than 1000 μg kg^{-1} are considered Se deficient (Fordyce, 2007). Indeed, in soil with Se concentration of 183 μg kg^{-1} the application of Se increased the availability of the element to maize plants (Feudis et al., 2019). Tissue Se concentrations in control plants were relatively small and the application of Se as sodium selenate caused a substantial increase in Se concentration in cowpea tissues (Fig. 2, 3, 4). This suggests that much of the Se in the natural soil was unlikely to be available to plants, while the applied Se was likely to be highly available for uptake and subsequent translocation to grains. The applied selenate is more available to plants, less adsorbed in the soil, and has been shown to influence Se accumulation in cowpea more readily than selenite, another Se source (Silva et al., 2019)

The concentration of Se in leaves + stems of cowpea when Se fertiliser was applied varied from 195 μg kg^{-1} dry weight in genotype 27 (Pingo de Ouro 1-2) to 1242 μg kg^{-1} dry weight in genotype 5 (BRS Guariba). When Se fertiliser was applied, the concentration of Se in grain was greater than in leaves + stems in all genotypes. Larger concentrations of Se in grains than in vegetative tissues was previously observed in cowpea by Silva et al (2019). By contrast, it was observed that Se concentrations in wheat tended to be larger in flag leaves than in grains (Filek et al., 2019; Souza et al., 2014) and shoot Se concentrations were also larger than grain Se concentration in rice (Zhang et al., 2019a)

Total grain Se concentration has also been reported to be larger in dicots than in monocots (White, 2018). In the present study, Se concentrations in grain of cowpea genotypes ranged from 549 µg kg^{-1} to 2462 µg kg^{-1} dry weight when Se fertiliser was applied at 12.5 µg Se kg^{-1} soil (Fig. 4). In three common bean genotypes grain Se concentration varied from 2000 µg $kg^{-1}$to 4000 µg $kg^{-1}$when 5 µM Se was applied in 10 L pots in nutrient solution [34], while in two rice cultivars, grain Se concentration was less than 1 mg kg^{-1} when 500 µg Se kg^{-1} soil was applied (Zhang et al., 2019a) and in a study comparing 20 wheat lines, Se concentration in grains was less than 500 µg kg^{-1} when 10 mM Se was applied in 10L pots nutrient solution (Souza et al., 2014)

Data for the partitioning of Se to shoots and grains provide valuable information on the ability of genotypes to accumulate Se in edible tissues. As observed in the decision matrix (Table A.6), genotype 5 (BRS Guariba) had the largest Se concentration in leaves + stems (Fig. 3) and grain (Fig. 4), and the highest partitioning to shoots (Fig. 5). The high partitioning of Se to shoots (Fig. 5), and the small Se concentration in roots (Fig. 2), of BRS Guariba indicate that this genotype has a high capacity to translocate Se from roots to leaves + stems and to grains, leading to a higher Se biofortification efficiency than other genotypes. Nevertheless, other genotypes had greater Se partitioning to grain than BRS Guariba (Fig. 6). For instance, genotype 24 (MNC04 795F 158), which had a larger Se partitioning to grain than BRS Guariba, had slightly smaller shoot and grain Se concentrations than BRS Guariba but a larger grain dry weight (Fig. 1c). Genotype 24 (MNC04 795F 158) had one of the largest concentrations of Se in roots among the genotypes studied (Fig. 2) and also a large Se concentration in leaves + stems and grain (Fig. 3 and 4). Genotypes such as 9 (BRS Milênio), 16 (BRS Xique xique), 21 (MNCO4 782F 108), 22 (MNCO4 792F 143) and 27 (Pingo de ouro 1 2) had among the smallest Se concentrations in roots, leaves + stems and grains under Se application (Fig. 2, 3 and 4), which indicates that the acquisition of Se by roots is relatively inefficient compared to other genotypes, although Se transport to shoots and grain is not necessarily the smallest in these genotypes (Fig. 5 and 6).

5. CONCLUSION

The screening of cowpea genotypes is important for selecting and breeding

genotypes with a high potential for Se biofortification of grain, either though agronomic or genetic approaches. Comparative studies of Se acquisition, partitioning and accumulation in edible portions among cowpea genotypes, or even genotypes of pulses in general, are scarce. This study provides information about how each of the 29 genotypes selected accumulate Se in its grains, as well as how of the Se partitioning occurs in the whole plant. The information obtained is an important resource for selecting better genotypes for breeding programs aiming to increase the concentrations of Se in human and animal diets.

6. REFERENCES

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7. APPENDICES MATERIAL – TABLES

Table A.1. F ratio and P value of analysis of covariance (ANCOVA). Sources of variation: Genotypes, Se application and the variation between the factors.

Root dry weight		
Source	F ratio	Pr (<F)
Genotypes (A)	4.01	0.0001**
Se application (B)	0.53	0.47^{NS}
A X B	1.82	0.01*
Leaves + stem dry weight		
Source	F ratio	Pr (<F)
Genotypes (A)	2.98	0.0001**
Se application (B)	1.01	0.30^{NS}
A X B	0.79	0.76^{NS}
Grains		
Source	F ratio	Pr (<F)
Genotypes (A)	2.06	0.004**
Se application (B)	0.00	0.95^{NS}
A X B	1.21	0.33^{NS}
Root Se concentration		
Source	F ratio	Pr (<F)
Genotypes (A)	3.72	0.0001**
Se application (B)	379.90	0.0001**
A X B	3.13	0.0001**
Stem + Leaves Se concentratio		
Source	F ratio	Pr (<F)
Genotypes (A)	3.01	0.0001**
Se application (B)	411.52	0.0001**
A X B	3.03	0.0001**
Grains Se concentration		
Source	F ratio	Pr (<F)
Genotypes (A)	3.23	0.0001**
Se application (B)	544.69	0.0001**
A X B	3.22	0.0001**
Se partitioning to Shoot		
Source	F ratio	Pr (<F)
Genotypes (A)	3.43	0.0001**
Se application (B)	785.63	0.0001**
A X B	4.2	0.0001**
Se partitioning to Grains		
Source	F ratio	Pr (<F)
Genotypes (A)	1.47	0.08^{NS}
Se application (B)	209.96	0.0001**
A X B	1.23	0.21^{NS}

Table A.2. Mean comparison of roots dry weight of cowpea genotypes with and without application of Se, and leaves + stems (L+S) and grains dry weight of cowpea genotypes.

Different letters indicate difference between means according to Scott Knott test (p≤0.05). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

Treatment	Root DW (g)		L+S DW (g)	Grain DW (g)
	Control	Se		
Genotype 1	13.52 a	6.72 c	12.09 b	2.3 b
Genotype 2	14.3 a	9.2 c	17.79 b	2.88 b
Genotype 3	10.23 b	11.2 c	13.69 b	3.14 b
Genotype 4	17.17 aA	7.83 cB	24.15 a	4.95 a
Genotype 5	12.97 a	4.6 c	15.89 b	3.17 b
Genotype 6	15.63 a	14.02 c	20.46 a	4.02 a
Genotype 7	17.63 a	9.73 c	15.12 b	2.84 b
Genotype 8	16 aB	31.22 aA	21.04 a	3.28 b
Genotype 9	7.17 b	15.94 b	14.83 b	2.27 b
Genotype 10	9.33 b	8.42 c	16 b	4.66 a
Genotype 11	6.17 b	7.13 c	16.54 b	2.69 b
Genotype 12	6.1 b	5.13 c	18.39 a	4.38 a
Genotype 13	11.58 a	4.71 c	17.88 b	2.94 b
Genotype 14	6.08 b	3.57 c	16.22 b	3.11 b
Genotype 15	10 b	12.53 c	16.92 b	3.82 a
Genotype 16	8.55 b	4.16 c	15.45 b	2.89 b
Genotype 17	6.14 b	6.33 c	18.86 a	4.67 a
Genotype 18	10.41 b	11.07 c	16.09 b	2.9 b
Genotype 19	7.7 bB	23.65 bA	19.63 a	4.81 a
Genotype 20	13.17 a	14.62 c	15.84 b	2.37 b
Genotype 21	15.43 a	21.32 b	19.3 a	3.42 b
Genotype 22	6.83 b	6.54 c	15.7 b	3.36 b
Genotype 23	4.57 b	8.2 c	20.66 a	5.49 a
Genotype 24	7.83 b	3.33 c	20.46 a	4.32 a
Genotype 25	6.13 b	4.4 c	21.08 a	5.2 a
Genotype 26	6.07 b	3.57 c	18.4 a	4.43 a
Genotype 27	10.59 b	8.01 c	15.15 b	3.28 b
Genotype 28	6.52 b	3.53 c	17.41 b	3 b
Genotype 29	15.88 a	11.32 c	16.44 b	2.98 b

Table A.3. Mean comparison of Se concentration in cowpea genotypes roots, leaves+stems (L+S) and grains with and without application of Se. Different letters indicate difference between means according to Scott-Knott test (p≤0.05). Uppercase

letters correspond to absence or presence of Se application, and lowercase letters correspond to genotypes.

Treatment	Roots (µg kg^{-1})		L+S (µg kg^{-1})		Grains (µg kg^{-1})	
	Control	Se	Control	Se	Control	Se
Genotype 1	390.86 c	722.02 c	16.02 aB	790.95 bA	18.99 aB	1494.33 cA
Genotype 2	411.56 bB	910.9 cA	18.45 aB	568.23 cA	18.03 aB	1293.1 cA
Genotype 3	325.22 dB	736.16 cA	29.93 aB	833.53 bA	28.35 aB	1430.99 cA
Genotype 4	230.02 eB	896.14 cA	15.67 aB	870.17 bA	4.31 aB	1160.43 dA
Genotype 5	406.36 bB	934.99 cA	27.95 aB	1242.98 aA	47.71 aB	2462.09 aA
Genotype 6	297.29 eB	1066.91 bA	10.33 aB	379.01 dA	19.21 aB	1058.9 dA
Genotype 7	247.54 eB	726.64 cA	17.53 aB	514.03 cA	47.29 aB	820.09 eA
Genotype 8	256.84 eB	624.59 cA	12.83 a	313.47 d	38.24 aB	949.81 dA
Genotype 9	413.82 bB	874.64 cA	17.16 a	273.55 d	17.98 aB	573.68 eA
Genotype 10	272.11 eB	731.33 cA	13.69 aB	465 cA	22.97 aB	781.73 eA
Genotype 11	394.88 cB	1190.31 bA	13.94 aB	636.5 cA	21.88 aB	1844.02 bA
Genotype 12	298.7 dB	1285.98 bA	11.68 aB	1183.51 aA	13.88 aB	1881.77 bA
Genotype 13	347.07 dB	1444.42 aA	9 aB	677.63 bA	13.36 aB	1103.5 dA
Genotype 14	299.05 dB	1193.79 bA	14.69 aB	885.14 bA	8.59 aB	1699.38 cA
Genotype 15	295.53 eB	1059.64 bA	16.17 aB	628.18 cA	20.96 aB	1371.28 cA
Genotype 16	256.64 eB	756.63 cA	8.66 aB	363.63 dA	10.49 aB	549.72 eA
Genotype 17	246.89 eB	1327.85 bA	38.8 aB	733.42 bA	55.73 aB	1037.78 dA
Genotype 18	372.21 c	674.02 c	25.41 aB	618.55 cA	19.13 aB	1073.24 dA
Genotype 19	338.04 dB	774.97 cA	13.85 aB	326.4 dA	22.12 aB	818.5 eA
Genotype 20	256.62 eB	668.38 cA	22.84 aB	392.57 dA	69.46 aB	652.16 eA
Genotype 21	322.23 d	550.08 c	14.24 a	243.91 d	8.6 aB	582.12 eA
Genotype 22	377.46 c	633.92 c	78.16 a	297.08 d	19.03 aB	597.59 eA
Genotype 23	301.91 dB	951.69 cA	8.42 aB	633.18 cA	6.15 aB	1613.25 cA
Genotype 24	430.65 aB	1582.07 aA	18.67 aB	857.11 bA	10.43 aB	1924.42 bA
Genotype 25	378.34 cB	1096.58 bA	13.4 aB	518.77 cA	3.59 aB	1448.57 cA
Genotype 26	355.62 cB	913.32 cA	11.8 aB	555.07 cA	15.03 aB	1371.66 cA
Genotype 27	387.07 cB	757.42 cA	11.24 a	195.29 d	8.85 aB	598.96 eA
Genotype 28	405.32 cB	1755.7 aA	20.03 aB	837.32 bA	159.91 aB	1368.87 cA
Genotype 29	378.91 c	630.35 c	45.85 aB	504.77 cA	62.92 aB	1157.27 dA

Table A.4. Mean comparison of Se partitioning to shoot of cowpea genotypes with and without application of Se. Different letters indicate difference between means according to Scott Knott test (p≤0.05). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

Treatment	Se partitioning to shoot (%)	
	Control	Se
Genotype 1	05.99 bB	70.28 aA
Genotype 2	07.09 bB	60.52 bA
Genotype 3	14.91 bB	64.75 aA
Genotype 4	09.54 bB	80.31 aA
Genotype 5	09.12 bB	90.92 aA
Genotype 6	08.37 bB	58.22 bA
Genotype 7	11.25 bB	53.66 bA
Genotype 8	07.86 bB	34.91 cA
Genotype 9	32.33 a	28.46 c
Genotype 10	12.85 bB	57.77 bA
Genotype 11	19.07 bB	66.69 aA
Genotype 12	14.02 bB	81.93 aA
Genotype 13	04.54 bB	68.27 aA
Genotype 14	16.32 bB	80.90 aA
Genotype 15	11.59bB	64.27 aA
Genotype 16	07.48 aB	72.50 aA
Genotype 17	37.29 bB	77.10 aA
Genotype 18	12.51 bB	61.00 bA
Genotype 19	13.65 bB	36.54 cA
Genotype 20	12.72 bB	47.07 bA
Genotype 21	05.54 bB	50.16 bA
Genotype 22	52.43 b	52.09 b
Genotype 23	14.39 bB	75.28 aA
Genotype 24	20.20 bB	80.23 aA
Genotype 25	11.90 bB	79.80 aA
Genotype 26	12.73 bB	83.04 aA
Genotype 27	05.62 bB	52.36 bA
Genotype 28	15.05 bB	81.00 aA
Genotype 29	10.46 bB	60.84 bA

Treatment	Se translocation to grains (%)
Se Application	64.51 A

Control	14.22 B

Table A.5. Mean comparison of Se partitioning to grains of cowpea genotypes. Different letters indicate difference between means according to Scott Knott test (p≤0.05). Uppercase letters correspond to absence or presence of Se application, lowercase letters correspond to genotypes.

Treatment	Se partitioning to grains (%)
Genotype 1	13.80 a
Genotype 2	06.64 b
Genotype 3	11.49 a
Genotype 4	09.50 b
Genotype 5	13.97 a
Genotype 6	07.25 b
Genotype 7	07.43 b
Genotype 8	06.01 b
Genotype 9	05.13 b
Genotype 10	13.08 a
Genotype 11	13.87 a
Genotype 12	14.68 a
Genotype 13	08.50 b
Genotype 14	11.10 a
Genotype 15	11.83 a
Genotype 16	12.08 a
Genotype 17	15.43 a
Genotype 18	07.73 b
Genotype 19	08.40 b
Genotype 20	05.68 b
Genotype 21	07.54 b
Genotype 22	15.25 a
Genotype 23	16.61 a

Genotype 24	15.93 a
Genotype 25	16.55 a
Genotype 26	17.79 a
Genotype 27	05.35 b
Genotype 28	15.84 a
Genotype 29	09.11 b

Treatment	**Se translocation to grains (%)**
Se application	19.99 A
Control	2.32 B

Table A.6. Decision Matrix for variables presenting Se and Genotype interaction. The "*" indicates genotypes that presented the highest result observed, for each specific variable.

Variable	**Genotypes**	
	5 (BRS Gruariba)	**28 (Pingo-De-Ouro-2)**
Grain Se Concentration	*	
Leaves + Stem Se Concentration	*	
Roots Se Concentration		*
Se Partitioning to Shoots	*	

8. APPENDICES MATERIAL – FIGURES

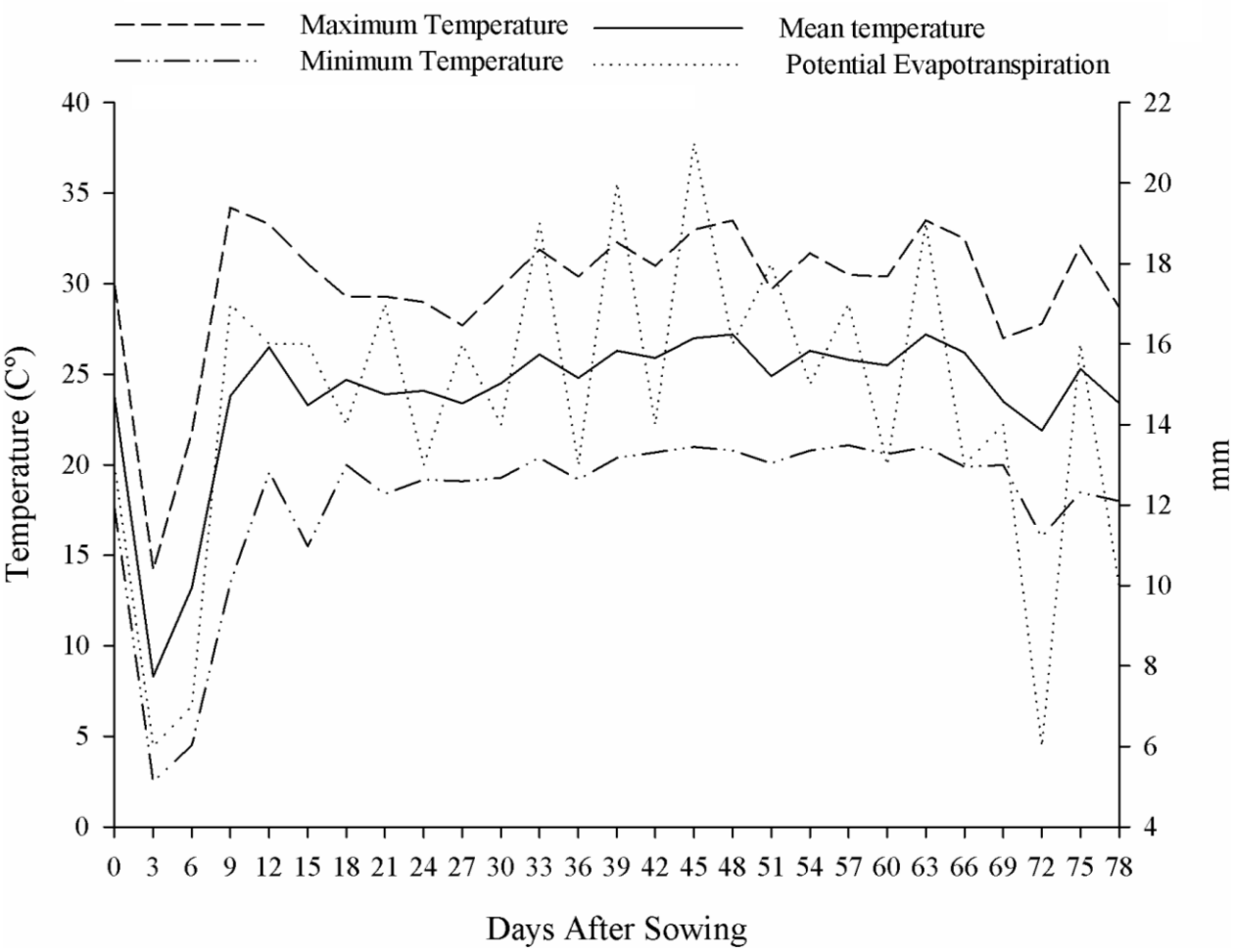

Figure A.1. Maximum, mean and minimum temperature, and potential evapotranspiration during cowpea greenhouse experiment.

CHAPTER 4 – DOES SELENIUM APPLICATION INCREASE THE YIELD OF COWPEA PLANTS? EVIDENCE FROM 29 GENOTYPES ON UREIDES AND SUGAR INDEX AFFECTING THE YIELD

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ABSTRACT

Purpose: Cowpea (*Vigna unguiculata* L. Walp) is a low-cost source of protein in developing countries. Selenium (Se) is a micronutrient for humans and animals but is considered a beneficial element for plants. This study aimed to evaluate the effect of Se on the yield, sugar, and ureides concentration of cowpea.

Methods: The experiment was comprised of 29 cowpea genotypes cultivated in a greenhouse fertilized with Se (control and 12.5 µg Se dm^{-3}). The plants were cultivated until harvest to the following evaluations: grains yield, total sugar accumulation in grains, and total ureides concentration in shoots. The results were used to estimate Se influence in each variable by index production selenium (IPS), index sugar selenium (ISS), and index ureide selenium (IUS). The genotypes were divided into classes according to the respective indexes.

Results: Selenium application provided an increase or decrease in yield, depending on genotypes. In genotypes with high IPS, Se application increases sugar in grains leading to greater yield, but no correlation between yield and ureides in shoots was observed.

Conclusions: The class in which genotypes were allocated is important since some low-yield genotypes presented a significant increase in yield under Se application. On the other hand, Se provided a low-yield increase in some high-yield genotypes. This study shows valuable information for future cowpea breeding programs, to enhance the nutritional quality of grains and alleviate human malnutrition.

Keywords: *Vigna unguiculata* L. Walp, sodium selenite, carbohydrates, N-compounds, allantoin, allantoic acid

1. INTRODUCTION

Among the legume plants, one that stands out is cowpea (*Vigna unguiculata* L. Walp), a crop of African origins (Nwokolo and Ilechukwu 1996). It is a very rustic plant, tolerant to semi-arid conditions (Gonçalves *et al.* 2016), adapted to high temperature and low fertility soils (Carvalho *et al.* 2019). Cowpea is more tolerant than common beans to infertile soils, drought, waterlogging, and acid stress (Islam *et al.* 2012). In addition, cowpea is rich in proteins and carbohydrates (Alidu *et al.* 2020). Thus, cowpea is a crop used to complement cereals, nuts, and tubers in developing countries of Africa, South America, and Asia (Manzeke *et al.* 2019; Silva *et al.* 2019) with the potential to reduce poverty and improve households' income (Manda *et al.* 2019).

As an essential micronutrient to humans and other animals, Selenium (Se) plays a role in thyroid functioning and immune systems (Ekuma *et al.* 2021), protects the human body against reactive oxygen species (ROS) (Spallholz, 2019), and contributes to DNA formation (Taylor and Radding, 2020). To plants, it is considered a beneficial element, providing tolerance to biotic and abiotic stress as an antioxidant (White, 2016; Gouveia *et al.* 2020; Silva *et al.* 2020; Lanza and Reis, 2021). Usually, Se effect on plants is related to growth promotion under stressful conditions, by mitigating lipid peroxidation (Gouveia *et al.* 2020; Hussain *et al.* 2020). Several beneficial effects of Se in plants were previously observed. In peanuts, Se enhances nodulation (Cunha *et al.* 2022), and in cowpea, Se was reported to increase yield and photosynthetic pigments (Lanza *et al.* 2021), and total sugars and sucrose (Silva *et al.* 2020)

In cowpea, agronomic biofortification with Se provided grains with safe concentrations of the element without impairing production (Silva *et al.* 2019). Some genotypes of lettuce and lentils, under Se application, presented increased yield in shoot biomass and grains, respectively (Ramos *et al.* 2011; Ekanayake *et al.* 2017). In peanuts, Se application can increase soluble sugars (Hebat-Allah *et al.* 2019). According to some reports, Se might increase sugar concentration in sink tissues, by enhancing the activity of enzymes related to sugar metabolism (Malik *et al.* 2011; Ren *et al.* 2020). In soybean, Se application can enhance sugar concentration in leaves (Cunha *et al.* 2023). Sorghum plants under Se application presented higher levels of Starch and sucrose (Cipriano *et al.* 2022).

Selenium could also be related to nitrogen metabolism in Fabaceae, in an extensive review studying legume plants, it was observed that Se application can enhance N accumulation up to 54% (Lei et *al.* 2022).The element is essential to *Rhizobium* (Ekanayake *et al.* 2017), the group of symbiotic diazotrophic bacteria that benefit Fabaceae plants by biological nitrogen fixation (BNF) in root nodules (Hoffman *et al.* 2014). Selenocysteine is a part of the active site of the enzyme hydrogenase, which is essential to BNF (Baltazar *et al.* 2011). Selenium application can promote nodulation in alfalfa (Hajiboland *et al.* 2015), peas (Poblaciones and Rengel 2018), and peanuts (Cunha *et al.* 2022). In maize, Se application enhances gluthatione and N metabolism (Wang *et al.* 2023). Under Se application, soybean presented higher ureides concentration in leaves as well as enhanced nitrate reductase activity (Cunha *et al.* 2023). However, further investigation on Se influence in N metabolism is required. The ureides (allantoic acid and allantoin) are the final products of BNF (Dunn, 2015), and the main molecules that are transported from nodules to roots and finally plant shoots (Irani and Todd 2016). However, the effect of Se in the accumulation of these N-compounds remains unclear.

Cowpea is a wide variable crop, with plenty of genotypes, lines, landraces, and varieties cultivated (Phillips, 2012). Its grains vary in color, texture, size (Rocha *et al.* 2017) and quality (Oliveira *et al.* 2004; Carvalho *et al.* 2012). In addition, the potential of Se to enhance some characteristics, such as sugar and ureides concentration, could be explored in distinct genotypes of cowpea. Thus, this study aimed to evaluate the effect of Se application on yield, sugar accumulation, and ureides concentration in genotypes of cowpea.

2. MATERIALS AND METHODS

2.1 Experiment description

The experiment was performed in a greenhouse at Tupã unity of São Paulo State University (UNESP), São Paulo State, Brazil. In response to 12.5 µg dm^{-3} of Se applied as sodium selenate, twenty-nine genotypes of cowpea (Table 1) were cultivated in 5 L pots, with two plants per pot.

Table 1. Characteristics of cowpea genotypes used in this study based on grain color, maturation cycle and geographical recommendation in Brazil.

ID	Genotype	Grain color	Maturation Cycle	Geographical recommendation
1	BR 17 - Gurguéia	Brown	Early medium	Wide
2	BR 3-Tracuateua	White	Early	Restrict
3	BRS Aracê	Green	Early médium	Restrict
4	BRS Cauamé	White	Early	Medium
5	BRS Guariba	White	Early	Wide
6	BRS Itaim	White	Early	Restrict
7	BRS Juruá	Green	Early medium	Restrict
8	BRS Marataoã	Brown	Early medium	Wide
9	BRS Milênio	White	Early medium	Restrict
10	BRS Novaera	White	Early	Wide
11	BRS Pajeú	Brown	Early Medium	Medium
12	BRS Potengi	White	Early	Medium
13	BRS Rouxinol	Brown	Early Medium	Restrict
14	BRS Tumucumaque	White	Early	Wide
15	BRS Urubuquara	White	Early Medium	Restrict
16	BRS Xiquexique	White	Early Medium	Wide
17	California CB-5	White	Early	Restrict
18	Inhuma	Brown	Early Medium	Wide
19	MNC01-631F-20-5	Brown	Early Medium	Medium
20	MNC04-769F-62	Brown	Early	Wide
21	MNC04-782F-108	Brown	Early Medium	Wide
22	MNC04-792F-143	Brown	Early	Wide
23	MNC04-792F-146	Brown	Early	Wide
24	MNC04-795F-158	Brown	Early Medium	Wide
25	Patativa	Brown	Early Medium	Restrict
26	Paulistinha	Brown	Early Medium	Restrict
27	Pingo de Ouro-1-2	Brown	Early Medium	Wide
28	Pingo de Ouro-2	Brown	Early Medium	Medium
29	Pretinho	Black	Medium	Wide

The experimental design was completely randomized, with three replications for each genotype, in the absence (control) and presence of Se, totaling 174 pots. In November 2016, the soil was collected from UNESP experimental farm and sieved using 5mm sieve to fill the 5 L pots and to evaluate soil chemical properties as it follows: pH ($CaCl_2$ 0.01 M) 4.6; phosphorus (resin): 6 mg dm^{-3}, sulfur: 3 mg dm^{-3} (calcium phosphate), boron (hot water): 0.07 mg dm^{-3}, copper (DTPA): 0.5 mg dm^{-3}, iron (DTPA): 11 mg dm^{-3}, manganese (DTPA): 12 mg dm^{-3}, zinc (DTPA): 0.2 mg dm^{-3}; potassium

(resin): 0.9 $mmol_c\ dm^{-3}$, calcium (resin): 5 $mmol_c\ dm^{-3}$, magnesium (resin): 3 $mmol_c\ dm^{-3}$, H+Al (SMP buffer): 16 $mmol_c\ dm^{-3}$, cation exchange capacity: 24.9 $mmol_c\ dm^{-3}$ and base saturation: 36%. Selenium concentrations in soil were 45 μg dm^{-3} (Total Se concentration) and 3.2 μg dm^{-3} (exchangeable Se concentration).

Chemical analyses of pH, macronutrients, micronutrients, and H+Al were performed according to Raij et al., (1997). Total and exchangeable Se concentrations was determined according to Silva et al. (2019).

2.2 Experiment conduction

On November 29, 2016, an application of 1.25 g of lime pot^{-1}, 0.46 g single superphosphate pot^{-1}, and 0.17 g KCl was performed to provide proper pH and fertility for cowpea plants. After the 30 days incubation period, the sowing was carried out, on December 29, 2016. Before sowing, seeds were inoculated with a peat inoculum specific for cowpea (SEMIA 6462, 2.0 x 109 colony forming units g^{-1}, São Joaquim da Barra city, Brazil, BIOMAX) at 8 g kg^{-1} of seed. The inoculum was dissolved using a 10% sugar solution, and then, the solution was mixed with the seeds. On January 5, 2017, 7 days after sowing, the emergence started. At 20 days after emergence (DAE), urea was applied at a rate of 0.10 g pot^{-1}. Irrigation was performed five times daily for one minute each time (100 ml min^{-1}) using low-density polyethylene (LDPE) pipes. The irrigation was carried out automatically using a custom computerized system. Further applications of urea (0.22 g pot^{-1}), KCl (0.17 g pot^{-1}), and single superphosphate (0.45 g pot^{-1}) were performed at 36 and 61 DAE. The application of treatment of 12.5 μg dm^{-3} of Se as sodium selenite was performed to the relevant pots at 44 DAE when all genotypes were at the flowering stage.

2.3 Harvest and sampling

Across a series of days, the harvest was performed according to the pod's maturity for each genotype. Plant material was separated into grains and shoots. Total grain weight of each pot was weighted to record the yield. The separated material was dried in an oven for 72 hours at 40 °C to a constant mass to measure the dry weight (DW) of grains and shoots. Then, the material was grounded and homogenized in a Wiley mill.

For analysis of total sugar concentration, 0.5 g of milled grain samples were extracted, and for ureides analysis (allantoin and allantoic acid) 0.3 g of milled shoot samples were extracted. In both cases, in 10 ml of MCW solution (60% methanol, 25% chloroform, and 15% water). The extract was prepared according to Bielesk & Turner (1996).

2.4 Laboratory analyses

Total ureides were estimated by summing the concentration of allantoin and allantoic acid, according to Vogels & Van Der Drift, (1970). To quantify both allantoin and allantoic acid, a standard allantoin curve was used.

The total sugar concentration in grains was determined according to Dubois et al., (1956). A standard sucrose curve was used to quantify total sugar.

To evaluate Se, samples of approx. 0.20 g dried, milled grain materials were weighed. The Se analysis was performed according to the methodology described by Thomas et al. (2016).

2.5 Data analysis

Data homogeneity was evaluated by Leven's test, and data normality was evaluated by Anderson-Darling test. Afterward, to classify the 29 genotypes according to selenium application response to yield, sugar accumulation, and ureides, the following indexes were used:

Index of production selenium (IPS):

$$IPS = \frac{Grains\ dry\ weight\ under\ Se\ application - Grains\ dry\ weight\ in\ control\ treatment}{Grains\ dry\ weight\ in\ control\ treatment}$$

Index of sugar selenium(ISS):

$$ISS = \frac{Total\ sugar\ under\ Se\ application - Total\ sugar\ in\ control\ treatment}{Total\ sugar\ in\ control\ treatment}$$

Index of ureides selenium (IUS):

$$IUS = \frac{Total\ ureides\ under\ Se\ application - Total\ ureides\ in\ control\ treatment}{Total\ ureides\ in\ control\ treatment}$$

The genotypes were classified according to each of the indexes (IPS, ISS and IUS). Aiming to identify the genotypes with higher of each index, as well as to group genotypes related to each index according to the studied variables. An analysis of variance (ANOVA, F test) was performed in the indexes, total sugar and total ureides data (Supplementary table 1).

All genotypes that presented an IPS higher equal or higher than 0.3, which means that Se application led to an increase of at least 30% in yield, the contour maps were performed to compare different results. For total sugar and ureides concentration, the Scott Knot test was performed for mean comparisons ($p \leq 5\%$).

This methodology made it possible to better understand each genotype behavior according to the Se indexes relating the variables among each other. The dataset was also submitted to linear regression analysis ($p \leq 5\%$). Thus, the main mathematical parameter evaluated in this study was the indexes, aiming mainly at the use and efficiency of Se to improve cowpea yield. The group in which each genotype is allocated is very important. Some are low-yield genotypes with high yield increase, and some are high-yield genotypes with low yield increase, under Se application.

3. RESULTS

According to the ANOVA test, significative differences were observed among the genotypes, for IPS, IUS, ISS, Total sugar, and Total ureides concentration ($P < 0.01$; supplementary table 1). Among the 29 genotypes, eight of them (genotypes 5, 9, 12, 13, 20, 21, 27, and 28) presented an IPS equal or higher than 0.3. Meaning that the Se application provided an increase of, at least, 30% in the yield. On the other hand, genotypes 1, 2, 6, 10, and 15 presented an IPS lower than -0.3. In this case, the Se application decreased yield by at least 30% (Table 2). For ISS, genotypes 5, 13, 20, 21, 27 and 28 presented an index higher than 0.3, and genotypes 2, 3, 6, 7, 15, and 29 presented an ISS lower than - 0.3 (Table 2). Genotypes 3 and 11 presented IUS higher than 0.3, and no genotypes presented IUS lower than - 0.3 (Table 2).

Table 2. Means of grain dry weight without Se application, and index of productivity, sugar and ureides for Se application. The highlighted lines represent genotypes with index production selenium > 0.3.

Genotypes	Grain dry weight g plant^{-1}		Index production selenium	Index sugar selenium	Index Ureides selenium
	Control	Se (12.5 µg dm^{-3})			
1	2.7	1.2	-0.54	-0.33	-0.11
2	3.9	1.7	-0.57	-0.42	0.32
3	2.9	2.3	-0.20	-0.49	0.37
4	4.4	5.5	0.25	0.13	0.19
5	2.6	3.8	0.47	0.41	-0.03
6	5.0	3.1	-0.38	-0.54	-0.10
7	3.4	2.3	-0.31	-0.37	0.00
8	3.6	4.0	0.12	-0.20	0.25
9	1.8	2.7	0.46	-0.18	-0.27
10	3.8	2.4	-0.36	-0.35	0.29
11	2.8	2.6	-0.09	-0.32	0.46
12	3.7	5.1	0.36	0.02	0.24
13	2.5	3.4	0.33	0.55	-0.10
14	3.4	2.8	-0.18	-0.17	0.02
15	4.4	2.2	-0.50	-0.46	0.29
16	3.4	2.4	-0.31	-0.29	-0.06
17	4.2	5.2	0.25	-0.13	-0.09
18	3.4	2.4	-0.31	-0.28	0.04
19	4.9	4.7	-0.03	0.03	0.00
20	1.9	2.9	0.53	0.74	-0.16
21	2.0	4.8	1.45	1.23	0.09
22	4.1	2.6	-0.38	-0.47	0.08
23	5.0	6.0	0.20	-0.30	-0.01
24	4.8	3.9	-0.19	-0.15	0.06
25	4.9	5.5	0.14	-0.32	0.12
26	4.1	4.7	0.14	0.23	-0.09
27	2.1	3.2	0.52	0.77	0.27
28	1.3	4.7	2.67	1.89	0.02
29	3.3	2.6	-0.21	-0.42	0.04

Total sugar concentration varied between 13.66 (genotype 28) and 71.95 mg g DW^{-1} (genotype 6) in control treatments, and between 15.17 (genotype 9) and 57.34 mg g DW^{-1} (genotype 21) under Se application (Table 3). Genotypes 5, 13, 20, 21, 26, 27, and 28 presented an increase in total sugar concentration under Se application. Among

genotypes that presented an increase in total sugar concentration under Se application, only genotype 26 did not present IPS higher than 0.3.

Table 3. Mean comparison of total ureides concentration and total sugar concentration in cowpea shoots without application of Se. Different letters indicate difference between means according to Scott-Knott test ($p \leq 0.05$). Uppercase letters correspond to absence or presence of Se application, and lowercase letters correspond to genotypes. The highlighted lines represent genotypes with selenium production index > 0.3.

Genotypes	Total Ureides (μmol g^{-1})		Total Sugar (mg g^{-1})	
	Control	Se (12.5 μg dm^{-3})	Control	Se (12.5 μg dm^{-3})
1	8959 aA	7952 bB	33,03 dA	22 eB
2	4377 eB	5567 dA	36,45 dA	20,99 eB
3	2986 gB	4093 fA	36,84 dA	18,66 eB
4	3263 g	3882 f	44,51 c	50,1 b
5	5959 c	5807 d	31,3 dB	44,17 bA
6	3987 f	3577 f	71,95 aA	32,95 dB
7	5750 c	5741 d	36,2 dA	22,68 eB
8	4412 eB	5786 dA	35,08 d	28,23 d
9	6063 cA	4417 eB	18,61 e	15,17 e
10	5163 dB	6686 cA	51,62 bA	33,72 dB
11	2931 gB	4293 eA	31,66 dA	21,59 eB
12	4830 eB	6008 dA	37,18 d	37,81 c
13	7180 bA	6468 cB	19,02 eB	29,57 dA
14	5374 d	5508 d	36,66 dA	30,57 dB
15	3217 gB	4142 fA	46,68 cA	25,28 eB
16	3839 f	3590 f	41,9 cA	29,74 dB
17	4388 e	3988 f	51,18 b	44,63 b
18	3600 g	3748 f	33,71 dA	24,25 eB
19	3924 f	3920 f	44,85 c	46,25 b
20	5263 dA	4425 eB	17,36 eB	30,24 dA
21	8714 aB	9533 aA	25,72 eB	57,34 aA
22	5367 d	5774 d	41,27 cA	21,69 eB
23	3901 f	3862 f	51,68 bA	36,34 cB
24	3675 g	3892 f	56,04 bA	47,68 bB
25	4353 e	4857 e	46,32 cA	31,61 dB
26	5646 c	5133 e	39,81 cB	49,13 bA
27	3722 gB	4744 eA	17,72 eB	31,41 dA
28	3508 g	3580 f	13,66 eB	39,47 cA
29	3320 g	3468 f	32,9 dA	19 eB

Considering the genotypes with ISS higher than 0.3 (5, 13, 20, 21, 27, and 28), according to Scott Knot test, in control plants for these genotypes were all observed in the groups with the lowest total sugar concentration (Groups d and e). On the other hand, under Se application, genotype 21 is in group a, while genotype 5 is in group b, and genotype 28 is in group c.

Among the genotypes that presented IPS higher than 0.3 (Table 2), only genotype 9 presented a low total sugar accumulation, around 15 mg plant^{-1}, and allocated in group e according to Skott Knot test. Under Se application, genotypes 13, 20 and 27, together in group d, presented a total sugar accumulation of around 30 mg plant^{-1} (Table 3). In group c, genotypes 12 and 28 presented aprox 38 mg total sugar plant^{-1} under Se application (Table 3), in addition, genotype 28 presented the highest IPS, 2.7. Under Se application, genotype 5 was in group b for total sugar concentration, presenting 44.17 mg sugar plant^{-1}, while genotype 21, in group a presented 57,3 mg sugar plant^{-1} under Se application (Table 3)

According to contour maps (Figure 2), genotype 28 is the only one in the group that accumulates high total sugar and presents a very high IPS (higher than 2.4). Genotype 21 also presented a high IPS (1.4) while presenting the highest total sugar accumulation (57.3 mg plant^{-1}; Figure 1).

Genotypes 21 and 28, presented similar responses to IPS and very high total sugar accumulation. However, presented very different total ureides concentrations: while genotype 21 presented the highest total ureides concentration among genotypes (9533 µmol g^{-1} DW), genotype 28 presented one of the lowest (3580 µmol g^{-1} DW, Figure 1). Genotype 21 was the only one with an IPS higher than 0.3, which also presented a high ureide concentration. The other genotypes presented low or average total ureides concentration (Figure 1).

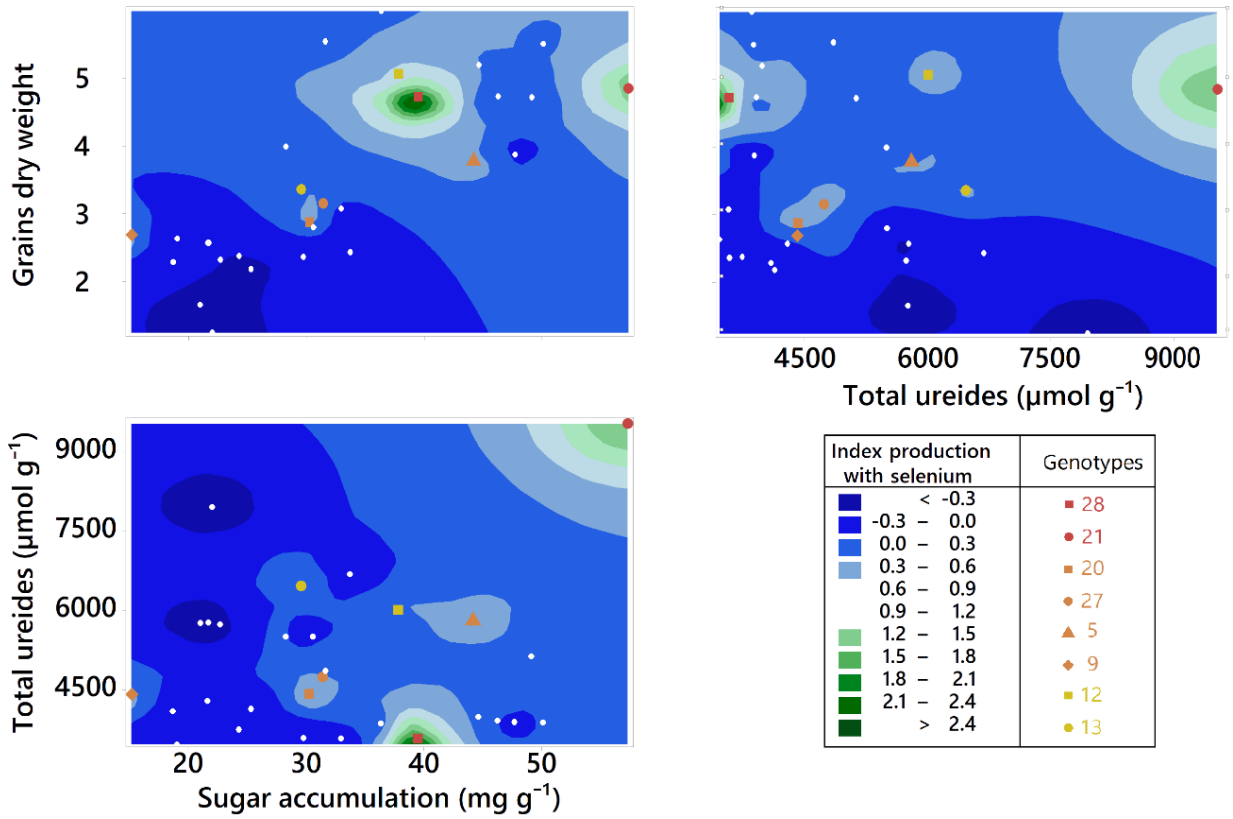

Fig 1. Contour Map of surface with variables Grains dry weight, Sugar accumulation and Total Ureides, according to the response to the variable Index production with selenium (IPS). The highlighted genotypes presented IPS of 0.3 or higher.

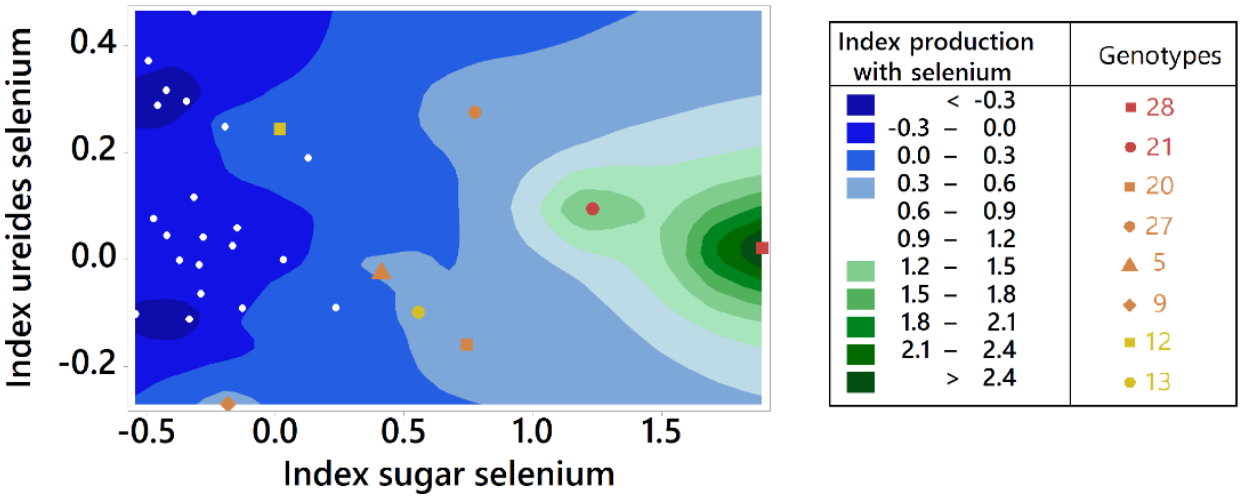

Fig 2. Contour Map of surface with variables Index Total Sugar (ISS) and Index Total Ureides (IUS), both with selenium, according to the response the variable Index production with selenium (IPS). The highlighted genotypes presented IPS of 0.3 or higher.

Total ureides concentration in control treatment varied between 2931 (genotype 11) and 8959 µmol g^{-1} DW (genotype 1), and between 3468 (genotype 29) and 9533 µmol g^{-1} DW (genotype 21) under Se application. Se application increased total ureides concentration in genotypes 2, 3, 8, 10, 11, 12, 15, 21, and 27, but decreased total ureides concentration in genotypes 1, 9, 13, and 20. Among genotypes that presented IPS higher than 0.3, only genotypes 12, 21, and 27 presented an increase in Total ureides concentration under Se application (Table 3). Is noteworthy that genotype 21, which presented the highest total ureides concentration under Se application, also presented a very high total ureides concentration in control treatments, resulting in a low IUS of 0.1 (Table 2).

Regarding ISS compared to IUS, under negative ISS, wide variability in IUS is observed. But the two genotypes that presented the highest ISS (21 and 28) presented IUS tending to zero. On the other hand, genotypes 20 and 27 presented similar ISS (0.7 and 0.8 respectively) presented very distinct IUS (-0.2 and 0.3, respectively; Figure 1).

The genotypes presented a strong positive correlation between IPS and ISS ($p<0.01$; Figure 3). The six genotypes that presented the highest ISS also presented IPS higher than 0.3. Genotype 9 was the only one that presented IPS higher than 0.3 but presented a negative ISS (Table 2).

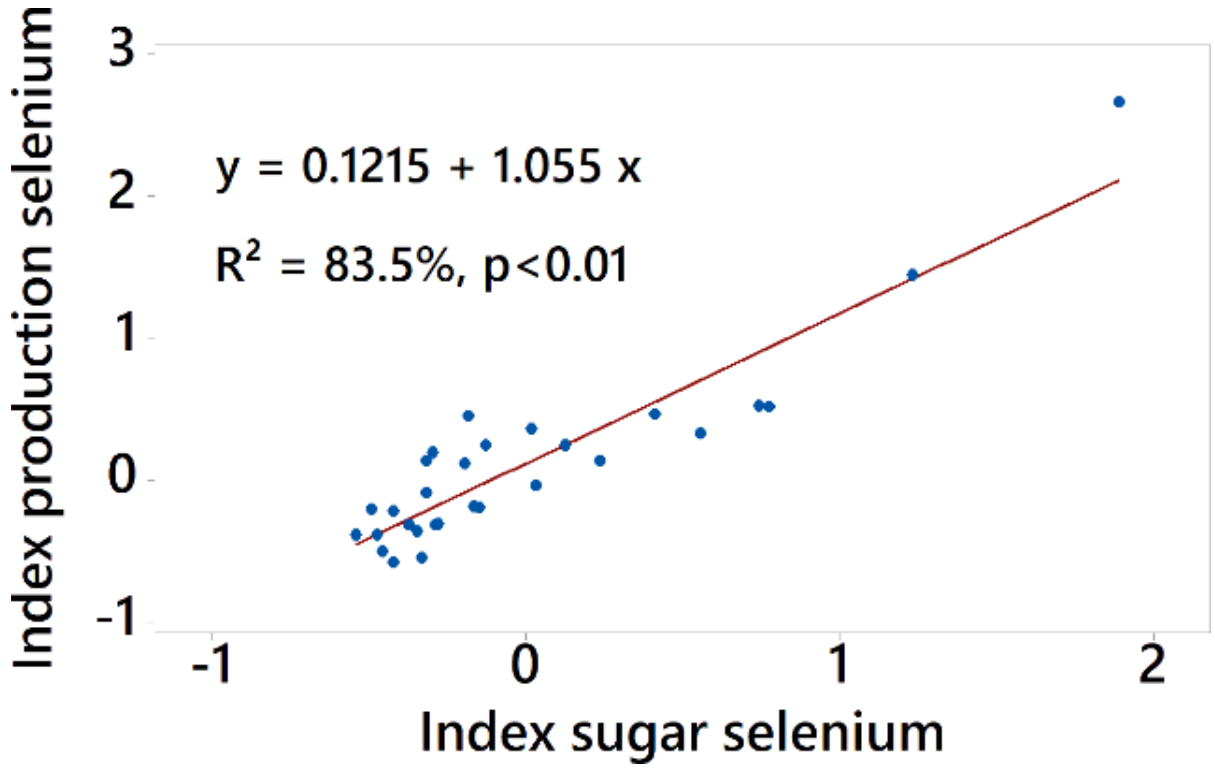

Fig 3. Linear regression of Index production selenium according to the Index Total Sugar. The Pearson correlation between IPS and ISS is 91,4%.

The consequences of Se application varied depending on each genotype. Genotype 12 (BRS Potengi) control treatment presented a yield above average, but the Se application increased its yield even more (Table 2). While genotypes 5 (Guariba) and 28 (Pingo de Ouro-2), presented Yield below average in control treatments and above-average under Se application (Table 2). And other genotypes: 2 (BR 3-Tracuateua) and 15 (BRS Urubuquara) presented above-average yield in control treatments, but a decrease under Se application (Table 2).

4. DISCUSSION

Selenium is a beneficial element for plants required in very small concentrations (White 2016; Silva *et al.* 2018), and the range between Se beneficial and detrimental effect is narrow (Schiavon and Pilon-Smits 2017) which could explain the variable results. Cowpea stands out due to its wide variation in its characteristics (Phillips *et al.* 2012; Rocha *et al.* 2017), thus the standard condition in our experiment might be stressful to

some genotypes, leading to low yields, as observed in genotypes 5 and 28. Selenium is well known for mitigating damage caused due to stress (Pandey and Gupta 2018; Jóźwiak and Politycka 2019; Hussain *et al.* 2020). Selenite enhances the activity of catalase, a ROS scavenging enzyme (Silva *et al.* 2020). Helping to reduce stress caused due to lipid peroxidation of cell membranes (Farooq *et al.* 2019), and thus, leading to positive IPS for genotypes as 5 and 28, in previous studies the application of selenite already provided an increase in the yield of lentils cultivated in fields of California (Ekanayake *et al.* 2017)

On the other hand, high Se concentrations might disrupt cell membranes and increase lipid peroxidation (Mostofa *et al.* 2017; Silva *et al.* 2018), causing a decrease in plant biomass (Gouveia *et al.* 2020) and yield (Reis *et al.* 2020). Genotypes such as 2 and 15, which presented a negative IPS under Se application, could be impaired by Se toxicity. Similar behavior was observed in 30 lettuce accessions under Se application, in which shoot biomass was increased in five accessions and decreased in 18 due to Se application (Ramos *et al.* 2011).

An increase in sugar accumulation in grains was observed in most of the genotypes in which the Se application provided an increase in yield (Figure 1). Index sugar selenium (ISS) and IPS presented a positive linear correlation (Figure 3). This is expected since Se application might be beneficial to increase sugar content, as observed in cowpea leaves (Silva *et al.* 2020) and rice (Gouveia *et al.* 2020). As the main product of photosynthesis, sugar is transported from leaves to grains depending on the activity of sucrose metabolism enzymes and starch degradation (Ren *et al.* 2020). In mung beans, the application of 0.75 ppm of Se as sodium selenate can increase α and β amylase activity in shoots (Malik *et al.* 2011). Foliar application of 100-150 mg L^{-1} of sodium selenite might increase the activity of sucrose synthase and sucrose phosphate synthase in apples (Ren *et al.* 2020). In peanuts, 40 ppm of Se nanoparticles increases soluble sugars (Hebat-Allah *et al.* 2019).

Sugars are the major carbon-transporting molecules, playing important roles in plants' growth and development to provide increases in yield by filling grains and other edible parts of plants (Halford *et al.* 2011; Zhao *et al.* 2019). And thus, is expected that yield and sugar concentration, are positively correlated, as observed in our data.

Total ureides concentration did not present any direct correlation to yield (Figures 2 and 3). Genotype 21 (“MNC04-782F-108”) was the only one that presented a high total ureides concentration and IPS higher than 0.3. Plenty of studies demonstrated the indirect effect of Se on BNF: The application of 0.045% (w/v) of sodium selenate increases root growth and nodulation of *Pisum sativum* (Poblaciones and Rengel 2018). In lentils, Se application can increase the nitrogen derived from the air by 44% compared to control (Ekanayake *et al.* 2017). In pot experiment, the application of 1 µM of sodium selenate increased the number and weight of nodules in alfalfa (Hajiboland *et al.* 2015).

Since ureides are the main N-compounds produced by nodules (Baral *et al.* 2016), the enhancements provided by Se, mostly in nodulation, could indirectly increase its biosynthesis. After its synthesis, ureides are loaded into the nodules-roots system and transported to shoots by xylem, and then are metabolized into amino acids, proteins, and other N-compounds (Baral *et al.* 2016). Thus, the medium-low ureides concentration in most genotypes might indicate that ureides are being metabolized in other N-compounds. On the other hand, in genotype “MNC04-782F-108”, Se might impair the conversion of ureides into other N-compounds. Therefore, the effect of Se in ureides concentration might be indirect, and further investigations are required to better understand the element’s role in allantoin and allantoic acid metabolism.

Is noteworthy that the Se application rate was performed on the same day (44DAE) in all genotypes, under the flowering stage. However, due to cowpea wide variability (Oliveira *et al*. 2004; Carvalho *et al*. 2012; Rocha *et al*. 2017), this might not be the optimal timing for Se application in all genotypes studied. Fertilization timing is an important factor in plant development (Efreteuei *et al*. 2016; Ali *et al*. 2019; Davies *et al*. 2020), there are no studies specifically addressing the Se application timing effect. Although the data provided in this work could be a starting point to further investigate more appropriate Se application timing in specific genotypes.

5. CONCLUSION

Selenium narrow range between toxicity and benefit was elucidated: genotypes “BRS Guariba”; “Pingo de Ouro-1-2” and “Pingo de Ouro-2” “presented the highest increase in yield under Se application, and genotypes “BR 17 – Gurguéia”, “BR 3-

Tracuateua" and "BRS Urubuquara" were the most impaired. Considering the increase in yield and sugar accumulation due to Se application, genotypes "MNC04-782F-108", and "Pingo-de-ouro-2" presented the best responses. Selenium provided a positive correlation on yield and sugar content. Ureides in shoots were not strongly related to the Se effect in yield. This study provides important information of Se on ureides and sugar metabolism in cowpea genotypes and its relation to grain yield and nutritional quality.

6. SUPPLEMENTARY TABLES

Table S1. F ratio and P value of analysis of variance (ANOVA). Sources of variation: genotypes (for IPS, ISS and IUS) and genotypes and Se application (For Total Ureides and Total Sugar).

Variable	F ratio	Pr (<F)
IPS	6.89	0.0001**
ISS	7.89	0.0001**
IUS	4.28	0.0001**
Total Ureides	6.35	0.0001**
Total Sugar	14.82	0.0001**

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CHAPTER 5 - INTERACTION BETWEEN SULFUR AND SELENIUM IN AGRONOMIC BIOFORTIFICATION OF COWPEA PLANTS UNDER FIELD CONDITIONS

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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. MATERIALS 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).

Table 1. Stoichiometry balance between, ammonium sulfate and urea for each treatment.

Treatment	Se (g ha^{-1})	Sulfur (kg ha^{-1})	Ammonium sulfate + Urea (g $plot^{-1}$)	N supply as ammonium sulfate (g $plot^{-1}$)*	N supply as urea (g $plot^{-1}$)**
T1	**0**	**0**	0.00 + 53.36	0	23.48
T2	**10**	**0**	0.00 + 53.36	0	23.48
T3	**25**	**0**	0.00 + 53.36	0	23.48
T4	**50**	**0**	0.00 + 53.36	0	23.48
T5	**0**	**15**	29.35 + 40.02	5.87	16.71
T6	**10**	**15**	29.35 + 40.02	5.87	16.71
T7	**25**	**15**	29.35 + 40.02	5.87	16.71
T8	**50**	**15**	29.35 + 40.02	5.87	16.71
T9	**0**	**30**	58.7 + 26.68	11.74	11.74
T10	**10**	**30**	58.7 + 26.68	11.74	11.74
T11	**25**	**30**	58.7 + 26.68	11.74	11.74
T12	**50**	**30**	58.7 + 26.68	11.74	11.74
T13	**0**	**60**	117.39 + 0	23.48	0
T14	**10**	**60**	117.39 + 0	23.48	0
T15	**25**	**60**	117.39 + 0	23.48	0
T16	**50**	**60**	117.39 + 0	23.48	0

* N concentration in the ammonium sulfate fertilizer used in the experiment: 20%.
*** N concentration in the urea fertilizer used in the experiment: 44%.

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).

Table 2. P values of analysis of Variance (ANOVA). Sources of variation: S application rates, Se application rates and the interaction between the factors regarding cowpea yield, plant height, leaf and seed Se concentration, leaf and seed S concentration in the first (2016) and second (2017) year.

Source of Variation	2016	2017
Cowpea Yield		
S application rates (A)	0.948 ^{NS}	0.473 ^{NS}
Se application rates (B)	0.060 ^{NS}	0.217 ^{NS}
A*B interaction	0.937 ^{NS}	0.039*
Plant Height		
S application rates (A)	0.713 ^{NS}	0.588 ^{NS}
Se application rates (B)	0.863 ^{NS}	0.466 ^{NS}
A*B interaction	0.193 ^{NS}	0.880 ^{NS}
Leaf Se Concentration		
S application rates (A)	0.148 ^{NS}	0.0001**
Se application rates (B)	0.0001**	0.0001**
A*B interaction	0.885 ^{NS}	0.0001**
Seed Se Concentration		
S application rates (A)	0.133 ^{NS}	0.0001**
Se application rates (B)	0.0001**	0.0001**
A*B interaction	0.334 ^{NS}	0.0007**
Leaf S Concentration		
S application rates (A)	0.0001**	0.003 **
Se application rates (B)	0.849 ^{NS}	0.196 ^{NS}
A*B interaction	0.208 ^{NS}	0.312 ^{NS}
Seed S concentration		
S application rates (A)	0.0001 **	0.0001 **
Se application rates (B)	0.293 ^{NS}	0.038 *
A*B interaction	0.0005 **	0.083 ^{NS}

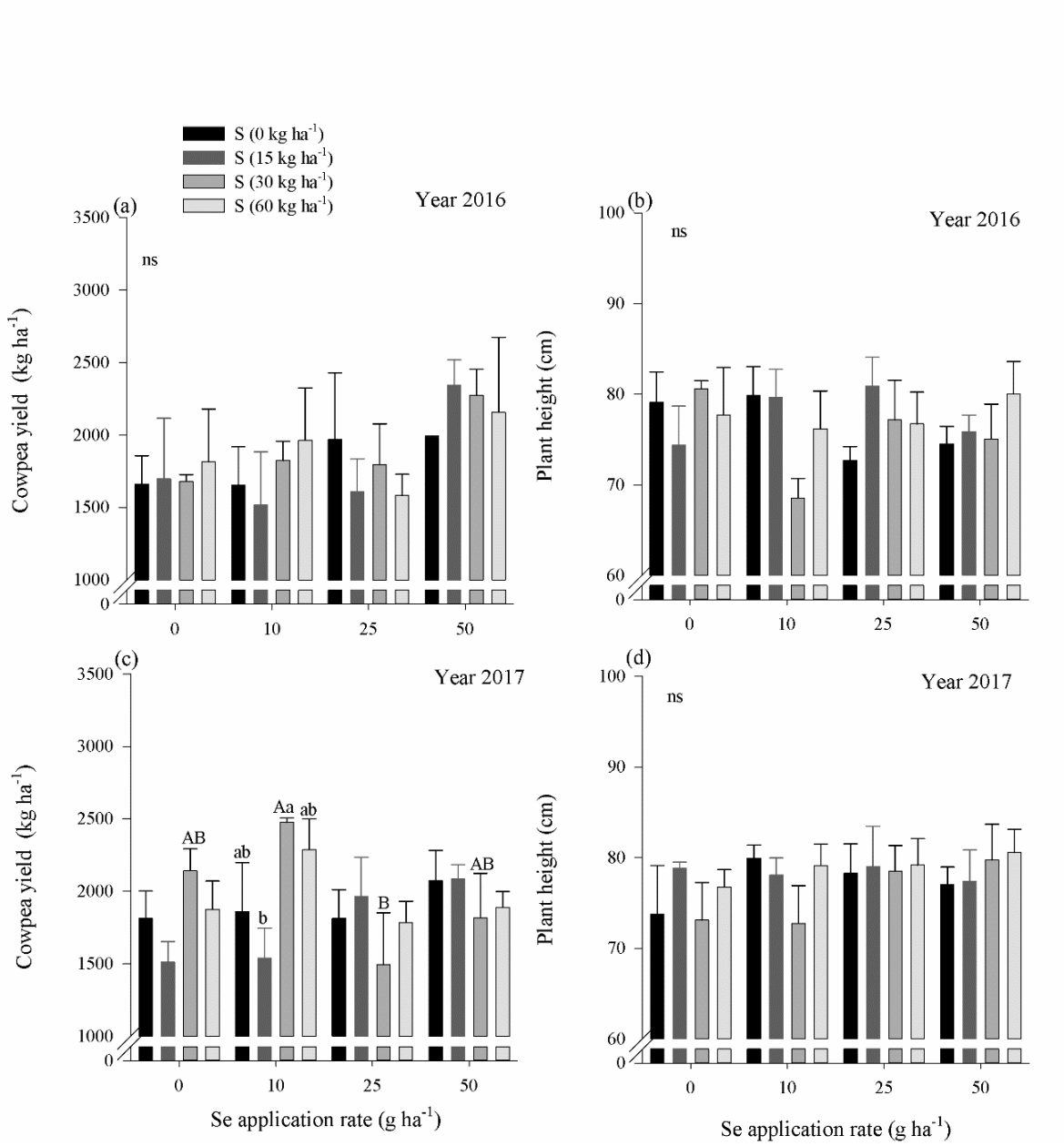

Figure 1. Yield and plant height in the first (a-b) and second (c-d) year of cowpea plants in response to Se and S combination. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test (p≤0.05). Uppercase letters correspond to Se rates, and lowercase letters correspond to S rates. ns = not significantly according to tukey test (p≤0.05). CV (%) = 30.47 (a); 8.59 (b); 21.13 (c); 8.13 (d). Interaction between Se and S according to tukey test (p≤0.05) observed in: c (S rates under 10 g ha^{-1} of Se and Se rates under 30 kg ha^{-1} of S).

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).

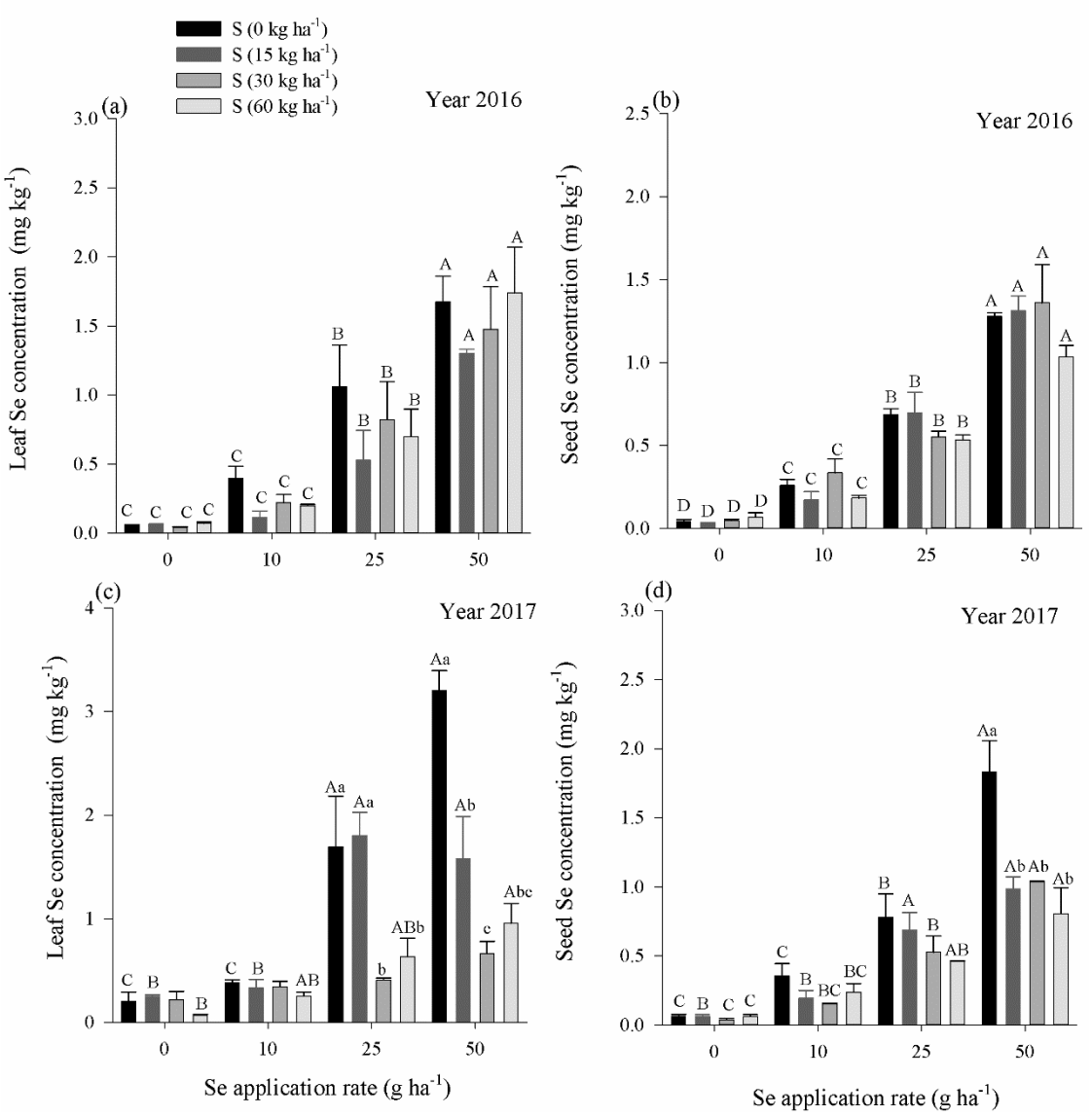

Figure 2. Leaf and seed Se concentration for the first (a-b) and second (c-d) year of cowpea plants in response to Se and S combination. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test (p≤0.05). Uppercase letters correspond to Se rates, and lowercase letters correspond to S rates. ns = not significantly according to tukey test (p≤0.05). CV (%) = 54.22 (a); 29.59 (b); 46.49 (c); 39.51 (d). Interaction between Se and S according to tukey test (p≤0,05) observed in: c (S rates under 25 and 50 g ha^{-1} of Se, and Se rates under 0, 15 and 60 kg ha^{-1} of S); d (S rates under 50 g ha^{-1} of Se, and Se rates under all S rates) .

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).

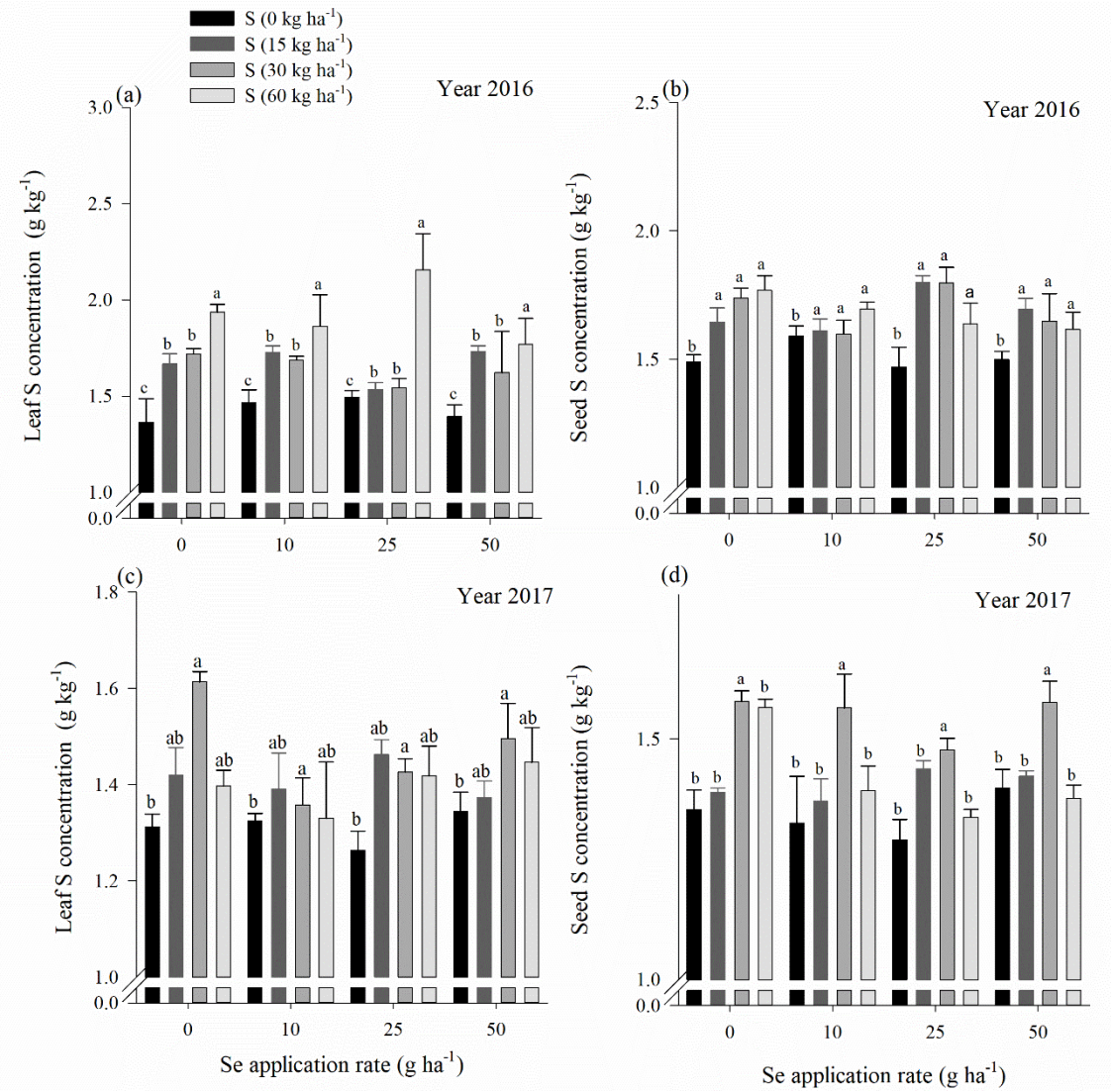

Figure 3. Leaf and seed S concentration for the first (a-b) and second (c-d) year of cowpea plants in response to Se and Se combination. Error bars indicates the standard error of mean (n=4). Different uppercase letters indicate difference between means according to tukey test (p≤0.05). ns = not significantly according to tukey test (p≤0.05). CV (%) = 11.95 (a); 7.71 (b); 8.15(c); 5.95 (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).

Table 3. P values of analysis of Variance (ANOVA). Sources of variation: S application rates, Se application rates and the interaction between the factors regarding total sugar, sucrose, total free amino acids, albumin, globulin, prolamin and glutelin concentration in seeds of cowpea for the first (2016) and second (2017) year.

Source of Variation	2016	2017
Total sugars		
S application rates (A)	0.001 **	0.291 ^{NS}
Se application rates (B)	0.0001 **	0.0001 **
A*B interaction	0.0003 **	0.0001 **
Sucrose		
S application rates (A)	0.0001 **	0.0001 **
Se application rates (B)	0.0001 **	0.0001 **
A*B interaction	0.0001 **	0.0001 **
Total free amino acids		
S application rates (A)	0.0001 **	0.0001 **
Se application rates (B)	0.0001 **	0.0001 **
A*B interaction	0.0001 **	0.0001 **
Albumin		
S application rates (A)	0.0001 **	0.025 *
Se application rates (B)	0.0001 **	0.0001 **
A*B interaction	0.0001 **	0.0001 **
Globulin		
S application rates (A)	0.0001 **	0.0001 **
Se application rates (B)	0.003 **	0.0001 **
A*B interaction	0.0001 **	0.0001 **
Prolamin		
S application rates (A)	0.0001 **	0.0001 **
Se application rates (B)	0.0001 **	0.0001 **
A*B interaction	0.0001 **	0.0001 **
Glutelin		
S application rates (A)	0.0001 **	0.0001 **
Se application rates (B)	0.0001 **	0.0001 **
A*B interaction	0.0001 **	0.0001 **

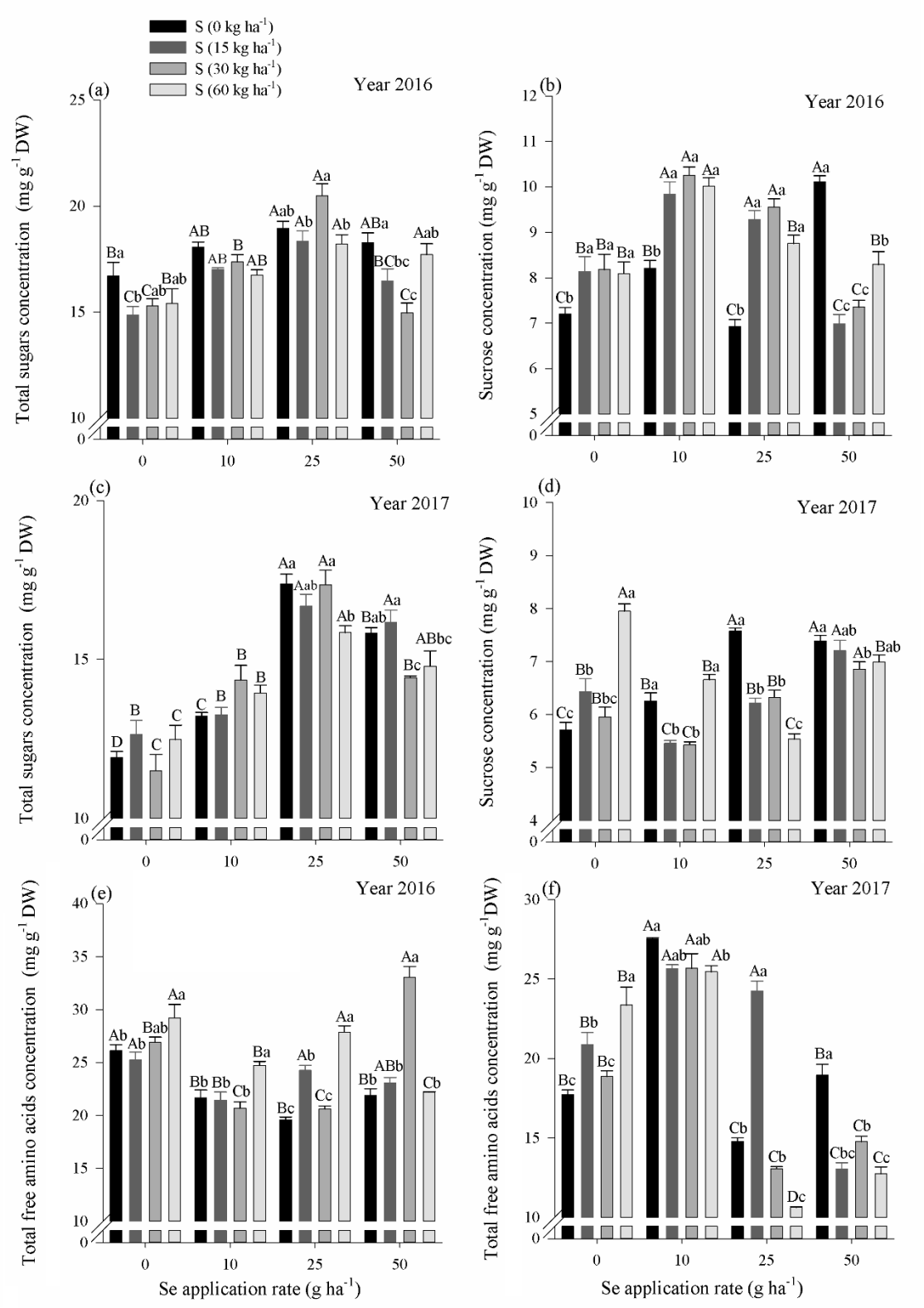

Figure 4. Sucrose, total sugar and total free amino acids concentration in seeds in the first (a-b and e) and second (c-d and f) year of cowpea plants in response to Se and S

combination. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test (p≤0.05). Uppercase letters correspond to Se rates, and lowercase letters correspond to S rates. ns = not significantly according to tukey test (p≤0,05). CV (%) = 5.35 (a); 5.07 (b); 4.61 (c); 3.97 (d); 5.42 (e); 5.40 (f). Interaction between Se and S according to tukey test (p≤0,05) observed in: a (S rates under 0, 25 and 50 g $ha^{-1}$of Se, and Se rates under all S rates); c (S rates under 25 and 50 g $ha^{-1}$of Se, and Se rates under all S rates); b, d, e and f (S rates under all Se rates, and Se rates under all S rates).

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).

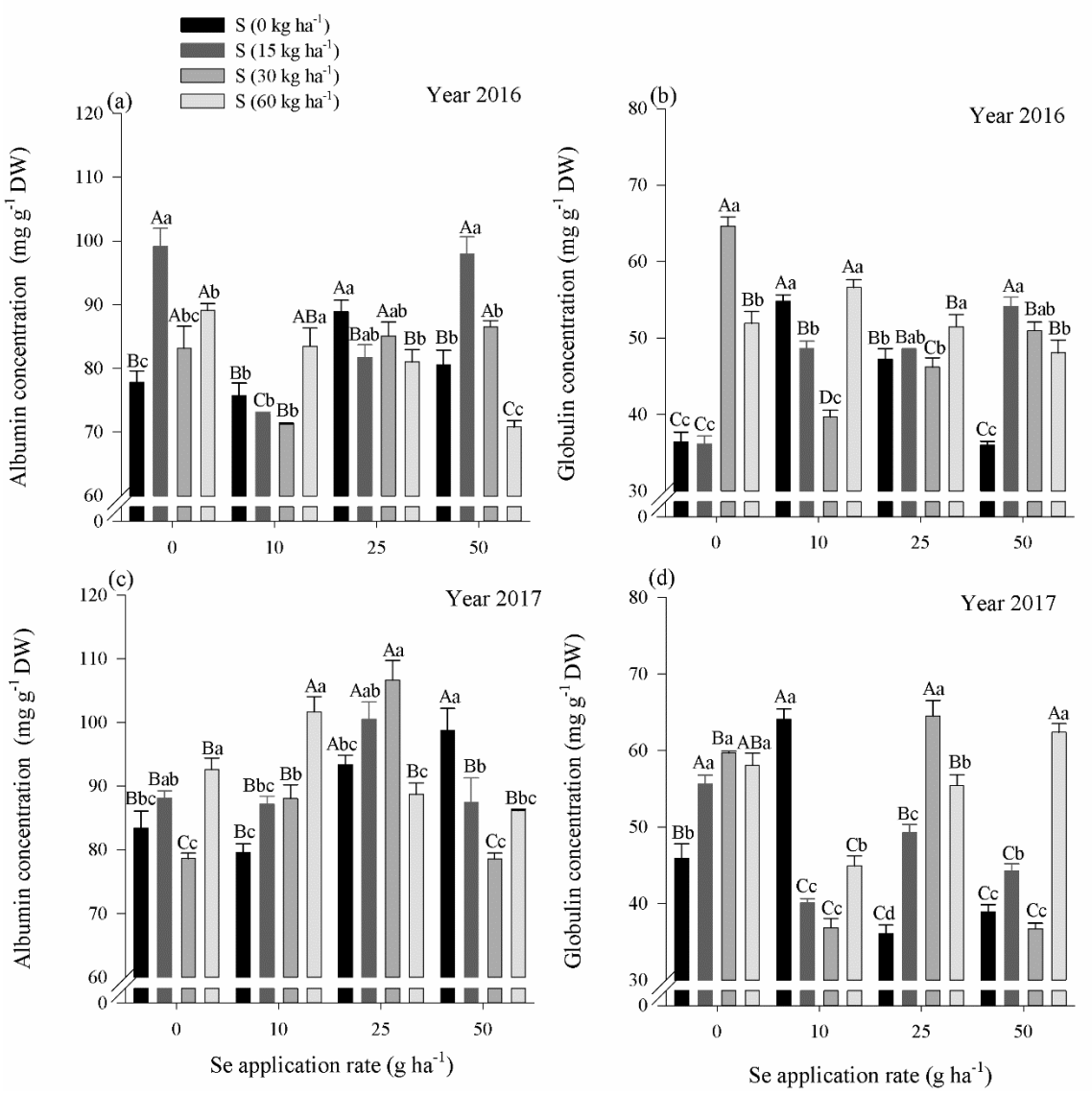

Figure 5. Albumin and globulin concentration in seeds for the first (a-b) and second (c-d) year of cowpea plants in response to Se and S combination. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test (p≤0.05). Uppercase letters correspond to Se rates, and lowercase letters correspond to S rates. ns = not significantly according to tukey test (p≤0.05). CV (%) = 4.95 (a); 4.47 (b); 4.71 (c); 4.74 (d). Interaction between Se and S according to tukey test (p≤0.05) observed in: a, b, c and d (S rates under all Se rates, and Se rates under all S rates).

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).

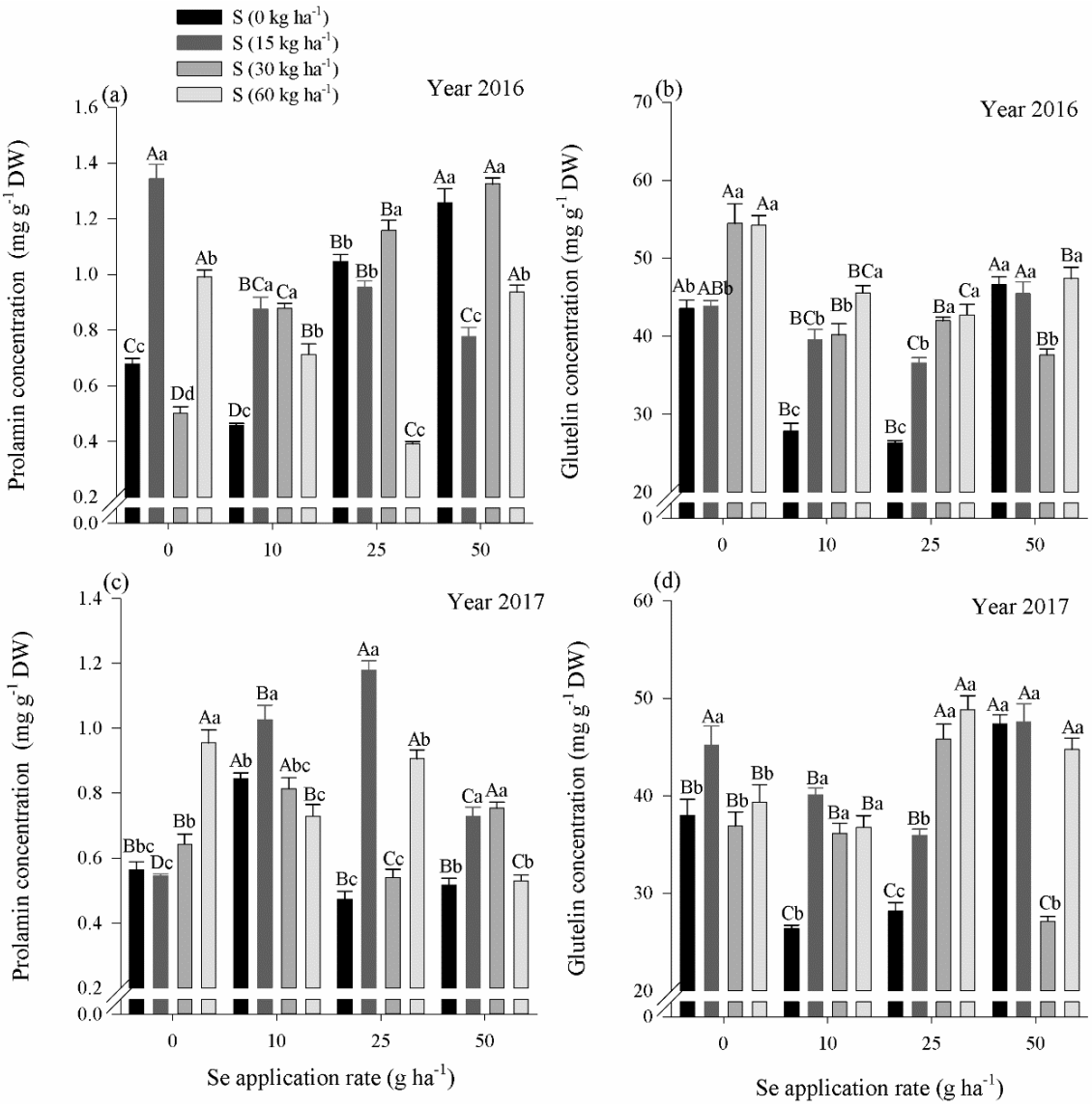

Figure 6. Prolamin and glutelin concentration in seeds for the first (a-b) and second (c-d) year of cowpea plants in response to Se and S combination. Error bars indicates the standard error of mean (n=4). Different letters indicate difference between means according to tukey test (p≤0.05). Uppercase letters correspond to Se rates, and lowercase letters correspond to S rates. ns = not significantly according to tukey test (p≤0,05). CV (%) = 6.50 (a); 5.74 (b); 7.02 (c); 6.63 (d). Interaction between Se and S according to tukey test (p≤0.05) observed in: a, b, c and d (S rates under all Se rates, and Se rates under all S rates).

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. CONCLUSION

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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7. SUPPLEMENTARY FIGURES

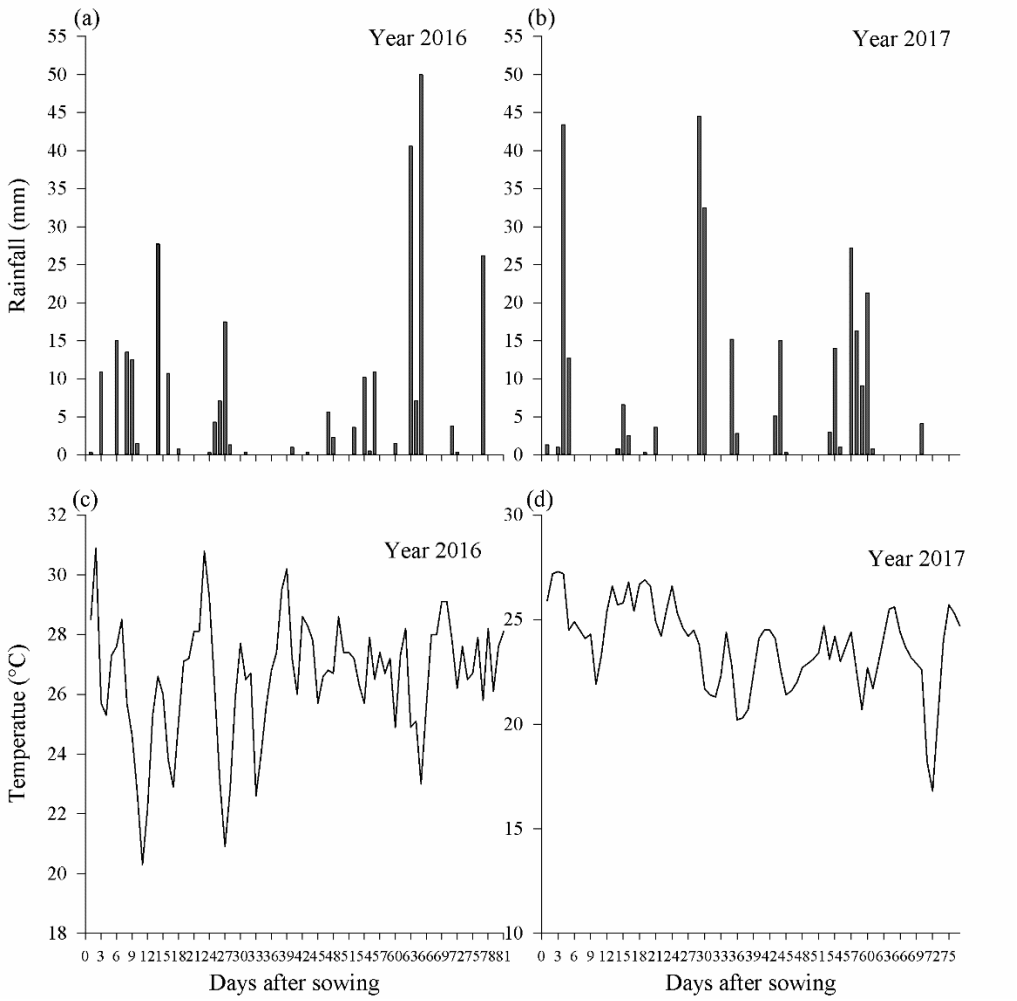

Figure S.1. Daily Rainfall (a-b) and mean temperature (c-d) recorded during cowpea cultivation period.

CHAPTER 6 - SELENATE AND SULFATE INTERACTION ON COWPEA FLAVONOIDS, GENE EXPRESSION, NODULATION AND METABOLITES.

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ABSTRACT

Selenium (Se) is a beneficial element to higher plants at low concentrations. As selenate, it shares similarities with sulfate in transport and assimilation by plants. Thus, Se can affect S uptake and assimilation and vice-versa, which could lead to alterations in plant responses related to S and Se, such as flavonoids biosynthesis, S and Se transport, and legume plants nodulation. This study aims to evaluate Se and S combination on flavonoid, nodulation, transport, and assimilation in cowpea (*Vigna unguiculata* (L.) Walp). Sulfur regulates flavonoid metabolism, which is related to Rhizobia bacteria and root communication, regulating the nodulation of legume plants. A pot experiment was carried out to evaluate the effect of Se x S interaction on flavonoids profile and its relations with nodulation. The experimental design was fully randomized and composed by four treatments: control, Se (100 g Se ha^{-1}), S (0 and 25 kg ha^{-1}) and Se + S, and four replications. Selenium enhanced nodulation but the interaction with S impaired it. Daidzein concentration was enhanced in nodules under Se application, however, the selenate and sulfate interaction impaired flavonoid concentration roots. Sulfate transporters were enhanced by Se and S interaction. Selenium application did not impair S concentration in plant tissues, but S application impairs Se concentration in leaves and roots of cowpea. The effect of Se and S on the expression of genes related to flavonoid biosynthesis was variable, further investigations are required to better understand how Se and S can affect flavonoid biosynthesis.

Keywords: *Vigna unguiculata* (L.) Walp; Sultr; selenium; sulfur; daidzein; nodulation

1. INTRODUCTION

Defined as the inadequate intake of nutrients and vitamins, hidden hunger affects around 2 billion people worldwide (Srivastav et al., 2022), the problem is more common in low and middle-income countries (FAO, 2019). Providing staple foods with high levels of key nutrients, such as Iron (Fe), Zinc (Zn), Selenium (Se) and Iodine (I), is crucial to alleviate hidden-hunger (Lenaertes & Demont, 2021), but most plant-breeding programs tend to focus mainly on yield enhancement, leading to a decrease in nutrients concentration in edible parts of crops (Schjoerring et al., 2018). To hinder this problem, biofortification programs are performed worldwide to increase key elements concentration in staple foods in low and middle-income countries (Stangoulis & Knez, 2022).

The Se is a nutrient for humans and other animals, and a beneficial element for plants. Selenium 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). The antioxidant proprieties provided by Se are crucial for human health, as the element plays a role in thyroid hormone synthesis (Ekuma et al. 2021) and in the immune system (Rayman 2000), Se deficiency can cause Keshan Disease (Shi et al., 2021), as well as gestational disorders (Hogan & Perkins, 2022).

In plants, most of the studied Se benefits are related to its antioxidant enhancements, provided by an increase in antioxidant enzymes activities (Shahid, 2019; Silva et al., 2020; Lanza & Reis 2021), leading to increased yield and development for plants under stressful conditions (Yao et al., 2013; Kaur & Nair, 2015; Manaf, 2018). However, other benefits for plants are also related to Se application: Se can enhance plant resistance to pathogens, and decrease attacks from insects (Schiavon & Pilon-Smits, 2017), and in recent studies, it was observed that Se can stimulate legume plants nodulation (Cunha et al., 2022).

To provide Se in well-oxygenized soils, sodium selenate is the most efficient and common source of Se (Silva et al., 2019), however selenate shares many similarities with sulfate (Cabannes et al., 2011). Due to these similarities, sulfate and selenate are transported and assimilated in plants by the same pathways (Natasha et al., 2018), leading to interactions between both elements. Under S deficiency, *Arabidopsis thaliana* plants presented higher expression of the sulfate transporter

AtSULTR1;1, leading to enhanced Se uptake (White, 2016). In field-cultivated cowpea, sulfate application can impair Se uptake and accumulation in leaves and seeds (Silva et al., 2022), and in rapeseed, it was also reported that S can impair Se uptake (Liu et al., 2017). But the contrary was also observed: in Tartary buckwheat S application increased Se uptake (Golob et al., 2016). Considering assimilation both elements share the same assimilation pathway, by enzymes adenosine triphosphate sulfurylase (APS) and adenosine triphosphate sulfurylase reductase (APR) that catalyzes the reduction of S and Se, which are further converted into the amino acids cysteine (Cys) and methionine (Met) or selenocysteine (Se-Cys) and selenomethionine (Se-Met), respectively (White, 2018). Other pathways are related to Se and S assimilation by plants too, such as glutathione assimilation pathway in which Cys + glutamine are converted in γ-glutamylcysteine (γ-EC) catalyzed by γ-EC synthetase, then γ-EC + glycine are converted into glutathione, catalyzed by glutathione synthase (Hasanuzzman et al., 2019)

Cowpea (*Vigna unguiculata* L. Walp) is a rustic Fabaceae and is one of the most important protein sources in low and middle-income countries and regions (Manzeke et al., 2017). This is due to its high tolerance to drought (Gomes et al, 2020), and high temperatures (Carvalho et al., 2012). In addition to its rusticity, cowpea present high content of protein in grains, surpassing common bean (Teka et al., 2020), and superior nutritional attributes (Tankari et al., 2021) such as high bioavailability of Zn and Fe (Coelho et al., 2021), and potential for agronomic biofortification with Se (Silva et al., 2019; 2022). According to Gomes et al., 2020, the crop is cultivated around the world in more than 100 countries (Gonçalves et al., 2016) providing a global yield of 9.8 million tons in 2020 with an estimated yield of 12.3 million tons by 2030. The application of Se on cowpea can increase Se concentration in grains to safe levels for human consumption (Silva et al., 2019) in addition to enhancing cowpea sugar concentration (Silva et al., 2020).

The biological N fixation (BNF) is a process that occurs in the plant nodule, structures assembled through isoflavonoids-induced chemotaxis (Bosse et al., 2021). The isoflavonoids such as genistein and daidzein induce the expressions of *nod* genes of rhizobia bacteria (Subramanian et al., 2007) leading to the synthesis and secretion of nodulation factors which stimulates physiological modifications on plant cells that

start the nodule formation (Bosse et al., 2021). In fully developed nodules, N_2 is assimilated and converted to ammonia and further converted to ureides, then transported to shoot tissues through the xylem (Soumare et al., 2020). Once in the cell cytosol, these compounds are converted into ammonium, amino acids, and proteins required for plant development (Bosse et al., 2021). As previously reported, Se can enhance flavonoid biosynthesis in peanuts, which led to an enhancement in nodulation (Cunha et al., 2022). This is noteworthy to further investigate Se influence on nodulation in legume plants.

This study hypothesizes that Se and S interaction leads to a variation in gene expression responsible for Se and S transport and metabolism, and flavonoid biosynthesis. The objective of this study is to evaluate the effect of Se and S interaction on Se and S transport and assimilation, as well as in flavonoid biosynthesis regulating the nodulation of cowpea plants.

2. MATERIALS AND METHODS

2.1 Growth condition and harvest

The experiment was carried out in a greenhouse at Cornell University, Ithaca, NY, USA. The environment inside the greenhouse was set to 28 °C, with 14 hours day^{-1} of light incidence. On May 2nd, 2023, the sowing of cowpea was performed in 2 L polyethylene pots, seeds from genotype BRS-GUARIBA were used, and the pots were filled with 2 dm^3 of a commercial growing medium PRO-MIX BK25 (Lot# 50280RGVE), composed by: Canadian Sphagnum Peat Moss, Vermiculite, Processed Pine Bark, Dolomitic Limestone, and Wetting Agent. The pots were then watered with deionized water until the humidity reach the bottom of the pots. Prior to sowing, the seeds were inoculated using solid inoculant VERDESIAN GARD-N (Lot# U108) composed by a mix with, *Bradyrhyzobium sp., B. japonicum, Rhizobium leguminosiarum biovar phaseoli and R. l. b. viceae*. Inoculum and seeds were mixed with water at the following rates: 3 g seeds, 0.03 g inoculum, and 3 mL water. After inoculation, sowing was performed, one seed was sowed per pot. Emergence occurred on May 10th, 2023, 8 days after sowing. Treatments were applied on May 22nd, 2023, at 12 days after emergence (12DAE), and on 19 DAE. The experiment was composed by the following treatments: 1 – Control; 2 - 100 g ha^{-1} Se as sodium selenate; 3 – 25 kg ha^{-1} S as

sodium sulfate; and 4 - 100 g ha^{-1} Se as sodium selenate + 25 kg ha^{-1} S as sodium sulfate. Each pot received 1 mL of solution with half the dosage at 12 DAE and half the dosage at 19 DAE, control plants received 1 mL of deionized water. Each treatment was composed of 4 replications, totaling 16 pots.

Plants were harvested at 33 DAE and 14 days after the second half of the treatment application. The second fully expanded trifoliate, from top to bottom, was collected and stored in liquid nitrogen for gene expression, and flavonoid analysis. Roots were rinsed and stored in liquid N for flavonoid analysis. Nodules were separated from roots, the total number of nodules was counted, and the total nodule's fresh weight was recorded, then nodules were stored in liquid N for flavonoid analysis. Plants were separated into roots and shoots, and the material was dried in an oven with forced air circulation at 60 °C until constant weight. The root and shoot dry weight were recorded, and then, the root and shoot dry material was collected for nutrient analyses.

2.2 Gene expression analyses

For RNA extraction, leaf samples were ground in liquid N, then approximately 100 mg material was weighted, in which 1 mL TRIzol was added, then the material was homogenized and kept at room temperature for 15 minutes. In sequence, 200 µL chloroform was added, and the mixture was homogenized and kept at room temperature for 3 minutes. After that, samples were centrifuged at 12000 g for 15 min at 4 °C, then the supernatant was extracted, placed in another tube, and stored at - 20 °C for 30 minutes. Then the samples were centrifuged at 12000 g for 10 min at 4 °C, the supernatant was discarded, and the RNA pellet was washed twice with 1 mL ethanol 75% in DEPC water. The ethanol was discarded, and samples were centrifuged at 7500 g for 5 min at 4 °C, the supernatant was removed aiming to fully discard the ethanol. Samples dried at room temperature for approximately 5 minutes, then the pellet was resuspended with 50 µL DEPC water.

For gDNA removal, in 1000ng of RNA, it was added 1µl DNAse, 1 µl buffer, and DEPC-water to complete 10 µl. The mixture was stored at 37 °C for 30 minutes, then 1 µl 50 mM EDTA added, and the mixture was submitted to 65 °C for 10 minutes.

After gDNA removal, a reverse transcription reaction process was performed by adding 2.5 µl of 20 µM oligo-dT primer, the mixture was submitted to 70 °C for 3

minutes and immediately put on ice. Afterward, it was added 5 µl of buffer, 2.5 µl of 10 mM DEPC-dNTPs, 2.5 µl of 100 mM DTT, 0.625 µl of MMLV-Rtase and 0.875 µl of DEPC water. The mixture was submitted to 42 °C for 60 minutes, and then to 70 °C for 15 minutes.

The formed cDNA was then used for qPCR to evaluate gene expression. A cowpea actin gene was used to check cDNA quality in a PCR. The qRT-PCR was performed using a Maxima qRT-PCR mix, in 5 µl of the mix it was added 1 µl of cDNA, 0.3 µl of reverse, and 0.3 µl of forward primer (Table 1). The actin gene was used to estimate the relative gene expression of the other genes. Primers were designed based on cowpea genome using Basic Local Alignment Search Tool (BLAST) from ncbi, whith PCR product size of 80 to 150 bp and preference for exon-exon junction.

Table 1. Primers used for gene expression analysis of cowpea

Gene Name	Gene ID	Primer sequence	ncbi RefSeq
Actin	Contig16004		*
F		TCAGGTGTCCAGAGGTGTTGTA	
R		ATGGTTGTGCCTCCTGAAAGTA	
SulTr 1;2	114193223		XM_028082945.1
F		AACACCAGAGCTGCAAGCAT	
R		CCGAAGCACTGAATCCCAAC	
SulTr 2;2	114193378		XM_028083144.1
F		TGGACGTGCTTTCTCATGCT	
R		GCTGAGGCTGGCAAGAGTTA	
CHR	114163850		XM_028048209.1
F		ACTCTCCTCTAGGGGCACCA	
R		CAACCATCTAAGAGATACCTGAGCA	
CHS	114173536		XM_028058002.1
F		CGTTGTTCTCCATAGTGTCGC	
R		GGCTAAAAGGCAGCCAAACC	
APS	114178424		XM_028064322.1
F		TCGTGGAATGTACAGCGAGC	
R		TATCCCTAGCTGTGCCCGAT	
APR	114187852"		XM_028076238.1
F		TGCGTCCAGGTTCAATCACA	
R		AAGAAGTGGTAGTGACGGCG	

* Actin gene used according to Amorin et al. (2018)

2.3 Flavonoid analyses

Samples of nodules, roots, and leaves were collected and stored in liquid N. In sequence, the material was ground with pistil and mortar in liquid N, the macerated material was transferred to a 1.5 mL polyethylene microtube (approx100 mg), and the exact weight was recorded. For extraction, 80% UPLC-grade methanol was used, for roots and nodules a 10 times ratio w/v was used, and for leaves, a 2 times ratio w/v was used. The samples were then put to sonicate in an ultrasonic cleaner (Model 150T, VWR Scientific-Aquasonic, West Chester, PA), with ice added to the water, for 30 minutes.

After the extraction, samples were centrifuged at 14000 rpm for 10 minutes at 4 °C, the supernatant was transferred to a new 1.5 mL polyethylene microtube and the samples were submitted to another centrifugation process (same setup as aforementioned). Then, 25 µL of supernatant and 25 µL of 2N HCl were added into a PCR tube for hydrolisys. The mixture was boiled on a thermal cycler (Model T100 Thermal Cycler, Bio-Rad, Singapore), for 60 min at 90 °C. Then, 40 µL were transferred to glass vials. The concentration of daidzein, genistein, and kaempferol in samples were analyzed using an ACQUITY UPLC equipped with a BEH C18 column (2.1 × 50 mm, 1.7 µm) (Waters, Milford, MA, USA). Samples (4 µL) were injected into the column and eluted using the mobile phases consisting of solvent A (0.1% aqueous formic acid) and solvent B (0.1% formic acid in acetonitrile) in a linear gradient of 15–85% solvent B over 4 min at flow rate of 0.6 mL min^{-1}. The column effluent was monitored at 360 nm. The observed results were expressed in ng mg FW^{-1} and were quantified using a standard curve with a max concentration of 10 µM daidzein, genistein, and kaempferol.

2.4 Nutrient analyses

The concentration of total Se and S in roots and leaves was evaluated by using an inductively coupled plasma (ICP) trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA). Roots and leaves were dried in an oven with forced air circulation at 60 °C until constant weight, the material was ground and weighed (~0.500g). The samples were acid digested in 1.0 mL HNO_3

+ 1.5 mL $HClO_4$ at 110°C for 1 h, and then at 220°C until $HClO_4$ fumes were observed. Samples were diluted until 15 mL using 18 mΩ water. The instrument calibration was performed using 7% $HClO_4$ as a low standard and 5.0 μg mL^{-1} of Se and 40.0 μg mL^{-1} of S in a multi-element standard as a high standard. The Se was determined by 196.0 nm line, and S was determined by 182.0 nm line. Three replications were used for each treatment.

2.5 Statistical analysis

The results were submitted to Anderson-Darling tests to evaluate normality; then to Leven's test to evaluate homogeneity, then, to variance analysis (F test). Differences between treatments were compared using a Tukey test at 5% probability. Analyses were performed using the software's Minitab and Agroestat, and graphs were prepared using SigmaPlot 12.5.

3. RESULTS

Cowpea shoot and root dry weight were not affected by Se or S application, while the number of nodules and nodules' fresh weight (FW) were affected by its interaction ($p < 0.05$; Table 2). Under no Se application, S application increased nodules' FW by around 30% while under 10 g ha^{-1} Se, S application decreased nodule FW by around 10%. And under no S application, Se application increased nodule FW by around 45% (Fig. 1a). Considering nodules number, under no Se application S provided an increase of 32%, and under 10 g ha^{-1} Se, S application decreased by 13% nodules number, while under no S, Se increased nodule number by 55% (Fig 2b).

The concentration of daidzein in roots and nodules was affected by Se and S interaction, while kaempferol concentration was affected by Se application ($p < 0.05$; Table 2). At no Se application, daidzein in nodules presented an increase of 28% under S application, compared to control, while at 10 g ha^{-1} Se, S application decreased daidzein in nodules by -19%. Under no S application, Se increased daidzein concentration by 33%, and under S application, Se decreased daidzein concentration by 16% (Fig 2a). In roots, under Se application, S decreased daidzein by 43%, while under S application, Se decreased daidzein concentration in roots by 34% (Fig 2b). Kaempferol concentration in leaves decreased by 74% under Se application, regardless of S application (Fig 2c).

Table 2. F ratio and P values of analysis of covariance (ANCOVA), regarding cowpea nodule number, nodule fresh weight (FW), shoot dry weight (DW), root DW, daidzein in nodules and roots, and kaempferol in leaves.

Source of Variation	F ratio	Pr(> F)
Nodule number		
Sulfur application (A)	0.05^{NS}	0.8351
Selenium application (B)	49.28**	<0.0001
A*B interaction	40.51**	<0.0001
Nodule FW		
Sulfur application (A)	1.35	0.2672
Selenium application (B)	10.93**	0.0063
A*B interaction	9.45**	0.0096
Shoot DW		
Sulfur application (A)	0.50^{NS}	0.4941
Selenium application (B)	3.00^{NS}	0.1090
A*B interaction	3.04^{NS}	0.1067
Root DW		
Sulfur application (A)	0.03^{NS}	0.8723
Selenium application (B)	1.78^{NS}	0.2069
A*B interaction	4.30^{NS}	0.0604
Daidzein (Nodules)		
Sulfur application (A)	0.26^{NS}	0.6203
Selenium application (B)	3.32^{NS}	0.0934
A*B interaction	64.32**	<0.0001
Daidzein (Roots)		
Sulfur application (A)	18.11**	0.0011
Selenium application (B)	3.73^{NS}	0.0744
A*B interaction	5.96*	0.0311
Kaempferol (Leaves)		
Sulfur application (A)	1.50^{NS}	0.2438
Selenium application (B)	384.74**	<0.0001
A*B interaction	1.74^{NS}	0.2122

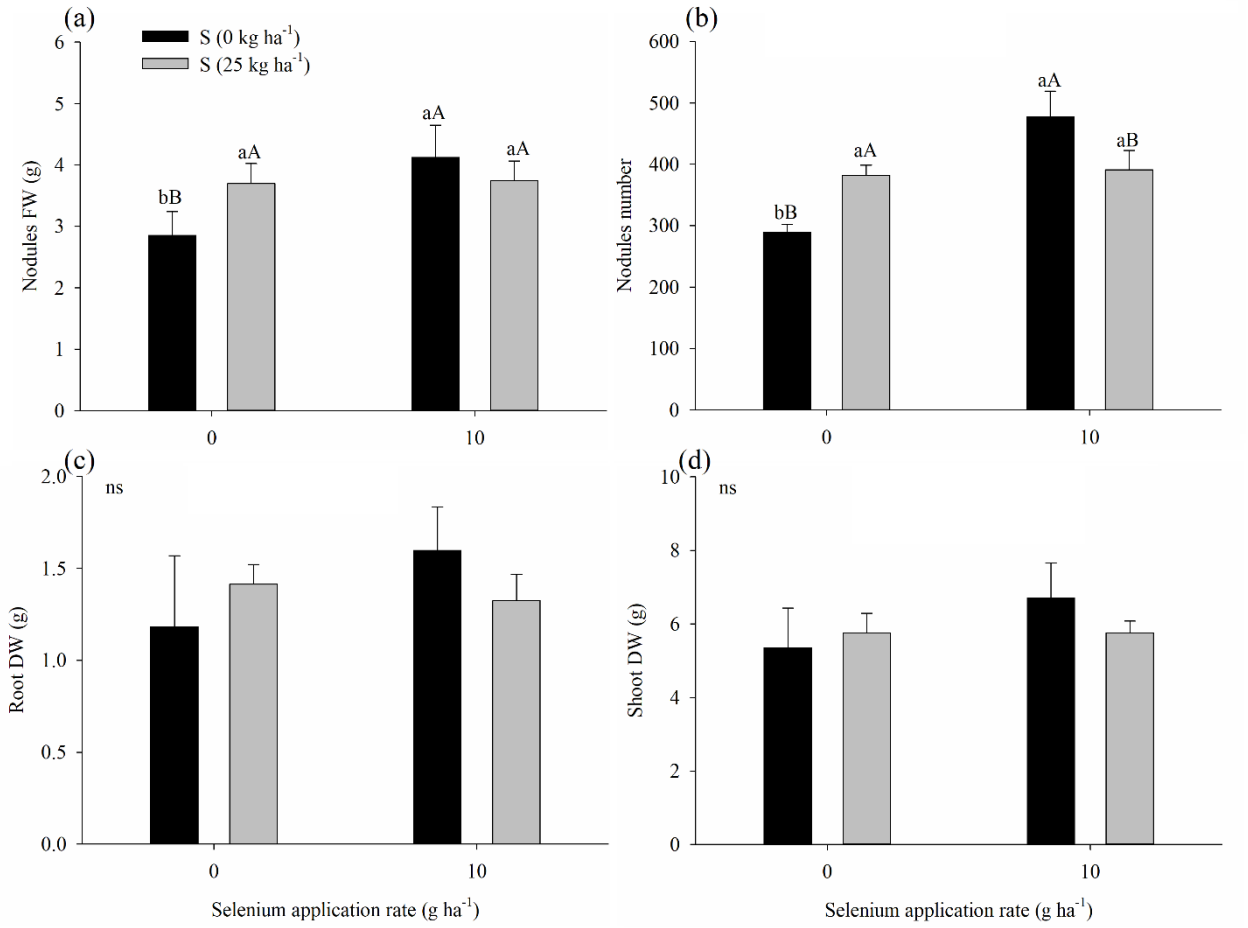

Figure 1. Nodules fresh weight (a), nodules number (b), root dry weight (c), and shoot dry weight (d) on cowpea under sodium selenate and sodium sulfate interaction. Different uppercase letters (for sulfate effect in each selenate application) and lowercase letters (for selenate effect in each sulfate application) indicate the difference between means according to the Tukey test ($p \leq 0.05$). ns = insignificant according to the Tukey test ($p \leq 0.05$). CV (%) = 11.11 (a); 7.33 (b); 17.65 (c); and 13.23 (d).

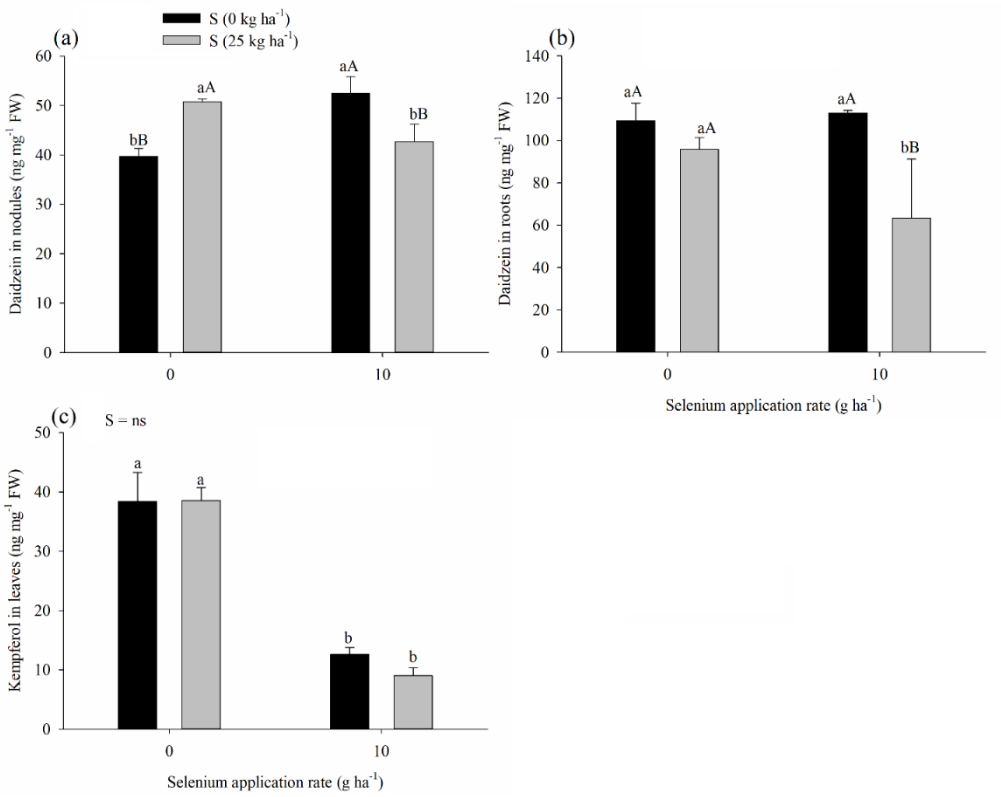

Figure 2. Daidzein in nodules (a), daidzein in roots (b), and kaempferol in leaves (c) on cowpea under sodium selenate and sodium sulfate interaction. Different uppercase letters (for sulfate effect in each selenate application) and lowercase letters (for selenate effect in each sulfate application) indicate the difference between means according to the Tukey test (p≤0.05). ns = insignificant according to the Tukey test (p≤0.05). CV (%) = 5.63 (a); 15.56 (b); and 11.45 (c).

Regarding the relative gene expression, SulTr 1;2 and PAS were affected by Se and S, but not by its interaction, CHS was affected by the S application, while SulTr 2;2, CHR, and APR were affected by Se and S interaction ($p < 0.05$; Table 3). The relative expression of SulTr 1;2 increased by 8% under S application and by 9% under Se application (Fig 3a). Regarding SulTr 2;2, under no Se application, S increased the gene relative expression by 9 times, and at 10 g ha^{-1} Se, S increased SulTr 2;2 relative expression by 16 times, while under S application, Se increased the gene relative expression by 2.6 times (Fig 3b). The sulfur application decreased CHS relative expression by 8%, while Se application did not affect this gene expression (Fig 3c). The relative expression of CHR was enhanced under S application by 11% (under no

Se application) and by 55% (under Se application), while Se provided an increase in the gene expression of 37%, under S application (Fig 3d). For APS, the relative expression increased by 25% under S application and by 9% under Se application (Fig 3e). For APR, S application decreased its gene expression by 15% under no Se application, but under Se application, S did not impair APR gene expression, similar behavior was observed for Se: under no S, Se application decreased APR expression by -14%, but under S application, Se did not impair APR expression (Fig 3f).

Table 3. F ratio and P values of analysis of covariance (ANCOVA), regarding cowpea relative gene expression of High-Affinity Sulfate Transporter (SulTr 1;2), Low-Affinity Sulfate Transporter (SulTr 2;2), Chalcone Reductase (CHR), Chalcone Synthase (CHS), ATP sulfurylase (APS), and ATP sulfurylase reductase (APR).

Source of Variation	F ratio	Pr(> F)
SulTr 1;2		
Sulfur application (A)	7.70*	0.0168
Selenium application (B)	9.15*	0.0106
A*B interaction	2.70^{NS}	0.1264
SulTr 2;2		
Sulfur application (A)	85.55**	<0.0001
Selenium application (B)	21.25**	0.0006
A*B interaction	18.45**	0.0010
CHR		
Sulfur application (A)	1427.21**	<0.0001
Selenium application (B)	523.29**	<0.0001
A*B interaction	624.51**	<0.0001
CHS		
Sulfur application (A)	9.12*	0.0107
Selenium application (B)	4.00^{NS}	0.0686
A*B interaction	3.63^{NS}	0.0810
APS		
Sulfur application (A)	51.58**	<0.0001
Selenium application (B)	8.06*	0.0149
A*B interaction	3.38^{NS}	0.0909
APR		
Sulfur application (A)	0.94^{NS}	0.3525
Selenium application (B)	0.44^{NS}	0.5213
A*B interaction	7.14*	0.0203

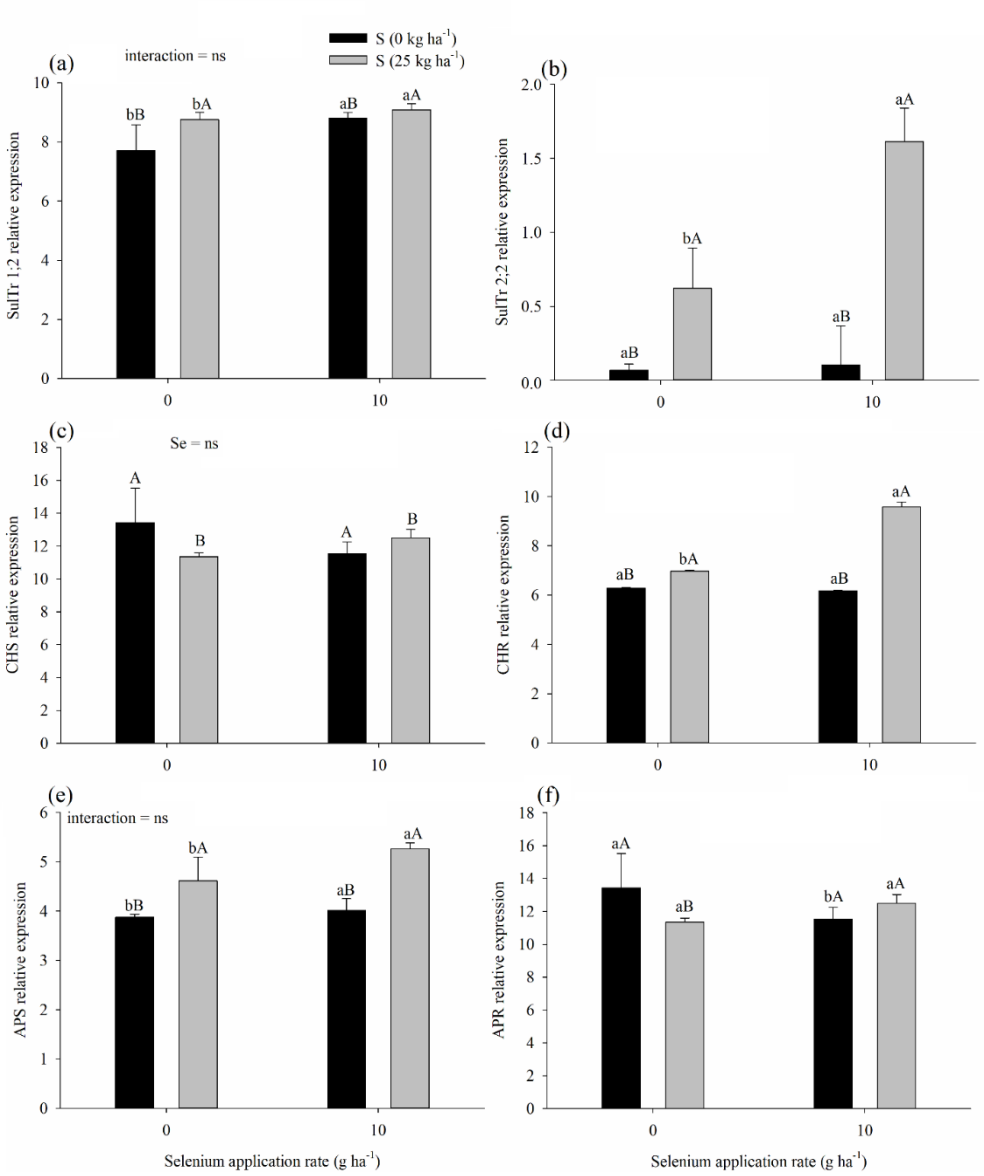

Figure 3. Relative gene expression of SulTr 1;2 (a), SulTr 2;2(b), CHS (c), CHR (d), APS (e), and APR (f) on cowpea under sodium selenate and sodium sulfate interaction. Different uppercase letters (for sulfate effect in each selenate application) and lowercase letters (for selenate effect in each sulfate application) indicate the difference between means according to the Tukey test (p≤0.05). ns = insignificant according to the Tukey test (p≤0.05). CV (%) = 5.51 (a); 37.15 (b); 5.26 (c); 1.50 (d); 6.22 (e); and 9.35 (f).

Cowpea leaf Se concentration was affected only by Se application, while leaf S concentration was affected only by S application. On the other hand, both Se and S root concentrations were affected by selenate and sulfate interaction ($p < 0.05$; Table

4). Leaf S concentration increased by 24% under S application (Fig 4a) while leaf Se concentration was increased by 10.7 times under Se application (Fig 4b). Under no Se application, S increased root S concentration by 51%, while under Se application, S application did not affect root S concentration, while under no S application, Se application increased root S concentration by 29%, and under S application Se also did not affect root S concentration (Fig 4c). Considering root Se concentration, under Se application, S decreased root Se concentration by 18%, Se increased root Se concentration by 72 times under no S application and by 19 times under S application (Fig 4d).

Table 4. F ratio and P values of analysis of covariance (ANCOVA), regarding cowpea Sulfur concentration in roots (SR) and leaves (SL), Selenium concentration in roots (SeR) and leaves (SeL), and Selenocysteine (SeCys), and Selenomethionine (SeMet) in leaves.

Source of Variation	F ratio	Pr(> F)
SL		
Sulfur application (A)	9.81*	0.0140
Selenium application (B)	3.64^{NS}	0.0930
A*B interaction	2.70^{NS}	0.1388
SeL		
Sulfur application (A)	1.88^{NS}	0.2078
Selenium application (B)	202.07**	<0.0001
A*B interaction	2.00^{NS}	0.1950
SR		
Sulfur application (A)	12.83**	0.0072
Selenium application (B)	0.88^{NS}	0.3749
A*B interaction	5.92*	0.0410
SeR		
Sulfur application (A)	8.07*	0.0218
Selenium application (B)	1243.22**	<0.0001
A*B interaction	17.83**	0.0029

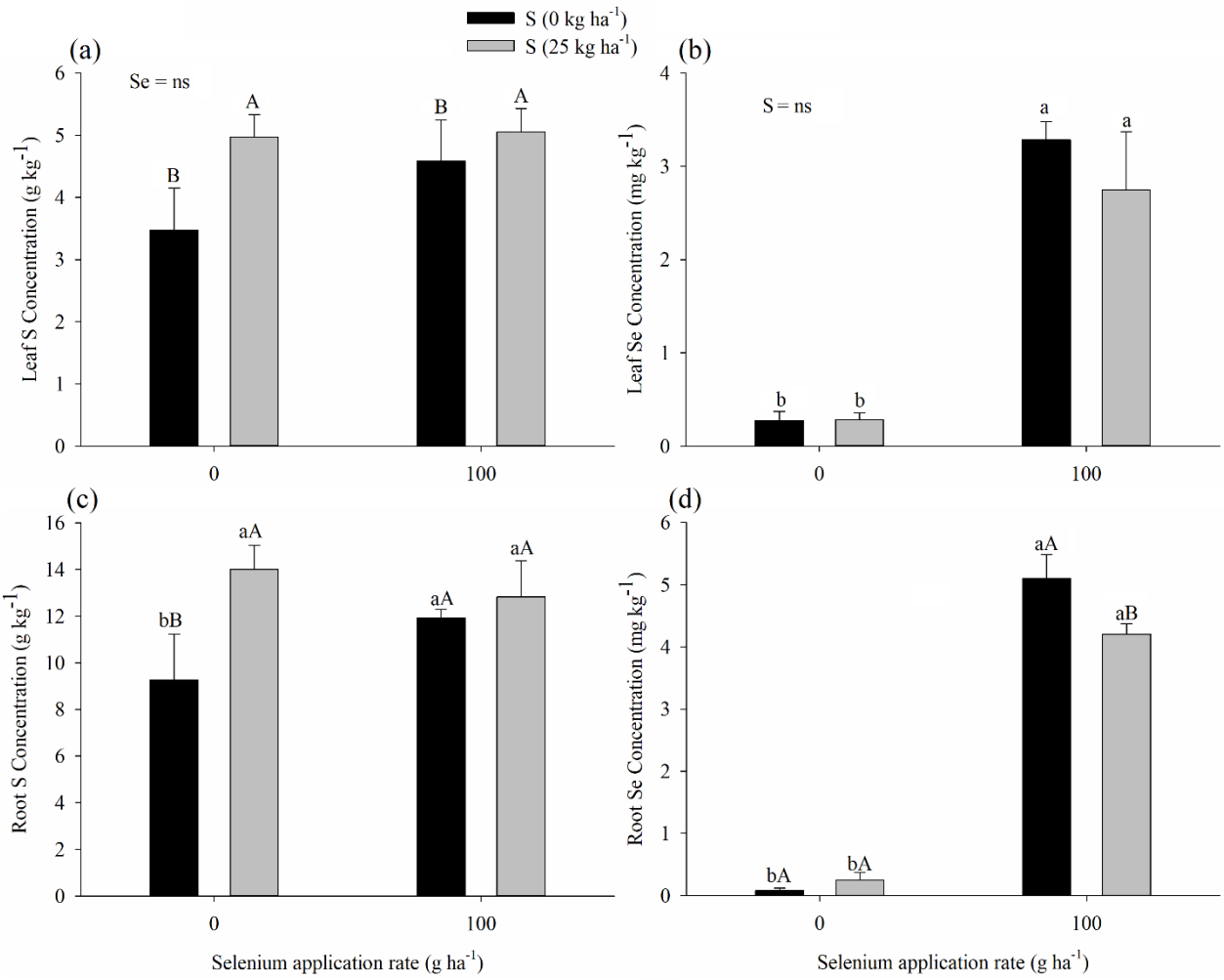

Figure 4. Leaf S concentration (a), leaf Se concentration (b), root S concentration (c), and root Se concentration (d) on cowpea under sodium selenate and sodium sulfate interaction. Different uppercase letters (for sulfate effect in each selenate application) and lowercase letters (for selenate effect in each sulfate application) indicate the difference between means according to the Tukey test (p≤0.05). ns = insignificant according to the Tukey test (p≤0.05). CV (%) = 12.03 (a); 20.23 (b); 11.38 (c); and 9.14 (d).

4. DISCUSSION

In the present study, Se or S application did not affect cowpea growth. Considering Selenium, similar results were observed before in cowpea (Silva et al., 2019) and in rice (Reis et al., 2018). Although Se can enhance plants' growth, as previously observed in soybean under Se application in the flowering stage (Dai et al.,

2020), this effect is more commonly reported in plants under stressful conditions: in wheat under ultraviolet stress, it was reported a yield increase under Se application (Yao et al., 2013), while regarding salt stress conditions, Se can enhance cowpea yield (Manaf, 2018) as well as mungbean (Kaur & Nair, 2015). Those beneficial Se effects on plant development are more common in stressed plants most likely due to Se's role in ROS scavenging systems and oxidant stress mitigation (Silva et al., 2020; Lanza & Reis 2021). It's also noteworthy that phytotoxicity caused by excessive Se is also reported in cowpea (Silva et al. 2018), thus the lack of growth response in the present study suggests that the Se application used is not detrimental to cowpea in the observed conditions.

In general, nodulation was enhanced by Se application while the interaction resulted in a decrease. Selenium effect on nodulation was observed in alfalfa, in addition, Se also provided an increase in sugars metabolism (Owusu-Sekyere et al., 2013; Hajiboland et al., 2014). Sulfur can increase nodule size in soybean (Cigelske et a., 2020) and under S starvation, legume plants present lower nodulation, decreased biological N fixation, and slower nodule metabolism (Becana et al., 2018). In functioning nodules, the bacteroids provide the plant with N-compounds, while the plant provides the bacteriods with photoassimilates that are used to obtain ATP which is used for N_2 fixation (Naya et al., 2007; Vance, 2008). Thus, the Se and S supply may lead to sugars increase, which can enhance nodulation as observed in the present study. The decrease in nodules number observed in the combination of Se and S might be related to Se and S competition uptake (Silva et al., 2022). However, is noteworthy that the nodulation decrease in Se and S combination occurred only compared to the treatment in which only Se was applied, and that the combination still provided higher nodules number than the control (Fig 1b).

Daidzein is an isoflavone that plays a role in nodulation due to inducing nod genes, that produces nodulation factors (Nod Factors), and the secretion of those compounds provides a series of physiological modification in root cells in plants, leading to nodule formation (Bosse et al, 2021). The daidzein concentration in nodules is more likely to be related to the nodulation directly since in nodules the daidzein concentration presents a similar behavior to the number of nodules. On the other hand, daidzein concentration in roots presents a different behavior to the results regarding

nodulation. A chemical gradient is formed in soil, by exudates from roots, such as sugars, amino acids, organic acids, and other metabolites, this gradient attracts rhizobia by chemotaxis (Baldri & Vivanco, 2009). The flavonoids stimulate the *nod* gene expression in rhizobia, which led to root infection and nodule organogenesis (Barbour et al., 1991; Kape et al., 1991; Hirsch, 1992). Thus, is possible that specific regions on root tissue with higher flavonoid concentration provide a higher stimulus to nodule formation, consequently, the root regions with higher daidzein content may undergo modifications to turn into nodules. Kaempferol is a leaf flavonoid not related to nodulation. However, the decrease in kaempferol concentration under Se application did not lead to any detrimental effect on plant growth.

It is well known that Se is transported by sulfur transporters (White, 2016), both Se and S individually increased SulTr 1;2 relative expression, while SulTr 2;2 relative expression was affected by the interaction between both elements, and the increase in its relative expression was more intense compared to SulTr 1;2. SulTr 1;2 is a high-affinity S transporter, which is already highly expressed under lack of S (Shibagaki & Grossman, 2004), in addition, according to some studies, SulTr 1;2 response to S changesin the medium is low (Yoshimoto et al., 2020), this could explain why the gene expression of Sult 1;2 was only creased by 8 and 9% under S and Se application, respectively, and this result is likely not related to substantial changes in those elements concentration in plat leaves. SulTr 2;2 on the other hand, is a low-affinity S transporter (Kirschner et al., 2018), thus its expression is more is upregulated when high levels of S are applied. In previous reports in *Arabidopsis thaliana,* it was observed that under low S application, Se uptake is more at to low S levels (El Kassis, 2007). However, in the transporters observed in the present study, the gene expression was enhanced by both Se and S supply, mainly regarding SulTr 2;2, which was even more enhanced by the combination between S and Se. This indicates that Se supply in the solution provides a similar stimulus to S, considering SulTr 2;2 expressions.

Sulfate application slightly decreased CHS, on the other hand, provided a more relevant increase in CHR gene expression (Fig 3c-d), Both CHS and CHR play a role in isoflavones synthesis, such as genistein and daidzein (Sepiol et al., 2017). The level of kaempferol in leaves decreased under Se application, in contrast to the increase in daidzein levels in nodules, and the increase in CHR gene expression under S

application. It's noteworthy that flavonoids are transported from leaves to roots (Buer et al., 2007), thus is possible that, the gene expression increase in leaves might be related to the daidzein increase in nodules, considering the possibility of flavonoids transport from shoots to roots.

The enzymes ATP sulfurylase (APS) and APS reductase (APR) are crucial to the conversion of S and Se uptake into cysteine and selenocysteine (González-Morales et al., 2017). The expression of APS was upregulated by Se and S application, while APR higher expression was observed in control plants. Although the higher expression of the genes related to these enzymes might be helpful to increase S and Se assimilation in plants, is noteworthy that Se under very high levels could be harmful to the plants (Silva et al. 2018). Is possible that the low APR gene expression, especially under Se application, might be a mechanism to decrease Se assimilation, aiming to decrease Se uptake by plants. Considering that no impairment in development was observed in the present study (Fig 1 and 5), and APS is still being upregulated by both Se and S (Fig 3e), the Se application rate used in this study is not toxic to the plant, since it is still promoting upregulation of genes related to Se assimilation.

The leaf concentrations of Se and S were affected only by Se and S respectively. Thus, although S and Se interaction provided a change in gene expression of transporters (Fig 3a-b), the actual transport of Se and S from roots to leaves was only affected directly by the element applied. The effect of sulfate transporters in selenate uptake by plants was previously observed (White, 2016, Natasha et al., 2018). In previous studies with cowpea crop, it was observed that different Se and S applications combined may or may not lead to Se and S interaction in those elements' concentration in leaves (Silva et al., 2022). On the other hand, root S and Se concentration were affected by Se and S interaction (Fig 3c-d): while Se lead to an increase in S concentration, S provided a decrease in Se concentration. In soybean, 100 mg kg^{-1} S inhibits Se uptake (Deng et al., 2020), while in repressed, the application of 60 kg ha^{-1} of S as sulfate can inhibit Se concentration in plants (Liu et al., 2017). Selenate and sulfate share the same transporters in plants (White, 2016), and considering the higher content of sulfate available compared to selenate, the saturation of transporters with S might led to a decrease in Se uptake, leading to a

decrease in Se concentration in roots. Is also noteworthy that besides the decrease in Se concentration in roots due to S, this element application did not impaired leaf transporters' gene expression (Fig 1a-b), which might explain why S application did not impair Se concentration in leaves.

5. CONCLUSION

Selenate and sulfate application in the present study did not affect cowpea growth, indicating that the Se application is not unsafe for the plant's development under the present condition. Although Se application was not detrimental to S accumulation in plants, S application impaired Se accumulation. In addition, a general increase in nodulation and flavonoids cowpea under Se application without additional S, but under interaction a decrease in nodulation was observed, indicating that while Se is beneficial to legume plants' nodulation, the interaction with Sulfate could hinder this effect.

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FINAL CONCLSIONS

1. GENERAL CONCLUSIONS

Selenium application in plants is performed usually with biofortification as well as to promote plant's tolerance to oxidative stress. However, the present thesis demonstrates the Se could be beneficial to cowpea in many ways when applied as selenate. At application rates as low as 2.5 g ha^{-1}, Se as selenate resulted in an increase in sugar content and pigment concentration in cowpea leaves, in addition to providing a typical antioxidant effect by enhancing Glutathione reductase activity. Many genotypes of cowpea presented a response to Se application by increasing Se partitioning to shoots, grains yield, sugar yield in grains and ureides concentration in leaves. The effect of Se on flavonoid biosynthesis in cowpea roots led to nodulation enhancement, reinforcing the previously mentioned result of ureides increase in leaves. All these results vary among genotypes and the data registered in this thesis could be used for breeding programs aiming to enhance cowpea response to Se application.

Although greenhouse experiments resulted in Sulfate impairing nodulation in cowpea roots, the interaction between S and Se might be helpful for cowpea production. In Brazil, cowpea mean yield varies between 200 and 1400 kg ha-1 depending on the region (CONAB, 2023), but in the present study, in field conditions, the combination of selenate and sulfate at application rates of 10 g Se ha-1 and 30 kg S ha-1 might result in a grain yield of 2500 kg ha-1. This crop is still widely cultivated in low-income regions, without the application of fertilizers in low technical conditions, but its cultivation has been expanding in the past years, and the results presented in

this thesis might be a starting point to further investigate Se and S effect on cowpea to improve this crop yield.

In general, the present study compiles a series of results that could be used in future research to improve cowpea response to Se, a better understanding of Se and S interaction as well as to improve cowpea development and yield.

2. REFERENCES

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