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

SELENIUM ABSORPTION KINETICS AND LOCALIZATION IN COWPEA GENOTYPES USING XRF TECHNIQUE ASSOCIATED WITH PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES

Maria Gabriela Dantas Bereta Lanza
Biosystems Engineering

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DADOS CURRICULARES DO AUTOR

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

- Marie Curie,
Physicist, chemist, and winner of the 1903 and 1911 Nobel Prize in Physics

DEDICO

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ANALYSIS OF SELENIUM ABSORPTION KINETICS AND LOCALIZATION IN COWPEA GENOTYPES USING THE XRF TECHNIQUE ASSOCIATED WITH PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES

RESUMO – O selênio (Se) é um elemento essencial para humanos e animais e benéfico para as plantas. Os solos são pobres em Se, tornando a dieta da população deficiente. A biofortificação agronômica é uma estratégia para aumentar os teores de Se nas plantas. Nesse trabalho, avaliou-se a eficiência da suplementação mineral dependente de fatores genotípicos e da fonte adequada de Se. Foram conduzidos 3 experimentos, sendo 2 experimentos preliminares, e 1 experimento crítico. No experimento 1 avaliou-se concentrações de Na_2SeO_4 (0, 20, 40, 80 e 120 µM) em feijão-caupi (*Vigna unguiculata* [L.] Walp cv. BRS Rouxinol), a fim de determinar a condição com melhor detecção de sinal para a avaliação *in vivo* das plantas pela técnica de fluorescência de raios x (XRF). No experimento 2, a melhor concentração resposta (40 µM) foi utilizada para avaliar a eficiência de absorção de Se de 20 genótipos comerciais de feijão-caupi, por meio da concentração de Se na parte aérea e raiz pela técnica de fluorescência de raios-X dispersiva de energia de bancada (EDXRF), afim de determinar genótipos com capacidade de absorção contrastante. No experimento 3, selecionou-se o genótipo com maior (BRS Xiquexique) e menor (BRS Urubuquara) capacidade de absorção de Se avaliando as respostas fisiológicas da aplicação de duas fontes de Se (Na_2SeO_4 e Na_2SeO_3), afim de identificar a fonte mais eficiente para a absorção e translocação de Se para a parte aérea das plantas, visando a geração de plantas com maior valor nutricional. Os resultados demonstram a presença de variação genotípica na cultura do feijão-caupi, e relaciona a capacidade de plantas eficientes em translocar Se das raízes para a parte aérea com uma alta eficiência do metabolismo fotossintético. Além disso, a fonte Na_2SeO_4 demonstrou ser uma ótima alternativa para garantir a translocação de Se raiz-parte aéra de plantas de feijão-caupi.

Palavras-chave: biofortificação agronômica, fluorescência de raios x, selenato, selenito, variação genotípica, *Vigna unguiculata,*

Selenium absorption kinetics and localization in cowpea genotypes using XRF technique associated with physiological and biochemical responses

ABSTRACT – Selenium (Se) is an essential element for humans and animals and is beneficial to plants. Soils are poor in Se, making the population's diet deficient. Agronomic biofortification is a strategy to increase Se levels in plants. In this work, we investigated the efficiency of mineral supplementation dependent on genotypic factors and the adequate source of Se. Three experiments were conducted, two preliminary experiments and one critical experiment. In experiment 1, concentrations of Na_2SeO_4 (0, 20, 40, 80, and 120 µM) in cowpea plants (*Vigna unguiculata* [L.] Walp cv. BRS Rouxinol), were evaluated to determine the best concentration-response for the in vivo evaluation of plants using the X-ray fluorescence (XRF) technique. In experiment 2, the best concentration-response (40 µM) was used to evaluate the Se absorption efficiency of 20 commercial cowpea genotypes, using Se concentration in shoots and roots by the X-ray fluorescence technique bench energy dispersive (EDXRF) to determine genotypes with contrasting absorption capacity. In experiment 3, the genotype with the highest (BRS Xiquexique) and lowest (BRS Urubuquara) Se absorption capacity was selected. Moreover, evaluating the physiological responses of the application of two Se sources (Na_2SeO_4 and Na_2SeO_3), to identify the most efficient source for the absorption and translocation of Se to the shoot of the plants, aiming at the generation of plants with greater nutritional value. Our results demonstrate the presence of genotypic variation in the cowpea crop and relate the ability of efficient plants to translocate Se from roots to shoots with the high efficiency of photosynthetic metabolism. Furthermore, the Na_2SeO_4 source proved to be an excellent alternative to ensure the root-shoot Se translocation in cowpea plants.

Keywords: Agronomic biofortification, x-ray fluorescence, selenate, selenite, genotypic variation, *Vigna unguiculata*.

CHAPTER 1 – Roles of selenium in mineral plant nutrition: physiological and biochemical responses

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ABSTRACT

Agronomic biofortification of crops with selenium (Se) is an important strategy to minimize hidden hunger and increase nutrient intake in poor populations. Se is a nutrient that has several physiological and biochemical characteristics, such as the mitigation of different types of abiotic stress. Selenoproteins act as powerful antioxidants in plant metabolism through the glutathione peroxidase (GSH) pathway, and provide an increased activity for enzymatic (SOD, CAT, and APX) and non-enzymatic (ascorbic acid, flavonoids, and tocopherols) compounds which combat reactive oxygen species (ROS) and cell detoxification. Se can mitigate the damage caused by climate changes such as drought, salinity, heavy metals, and temperature. Also, it is related to the regulation of the antenna complex of photosynthesis, protecting chlorophylls by raising photosynthetic pigments. However, Se concentrations in soils vary widely in the earth's crust. Soil availability influences the concentrations in food, in addition to the capture, transport, accumulation, and speciation of Se in plants. The foliar application of Se until the concentration of 50 g ha^{-1} in the sodium selenate source increases the antioxidant, photosynthetic metabolism, and productivity of the cultures. The foliar application of Se is a strategy to minimize soil adsorption and root accumulation. However, the limit between the beneficial and toxic effects of Se requires research to establish an optimal concentration for each plant species in different edaphoclimatic conditions. In this review, we present the compilation of several studies on agronomic biofortification of plants with Se to ensure food production and food security to mitigate hidden hunger and improve the health of the population.

Keywords: abiotic stress, antioxidant metabolism, food security, biofortification, hidden hunger, selenium.

1. INTRODUCTION

According to FAO (2017), there are about 2 billion people with nutritional deficiencies in the world. The nutritional deficit is caused by the production of staple foods in soils with low mineral availability. The deficiencies caused by the lack of iron (Fe), iodine (I), selenium (Se), zinc (Zn), and vitamin A are currently the ones that cause the greatest concern regarding human health, especially in developing countries (White, 2016).

Eating vegetables rich in Se can be a safe way to combat Se deficiency in humans, however, most soils are considered poor in Se, and nutrient absorption depends on several factors that reduce their incorporation. (Yang et al., 2019). Agronomic biofortification is an agricultural practice used to enrich food production with nutrients, such as Se, to increase food intake by the population (Adebayo et al., 2020).

The application of exogenous Se can increase the concentration of Se in the edible parts of plants (Oliveira et al., 2019). Se is beneficial for crop productivity, promoting increased photosynthesis by increasing secondary metabolites and accumulating carbohydrates (Andrade et al., 2018). Also, their synergistic and antagonistic relationships with other nutrients favor plant nutrition and mitigate abiotic stresses such as drought, high temperatures, salinity, and heavy metals (Gupta and Gupta, 2017).

According to Hartikainen et al. (2000), selenium has a wide antioxidant capacity due to the promotion of selenoproteins such as glutathione peroxities, which act to combat reactive oxygen species (ROS) promoted in plant osmotic imbalance under stressful situations. It may be an osmoprotective strategy to mitigate the harmful effects of abiotic stresses such as drought (Rady et al., 2020), salinity (Habibi, 2017), heavy metals (Shekari et al., 2019), temperature (Seliem et al., 2020) and light (Jaiswal et al., 2018).

Excess Se can be toxic to plants and humans (Gouveia et al., 2020; Martens et al., 2015). Characterized by the presence of chlorosis in the leaves of vegetables, and hormonal changes in the human body (Vinceti et al., 2018; Lanza et al., 2021). The determination of ideal doses and sources of Se is extremely important to ensure the food security of the population through the consumption of legumes (Silva et al., 2018),

cereals (Lara et al., 2019), fruits (Deng et al., 2019), vegetables (Tian et al., 2018), and even medicinal plants used as herbal medicines (Stonehouse et al., 2020).

Thus, the present literature review seeks to present the most recent discoveries about Se, analyzing its reactions in different living organisms and their interactions with the abiotic environment. Demonstrating that the element is widely explored as a strategy to increase the metabolism of plants, but that it also has several opportunities and challenges to be unveiled.

2. AVAILABILITY AND SE DISTRIBUTION IN SOIL, PLANT, ATMOSPHERE, AND HUMANS HEALTHY

Se concentration generally ranges between 10-200 µg kg^{-1} in most non-seleniferous soils (Yang et al., 2019). Thus, many factors can affect the content of Se in different foods, including different rates of absorption by plants, which may be related to the type of plant species, soil, pH, microbial activity, precipitation, and some other biogeochemical parameters (Reis et al., 2017)**.**

The biogeochemical cycle of Selenium is based on its availability to plants by removing the available element from the soil, and consequently the absorption in their tissues and translocation to the edible parts of food. There is also the possibility that the element can be converted into volatile metabolites and enter the atmosphere, being able to return to vegetables through the leaf surface. Anthropogenic activities are a major source of Se availability in the atmosphere, such as burning fossil fuels and forest fires (Terry et al., 2000; Combs, 2001; Gupta and Gupta, 2017).

The antioxidant role of Se helps to improve the immune system of patients, minimizing the risk of infections, protecting the body against the action of molecules that damage metabolic functions, and mainly helping to fight free radicals and prevent cardiovascular diseases (Adebayo et al., 2020). Recently, adequate levels of Se have been associated with reduced mortality risks from the coronavirus disease pandemic (COVID-19), where Se status was higher in patients who survived SARS-CoV-2 infection (Moghaddam et al., 2020).

The presence of Se in soils around the world is highly variable, with poor soils in Se (0,01 e 2 mg kg^{-1}) or seleniferous soils (<100 mg kg^{-1} de Se) (Chauhan et al., 2019). Brazil is a country with a vast territorial extension and wide biodiversity,

reflecting that the levels of Se in soils of only one state (São Paulo) can vary from 10-650 μg kg^{-1} (Reis et al., 2017). In China, 72% of the mainland is reported to suffer from Se deficiencies, which is associated with an increase in cardiovascular and osteopathic diseases. The low presence of Se in the soil (0.10-87.3 mg kg^{-1}) is reflected in an average intake below 10 μg day^{-1} (Lin et al., 2017)

Africa is estimated to have 28% Se deficiency in its total area, and studies carried out on the African continent also highlight that the Se deficiencies found in the population also involve socio-economic factors, where a large part of the Se sources are found in foods with high added value such as meat, nuts, and seafood. And because of this, given that a large part of the continent feeds primarily on vegetables, such as rice, corn, potato, and cassava, the low levels and Se in the soils are combined with the increase in cases of infection and low immunity in the country (Ligowe et al., 2020).

According to Combs (2001), foods need to provide content greater than 40 μg per day of Se for the element to be used as a cofactor for antioxidant enzymes, and values above 300 μg per day to reduce the risks of severe diseases, such as cancer. However, high values can induce Se toxicity and may vary about the absorption of these nutrients in each food, and their absorption in human metabolism.

A study conducted in Brazil induced children to eat 15-30 g of Brazil nuts (three to six nuts), which is the amount allowed by public health policies. However, the results of Se concentrations found in plasma and urine were above the allowed, with 155.3 μg day^{-1} for the city of Macapá and 44.4 μg day^{-1} for Belém (Martens et al., 2015). On the other hand, in India, it has been found that the consumption of food grown in seleniferous soils causes dystrophic changes in the nails, hair loss, strong odors, dysfunctions in the liver, kidney, and pancreas (Vinceti et al., 2018).

3. UPTAKE, TRANSPORTATION, AND ACCUMULATION OF SE IN PLANTS

In nature there are organic and inorganic forms of Se, however, the elemental bioavailability has a direct influence on the absorption and translocation of the element in plant species. The organic forms most found in soils are selenate and selenite. Several studies demonstrate the efficiency of application of both forms via soil and report that the best form of application to increase the Se content in grains is selenate,

because selenite is more adsorbed on soil particles (Deng et al., 2019; Silva et al., 2019).

Hawrylak-Nowak et al. (2015) demonstrated that while selenite accumulate to a higher concentration in roots (967.2 mg kg^{-1} DW) compared to shoots (120.6 mg kg^{-1} DW), selenate had a translocation factor of Se of approximately 8 times greater.

Also, studies evaluating the absorption, translocation, and speciation of Se in wheat (*Triticum aestivum*) revealed by the analysis of plant tissues that plants supplemented with sodium selenate have primarily the same elemental form in the xylem, while plants with the application of sodium selenite generated several compounds such as SeMet, SeOMet, and MeSeCys in the roots. In the same study, it was noticed that plants supplemented with selenate or selenite have an inversely proportional relationship with sulfur (S) and phosphorus (P), respectively (Li et al., 2008).

3.1 Selenium supply by soil or foliar

Selenate is the main soluble form of Se in soils and, due to its chemical similarity to sulfur (S), it is transported as via sulfate transporters (SULTRs) present in the root plasma membrane. Where it is translocated to leaves and synthesized in plastids (Gup and Gupta, 2017). Selenite is absorbed by phosphate transporters and aquaporins (OsNIP2; 1) (Zhao et al., 2010). According to (Oliveira et al., 2019), selenate has greater mobility within the plant, in contrast to selenite, which is more concentrated in the root system instead of being transported to the shoot due to the rapid transformation into organic forms of Se.

The reduction of selenate occurs through a series of consecutive steps that convert selenate> selenite> selenide> Secys. Figure 1A illustrates the scheme of the metabolic reduction of inorganic forms of Se and its conversion to organic forms from the soil. Selenate is converted to selenite through the enzymes ATP sulfurylase (APS) and APS reductase (APR). Then, selenite becomes Se^{2-} through the enzymes sulfite reductase, glutathione, or glutaredoxins. The Se^{2-}, in turn, is then converted to SeCys by coupling with O-acetylserine (OAS) in the presence of a cysteine synthase enzyme. The last stages of synthesis are responsible for the formation of elementary Se, methyl-selenocysteine (Me-SeCys), and selenomethionine (SeMet), the latter being

responsible for the origin of selenoproteins (Schiavon et al., 2017). In plants, selenoproteins act in the transport of Se and regulation of cellular redox balance, the main example of this class being glutathione peroxidase (Malik et al., 2011)

Scientific advances point to problems in Se supplementation via soil, indicating that the element is not efficiently absorbed by plants and indicating economic losses with the application (Deng et al., 2019). Selenate is more absorbed by plants than selenite. Selenite is strongly adsorbed on soil components such as Fe / Al oxyhydroxides, having low mobility in the soil (Lopes et al., 2017).

The application of Se via foliar provides the deposit of droplets containing elements on the leaves, which in turn allows entry through the various physiological barriers of the plant. The main pathway for nutrients to enter the leaves is through a passive process due to difference in concentration, which occurs on the external surface, where there is a higher concentration of solute, towards the internal, with a lower concentration of solutes. Selenite passively enters wheat leaf blades via cuticle and stomata, and then enters mesophyll cells via Pi transporters (Yang et al., 2019).

Although entry through leaf cuticles is the process with the highest absorption capacity, according to Wang et al. (2016), elementary particles, such nanoparticles (NPs), can enter plant tissues through either the root tissues or the aboveground organs and tissues (e.g., cuticles, trichomes, stomata, stigma, and hydathodes), including through wounds and root junctions. The cuticles have limitations regarding the size of the particles, and the entry through the stomata depends on stomatal permeability, and as a consequence of the opening and closing of the stomata, in addition to the hydraulic conductivity of the cells. (Yang et al., 2019).

4. PHYSIOLOGICAL AND BIOCHEMICAL ROLES OF SE IN PLANTS: ANTIOXIDANT METABOLISM RESPONSES

Plants subjected to high-stress intensities produce high amounts of ROSs, which can produced as a superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl free radical ($OH\cdot$), singlet oxygen ($^1O_2$), methyl radical ($CH_3\cdot$) and lipid peroxidation free radicals ($LOO\cdot$, $ROO\cdot$) (Feng et al., 2013).

ROS are molecules that are part of the metabolic daily life of cells, and when produced properly do not present cellular risks. Under stress conditions, there is an

overproduction of ROSs promoting the oxidation of plant cell membranes promoting cell damage (Foyer and Noctor, 2012). These molecules are formed from the transfer of one ($O_2^{\cdot-}$), two (H_2O_2) or three ($OH\cdot$) electrons for the O_2 molecule (Schiavon et al., 2017).

In this sense, oxidative stress occurs when there is an imbalance of antioxidant activity and the production of ROS, and these elements overlap the defense mechanisms, causing damage to cellular components, such as lipids, nucleic acids, metabolites, and proteins, and can cause cell death in plants (Xie et al., 2019). The production of ROS in chloroplasts can vary from low levels (<240 μM s^{-1} $O_2^{\cdot-}$ e 0,5 μM de H_2O_2), down to levels under high stress concentrations (240–720 μM s^{-1} $O_2^{\cdot-}$ e 5–15 μM H_2O_2) (Mittler, 2002).

High concentrations of ROS cause the peroxidation of membrane lipids, and one of the main meters is through the quantification of malondialdehyde (MDA), which is a widely used marker of oxidative lipid damage caused by environmental stress. Also, 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) are other useful markers to assess the fight against oxidative stress in plant metabolism (Silva et al., 2018).

Several studies demonstrate the ability of Se in low concentrations to improve plant defense systems by detoxifying intracellular free radicals, and increasing enzymatic and non-enzymatic activity, which can help plants eliminate ROS and prevent oxidative stress (Schiavon et al., 2017; Silva et al., 2018).

According to Chauhan et al. (2019), selenium can mitigate oxidative stress by regulating ROS, acting in the following ways: (1) stimulation of $O_2^{\cdot-}$dismutation into H_2O_2, (2) regulation of enzymatic and non-enzymatic antioxidant systems, (3) direct extinction of ROS through Se species, and (4) regulation of photosynthetic complexes.

Non-enzymatic molecules act in minimizing ROS and are also important for preserving the cellular redox state, being of the type: glutathione (GSH), ascorbate, phytochelatins (PCs), proline, flavonoids, alkaloids, and carotenoids (Foyer and Noctor, 2012).

The two amino acids produced in Se biosynthesis via the S assimilation pathway are selenocysteine (SeCys) and selenomethionine (SeMet). Cysteine is a component

of glutathione (GSH), a central molecule in plant responses to various types of stress, while Met is a precursor to aliphatic glucosinolates, which are compounds involved in plant-pathogen / herbivore interactions (Schiavon et al., 2017).

According to Hunter (2014), the antioxidant capacity of Se is incorporated into the mechanism of glutathione peroxidase (GSH) in the reduction of hydrogen peroxide through the use of ascorbate (AsA). The GSH-Px enzyme is key that can be strongly activated by Se in several plants exposed to different environmental stresses (Feng et al., 2013). The increase in GSH-Px, which sequesters of H_2O_2 and lipid hydroperoxides, results in a reduction in the formation of superoxide anions ($O_2^{\cdot-}$) among oxygen species (Hartikainen et al., 2000).

High stressful conditions can induce a significant reduction in the levels of GSH, and consequently the enzyme GSH-PX, making it viable via Ascorbate-Glutathione, and it is necessary to use antioxidant enzymes to reestablish the metabolism, as occurs with SOD activity (Hartikainen et al., 2000). Thus, the ideal levels of Se primarily induce antioxidant activity through spontaneous reduction of $O_2^{\cdot-}$ by GSH-Px, however, under severe stress, high levels of ROS will be produced, requiring specific enzymatic activities, such as SOD, and subsequently of other enzymatic and non-enzymatic antioxidants (Feng et al., 2013).

4.1 Selenium against lipid peroxidation – Non-enzymatic antioxidants

In addition to being a component of the non-enzymatic process of ROS elimination, the forms of ascorbate (AsA) in plants suggest a multitude of benefits, such as accumulation of secondary metabolites, growth control, sugar increase, and control pathogens. The application of Se influences the increase in the accumulation of AsA mobility, resulting in high elimination of H_2O_2 through various reactions such as maintenance of reduced α-tocopherol, zeaxanthin biosynthesis, reduction of the state of prosthetic metal ions, and maintenance of activity of antioxidant enzymes (Foyer and Noctor, 2012; Hasanuzzaman et al., 2019).

According to Hartikainen et al., (2000) tocopherols, popularly known as vitamin E, can eliminate the radicals of lipid peroxide ($LOO\cdot$), in addition to their performance in cooperation with selenium to positively influence the detoxification of $O_2^{\cdot-}$. Thus, the

main antioxidant function of vitamin E is to protect the phospholipid bilayer of the cell membrane, preventing lipid peroxidation (Hunter, 2014).

Carotenoids can directly eliminate free radicals and extinguish species of $O_2^{\cdot -}$ (Hunter, 2014). In plants, carotenoids participate in the maximum photochemical efficiency of PSII, indicating their photoprotective action during photosynthesis (Habibi, 2017). For human health, lycopene and β-carotene are considered major natural antioxidants, acting as inhibitors of the most important carotenoids and acting as inhibitors of $O_2^{\cdot -}$ and protecting cells against free radicals (Morales-Espinoza et al., 2019).

The Se affects the metabolism of nitrogen biosynthesis, proteins, and amino acids, and, in particular, the amino acid phenylalanine which is a precursor to phenolic compounds, such as flavonoids (Golubkina et al., 2018). Flavonoids have been widely recognized as beneficial antioxidants due to their ability to capture free radical ions and protect plants from the adverse effects of abiotic stress (Chauhan et al., 2017; Jaiswal et al., 2018).

Oliveira et al. (2019) noted that increasing proline content is a process that plants use to adapt to abiotic stress, reducing excess free radicals. Thus, several studies claim that proline can be used as a strategy to maintain the fluidity of the membrane during water stress (Rady et al., 2020). Or, also, act as a stress signal, or as a metal chelating osmolyte (Dai et al., 2020)

Sugars are used as biomarkers of ROS, and in large quantities, can influence the increase in cytosolic H_2O_2, but in ideal doses, it works to combat oxidative stress, mainly through the pentose phosphate (PPP) pathway. Se also increases a very important element of analysis, especially for fruits, which are total soluble solids (TSS), and which are composed of sugars, playing an important role in metabolism, photosynthesis, and carbohydrate production (Golubkina et al., 2018; Gouveia et al., 2020).

4.2 Selenium against lipid peroxidation – Enzymatic antioxidants

Superoxide dismutase (SOD) can be considered the first enzymatic barrier against oxidative stress by the $O_2^{\cdot -}$ dismutation reaction to form O_2 and H_2O_2. Thereafter, the H_2O_2 can be quickly converted to H_2O and O_2 by the direct or indirect

action of enzymes such as catalase, guaiacol peroxidase (GPX), ascorbate peroxidase (APX), guaiacol peroxidase (GPOX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and glutathione S-transferases (GST) and non-enzymatic antioxidants that are ascorbic acid, glutathione, carotenoids, tocopherols, proline, glycine betaine and flavonoids (Schiavon et al., 2017; Xie et al., 2019).

Specifically, CAT is an enzyme that accelerates the breakdown of H_2O_2 to H_2O and O_2 due to the high affinity with this substrate. While APX uses ascorbate as an electron donor to reduce H_2O_2 to H_2O and $O_2^{\cdot-}$. Thus, the GR maintains an intracellular reduction state by converting glutathione disulfide (GSSG) to reduced glutathione (GSH) (Reis et al., 2017; Silva et al., 2018).

4.3 Selenium in chlorophyll and photosynthesis

The application of low concentrations of Se can improve the photosynthetic process by increasing the production of chlorophyll (Ashraf et al., 2018; Hemmati et al., 2019; Seliem et al., 2020), stomatal conductance (Bian et al., 2020; Elkelish et al., 2019; Oliveira et al., 2019), and internal CO_2 concentration (Zhang et al., 2020).

The benefits of Se in photosynthesis may be related to its relationship with the Fe-S complex in chloroplasts, which have a vital role in the electron transport chain, making the high excitations of electronic levels have enough substrates to remain organized. This process helps to reduce the generation of ROSs, mainly $O_2^{\cdot-}$ and H_2O, in plants subjected to stress (Feng et al., 2013).

When plants are subjected to environmental stress, their chloroplasts are damaged, leading to the interruption of photosynthesis. However, adding appropriate levels of Se can reduce damage to chloroplasts and increase the content of chlorophyll and other photosynthetic pigments that act as non-enzymatic antioxidants, such as carotenoids (Hasanuzzaman et al., 2019). Chloroplasts and peroxisomes are the main generators of ROS in the presence of light, while mitochondria are the main sources of ROS production in dark conditions (Xie et al., 2019).

The experiments of Silva et al. (2020) suggest that the application of Se promotes a rearrangement of the antenna complex to improve energy uptake and

protect the system against oxidative stress, with the application of selenite providing greater production of Chl b, while that of carotenoid selenate.

According to Mittler (2002), antioxidants influence the molecular mechanisms to regulate the photosynthetic apparatus and the antenna complex according to the quality and intensity of the light, and may even promote the suppression of photosynthesis. In the chlorophyll cycle, the proportion of chlorophylls can change to stabilize the antenna system, given that each photosynthetic component can absorb light in a different spectrum, and therefore, plants can use Se as a precursor of this system of adaptation to stressful situations (Chauhan et al., 2017).

Photosynthetic pigments act as protectors of light capture, preserving chlorophylls, which are the central molecules of photosynthesis, from undergoing disruption and degradation of their structures. The synthesis of these metabolites (anthocyanin, ascorbates, and carotenoids) provides that plants can carry out their photosynthetic cycle, and provide the plant with the energy necessary to complete their life cycle. The result of the action of these compounds is seen in increased synthesis of sugars and carbohydrates, proteins, productivity, among others (Saleem et al., 2020; Schiavon et al., 2020).

5. SELENIUM AS MITIGATION OF ABIOTIC STRESSES

Plants are sessile beings that are exposed to various stresses from the environment, such as salinity, drought, contrasting temperatures, concentrations of toxic elements, and excess light. Vegetables express complex strategies to release damage and return to their homeostasis as a way of mitigating stress (Xie et al., 2019). In the following section, the role of Se as a mitigator of different abiotic stresses will be specifically addressed (Table 1), its main routes of action according to the intrinsic characteristics of each stress-promoting source in plants.

5.1 Drought

Plants have physiological mechanisms to acclimate to water stress or even to resist periods of water deficiency, such as stomatal closure. This process is responsible for reducing the rate of CO_2 assimilation and the stomatal conductance (Gs) that is directly linked to the degradation and fragmentation of chlorophyll, caused

by the overexpression of the enzyme Rubisco, caused by the photorespiration process (Andrade et al., 2018). Antioxidants, such as Se can protect chloroplasts and increase chlorophyll content, regulate redox reactions, and stimulate the action of antioxidant enzymes (Rady et al., 2020).

However, the availability of nutrients for plants under water restriction is another challenge to be overcome, since, under conditions of water stress, the roots are unable to absorb many nutrients from the soil due to the decrease in root activity, which is associated with the decreased movement of water towards the roots and the slower ion diffusion (Andrade et al., 2018).

Rady et al. (2020) demonstrated that the application of 20-40 mM of selenate to tomatoes subjected to irrigation with only 60% of the field capacity resulted in a significant increase in photosynthesis quantification parameters, such as the efficiency of photosystem II (Fv / Fm), the rate photosynthetic (Pn), and the SPAD index, which is relative to the chlorophyll content present in the leaves. Also, Se induced an increase in non-enzymatic antioxidants (water content, lycopene, total soluble sugars, proline, AsA, GSH, and α-tocopherol) and enzymatic (SOD, CAT, and APX), resulting in a decrease in $O_2^{\cdot-}$, H_2O_2, and MDA.

Canola submitted to severe water deficit during the development stage showed a reduction in plant size (Table 1), while the application of Se in the dosage of 30 g L^{-1} of Se in the sodium selenate source induced gains in productivity (plant height, number of pods and seeds, the weight of 100 seeds, seed yield and seed oil yield). This result is explained by the increase in photosynthetic efficiency (chlorophylls a and b) and of substances such as proline (Hemmati et al., 2019).

Cucumber seeds treated with sodium selenite demonstrated the narrow limit between the benefits and toxicity of Se and its influence on the generation of ROSs (Table 1). The application of Se in low concentrations (1 and 5 µM) increases the antioxidant capacity through the action of the enzymes SOD, CAT, and APX, and reduces the ROS ($O_2^{\cdot-}$, H_2O_2, $OH\cdot$ and MDA), while Se in high concentrations (10 µM) increases lipid peroxidation (Jóźwiak and Politycka, 2019).

The application of Se as a mitigator for water stress, in addition to increasing physiological characteristics, is also demonstrated as a commercial strategy. The treatment with 100 mg L^{-1} of Se in the sodium selenate source increased molecules

such as chlorophylls, carotenoids, phenols, and SeMet, and provided an increase in the antioxidant capacity of extra virgin olive oil (Table 1), by increasing oxidative stability of the product, favoring shelf life for commercialization (D'Amato et al., 2018).

5.2 Salinity

According to Ashraf et al., (2018), the use of mineral elements is an approach widely used by researchers to improve tolerance to salinity in crops. In the studies conducted by the authors, the application of 20 and 40 mg L^{-1} of Se in the sodium selenate source via leaf decreased the accumulation of toxic Na^+ in different parts of the plant and, thus, regulated important physicochemical attributes under salinity (Table 1). These results suggest that Se reduced Na^+ due to the compartmentalization of Na^+ in vacuoles, as well as increasing the bond with the cell wall.

Regarding stomatal opening, treatments of 1-25 µM Se in the sodium selenite source in corn exposed to 100 mM NaCl resulted in an increase in stomatal conductance (Gs), transpiration rate, and, consequently, photosynthetic efficiency (Table 1). The increase in carotenoid levels may also be related to better absorption and regulation of the antenna complex, leading to a reduction in lipid peroxidation (Jiang et al., 2017; Silva et al., 2020).

High concentrations of Na^+ have an antagonistic relationship with the ions K^+ and Ca_2^+, causing an imbalance in cell homeostasis, nutrient deficiency, oxidative stress, delayed growth, and cell death (Yu and Assmann, 2016). For wheat exposed to 100 mM NaCl, the treatment of 5 µM of Se in the sodium selenate source provided a reduction of Na in plant tissues, and an increase in K, Ca, and N, reflecting an increase in productivity (Elkelish et al., 2019).

Elkelish et al. (2019) observed that the increase in the levels of non-enzymatic antioxidants (AsA GSH, phenolic compounds) and the accumulation of osmolytes (proline, sugars, and K^+) besides acting in the fight against ROS, and the fight against lipid peroxidation, also act in the gene expression from vegetables, increasing Na^+ transporters to vacuoles, as a way to avoid excess salinity in the other parts of the plant.

The results found by Habibi (2017) suggest that Se decreases salt-induced damage by stimulating antioxidant activities. Sunflower seeds subjected to salt stress

(15 dS m^{-1} NaCl) and treated with 5 mg kg^{-1} of Se in the sodium selenate source demonstrate an increase in carotenoids and anthocyanins, which are photosynthetic pigments that help in the capture and regulation of the photosynthetic apparatus. Consequently, there were several positive results of photosynthetic components, such as the increase in chlorophylls a and b, the efficiency of photosystem II (Fv / Fm), photosynthetic performance rate (PI), and the reduction of oxidative stress (H_2O_2 and MDA).

Another point raised in the literature is that under saline stress, in addition to biochemical modifications, plants can undergo morphological modifications, such as reduced leaf area, or biochemical, which can also be aggravated by harmful doses of Se. In this sense, the treatment of tomatoes with 1-20 mg L^{-1} of Se in the nano-Se source demonstrated that the nanoparticles present low risks of toxicity, and increase the antioxidant capacity of the element, as seen in the levels of flavonoids, phenols, lycopene, β-carotene, titratable acidity, soluble solids (Morales-Espinoza et al., 2019).

5.3 Heavy Metals

Plants can absorb elements from the soil, and assimilate them for use in their essential metabolic processes. However, this process can occur without specific selectivity, attributing to plants the risks of absorbing elements harmful to plant metabolism. However, the biological activity of metal ions can be markedly affected by the presence of other ions in the root zone (Hawrylak-Nowak et al., 2014).

Chromium (Cr) mainly affects plant morphology, causing irreversible anatomical and ultrastructural changes and interfering with mineral nutrition. Pumpkins under Cr toxicity (1 mg L^{-1} $K_2Cr_2O_7$) demonstrated that the application of Se in low doses (0.1 mg L^{-1} of Se in the sodium selenate source) can reduce the concentration of the element in the roots (Na, Ca, Fe, Mn, Cu, and Zn), in addition to increasing the absorption of other elements, demonstrating that there was protection or regeneration of the root system (Table 1) (Zhao et al., 2019).

Cadmium (Cd) is one of the most dangerous heavy metals due to its high toxicity, mobility, and availability to all living organisms. Vegetables like cucumber exposed to different levels of Cd (25 and 50 µM) have problems with water balance, mineral nutrition, photosynthesis, and respiration. However, treatment with Se (5 and

10 µM) provided an increase in the fresh matter, chlorophylls, carotenoids, and phytochelatins, which are recognized by the ability to inactivate metal ions (Table 1) (Hawrylak-Nowak et al., 2014).

The same effect of Se on Cd mitigation was seen by Zhang et al. (2020) for rapeseed (*Brassica napus*) treated with 50 µM L^{-1} of Cd, which is a Cd hyperaccumulator. The experiment focused on the speciation of Se (3 µM L^{-1} Na_2SeO_4, Na_2SeO_3, and Se-Met), and it turned out that the treatments were beneficial for photosynthesis (photosynthetic rate, stomatal conductance, internal CO_2 concentration, and respiration rate). Also, there was a reduction in H_2O_2 *and* $O_2^{\cdot-}$ (Zhang et al., 2020).

The stress of arsenic rice plants (25 µM $NaAsO_2$) was remedied with the application of 5 - 25 µM An increase in other nutrients (Mn, Fe, Co, Cu, Zn, and Mo) was provided, demonstrating benefits in the mineral nutrition of plants (Table 1). Also, the doses were responsible for increasing the content of antioxidants, such as carotenoids and phenolic compounds, and consequently improving the photosynthetic apparatus. In this experiment, a reduction in SOD activity was observed, and an increase in enzymes involved in the ascorbate-glutathione pathway, suggesting that stress may have been alleviated through spontaneous stimulation mechanisms of $O_2^{\cdot-}$ dismutation in H_2O_2 and through non-enzymatic antioxidants. These results were associated with the similar electron configuration between arsenite (AsIII) and selenite (SeIV) and with the sharing of a common carrier for its uptake in plants, where AsIII enters through the NIP (Nodulin 26-type intrinsic protein) transporter of aquaporins. family in rice (Chauhan et al., 2017).

5.4 Temperature

Each region of the planet has specific thermal characteristics, and as a consequence, plant species have created networks of adaptations over the generations to ensure productivity. Temperature stress is one of the items of greatest concern for future projections, taking into account climate changes and the imbalances in ecosystems caused by anthropic action in recent decades (Bouis et al., 2019).

Vegetables are highly sensitive to high temperatures, requiring the management of ideal conditions for their growth and development. Thus, the application of 50 mg

dm^{-3} of Se via leaf in lettuce plants submitted to 35°C, was more efficient than through the soil, increasing fresh matter, and antioxidant enzymes (GPx, CAT, GSH). In contrast, the reduction in proline levels indicates that Se did not exert its action through the increase in antioxidants, but again via the Ascorbate-Glutathione pathway (Hawrylak-Nowak et al., 2018).

On the other hand, under cold stress (0°C), the application of 5 mg L^{-1} Se in strawberry induced the antioxidant action by increasing enzymatic compounds, mainly SOD, and non-enzymatic compounds such as ascorbic acid. The treatment had a great influence on the photosynthetic processes, increasing the photosynthetic rate, chlorophyll content, and stomatal conductance (Huang et al., 2018).

Flowers are also species that are very sensitive to thermal imbalance, suffering losses in their production due to temperature fluctuations, and also requiring high nutritional control, being vulnerable to toxicity. Thus, the use of Nano-Se also demonstrates positive effects in the control of thermal stress, reducing lipid peroxidation through doses that ensure the benefits of Se, reducing the amount of application, but expanding the antioxidant capacity. The application of 150 mg nano-Se L^{-1} in Chrysanthemum subjected to 41.6C increased the activity of antioxidative enzymes, such as CAT and SOD, and increased the concentration of chlorophylls.

5.5 Light

According to Jaiswal et al. (2018), exposure to UVB stress accelerates the level of reactive oxygen species (ROS) and can cause oxidative damage to proteins, lipids, and nucleic acids in plants. In their studies, it was found that the cultivation of wheatgrass in soils with high concentrations of Se (seleniferous) induces plants to increase their ability to synthesize antioxidant compounds (phenolic compounds and flavonoids) as a way to mitigate excess light energy. Also, the high concentrations of Se induced a reduction in lipid peroxidation through the action of the enzymes SOD and CAT.

Soy has compounds that attribute antioxidant and anti-inflammatory characteristics to its grains, such as isoflavones (genistein, daidzein, and glycitein). However, under high intensity of UVB light changes in its biochemical and physiological routes. Therefore, the application of 5-20 mg L^{-1} can mitigate the effects

of excess light by increasing secondary metabolism compounds (Mata-Ramírez et al., 2019).

The increase in the amount of light energy acts directly on the photosynthetic mechanisms, overloading the complex antennas, and causing a disruption in the electron transport chain, generating the production of ROS and consequently damage to cellular components. The application of 100 µM L^{-1} If in broccoli treatments with different types of LED light it was responsible for reducing the levels of chlorophylls (a and b), in addition to photosynthetic pigments (He et al., 2020). This result may be associated with the regulation of the ability to capture light under stress (Chauhan et al., 2017).

6. PHYSIOLOGICAL ROLES OF SE ON CROP YIELDS: AGRONOMIC BIOFORTIFICATION PERSPECTIVE

Functional foods contain nutrients and bioactive compounds that provide benefits to the human and animal organisms, and are used to combat pathologies and regulate metabolism, and can be included in diets such as fruits, vegetables, legumes, nuts, spices, olive oils, mustards, canola, and flaxseed) (Tripathi et al., 2018).

However, according to Bouis et al. (2019), these foods are increasingly being threatened by the loss of biodiversity and poor population habits and emphasize that agricultural foods have the function of providing the macro and micronutrients necessary to maintain life, support physical activity and achieve a healthy body composition.

The Codex Alimentarius indicates that the production of biofortified foods should not have Se levels greater than 0.3 mg kg^{-1}. However, diets with an intake of less than <50 µg day^{-1} of Se are considered a nutritional deficiency (Reis et al., 2017). In the next section, the effect of agronomic biofortification with Se will be addressed in the main food classes that make up the food routine of the world population through the most recent research in the scientific scenario (Figure 3).

6.1 Legume

Legumes are of great importance for food for humans and animals, providing high levels of protein, minerals, dietary fibers, and low levels of fats, and being an

alternative for products with high added value, such as meat and seafood. This food class has consolidated its importance in combating the eradication of world hunger with the declaration of the United Nations General Assembly (UNGA) in 2016 with the establishment of the International Year of Pulses (IYP) (FAO, 2019).

Studies with cowpea have shown that application via leaf up to a dose of 50 g ha^{-1} Se is responsible for increasing the levels of the element in the shoot and the grains, without causing symptoms of toxicity of the element and causing oxidative damage in the crop (Table 2). Also, the intake of more than 50 g per day of biofortified cowpea with Se in the mentioned dosage can meet the recommended recommendation for adults (0.1 mg Se per day). This application limit also increases photosynthetic pigments, sugars and reduces oxidative damage (Silva et al., 2018, 2020; Lanza et al., 2021).

In a study with soy, Dai et al. (2020) demonstrated that the application of Se in the dosages of 0.5, 1.5, and 2 mg kg^{-1} Se in the form of L-selenomethionine ($C_5H_{11}NO_2Se$) applied via soil, it was able to increase Se concentration in the grains by about 27 times, obtaining a maximum concentration of 0.23 mg kg^{-1} of Se. The application also increased productivity, content of photosynthetic pigments, proline and reduced significant damage. Hussein et al. (2019) did not describe the concentration of Se in his work with the application of Nanoparticles, but highlighted the role of the element in the increase of fatty acids and proteins, and indicates the consumption of peanuts biofortified with Nano-Se to avoid the phytotoxicity of the element and increase the quality and quantity of the crop giving priority to food biosafety.

6.2 Cereal Crop

Cereals are a food class widely used for human consumption, but with great emphasis on animal supplementation. However, over the years, cereal production is being affected by the environmental conditions of the main producing countries (Tripathi et al., 2018). According to FAO (2020), the consumption of cereals is expected to increase by 2.6% in 2020/2021 concerning the previous year, this increase is attributed mainly to the increase in the use of feed, especially corn and sorghum, and for the production of corn ethanol. Thus, strategies to increase cereal productivity should be a necessity for the coming years.

The average consumption of wheat in Brazil is based on the product's derivatives, such as bread, pasta, and cakes, equivalent to a consumption of 60 kg $year^{-1}$, or 164 g day^{-1} per person. Thus, the application of Se at a dose of 21 g ha^{-1} is the most suitable for biofortification of wheat with Se, providing the grains with a grain concentration of 0.71 mg kg^{-1} of Se. The stipulated dosage also favored the increase in productivity, photosynthetic pigments, and carbohydrate metabolism (Lara et al., 2019).

Biofortification of rice with Se results in grains with higher nutritional quality (proteins, sugars, amino acids, nitrate, and ammonia), in addition to a significant increase in Se in grains. Considering that the average consumption of rice by the Brazilian population is 25 kg $year^{-1}$, that is, 68.5 g day^{-1} per capita, the dosage of 25 g Se ha^{-1} applied via leaf in the sodium selenate source was able to increase up to 50% the supply of Se to meet the daily requirement, obtaining a value of 0.35 mg kg^{-1} (Reis et al., 2018). Higher values do not perform well for the development of culture and are not suitable for human and animal consumption (Gouveia et al., 2020; Reis et al., 2020).

6.3 Fruit Tree

According to Bouis et al. (2019), the low consumption of specific food groups in the past reveals high indications of food malnutrition in the present, and the low consumption of fruits is reported as one of the limiting factors in human food. Studies show that fruit biofortification, in addition to increasing nutritional levels, also adds several benefits to the commercial characteristics of foods, such as sweetness, firmness, pH (Zahedi et al., 2019)**.**

The consumption of 150 g of fresh strawberries grown hydroponically and treated with 10 µM Se (Table 2) provides the human metabolism with 60 µg Se per day and provides a diet with a higher content of sweetness in the fruits and antioxidant compounds (flavonoids and polyphenols) beneficial to health (Mimmo et al., 2017). The foliar application of selenate increased more than 70% of Se in the Pear juice (Table 2), with the element mainly concentrated in the peel and juice, accounting for 41–58% and 31–48% of the total Se concentration of the fruit, respectively (Deng et al., 2019). For tomatoes, the application of 2 and 5 mg L^{-1} Se provided a concentration

of 24.5 - 35.8 µg g^{-1} of Se (Table 2), revealing that the application of Se in low concentrations can improve harvest yield and fruit quality (Rahim et al., 2020).

6.4 Vegetables

According to Bian et al. (2020) supplementing vegetables with Se is a safe and effective way to combat Se deficiency in humans, as vegetables play an important role in the human diet. The application of 25 µM of Se in Broccoli was able to increase 10-12 mg kg^{-1} in its flowers and 17-30 mg kg – 1 in the stems and leaves, indicating that in addition to being a vegetable highly consumed for its nutritional density (Table 2). Broccoli has high attractiveness because it is entirely used for consumption, and consequently has a larger area of supply of Se (Tian et al., 2018).

Oliveira et al. (2018) report that the consumption of 50 g of carrots biofortified with 50 µm L^{-1} Se, applied via leaf, corresponds to a daily intake of 105 µg Se, considering that the treatment concentrated about 14 mg kg^{-1} of dry mass and the 85% humidity value for the carrot (Table 2). This application also favored increased productivity, titratable acidity, and reduced root maturation.

Selenate is the most efficient source for potato biofortification with Se in tropical conditions when supplied in low doses (Table 2). The application of 0.75 mg kg^{-1} of Se via soil concentrated 5 mg kg^{-1} of Se in the tubers, being able to supply 90 µg of Se, considering the consumption of 100 g of fresh tuber (80% water), also, to increase the content of other nutrients, such as Ca (Oliveira et al., 2019).

6.5 Medicinal

Garlic (Allium sativum) is probably the most cited herb in the literature for medicinal properties (Tripathi et al., 2018). The biofortification of garlic with Se in the dosage of 50 g ha-1 in the sodium selenate source can supply the daily need for Se in humans from the consumption of 16 g of the bulb, and the dosage was able to generate a concentration of 3.23 mg kg-1 in the edible parts (Table 2). Also, it was responsible for increasing the total phenolic content, total flavonoid content, and total antioxidant capacity (Shafiq et al., 2019).

Mushrooms, in addition to food interest, also have high value as a medicinal product, such as *Cordyceps militaris* which is one of the most popular edible and

medicinal mushrooms worldwide, especially in Asian countries, given its pharmacological functions that affect the circulatory system and anti-inflammatory (Hu et al., 2019).

Cannabis sativa L. seeds is one of the functional foods of the century, and besides being associated with several medicinal benefits, it also has great potential for phytoremediation of soil elements, given its large accumulation of nutrients in its seeds and leaves. An experiment conducted in Colorado (USA) determined that the average dosage of sodium selenate could provide the recommended daily intake of Se (55 to 75 µg of Se) recommended through approximately 4 g of seeds. According to USDA (2020), the daily recommendation for seeds is 30g, which would provide an average of 450-750 µg of Se to human metabolism, which corresponds to the maximum allowed intake (Table 2). Another product based on *Cannabis sativa L.* seeds is beer, which, if produced based on biofortification, may offer 42 µg Se L^{-1}, which corresponds to 15 µg Se, about 25% of daily needs (Stonehouse et al., 2020).

Natural spices and sweeteners are also featured in medicinal plants and have a high capacity of biofortification with Se, which can be used as a food supplement. Szarka et al. (2020) noticed that the application of 1 mg kg^{-1} Se in *Stevia rebaudiana (Bert.)* results in approximately 0.1 mg kg^{-1} Se in water drinks, coffee, or green tea (Table 2). Stevia is known for the high level of sweetness found in its leaves, being used as a substitute for conventional sugar, which in turn is highly harmful to human health and well-being. Basil is also another element widely used in tropical cuisine, and the use of 9 g of basil seeds with the application of 5 mg m^{-2} of Se can supply the recommended daily dose, while non-biofortified seeds should be consumed at an average of 120 g per person (Table 2). *Ocimum basilicum* also contains a significant amount of biological compounds with strong healing properties, and the application of Se was responsible for increasing the content of polyphenols and the antioxidant capacity.

7. CONCLUSION AND FUTURE PERSPECTIVES

The Se is an element that has a wide cycle of action, influencing mechanisms in plants, humans, and animals, and presenting risks to these organisms both with their lack and excess. However, there is conclusive evidence that supplementation is safely

possible, and that the benefits gained from the exogenous application of Se help in improving the nutritional quality of food, mitigating hidden hunger.

As perspectives, we have that the improvements of the technologies, as the use of nanotechnology, help in the regulation of the limits between the toxic and the beneficial for the supplementation of plants, minimizing the necessary amount of application to reach the established daily consumption and the benefits for the plant metabolism. The foliar application of Se in low concentrations can be used to improve the antioxidant (SOD, CAT, APX, GR) and non-oxidant (ascorbate, carotenoids, flavonoids, tocopherol) metabolism of plants to induce tolerance to abiotic stresses (drought, temperature, salinity and heavy metals). Studies are necessary to establish an optimal dose-response for different plant species in different edaphoclimatic conditions aiming at agronomic biofortification. The practice of agronomic biofortification with Se in plants can increase productivity, improve the nutritional and biochemical quality of agricultural products, and can bring benefits to human health.

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Abiotic Stress	Common Name	Ordem	Family	Species	Genotype	Location	Trial	Stress	Se doses/source	Application	Yield	Photosynthesis	Nutrients	Non-enzymatic antioxidants	Enzymatic antioxidants	ROS and lipidic peroxidation	Conclusion	Reference
Drought	Rice	Poales	Poaceae	*Oryza sativa L.*	IAC 202	Lavras, Brazil	Greenhouse	−50 kPa	0.5 - 2.0 mg kg^{-1} Na_2SeO_4	Soil	↑ Plant height	↑ SPAD index, A, Gs, Pn, Ec and WUE.	↑ S, Cu and Se in grains (0 to 20.78 mg kg−1)	-	↑ SOD	↓ H_2O_2	The antioxidant capacity of Se in rice under water deficit minimizes damage to productivity by increasing photosynthetic capacity and reducing lipid peroxidation.	Andrade et al. (2018)
Drought	Canola	Brassicales	*Brassicaceae*	*Brassica napus L.*	Ahmadi, SW102, Okapi, GKH2624, GK-Gabriella, and Elvis	Karaj, Iran	Field	Irrigation and development stage	30 g L^{-1} Na_2SeO_4	Foliar	↑ Plant height, number of poods and seeds, 100 seeds weight, seed yield and seed oil yield.	↑ Chlorophylls (a and b)	-	↑ Proline and relative water content	-	-	The interference of parameters under drought stress may have been generated by the increase in ROS, and the application of Se was useful to reduce harmful effects.	Hemmati et al. (2019)
Drought	Cucumber	Cucurbitales	*Cucurbitaceae*	*Cucumis sativus L.*	Dar	Poznan, Poland	Hydroponics	Total water deficit after 96h of Se treatment	1 -10 µM Na_2SeO_3	Solution	-	-	Se in roots (524.06 - 759.4 µg kg−1 DW)	↑ Water content	↑ APX, POD and SOD.	↓ $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$ and MDA	Se at lower concentrations (1 and 5 µM) increased antioxidant capacity and reduces the ROS, while Se at higher concentrations (10 µM) enhances lipid peroxidation.	Jóźwiak et al. (2019)
Drought	Olive	Lamiales	*Oleaceae*	*Olea europaea L.*	Leccino	Perugia. Italy	Field	Total water deficit and 110 m^3 ha^{-1}	100 mg L^{-1} Na_2SeO_4	Foliar	↑ Dry, fresh weight and oil content	↑ Chlorophylls	Se in oil (149 - 529 µg kg−1)	↑ Carotenoids and Phenols	-	-	The application of Se can increase the nutritional properties, increasing antioxidant compounds and providing longer shelf life.	D'Amato et al. (2018)
Drought	Tomato	Solanales	Solanaceae	*Solanum lycopersicum*	Login 935	Fayoum, Egypt	Greenhouse	100 and 60% Soil field capacity	20-40 mM Na_2SeO_4	Soil and Foliar	↑ Number of leaves, dry and fresh weight	↑ Fv/Fm, Pn, and SPAD index.	Se in fruits (1.96 - 3.74 mg kg-1 FW)	↑ Water content, lycopene, total soluble sugars, proline, AsA, GSH and α.TOC	↑ SOD, CAT, and APX	↓ MDA, H_2O_2, and $O_2^{\bullet-}$	The supplementation of Se in the soil is more efficient to increase the enzymatic and non-enzymatic antioxidants and minimize the effects of drought stress through antioxidant metabolism.	Rady et al. (2020)
Salinity	Maize	Poales	Poaceae	*Zea mays L.*	Agaiti-2002	Faisalabad, Pakistan	Greenhouse	4 dSm^{-1} NaCL	20 and 40 mg/L Na_2SeO_4	Foliar	↑ Dry and fresh weight	↑ Chlorophylls (a and b)	↓ Na ↑ K and Ca	↑ Total solubre proteins, phenolics and flavonoids	↑ CAT, POD and SOD	↓ MDA and H_2O_2	The antioxidant action of Se in salinity stress depends on the form of application, low doses and vegetative stage, promoting a reduction in lipid peroxidation,	Ashraf et al. (2018)

Salinity	Wheat	Poales	Poaceae	*Triticum aestivum L*	-	Ismailia, Egypt	Greenhouse	100mM NaCl	5 and 10 µM Na_2SeO_4	Soil	↑ Length, fresh and dry biomass	↑ Chlorophylls (a and b), total pigments, Pn, and Gs.	↓ Na ↑ N, K and Ca	↑ Carotenoids, proline, soluble sugars and water content.	↓ Proline oxidase and ↑ γ-glutamyl kinase, SOD, CAT, GST, APX, GR.	↓ H_2O_2 and MDA	increased photosynthetic capacity and Na inhibition. Low concentration Se (5 µM) was shown to be beneficial in increasing non-enzymatic antioxidants and reducing ROS, and therefore, minimizing the deleterious effects of salt stress.	Elkelish et al. (2019)
Salinity	Sunflower	Asterales	Asteraceae	*H. annuus L.*	Hysun33	Tehran, Iran	Greenhouse	15 dS m^{-1} NaCl	5 mg kg^{-1} Na_2SeO_4	Soil	↑ Dry weight	↑ Chlorophylls (a and b), Fv/Fm, PI, F_v/F_o, F_v/F_M and ↓ ABS/RC and F_o	↓ Na and ↑Ca, K and Se in seeds (2.40-2.58 mg kg-1 DW)	↑ Carotenoids and Anthocyanin	↑ GPx	↓ H_2O_2 and MDA	Se minimizes oxidative damage caused by salt stress by increasing photosynthetic parameters, inhibiting Na translocation and reducing oxidative stress.	Habibi et al. (2017)
Salinity	Tomato	Solanales	Solanaceae	*Solanum lycopersicum L.*	El Cid F1	Saltillo, Mexico	Greenhouse	50 mM NaCl	1-20 mg L^{-1} Nano-Se	Soil	↓ Plant height, number of leaves and ↑ Number of fruits,	↑ Chlorophylls (total, a and b)		↑ Flavonoids, Phenols, Lycopene, and β-carotene, titratable acidity, soluble solids ↓pH	↑ APX, GPx, CAT, SOD	-	Nano-Se increased the enzymatic activity and resulted in the improvement of the photosynthetic capacity of the plants, in addition to increasing the antioxidant compounds.	Morales-Espinoza et al. (2019)
Salinity	Maize	Poales	Poaceae	*Zea mays L.*	Nongda 108	Hefei, China	Growth chamber	100 mM NaCl	1-25 µM Na_2SeO_3	Soil	↑ Dry Weight	↑ Chlorophylls, Pn, Gs and Tr.	↓ Cl and Na, and ↑ K	↑ Carotenoids	↑ SOD, CAT and APX	↓ Lipidic Peroxidation	Low Se treatment activated the antioxidant defence system, preserve the chloroplasts ultrastructure promote alleviate ROS damage, and reduced Na concentration.	Jiang et al. (2017)
Heavy metals	Rice	Poales	Poaceae	*Oryza sativa L.*	Triguna	Chinsurah, índia	Hydroponics	25 µM $NaAsO_2$	5 - 25 µM Na_2SeO_3	Solution	↓ Plant Growth	↑ Total chl, Chl a, Chl b	↑ Mn, Fe, Co, Cu, Zn and Mo	↑ Carotenoids and phenolic compounds	↓ SOD and CAT ↑ GPX, GR, APX, POD	↓ $O_2^{\bullet-}$, H_2O_2 and MDA,	Se supplementation ameliorated As toxicity in rice plant by reducing As accumulation and by retrieving As induced nutrient deficience.	Chauhan et al. (2017)
Heavy metals	Cucumber	Cucurbitales	Cucurbitaceae	*Cucumis sativus L.*	Polan F1	Lublin, Poland	Growth chamber	25 and 50 µM $CdCl_2 \cdot 2.5 H_2O$	5 and 10 µM Na_2SeO_4	Solutions	↑ Fresh weight	↑ Chl a, Chl b		↑ Phytochelatins and carotenoids		↓ MDA	Treatment with Se can relieve the toxicity of Cd and its destructive physiological effects on plants, however, depends on the proportion of these two elements in the nutrient solution.	Hawrylak-Nowak et al. (2014)
Heavy metals	Chinese cabbage	Brassicales	Brassicaceae	*Brassica campestris L.*	Pekinensis		Hydroponics	1 mg L^{-1} $K_2Cr_2O_7$	0.1 mg L^{-1} Na_2SeO_3	Solution	↑ Increased the total length, surface	-	↓ Cr and ↑ Na, Ca, Fe, Mn, Cu and Zn	-	-	-	Se application reduced Cr uptake and increasing the negative effects of Cr toxicicity.	Zhao et al. (2019)

										area, volume and tip numbers							
Heavy metals	Oilseed rape	Brassicales	Brassicaceae	*Brassica napus and Brassica juncea*	Zhongshuang and Yongbao 2	Sichuan, China	Growth chamber	50 µM/L $CdCl_2$	3 µM/L Na_2SeO_4, Na_2SeO_3 and Se-Met	Soil	↑ Dry Weight	↑ Pn, Gs, Ci and Tr	↓ Cr	-	↑ CAT and ↓ SOD and POD	↓ H_2O_2 and $O2^{\bullet-}$	Cadmium stress was combated by the application of Se by reducing reactive oxygen species by increasing antioxidant activity and photosynthesis.	Zhang et al. (2020)
Heavy metals	Cucumber	Cucurbitales	Cucurbitaceae	*Cucumis sativus L.*	-	Mashhad, Iran[1]	Hydroponics	20-25 mM $CdCl_2$ and 60-100 mM $PbCl_2$	4-8 mg L^{-1} Na_2SeO_3	Soution	↑ Fruiting, accelerated flowering, number of fruits, fruit length and diameter, single fruit weight and total yield.	-	-	-	-	-	The application of Se relieves the symptoms of stress by cadmium and lead, increasing the fruit productivity parameters.	Shekari et al. (2019)
Temperature	Lamb's lettuce	Dipsacales	Caprifoliaceae	*Valerianella locusta L.*	Verte de cambrai	Lublin, Poland	Greenhouse	35/22 °C day/night	50 mg dm^{-3} Na_2SeO_4	Soil and Foliar	↑ DW	-		↓ Proline	↓ APX ↑ GPx, CAT, GSH	↓ H2O2	Foliar Se treatment was more efficient than soil application, and Se efficiently alleviated HS-induced disorders in the metabolism.	Hawrylak-Nowak at al. (2018)
Temperature	Strawberry	Rosales	Rosaceae	*Fragaria × ananassa*	Akihime and Benihoppe	Zhejiang, China	Growth chamber	0°C (6-12 h)	2.5-10 mg L^{-1} Na_2SeO_3	Foliar	-	↑ Pn, Gs, total Chl and ↓ intercellular CO_2 concentration	-	↑ Ascorbic Ácid	↓ APX, GR, and MDHAR, and ↑ SOD, CAT, POD, DHAR	↓ MDA, and H_2O_2	Se applications significantly alleviated the adverse impacts of chilling stress on changes in stomatal conductance and intercellular CO2 concentration.	Huang et al. (2018)
Temperature	Mize	Poales	Poaceae	*Zea mays*	OSSK-713		Hydroponics	24-44°C	5–15 µM Na_2SeO_3	Solution	-	-	-	-	↑ SOD, APX, GR, MDHAR, DHAR, GPX, and ↓ POX and TBARS.	↑$O_2^{\bullet-}$ and H_2O_2	Se can control the production and quenching of ROS.	Yildiztugay et al. (2017)
Temperature	Chrysanthemum	Asterales	Asteraceae	*Chrysanthemum morifolium Ramat.*	Sensual and Francofone	Tanta, Egypt	Greenhouse	37.3-41.6 °C	50-200 mg nano-Se L^{-1}	Foliar	↑ Number of opening flowers, plant height and weight	↑ Chlorophylls (a and b)	-	-	Low Se ↓POX, CAT and ↑ PPO	Low Se ↑ H_2O_2 and ↓ electrolyte leakage	The application of Nano-Se has the ability to protect flowers from the negative effects of thermal stress, providing the activation of the antioxidant system and ensuring the functioning of the photosynthetic apparatus.	Seliem et al. (2020)

Temperature	Cotton	Malvales	Malvaceae	*Gossypium hirsutum L.*	MNH-886	Faisalabad, Pakistan.	Field	Heat imposed at squaring or flowering (±40°C)	50-150 mg/L Se	Foliar	↑ Seed cotton yield and fiber quality	-	-	-	-	-	There was a strong correlations of phenological and quality attributes with seed cotton yield with Se treatment.	Saleem et al. (2020)
Light	Lettuce	Asterales	Asteraceae	*Lactuca sativa L.*	Butterhead	Beijing, China[1]	Hydroponics	Fluorescent light, red LED light, blue LED, and mixed red and blue LED.	10 mmol L^{-1} Na_2SeO_4 and 0.5 mmol L^{-1} Na_2SeO_3	Solution	↑ Fresh and dry weight and lenght	↑ Pn, Gs and Tr	↑ Se in leaves (±0.4 - 40 mg kg-1 FW), roots (4 - 45 mg kg-1 FW) and ↓ NO_3^-	-	↑ NR, NiR, GS and GOGAT	-	Se forms has a strong interaction with the light spectra, and promotes influence on the Se accumulation and the regulation of nitrogen metabolism.	Bian et al. (2020)
Light	Wheat	Poales	Poaceae	*Triticum aestivum L.*	Reska	Ljubljana, Slovenia	Field	UV+ and UV-	10 mg L^{-1} Na_2SeO_4	Foliar	↑ Biomass ↓ Plant height	↑ Chlorophylls (a and b)	↑ Se in leaves (3885 - 4697 mg g−1 DM)	↓ Carotenoids and Anthocyanins	-	-	The combined treatment of UV light and Se promoted an increase in the defense metabolism of plants through the increase of compounds that absorb UV, light reflectance and transmittance, combating oxidative stress.	Golob et al. (2017)
Light	Broccoli	Brassicales	Brassicaceae	*Brassica oleracea L.*	Ivhua	Guangzhou, China[1]	Growth chamber	CK:1R1B1G LED, 1R1B1G+Se, 1R1B+Se, 1R2B+Se, 2R1B+Se	100 µM L^{-1} Na_2SeO_3	Solution	↓ Hypocotyl Length, Fresh Weight and ↑ Dry Weight	↓ Chlorophylls (a and b)	↓ P, K, Ca, Mg, Zn and ↑ N, S Fe and Se (4.48-110.33 mg/kg DW)	↑ Carotenoids, Anthocyanins, Soluble sugar, Protein, Vitamin C, Total Phenolic Compounds, Total Flavonoids and Glucosinolate	-	-	The combination of Se with different light treatments has been shown to be effective in increasing the nutritional quality and protective compounds of broccoli sprouts for plants and humans.	He et al. (2020)
Light	Soybean	Fabales	Fabaceae	*Glycine max L.*	Suaqui'86	Monterrey NL, Mexico[1]	Growth chamber (in vitro)	UVC-light (30 or 60 min)	5, 10 or 20 mg/L Na_2SeO_3	Solution	-	-	↑ Se (0.18-6.40 mg L^{-1})	↑ Isoflavonoids, inhibition of nitric oxide production and Cellular antioxidant activity	-	-	Se stress increased the concentrations of isoflavonoids (glycitein and genistein) in soybean calluses, demonstrating higher levels of nitric oxide inhibition and cellular antioxidant activity.	Mata-Ramírez et al. (2019)
Light	Wheat	Poales	Poaceae	*Triticum aestivum*	PBW343	Punjab, India	Growth chamber (in vitro)	Visible light and UV-B light	Seleniferous and nonseleniferous soil	Soil	-	-	↑ Se (0.48 - 151.3 µg/g)	↑ Phenolic content, flavonoids and DPPH radical scavenging	↑ SOD, CAT and ↓ POD, APX, GR, GPx.	↓ Lipid peroxidation	Se treatment increased different secondary metabolites in wheatgrass and protect against lipidic peroxidation.	Jaiswal et al. (2018)

Table 1. Selenium against abiotic stress. **PHOTOSSYNTESIS:** CO_2 assimilation rate (A), stomatal conductance (gs), transpiration rate (E), internal CO_2 concentration (Ci), instantaneous efficiency of carboxylation (EiC), water use efficiency (WUE), Photosynthetic rate (Pn), transpiration rate (Tr), Chlorophyll (Chl) or SPAD index, ion leakage (EC), nitric oxide (NO), NADPH Oxidase (NOX), , thiobarbituric acid-reactive substances (TBARS), maximum quantum yield of photosystem II (FV/FM), Performance Index (PIABS), Light absorption flux (ABS/RC), Minimal fluorescence

(Fo), Variable chlorophyll fluorescence (Fv), Electrolyte leakage (REL %), soil field capacity (SFC). **ROS AND LIPIDIC PEROXIDATION:** Superoxide anion radical ($O2^{\bullet-}$), Hydroxyl radical ($HO^{\bullet}$), Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA). **NON-ENZYMATIC ANTIOXIDANTS:** Selenium (Se), Ascorbic acid or vitamin C (AsA), Glutathione (GSH), α-tocopherol (α.TOC), Carotenoids (β-caroteno). **ENZYMATIC ANTIOXIDANTS**: Superoxide dismutase (SOD; EC 1.15.1.1), Catalases (CAT, EC 1.11.1.6), Ascorbate peroxidase (APX, EC 1.11.1.1), Guaiacol peroxidase (POD, EC 1.11.1.7), Glutathione peroxidases (GPX, EC 1.11.1.9), polyphenol oxidase (PPO), Tetra guaiacol (POX), Glutathione reductase (GR, EC 1.6.4.2), Glutamate synthase (GOGAT), Nitrate reductase (NR), Nitrite reductase (NiR), Glutamine synthetase (GS).

ID	Common Name	Order	Family	*Species*	Genotypes	Location	Trial	Se biofortification	Se source	Application	Se shoot*	Se edible part	Se roots*	Observation	Conclusion	Reference
Legume	Cowpea	Fabales	Fabaceae	*Vigna unguiculata (L.) Walp.*	BRS XiqueXique	Tupã (SP), Brazil	Field	10 - 150 g ha^{-1}	Na_2SeO_4	Foliar	0.04 - 17 mg kg^{-1}	0.04 - 10.4 mg kg^{-1}	-	Above 50 g ha^{-1} resulted in visual symptoms of Se toxicity	The dose of 50 g ha^{-1} ↑ photosynthetic pigments and yield.	Lanza et al. (2020)
Legume	Cowpea	Fabales	Fabaceae	*Vigna unguiculata (L.) Walp.*	BRS Tumucumaque	Selvíria (MS), Brazil	Field	50 - 1600 g ha^{-1}	Na_2SeO_3	Foliar	± 0.01 - 100 mg kg^{-1}	± 1.5 - 12.2 mg kg^{-1}	-	Concentrations higher than 50 g ha^{-1} caused leaf toxicity symptoms	The doses of 1200 and 1600 g ha^{-1} ↑ lipid peroxidation and ↓ total sugars, sucrose, and carotenoid concentration.	Silva et al. (2018)
Legume	Cowpea	Fabales	Fabaceae	*Vigna unguiculata (L.) Walp.*	BRS Tumucumaque	Selvíria (MS), Brazil	Field	2.5 - 60 g ha^{-1}	Na_2SeO_3 and Na_2SeO_4	Soil	Selenate (0.12 -0.39) and Selenite (0.09 - 0.29 mg kg^{-1})	Selenate (0.09-0.67) and Selenite (0.09 - 0.32 mg kg^{-1})	-	The effect of Se application differed between source.	Both sources ↑ leaf sucrose and total sugars concentration in cowpea leaves (indicator of ROS scavenging).	Silva et al. (2020)
Legume	Soybean	Fabales	Fabaceae	*Glycine max (L.)*	Qindou	Hanzhong, China	Greenhouse	0. 1.5 and 2 mg kg^{-1}	L-selenomethionine ($C_5H_{11}NO_2Se$)	Soil	1.05 - 2.89 mg kg^{-1}	0.08 - 0.23 mg kg^{-1}	1.36 - 3.74 mg kg^{-1}	Application of Se reduced Zn uptake from soil.	All tretaments ↑ biomass, photossynthetic pigments, proline and ↓ MDA.	Dai et al. (2020)
Legume	Penaut	Fabales	Fabaceae	*Arachis hypogaea L.*	NC, Gregory and Giza 6	Cairo, Egypt	Greenhouse	0, 20 and 40 ppm	N-Se	Foliar	-	-	-	The doses altered fatty acid composition and protein signatures.	The treatments ↑ yield components, seeds oil and antioxidant metabolism.	Hussein et al. (2019)
Cereal Crops	Wheat	Poales	Poaceae	*Triticum aestivum L.*	BRS 264	Lambari (MG), Brazil	Field	12 - 120 g ha^{-1}	Na_2SeO_4	Foliar	0.3 - 2.35 mg kg^{-1}	0.6 - 2.86 mg kg−1	-	Increasing doses of Se did not influence S contents.	The dose of 21 g ha^{-1} ↑ photossynthetic rate, starch, total soluble sugars, reducing sugars, sucrose, and yield.	Lara et al. (2019)
Cereal Crops	Rice	Poales	Poaceae	*Oryza sativa (L.)*	BRS Esmeralda	Selvíria (MS), Brazil	Field	250 - 2000 g ha^{-1}	Na_2SeO_4	Foliar	-	± 3.5 - 25 mg kg^{-1}	-	Se acumullation occurred in the endosperm and aleurone/pericarp of the seeds.	All treatments ↓ yield and physiological seed quality.	Reis et al. (2018)
Cereal Crops	Rice	Poales	Poaceae	*Oryza sativa (L.)*	ANa 5015, AN Cambará and ANa 7007	Selvíria (MS), Brazil	Field	10 - 100 g ha^{-1}	Na_2SeO_4	Soil	0.014 - 2.97 mg kg^{-1}	0.099 - 2.16 mg kg^{-1}	-	There was a high translocation of Se from leaves to grains.	All treatments ↑ Se concentrations in leaves and grains, and the concentrations of storage proteins in rice grains.	Reis et al. (2020)
Cereal Crops	Rice	Poales	Poaceae	*Oryza sativa (L.)*	BRS Esmeralda, BRSMG Relâmpago, BRS Bonança and Bico Ganga	Ilha Solteira (SP), Brazil	Hydroponics	1.5 mM L^{-1}	Na_2SeO_4	Solution	± 4.2 - 5.1 mg kg^{-1}	-	± 8.2 - 11 mg kg^{-1}	The treatment cause visual symptoms of toxicity characterized as leaf chlorosis and necrosis.	The treatments ↑ leaf sucrose, total sugars, nitrate, ammonia, and activity of antioxidative enzymes.	Gouveia et al. (2020)
Cereal Crops	Corn	Poales	Poaceae	*Zea mays L.*	PMH-1	Ludhiana, India	Greenhouse	1,2,4, 8,16, 32 mg kg^{-1}	Na_2SeO_4	Soil	95.4 - 318.4 mg kg^{-1}	-	40.2 - 167.9 mg kg^{-1}	The treatments bove 4 mg kg^{-1} soil was toxic to maize plants.	Lower Se doses ↑ dry matter, chlorophyll, proline and activities of defence enzymes.	Sharma et al. (2018)
Fruit trees	Coffe	Gentianales	Rubiaceae	*Coffea Arabica*	Obatã, IPR99, IAC125, IPR100 and Catucaí	Ilha Solteira (SP), Brazil	Hydroponics	1.0 mmol L^{-1}	Na_2SeO_4	Solution	± 200 - 383.27 mg kg^{-1}	-	± 500-849.92 mg kg^{-1}	The treatment promoted root browning in all genotypes and influence P and S contents in the shoots.	The treatment ↓ photosynthetic pigments, antioxidant enzymes activities and ↑ sucrose.	Mateus et al. (2020)
Fruit trees	Strawberry	Rosales	Rosaceae	*Fragaria × ananassa*	Elsanta	Bolzano, Italy	Hydroponics	10 and 100 µM	Na_2SeO_4	Solution	10.48 - 125.08	3.95 - 46.04 µg g^{-1} DW	19.02 - 174.42	There was involvement between phenolic compounds	Both treatments ↑ mineral nutrient content (Ca, Mg, Mn and K), quality parameters,	Mimmo et al. (2017)

											$\mu g\ g^{-1}$ DW		$\mu g\ g^{-1}$ DW	and Se supplementation, and alteration of secondary metabolites (cytokinins and amino acids).	soluble sugars, organic acids (Citric and Malic).	
Fruit trees	Pomegranate	Myrtales	Lythraceae	*Punica granatum*	Malase Saveh	Nemahil village, Iran	Field	1 and 2 µM	Na_2SeO_4 and N-Se	Foliar	N-Se (2-2.5) and Selenate (1.5-2 $\mu g\ g^{-1}$ DW)	-	-	Se and N-Se controlled fruit cracking.	The dose of 2µm (N-Se) increased chlorophyll content, yield, leaf mineral content (N, P, K, Ca, Fe, Zn), total sugars, phenolic compounds, antioxidants and anthocyanins.	Zahedi et al. (2019)
Fruit trees	Pear	Rosales	Rosaceae	*Pyrus communis L.*	Liuyuexueli	Jianshi, China.	Field	20 - 200 mg L^{-1} and different stages.	Na_2SeO_3 and Na_2SeO_4	Foliar	Selenite (0.63-7.85) and Selenate (0.72-8.92 mg kg^{-1} DW)	Selenite (7.04-27.30) and Selenate (61.77-127.32 µg kg^{-1} FW)	-	Above 40 mg L^{-1} resulted resulted in toxicity symptoms.	Selenate accumulate more Se in the fruit.	Deng et al. (2019)
Fruit trees	Tomato	Solanales	Solanaceae	*Solanum lycopersicon L. Mill*	Rio Grande	Saltillo, Mexico	Greenhouse	2 and 5 mg L^{-1}	Na_2SeO_3	Soil	20.9 - 20.4 µg g^{-1}	24.5 - 35.8 µg g^{-1}	-	The solution doses did not interfere with the absorption of macronutrients.	The treatments ↑ agronomic values.	Rahim et al. (2020)
Vegetables	Cucumber	Cucurbitales	Cucurbitaceae	*Cucumis sativus*	Polan F1	Lublin, Poland	Hydroponics	Selenate (2 - 80) and Selenite (2 - 60 µM)	Na_2SeO_3 and Na_2SeO_4	Solution	Selenate (10.2 - 648.0) and Selenite (11.2 - 120.6 mg kg^{-1} DW)	-	Selenate (7.75-603.5) and Selenite (68.5-967.2 mg kg^{-1} DW)	There was symptoms of Se toxicity at the concentrations of 80 (Selenate) and 20 µM (Selenite).	Low doses of Se promoted ↑ growth and ↓ lipid peroxidation in roots.	Hawrylak-Nowak et al. (2015)
Vegetables	Carrot	Apiales	Apiaceae	*Daucus carota L.*	Brasília	Lavras, Brazil	Greenhouse	Soil (1.0 mg dm^{-3}) and Foliar (50 µm L^{-1})	Na_2SeO_3 and Na_2SeO_4	Soil and Foliar	Selenate (±10-15) and Selenite (±3-19 mg kg^{-1})	-	Selenate (±10-15) and Selenite (±7-8 mg kg^{-1})	Foliar application of selenate was the most effective source and form of application.	Foliar application ↑ dry mass, yield, titratable acidity and ↓ root maturation.	Oliveira et al. (2018)
Vegetables	Broccoli	Brassicales	Brassicaceae	*Brassica oleracea L.*	Diplomat and Gypsy	Ithaca, USA	Greenhouse	25 µM	Na_2SeO_4	Soil	±17-30 mg kg^{-1}	±10-12 mg kg^{-1}	-	The total amino acid levels were not significantly affected by treatment	The treatment ↓ the secundary metabolism	Tian et al. (2018)
Vegetables	Mustard	Brassicales	Brassicaceae	*Brassica juncea L.*	Volnushka	Moscow, Russia	Field	50 mg Se L^{-1}	Na_2SeO_4	Foliar	8922 ± 870 µg kg^{-1} DW**	-	-	There was a synergism between Se and I that provided inhibiting nitrate accumulation.	The treatment ↑ choropphyll, carotene, ascorbic acid, flavonoids, total soluble solids, Al, B, and ↓ Cd and Sr concentrations.	Golubkina et al. (2018)
Vegetables	Potato	Solanales	Solanaceae	*Solanum tuberosum L.*	Agata	Lavras, Brazil	Greenhouse	0.75 - 5.0 mg kg^{-1}	Na_2SeO_3 and Na_2SeO_4	Soil	Selenate (±0.8-5.63) and Selenite (±0.5-6.20 mg kg^{-1})	Selenate (±5-10) and Selenite (±1.6-5 mg kg^{-1})	-	In low doses selenate is the most efficient source	Low doses ↑ production of tubers, Ca content and antioxidant system. High doses ↓ production, S content, pH and peroxidase activity.	Oliveira et al. (2019)
Medicinal	Garlic	Asparagales	Amaryllidaceae	Allium sativum L.	-	Lahore, Pakistan	Field	20 and 50 g ha^{-1}	Na_2SeO_4	Foliar and Soil	8.020 - 15.46 mg Kg^{-1}	1.732 - 3.23 mg Kg^{-1}	-	Foliar application was more positive than soil.	The dose of 50 g ha^{-1} applied by leaf ↑ total phenolic content, total flavonoid content, and total antioxidant capacity.	Shafiq et al. (2019)
Medicinal	Hamp	Rosales	Cannabaceae	*Cannabis sativa L.*	Workhorse	Colorado, EUA	Hydroponics	5 - 80 µM	Na_2SeO4	Solution	±8 - 200 mg	±9 - 150 mg kg^{-1} DW	±12 - 80 mg	The dose above 40 µM was toxic to hamp plants	In the mature plant stage the doses of 5 to 40 µM ↑ yield,	Stonehouse et al. (2020)

											kg^{-1} DW		$Se\ kg^{-1}$ DW	plant height and photosynthetic performance	
Medicinal	Stevia	Asterales	Asteraceae	*Stevia rebaudiana Bert.*	-	Budapest, Hungary	Greenhouse	1 - 10 mg kg^{-1}	Na_2SeO_3 and Na_2SeO_4	Soil	Selenite (11.4-96.7) and Selenate (77.9 - 415 mg kg^{-1})	-	-	Selenate and Selenite treatments did not affect the growth and shoot biomass	Stevia leaves fortified with 1 mg kg^{-1} of selenate can be used as sweetener for some drinks (water, coffee, and green tea).	Szarka et al. (2020)
Medicinal	Basil	Lamiales	Lamiaceae	*Ocimum spp.*	Tulsi, Cinamonette, Dark Green	Nitra, Slovak	Greenhouse	50 g ha^{-1}	Na_2SeO_4	Foliar	-	0.370-0.823 mg kg^{-1} DW	-	The consuming of 9 g selenised basil seeds the daily recommended dose of selenium could be covered for human beings.	The treatment ↑ the content of selenium in basil seeds that is a source of polyphenols with multiple times higher antioxidant activity.	Mezeyová et al. (2020)
Medicinal	Mushroom	Hypocreales	Cordycipitaceae.	*Cordyceps militaris*	-	Beijing, China	Greenhouse	5 - 40 µg g^{-1}	Na_2SeO_3 , Na_2SeO_4 and Se-Met	Solution	-	Selenate (±17 - 130.940), Selenite (±22 - 125) and Se-Met (12- 58 µg g^{-1})	-	The predominant Se species in Se-biofortified fruiting bodies were SeCys2 and SeMet.	The application of 40 µg g^{-1} as source of selenate and selenite ↑ the Se concentration in fruiting bodie.	Hu et al. (2019)

Table 2. Selenium biofortification

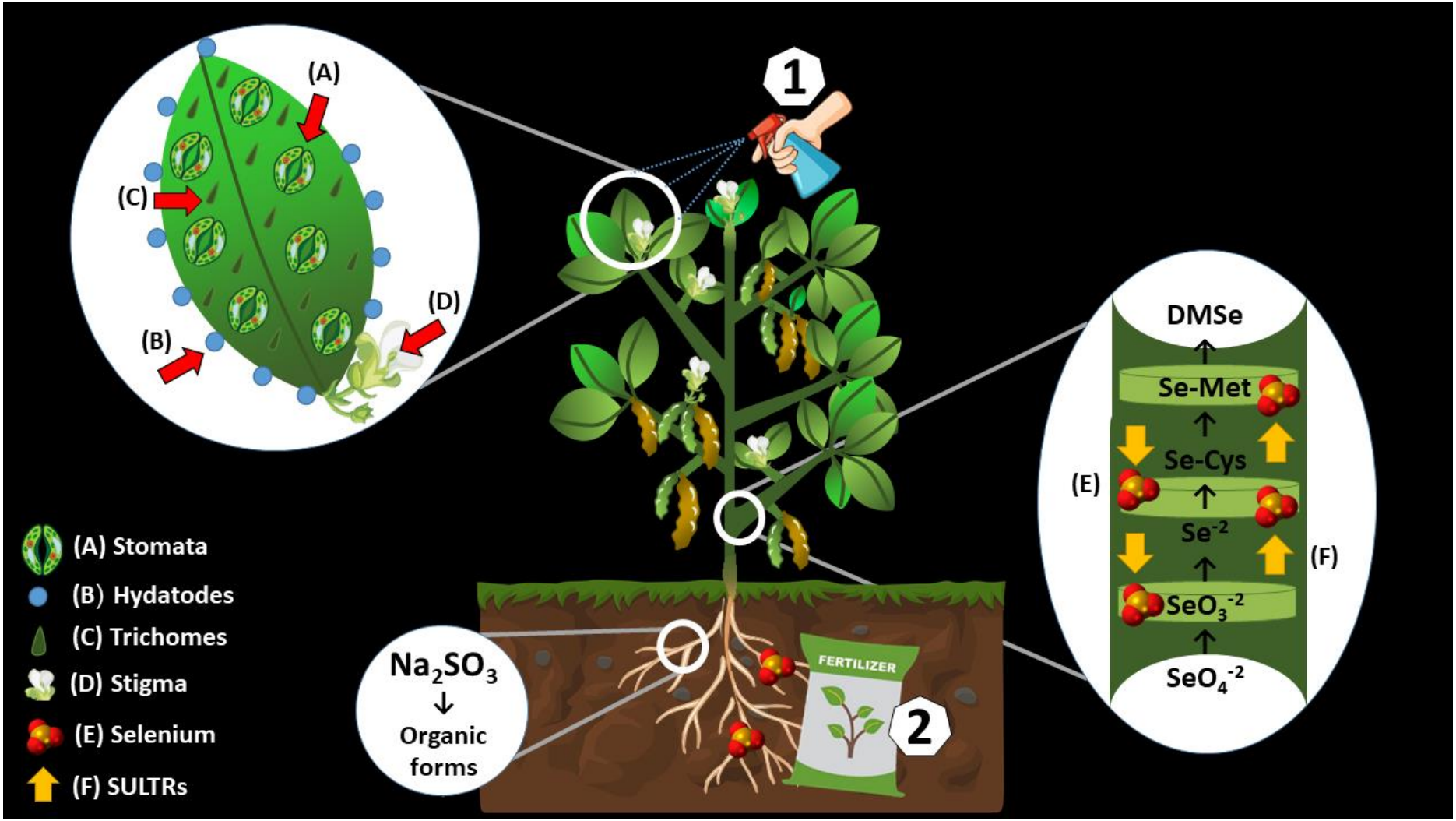


Figure 1. Selenium uptake and assimilation mechanisms by plants. (1) Foliar selenium application. (2) Selenium application by soil.

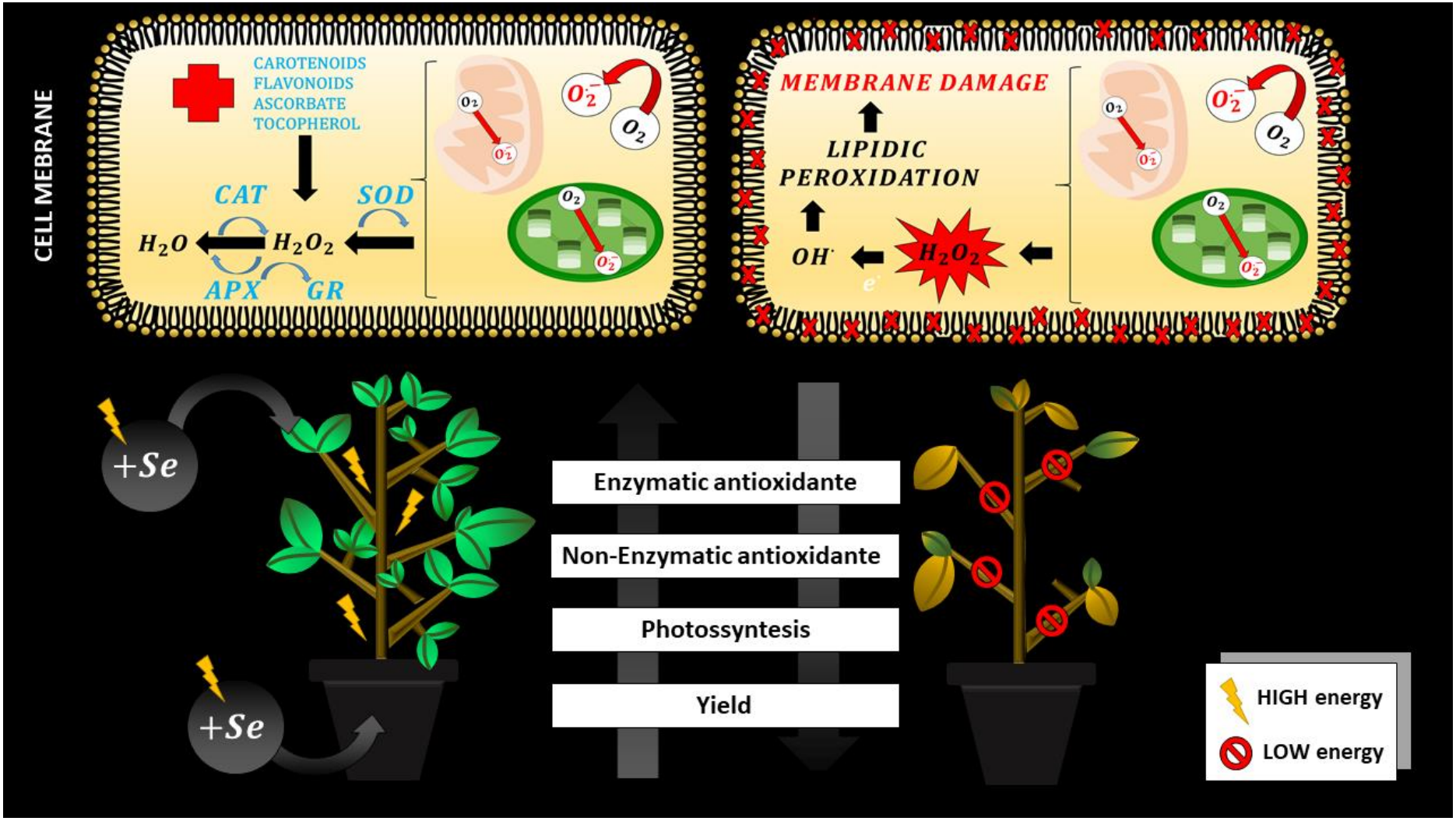


Figure 2. Selenium role in antioxidant metabolism (enzymatic and non-enzymatic) as a mitigator factor of abiotic stress and ROS scavenging system.

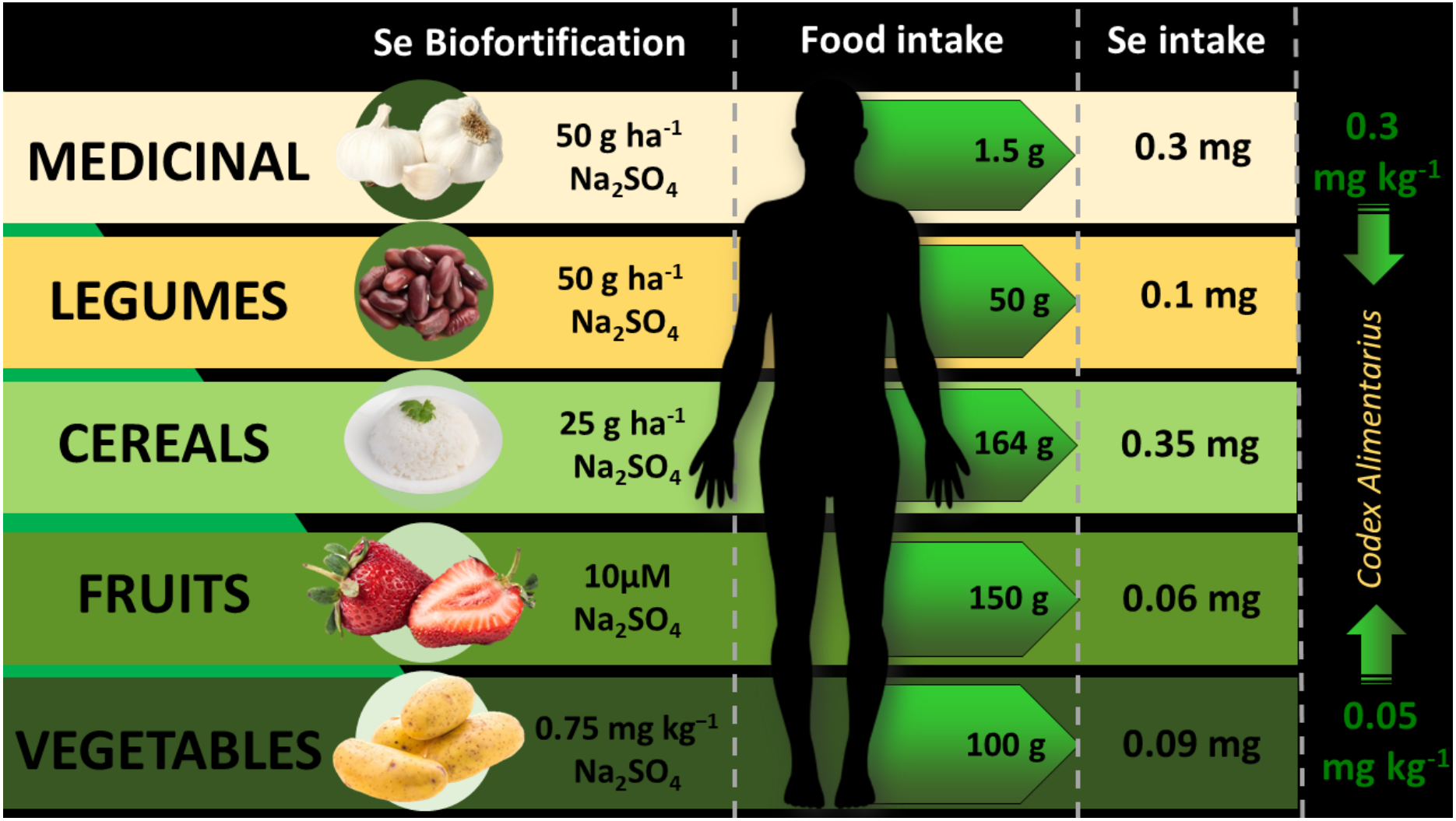


Figure 3. Se intake in the human body. Maximum (0.3 mg kg^{-1}) and minimum (0.05 mg kg^{-1}) limite range of Codex Alimentarius for biofortified foods.

CHAPTER 2 - Root-to-shoot selenium transport and uptake kinetics in cowpea plants by *in vivo* XRF

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ABSTRACT

Legumes with high protein and mineral contents, such as cowpea, are an opportunity to provide food security for the population. Selenium (Se) is an essential element with high antioxidant capacity in plant metabolism and immune response for humans and animals. However, Se levels in plant tissues are highly sensitive due to the high antioxidant potential and conversion of volatile forms. X-ray fluorescence spectroscopy (XRF) is a direct solid analysis technique that allows *in vivo* evaluation of plants. In this study, we used a portable XRF to evaluate the kinetics of Se uptake in young cowpea (*Vigna unguiculata [L.] Walp* cv. BRS Rouxinol) plants during 60 hours of experiment. Sodium selenate was utilized as the selenium source at five Se concentrations (0, 20, 40, 80, and 120 µM). Se concentration was quantified by benchtop energy dispersive XRF. The analysis of oxidative stress and activity of antioxidant enzymes (SOD, CAT, and APX) evaluated the beneficial and toxic effects of Se. The Se intensities were monitored in the second cowpea trifolium. Se uptake kinetics revealed that cowpea plants demonstrate mechanisms for limiting the uptake of this beneficial element. Interestingly, the Se-Kα XRF signal at the concentration of 120 µM was lower than 80 µM. The greatest increases in H_2O_2, MDA, and accumulation of total sugars were seen for 80 µM. All concentrations applied reduced the activity of antioxidant enzymes, being strongly influenced by the concentration of soluble proteins. The application of 40 µM of Se showed the greatest synergism with the elements K, Ca, Mn, and Zn. The lowest concentration of Se (20 µM) had the highest translocation capacity from roots to shoot (81.7%). Thus, XRF combined with the analysis of antioxidant metabolism proved to be a non-destructive and effective methodology to assess in vivo plant monitoring for kinetic studies.

Keywords: sodium selenate, *Vigna unguiculata [L.] Walp*, mineral nutrient uptake, absorption, and transport of nutrients, X-ray fluorescence spectroscopy.

1. INTRODUCTION

Agronomic biofortification is a strategy employed to produce varieties with higher minerals and vitamins content in a sustainable and low-cost way to reach populations with limited access to formal market and health systems (Adebayo et al., 2020). Amongst the concerns regarding global food insecurity, selenium, zinc, iron, vitamin A, and vitamin D deficiencies affect a significant fraction of the population (Prasad and Shivay, 2020) which are propagated by vegetable production with low nutritional value causing hidden hunger (Eggersdorfer et al., 2018).

Several studies indicate legumes with high nutritional indices to achieve sustainable development goals (SDGs) and minimize nutritional deficiencies due to the lack of accessibility to food (Maphosa and Jideani, 2017; Gerrano et al., 2019; Rehman et al., 2019). In this context, the cowpea (*Vigna unguiculata* [L.] Walp) seeds outstand as the main source of dietary protein, calories, fibers, vitamins, and minerals of a large segment of the world population (Phillips et al., 2003; Silva et al., 2018; Vasconcelos et al., 2010). Furthermore, it is a model species for phytotoxicity studies in the semiarid tropics, where Se toxicity is often a concern (Wang et al., 2013b). Therefore, cowpea can also become a biofortification model for testing Se doses and sources (Singh, 2020).

Due to the low Se content in agricultural soils, Se fertilization has already become a public health measure in many countries, as seen in Finland (Eurola et al., 2001). In recent years, emerging tropical countries, such as Brazil, have also allowed Se addition in fertilizers (regulation N° 46/2016 of the Agriculture, Livestock and Food Supply, Brazil, 2016). Selenium is considered a beneficial element to plants (Pilon-Smits and Quinn, 2010). According to Lanza and Reis (2021), Se can help to alleviate stresses related to drought, salinity, potentially toxic elements exposure, and extreme temperature by increasing activity of enzymatic compounds, e.g., SOD, CAT, and APX that act in reactive oxygen species (ROS) scavenging system.

Besides, due to its antioxidant role in the immune response of the defense system, Se is considered essential in both human and animal metabolism (Rayman, 2012; Moghaddam et al., 2020). Doses of up to 50 g ha^{-1} of Se applied as sodium selenate were indicated as ideal for the biofortification of cowpea (Lanza et al., 2020). These values correspond to an intake of about 0.1 mg of Se per day, which is recommended by the

Codex Alimentarius (Silva et al., 2018; Lewis, 2019). However, toxicity symptoms were observed even in crops under low Se doses in hydroponic conditions (Mateus et al., 2021), thus showing a narrow margin between the beneficial and toxic role of Se. Furthermore, Se levels in leaf tissues are sensitive to volatilization. At high concentrations, plants have the phytovolatilization mechanism to detoxify the metabolism, causing assimilated selenium to be converted quickly into volatile forms. (Dumont et al., 2006).

There several techniques able to reveal the Se concentration in plant tissue. One can highlight laser-Induced breakdown spectroscopy (LIBS) and inductively coupled plasma mass spectrometry with laser ablation/optical emission spectroscopy (LA-ICP-MS/OES), which offer low limits of detection and afford direct solid analysis, nonetheless they are destructive. On the other hand, the X-ray fluorescence spectroscopy (XRF) is a non-destructive and multielemental technique that allows spatial and time-resolved analysis in a myriad of sample tissues, even under *in vivo* and *in situ* conditions (Montanha et al., 2020a, Gomes et al., 2019, Bachiega et al., 2019; Reis et al., 2020).

Since it enables a real-time assessment of the nutrient dynamics within plant tissues, the *in viv*o XRF analysis has the potential to improve our understanding of plant metabolism and development (Montanha et al., 2020b, Rodrigues et al., 2018). Therefore, the present study aims to investigate kinetics of Se uptake and transport from roots to the shoot in conjunction with the responses of the antioxidant metabolism. The results can provide relevant and unprecedented information for the in vivo Se absorption kinetic studies in cowpea plants using portable XRF.

2. MATERIALS AND METHODS

2.1 Cultivation assay

The greenhouse experiment was conducted at Sao Paulo State University, UNESP, in the municipality of Tupã, Sao Paulo State, Brazil (509 m; 21°55′45″S; 50°29′32″W). Cowpea seeds (*Vigna unguiculata* (L.) Walp.) *cv*. BRS-Rouxinol were sowed in black polyethene trays containing 98 cells filled containing substrate and irrigated with deionized water. The seeds were treated with Sementhiran® (300 mL/100 kg) and Maxim xl cruser® (100 mL/100 kg).

After 14 days of germination, twenty-five plants were acclimated in five black paint-coated plastic pots (5 L) with automatic aeration, content Hoagland nutrient solution (see the solution composition in Table S1), according to Taiz et al. (2006). Firstly, the plants were acclimated with 25% of Hoagland Solution (Table S1), then on the 19th day of cultivation, the ionic force of solution was increased to 50%. After four days under the 50% ionic strength solution, the plant pots were transported to the Laboratory of Nuclear Instrumentation (LIN) (University of São Paulo), Piracicaba, Sao Paulo State, Brazil (22º 42' 24" N; 47º 38' 43" W).

2.2 *In vivo* XRF Se kinetics

Cowpea plants with twenty-one days of cultivation were placed in a growth room at 25 ± 2 ºC, and 12 h-photoperiod at 250 µmol of photons m^{-2} s^{-1} provided by 3000 K LED lights. The plants were acclimated with 80% of Hoagland Solution (Table S1) during 48 h. Moreover, at the beginning of the Se absorption kinetic study, a Hoagland solution at 0, 20, 40, 80, or 120 µM of Se were applied. The source of Se was sodium selenate (Na_2SeO_4, Merck KGaA, Darmstadt, Germany). The solution was monitored daily to volume with purified water and adjusted to pH (6.00).

The central leaflet of the second completely expanded trifoliate leaves was analyzed by a handheld XRF spectrometer (Bruker, Tracer III SD, Germany). The XRF analysis was carried out using a 4 W Rh anode at 40 kV and 30 µA, with a primary filter (304.8 µm Al + 25.4 µm Ti + 25.4 µm Cu) and the X-ray spectrum recorded by a 10 mm^2 X-Flash detector. The XRF measurement time was 60 s (Figure S1 and S2).

The leaves were marked using a pen, and the same sample region was monitored along 64 h by the portable XRF. The detector dead-time was lower than 1%. The instrumental stability was checked with a Se thin-film standard at 49.5 µg cm^{-2} Se (Micromatter™, serial number 30112, Canada) under the same analytical conditions as the leaf samples just before each measurement along the experiment. Five biological replicates were employed. Only Se XRF intensities whose signal with statistical significance above the noise were considered. Besides, the XRF Se signals were normalized by Rh-Kα Compton scattering to correct the effects of the cowpea leaf thickness variation.

2.3 Se quantification by Benchtop EDXRF

Right after the kinetics assay, the cowpea root and shoot were separated. The roots were rinsed in a sequence of HCl 0.3 M, and Na_2HPO_4 0.1 M solutions for 1-min each for removing the Se adhered to outer root surfaces. The sampled tissues were then dried in a laboratory over at 60°C for 24 hours (Carmo et al., 2000).

The quantification of Se in the plant tissue was performed by energy dispersive X-ray fluorescence spectroscopy (EDXRF, Shimadzu EDX-720, Japan) according to a modified method described by Bachiega et al. (2019). The EDXRF analysis was carried out using a 50 W Rh X-ray at 50 kV and auto-adjusted current (maximum of 30% of the detector dead time), 3 mm collimator and Ag primary filter. A Si (Li) detector was used for recording the sample X-ray spectrum. The acquisition time was 200 s.

Selenium quantification was based on a broccolis-based external calibration curve at 0, 50, 100, 150, 200 e 250 mg kg^{-1} Se, according to Bachiega et al. 2019. The trueness of the method was evaluated by spike-and-recovery assay, in which 300 □L of a 100 µg mL^{-1} Se aqueous standard (from 1:10 dilution 1 mg mL^{-1} of Se Acros Organics) were added in 300 mg of cowpea leaf, previously weighed in a petri dish. Subsequently, the spiked standard was dried in a laboratory oven (Tecnal TE-393) at 60 °C for 12 h and homogenized utilizing an agate mortar.

The dried samples were ground in a mortar with the aid of a pistil, and refined through an 80-mesh stainless steel sieve. One hundred micrograms of sample and standard were transferred into a 6.3 mm window XRF cuvette (cat. no. 3577, Spex) sealed with 6.35 µm thick Mylar® film (no. 3517, Spex). The powder sample was pressed manually with a glass stick to avoid voids in the sample. The typical sample thickness was ca. 7 mm. Three biological replicates were analyzed.

2.4 Se translocation index

The translocation index was calculated using the Se concentrations (mg kg^{-1}) values of the roots, and the shoot quantified in Benchtop EDXRF. Equation 1 was applied according to Abichequer and Bohnen (1998).

$$Equation\ 1. Root\ to\ Shoot\ Se\ translocation\ index\ (\%) = \frac{Se\ content\ in\ the\ shoot}{(Se\ content\ in\ plant)}\ x\ 100$$

The Se content in the plant is the sum of its concentration in the shoot and the root.

2.5 Determination of antioxidant metabolism

The determination of antioxidant metabolism was performed by analyzing following enzymatic activities: superoxide dismutase activity (SOD, EC:1.15.1.1), catalase activity (CAT, EC:1.11.1.6), ascorbate peroxidase activity (APX, EC:1.11.1.11), hydrogen peroxide concentration (H_2O_2), and lipid peroxidation (malondialdehyde). After 64 h of Se exposure, two leaves per plant were collected and quickly stored in plastic bags in liquid nitrogen for all analyses. A composing sample of leaf material replicates of each treatment was macerated in nitrogen liquid and stored in a freezer at −80 °C.

The extract of the enzymatic activity was performed using 1.5 g of plant material and adding 1 mL of phosphate buffer pH 7.5 0.1M + 1mM EDTA + 3mM DTT with 4% PVPP, and stored in a freezer (Gomes-Junior et al., 2006). To determine the SOD activity were added: 2 ml of Na buffer at pH 7.8 0.05 M, 50 µL of extract, 200 µL of nitro blue tetrazolium chloride (NBT) 75 µM, 250 µL of methionine, and 250 µL of riboflavin (Giannopolitis and Ries, 1977). The mix was vortexed and exposed to light for 20 minutes at room temperature. Samples were read on a spectrophotometer (UV VIS SPECTRO 580UVP) with λ = 560nm.

CAT activity was determined by adding 25 µL of supernatant and 1 mL of buffer K pH 7.5 0.1M with H_2O_2 in the cuvette. Immediately, spectrometer measurement began at λ = 240 nm (absorbance). The reading was monitored during the 1-minute interval, and the absorbance was recorded every 10 seconds to observe the H_2O_2 degradation (Azevedo et al., 1998). APX activity was measured by adding in a glass tube: 600 µL of Buffer K pH 7 0.08M and 100 µL of EDTA 1 mM. The vial was heated at 30°C for 10 min and added 100 µL of extract and 101 µL of H_2O_2 1 mM. The sample was measured at λ = 290 nm (Nakano and Asada, 1981; Moldes et al., 2008). The determination of the antioxidant enzyme was performed based on the total protein concentration, which was

determined according to the Bradford method, using 5 µL of extract and 1 mL of Bradford Solution (Bradford, 1976).

The extract of MDA and H_2O_2 was made using 0.4 g of fresh leaves macerated in liquid nitrogen and 4 mL of 0.1% trichloroacetic acid. Lipid peroxidation was estimated based on malondialdehyde (MDA) (Heath and Packer, 1968). MDA test was performed using 1 mL of TCA 20% + Thiobarbituric acid (TBA) 0.5%. The assay was heated to 95°C for 30 min, and then cooled for 10 min in ice bath. The sample was measured at λ = 600 and λ = 535 nm, in which the difference between the absorbances provides the results. The H_2O_2 teste was performed using 200 µL of extract, 200 of K buffer pH 7.5 0.1 M, 800 KI 1 M. Then, the vial was cooled in the dark for 1 hour and measured at λ = 39 0nm. This method was adapted from (Alexieva et al., 2001).

2.6 Total sugars

The total sugars was extracted using 1 g of plant material and 10 mL of MCW solution (methanol:chloroform:water) (Dicksson and Haissig, 1979; Rose et al., 1991). The test was performed by adding i) 50 µL of MCW extract diluted in 450 µL of deionized water, ii) 500 µL of 5% phenol solution and iii) 2 mL of 100% H_2SO_4 (Dubois et al., 1956). The solution was homogenized by vortexing, and the absorbance was measured at 490 nm. The total sugar concentrations were based on the standard sucrose curve, and the results are expressed in mg g^{-1} FW.

2.7 Morphology

Diagnostic trefoil sheets were collected for each treatment and fixed at FAA 70% (formalin, acetic acid, and ethyl alcohol) (Johansen and McCracken, 1940). The samples were transferred to a 70% ethanol solution after 5 days of fixation, where they remained stored until cross-sections of the plant tissues were performed.

The plant tissues were cross-sectioned using the free-hand cutting technique, with the aid of styrofoam blocks, blades, and scalpel. For the histochemical test of starch, freehand cuts were made, and the plant material was subjected to a sodium hypochlorite solution (6%) for 60 minutes and washed with distilled water in three batteries for 10

minutes. The test for starch detection was performed with a 10% Lugol solution (Berlyn and Miksche, 1977).

For permanent preparation, the cuts underwent an ethanolic dehydration series (10, 20, 30, 50, 70, 80, 90 e 100%), for 10 minutes each. Then, plastic resin (Leica Biosystems Nussloch GmbH, Germany) was added to the material. The samples were submitted to a vacuum pump for 7 days to facilitate the infiltration. During the infiltration process, the resin samples were kept in a closed place with silica, to avoid humidity. After complete infiltration, the samples were added to rectangular wooden blocks (blocking), as preparation for micrometer sectioning.

The samples were sectioned, 5-7 µm thick, using a rotational microtome (Leica RM 2045). The histological analysis using photomicrographs. Cross-sections were mounted on glass slides and stained with toluidine blue (Sakai, 1973). The images were processed and the biometric parameters were calculated using the ImageJ® software.

2.8 Statistical analysis of results

The results of the antioxidant metabolism were submitted to the outliers test (Grubbs Test) in the Minitab® Statistical Software to identify extreme points in the data dispersion. Then, on the same platform, the data normality test was performed by the Anderson-Darling method, using a significance level p>0.05 to verify the trend of the values. The experimental design was considered five treatments with four replicates in the R-Project® software (Bell Laboratories). The variance analysis of data was analyzed using the Tukey method at 5% probability.

Linear regression analyses were performed for Se concentration in response to the applied concentrations. Regression models were selected based on the significance of the regression coefficients and the F test ($p \leq 0.05$). The graphics were built from the SigmaPlot 12.5 software.

The percentage variation was used to identify the variation in nutrient concentration (Figure S2). The formula used is:

$$Equation\ 2.\ PV\ (\%) = \frac{(a-b)}{b}\ x100$$

Where:

A = Baseline

B = Reference value obtained by applying treatments

The calculation of variation rate (Table S2) was used for determining the variation of the treatment data is between the time of analysis, according to equation 3

$$Equation\ 3.\quad \frac{f(b) - f(a)}{b - a}$$

Where: f is the recorded XRF elemental intensity (counts) and b and a is the difference between two consecutive times of measure.

3. RESULTS

3.1 *In vivo* Se kinetics

Figure 1 presents the normalized XRF Se intensities recorded on cowpea leaves during the 60-h *in vivo* assessment. It is noteworthy to highlight that the Se XRF intensities are directly proportional to Se concentration. Although Se signals were firstly detected *ca*. 4-h after the beginning of the experiment, increasing of Se intensities were observed in plants subjected to the highest concentrations of Se, *i.e.*, 40, 80 and 120 µM, from 12 to 16-h after the initial exposure. For 20 µM of Se, however, such increase was verified only after 20 h the initial exposure. Nevertheless, one should notice that the recorded values were close to those found in the control group.

The point-by-point and linear regression analysis of Se absorption (Table S2 and Figure S3) unveil points where Se absorption was significantly higher compared to the previous point. Between the 4-12 hours points, the intensities increased more than four-fold for 80 µM and six-fold for 120 µM. On the other hand, the intensities for the 40 µM treatments increased in the following hours (12-16 hours). Moreover, it was seen that the dosage of 20 µM varied by more than 160% at the 16-20 hour points. The calculation of the percentage variation of the normalized intensity of Se demonstrates that the lower the

concentration of the element in the solution, the longer the time to increase the absorption by the roots.

The stabilization of Se intensity was seen only for the smallest treatments (20-40 µM) in 60 hours of analysis. This analysis was performed by evaluating the last two monitoring points (48 and 60 hours), which suggested drops of -25% for 20 µM and -9% for 40 µM of Se. As for the highest dosages, no evident stabilization was seen in the last hour of monitoring. However, the low variations between the last hours of the analysis suggest a stabilization trend in plant absorption.

Figure S4 shows the intensities of Ca, K, Mn, and Zn. The fluctuations of the elemental intensities do not present a clear trend, neither indicating to be affected by Se uptake nor as a function of the time.

3.2 Se concentration

Figure 2 demonstrates Se concentration in shoots (Figure 2A) and roots (Figure 2B) of cowpea plants and then root-to-shoot Se translocation factor (Figure 2C). The control treatment was not shown because the Se concentration was below the limit of detection (LOD =0.9 mg kg^{-1} Se). For the sake of information, the recovery of the method was 111 %.

For the shoot (Figure 2A), the concentration of samples treated with 20 µM of Se was 29.26 mg kg^{-1}. The 40 µM concentration corresponded only 14.26 mg kg^{-1}, which is about half of the 20 µM concentration. While, although the 80 µM leaf intensities were higher (Figure 1A), the 120 µM dosage resulted in a concentration around 13% higher compared to the 80 µM treatment.

For the roots (Figure 2B), linear behavior was observed. The amount of Se seen for the 20, 40, 80 and 120 µM treatments was 6.54 mg kg^{-1}, 20.58 mg kg^{-1}, 34.68 mg kg^{-1} and 58.26 mg kg^{-1}, respectively. Comparing the concentration found for the shoot (Figure 2A) and the roots (Figure 2B), it can be seen that the concentration of 20 µM was the only one with the highest concentration in the shoot. The other treatments accumulated more Se in the roots.

In this regard, Figure 2C shows the percentage of translocation of each treatment from roots to shoot. The treatment of 20 µM of Se had the highest translocation rate,

corresponding to 81.7%. Then, the 40 and 80 µM treatment had similar translocations, with 41% and 48.6%, respectively. Moreover, the smallest translocation was retained in the 120 µM of Se treatment, with 38.7%. Thus, the analysis of the results expressed in Figure 2 reveals that low concentrations (20µM) have greater translocation capacity from roots to shoots. In the opposite situation, the application of high concentrations of Se (120 µM) is retained in the roots.

3.3 Antioxidant metabolism

Lipid peroxidation was assessed through the concentration of malondialdehyde (MDA) (Figure 3A), the hydrogen peroxide content present in cowpea leaves (Figure 3B), and the accumulation of sugar in the tissues (Figure 4E). The analysis aimed to evaluate the health of cowpea plants in response to Se concentrations.

MDA concentration (Figure 3A) increased proportionally to the concentration of the Se applied. Compared to the control, the increase in MDA concentration ranged from 4% for the application of 20 µM to 28% for 120 µM of Se. The lowest concentration used (20 µM) reduced the H_2O_2 content by 15% (Figure 3B), and the concentration that increased the most was 80 µM, with about 47% compared to the control. Similarly, for sugars (Figure 4E), the 80 µM treatment was the one that most stimulated the accumulation in plant tissues. The other treatments of the treatments indicated less sugar (20 and 40 µM) and an amount similar to that found in control (120 µM).

The activity of the antioxidant enzymes APX (Figure 4A), CAT (Figure 4B), and SOD (Figure 4C) were measured concerning the amount of protein in the samples (Figure 4D). For all enzymatic activities analyzed, the control treatment showed the highest performance. Since the lowest concentration was applied, there was already a significant decrease in the action of enzymes in tissues.

APX (Figure 4A) demonstrated a reduction in its activity inversely proportional to Se concentrations. Since the higher the Se concentration, the lower the enzyme activity. In Figure 4B, CAT showed increased activity at 80 µM of Se. However, concentration at 120 µM showed the lowest CAT activity. Likewise, SOD (Figure 4C) reduced its activity by more than 90% at the highest concentration, compared to the control treatment. The reduction in enzymatic activities is directly linked to the accumulation of proteins in plant

tissues, which gradually increased with the concentrations used. Figure 5 shows the profile of the plants after each hour of monitoring. It demonstrates that there was no visual modification on the leaf surfaces, mainly related to phytotoxicity symptoms.

3.4 Morphology analysis

Cytochemical testing revealed that starch granules were found in greater quantity in midrib parenchyma cells in the control group (Figure 6A), as well as in the palisade parenchyma of those plants treated with 80 µM (6K). The distribution of starch granules was also observed in the guard cells in the stomata (Figure 6 C, F, I, L, O). Besides, the cowpea has a dorsiventral mesophyll, a layer of palisade parenchyma facing the adaxial surface, and a layer of spongy parenchyma closer to the abaxial epidermis.

Moreover, the analysis of cowpea leaf biometry showed a decrease in the thickness of the adaxial epidermis (Figure 7A) in those plants exposed to 40 µM Se, whereas no significant differences were observed in the other treatments. Figure 8A reveals a *ca*. 30% reduction compared to the control. The thickness of the abaxial epidermis (Figure 7B) showed no difference between treatments (Figure 8B).

On the other hand, a significant increase in the palisade parenchyma (Figure 7C) and the spongy parenchyma (Figure 7D) thickness were observed in the mesophyll cells. The 120 µM Se concentration induced a 20% increase in the palisade parenchyma compared to the control treatment (Figure 8C), whereas the spongy parenchyma thickness increased by 34% and 46% in those plants treated with 80 and 120 µM Se, respectively (Figure 8D). Plants present in treatments with 80 and 120 µM Se also presented increased growth in leaf thickness (Figure 7E) compared to the other treatments. The leaf thickness of the plants was increased by 18% for the application of 80 µM Se and 25% for 120 µM Se (Figure 8G)

Regarding the analysis of the vascular system, an increase in the diameter of the vessel element in the leaf was observed (Figure 7F) with the application of 20 µM Se, which corresponds to approximately 30% (Figure 7E). The cross-sectional area of the vessel element also suffered a significant effect from the treatments, where the application of 40 µM Se increased the variable under analysis by 64% (Figure 8F).

4. DISCUSSION

4.1 Optimization of experimental parameters

The optimization of the instrumental parameters is crucial to improve the analyte LOD (lower values) and reduce the error of the measurement. For that, the signal-to-noise ratio (SNR) was evaluated utilizing different primary filters (Tadeu Costa Junior et al., 2020).

Higher SNR results from a higher analyte counts or/and lower background values. The primary filters are often very helpful to increase SNR, since it reduces the background values to a greater extent, thus improving the detection of the element of interest (Byers et al., 2019). Herein, the highest SNR was achieved by using a 304.8 µm Al + 25.4 µm Ti + 25.4 µm Cu filter, as shown in Fig. S1. The same filter was proven to be the more appropriate for Se detection in broccoli (Bachiega et al. (2019).

Another important parameter regarding the coefficient of variance among measurements as a function of measurement time. Previous studies have shown that 60 s (CV < 5%) and 180 s (CV < 15%) are suitable for the simultaneous detection of macro and micronutrients, respectively, in living soybean leaves (Tadeu Costa Junior et al., 2020), whereas 120 s was used for specifically Mn determination (Machado et al., 2019). However, similar results were found to detect P in sugarcane with a time of 60 s (Soares et al., 2021). According to the authors, coefficients of variation <5% are acceptable for *in vivo* analysis of plants. Thus, the analysis measurement time should be established according to the sample and analyte investigated.

On the other hand, one should keep in mind a shorter exposure time can be positive to preserve plant integrity, since X-ray radiation can damage biological tissues due to photolysis or heating (Rodrigues et al., 2018) and high-throughput analysis. According to Jones et al. (2017), damage caused by radiation from X-ray fluorescence microscopy may invalidate its usefulness. In Montanha et al. (2020a) studies, no tissue damage was observed in a soybean leaf irradiated by a 1 mm-collimated X-ray beam at 45 kV and 900 µA during 60-min. Although it is not possible to compare the beam fluxes between the conditions since each plant was herein irradiated for a total of 11 min using an X-ray source at least 33-fold smaller power rate. It is possible to ensure that plant

elemental composition and structure were not affected by the X-ray exposure, as can also be seen in Figure S4.

Besides filter and exposure time, the Se intensity might also be affected as a function of leaf position. As illustrated in Fig. S5, significant differences in the Se region of interest (ROI) spectra was observed on measurements carried out on the youngest trefoil and cotyledonary leaves of cowpea plants, thus indicating that there are significant differences between the transport and accumulation of Se from the leaves of the same plant.

Studies show that the concentration of Se found is higher in young leaves than older ones during seedling growth (Harris et al., 2013; Cappa et al., 2014). (Harvey et al., 2020) demonstrated that the highest concentrations are found in younger leaves (13.600 µg Se g^{-1} DW) than older leaves (460 µg Se g^{-1} DW). In addition, the greater accumulation of Se in the younger leaves is related to the highest transpiration rates, given the stage of plant development (Pickering et al., 2000).

Plant development goes through several stages, where the sink and source function are vary between the leaves of the plant. Cotyledon leaves are initially used as a nutrient reserve to support heterotrophic plant development. However, with the maturity of the phenological stages, the translocation of metabolites to the trifoliate leaves occurs. Which, in turn, will be responsible for providing the nutrients for the grain filling process. In this process, when a leaf is no longer the source, it starts its senescence process, reducing its metabolic functions (Brown and Hudson, 2015).

In this sense, the low levels of Se intensity in the cotyledonary leaves are understood compared to the trefoil leaves, were plants already in an advanced phenological stage, which justifies the accumulation of Se in the upper leaves (trifol) compared to the cotyledonary leaves. Considering that the cowpea plants were already between the phenological stages V5 (third trifoliate leaf with separate and completely open leaflets) and V6 (primary of the second branch in the axils of the unifoliate leaves, and the axils of the first trifoliate leaves) (Campos et al., 2000). Thus, the elemental evaluation in plants *in vivo* must consider the physiology and plant development to maximize benefit from the results.

4.2 Se metabolism in cowpea plants

Increasing Se concentration in the solutions resulted in faster uptake by cowpea plants (Figure 1). This fact can be attributed to the excess of solutes favoured by the water flow from the solution to the xylem, provided by the mass flow phenomenon (Zhang et al., 2019). Furthermore, organic forms of Se are absorbed faster than inorganic forms (Dhir, 2020). Moreover, selenate is the most mobile form of Se in the plant, established by the order selenate> SeMet> selenite/SeCys, evaluated in wheat and canola (Renkema et al., 2012).

Thus, besides the absorption of selenate being an important fact for understanding the kinetics, the assimilation and transformation of its isoforms also define the transport and volatilization of the element. Moreover, they are also crucial to understanding the beneficial and toxic role of the element within cells. For a better understanding, the selenate assimilation process will be discussed below.

The available forms of Se are absorbed by the plant through active processes, and therefore, with energy expenditure (Schiavon et al., 2017). The roots absorb selenate via sulfate transporters (SULTR1.1 and SULTR1,2) (Gigolashvili and Kopriva, 2014). These transporters remain active in the passage of selenate into the root xylem via group 2 low-affinity transporters (SULTR2; 1 and SULTR2; 2), aided by SULTR3; (Kataoka et al., 2004). After its absorption by the root cells, selenate quickly moves through the symplast from the root to the stele and is translocated to the shoot (White, 2018). Selenate enters leaf mesophyll cells, hence chloroplasts, via SULTR3; 1 (Cao et al., 2012).

The results of Se concentration indicate that only the lowest concentration (20 µM) accumulated more Se in shoots than in roots (Figure 2A and B). Revealing that there was an intercept of the element's translocation. As shown by de Souza et al., 1998, concentrations from 0 to 20 µM are transported to the shoot, but concentrations from 50 to 200 µM remain primarily in the roots. For rice plants grown at 1 µM selenate for one week, Se concentrations were 6.25 µg g^{-1} in the roots and 66.5 µg g^{-1} in the leaves (Wang et al., 2015). This study shows that the lower the concentration of Se applied, the greater the translocation factor (about 89%), similar to that seen in Figure 2C.

A fact associated with low translocation is that after the roots assimilate selenate, it is converted into selenite, that is incorporated into organic molecules such as Se-

methionine and Se-cysteine (Adebayo et al., 2020). Se can also be incorporated into S derivatives (glutathione) (Dawood et al., 2020), glucosinolates (Matich et al., 2015), and iron agglomerates (Fe) –Se (Van Hoewyk, 2013). All these components being possible reasons for the low translocation of the element to the shoot. The studies of Kikkert and Berkelaar (2013) revealed that plants exhibited higher Se concentrations in shoots when exposed to higher Se concentrations for longer periods of exposure. The exposure time may be related to the fact that even after 60 hours of contact with Se, the highest dosages (80 and 120 µM) still showed signs of absorption of the element (Figure 1).

To avoid plant toxicity, methylated non-protein amino acids, SeMet and SeCys, can be converted into volatile compounds, such as dimethyl selenide (DMSe), which evaporates and moves into the atmosphere (Pilon-Smits and Quinn, 2010). Otherwise, excess Se may accumulate in the vacuoles of cells within the leaf vasculature and mesophyll (Wang et al., 2015; Lanza et al., 2020). Excess organic forms of Se can generate nonspecific selenoproteins. According to the pioneering work of Brown and Shrift (1980), Se toxicity occurs when a tRNAcys binds to selenocysteine instead of cysteine during translation, forming nonspecific and plant-toxic selenoproteins.

Thus, the limitation of Se absorption at the highest concentration (120 µM) (Figure 1) and the low translocation of the element to the shoot (Figure 2C) can be explained by the fact that high Se concentrations lead to rapid conversions of elementals forms by plants (Renkema et al., 2012). Alternatively, the efflux mechanism may occur, which is a form of defense by plants to reduce the entry of metalloid ions into cells (Dhir, 2020). The limitation of Se was seen for mustard under the application of 20 µM of sodium selenite and was justified by the physiological mechanisms explained above (de Souza et al., 1998). However, studies indicate that selenite is a more toxic form for plants than selenate (Wang et al., 2013b). Moreover, there are no reports in the literature of the limitation of selenate by plants observed *in vivo*, as seen in this study.

Thus, the accumulation of Se ions generated rapid transformations of selenate into organic forms, which may have gone through the process of (i) incorporation/volatilization or transformed into substances (ii) toxic to plants. This information can be monitored by the results of antioxidant metabolism (Figure 3), which revealed that the greater the Se intensity (Figure 1), the greater were the indicatives of lipid peroxidation, seen in the H_2O_2

content (Figure 3A), the accumulation of MDA (Figure 3B), and tissue sugars (Figure 4E). The literature has also reported lipid peroxidation caused by Se toxicity (Cabral Gouveia et al., 2020; Silva et al., 2020; Mateus et al., 2021).

ROS are the product of aerobic metabolism in a plant that acts as signaling molecules. However, excess ROS can cause oxidative stress, which arises when the ROS level exceeds the defense mechanisms (Sharma et al., 2012). The overproduction of ROS causes peroxidation of membrane lipids, and one of the main meters is through the quantification of malondialdehyde (MDA), a widely used marker of oxidative lipid damage caused by environmental stress (Lanza and Reis, 2021).

Selenium was able to reduce H_2O_2 in cells at a dosage of 20 µM (Figure 3B). However, all concentrations of Se increased MDA levels (Figure 3A) compared to the control, indicating possible damage to the cell membrane of cowpea plants. On the other hand, the sugar contents (Figure 4E) were increased only at the highest dosages (80 and 120µM), and they are a biomarker indicator of ROS (Mostofa et al., 2017). According to Malik et al. (2011) Se has a positive relationship with carbohydrate metabolism, being able to stimulate the activity of starch hydrolysis enzymes, such as amylases and sucrose hydrolysis enzymes (Figure 6 and 7).

The studies of Cabral Gouveia et al. (2020) demonstrate that Se induces oxidative stress in rice plants, and the data indicate increases of 3 times more MDA and twice as much H_2O_2 for plants treated with Se. In our studies, the maximum differences were ±4 nM g^{-1} FW for MDA (Figure 3A) and ±3 µM g^{-1} FW for H_2O_2 (Figure 3B). Indicating small variations may explain the low activation of the enzymatic antioxidant system, which is composed of antioxidant enzymes.

Lanza et al. (2020) demonstrate the visual aspects of Se toxicity in cowpea plants treated with high concentrations of Se. Souza et al. (1998) showed in their studies that mustard plants treated with Se for seven days began to show symptoms of toxicity marked by chlorosis at concentrations above 200 µM. However, Figure 5 demonstrates that there were no visible symptoms of toxicity in the cowpea plants.

The results reveal that the most active enzyme was CAT (Figure 4B) at the 80 µM and that it is also associated with higher intensity of the elements detected in plants. Indicating that the enzyme acted, which is known to convert H_2O_2 into H_2O and APX

(Figure 4A). SOD (Figure 4C), the barrier enzyme in the defense mechanism acting in the dismutation of O_2^- into O_2 and H_2O_2, had its activity linearly reduced in response to the application of concentrations of Se. Enzymes are expected to act in the following process: $O2^{\cdot-} \rightarrow H_2O_2 \rightarrow H2O$ (Silva et al., 2018; Reis et al., 2020)

The behavior of antioxidant enzymes agrees with the study of Mateus et al. (2020) for coffee. In the present study, the concentration of 1 mmol L^{-1} reduced SOD and CAT activity, which was clearly explained by the increased concentration of soluble proteins (Figure 4D), possibly induced by Se stress. The authors state that the dosage of Se was not sufficient to cause disorder in the coffee tree's antioxidant system. Furthermore, antioxidant enzymes can also be related to plant nutrition (Tavanti et al., 2021).

The increase in Ca concentration (Figure S4-B) in plant tissues is one of the main indicators of plant stress. Lanza et al. (2020) demonstrated by the X-ray microfluorescence technique (µXRF) that leaf lesions caused by mineral toxicity had higher Ca concentrations than uninjured regions. In this study, there was no relationship between the increase of Ca and the high Se concentration, and it was stimulated precisely by the lower Se concentration (20 and 40 µM).

5. CONCLUSION

This study demonstrates that young cowpea plants have mechanisms for limiting high Se concentrations, as shown at concentrations above 80 µM. In addition, low Se concentrations are more efficient for translocation from root to shoot. Indicating that studies aimed at enriching grains with Se should choose rates that perform high translocation rates. Thus, the portable XRF has proven as an effective tool for investigating the Se uptake transport under *in vivo* conditions in plants through specific experimental conditions for the element of interest. The analysis of antioxidant metabolism proved to apply to the understanding of Se levels in the analysis of absorption kinetics. Revealing that the use of integrated laboratory practices demonstrates satisfactory results on plant metabolism.

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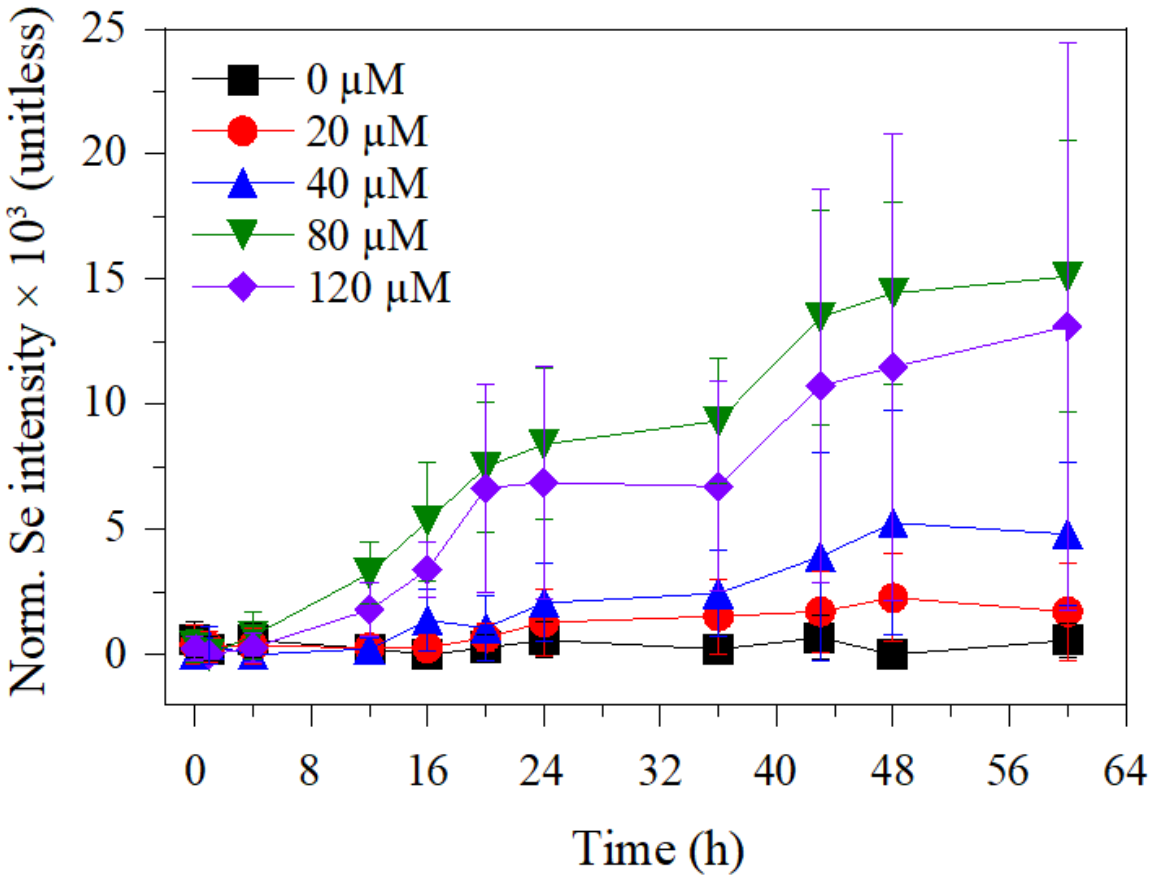


Figure 1. *In vivo* monitoring Se XRF intensities in cowpea leaf in response to Se application rates.

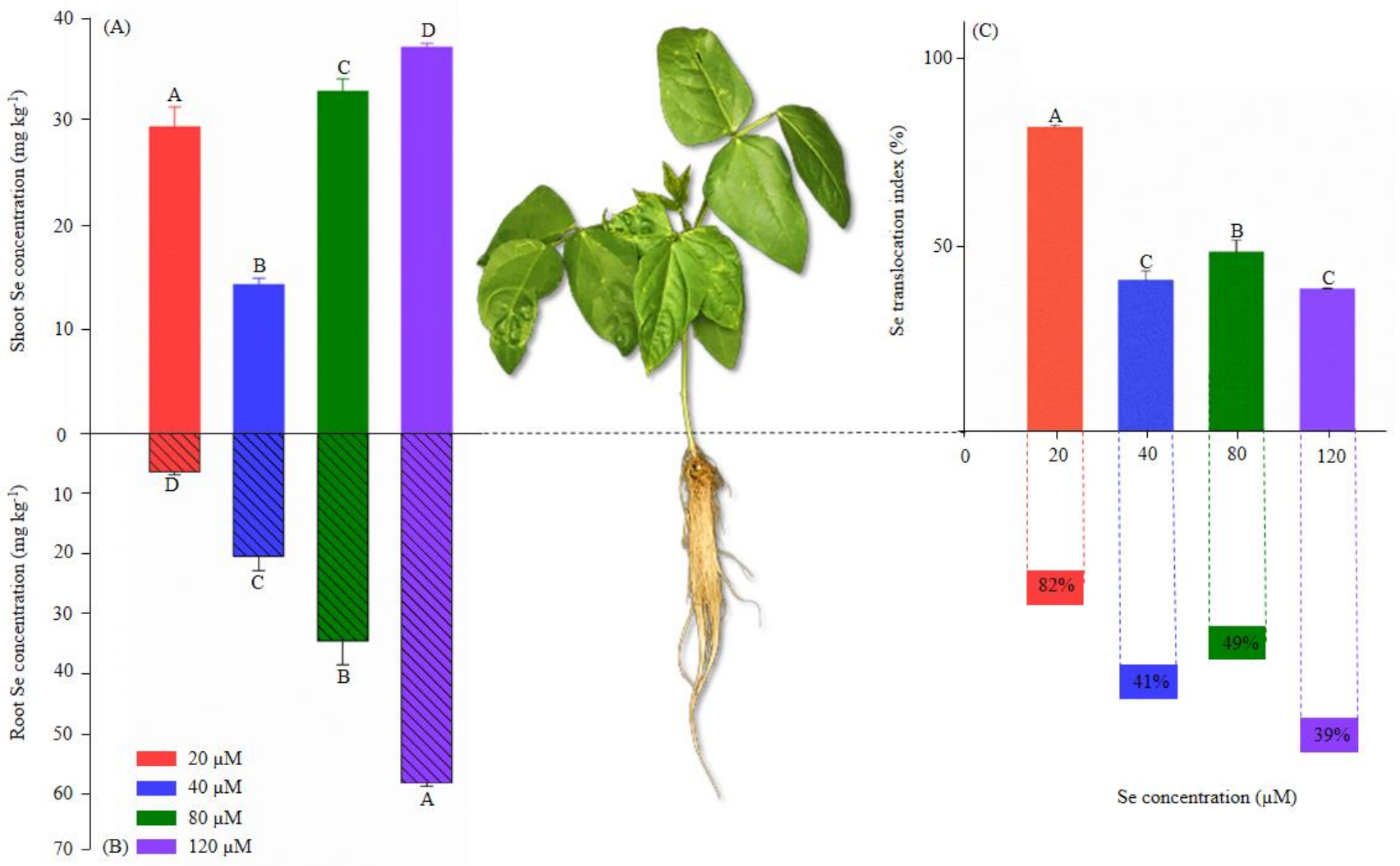


Figure 2. Se concentration determined by Benchtop EDXRF in (A) Shoots and (B) Roots, and (C) determination of Se translocation index (%). Error bars indicate the standard error of the mean (n=4 replicates). Different letters indicate the difference between treatments means according to a Tukey test (P≤ 0.05).

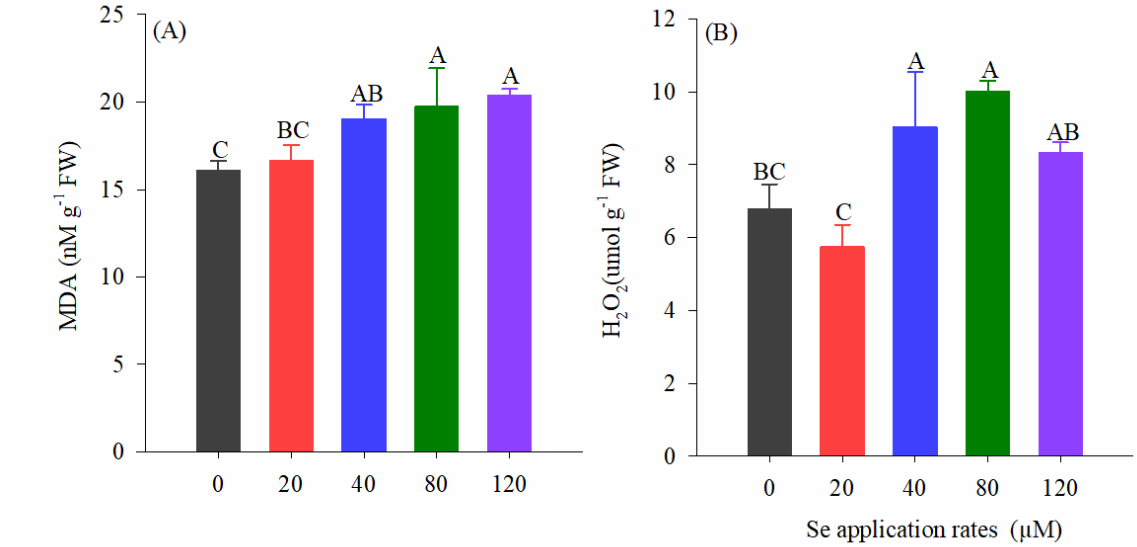


Figure 3. Antioxidant metabolism in cowpea plants. (A) Malondyaldeide and (B) Hydrogen Peroxide. Error bars indicate the standard error of the mean (n=4 replicates). Different letters indicate the difference between treatments means according to a Tukey test (P≤ 0.05).

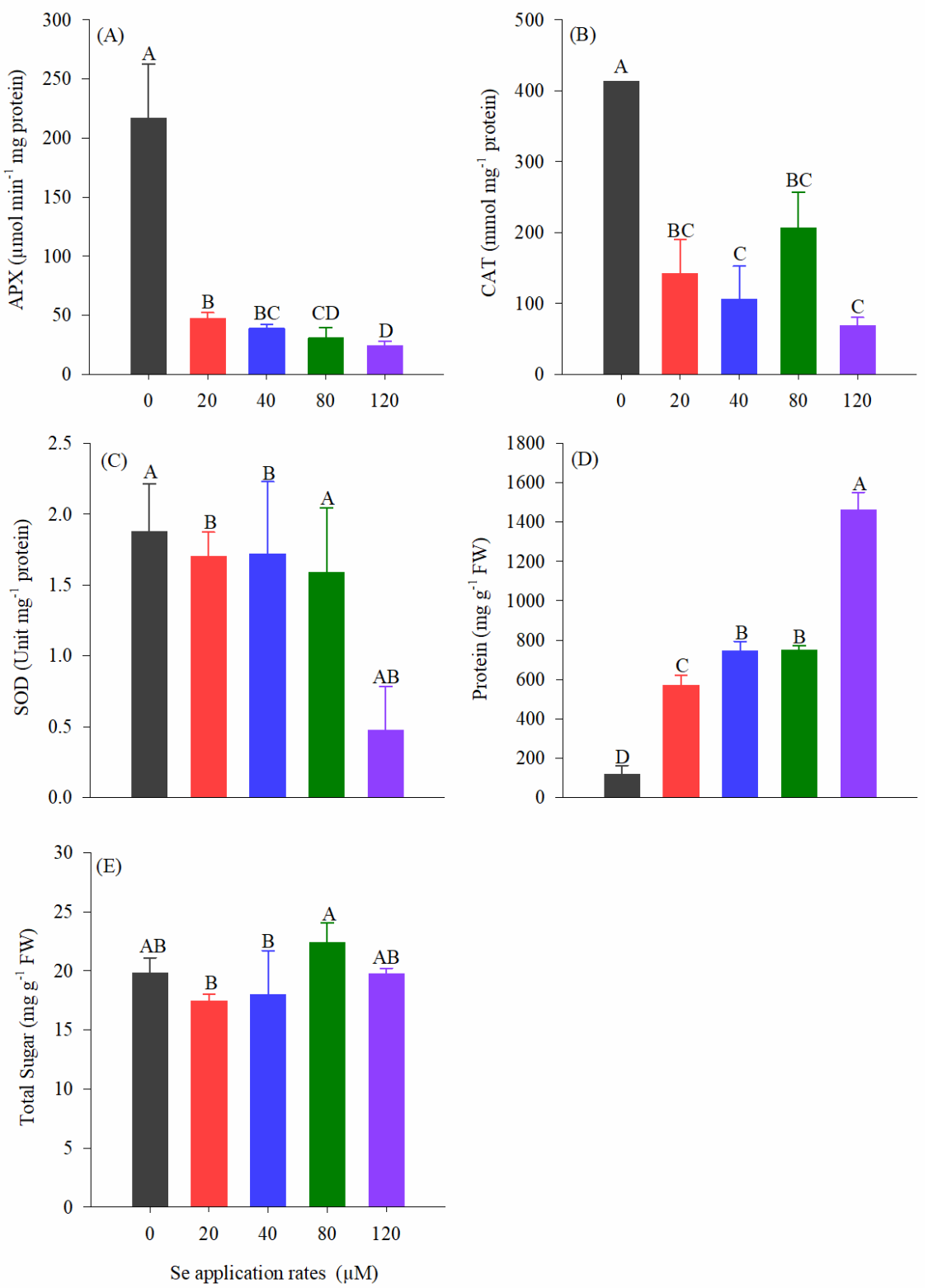


Figure 4. Antioxidant Enzymes and compounds. Error bars indicate the standard error of the mean (n=4 replicates). Different letters indicate the difference between treatments means according to a Tukey test (P≤ 0.05).

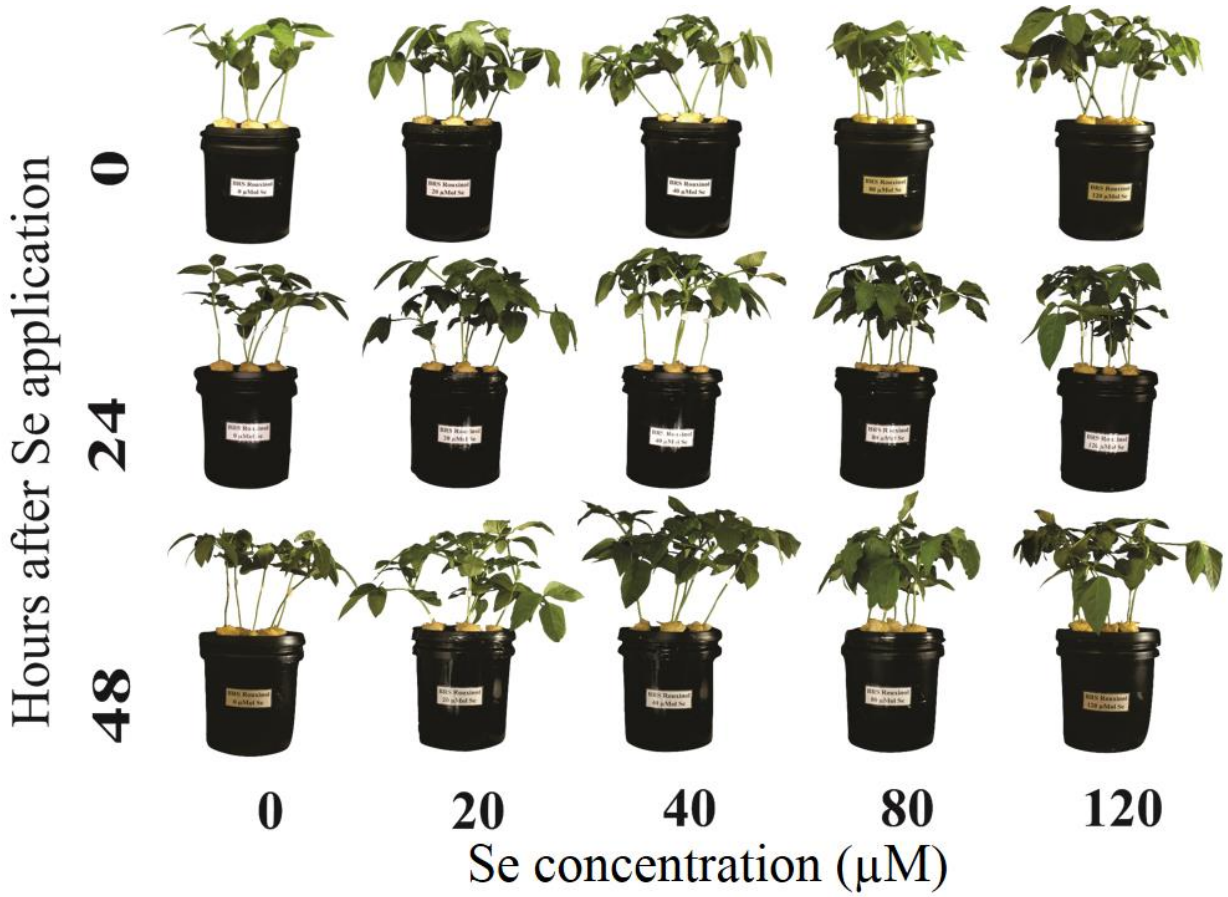


Figure 5. Visual analyses of selenium in cowpea plants after 48 hours

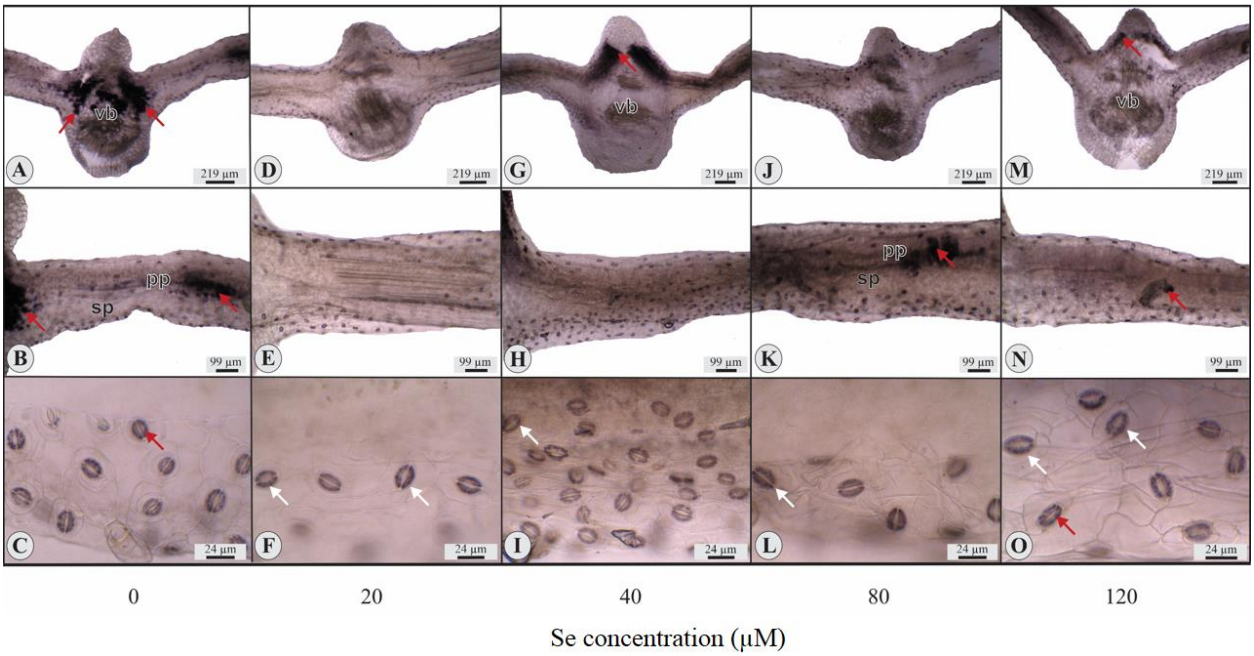


Figure 6. Histochemical test of starch. The letters mean: vb – vessel bundle; pp – palisade parenchyma; sp – spongy parenchyma

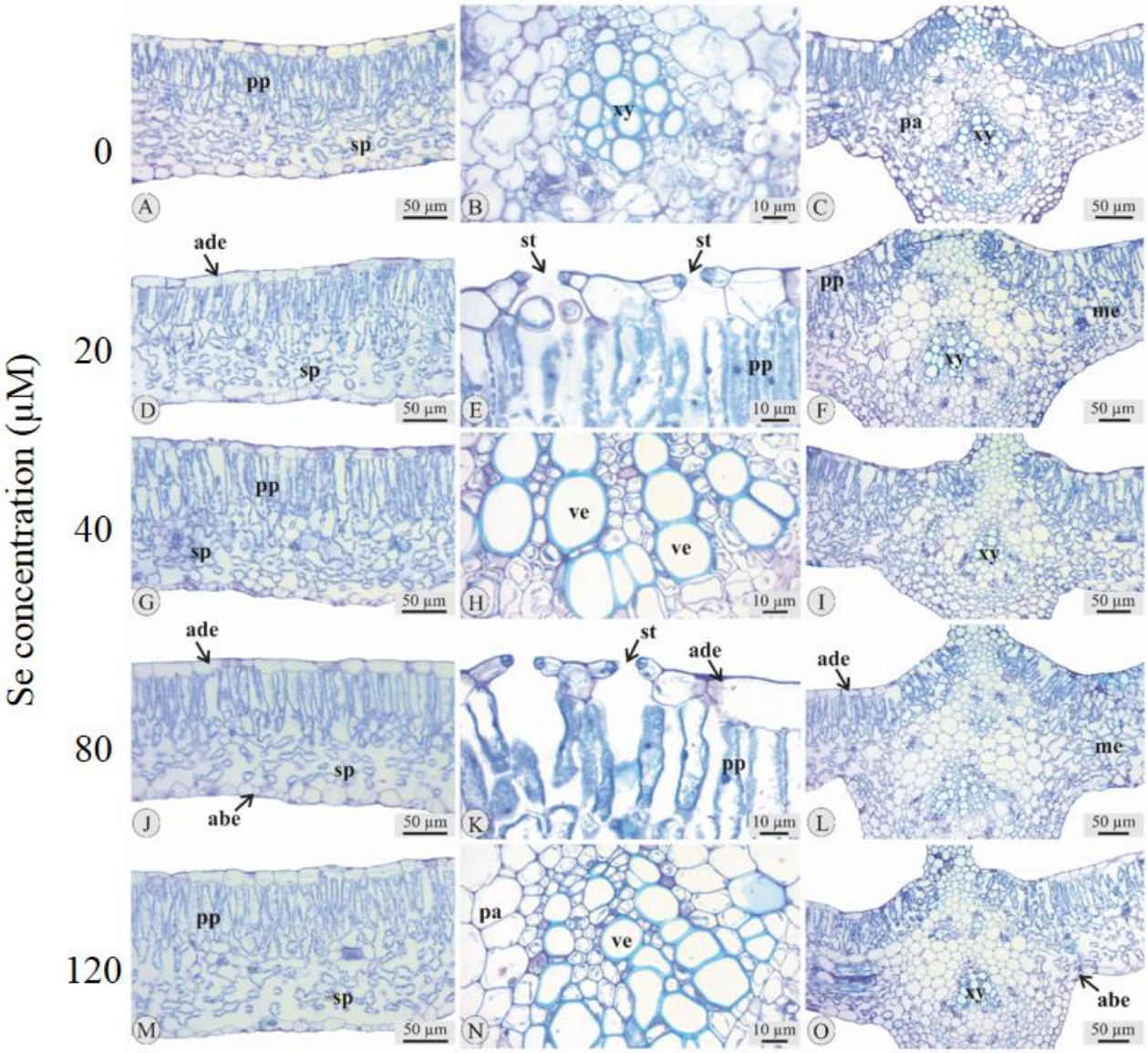


Figure 7. Analysis of leaf biometry by light microscopy. The letters mean: pp – palisade parenchyma; sp – spongy parenchyma; ade – adaxial epidermis; abe – abaxial epidermis; xy – xylem; st – stoma; ve – vessel element; pa – parenchyma; me – mesophyll.

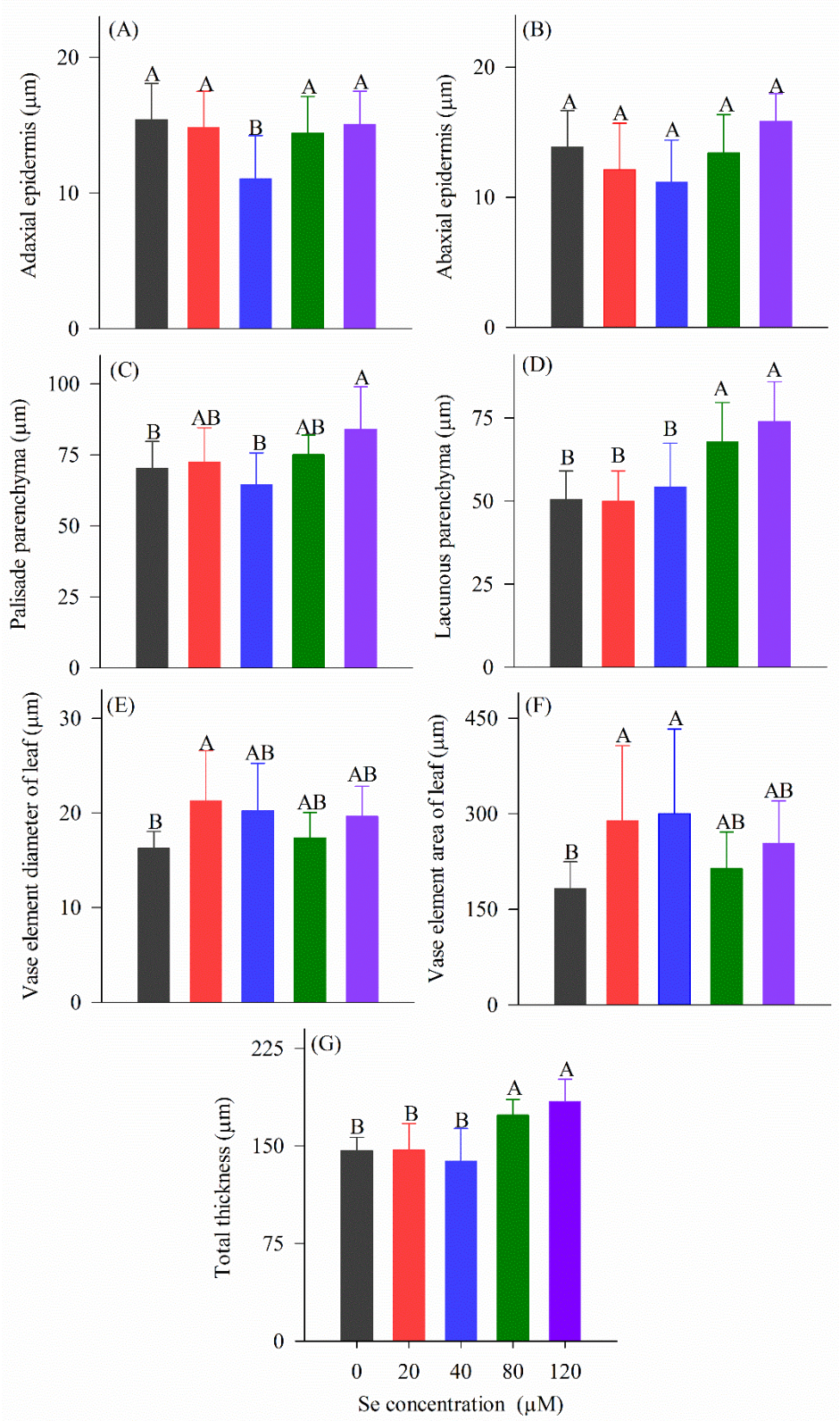


Figure 8. Quantification of leaf biometry parameters by light microscopy Error bars indicates the standard error of the mean (n=4 replicates). Different letters indicate the difference between treatments means according to a Tukey test (P≤ 0.05).

7. SUPPLEMENTARY MATERIAL

	Compound	Molecular weight	Concentration of stock solution	Concentration of stock solution	Volume of stock solution per liter of the final solution (mL)			
		(g mol^{-1})	(mM)	(g L^{-1})	100%	80%	50%	25%
Macronutrients	KNO_3	101.10	1000	101.10	6	4.8	3	1.5
	$Ca\,(NO_3)_2\,.\,4H_2O$	236.16	1000	236.16	4	3.2	2	1
	$NH_4H_2PO_4$	115.08	1000	115.08	2	1.6	1	0.5
	$MgSO_4.7H_2O$	246.47	500	123.24	2	1.6	1	0.5
Micronutrients	KCL	74.55	25	1.864	2	1.6	1	0.5
	H_3BO_3	61.83	12.5	0.773	2	1.6	1	0.5
	$MnSO_4.H_2O$	169.01	1.0	0.169	2	1.6	1	0.5
	$ZnSO_4.7H_2O$	287.54	1.0	0.288	2	1.6	1	0.5
	$CuSO_4.5H_2O$	249.68	0.25	0.062	2	1.6	1	0.5
	H_2MoO_4	161.97	0.25	0.040	2	1.6	1	0.5
	NaFeDTPA	558.50	53.7	30	1	0.8	0.5	0.25

Table S1. Hoagland Solution (Taiz et al., 2006).

PV of Se absorption kinetics to two consecutive points (%)											
	0-0	0-1	1-4	4-12	12-16	16-20	20-24	24-36	36-43	43-48	48-60
µM	Time (h)										
0	****	-58%	137%	-58%	-100%	****	113%	-63%	215%	-100%	****
20	****	-23%	13%	-22%	-3%	161%	88%	19%	12%	32%	-25%
40	****	****	-100%	****	502%	-23%	95%	19%	60%	35%	-9%
80	****	-100%	****	333%	65%	40%	12%	11%	45%	7%	5%
120	****	-100%	****	524%	90%	96%	3%	-2%	60%	7%	14%

Table S2. Calculation values of the percentage change in the absorption kinetics of Se and other elements. The results are described in the results and discussions sections. The formula is described in the materials and methods (equation 4).

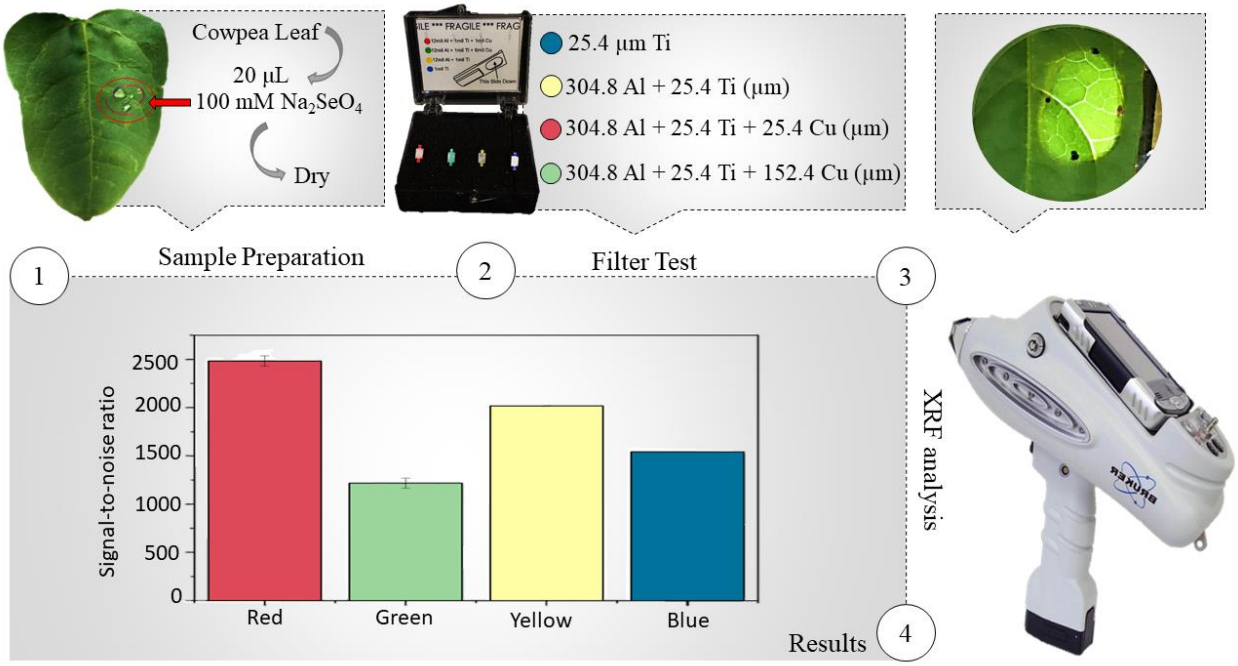


Figure S1. Evaluation of signal-to-noise ratio (SNR) for selenium using different filters with 60 s of time analysis.

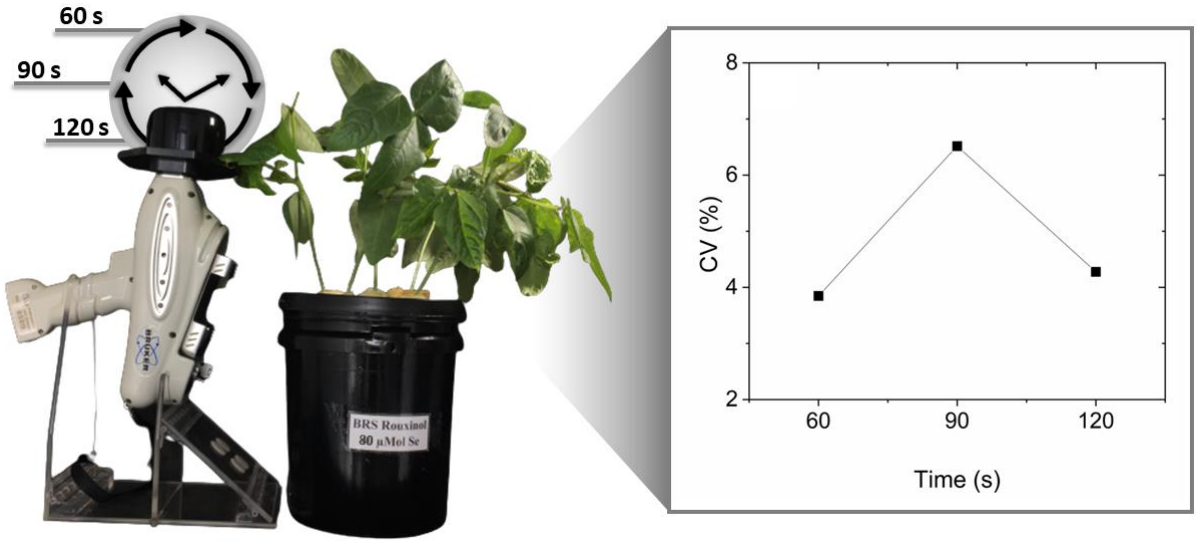


Figure S2. Evaluation of coefficient of variation (CV) as a function of time for selenium in cowpea plants at handheld energy-dispersive X-ray fluorescence spectrometry (EDXRF).

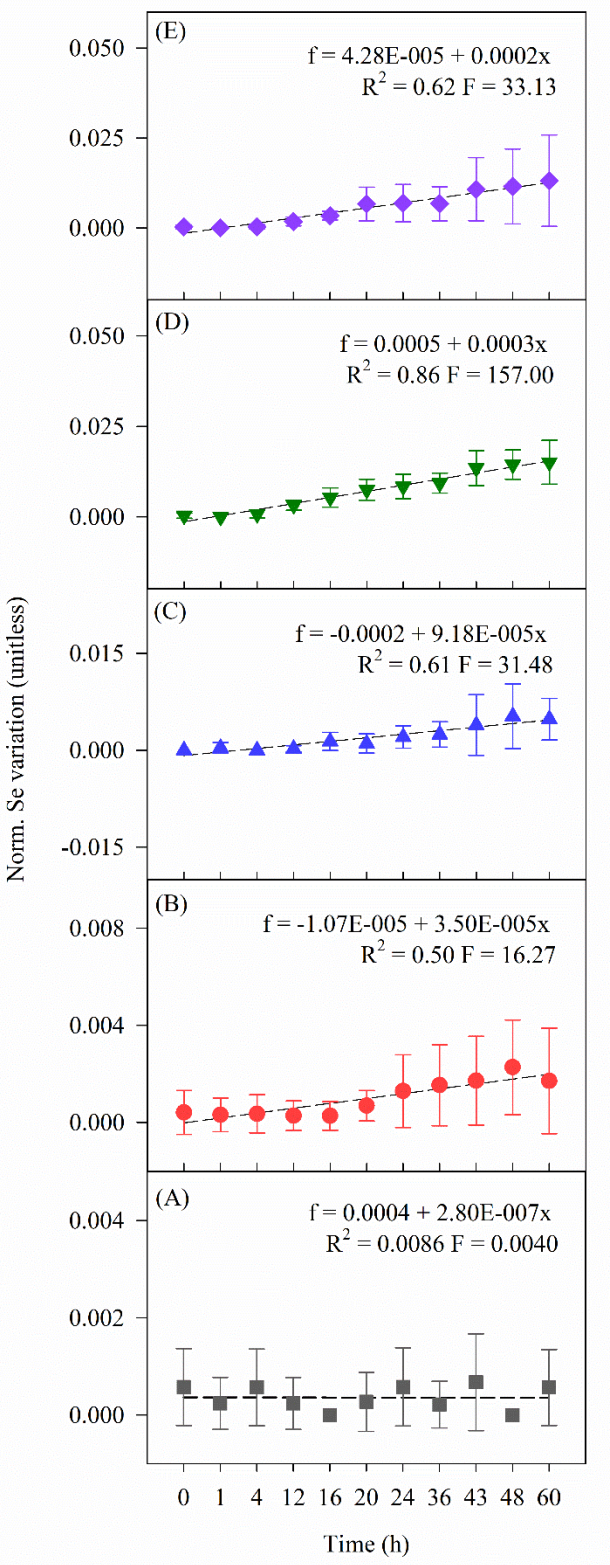


Figure S3. Linear regression for the respective treatments: 0, 20, 40, 80, and 120 µM.

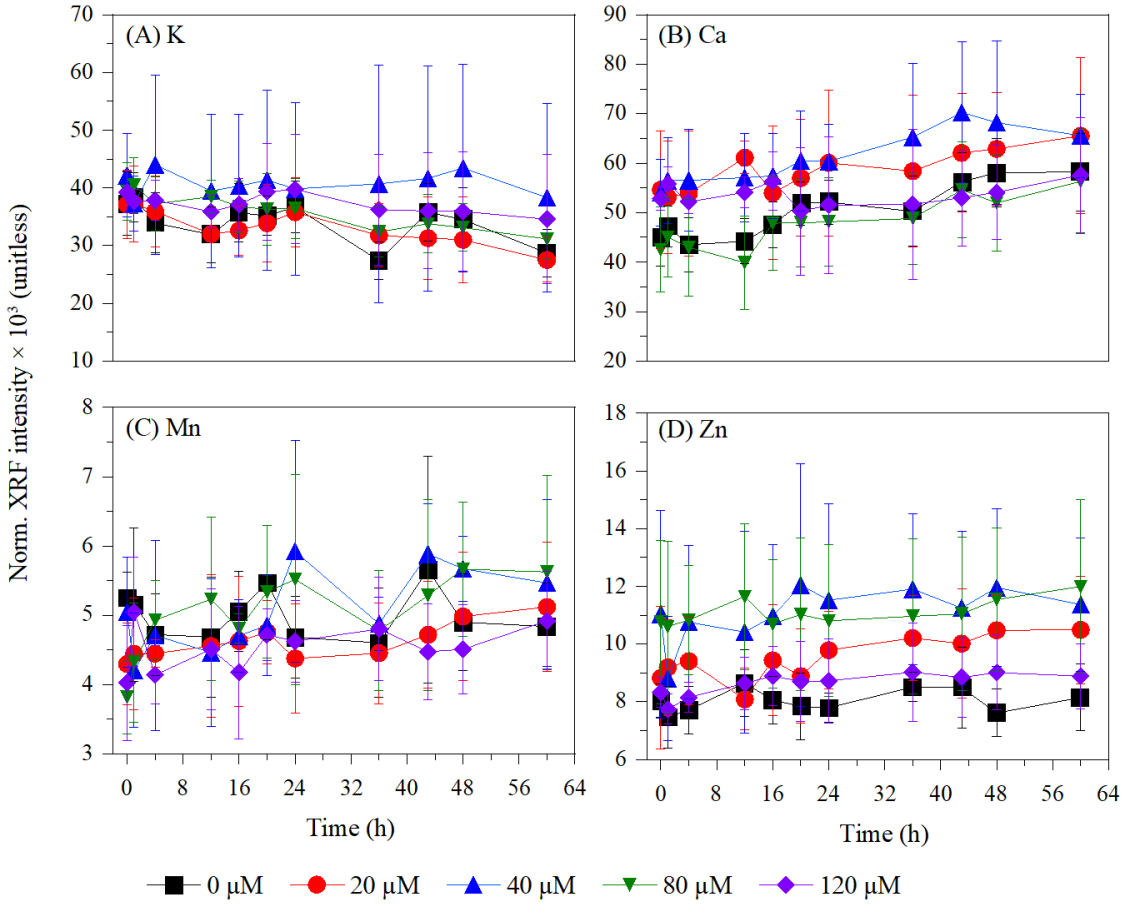


Figure S4. *In vivo* monitoring of K, Ca, Mn, and Zn XRF intensities in cowpea plants in response to Se application rates.

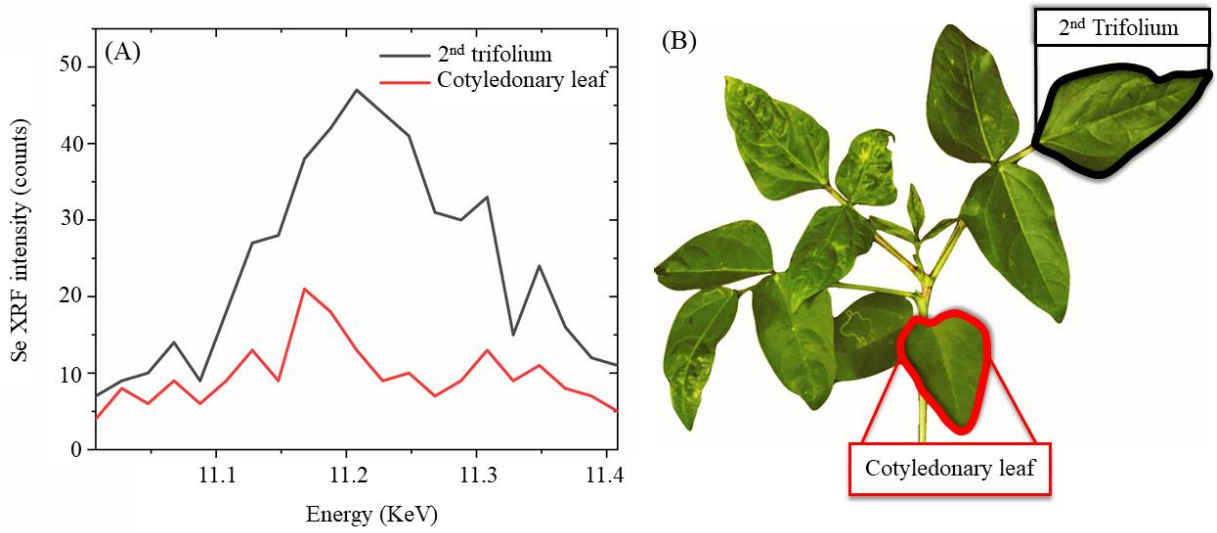


Figure S5. Se spectral profile among young and cotyledonary leaves at handheld energy-dispersive X-ray fluorescence spectrometry (EDXRF). This image shows a real plant using in this experiment.

XRF parameters optimization for *in vivo* Se uptake analysis

The portable XRF instrumental conditions were optimized for Se detection in living plant tissues. In this scenario, a 20 µL droplet of 100 mM Se Na_2SeO_4 solution was applied to a cowpea leaf, and *e.g.*, the primary filter and measurement time were tested. Four primary filters: (a), 25.4 µm Ti; (b), 304.8 µm Al + 25.4 µm Ti; (c) 304.8 µm Al + 25.4 µm Cu; (d) 304.8 µm Al + 25.4 µm Ti + 152.4 µm and three measurement times, *i.e.*, 60, 90, and 120 s were tested. The filter was selected based on the signal-to-noise ratio (SNR), according to Equation 1, in which the better Se instrumetnal sensitivity the higher SNR value. The measurement time were selected as a function of the coefficient of variance of three independent Se intensities recorded on cowpea leaves of each measurement time (Figures S1-S2)

$$Equation\ 1.\ SNR = \frac{I}{(BG)^{1/2}}$$

Where: SNR is the signal-to-noise ratio, *I* is the XRF elemental intensity (counts) and *BG* is the background counts(counts) under the corresponding analytic signal.

CHAPTER 3 – Genotypic variation of cowpea genotypes in selenium uptake and translocation analyzed by Benchtop Energy-Dispersive X-Ray Fluorescence (EDXRF) technique: physiological and biochemical responses

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ABSTRACT

Cowpea is a legume of great interest for plant improvement due to its adaptation to dry environments and high temperatures. Agronomic biofortification of crops with selenium (Se) growing in emerging countries to fight malnutrition and hidden hunger. However, in addition to recommending Se concentrations and sources for supplementing cultures, it is necessary to understand the mechanisms involved in Se uptake, translocation, and assimilation. This study demonstrates the existence of genotypic variation in Se absorption by cowpea plants. The photosynthetic metabolism is directly related to this mechanism, an absorption force of soil solutes coordinated by the stomatal opening. Herein, the concentration of Se in roots and shoots and the rate of element translocation in 20 commercial cowpea genotypes subjected to 40 µM of Se. Energy-Dispersive X-Ray Fluorescence (EDXRF) technique was used to determine Se concentration. In addition, photosynthetic metabolism parameters, net photosynthetic rate (A), stomatal conductance (Gs), internal CO_2 concentration in the stomatal sub-chamber (Ci), and transpiration rate (E), were analyzed using an infrared gas analyzer. It revealed that the genotypes that retain more Se in the roots are the ones that translocate fewer nutrients to the shoot, and this mechanism has a strong correlation with low stomatal conductance, whereas plants with high Gs, Ci, and E presents the highest translocation rates of Se to the shoot. It was also be noted that genotypes unresponsive to Se application have low photosynthetic efficiency, and therefore do not present advantages for modulating plant metabolism. Thus, for the validation of agronomic biofortification of cowpea with Se, the genotypes with the best opportunities for increasing productivity must be evaluated to guarantee the efficiency of the application of Se.

Keywords: tolerant genotypes, sodium selenate, *Vigna unguiculata [L.] Walp*, plant nutrition cowpea uptake, X-ray fluorescence spectroscopy.

1. INTRODUCTION

Cowpea (*Vigna unguiculata (L.) Walp*.) is an important legume species widely adapted to hot and dry regions (Boukar et al. 2019). Thereby, its agronomic biofortification has been explored by several programs aiming at reducing malnutrition and hidden hunger altogether with a plentiful supply of agricultural products worldwide (Shafiq et al. 2019). In this scenario, enhancing the photosynthetic metabolism of cowpea plants is vital to increasing their productivity and promoting higher assimilation of nutrients and carbohydrates (Munjonji et al. 2017).

Cowpea beans are a remarkable source of Ca, Cu, Fe, K, Mg, Mn, Na, P, and vitamins, *e.g.*, folic acid and vitamin B (Carvalho et al. 2017), as well as carbohydrates (50–60%) and proteins (20–35%) (Jayashankar et al. 2018). Its consumption is currently more accentuated in emerging countries due to the strategies of high value-added proteins replacement (Singh 2020). In this scenario, improved varieties fostering to overcome nutritional deficiencies, such as Zn are being extensively studied (Silva et al. 2021a). However, there is also an urge for cultivars able to efficiently assimilate those essential nutrients widely scarce in the soils and supplemented through commercial fertilizers, such as selenium (Se) (Lanza and Reis 2021).

Due to its antioxidant capacity, Se acts as an important abiotic stress attenuator in plant's metabolism. At low concentrations, it can improve plant defense systems, increasing both enzymatic and non-enzymatic activities that counter reactive oxygen species (ROS) (Schiavon et al. 2017). Besides, Se is associated with the maintenance of the photosynthetic metabolism by preserving the electron transport chain (Jiang et al. 2017), and might even increase the concentration of photosynthetic pigments, thereby enhancing photosynthetic efficiency and, accumulation of sugars in tissues (Silva et al. 2020).

Previous studies revealed that a fine-tuning between Se doses and sources is essential to avoid toxicity (Cabral Gouveia et al. 2020; Lanza et al. 2021; Mateus et al. 2021). On the other hand, mostly due to the wide breeding-induced genotypic variation of cowpea plants, a proper selection of those genotypes with high potential for translocation and accumulation of Se must be taken into account by the agronomic biofortification programs (Silva et al. 2021b). In this scenario, the present study

investigated the potentials of root and shoot Se absorption by 20 commercial genotypes of cowpea, to provide more nutritious material with advantages for production in stressful environments.

2. MATERIALS AND METHODS

2.1 Plant Material

Twenty commercial genotypes of cowpea (*Vigna unguiculata* (L.) Walp.) provided by Brazilian Agricultural Research Corporation (Embrapa Mid-North, Teresina, Brazil), were used in this experiment: (1) BR 17 – Gurguéia, (2) BR 3-Tracuateua, (3) BRS Aracê, (4) BRS Cauamé, (5) BRS Guariba, (6) BRS Itaim, (7) BRS Juruá, (8) BRS Marataoã, (9) BRS Milênio, (10) BRS Novaera, (11) BRS Pajeú, (12) BRS Potengi, (13) BRS Rouxinol, (14) BRS Tumucumaque, (15) BRS Urubuquara, (16) BRS Xiquexique, (17) California CB-5, (18) Inhuma, (19) Epace-10 and (20) Pretinho.

2.2 Cultivation Assay

The cowpea seeds were treated with Sementhiran® (300 mL100 kg^{-1} seeds) and Maxim xl cruser® (100 mL 100 kg^{-1} seeds) and sown in black polyethylene trays containing 98 cells filled with Carolina Soil® substrate. The genotypes were sown on December 31, 2020, and after 10 days, they were transferred to an Arnon and Hoagland's (AH) hydroponic solution, then acclimated for 4 days. The hydroponic solution was prepared based on the follow concentrations of the stock solution: i) 101.10 g L^{-1} KNO_3; ii) 236.16 g L^{-1} $Ca(NO_3)_2.4H_2O$; iii) 115.08 g L^{-1} $NH_4H_2PO_4$; iv) 123.24 g L^{-1} $MgSO_4.7H_2O$; v) 1.864 g L^{-1} KCl; 0.773 g L^{-1} H_3BO_3 + 0.169 g L^{-1} $MnCl_2.4H_2O$ + 0.288 g L^{-1} $ZnSO_4.7H_2O$ + 0.062 g L^{-1} $CuSO_4.4H_2O$ + 0.040 g L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$; and vi) 30 g L^{-1} $FeO_4S.7H_2O$.

During the first two days of acclimation, the plants remained in the solution at 25% of the Arnon's and Hoagland solution, with the following amounts of the volume of stock solution per liter of the final solution: i) 1.5 mL L^{-1}, ii) 1 mL L^{-1}, iii) 0.5 mL L^{-1}, iv) 0.5 mL L^{-1}, v) 0.5 ml L^{-1}, vi) 0.25 ml L^{-1}, whereas it was increased to 50%, *i.e.*, with the respective volume of stock solution per liter of the final solution: i) 3 mL L^{-1}, ii) 2 mL L^{-1}, iii) 1 mL L^{-1}, iv) 1 mL L^{-1}, v) 1 ml L^{-1}, vi) 0.5 ml L^{-1} in the next 2 days.

On the 15th day of cultivation, 40 µM of selenium was applied, using sodium selenate as Na_2SO_4, with the 80% of Hoagland content the respective volume of stock solution per liter of final solution, *i.e.*, i) 4.8 mL L^{-1}, ii) 3.2 mL L^{-1}, iii) 1.6 mL L^{-1}, iv) 1.6 mL L^{-1}, v) 1.6 mL L^{-1}, vi) 0.8 mL L^{-1}. Both Se source and dosage were chosen based on previous experiments (chapter 2). Control groups did not receive the Se spike.

All genotypes were kept in contact with the treatment during the 48 hours. After the time of exposure to the treatment, to remove excess Se on the external surface of the plants, the roots were removed from the nutrient solution sequentially immersed in 0.3 mol L^{-1} HCl and 0.01 mol L^{-1} Na_2HPO_4, respectively, for *ca*. 1 minute each, then gently washed with deionized water (Carmo et al. 2000). Both root and shoot tissues were stored in paper bags and dried in a laboratory oven (Tecnal TE-393) with forced air circulation at 60 °C for 24 hours. The dried vegetable material was ground with a pistol and mortar, and then processed with an 80-mesh sieve, with a particle size <100 µm.

2.3 Determination of Se concentration by Benchtop EDXRF Analysis

Se concentration in the roots and shoots was determined by energy dispersive X-ray fluorescence spectroscopy (EDXRF, Shimadzu EDX-720, Japan) according to a method previously described (Bachiega et al. 2019), with minor modifications. One hundred micrograms of powdered sample tissues were transferred into a 6.3 mm window XRF cuvette (cat. no. 3577, Spex) sealed with 6.35 µm thick Mylar® film (no. 3517, Spex), and gently pressed with a glass stick to avert voids in the sample. In this process, sample thickness is usually *ca*. 7 mm. The EDXRF analysis was carried out using a 3 mm collimated X-ray beam generated by a 50W Rh X-ray tube operation at 50 kV and auto-adjusted current (maximum of 30% of the detector dead time) with an Ag primary filter selected. The acquisition time was 200 s $sample^{-1}$, and a S i(Li) detector was used for recording the sample X-ray spectrum. Three biological replicates per sample were analyzed.

The trueness of the method was evaluated through a spike-and-recovery assay. In which 300 µL of a 100 µg mL^{-1} Se solution (1:10 dilution from a 1 mg mL^{-1} Se standard solution (Acros Organics, Acros Organics, New Jersey, USA) were added in 300 mg of cowpea leaf powder from BRS Urubuquara (those with the lowest Se concentration

recorded), previously weighed in a petri dish. Subsequently, the spiked standard was dried in a laboratory oven (Tecnal TE-393, Brazil) at 60 °C for 12 h and homogenized utilizing an agate mortar during *ca*. 30 min. The dried samples were ground in a mortar with the aid of a pistil and refined through an 80-mesh stainless steel sieve. Then, the same sample analysis procedure described above was performed.

The detection limit (LOD) was calculated according to Equation 1:(equation 1):

$$Equation\ 1. LOD = \frac{3}{Si}\sqrt{\frac{I_{i(BG)}}{t}}$$

Where $I_{i(BG)}$ is the background intensity of selenium (cps μA^{-1}), t is the acquisition time (s), and Si is the analytical sensitivities to the analyte (cps µg g^{-1}). The recovery found for Se was 111%.

2.4 Gas exchange measurements - LI-6400 Portable Photosynthesis System

The determination of gas exchange was performed by a portable infrared gas analyzer system (Li-6400XTR, LI-COR Biosciences, Lincoln, NE, USA). 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 stomatal sub-chamber (µmol CO_2 mol^{-1}) and transpiration rate (mmol H_2O m^{-2} s^{-1}). For the measurements, the standard conditions used were: 1200 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), provided by LED lamps, 400 ppm CO_2 chamber,28 °C temperature, and 40-45% humidity, according to Lima et al. (2019).

2.5 Translocation index

The translocation index was calculated using the Se concentrations (mg kg^{-1}) values of the roots, and the shoot quantified in Benchtop EDXRF. Equation 2 was applied according to Abichequer and Bohnen (1998). The Se content in the plant is the sum of its concentration in the shoot and the root.

$$Equation\ 2\ .\ Root - to - Shoot\ Se\ translocation\ index\ (\%) = \frac{Se\ content\ in\ the\ shoot}{(Se\ content\ in\ plant)}\ x\ 100$$

2.6 Statistical analysis

All data were first submitted to the Outlier test (Grubbs' test). Then, the normality of the data population was determined according to the Anderson-Darling method with a 0.05 significance level. For the statistical analysis of the data, the experimental design was considered as a completely randomized design (DIC), with 2 factors (double factorial), considering factor A being the treatments (control and 40µM Se) and factor B the 20 genotypes. Data variance analysis was analyzed using the Scott and Knott (1974) method at 5% probability.

For grouping treatments, a UPGMA (unweighted pair group method using arithmetic averages) hierarchical clustering cluster analysis was performed using the Euclidean distance. To verify the correlation between the variables Pearson's product-moment correlations were determined All data analyses were carried out using Minitab® Statistical Software, SigmaPlot 12.5 software, and R software (version 4.0.3).

To identify the variation in nutrient concentration, the percentage variation formula was used based on the values found in the control treatment (0 g ha^{-1}). The formula used is expressed in equation 3:

$$Equation\ 3. \qquad PV\ (\%) = \frac{(A - B)}{B}\ x100$$

Where:

A = Baseline (control treatment)

B = Reference value obtained by applying treatments

3. RESULTS

3.1 Selenium absorption by cowpea shoot, roots, and root/shoot translocation

Figure 1 shows the Se concentration found in the shoot tissues of 20 commercial genotypes 48-h past root Se application at 40 µM. According to the Scott-Knott test ($p \leq$

0.05), it was possible to classify the shoot Se uptake into nine distinct groups. The genotypes (16) BRS Xiquexique (224.83 mg kg^{-1}) and (11) BRS Pajeu (219.18 mg kg^{-1}) presented the highest Se concentration, whereas the genotype (15) BRS Urubuquara showed the lowest (65.85 mg kg^{-1}).

Furthermore, twelve groups were identified (Figure 2) to Se concentration in the roots. The genotype (20) Pretinho led to the highest Se retention in the root tissues ((142.77 mg kg^{-1}), whereas the genotypes (4) BRS Cauamé (26.05 mg kg^{-1}) and (3) BRS Aracê (28.94 mg kg^{-1}) presented the lowest values. The control treatments are not shown in figures 1 and 2, as the Se levels were below the detection limit (LOD).

These results indicate an inverse relationship between the accumulation of Se in the shoot and root, which is reinforced by root-to-shoot Se translocation index shown in Figure 3, which demonstrates the higher is root Se accumulation, the lower is translocation. It was classified into 14 distinct positions by the Scott-Knott test, and the most significant translocation rate was seen for the genotypes (3) BRS Aracê and (4) BRS Cauamé, respectively, whereas the (20) Pretinho encompassed the smallest translocation, which was *ca*. 57% lower compared to BRS Cauamé genotype (4).

3.2 Variation in photosynthetic metabolism responses of 20 cowpea genotypes in response to Se exposure

Although no statistical differences were observed for net photosynthesis values observed in the control and Se exposed plants (Figure 4), the Scott-Knott test ($P \leq 0.05$) classified the photosynthetic activity into two groups, where the genotypes 4, 7, 20, 3, 17, 18, 1, 9, 2, 5, and 8 showed the highest rates of photosynthetic activity.

Stomatal conductance (Gs) were classified the genotypes into three different groups (Figure 5), and the two genotypes (3 and 1) with the highest Gs (Figure 5A) showed statistically different from the control groups, with an increase in the order of 71.03% and 47.14%, respectively ((Figure 5B). Furthermore, the genotype (19) showed a more significant percentage difference compared to the control (183.01%).

Regarding the internal CO_2 concentration (Ci), only genotypes 16, 20, and 19 (Figure 6A) showed differences an increase higher than 10% between the 40 µM compared to the control groups. Scott-Knott's test resulted in three distinct groups, with

the genotype (8) BRS Marataoã displaying the smallest Ci values. A 39% increase in the Ci was observed comparing the genotype 8 with that one with the highest value (1). Nevertheless, one should notice that Ci was not responsive to the application of Se in genotype 1. Conversely, the BRS Xiquexique genotype (16) presented high levels of Ci and a significative percentage difference compared to the control treatment (11.63%) (Figure 6B).

Finally, the transpiration rate (E) classified the genotypes into two groups according to Scott-Knott's test (Figure 7). From all the 20 genotypes evaluated, only the genotypes 20 and 19 exposed to 40 µM of Se statistically differed from control groups. However, both genotypes 1 and 3 also maintained the highest transpiration rates values, positively responding (13.24% and 8.23%, respectively) to Se application Herein, genotype 8 also presented the lowest E rate, thereby presenting the same trend seen for Gs and Ci, then followed by genotype 10. On the other hand, genotype 3 also showed greater transpiration.

3.3 Statistical tools for analyzing the results of biofortified genotypes with Se

Aiming at understanding the dynamics of physiological responses of the 20 different cowpea genotypes to Se application in a comprehensive mode, all results were subjected to a hierarchical grouping of genotypes, shown in Figure 9. Due to the low intensity of Se concentrations in the root and shoot tissues, it is evident that all control treatments remained in the same group. In addition, even without Se application, some genotypes present genetic characteristics that induce a higher photosynthetic metabolism. For example, the genotypes 17, 1, 3, 4, 7 expressed the variables E, Gs, A Ci. There are genotypes with low photosynthetic activity, as seen for 20, likely due to the lower intensity of internal Ci concentration.

On the other hand, the cluster analysis showed that although genotypes 8, 10, 14, 11, 12, 2, and 15 were responsive to Se assimilation by roots, translocation, and accumulation in the shoot, it did not present significant intensities for the variables of photosynthetic metabolism. In this scenario, the genotypes responsive to the application of Se were 3, 1, 20, 16, 17, 19, 4, 5, 6, 13, 18, 7, and 9. Of this group, genotypes 1 and

20 were the most expressed for Se concentration in the roots, while 16 stood out in shoot accumulation and photosynthesis, and 3 and 1 in stomatal conductance.

Pearson's correlation (Figure 10) demonstrates that Se concentrations in the root and shoot tissues, as well as the translocation, were positively correlated with photosynthetic parameters (A, Gs, Ci, and E), with emphasis to the highest relationship between Ci and Se concentration in the shoot (0.18), and the smallest between photosynthesis (A) and Se concentration in the roots (0.03).

4. DISCUSSION

The supplementation of Se into commercial fertilizers to combat its deficiency in humans and animals nutrition is already a reality in several countries (Yläranta 2019). However, genotypic regulatory mechanisms can influence the performance of the biofortification of crops. The genetic disparities in Se accumulation have proven to be especially important in soils with high Se availability (Raina et al. 2020), thereby proving that only increasing Se input might not be suitable for biofortification programs. In the present study, the genotype that most accumulated Se in the shoot was (16) BRS Xiquexique (Figure 1). Although investigations of Brazilian varieties are scarce in the literature, the evaluation of Bandeira and Sousa et al. (2017) with cowpea lines in the Brazilian Cerrado showed that this cultivar presents high stability and adaptability in unfavorable environments. On the other hand, Galvão et al. (2013) demonstrated that the cultivar (15) BRS-Urubuquara has an inadequate response to nutritional treatments and low productivity, which is in line with the Se assimilation recorded for this genotype in our study.

Silva et al. (2021b) evaluated the efficiency of 29 cowpea genotypes with the application of 12.5 μg Se kg^{-1} of sodium selenate via soil. And found the genotype BRS Guariba as the best potential candidate for biofortification displaying a high concentration of Se in the shoot (1242.98 μg kg^{-1}), grains (2462.09 μg kg^{-1}), as well as root-to-shoot translocation index (90.92%). The differences found across genotypes are likely related to the differences in the phenological phase of the application, dose, and cultivation method (soil/hydroponics), since the concentration of Se in plants tissues might largely depend on the plant's stage of development (Harris et al. 2014).

Besides, the ability of plants to translocate the elements present in the soil to the shoot, where the filling of the grains will take place, stands out as one of the factors of great relevance for the success of the biofortification process". The literature demonstrates that Se has significant problems in the adsorption of particles in the soil colloid and competition of transport sites (Zhou et al. 2020). Due to the chemical similarity of S and Se, Sulphur transporters present in the plasmatic membrane of the roots are usually responsible for the transport of selenate. Thereafter, Se is translocated to the leaves and later metabolized in the plastids, where it is converted to selenoaminoacids, *i.e.*, SeCys and SeMet. These compounds can then be assimilated or methylated and vaporized into the atmosphere in non-toxic forms (Trippe and Pilon-Smits 2021).

Therefore, plants that present high concentrations of Se in the root and low concentrations in the shoot indicates low translocation capacity associated with visual impairment in the photosynthetic process. It can be seen observed in the genotype (20) Pretinho, which has the highest concentration of Se in the roots (Figure 2), but the lowest translocation rate of the element (Figure 3). Cluster analysis showed that this genotype has low photosynthetic efficiency, even without the application of Se (Figure 10). The studies of Gouveia et al. (2020) demonstrate that the application of 1.5mM Na_2SeO_4 induced harmful effects in some s rice cultivars.

The stomatic conductance (Gs) is directly related to the soil availability of water and nutrients to plants. The greater genotype's ability to maintain high Gs, the higher are the productivity gains (Alidu et al. 2020). In this regard, cowpea genotypes with high photosynthetic metabolism demonstrate better efficiency in accumulating Se in shoots, thereby being more suitable for plant breeding programs. Figure 10 demonstrates the positive correlation between stomatal conductance and shoot Se concentration. Moreover, this fact can be highlighted by the excellent performance of the genotype (3) BRS Aracê, which presented the highest Se translocation from roots to shoots, and also the highest values of Gs, Ci, and E (Figures 5, 6 and 7).

Photosynthesis is the physiological backbone of all plants. Therefore, plants with efficient photosynthetic metabolism are more productive and have a higher quality of their plant materials, especially in criticals scenarios, such as high temperatures, droughts, high light intensity, frost, and flooding. In this scenario, low Se concentration has proven

to benefit plant growth and development by increasing photosynthetic efficiency (Andrade et al. 2018).

Wang et al. (2021) demonstrated that the application of Se led to a higher expression of proteins in the chloroplasts of alfalfa (*Medicago sativa* L.) plants. In this study, proteins involved in the light-energy collection during the photosynthetic process, such as chlorophyll-binding protein a - b CP29.3 (Q9S7W1), found in photosystem I and II, respectively were up-regulated. Moreover, the expression of fructose-1,6-bisphosphatase (P46276) is directly linked to carbon fixation, providing substrates for the photosynthetic process. Furthermore, a 4 μM kg^{-1} Na_2SeO_4 application was beneficial for all gas exchange parameters in *Brassica juncea* L. plants (Handa et al. (2019). This result agrees with Silva et al. (2020a), which states Se has a high antioxidant capacity and can regulate the photosynthetic machinery by stabilizing electron transport, preserving chloroplasts, and photosynthetic pigments, which help plants withstand abiotic stresses.

Although the cowpea genotypes did not show statistical differences for the net photosynthesis variable (A) when submitted to 40 μM of Se (Figure 4) significant differences in the photosynthetic efficiency amongst the 20 genotypes were shown, especially for the genotype (16) BRS Xiquexique (Figure 9). Similar results were seen by Reddy et al. (2017), demonstrating that strains of mulberry have genetic characteristics more favorable to photosynthesis than others.

Herein, the genotypes with better photosynthesis rates also presented higher Gs, Ci, and E. Therefore, it can suggest that the genotypes (3) BRS Arace and (1) BR 17-Gurgueia can keep their stomata open for longer (Figure 5), and consequently, greater uptake of CO_2 (Figure 6) for photosynthetic process use. In addition, the stomatal opening allows a range of gas exchange and the transpiration process as a way to eliminate cellular heat induced by high temperatures (Figure 7), a strategy to avoid overloading the electron transport chain and the generation of reactive oxygen species (ROS) (Jiang et al. 2017).

Our previous studies demonstrate that the application of low doses of Se (<50 g ha^{-1}) can increase the activity of the enzymes SOD, CAT, POD, and APX and reduce the hydrogen peroxide (H_2O_2) content of cowpea cells (Lanza et al. 2020), thereby supporting that the application of Se is indeed useful for countering ROS accumulation supports this

process. On the other hand, one should notice that high Se concentrations (>1.5 Mm Na_2SeO_4) reduce chlorophylls and photosynthetic pigments, in addition to impairing carbohydrate metabolism (Gouveia et al. 2020), which reinforces that a fine-tuning between Se doses and sources are required to avoid toxicity.

Abiotic stresses influence the photosynthetic metabolism of plants with water availability and temperature issues (Dubey 2016). When soils are below their field capacity, plants close their stomata to prevent internal loss of water in the form of vapor molecules, leading to a reduction in the water potential of the cells and the conductance of solutes, directly affecting impacting the transport of nutrients, mainly dependent of water for absorption and translocation (Taiz et al. 2017). Therefore, plants with low Gs have impairments in cell division rate and elongation, leaf area, root and stem growth, and water use efficiency (Hussain et al. 2018). A 5 mg L^{-1} foliar application of Se increased the Gs of strawberry plants subjected to low-temperature stress (Huang et al. 2018).

According to Hajiboland et al. (2015), the stomatal opening is induced by regulating the pumping of protons from the guard cells by promoting the entry of potassium ions (K^+). This phenomenon generates a more negative potential and induces water absorption by the cell, increasing its turgor pressure. Correlating the variables Gs, E, and Ci, it is evident that the greater the capacity of the plants to keep the stomata open (Figure 5), the greater the transpiration and the internal concentration of CO_2 (Figure 6).

On the other hand, one should keep in mind that not all plants are responsive to the Se application, which might be likely due to genetic traits. For example, Souza et al. (2020) demonstrated that the BR3-Tracuateua genotype prioritizes the maintenance of its water status over the photosynthetic activity through modulating stomatal closure. Thus, it did not show an expressive reaction as a function of Se application to all photosynthetic parameters. Furthermore, this genotype also showed a high Se retention in the roots and thereby, low translocation to the shoot, indicating deficient stomatal conductance, transpiration, and photosynthesis.

In this study, BR17 Gurgueia genotype showed the highest Ci but was unresponsive to Se application (Figure 6A). On the other hand, BRS Xiquexique, in addition to maintaining high levels of Ci, demonstrated that the Se application could be

positively modulated (Figure 6B), thus demonstrating that high performance of photosynthetic metabolism should not be the only factor to be taken into account during a selection for an agronomic biofortification program.

5. CONCLUSION

The data herein presented revealed that those genotypes which retain more Se in their roots due to the insufficient translocation mechanisms present low photosynthetic efficiency, characterized by the reduced Gs, Ci, and E parameters. It suggests that choosing materials with high stomatal conductance - and consequently, high Se absorption of Se -is a way to ensure the efficiency of suitable agronomic biofortification of cowpea plants with Se. Furthermore, this study provided a comprehensive classification of the 20 cowpea genotypes with the highest and lowest Se absorption capacities. This study suggested that genotypes showing higher Se accumulation in shoots might have greater antioxidant capacity for modulating plant metabolism to against abiotic stresses and achieve higher yields. Future studies should be carried out to under field conditions to evaluate the genetic variables involved in the absorption, transport, and assimilation of Se in cowpea genotypes.

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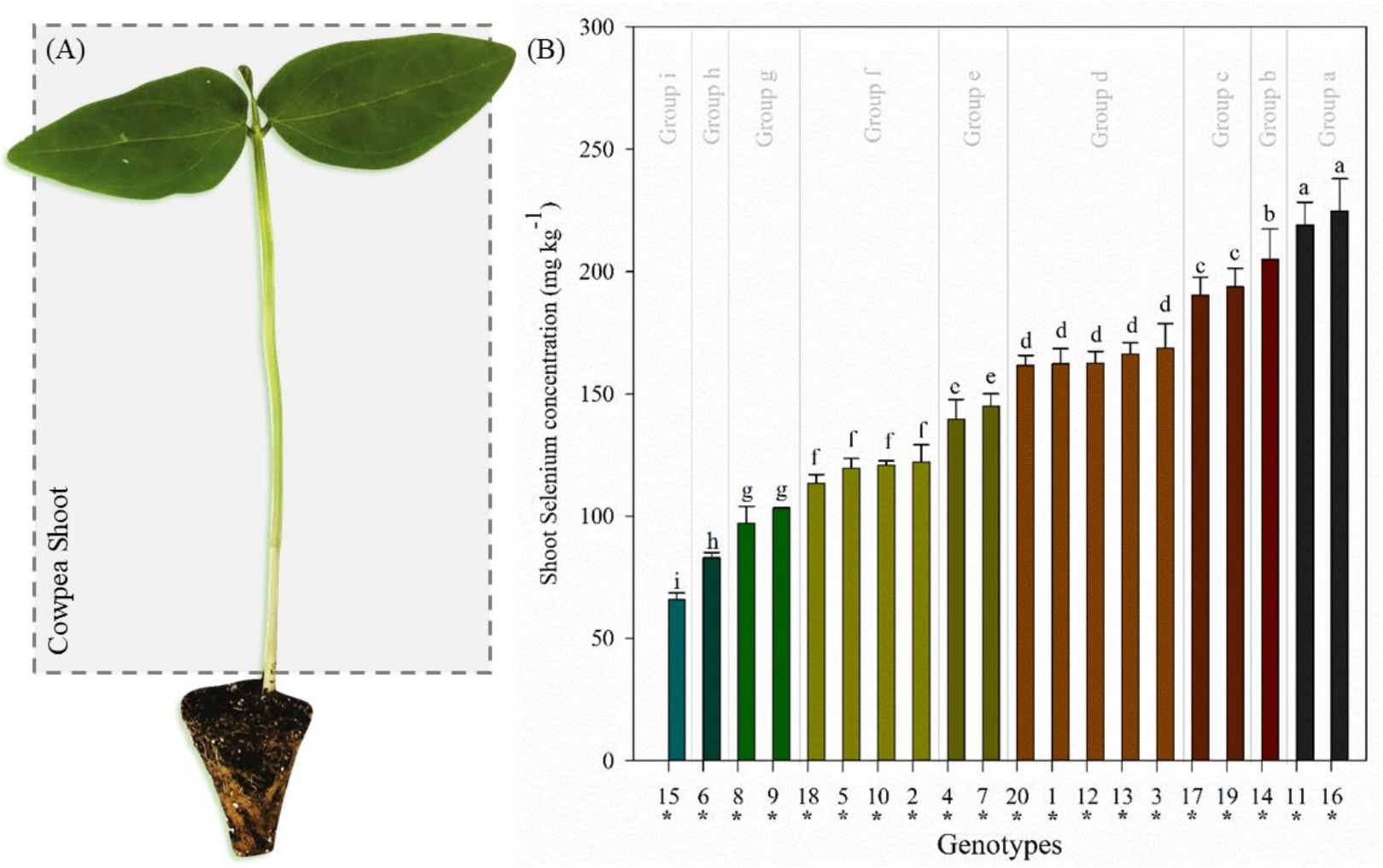


Figure 1. Selenium absorption by shoots of cowpea genotypes. The different colors indicated different groups for Se concentration. According to a Scott-Knott test, the difference between means and groups ($P \leq 0.05$). The standard error of the mean (n = 4) is indicated by error bars. Lowercase letters indicate genotypes. Different letters indicate correspond to Se application rates. CV (%) = 6.60. All The controls are lower de detection limit for Selenium at benchtop equipment. * indicate a statistical difference between treatments.

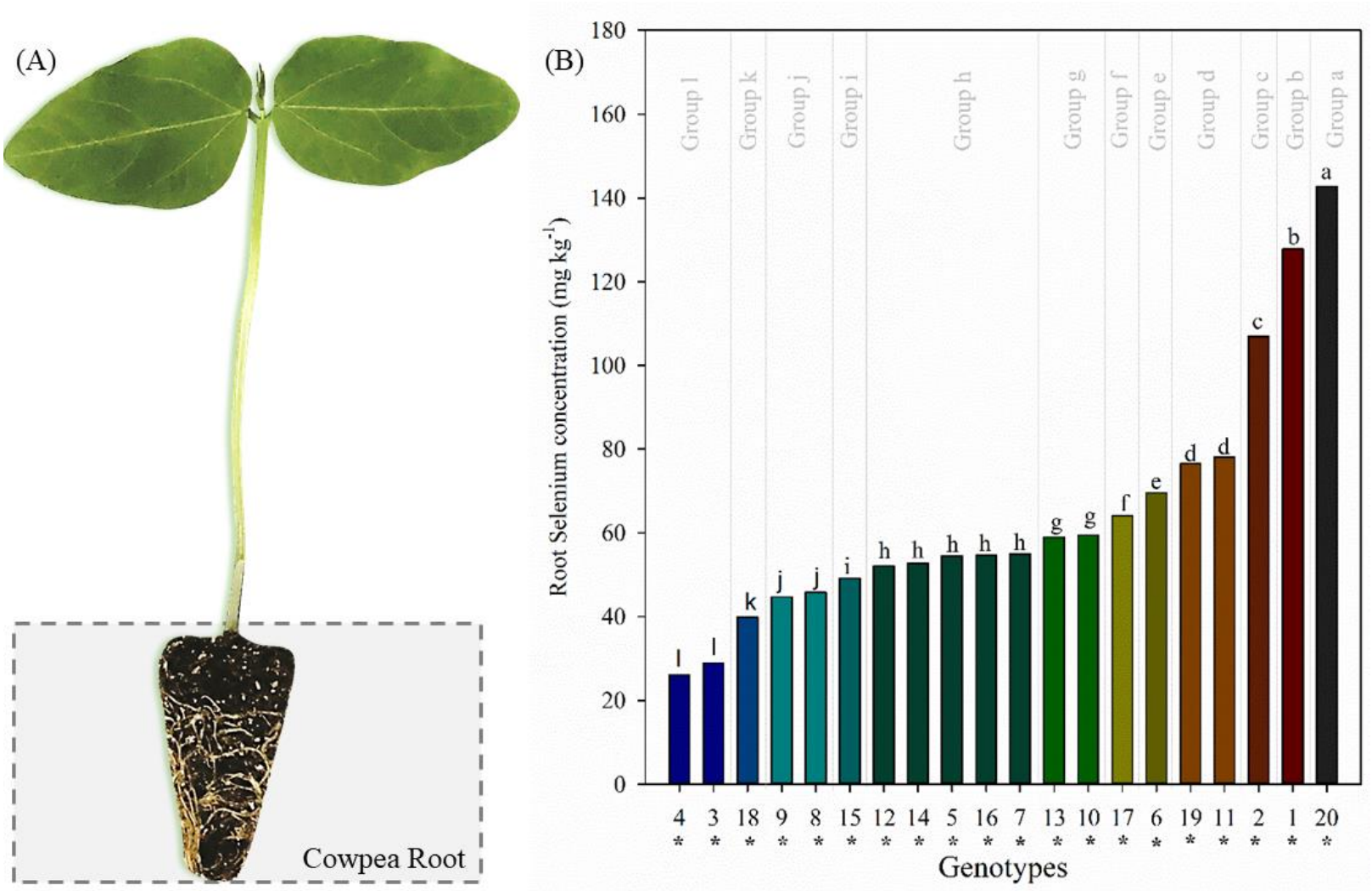


Figure 2. Selenium absorption by roots of cowpea genotypes. The different colors indicated different groups for Se concentration. According to a Scott-Knott test, the difference between means and groups ($P \leq 0.05$). The standard error of the mean (n = 4) is indicated by error bars. Lowercase letters indicate genotypes. Different letters indicate correspond to Se application rates. CV (%) = 6.36. All The controls are lower de detection limit for Selenium at benchtop equipment. * indicate the statistical difference between treatments.

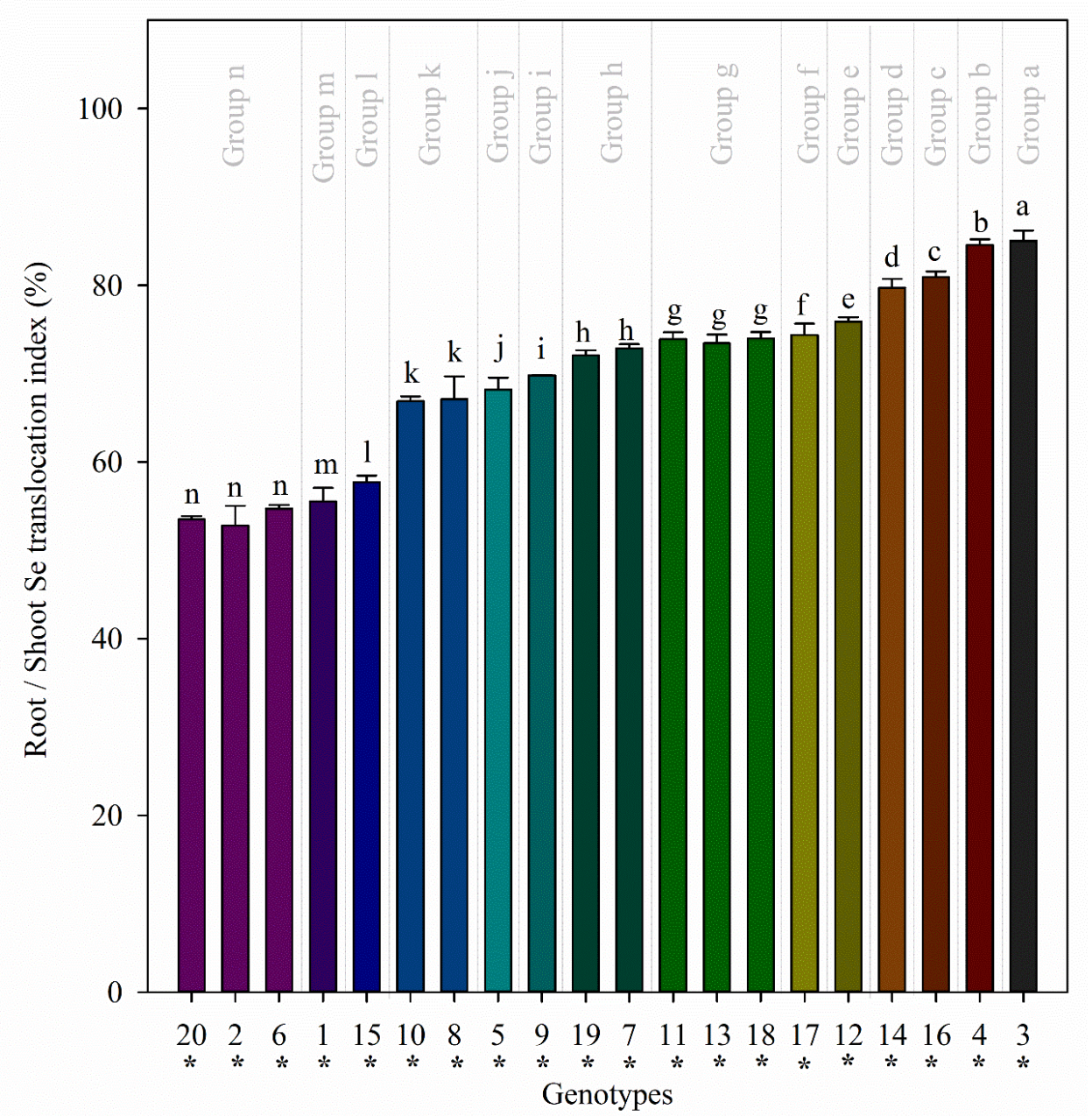


Figure 3. Root to shoot selenium translocation index. The different colors indicated different groups for Se concentration. According to a Scott-Knott test, the difference between means and groups (P ≤ 0.05). The standard error of the mean (n = 4) is indicated by error bars. Lowercase letters indicate genotypes. Different letters indicate correspond to Se application rates. NS = not significant according to a Scott-Knott test (P ≤ 0.05). CV (%) = 1.81. All The controls are lower de detection limit for Selenium at benchtop equipment. * indicate a statistical difference between treatments.

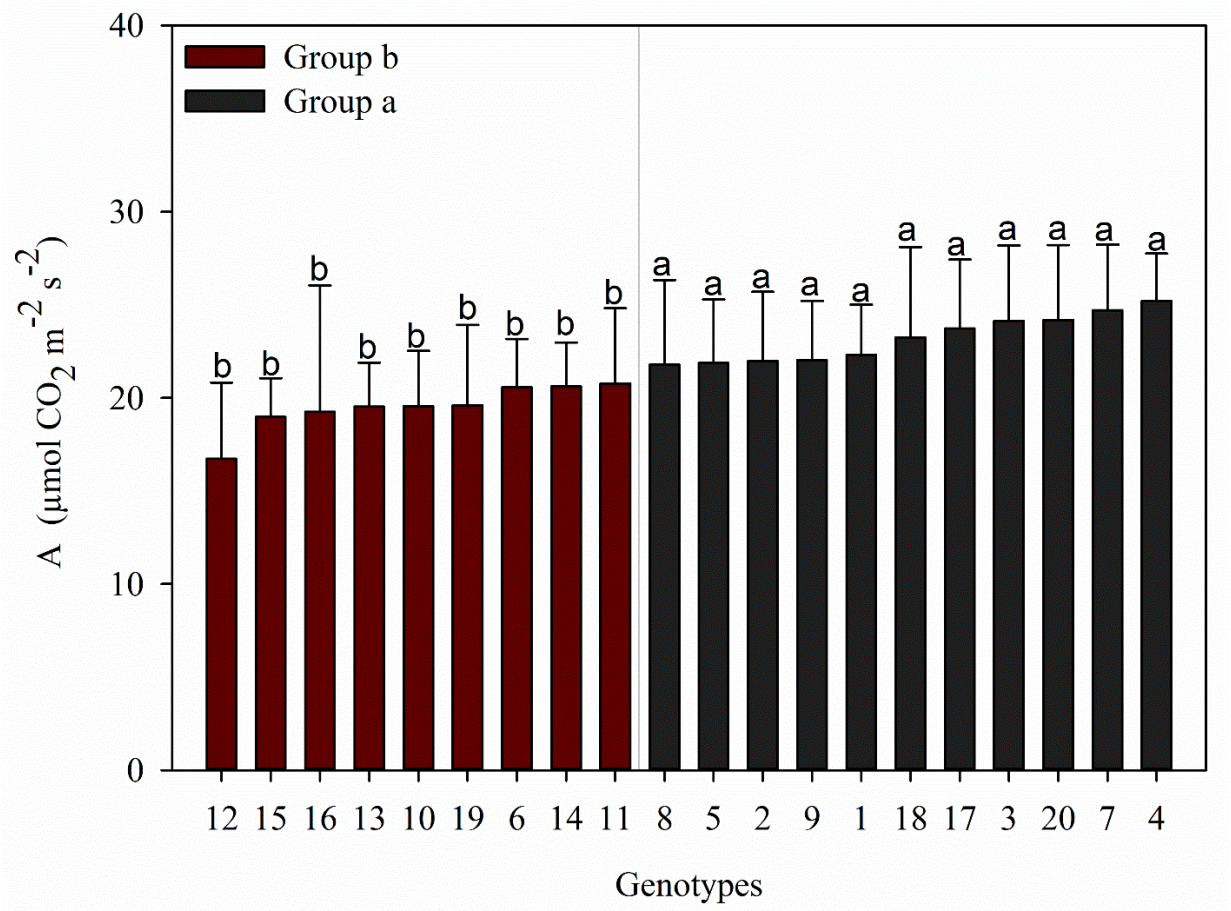


Figure 4. Cowpea photosynthesis rate in response to Se treatment. Grouping of genotypes according to a Scott-Knott test (P ≤ 0.05). The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate correspond to genotypes. CV (%) = 16.74.

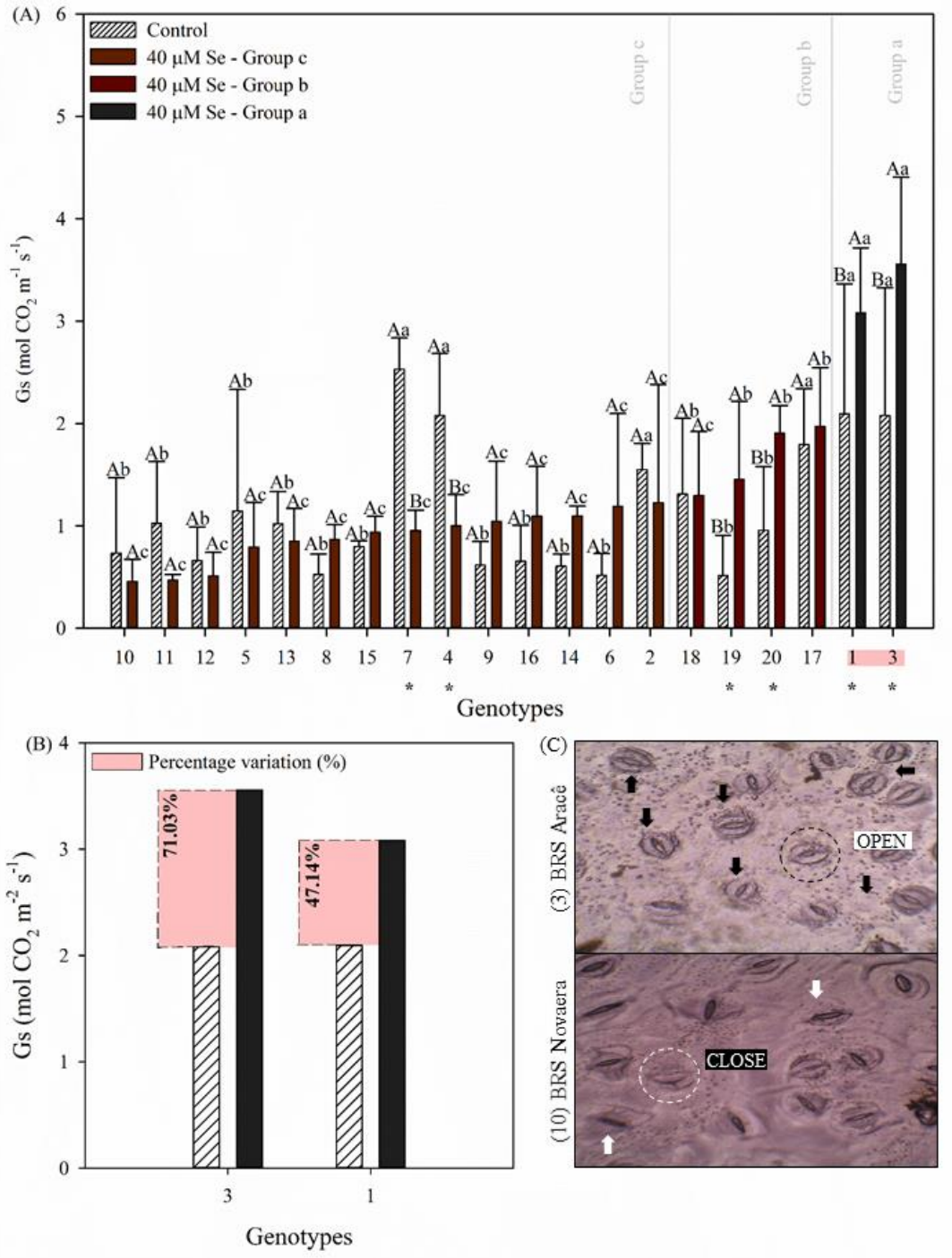


Figure 5. Stomatic Conductance (Gs) rate in response to Se treatment. (A) Relationship between genotypes and treatments. (B) Clip indicates the percentage variation of genotypes with the greatest difference between control and treatment (>40%). (C) Stomata printing of control. The difference between means according to a Scott-Knott test ($P \leq 0.05$). Uppercase letters correspond to Se treatments, and lowercase letters to genotypes. The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate correspond to Se application rates. CV (%) = 47.68. * indicate a statistical difference between treatments.

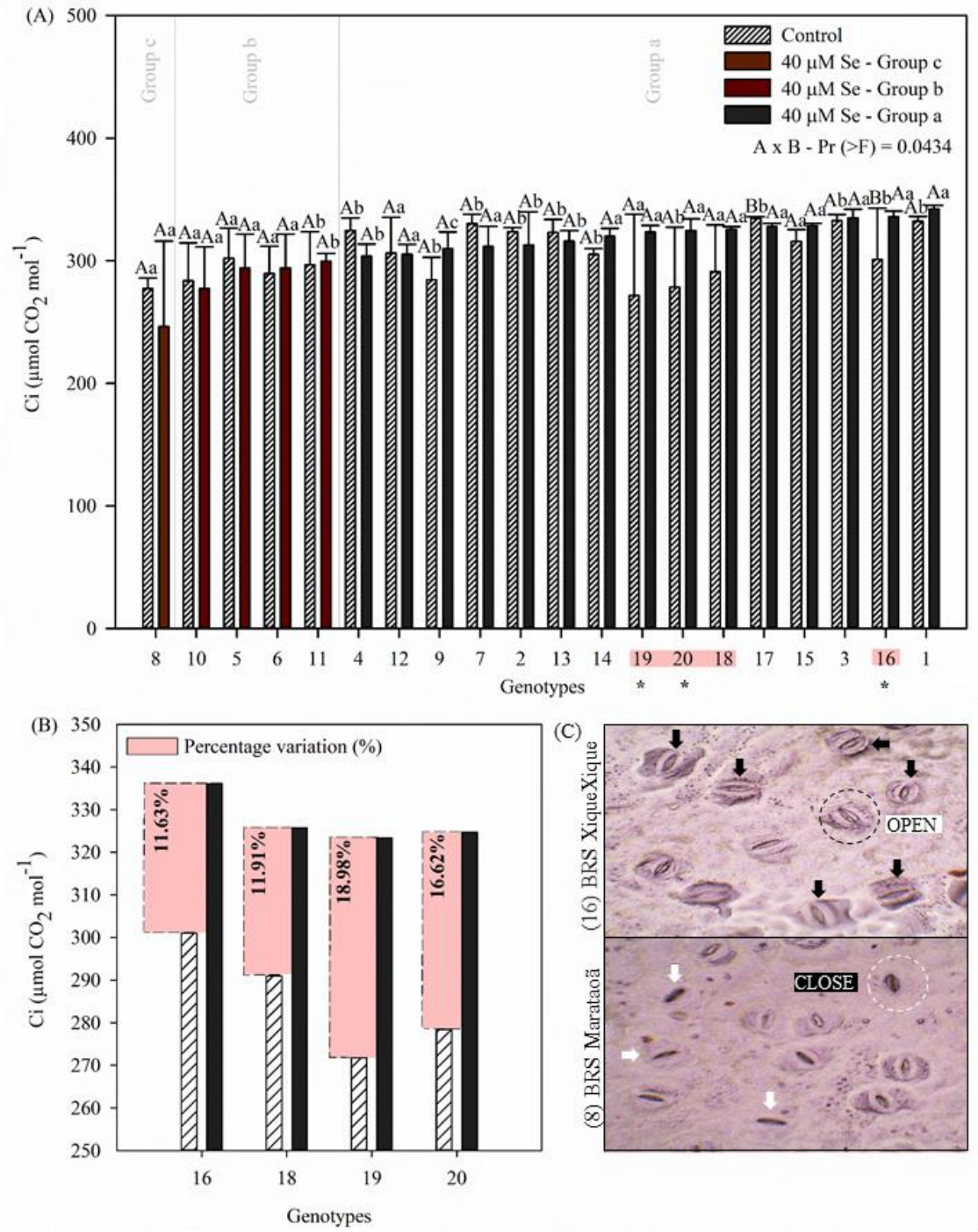


Figure 6. Internal CO_2 concentration (Ci) rate in response to Se treatment. (A) Relationship between genotypes and treatments. (B) Clip indicating the percentage variation of genotypes with the greatest difference between control and treatment (>10%). (C) Stomata printing of control. The difference between means according to a Scott-Knott test ($P \leq 0.05$). Uppercase letters correspond to Se treatments, and lowercase letters to genotypes. The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate correspond to Se application rates. CV (%) = 7.89. * indicate a statistical difference between treatments.

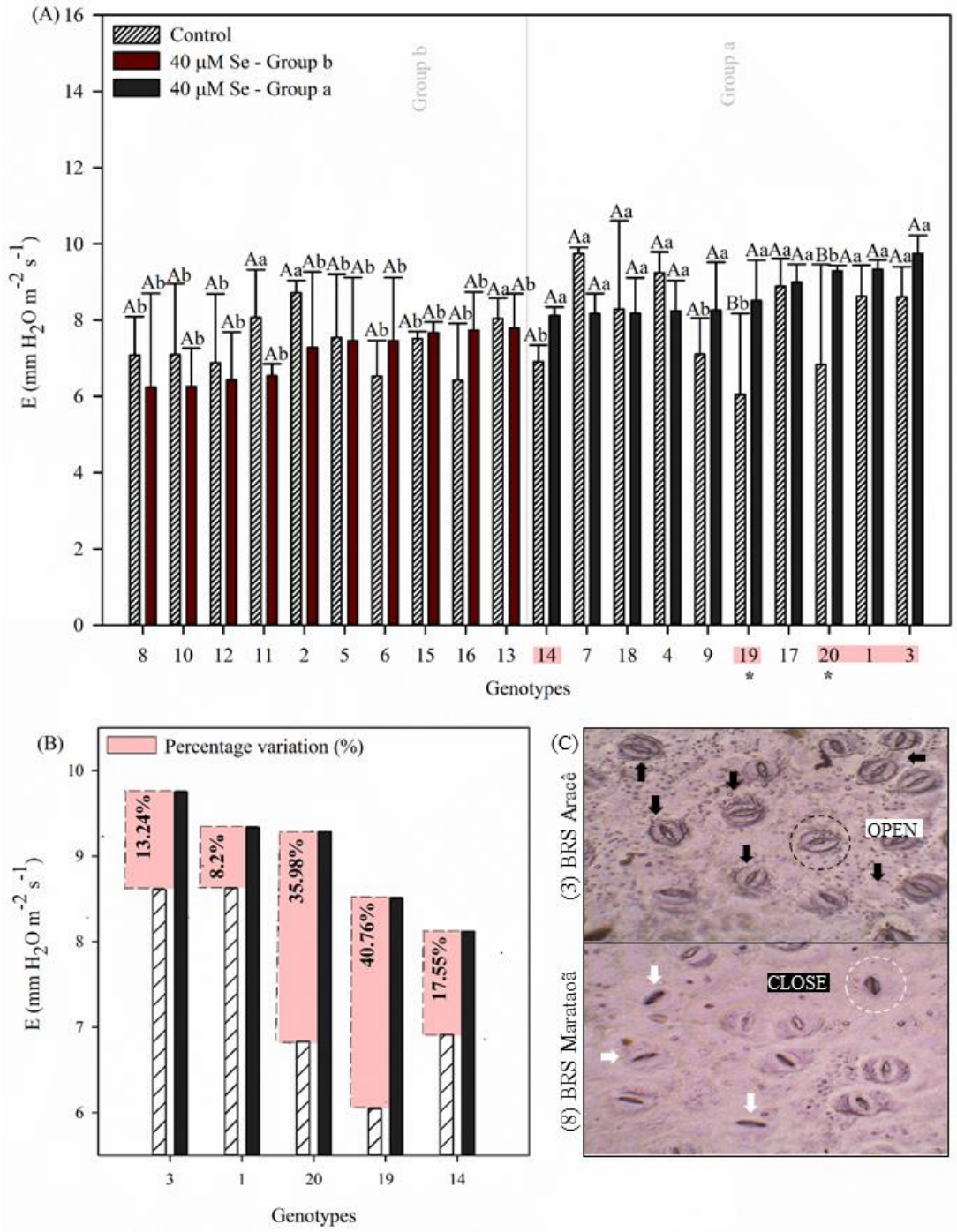


Figure 7. Transpiration (E) rate in response to Se treatment. (A) Relationship between genotypes and treatments. (B) Clip indicates the percentage variation of genotypes with the greatest difference between control and treatment (>10%). (C) Stomata printing of control. The difference between means according to a Scott-Knott test ($P \leq 0.05$). Uppercase letters correspond to Se treatments, and lowercase letters to genotypes The standard error of the mean (n = 4) is indicated by error bars. Different letters indicate correspond to Se application rates. CV (%) = 15.81. * indicate a statistical difference between treatments.

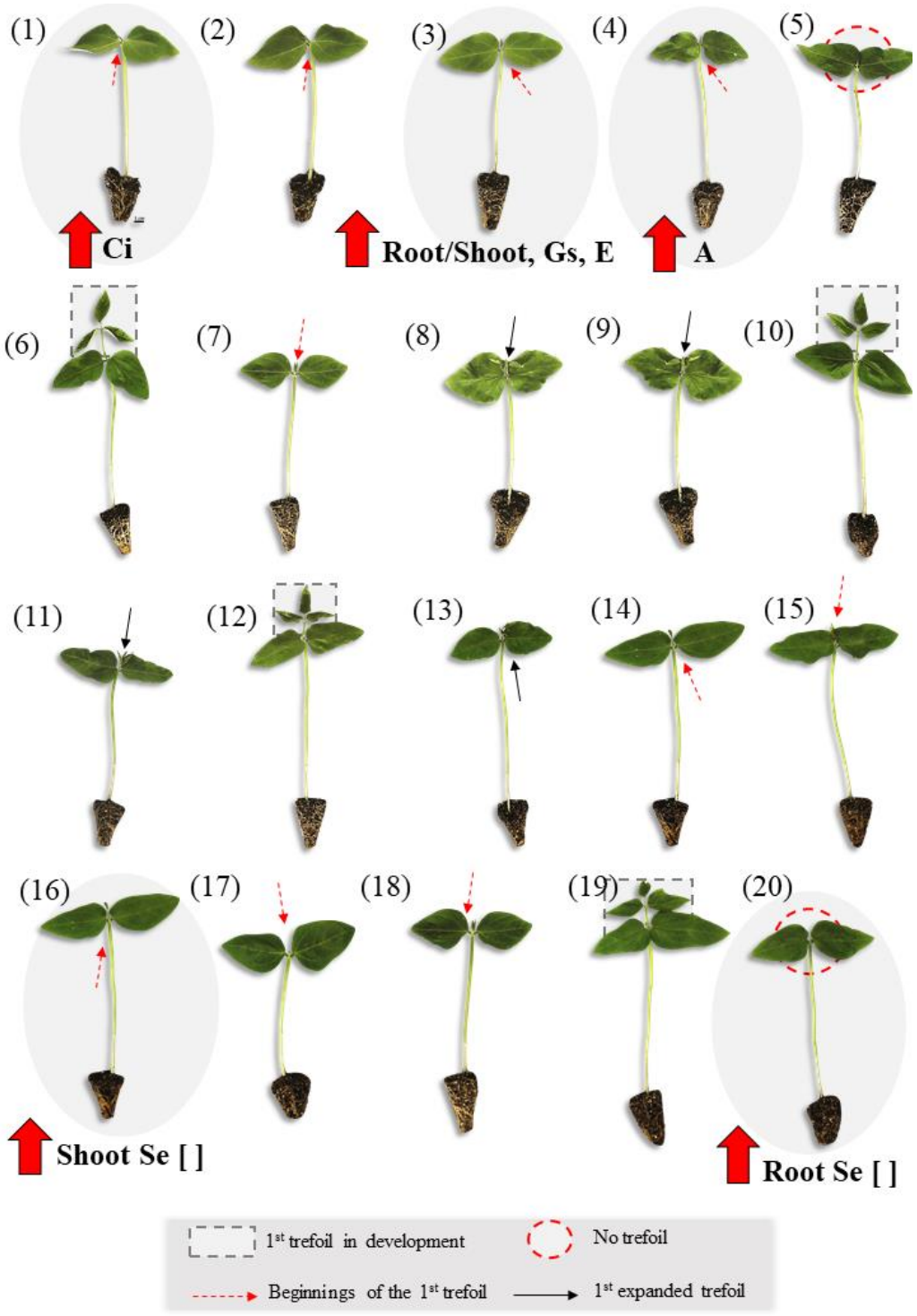


Figure 8. Phenological characteristics of the development stage of the cowpea genotypes used, as follows: (1) BR17 Gurgeia, (2) BRS Tracuateua,(3) BRS Arace, (4) BRS Cauame, (5) BRS Guariba, (6) BRS Itaim, (7) BRS Juruá, (8) BRS Marataoã, (9) BRS Milênio, (10) BRS Novaera, (11) BRS Pajeu, (12) BRS Potengi, (13) BRS Rouxinol, (14) BRS Tumucumaque, (15) BRS 15) Urubuquara, (16) BRS Xique xique, (17) California CBS, (18) Inhuma, (19) Epace 10 and (20) Pretinho.

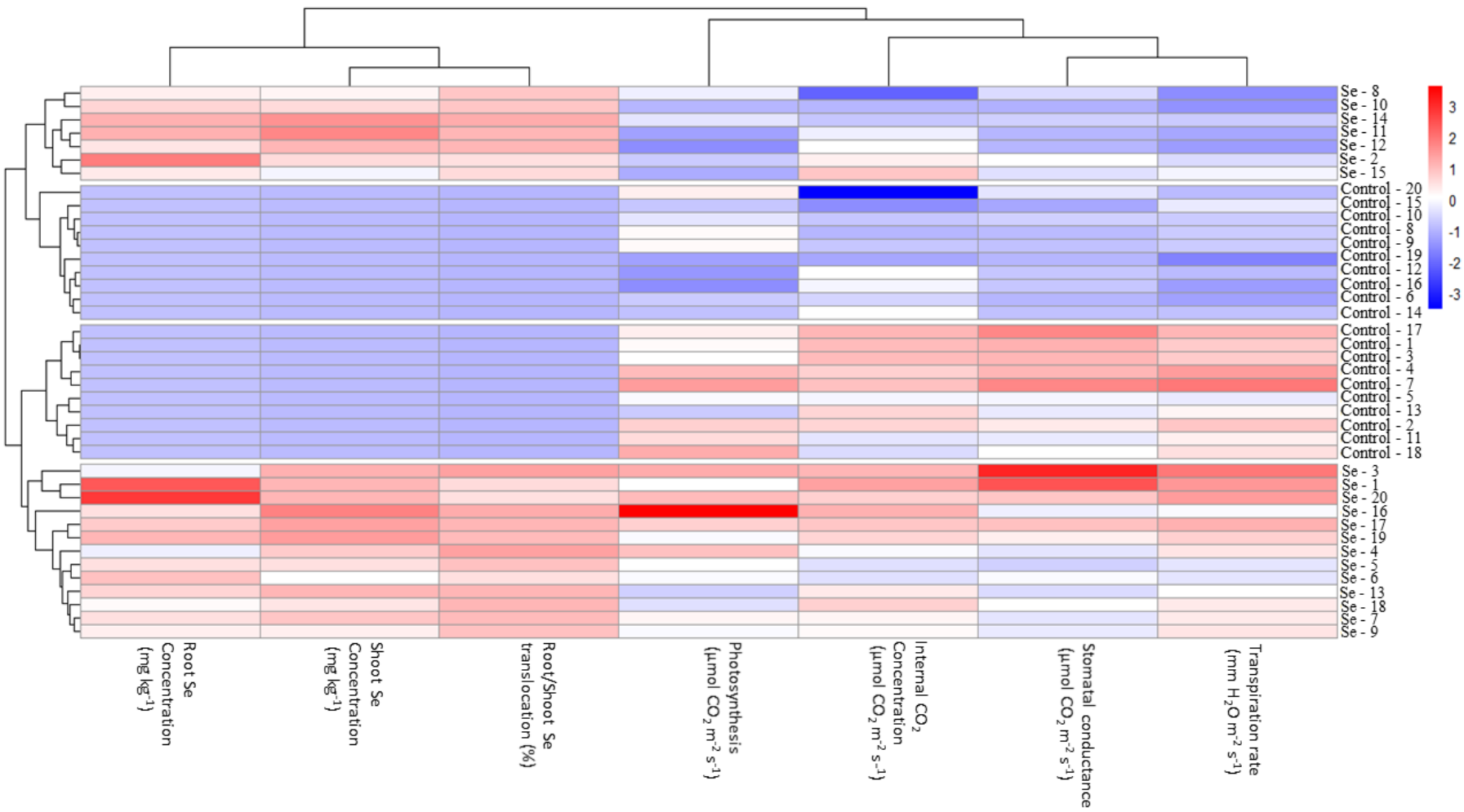


Figure 9. Hierarchical clustering of cowpea cluster subjected to Se solution by Euclidean distance using UPGMA method.

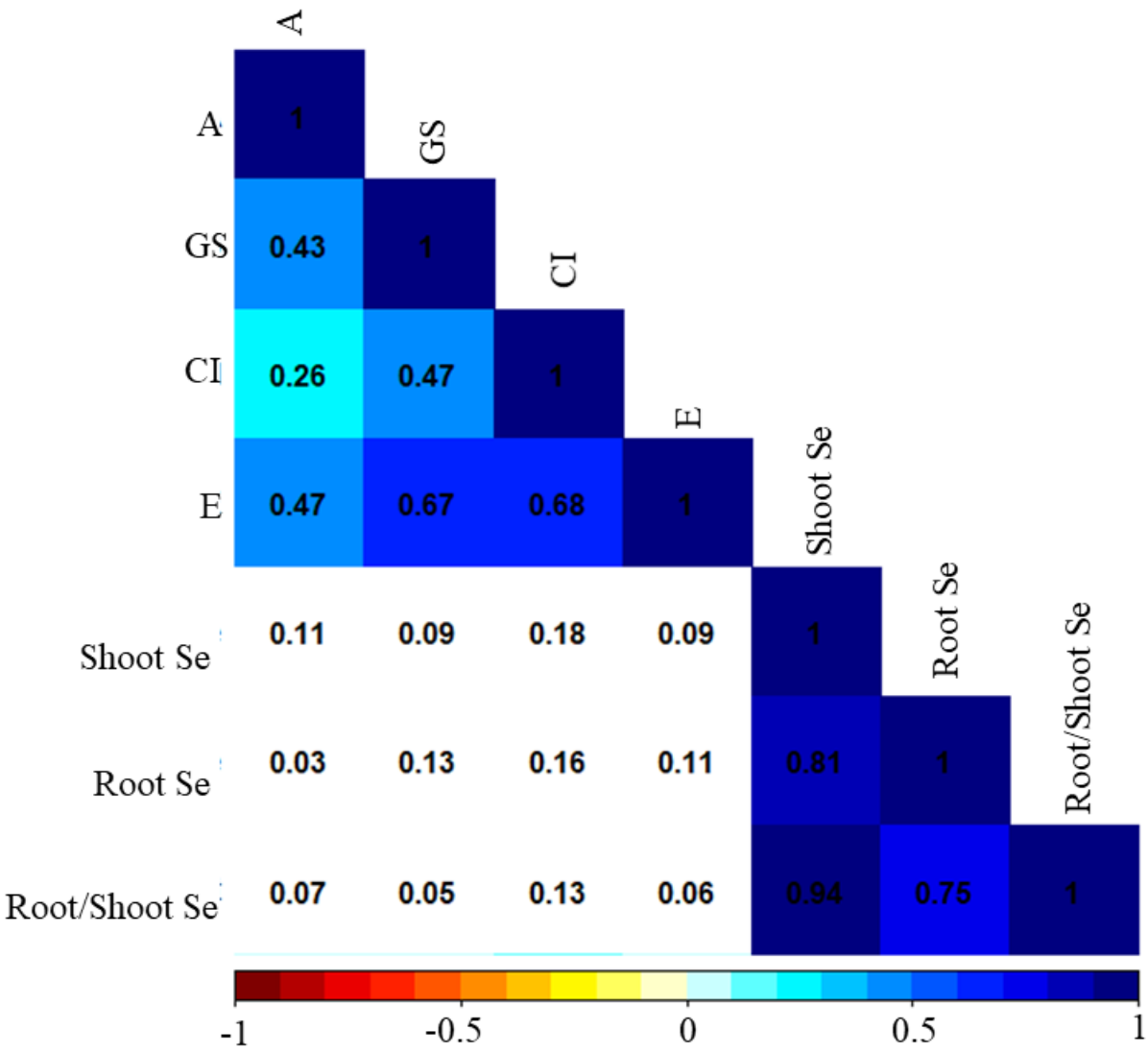


Figure 10. Pearson linear correlation of cowpea variables subjected to selenium application.

SUPPLEMENTARY MATERIAL

Table S1. Results and statistical analysis of gas exchange measurements (LI-6400 Portable Photosynthesis System) parameters.

Genotypes	A (μM CO_2 m^{-2} s^{-1})	GS (mol CO_2 m^{-2} s^{-1})	CI (μM CO_2 mol^{-1})	E (mm H_2O m^{-2} s^{-1})	Shoot Se concentration (mg kg-1)	Root Se concentration (mg kg-1)	Root/Shoot Se translocation (%)
			Control				
1	22.5 Aa	2.1 Ba	332.25 Aa	8.63 Aa	0 Ba	0 Ba	0 Ba
2	24.35 Aa	1.55 Aa	323.75 Aa	8.72 Aa	0 Ba	0 Ba	0 Ba
3	22.2 Aa	2.08 Ba	332.75 Aa	8.61 Aa	0 Ba	0 Ba	0 Ba
4	25.33 Aa	2.08 Aa	324.75 Aa	9.24 Aa	0 Ba	0 Ba	0 Ba
5	21.8 Aa	1.15 Ab	302 Ab	7.54 Ab	0 Ba	0 Ba	0 Ba
6	19.38 Ab	0.52 Ab	289.5 Ab	6.53 Ab	0 Ba	0 Ba	0 Ba
7	26.77 Aa	2.53 Aa	330.33 Aa	9.75 Aa	0 Ba	0 Ba	0 Ba
8	22.3 Aa	0.53 Ab	277.33 Ab	7.08 Ab	0 Ba	0 Ba	0 Ba
9	22.3 Aa	0.62 Ab	284.33 Ab	7.11 Ab	0 Ba	0 Ba	0 Ba
10	20.63 Ab	0.74 Ab	283.75 Ab	7.1 Ab	0 Ba	0 Ba	0 Ba
11	23.93 Ab	1.03 Ab	296.67 Ab	8.07 Aa	0 Ba	0 Ba	0 Ba
12	17 Ab	0.67 Ab	306.25 Ab	6.88 Ab	0 Ba	0 Ba	0 Ba
13	19.4 Ab	1.02 Ab	323 Aa	8.04 Aa	0 Ba	0 Ba	0 Ba
14	19 Ab	0.61 Ab	305.33 Ab	6.91 Ab	0 Ba	0 Ba	0 Ba
15	19.97 Ab	0.8 Ab	315.67 Aa	7.51 Ab	0 Ba	0 Ba	0 Ba
16	16.57 Ab	0.66 Ab	301 Bb	6.42 Ab	0 Ba	0 Ba	0 Ba
17	23 Aa	1.8 Aa	334.67 Aa	8.89 Aa	0 Ba	0 Ba	0 Ba
18	25.97 Aa	1.31 Ab	291 Ab	8.29 Aa	0 Ba	0 Ba	0 Ba
19	17.58 Ab	0.52 Bb	271.75 Bb	6.05 Bb	0 Ba	0 Ba	0 Ba
20	22.97 Aa	0.96 Bb	278.4 Ab	6.83 Bb	0 Ba	0 Ba	0 Ba
			40uMol Se				
1	22.13 Aa	3.08 Aa	342.25 Aa	9.34 Aa	162.33 Ad	127.82 Ab	55.93 Am
2	19.6 Aa	1.23 Ac	312.75 Aa	7.28 Ab	122.17 Af	106.83 Ac	53.29 An
3	26.1 Aa	3.56 Aa	335 Aa	9.75 Aa	168.62 Ad	28.94 Al	85.31 Aa
4	25.1 Aa	1 Bc	303.75 Aa	8.24 Aa	139.61 Ae	26.05 Al	84.24 Ab
5	21.98 Aa	0.8 Ac	294 Ab	7.46 Ab	119.55 Af	54.51 Ah	68.67 Aj
6	21.78 Ab	1.19 Ac	294 Ab	7.46 Ab	83.02 Ai	69.62 Ae	54.38 An
7	22.67 Aa	0.96 Bc	311.67 Aa	8.17 Aa	144.94 Ae	54.99 Ah	72.48 Ah
8	21.3 Aa	0.87 Ac	246.33 Ac	6.24 Ab	97.15 Ah	45.82 Aj	67.88 Ak
9	21.77 Aa	1.05 Ac	310 Aa	8.26 Aa	103.07 Ah	44.7 Aj	69.75 Ai
10	18.5 Ab	0.46 Ac	277.33 Ab	6.26 Ab	120.83 Af	59.49 Ag	67.01 Ak

11	17.63 Ab	0.47 Ac	299.67 Ab	6.54 Ab	219.18 Aa	78.14 Ad	73.7 Ag
12	16.45 Ab	0.52 Ac	305.25 Aa	6.43 Ab	162.59 Ad	52.09 Ah	75.72 Ae
13	19.67 Ab	0.85 Ac	316 Aa	7.8 Ab	166.33 Ad	59.01 Ag	73.8 Ag
14	22.23 Ab	1.1 Ac	320 Aa	8.12 Aa	204.99 Ab	52.74 Ah	79.49 Ad
15	18 Ab	0.94 Ac	328.67 Aa	7.67 Ab	65.85 Aj	49.2 Ai	57.21 Al
16	21.94 Ab	1.09 Ac	336 Aa	7.74 Ab	224.83 Aa	54.67 Ah	80.4 Ac
17	24.47 Aa	1.97 Ab	328 Aa	9 Aa	190.33 Ac	64.15 Af	74.77 Af
18	20.53 Aa	1.3 Ac	325.67 Aa	8.18 Aa	113.37 Ag	39.88 Ak	73.96 Ag
19	21.67 Ab	1.46 Ab	323.33 Aa	8.51 Aa	193.85 Ac	76.53 Ad	71.67 Ah
20	25.4 Aa	1.91 Ab	324.67 Aa	9.29 Aa	161.63 Ad	142.77 Aa	53.8 Na

Different letters indicate a difference between means according to a Tukey test ($p \leq 0.05$). Uppercase letters correspond to treatments. and lowercase letters correspond to genotypes.

Table S2. F ratio and P-value of analysis of covariance (ANCOVA) for photosynthesis. stomatic conductance. internal CO_2 concentration. transpiration rate. and Se concentrations in shoots. roots. and root/shoot translocation. Sources of variation: treatments (control x 40 µM Se). genotypes. and the variation between the factors.

Photosynthesis (A)		
Factor	F ratio	Pr (<F)
Treatment (A)	0.12^{NS}	0.7255
Genotypes (B)	3.08**	<0.0001
A X B	1.64^{NS}	0.0563
Stomatic Condutance (Gs)		
Factor	F ratio	Pr (<F)
Treatment (A)	1.91^{NS}	0.1698
Genotypes (B)	9.21**	< 0.0001
A X B	3.13**	< 0.0001
Internal CO2 concentration (Ci)		
Factor	F ratio	Pr (<F)
Treatment (A)	2.84^{NS}	0.00944
Genotypes (B)	4.59**	< 0.0001
A X B	1.71*	0.0434
Transpiration rate (E)		
Factor	F ratio	Pr (<F)
Treatment (A)	0.83^{NS}	0.3638
Genotypes (B)	3.58**	< 0.0001
A X B	1.96**	0.0155
Se concentration (Shoots)		
Factor	F ratio	Pr (<F)
Treatment (A)	36743.06**	< 0.0001
Genotypes (B)	169.67**	< 0.0001
A X B	169.67**	< 0.0001
Se concentration (Roots)		
Factor	F ratio	Pr (<F)
Treatment (A)	39504.41**	< 0.0001
Genotypes (B)	432.25**	< 0.0001
A X B	432.25**	< 0.0001
Root/Shoot Se translocation		
Factor	F ratio	Pr (<F)
Treatment (A)	487143.48**	502.83**
Genotypes (B)	502.83**	< 0.0001
A X B	502.83**	< 0.0001

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

CHAPTER 4 - Selenate or selenite: who wins the selenium absorption race?

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ABSTRACT

Selenium (Se) is an essential element for humans and animals and its deficiency in the diet has serious consequences for global health. The Se content in the plant is directly related to the Se concentrations in the soil. However, the presence of Se in the edible parts of plants depends directly on the source of Se present in the soil. The sources of Se most used in crop supplementation are selenate (SeO_4) and selenite (SeO_3). This work aimed to understand the factors that govern the accumulation and distribution of Se in tissues of cowpea (*Vigna unguiculata* L. Walp.) plants cultivated with 40 µM of Na_2SeO_4 and Na_2SeO_3 in genotypes characterized by high Se assimilation (BRS Xiquexique) and low Se assimilation (BRS Urubuquara). Plants evaluated in vivo by the XRF technique demonstrated to absorb more Se with Na_2SeO_4 source compared to Na_2SeO_3 throughout the entire exposure period. At the end of the experiment, the application of Na_2SeO_4 from the BRS Xiquexique genotype resulted in a concentration of 166.33 mg kg^{-1}, while the treatment with Na_2SeO_3 was 45.83 mg kg^{-1} of Se in the shoot. Furthermore, with the application of Na_2SeO_4, there was a translocation of 3 times more Se compared to plants treated with Na_2SeO_3. Regarding photosynthetic metabolism, plants treated with Na_2SeO_3 showed higher rates of accessory pigments (pheophytin and chlorophyll), related to cell protection and maintenance. While plants treated with Na_2SeO_4 increased the levels of chlorophylls, Gs, E, Ca, and K, related to the increase in carbohydrates. Nitrogen metabolism was affected by the antagonism between Se and Mn. Therefore, plants treated with molecules that have greater translocation and assimilation capacity in edible parts have greater possibilities of generating more nutritious food to feed populations. Therefore, the results presented demonstrate that the use of Na_2SeO_4 is away with better performance to biofortify the cowpea crop with Se.

Keywords: *Vigna Unguiculata*, sodium selenate, sodium selenite, Se kinetics, X-ray fluorescence, translocation index.

1. INTRODUCTION

Selenium (Se) is considered a beneficial element for plants, and essential for humans and animals (Adebayo et al. 2020). It is estimated that around 1 billion people worldwide are affected by Se deficiency (Jones et al. 2017). Se deficiency is directly related to low levels of the element in food (White 2018). This low intake has consequences on the autoimmune system (Schomburg 2021), cardiovascular (Zhou et al. 2018), in post-traumatic responses (Braunstein et al. 2020), and even in the increased risk of death from viral infections, such as SARS-CoV-2 (Covid-19) (Moghaddam et al. 2020).

Several improvement programs have been launched to increase the nutrient content in legume grains in response to the Sustainable Development Goals (SDGs), such as agronomic biofortification of crops (FAO 2017). Cowpea *(Vigna unguiculata L. Walp.)* is one of the most drought-tolerant legumes, with high nutritional potential, and an economically viable alternative for obtaining vitamins and minerals (Singh 2020). Deng et al. (2022) state that it is still not clear which source of Se can be used to efficiently and economically obtain a legume fortified with Se. Our previous researches revealed advances in the studies of doses, types of application, and sources of Se to guarantee food security and minimize hidden hunger (Silva et al. 2018; Reis et al. 2020; Lanza et al. 2021).

Selenium is a powerful antioxidant agent in higher plants with the main focus being selenoproteins (SeMet and SeCys) which act in plant metabolism through the glutathione peroxidase (GSH) pathway and provide increased activity to antioxidant enzymes (SOD, CAT, and APX) which act reactively in the reactive oxygen species (ROS) elimination system and cell detoxification (Lanza and Reis 2021). Furthermore, compounds from primary metabolisms, such as sucrose, total sugars, nitrate, ammonia, and amino acids increase in Se-treated plants, being a defense mechanism for osmotic adjustment (Gouveia et al. 2020).

Plants can absorb Se in the form of selenate, selenite, and organic Se species (Wang et al. 2019). However, selenite and selenate are recognized as the main sources of Se in mineral fertilizers applied via foliar or soil due to their high bioavailability (Chauhan et al. 2019). These two main forms have characteristics that can limit the absorption,

translocation, and assimilation of Se by cultures, directly affecting the nutrition of plants, and consequently humans and animals (Deng et al. 2017; Li et al. 2018; da Silva et al. 2020).

Selenate (SeO_4) has a high similarity to S and therefore competes for sites that carry the element in the process of translocation from roots to shoot (Shin and Moon 2021). This characteristic is associated with its efficiency in translocating Se to leaves, accumulating Se mainly in shoots and fruits, and being poorly metabolized as selenoamino acids (Izydorczyk et al. 2021). On the other hand, selenite (SeO_3) is more strongly retained in the roots, and its low translocation to edible parts is associated with its rapid conversion into organic compounds (SeMet, SeOMet, and MeSeCys) that are less metabolized by plants due to their high volatilization (Li et al. 2020). Plants treated with selenite have less risk of toxicity compared to plants treated with selenate, which demonstrate a narrow threshold capable of harming plant metabolism (Mateus et al. 2020), polluting the environment (Gan et al. 2021), and causing toxicity in mammals (Hadrup and Ravn-Haren 2020).

To verify with high efficiency the relationship between elementary Se sources and the physiological reactions of plants, techniques such as X-ray fluorescence spectroscopy (XRF) are an alternative for the qualitative and quantitative elementary assessment of the plant in vivo, preventing the responses from defenses caused by the extensive manipulation of the samples interfere in the results (Gomes et al. 2019). In addition to Se being an element with high oxidizing power, there is a wide variation in the assimilation of Se genotypes from the same plant family (Wang et al. 2021). Therefore, through the use of technological resources, such as the studies by (Li et al. 2020), it is possible to understand how the forms of Se are assimilated by each plant material, and what is the speciation of the element in plant organs.

Hypothetically, the analysis of Se uptake kinetics by the XRF technique is an efficient way to support the element concentration data in plant tissues and to evaluate in real-time the physiological status of Se uptake by cowpea plants. Therefore, the present work aimed to understand i) which is the most efficient source of Se for plant nutrition aiming economic viability? ii) How sources of Se interfere in the antioxidant and

photosynthetic metabolism of cowpea plants? and iii) What is the relationship between genotypic absorption capacity and Se sources?

2. MATERIALS AND METHODS

2.1 Cultivation Assay

Two cowpea genotypes were used, BRS Xiquexique characterized with high Se uptake potential and the BRS Urubuquara genotype with low Se uptake potential in our previous studies (chapter 3). The cowpea seeds were treated with Sementhiran® (300 mL/100 kg) and Maxim XL® (100 mL/100 kg) and sown in black polyethylene trays containing 98 cells filled with Caroline Soil® substrate. The genotypes were cultivated on December 31, 2020, and after 10 days (January 10, 2020) they were acclimated for 4 days in a Hoagland nutrient solution. The Hoagland solution was prepared based on the fallow concentrations of the stock solution: 1) 101,10 g L^{-1} KNO_3, 2) 236.16 g L^{-1} $Ca\,(NO_3)_2\,.\,4H_2O$, 3) 115.08 g L^{-1} $NH_4H_2PO_4$, 4) 123.24 g L^{-1} $MgSO_4.7H_2O$, 5) 1.864 g L^{-1} KCL, 0.773 g L^{-1} H_3BO_3, 0.169 g L^{-1} $MnCl_2.4H_2O$, 0.288 g L^{-1} $ZnSO_4.7H_2O$, 0.062 g L^{-1} $CuSO_4.4H_2O$, 0.040 g L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$ and 6) 30 g L^{-1} $FeO_4S.7H_2O$.

On January 10th and 11th, 2020 the plants remained in the solution at 25% of the hoagland solution, with the following amounts of volume of stock solution per liter of final of final solution:) 1.5 mL L^{-1}, 2) 1 mL L^{-1}, 3) 0.5 mL L^{-1}, 4) 0.5 mL L^{-1}, 5) 0.5 ml L^{-1}, 6) 0.25 ml L^{-1} . After, on January 12th and 13th, 2021 it was changed to 50% of the Hoagland solution, content the respective volume of stock solution per liter of final of final solution: 1) 3 mL L^{-1}, 2) 2 mL L^{-1}, 3) 1 mL L^{-1}, 4) 1 mL L^{-1}, 5) 1 ml L^{-1}, 6) 0.5 ml L^{-1}.

On the 15th day of cultivation, 40µM of Se was applied, using sodium selenate (Na_2SO_4), with the 80% of Hoagland content the respective volume of stock solution per liter of final of final solution: 1) 4.8 mL L^{-1}, 2) 3.2 mL L^{-1}, 3) 1.6 mL L^{-1}, 4) 1.6 mL L^{-1}, 5) 1.6 mL L^{-1}, 6) 0.8 mL L^{-1}. The genotypes were 14 days old. The dosage of Se was chosen based on previous experiments.

All genotypes remained in contact with the treatment during the 48 hours. After the time of exposure to the treatment, to remove excess Se on the external surface of the plants, the roots were removed from the nutrient solution and immersed in the sequence of solutions: HCl 0.3 M and Na_2HPO_4 0.1 M for about 1 minute (Carmo et al. 2000).

In the sample preparation, the roots of genotypes were separated from the shoots stored in paper bags and dried in a laboratory oven (Tecnal TE-393) with forced air circulation at 60 ° C for 24 hours. The dried vegetable material was ground with a pistol and mortar, and then processed with an 80 mesh sieve, with a particle size <100 µm.

2.2 *In vivo* XRF Se kinetics

Cowpea plants with twenty-one days of cultivation were placed in a growth room at 25 ± 2 ºC, and 12 h-photoperiod at 250 µmol of photons m^{-2} s^{-1} provided by 3000 K LED lights. The plants were acclimated with 80% of Hoagland Solution during 48 h. Moreover, at the beginning of the Se absorption kinetic study, a Hoagland solution at 40µM of Se was applied. The source of Se is sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3) (Merck KGaA, Darmstadt, Germany). The solution was volume adjusted with purified water and adjusted to pH 6.0 daily.

The central leaflet of the second completely expanded trifoliate leaves was analyzed by a handheld XRF spectrometer (Bruker, Tracer III SD, Germany). The XRF analysis was carried out using a 4 W Rh anode at 40 kV and 30 µA, with a primary filter (304.8 µm Al + 25.4 µm Ti + 25.4 µm Cu) and the X-ray spectrum recorded by a 10 mm^2 X-Flash detector. The XRF measurement time was 60 s (Figure S1 and S2).

The leaves were marked using a pen, and the same sample region was monitored along 64 h by the portable XRF. The detector dead-time was lower than 1%. The instrumental stability was checked with a Se thin-film standard at 49.5 µg cm^{-2} Se (Micromatter™, serial number 30112, Canada) under the same analytical conditions as the leaf samples just before each measurement along with the experiment. Five biological replicates were employed. Only Se XRF intensities whose signal with statistical significance above the noise were considered. Besides, the XRF Se signals were normalized by Rh-Kα Compton scattering to correct the effects of the cowpea leaf thickness variation.

2.3 Determination of Se concentration by Benchtop EDXRF Analysis

Se concentration in the roots and shoots was determined by energy dispersive X-ray fluorescence spectroscopy (EDXRF, Shimadzu EDX-720, Japan) according to a

modified method described by Bachiega et al. (2019). One hundred micrograms of sample and standard were transferred into a 6.3 mm window XRF cuvette (cat. no. 3577, Spex) sealed with 6.35 µm thick Mylar® film (no. 3517, Spex). The powder sample was pressed manually with a glass stick to avoid voids in the sample. The typical sample thickness was ca. 7 mm. Three biological replicates were analyzed. The EDXRF analysis was carried out using a 50 W Rh X-ray at 50 kV and auto-adjusted current (maximum of 30% of the detector dead time), 3 mm collimator, and Ag primary filter. A Si (Li) detector was used for recording the sample X-ray spectrum. The acquisition time was 200 s.

After all samples measurements, the genotype with the lowest Se concentration (BRS Urubuquara) was chosen for the test of recovery. The trueness of the method was evaluated by spike-and-recovery assay, in which 300 µL of a 100 µg mL^{-1} Se aqueous standard (from 1:10 dilution 1 mg mL^{-1} of Se Acros Organics) were added in 300 mg of cowpea leaf, previously weighed in a petri dish. Subsequently, the spiked standard was dried in a laboratory oven (Tecnal TE-393) at 60 °C for 12 h and homogenized utilizing an agate mortar. The dried samples were ground in a mortar with the aid of a pistil, and refined through an 80-mesh stainless steel sieve. Then, the same sample analysis procedure described above was performed.

The detection limit (LOD) was calculated based on the average of BG intensity (cps/µA), Current (µA), Time (s), and Sensitivity of equipment. The LOD (mg kg) was calculated as (equation 1):

$$Equation\ 1.\ LD = \frac{3}{Si}\sqrt{\frac{I_{i(BG)}}{t}}$$

Where $I_{i(BG)}$ is the background intensity of selenium (cps/µA), t is the acquisition time (s), and Si is the analytical sensitivities to an analyte (cps µg g^{-1}).

All Compton's of the samples were compared to verify that the beam was focused on the sample and that there was no deviation through the sample holder. The X-ray spectrum processing was carried out utilizing the EDX-720 software package provided by Shimadzu.

2.4 Gas exchange measurements - LI-6400 Portable Photosynthesis System

The determination of gas exchange was performed by a portable infrared gas analyzer system (Li-6400XTR, LI-COR Biosciences, Lincoln, NE, USA). 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 stomatal subchamber (μmol CO_2 mol^{-1}) and transpiration rate (mmol H_2O m^{-2} s^{-1}). For the measurements, the standard conditions used were: 1200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), provided by LED lamps, 400 ppm CO_2 chamber temperature 28 °C, and humidity 40-45%, according (Lima et al. 2019).

2.5 Determination of antioxidant metabolism

The determination of antioxidant metabolism was performed by analyzing the following enzymatic activities: superoxide dismutase activity (SOD, EC:1.15.1.1), catalase activity (CAT, EC:1.11.1.6), ascorbate peroxidase activity (APX, EC:1.11.1.11), hydrogen peroxide concentration (H_2O_2), and lipid peroxidation (malondialdehyde). After 64 h of Se exposure, two leaves per plant were collected and quickly stored in plastic bags in liquid nitrogen for all analyses. A composing sample of leaf material from the repetitions of each treatment was macerated in nitrogen liquid and stored in a freezer at −80 °C.

The extract of the enzymatic activity was performed using 1.5 g of plant material and adding 1 mL of phosphate buffer pH 7.5 0.1M + 1mM EDTA + 3mM DTT with 4% PVPP, and stored in a freeze (Gomes-Junior et al. 2006). To determine the SOD activity were added: 2 ml of Na buffer at pH 7.8 0.05 M, 50 μL of extract, 200 μL of nitro blue tetrazolium chloride (NBT) 75 μM, 250 μL of methionine, and 250 μL of riboflavin (Giannopolitis and Ries 1977). The test was vortexed and exposed to light for 20 minutes at room temperature. The samples were read on a spectrophotometer (UV VIS SPECTRO 580UVP) with λ = 560nm.

The CAT activity was determined by adding 25 μL of supernatant and 1 mL of buffer K pH 7.5 0.1M with H_2O_2 in the cuvette. Immediately, the spectrometer measurement began at λ = 240 nm (absorbance). The reading was monitored during the 1-minute interval, and the absorbance was recorded every 10 seconds to observe the

H_2O_2 degradation (Azevedo et al. 1998). The APX activity was measured by adding in a glass tube: 600 µL of Buffer K pH 7 0.08M and 100 µL of EDTA 1 mM. The vial was heated at 30°C for 10 min and added 100 µL of extract and 101 µL of H_2O_2 1 mM. The sample was measured at λ = 290 nm (Nakano and Asada 1981; Moldes et al. 2008). The determination of the antioxidant enzyme was performed based on the total protein concentration, which was determined according to the Bradford method, using 5 µL of extract and 1 mL of Bradford Solution (Bradford 1976).

The extract of MDA and H_2O_2 was made using 0.4 g of fresh leaves macerated in liquid nitrogen and 4 mL of 0.1% trichloroacetic acid. Lipid peroxidation was estimated based on malondialdehyde (MDA) (Heath and Packer 1968). The MDA test was performed using 1 mL of TCA 20% + Thiobarbituric acid (TBA) 0.5%. The assay was heated to 95°C for 30 min and then cooled for 10 min in an ice bath. The sample was measured at λ = 600 and λ = 535 nm, in which the difference between the absorbances provides the results. The H_2O_2 test was performed using 200 uL of extract, 200 of K buffer pH 7.5 0.1 M, 800 KI 1 M. Then, the vial was cooled in the dark for 1 hour and measured at λ = 39 0nm. This method was adapted from (Alexieva et al. 2001).

2.6 Total sugars and sucrose

The total sugars were extracted using 1 g of plant material and 10 mL of MCW solution (methanol:chloroform: water) (Dicksson and Haissig 1979; Rose et al. 1991). After 48 h in the refrigerator, an aliquot of 4 mL of the supernatant was removed, and 1 mL of chloroform and 1.5 mL of deionized water were added to another tube. After phase separation, the total sugars and sucrose were determined in the aqueous phase according to the method described by (Bieleski and Turner 1966) using i) 200 µL of MCW extract diluted in 400 µL of deionized water, ii) 500 µL of KOH 30% solution and iii) 2 mL of 100% H_2SO_4. The total sugars were determined, according to (Dubois et al. 1956), performed by adding i) 50 µL of MCW extract diluted in 450 µL of deionized water, ii) 500 µL of 5% phenol solution and iii) 2 mL of 100% H_2SO_4. The solution was homogenized by vortexing, and the absorbance was measured at 490 nm for total sugar and xx for sucrose. The total sugar and sucrose concentrations were based on the standard sucrose curve, and the results are expressed in mg g^{-1} FW.

2.7 Nitrogen metabolism

Nitrate (NO_3^-) was determined according to the modified method of Cataldo et al. (1975). From the extract of the water-soluble phase, an aliquot of 200 µL was added with 40 µL of 5% salicylic acid in H_2SO_4 (p/v). After 20 minutes at room temperature, 1 mL of 2N NaOH was added slowly. After cooling to room temperature, readings were carried out on a Mars spectrophotometer model UV VIS SPECTRO 580UVP at λ = 410 nm. The nitrate concentration was determined using a standard curve of calcium nitrate solution. The results expressed in µmol $plant^{-1}$.

The ammonia (NH_4) concentration was quantified according to the methodology described by McCullough (1967). In a glass tube was added 500 µL of water-soluble extract, 500 µL of phenol solution, and 500 µL of phosphate solution. The samples were incubated at 37 ºC for 1 h. The reading was performed by spectrometry at wavelength λ = 630 nm in absorbance. The ammonia concentration was determined using a standard curve of ammonium sulfate solution and the results are expressed in µmol per $plant^{-1}$.

Total free amino acids were determined according to (Yemm et al. 1955), performed with an aliquot of 500 µL from a water-soluble extract of MCW, 500 µL of 0.2 M sodium citrate, 200 µL of 5% ninhydrin in ethylene glycol monomethyl ether, and 1 mL of KCN 0.2 mM. The assay was heated to 100 °C for 15 minutes and then cooled for 10 minutes under running water. Then, 1 mL of 60% ethanol was added, followed by a reading on a spectrophotometer at λ = 570 nm. The concentration of total free amino acids was evaluated using the standard curve for methionine. The results were expressed in µmol g^{-1} DW.

2.8 Photosynthetic pigments

The determinations of photosynthetic pigments (chlorophyll, carotenoids, and pheophytin) were based on the description of (Lichtenthaler 1988) using 80% acetone as the solvent. The extraction was performed with 0.5 g of fresh matter in 5 mL of 80% acetone. The concentrations of total chlorophyll, total pheophytin, and carotenoids are expressed in µg mL^{-1}.

2.9 Translocation index

The translocation index was calculated using the Se concentrations (mg kg^{-1}) values of the roots, and the shoot quantified in Benchtop EDXRF. Equation 2 was applied according to (Abichequer and Bohnen 1998). The Se content in the plant is the sum of its concentration in the shoot and the root.

$$Equation\ 2\ .\ Root\ to\ Shoot\ Se\ translocation\ index\ (\%) = \frac{Se\ content\ in\ the\ shoot}{(Se\ content\ in\ plant)}\ x\ 100$$

2.10 Statistical analysis

All data were first submitted to the Outlier test (Grubbs's test) in the Minitab® Statistical Software. And on the same platform, the data normality test was performed by the Anderson-Darling method, using a significance level of 0.005. For the statistical analysis of the data, the experimental design was considered as a completely randomized design (DIC), with 2 factors (double factorial), considering factor A being the treatments (control, Na_2SeO_3 and Na_2SeO_4) and factor B the genotypes (BRS Xiquexique and BRS Urubuquara). The variance analysis of data was analyzed using the Tukey method at 5% probability. The graphics were built from the SigmaPlot 12.5 software

To identify the variation in nutrient concentration, the percentage variation formula was used based on the values found in the control treatment (0 g ha^{-1}). The formula used is expressed in equation 3:

$$Equantion\ 3.\ PV\ (\%) = \frac{(A - B)}{B}\ x100$$

Where:

A = Baseline (control treatment)

B = Reference value obtained by applying treatments

3. RESULTS

3.1 Selenium absorption kinetics in cowpea plants

The absorption of sodium selenate (Na_2SeO_4) was highest after 110 hours of monitoring compared to sodium selenite (Na_2SeO_3) (Figure 1). This evidence was seen for the two genotypes evaluated. BRS Xiquexique (Figure 1A) demonstrated a clear absorption of Na_2SeO_4 from a time interval of 0-15 hours, while BRS Urubuquara (Figure 1B) still showed a low absorption of Se sources, equaling the control treatment. Then, from the interval of 15-40 hours onwards, there was a clear leap in increasing absorption for both genotypes. The difference between selenite absorption and the control was minimal.

Comparing the two sources of Se used, it is measured through the percentage variation for BRS Xiquexique that Na_2SeO_4 was greater than Na_2SeO_3 showing an increase of 71.84 %, 221.89%, 763.77%, 767.56%, 2587,76%, and 701.6%, respectively. Thus, it can be seen that the highest peak of Na_2SeO_4 absorption occurred after 87 hours of application of the treatment. The same occurred for BRS Urubuquara, with an increase of 447.74% of Na_2SeO_4 absorption in 87 hours. BRS Xiquexique had an absorption 66.86% greater than the BRS Urubuquara at 87 hours.

About the other nutrients detected in the analysis of the Se absorption kinetics, the synergism between Se, potassium (K) and calcium (Ca) and the antagonism with manganese (Mn) is highlighted. Comparing Figure 2A (BRS Xiquexique) with Figure 2B (BRS Urubuquara) we observed that BRS Xiquexique genotype absorbed more K in all treatments applied including the control. For BRS Urubuquara, Na_2SeO_4 supply increased by more than 20% the absorption of K after 110 hours compared to untreated plants.

The application of Na_2SeO_4 and Na_2SeO_3 gradually increased the concentration of Ca in the two genotypes evaluated. For BRS Xiquexique, the gradual increase in absorption over time continued at an increasing pace until the last assessment point. For BRS Urubuquara (Figure 2D) there was a drop of about 2% in the Na_2SeO_3 treatment between the range of 80-110. The application of Se sources showed antagonistic results with Mn levels for both genotypes (Figure 2 E and F), resulting in higher Mn concentrations for the control treatment.

3.2 Gas exchange measurements related to Se absorption kinetics

The stomatal conductance (Gs) were greater at 87 hours for BRS Xiquexique (Figure 3A) in the application of Na_2SeO_3 and at 110 hours with the application of Na_2SeO_4, with an increase of 68.65% and 118.47%, for the respective treatments compared to the levels at the beginning of treatment. For BRS Urubuquara (Figure 3B) the greatest increase in GS was seen with 40 hours of treatment, corresponding to an increase of 88.28% for Na_2SeO_3 and 40.53% for Na_2SeO_4 compared to the beginning of treatment. At the same time (40 hours) there was a synchronism in the increase of GS for all treatments in the BRS Urubuquara genotype.

The transpiration rate (E) was also notably higher after 40 hours of exposure to the two treatment genotypes. For BRS Xiquexique (Figure 3C) the increase was 28.90% for Na_2SeO_3 and 86.76% for Na_2SeO_4 in relation to time 0. The BRS Urubuquara increased by 209.92% for Na_2SeO_3 and by 107.90% for Na_2SeO_4. Although the percentage variation of E was greater for the Urubuquara, compared to the control treatment. The BRS Xiquexique had the highest E rate for the two Se sources, being 4.07 mm H_2O m^{-2} s^{-1} for Na_2SeO_3 and 4.20 mm H_2O m^{-2} s^{-1} for Na_2SeO_4.

The internal CO_2 concentration (Ci) was more notable in the BRS Urubuquara (Figure 3F) in the control treatment, going from 50 µM CO_2 mol^{-1} at time 0 to 394.06 µM CO_2 mol^{-1} at 64 hours. The application of Na_2SeO_3 resulted in a reduction in Ci compared to time 0 after 15, 64, 87, and 110 hours for BRS Xiquexique (Figure 3E). For BRS Urubuquara, the same treatment reduced all Ci compared to time 0. Na_2SeO_4 was positive to increase the Ci of the BRS Xiquexique and BRS Urubuquara at 40 hours (62.01 and 131.65 respectively).

The highest net photosynthetic rate (A) was observed for BRS Xiquexique (Figure 3G) with Na_2SeO_3 treatment, reaching the maximum A value of 13.86 µM CO_2 m^{-2} s^{-1}. Comparing the two Se sources, BRS Urubuquara showed the lowest A rate in 15 hours with 10.13 µM CO2 m^{-2} s^{-1} with the application of Na_2SeO_4. The application of Na_2SeO_4 reduced throughout the evaluation period to A compared to time 0. On the other hand, after 40 hours of exposure to the treatments, it was noted that the application of Na_2SeO_3 increased by 16.47 and 5.05% the rates of A of BRS Xiquexique and BRS Urubuquara, respectively, compared to time 0. And that the application of Na_2SeO_4 increased in the BRS Xiquexique from 11 µM CO_2 m^{-2} s^{-1} to 12.36 µM CO_2 m^{-2} s^{-1} at the same time.

3.3 Selenium concentration in cowpea plants by Benchtop EDXRF

The concentration of Se in the shoot (Figure 4A) was higher for the BRS Xiquexique genotype in all treatments applied, with an increase of 97.49% for the application of Na_2SeO_3 and an increase of 18.14% for the Na_2SeO_4 compared to the concentrations for BRS Urubuquara. The Na_2SeO_4 concentration for BRS Xiquexique was 166.33 mg kg^{-1}, while the Na_2SeO_3 concentration was 45.83 mg kg^{-1}, resulting in an increase of more than 200% for the application of sodium selenate in the shoot of the genotype.

The concentration of Se in the roots (Figure 4C) showed an inverse relationship to that observed for the shoots. The BRS Urubuquara genotype retained about 42.57% more Na_2SeO_4 and 35.77% more Na_2SeO_3 in the roots than the BRS Xiquexique genotype. In the BRS Xiquexique genotype, the application of Na_2SeO_4 resulted in a concentration of 69 mg kg^{-1} in the roots, while the application of Na_2SeO_3 resulted in 246.50 mg kg^{-1}. While the BRS Urubuquara genotype retained 98.71 mg kg^{-1} with the application of Na_2SeO_4 and 334.57 mg kg^{-1} of Na_2SeO_3. These values indicate that the treatment with Na_2SeO_3 retained about 200% more selenite in the roots compared to selenate.

The translocation rate of the element between root and shoot (Figure 4B) demonstrated that the BRS Xiquexique has a greater capacity to translocate Se and that treatment with Na_2SeO_4 is translocated in greater proportions in the two genotypes evaluated. The BRS Xiquexique genotype translocated 70.65% of Na_2SeO_4, while the BRS Urubuquara translocated 58.86%. Regarding the treatment with Na_2SeO_3, BRS Xiquexique translocated 15.65% and BRS Urubuquara 6.49%.

3.4 Antioxidant metabolism

The concentration of hydrogen peroxide (Figure 5A) were not statistically significant. Lipid peroxidation (Figure 5B) evaluations revealed that the highest levels were observed for BRS Urubuquara. In the control treatment, about 50% more MDA and 68% more sucrose were observed for BRS Urubuquara compared to BRS Xiquexique. The application of Na_2SeO_3 resulted in 26.86 nM g^{-1} FW for the Xiquexique genotype and

33.86 nM g^{-1} FW for the BRS Urubuquara. While the application of Na_2SeO_4 resulted in 28.26 nM g^{-1} FW and 30.48 nM g^{-1} FW, respectively. Regarding sucrose, the Na_2SeO_3 treatment concentrated more than 200% in the BRS Urubuquara genotype and the treatment with Na_2SeO_4 about 30% more sucrose for the BRS Xiquexique genotype.

The enzyme superoxide dismutase (SOD) (Figure 6A) showed greater activity in the control and with the application of Na_2SeO_3 of the BRS Urubuquara genotype, with an increase of 14.29% compared to the BRS Xiquexique genotype. On the other hand, treatment with Na_2SeO_4 increased by 90.67% the SOD activity in the BRS Xiquexique genotype, compared to BRS Urubuquara. The catalase enzyme (Figure 6B) had its greatest activity in the control of the BRS Urubuquara genotype, being about 76% more active compared to the same treatment of the BRS Xiquexique genotype.

Na_2SeO_3 increased CAT activity in the BRS Urubuquara genotype, while Na_2SeO_4 increased the activity of BRS Xiquexique. Likewise, the enzyme Ascorbate peroxidase (APX) (Figure 6C) was more active in the control treatment of the BRS Urubuquara genotype, and for the Se treatments, it showed greater activity for the BRS Xiquexique genotype, with an increase of about 11% and 13% for the respective treatments with Na_2SeO_3 and Na_2SeO_4, compared to the BRS Urubuquara genotype. The activities of SOD, CAT, and APX enzymes were calculated according to the levels of proteins expressed in figure 6D.

There was a trend observed in photosynthetic pigments (Figure 7), where the BRS Xiquexique genotype increased all levels in the control and Na_2SeO_3 treatment, but the BRS Urubuquara genotype was more responsive to treatment with Na_2SeO_4. Chlorophyll A and B increased by 71% and 40%, respectively, compared to the control in the BRS Urubuquara genotype for the application of Na_2SeO_4, generating a 60% increase in total chlorophylls. For pheophytins, the application of Na_2SeO_4 generated an increase of about 53% for pheophytin A, 29% for pheophytin B, and consequently, a 45% increase for total pheophytins. Regarding carotenoids, the application of Na_2SeO_4 in the BRS Urubuquara genotype stood out, with an increase of 26% compared to the control treatment.

In the sugars metabolism the concentration of sucrose (Figure 8A) were not statistically significant, and the total sugars concentrations (Figure 8B) evaluations revealed that the highest levels were seen for the BRS Urubuquara genotype. The

nitrogen metabolism reveals that the concentration of ammonia (NH_4) did not show statistical significance (Figure 8C). On the other hand, the concentration of nitrate (NO_3^-) (Figure 8D) was more expressed in the BRS Xiquexique genotype, in the control treatment. The amino acid content (Figure 8E) was drastically reduced with the application of Na_2SeO_4 in the BRS Urubuquara genotype, with the BRS Xiquexique genotype showing 148% more amino acids.

4. DISCUSSION

Cowpea plants absorb Na_2SeO_4 more efficiently than Na_2SeO_3 (Figure 1). Na_2SeO_4 is more efficient due to the faster absorption of selenate and translocation from the root to the stem and leaves, in addition to faster transformation into its organic form, while selenite is more easily adsorbed to the soil, which makes it more difficult to be absorbed by the roots (Izydorczyk et al. 2021). Therefore, our studies indicated that the application of Na_2SeO_4 has greater translocation capacity (Figure 4B) and Se accumulation in the shoot of plants (Figure 4A). Whereas plants treated with Na_2SeO_3 more strongly retain Se molecules in plant roots (Figure 4C).

Experiments conducted by Longchamp et al. (2012) demonstrated that maize plants treated with Se sources accumulate more Se in shoots when treated with Na_2SeO_4, and retain more Se in roots when treated with Na_2SeO_3. In addition, ryegrass plants subjected to both Se sources exhibited higher root-to-shoot Se translocation rate and total Se uptake rate after treatment with Na_2SeO_4 compared to Na_2SeO_3 (Versini et al. 2016).

In addition to the higher concentration of the element in the shoot, the translocation rate of Na_2SeO_4 showed a greater capacity to translocate Se from the roots to the shoot. (Hawrylak-Nowak et al. 2015) demonstrated that while selenite retained a high concentration in the roots (967.2 mg kg^{-1} DW) and low in the shoot (120.6 mg kg^{-1} DW), selenate had an approximately 8-fold higher Se translocation factor. The results of Li et al. (2020) using the SR-XRF technique demonstrate that plants exposed to selenite concentrate Se mainly in the epidermis, while plants exposed to selenate concentrate Se in the vascular elements.

According to Oliveira et al. (2019), selenate has greater mobility within the plant, unlike selenite, which is more concentrated in the root system rather than transported to

the shoot, due to its rapid transformation into organic forms of Se. Furthermore, selenite retention is strongly associated with its adsorption on soil components such as iron and aluminum oxides and hydroxides located in the root epidermis and the environment (Lopes et al. 2017). Our experiments under hydroponic conditions also demonstrate the difficulty of the molecule to translocate to the shoot of plants. Furthermore, the lower translocation rate of selenite can also be associated with its different xylem transport pathways, as well as different selenite and selenate transporters, indicating that there is notable interspecific variation in the absorption and translocation of organic forms of Se in the plants (Li et al. 2020).

Selenate has a high chemical similarity with S, and therefore it is transported to plants through sulfate transporters (SULTRs) (Gupta and Gupta 2017). This fact is supported by studies that using the same concentration of the present work (40 µM of Na_2SeO_4) in broccoli demonstrated that there was an expression of the S transporter (SULTR 1; 1). And an increase in the activity of the enzymes ATP-sulfurylase and APS reductase, which are two key enzymes in the S/Se assimilation pathway (Tian et al. 2017). Furthermore, the application of 10 µM Na_2SeO_4 increased the transcription of SULTR1; 1, SULTR1; 3 and SULTR4; 1 in wheat roots (Boldrin et al. 2016). This transporter is expressed in xylem and phloem parenchyma cells of leaves and xylem parenchyma and cells of the pericycle of roots in Arabidopsis thaliana and mediates the movement of S in the vascular bundle, being a strong indication for the high translocation of Na_2SeO_4.

On the other hand, selenite is absorbed by phosphate and aquaporin transporters (OsNIP2;1) (Zhao et al. 2010). Selenite has similar characteristics to phosphate, corroborating its easy adsorption to soil surfaces compared to selenate. Furthermore, selenite is also shown to be converted to organic forms more easily than selenate. Another analysis performed by (Li et al. 2020) using Se speciation by SR-XANES revealed that garlic plants treated with Na_2SeO_3 have only 17% of selenite in the root, 12% in the bulb, and 8% in the leaf, with the remainder of the Se forms converted into various organic compounds such as SeMet, SeOMet, and MeSeCys. In this study, the concentration of Se in the roots of plants treated with selenite corresponded to 51% of MeSeCys, and 32% of SeCys.

The problem related to the conversion of $Na2SeO3$ into organic forms is that it turns into molecules with greater capacity to be volatilized by plants. According to the Se metabolism in plants, SeO_4 is first converted to SeO_3 through the action of ATP sulfurylase and APS reductase. Subsequently, SeO_3 is converted to SeO_2 by sulfite reductase. SeO_2 eventually couples to O-acetylserine through the action of cysteine synthetase to form SeCys. SeCys can be methylated to methyl-SeCys through the action of selenocysteine methyltransferase or to elemental selenium via SeCys lyase. Or converted by a series of enzymes to selenomethionine. This molecule can then be methylated and volatilized to a non-toxic form in the atmosphere (Adebayo et al. 2020).

Na_2SeO_4 is better absorbed even by genotypes with low Se assimilation capacity. Our previous studies (chapter 3) demonstrated that the BRS Xiquexique genotype has greater Se assimilation and translocation capacity than the BRS Urubuquara genotype, which retains more Se in the roots. Wang et al. (2021) evaluated low (1 mg kg^{-1}) and high (10 mg kg^{-1}) doses of the two inorganic sources of Se in eight wheat cultivars, and found that even cultivars with lower Se assimilation and translocation capacities still presented more significant results for the application of Na_2SeO_4 compared to Na_2SeO_3.

Another notable fact is that there is a synergism between the application of Se sources and the elements K (Figure 2A and B) and Ca (Figure 2C and D). Several reports demonstrate that Se can osmotically regulate cells, accumulating substances capable of stabilizing cell metabolism. Lanza et al. (2021) demonstrated that plants with high levels of lipid peroxidation accumulate Ca in their lesions. Furthermore, it was detected for *N. tabacum* that the application of Na_2SeO_4 had the greatest potential to increase the content of osmotic regulatory substances (Na^+, K^+, Ca^{2+}, Mg^{2+}) to protect the cell structure and regulate the ionic balance of leaves under water stress (Han et al. 2022). Hasanuzzaman et al. (2020) report that Se helps to increase K^+ accumulation in plants and also mediates the increase in chlorophyll a, chlorophyll b, and total chlorophyll content by 65%, 39%, and 56%, respectively, as Se absorption is linked to increased absorption of Mg and Fe.

Furthermore, there is antagonism between S and Mn (Figure 2E and F). Se disrupts plant uptake of Mn, which is necessary for the biosynthesis of molybdopterin, an essential cofactor for nitrate reductase activity and nitrate N assimilation (Schiavon et al. 2017). This fact may be related to the reduction in N metabolism, evidenced in the present

study. Nitrogen metabolism was shown to be strongly influenced by the application of Se sources. Genotypes that absorb more Se (BRS xiquexique) reduced the concentration of nitrate (Figure 8B) and amino acids (Figure 8C) of the leaves. While genotypes with lower Se absorption capacity (BRS Urubuquara) increased the nitrate content.

Se can influence N metabolism by activating enzymes that participate in nitrate reduction (NO_3^-) and ammonia assimilation (NH_4^+) (Bian et al. 2020). The application of Se can activate the enzymes nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT) due to the relationship between the metabolism of nitrogen (N) and sulfur (S) that results in greater production of amino acids, proteins, and availability of organic N (Li et al. 2021).

The application of Na_2SeO_4 was efficient to increase the chlorophyll content (Figure 7A, B, and C) of the plants. And treatment with Na_2SeO_3 was effective in increasing pheophytins (Figure 7D, E, and F) and carotenoids (Figure 7G) of the BRS Xiquexique genotype. The same phenomenon was seen by Silva et al. (2020), suggesting that the application of Se promotes a rearrangement of the antenna complex to improve energy capture and protect the system against oxidative stress. Photosynthetic pigments such as carotenoids and pheophytins can be considered the "sunscreens" of plant cells, acting in cellular protection against membrane degradation by ROS (Lanza et al. 2021)

According to Feng et al. (2013) the benefits of Se in photosynthesis may be related to its relationship with the Fe-S complex in chloroplasts, which plays a vital role in the electron transport chain, causing high excitations of electronic levels to have enough substrates to remain organized. This process helps to reduce the generation of ROS, mainly $O2^{\cdot-}$ and H_2O_2, in plants subjected to stress. Furthermore, Li et al. (2018) reported that Se increased photosynthesis by increasing photosynthesis rate, stomatal conductance, and transpiration efficiency.

Gas exchange measurements provided data to assess the photosynthetic physiology of plants, and its relationship to the uptake of Se sources by genotypes (Figure 3). The genotype with the highest Se absorption capacity (BRS Xiquexique) had the highest gas exchange rates in all evaluated variables. The increase in A, E, GS, and Ci has been seen in *Brassica juncea* under 10 times less application (4 µM) of Na_2SeO_4 compared to the concentration used in the present work (40 µM) (Handa et al. 2019).

Increased photosynthesis and concentration of photosynthetic pigments in Se-treated plants are associated with stress conditions (Silva et al. 2018). In mayze plants under salt stress, the addition of only one µM of Na_2SeO_3 increased plant chlorophyll and carotenoid concentration, net photosynthetic rate, and stomatal conductance (Jiang et al. 2017).

Overproduction of reactive oxygen species (ROS) by higher plants can increase lipid peroxidation of cell membranes (Silva et al. 2018). According to Lanza and Reis (2021) ROS production in chloroplasts can vary from low levels (<240 µM s^{-1} $O_2^{\cdot -}$ and 0.5 µM of H_2O_2 to levels under high-stress concentrations (240–720 µM s^{-1} $O_2^{\cdot -}$ and 5–15 µM H_2O_2). Our results show that the H_2O_2 values found (Figure 5A) were lower than the levels considered as low, which can be associated with the age of the plants, but which demonstrates that the applied dose of Se did not reach the levels recognized as high stress. Furthermore, plants treated with Se sources had lower rates of malondialdehyde (MDA), which is an indicator parameter of lipid peroxidation in plant cells (Figure 5B).

The reduction in MDA levels can be associated with the action of antioxidant enzymes. Enzyme antioxidant metabolism was stimulated by the application of both Se sources. The application of Na_2SeO_4 increased the activities of SOD (Figure 6A), CAT (Figure 6B), and APX (Figure 6C) for the BRS Xiquexique genotype, which showed the highest capacity to absorb Se. According to Silva et al. (2018) SOD is the first line of action enzyme against ROS, acting in the dismutation of singlet oxygen into H_2O_2, which is later transformed into H_2O by the action of the enzymes CAT and APX ($O2^{\cdot -} \rightarrow H_2O_2 \rightarrow H_2O$). However, the affinity of CAT with H_2O_2 is weaker compared to other antioxidant enzymes (Gouveia et al. 2020).

Furthermore, even about non-enzymatic antioxidant metabolism, the application of Se sources reduced the accumulation of sucrose in the leaves (Figure 8A), which is indicative of oxidative stress. Sugars are used as biomarkers of ROS, and in ideal doses, they work to combat oxidative stress, mainly via the pentose phosphate (PPP) pathway (Lanza and Reis 2021).

Several studies demonstrate that the application of Se can reduce lipid peroxidation through the induction of enzymatic and non-enzymatic antioxidant metabolism, seen in wheat (Jaiswal et al. 2018), rice (Yildiztugay et al. 2017), Brassica (Zhang et al. 2020), and *Zea Mays* (Jiang et al. 2017). According to (Chauhan et al. 2019),

o Se can mitigate oxidative stress by regulating photosynthetic complexes, stimulating $O2^{\cdot -}$ to H_2O_2 dismutation, regulating enzymatic and non-enzymatic antioxidant systems, and by direct extinction of ROS through Se species.

5. CONCLUSION

The results show that i) the application of one µM of Na_2SeO_4 was more efficient in assimilation, translocation, and concentration of Se in shoots compared to the performance of plants treated with Na_2SeO_3. Thus, the use of selenate proves to be a source with a better use for use in mineral supplementation of crops, and the objective of these programs is to increase the Se content in the edible parts of plants. Se sources that are trapped in plant roots can be an inefficient and cost-effective way of crop nutrition. Furthermore, it was found that ii) plants treated with selenate increase the levels of Chlorophylls, Gs, E, Ca, and K, demonstrating a strong correlation with stomatal opening, osmotic adjustment, and greater uptake of solar energy, which is one of the indispensable factors for increased productivity. On the other hand, plants supplemented with selenite tend to modulate their metabolism to increase accessory pigments (pheophytin and carotenoids), which are associated with protecting plants against intracellular damage. Finally, the use of iii) genotypes with high (BRS Xiquexique) and low (BRS Urubuquara) ability to assimilate Se demonstrated that the two Se sources have the same behavior about the evaluation of the absorption efficiency of the element. And that the union between more responsive genotypes with sources of greater assimilation of Se generates the highest concentration of the element in the shoot of the plant, and consequently, proves to be an effective combination in the nutrition of cowpea with Se.

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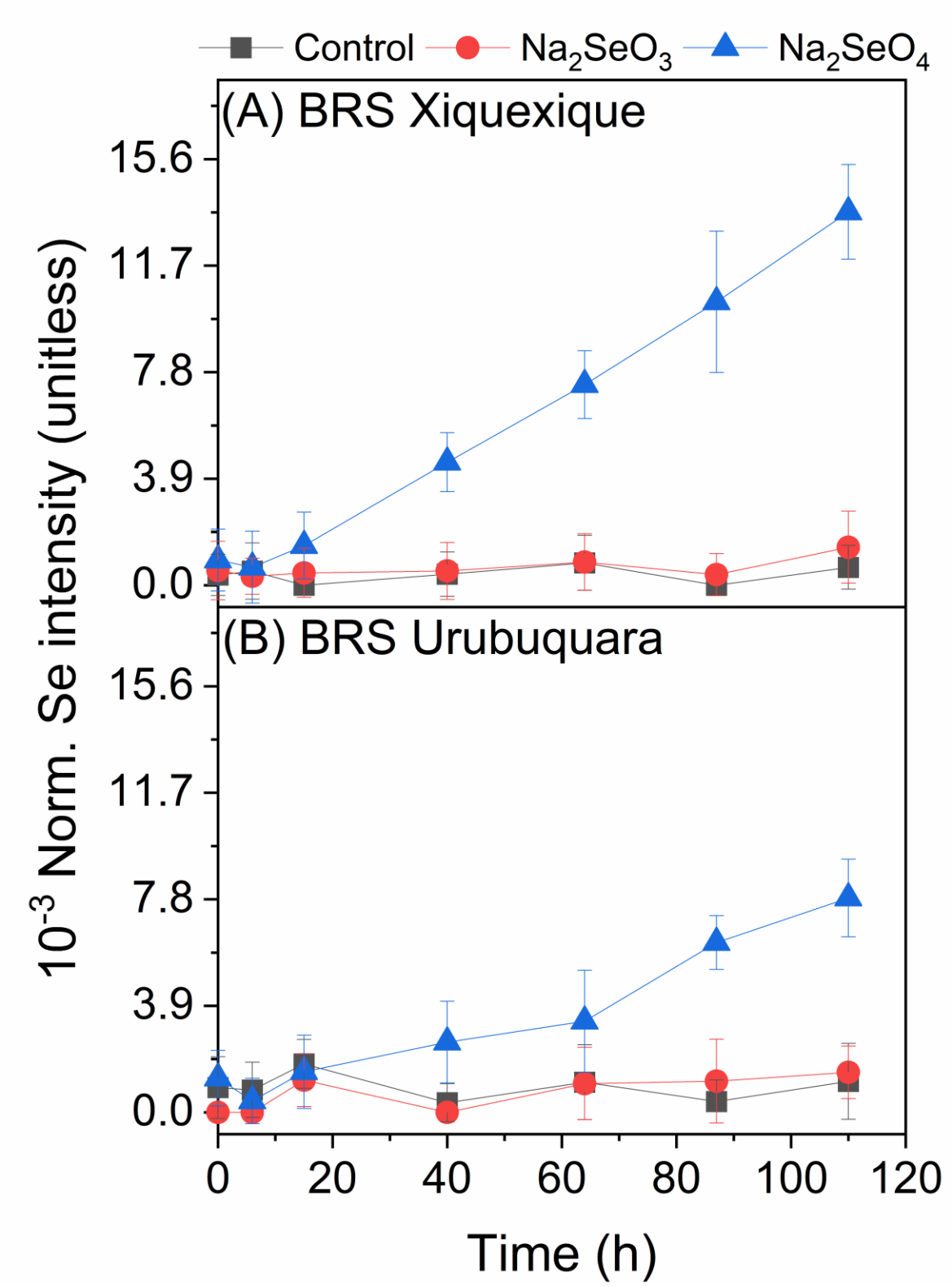


Figure 1. *In vivo* monitoring Se XRF intensities in cowpea plants in response to application of 40 µM of sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3).

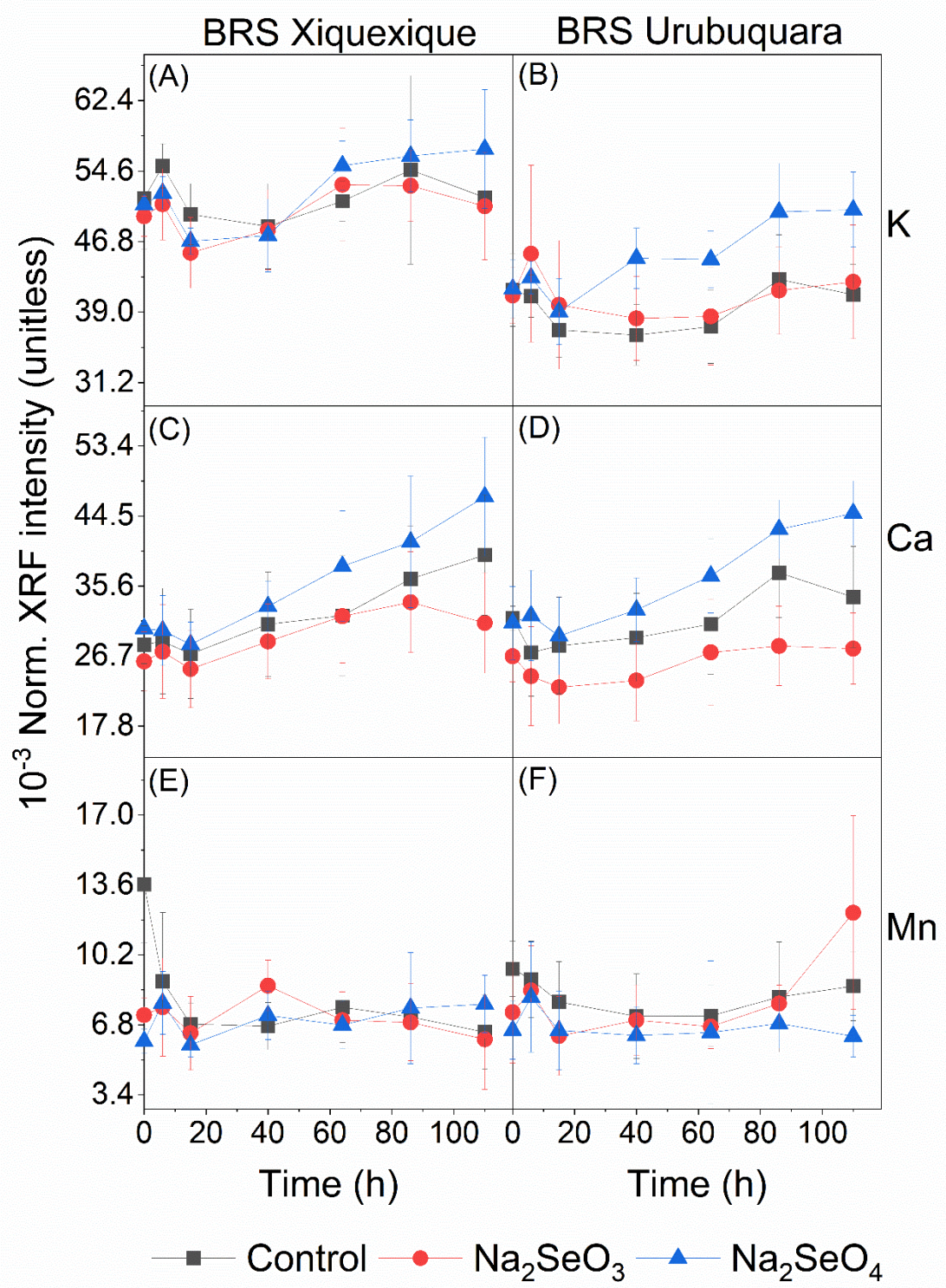


Figure 2. *In vivo* monitoring, K, Ca, and Mn XRF intensities in cowpea plants in response to application of 40 µM of sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3).

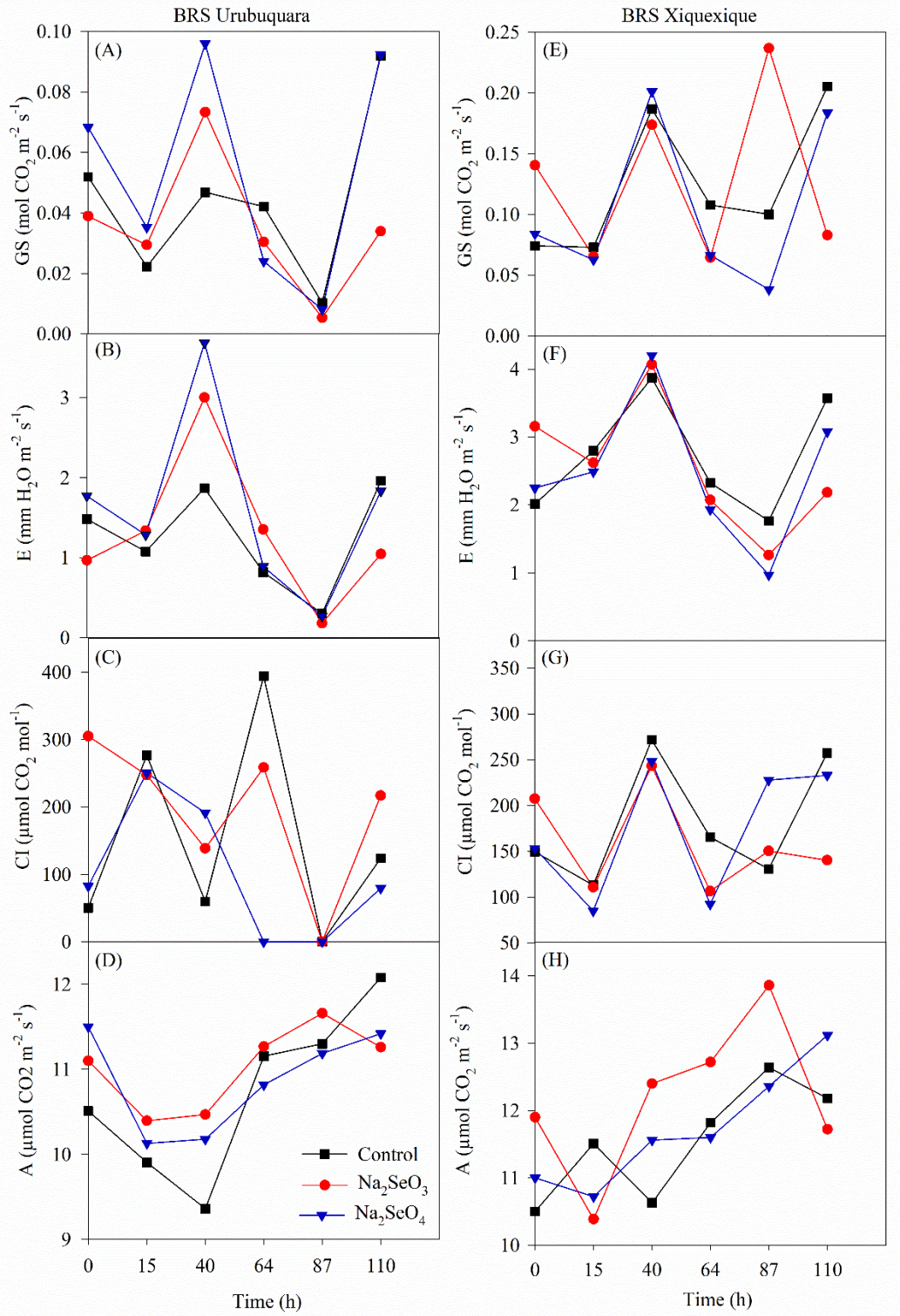


Figure 3. *In vivo* monitoring gas exchange measurements in cowpea plants in response to application of 40 µM of sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3).

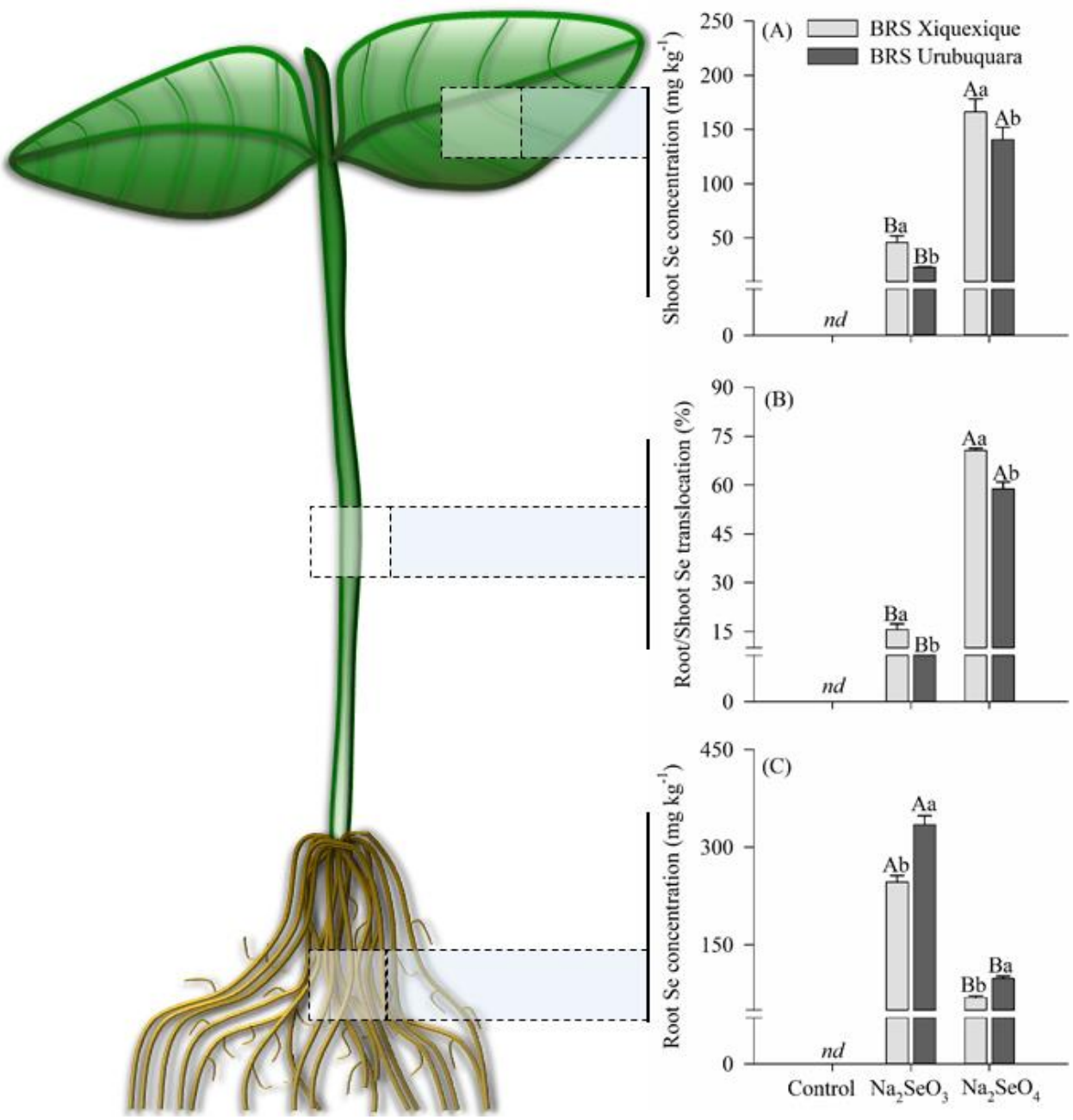


Figure 4. Root to shoot selenium translocation index. The difference between means and groups according to the Tukey test ($P \leq 0.05$). The standard error of the mean ($n = 4$) is indicated by error bars. Uppercase letters indicate selenium sources (Na_2SeO_3 and Na_2SeO_4), and Lowercase letters indicate genotypes (BRS Xiquexique and BRS Urubuquara). All The controls are lower de detection limit for Selenium at benchtop equipment.

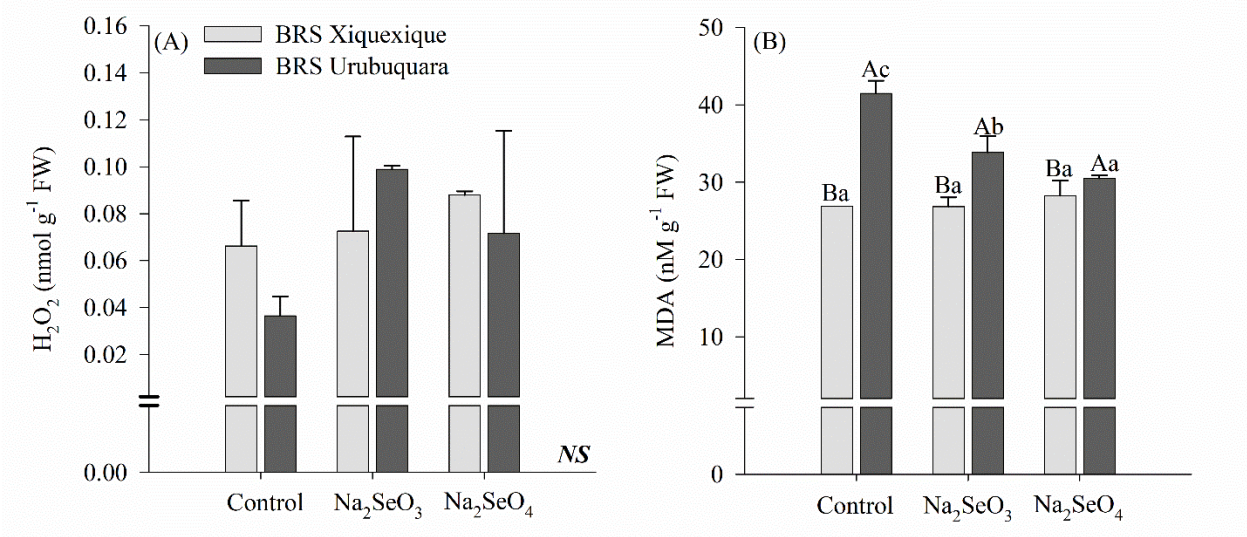


Figure 5. Compounds of lipidic peroxidation (H_2O_2 and MDA). The difference between means and groups according to the Tukey test ($P \leq 0.05$). The standard error of the mean ($n = 4$) is indicated by error bars. Uppercase letters indicate selenium sources (Na_2SeO_3 and Na_2SeO_4), and Lowercase letters indicate genotypes (BRS Xiquexique and BRS Urubuquara). All The controls are lower de detection limit for Selenium at benchtop equipment. NS = not significative.

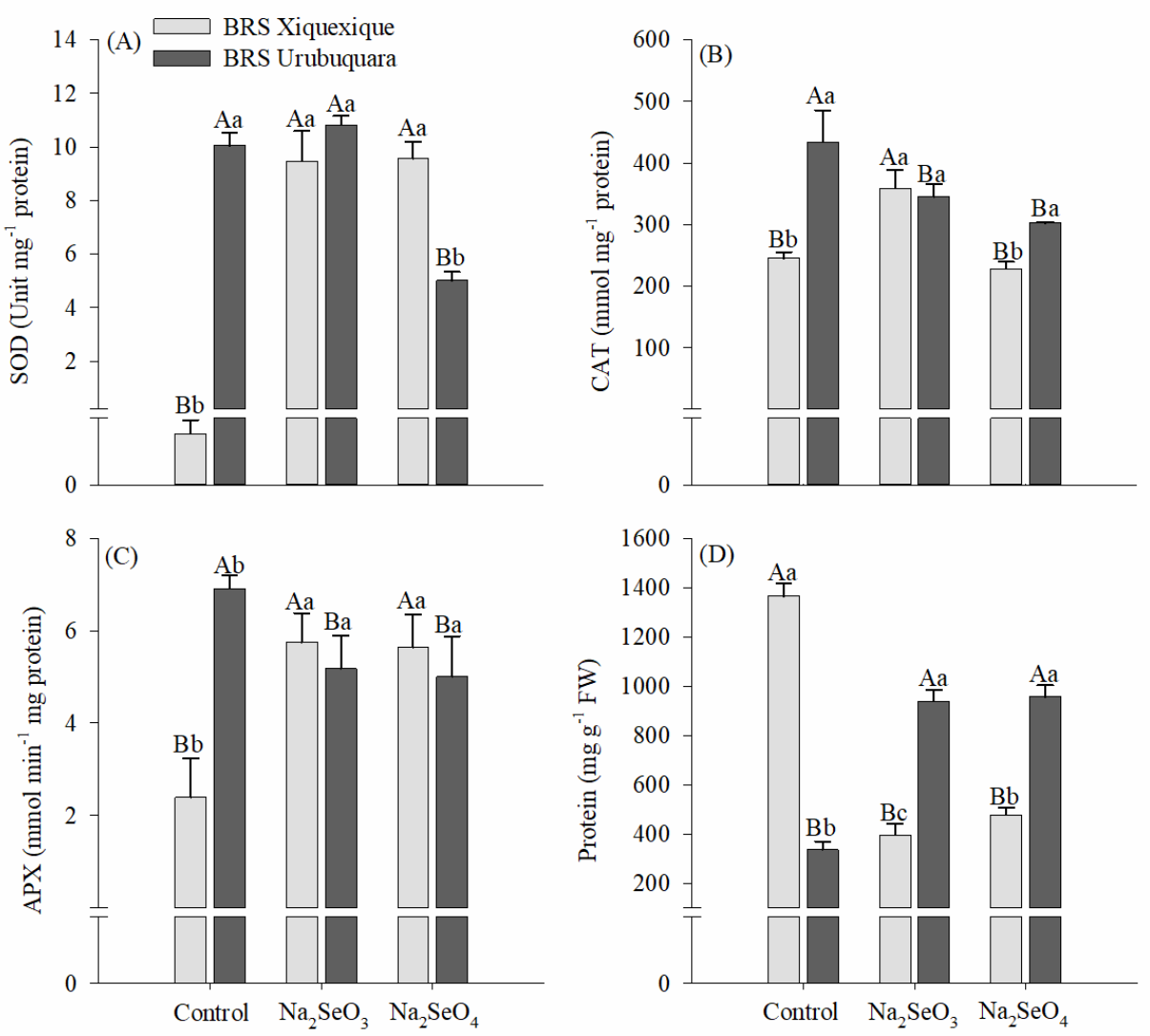


Figure 6. Antioxidant enzymes. The difference between means and groups according to the Tukey test ($P \leq 0.05$). The standard error of the mean ($n = 4$) is indicated by error bars. Uppercase letters indicate selenium sources (Na_2SeO_3 and Na_2SeO_4), and Lowercase letters indicate genotypes (BRS Xiquexique and BRS Urubuquara). All The controls are lower de detection limit for Selenium at benchtop equipment.

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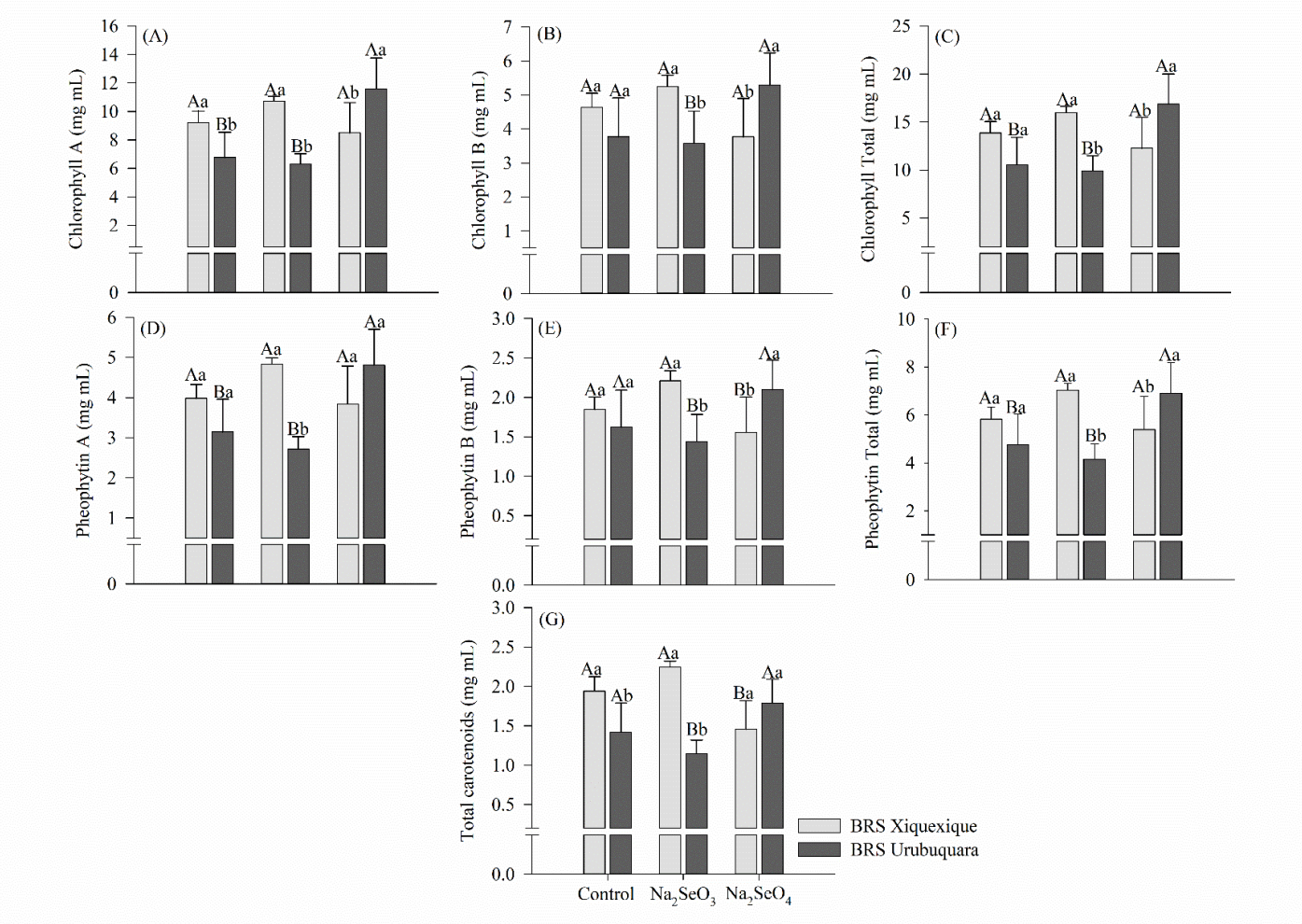


Figure 7. Photosynthetic pigments. The difference between means and groups according to the Tukey test ($P \leq 0.05$). The standard error of the mean (n = 4) is indicated by error bars. Uppercase letters indicate selenium sources (Na_2SeO_3 and Na_2SeO_4), and Lowercase letters indicate genotypes (BRS Xiquexique and BRS Urubuquara). All The controls are lower de detection limit for Selenium at benchtop equipment.

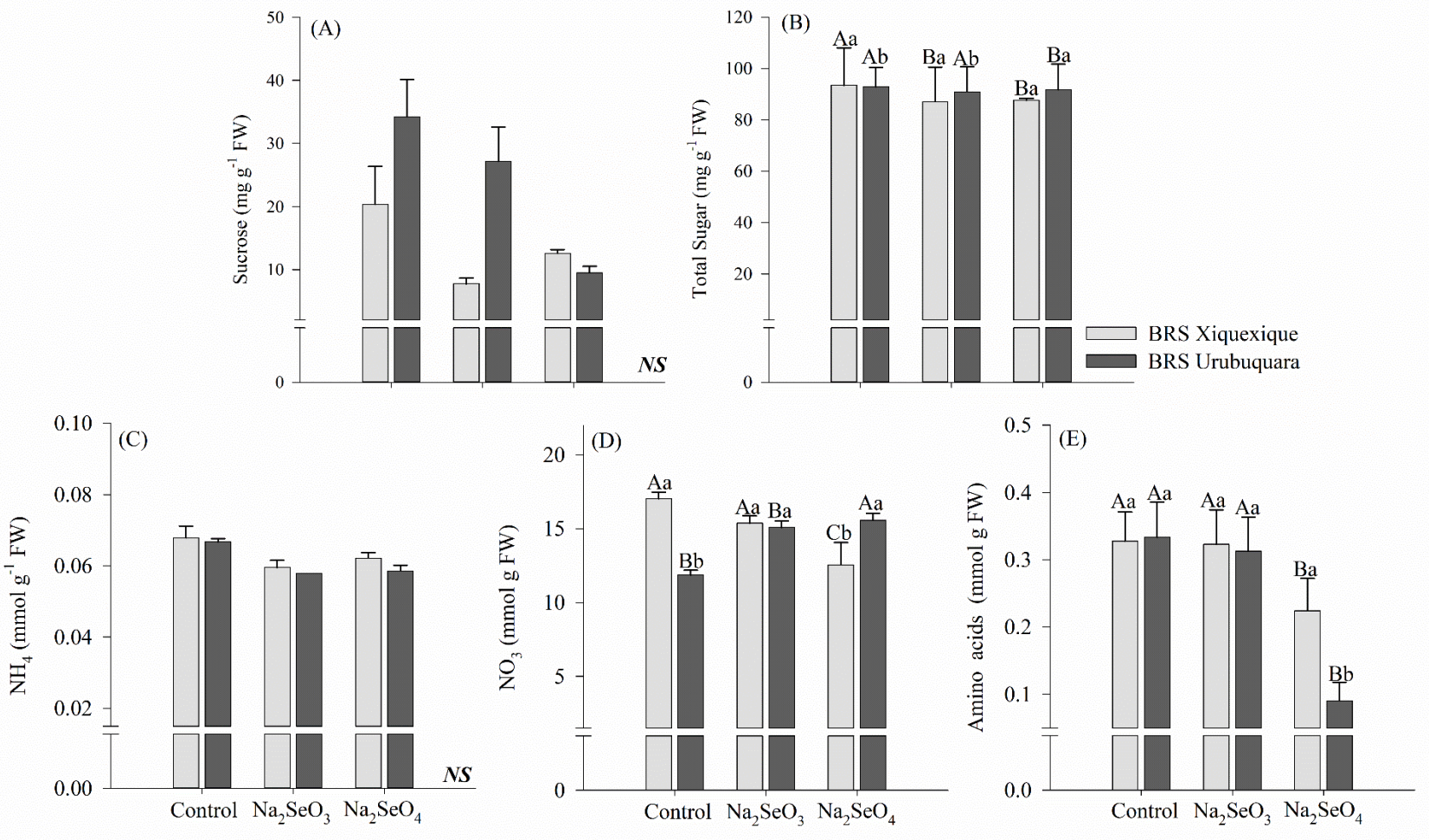


Figure 8. Sugars and nitrogen metabolism. The difference between means and groups according to the Tukey test ($P \leq 0.05$). The standard error of the mean (n = 4) is indicated by error bars. Uppercase letters indicate selenium sources (Na_2SeO_3 and Na_2SeO_4), and Lowercase letters indicate genotypes (BRS Xiquexique and BRS Urubuquara). NS = not significative.