**GUSTAVO FERREIRA DE SOUSA**

# SELENIUM SUPPLY IN COFFEE PLANTS AND ITS EFFECTS ON CHILLING AND DROUGHT STRESS TOLERANCE

**LAVRAS – MG**
**2023**

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Thesis presented to the Federal University of Lavras, as part of the requirements of the Graduate Program in Soil Science area of concentration in Soil Fertility and Plant Nutrition, to obtain the title of Doctor.

Professor Ph.D. Luiz Roberto Guimarães Guilherme
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**GUSTAVO FERREIRA DE SOUSA**

# SELENIUM SUPPLY IN COFFEE PLANTS AND ITS EFFECTS ON CHILLING AND DROUGHT STRESS TOLERANCE

# SUPRIMENTO DE SELÊNIO EM PLANTAS DE CAFÉ E SEUS EFEITOS NA TOLERÂNCIA A ESTRESSE POR BAIXA TEMPERATURA E HÍDRICO

Thesis presented to the Federal University of Lavras, as part of the requirements of the Graduate Program in Soil Science area of concentration in Soil Fertility and Plant Nutrition, to obtain the title of Doctor.

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**LAVRAS – MG**
**2023**

*To the lord of everything, God*
*To my parents, Vicente, and Márcia.*
*To my lovely fiancé, Maila*
*To my siblings, Kamilla and Mateus.*
*I dedicate!*

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*“In the end, everything is about people.”*

*Phill Knight - “Shoe dog”*

## RESUMO GERAL

Estresses abióticos são definidos como qualquer modificação dos recursos do meio que altere as condições ótimas para o desenvolvimento das culturas, de modo que elas não atinjam seu máximo potencial produtivo. A cultura do café está entre aquelas que são altamente afetadas pelos estresses abióticos, enfatizando-se que condições de baixa temperatura e falta de água são as principais causas de redução de produtividade nas lavouras brasileiras. Nos últimos anos, a incidência destes eventos tem aumentado nas regiões produtoras no Brasil, podendo inclusive acarretar mudança do zoneamento climático para a cultura. O manejo adequado da nutrição vegetal tem se mostrado eficaz na mitigação dos efeitos negativos promovidos por tais adversidades, e o uso de selênio (Se) tem se destacado no combate a tais condições adversas. Alguns estudos têm relacionado a aplicação de Se com melhorias do sistema antioxidante, relações hídricas e metabolismo de açúcares em plantas, porém, a resposta de sua aplicação na cultura do café ainda permanece pouco explorada na literatura. Esta tese teve como objetivo avaliar os efeitos da aplicação de Se nas respostas metabólicas de plantas de café submetidas aos estresses ocasionados pela baixa temperatura e falta de água, bem como fornecer informações que contribuam para o manejo eficiente da nutrição de Se nestas condições. No primeiro experimento, mudas de duas espécies de café (*Coffea arabica* cv. Arara e *Coffea canephora* clone 31) previamente supridas com Se via foliar foram submetidas à baixa temperatura (10°C dia/4°C noite), com posterior aumento (25°C dia/20°C noite), em condições de ambiente controlado. Verificou-se que as espécies avaliadas possuem respostas distintas à baixa temperatura e também à aplicação de Se, principalmente durante o retorno às condições ideais de temperatura. Ao mesmo tempo, a aplicação de Se aumentou o teor de açúcares e prolina nas folhas após o período de estresse, conferindo maior potencial de superação às condições adversas. No segundo experimento, avaliou-se a melhor época de aplicação de Se em plantas de café (*Coffea arabica* cv. Catuai) submetidas ao estresse osmótico induzido por polietileno glicol 6000. A aplicação de Se previamente ao período de estresse resultou em maior atividade de enzimas do sistema antioxidante e melhorou as relações hídricas nas folhas quando comparadas aos tratamentos sem aplicação de Se. Apesar disto, a aplicação de Se após o período de estresse atuou como um agente estressor, reduzindo o potencial hídrico das plantas. De modo geral, a aplicação foliar de Se promoveu a ativação de respostas metabólicas em plantas de café como forma de mitigação de estresses abióticos. Assim, os resultados obtidos a partir desta tese poderão dar suporte para a introdução de novas tecnologias de manejo nutricional que visam elevar a tolerância aos estresses abióticos que acometem a cultura do cafeeiro.



**Palavras-chave:** Nutrição de plantas. Elementos benéficos. Aquecimento global. Café.

## GENERAL ABSTRACT

Environmental stress refers to any change in environmental resources needed for optimal plant development, preventing them from reaching their maximum production capacity. The coffee crop is overly sensitive to stress, with low temperatures and drought being the main constraints on its global production. There has been an increase in extreme natural events in Brazilian coffee-growing areas over the past few decades, which raises concerns about coffee production in Brazil. The use of plant nutrition has been proven effective in mitigating the negative effects of these adversities, with selenium (Se) being highlighted as a valuable tool in combating such adverse conditions. Previous studies have demonstrated that Se supply leads to a more effective antioxidant system, improved water relations, and modulated carbohydrate production and breakdown in plants. However, the impact of Se application on the coffee crop has not been thoroughly addressed in the literature. The objective of this thesis was to explore whether foliar application of Se to coffee plants under low temperatures and drought stress can alleviate the negative effects and contribute to more efficient plant nutrition strategies under such challenging conditions. The first trial evaluated the plant responses of two coffee species (*Coffea arabica* cv. Arara and *Coffea canephora* clone 31) to low temperatures (10°C day/4°C night) and during the rewarming period temperatures (25°C day/20°C night). Notable variations in plant responses were observed among species, with *Coffea canephora* being more sensitive to low temperatures. Plant responses were more pronounced during the rewarming period. Selenium application increased carbohydrate and proline contents in the leaves after stress, enhancing the plant's ability to overcome the stress. In the second trial, the optimal timing for Se application in *Coffea arabica* cv. Catuai plants under osmotic stress induced by PEG-6000 was assessed. The results showed that pre-application of foliar Se promoted higher activities of antioxidant enzymes and improved water relations in the leaves compared to the control. However, Se application after the osmotic stress appeared to induce additional stress in the plants, resulting in a reduction of leaf water potential. Overall, Se application stimulated metabolic responses to tackle abiotic stress in coffee plants, and the findings of this thesis may provide support for nutritional management techniques to mitigate the negative effects of stresses on coffee trees.



**Keywords:** Plant nutrition. Beneficial elements. Global warming. Coffee.

**SUMMARY**

# FIRST PART

## 1 GENERAL INTRODUCTION

Coffee is a plant from the *Coffea* genus and there are more than 100 species worldwide. *Coffea arabica* and *Coffea canephora* are the most representative among them (FERNANDES et al., 2012). Although both coffee species are widely cultivated, they have different requirements for altitude, rainfall, fertilization, and pest and pathogen resistance. The traits related to coffee beverage quality are another contrast between them (LIMA FILHO et al., 2015).

Brazil has been the largest coffee producer in the world, and USDA estimates the marketing year 2022/23 (July-June) total Brazilian coffee production at 62.4 million bags (60-kg bags), followed by Vietnam, with 30.2 million bags, and Colombia, with 12.6 million bags (USDA, 2022). *Coffea arabica* beans are expected to account for 63 percent of all coffee beans production in Brazil. Meanwhile, Minas Gerais state is the highest producer in Brazil and accounts for over 43 percent of Brazil's coffee production.

The centers of origin of coffee are in areas with specific features of forest regions. As a result, the need for abiotic resources (*e.g.*, temperature, luminosity, soil fertility, and water demand) can be considered as restrictive characteristics of its production in not prone areas (AMARASINGHE et al., 2015; CHEMURA; MUTANGA; DUBE, 2017). Two of the most restrictive natural resources to coffee production are water and temperature availability (MANTRI et al., 2012) and they can contribute to defining the coffee production zone (CAMARGO, 2010).

Climate zoning is a tool that assists in agricultural management by providing information on the positioning of crops according to the most suitable climatic conditions for growing a particular crop (MCCARL; THAYER; JONES, 2016). Due to constant climate change and global warming, areas suitable for its cultivation increasingly present production risks (MELKE; FETENE, 2014). Thus, studies have been carried out to mitigate the negative effects of stresses caused by changing the environmental conditions of coffee cultivation.

*Coffea arabica* is originated in Ethiopia, growing into tropical forests where the rainfall is well distributed. For this species, the optimal growth temperature ranges from 18°C during the day and 22°C during the night, with at least 1400 mm of rainfall (precipitation). Due to these environmental conditions' requirements, *C. arabica* is primarily grown in places

close to the equator, with the Brazilian highlands, Central America, and Colombia serving as the primary production zones (DESCROIX; SNOECK, 2009).

*Coffea canephora* is native to forests at lower altitudes surrounding central and western sub-Saharan Africa. The rainfall in this area is around 2000 mm and the optimum rainfall is in between 2000 and 2500 mm (DESCROIX; SNOECK, 2009). Although it is more resistant to higher temperatures than *Coffea arabica* (optimum temperatures being between 22 and 28°C), it is more sensitive to lower temperatures (DAMATTA; RAMALHO, 2006; SOUSA et al., 2022). For the most common coffee genotypes – *C. arabica* and *C. canephora*, the optimal growth latitude ranges from 22° and 28° of latitude.

Plants cultivated under sub-optimal natural resources experience abiotic stress. Abiotic stress is defined as any adverse abiotic environmental conditions that trigger metabolic changes and can decrease the crop yield (KRASENSKY; JONAK, 2012; SAIBI; BRINI, 2018). Some authors consider that the more generalized and conserved cellular defense responses to the stress is the desaturation of membrane lipids, activation of reactive species scavengers, induction of the chaperones, and accumulation of compatible solutes (GHOSH et al., 2021; HASANUZZAMAN et al., 2020).

As an alternative to combat stress caused by abiotic agents, plants can be "conditioned" so that they develop tolerance mechanisms to the effects of stress (SAVVIDES et al., 2016). This induction of stress tolerance is called the "priming effect". The priming effect has been reported as a tool that helps combat adversities caused by environmental stress. It can be performed to promote physiological responses that will tackle imminent environmental stresses more quickly or more effectively (HESSINI et al., 2013; SAVVIDES et al., 2016). As an example of the priming effect, the application of nutrients or beneficial elements that promote greater antioxidant activity in plants can be cited (SHAHVERDI; OMIDI; TABATABAEI, 2017).

Selenium (Se) is an element that has shown great importance in reducing plant stress (AHMAD, Rashid et al., 2016; ANDRADE et al., 2018; NAWAZ et al., 2015). Selenium has been reported to regulate water use and promote the activity of enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and ascorbate peroxidase (APX) (LARA et al., 2019; RAMOS et al., 2010). These enzymes play an important role in the antioxidant system of plants, reducing the deleterious effects caused by environmental stresses and promoting better plant growth under adverse conditions (NATASHA et al., 2017).

Se has great chemical similarities with sulfur (S) and shares a similar route for absorption and translocation in plants. For example, selenite ($SeO_3^{2-}$) e selenate ($SeO_4^{2-}$) are analogues of sulfite ($SeO_3^{2-}$) and sulfate ($SeO_4^{2-}$), respectively (HASANUZZAMAN et al., 2020). Due to its similarity to S, Se can partially replace it in the composition of amino acids, forming seleno-amino acids, which will consequently form seleno-proteins. Some examples are Se-cysteine and Se-methionine (PILON-SMITS; WINKEL; LIN, 2017). Although the substitution of S for Se can occur, most of the symptoms of Se toxicity are related to S deficiency, so that there is a close relationship of synergism and antagonism between the two elements.

According to Jóźwiak and Politycka (2019), the application of Se in low concentrations increases the antioxidant activity of plants and hence, reduces ROS in plant tissue. Despite this, the authors found that in high quantities, the concentration of ROS can increase, mainly $O2•^{-}$, $H_2O_2$, and •OH. It is explained because both elements use the S route to be absorbed, so there must be a nutritional balance between them. Boldrin et al. (2016) state that the application of low doses of Se can promote greater absorption of S. The authors attribute this result to the induction of the expression of the SULTR1;1, SULTR1;3 and SULTR4;1 genes by the application of Se, which stimulates the absorption of both elements.

Selenium acts directly on the $H_2O_2$ detoxification route through the action of GPX, which acts on the oxidation of GSH. According to Banerjee and Roychoudhury (2019), an S-cysteine molecule is associated with the glutamate molecule and catalyzed by glutamylcysteine synthetase to form GSH. Therefore, the greater formation of Se-cysteine favors the formation of Se-GSH, which becomes the main route for Se to act in combating oxidative stress (NATASHA et al., 2017).

Selenium can be applied via soil or as a foliar spray. When applied via soil, it is subject to precipitation losses and adsorption in soil colloids, and this can occur in oxidic soils (LESSA, J. H. L. et al., 2016; SILVA, M. A. et al., 2022). One way to improve the use of Se is the application via foliar spraying. Foliar application allows less contact of Se with the soil, thus reducing problems with adsorption (NAWAZ et al., 2015). After foliar application, Se enters the plant through the leaves and is metabolized in plastids through the sulfur assimilation route, forming seleno-amino acids, such as Se-cysteine (SeCys) and Se-methionine (Se-Met) (SCHIAVON et al., 2017).

In a work carried out by Mateus et al. (2021), the authors observed a positive effect of Se application on the metabolism of Arabica coffee plants. In this work, there was an increase in photosynthetic pigments and enzymatic activity, reduction of lipid peroxidation, and reactive oxygen species (ROS). The authors also observed an increase in yield of coffee beans by up to 38%. It can be attributed to the improvement of the enzymatic system. Although the physiological responses of Se application in plants are observed in the literature, aspects concerning the establishment of Se application strategies, as well as its interaction with other antioxidant compounds are little explored in the context of abiotic stresses in coffee crops.

## 2 JUSTIFICATION

The seasonal rainfall patterns and the high occurrence of cold waves in areas not typically prone to these events have put the climatic zone for coffee production in question. Consequently, our team chose to investigate selenium supply strategies in coffee plants to help them overcome the negative effects of these abiotic stresses. With that, we hope this thesis can provide valuable insights into selenium pathways that trigger metabolic responses and highlight strategies to manage selenium in coffee plants to mitigate abiotic stresses.

## 3 GENERAL AIM

- Assessing the Se application via foliar for improving low temperature and drought stress in coffee seedlings;

# 4 SPECIFIC AIMS

- Assessing the application Se for reducing the leaf injures in *Coffea arabica* and *Coffea canephora* under induces low temperature stress;
- Assessing the changes in carbohydrate, enzymatic activity, and nutrient content in coffee leaves under low temperature and drought stress;
- Assessing the role of the Se supply in improving osmotic tolerance in coffee seedlings
- Assessing the best time for Se application in coffee under induced osmotic stress;

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**SECOND PART – ARTICLES**

## MANUSCRIPT 1: Selenium enhances chilling stress tolerance in coffee species by modulating nutrient, carbohydrates, and amino acids content

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**Abstract:** The effects of selenium (Se) on plant metabolism have been reported in several studies triggering plant tolerance to abiotic stresses, yet the effects of Se on coffee plants under chilling stress are unclear. This study aimed to evaluate the effects of foliar Se application on coffee seedlings submitted to chilling stress and subsequent plant recovery. Two Coffea species, *C. arabica* cv. Arara and *C. canephora* clone 31, were submitted to foliar application of sodium selenate solution (0.4 mg plant$^{-1}$) or a control foliar solution, then on day 2 plants were submitted to low-temperature (10°C day/4°C night) for two days. After that, the temperature was restored to optimal (25°C day/20°C night) for two days. Leaf samples were collected three times (before, during, and after the chilling stress) to perform analyses. After the chilling stress, visual leaf injury was observed in both species, however, the damage was 2-fold higher in *C. canephora*. The lower effect of cold on *C. arabica* was correlated to the increase in ascorbate peroxidase and higher content of starch, sucrose, and total soluble sugars compared with *C. canephora*, as well as a reduction in reducing sugars

and proline content during the stress and rewarming. Selenium increased the nitrogen and sulfur content before stress but reduced their content during low-temperature. The reduced content of nitrogen and sulfur during stress indicates that they were remobilized to stem and roots. Selenium supply reduced the damage in *C. canephora* leaves by 24% compared with the control. However, there was no evidence of the Se effects on antioxidant enzymatic pathways or ROS activity during stress as previously reported in the literature. Se increased the content of catalase during the rewarming. Se foliar supply also increased starch, amino acids, and proline, which may have reduced symptom expression in *C. canephora* in response to low temperature. In conclusion, Se foliar application can be used as a strategy to improve coffee tolerance under low-temperature changing nutrient remobilization, carbohydrate metabolism, and catalase activity in response to rewarming stress, but *C. arabica* and *C. canephora* respond differently to chilling stress and Se supply.

**Keywords:** environmental changes, beneficial elements, abiotic stress, low temperature, tropical agriculture, plant nutrition, coffee belt

## 1 Introduction

Coffee is one of the most important commodities worldwide with a significant economic impact on over 25 million, mostly smallholder farmers in more than 60 countries throughout the tropics (Jayakumar et al., 2017). Coffee plants are highly sensitive to the growing environment and are generally restricted to the 'Coffee Belt' - between 25 degrees North and 30 degrees South with an average temperature between 18 and 22°C for *C. arabica* and 22 and 28°C for *C. canephora* (Bliss, 2017; Descroix and Snoeck, 2014; DaMatta and Ramalho, 2006; Bunn et al., 2015). Among the 104 Coffea species described (Davis and Rakotonasolo, 2008), the two most economically important species are *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta). These two species are responsible for 99% of the world's green-bean production (Jayakumar et al., 2017).

Changes in the temperature due to climate change might adversely affect coffee plants because each species and genotype requires specific environmental conditions for successful production (Ramalho et al., 2014; Ebisa, 2017). Low-temperature stress may be denominated as i) cold stress - when plants suffer from sub-zero temperatures, and ii) chilling stress – when plants suffer from low but non-freezing temperatures (Graves, 1995). As a result of chilling stress, plants have shown reduced stomatal conductance, changes in the pigment complexes and losses of photochemical efficiency, restricted electron transportation, and changes in carbon metabolism, allocation, and partitioning (Ensminger et al., 2006; Partell et al., 2010; Batista-Santos et al., 2011).

Acclimation to low-temperature is usually initiated by a short-term fluctuation in temperature, which affects metabolic homeostasis and induces a stress response (Ensminger et

al., 2006). A sudden drop in temperature limits the ability of plants to induce protective metabolic responses. Severe frosts in 2021 were experienced in coffee areas in the southeast of Brazil, the highest production region of Brazil, with almost 8 - 10% of the arabica coffee affected, reducing the production in the order of 17% below recent on-year crops (USDA, 2021). Exogenous application of beneficial elements such as selenium (Se) has emerged as a tool to compensate for the negative impacts of many stresses, including chilling (Brown et al., 2021; Zellner et al., 2021).

Although Se is not an essential element for higher plants, it has been shown to increase antioxidant activity (Ekanayake et al., 2015), change carbohydrate metabolism (Lara et al., 2019; Silva et al., 2020), protect chlorophyll and modulate water relations (Zhang et al., 2014). Selenium application has reduced the side effects of abiotic stress in a wide range of staple crops, such as drought in common beans and rice (Andrade et al., 2018; Ravello et al., 2021), heavy metal exposure in wheat (Liu et al., 2021; Hasanuzzaman et al., 2022), and salinity in maize and garlic (Ashraf et al., 2018; Astaneh et al., 2019).

Previous studies resulted in higher coffee yield in response to selenium supply by increasing antioxidant metabolism (Mateus et al., 2021) however, there have been no studies that explore the influence of Se application in coffee species under chilling stress. Here, the effects of Se supply to coffee plants under chilling on plant metabolic responses and plant tolerance were examined.

## 2 Materials and methods

### 2.1 Plant material

The trial was performed using two different coffee species, *C. arabica* cv. Arara and *C. canephora* clone 31, differing in tolerance to low-temperature (DaMatta and Ramalho, 2006). According to the authors, low-temperature tolerance is related to the specie's ability to change its metabolism in order to trigger the adverse condition (e. g. increases the enzymatic activity, quantitative and qualitative changes in the lipid, protects the protein in cells membrane). The plants were provided by the National Institute of Science and Technology of Coffee (INCT Café). Plants with 5-6 pairs of fully expanded leaves were used. They were selected for high health and uniformity and allowed to acclimate under optimal conditions for 14 days in a Conviron® growth chamber (12 h of photoperiod, 60% relative humidity (RH), 260 μmol·m−2·s−1 of light intensity (during day), and optimal temperature

(25°Cday/20°Cnight)). Coffee seedlings were grown on 1 liter of a substrate composed of subsoil + cattle manure at a ratio of 3:1, with 5 g of single superphosphate being added to each kg of the mixture. The irrigation was made dairy with 80mL of deionized water during the optimal temperature and 15mL of deionized water during the chilling temperature.

## 2.2 Experimental design and treatments

The experiment was arranged in a randomized block design and a 2 × 2 factorial scheme with five replicas of seedlings for each treatment, with the experimental unit consisting of three pots totaling 60 pots. The factorial scheme was composed of two species (*C. arabica* cv. Arara and *C. canephora* clone 31), in the absence and presence of Se (0 and 80 mg $L^{-1}$ Se). Samples were collected three times to evaluate the plant responses before, during, and after exposure to chilling stress. Since a great number of leaves needed to be collected at each time of evaluation, each replication was composed of three seedlings. The Se rate used in the trial was based on unpublished data in coffee seedlings and also other crops. The control treatment was here described as the plants of the respective species analyzed before being submitted to the chilling stress.

Fourteen days after transfer to the growth chamber, the plants were transferred to a spray chamber in order to avoid contamination during the foliar treatment application. Thus, the respective Se treatments plants were sprayed manually to drip with 5 mL of a foliar solution of Se (80 mg $L^{-1}$ Se + 0.5 % v/v of mineral oil) and the remaining plants were sprayed with mineral oil solution (0.5 % v/v of mineral oil). Plants were then returned to the growth chamber. The Se source used was sodium selenate ($Na_2SeO_4$ - Sigma Aldrich 98.9%).

The first foliar sampling was performed seven days after the foliar treatment application. All plants were then exposed to chilling temperatures, which were decreased by 5 °C/hour from 25°C to 10°C during the first day. The temperature was set to 4°C during the night and 10°C during the day (12 h of photoperiod, 60% RH, 260 μmol·m−2·s−1 of light intensity). The temperature regime was defined as suboptimal for coffee growing (Ramalho et al., 2003; DaMatta and Ramalho, 2006).

The second foliar sampling was performed two days after low-temperature stress treatment. The temperature was then returned to optimal conditions (25°Cday/20°Cnight), and the third sampling was performed two days later (post-stress).

## 2.3 Assessments

### 2.3.1 Visual damage scale

The visual damage from low-temperature exposure in the leaves was carried out according to Manetti Filho e Caramori (1986). The scale of damage ranged from 1 to 5 – 1) no damage; 2) 0 to 25% of the total leaf area damaged; 3) 25 to 50% of the total leaf area damaged; 4) 50 to 75% of the total leaf area damaged, and 5) representing visual damages from 75 to 100% of the total leaf area. The visual damage scale from low-temperature exposure in the leaves was performed considering the general appearance of all leaves.

### 2.3.2 Sample collection and preparation

Two leaf samples were collected for different groups of analyses. i) The third and fourth fully expanded pairs of leaves from top to bottom of coffee plants were collected and washed three times with distilled water. Then, the samples were dried for 72 hours at 60°C and ground in a Willey mill to obtain the dried leaf tissue. The dried samples were used to quantify the parameters described in items 2.3.3 (total content of selenium, nitrogen, and sulfur), 2.3.7 (carbohydrates, total protein, total free amino acids), and item 2.3.8 (proline). ii) The second fully expanded pair of leaves from top to bottom of coffee leaves were collected two hours after lights-on then immediately snap-frozen in liquid nitrogen, individually macerated in liquid nitrogen, homogenized in a cooled mortar using 100 mg PVPP (antioxidant), and stored at −80°C. The dried tissue was used to perform the analysis of the content of selenium, sulfur, nitrogen, carbohydrates, total protein, total free amino acids, and proline. The frozen tissue was used to quantify the parameters described in items 2.3.4 (antioxidant enzymes) and 2.3.5 (hydrogen peroxide and lipid peroxidation).

The sample collection was repeated in every sample collection (before, during, and after chilling stress). Since that the sample collection is a destructive analysis, one plant of the experimental unit was used in each sample collection.

### 2.3.3 Total content of selenium, sulfur, and nitrogen

The extracts for the quantification of Se and S in leaves were obtained by acid digestion of 0.5 g of the dried sample according to the USEPA 3051A protocol (USEPA,

2007) in a microwave (Mars 5, CEM Corporation, Matthews, NC, USA). A blank and a certified reference material for Se (White clover, BCR402-IRMM) were included in each batch of samples. The Se content in the leaves was measured using GFAAS (Graphite Furnace Atomic Absorption Spectrometry, Atomic Absorption Spectrometry with Zeeman background correction and EDL lamp for Se; Analyst™ 800 AAS, Perkin Elmer), and the S content was measured using ICP-OES (Inductive Coupled Plasma Emission Spectrometry, Spectro, Blue model, Germany). Total N contents were determined by sulfur digestion and Kjeldahl distillation (Tecnal, TE-136, Brazil) (Malavolta et al., 1997).

### 2.3.4 Calculation of LOD, LOQ, and reference material recovery

The detection and quantification limits (LOD and LOQ) were calculated with three and ten times the standard deviation (LOD and LOQ, respectively) of ten individually prepared blank solutions (Silva Junior et al., 2017). The LOD and LOQ for Se were respectively 4.26 and 12.2 μg kg−1. The Se recovery rate in the reference material was 95.2% ± 4.1.

### 2.3.5 Antioxidant enzymes (SOD, CAT, APX, GR)

Frozen leaf tissue was weighed (0.2 g) and mixed with 1.5 mL of potassium phosphate buffer solution (0.1 mol $L^{-1}$, pH 7.8 + 0.1 mol $L^{-1}$ EDTA, pH 7.0, 0.01 mol $L^{-1}$ ascorbic acid, and 22 mg polyvinylpolypyrrolidone-PVPP). The suspension was centrifuged at 14,000 g for 10 min at 4°C (Biemelt et al., 1998). The supernatant was used to assess the activity of the antioxidant enzymes. Quality assurance and quality control of the enzymatic analysis were warranted by using two blanks on each reading plate and operating the samples at 0-4 °C. In addition, the enzyme extraction was performed on the day of the analysis in order to avoid the oxidation of the enzyme extract.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium at 560nm (Giannopolitis and Ries, 1977). The reading sample was composed of 50 mM of potassium phosphate buffer, pH 7.8, 14 mM methionine, 0.1 μM EDTA, 75 μM NBT, 2 μL enzyme extract, and 2 μM riboflavin.

Catalase (CAT, EC:1.11.1.6) activity was assayed by measuring the rate of decomposition of $H_2O_2$ at 240 nm (Havir and McHale, 1987). For this, we used a 200 mM

buffer solution of potassium phosphate pH 7.0, 12.5 mM $H_2O_2$, and 3 μL enzyme extract. The CAT activity was read every 15 seconds for 3 minutes and was defined as the amount of enzyme necessary to reduce 1 μmol $H_2O_2$ $min^{-1}$.

Ascorbate peroxidase (APX, EC:1.11.1.11) was determined by the method of reduction of ascorbate at 290 nm (Nakano and Asada, 1981). The reaction mixture consisted of 50 mM potassium phosphate 100 mM pH 6.0, 0.8 mM ascorbic acid, 1 mM $H_2O_2$, and 3 μL of enzyme extract. The APX activity was read every 15 seconds for 3 minutes and was defined as the amount of the enzyme required to oxidize 1 mmol (ascorbate) $min^{-1}$.

Glutathione reductase (GR, EC:1.6.4.2) was assayed according to the methodology proposed by Schaedle and Bassham (1977) and adapted by García-Limones et al. (2002). The GR activity was read at 340 nm. The reaction medium consisted of 50 mM buffer solution of potassium phosphate 50 mM pH 7.8, 0.5 mM oxidized glutathione, 3.0 mM $MgCl_2$, 0.15 mM NADPH, and 15 μL enzyme extract. One GR unit is defined as the amount of enzyme that oxidizes 1 mmol $min^{-1}$ NADPH.

The analyses were carried out in triplicates and were measured using an Epoch® Microplate Spectrophotometer (Biotek, USA).

### 2.3.6 Hydrogen peroxide ($H_2O_2$) and lipid peroxidation (MDA)

Frozen leaf tissue (0.2 g) was ground in liquid nitrogen, homogenized in 5 mL of trichloroacetic acid (TCA), and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was collected to determine hydrogen peroxide (Velikova et al., 2000) with adaptations of Loreto and Velikova (2001). Lipid peroxidation (MDA) was assayed according to (Buege and Aust, 1978) and Silva et al. (2020).

For the determination of hydrogen peroxide, 0.45 mL of supernatant was added to 2.5 mM potassium phosphate buffer pH 7.0 and 0.5 mM potassium iodate. The absorbance of the supernatant was read at 390 nm. The content of $H_2O_2$ was calculated by comparison with a standard calibration curve previously made by using different concentrations of $H_2O_2$.

The assay of lipid peroxidation (MDA) was carried out by the thiobarbituric acid (TBA) test, which determines the MDA as an end product of lipid peroxidation. Then, 0.125 mL of the supernatant was added to 0.25 mL of a mixed solution of TBA (0.5%) and TCA (10%). The mixture was incubated in a water bath at 95°C for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The absorbance of the supernatant was

measured at 532 nm, subtracting the value for non-specific absorption at 600 nm. This procedure was made in duplicates.

### 2.3.7 Carbohydrates, total protein (Prt), total free amino acids (AA)

The extraction of carbohydrates and proteins was based on Zanandrea et al. (2010). Individual dried leaf samples were weighed (0.2 g), mixed with 5 mL of potassium phosphate buffer (pH 7.0), and heated in a water bath at 30°C for 40 min. Then, the suspension was centrifuged at 10,000 g for 20 min and the supernatant was collected. This procedure was done twice and both supernatants were mixed. The same pellet was used for starch extraction mixing 8 mL of potassium acetate buffer (200 mM pH 4.8) and 2 mL of amyloglucosidase (1 mg $mL^{-1}$; 16 units of enzyme). Then, the samples were heated in a water bath at 40°C for 120 min and centrifuged for 20 min at 10,000 g. The supernatants were collected for measurements. The content of starch, sucrose (Suc), and total soluble sugars (TSS) was determined using the anthrone method (Dische, 1962). Reducing sugars were determined according to the DNS method (Miller, 1959), and total free amino acids (AA) was determined according to the ninhydrin method (Yemm et al., 1955). The protein content (Prt) in the leaves was also determined (Bradford, 1976).

### 2.3.8 Proline (Pro)

Proline content was assessed by the colorimetric method originally described by Bates et al. (1973) with minor modifications. The dried leaf tissue (0.1 g) was weighed and macerated with sulfosalicylic acid 3%. Next, samples were mixed for 60 min at environmental temperature. After the extraction, the content of Pro in the leaves was determined by adding 0.5 mL of extract, 1.5 mL deionized water, 2 mL freshly prepared acid-ninhydrin solution, and 2 mL of pure acetic acid. Tubes were incubated in a water bath at 100°C for 60 min. The reaction was stopped by placing the reaction tubes in an ice bath. The supernatant was carefully collected and read at 520nm.

### 2.3.9 Statistical Analysis

The statistical analyses were performed using the R software (R Core Team, 2021). An exploratory analysis of data was first performed to verify the existence of outliers. Then,

the analysis of variance (ANOVA) was conducted on the data after the validation of the model and tests of assumptions (normality, homoscedasticity, independence, and additivity of residuals). When significative ($p < 0.05$), the interaction of the studied factors (Se supply and coffee genotypes) was compared. When there was no interaction between tested factors ($p > 0.05$), the means of the treatments were compared at each factor. Means were compared using the Tukey test ($p < 0.05$). In addition, principal component analyses (PCAs) were performed to determine the relationships of the measured variables. Pearson´s correlation analysis ($p < 0.05$) was performed to validate the relationships observed in PCA. PCA and correlation analysis was performed for each species and time of evaluation (before, during, and after stress). The correlation matrices among variables are reported in the supplementary material.

## 3 Results

### 3.1 Visual damage scale

Leaf visual damage was influenced by species and Se supply (Figure 1). *C. canephora* was statistically ($p < 0.05$) more affected than *C. arabica* at both evaluation times. During the stress, the damage to *C. canephora* was two-fold higher than in *C. arabica* (Figure 1a). Selenium supply reduced the damage by low-temperature in *C. canephora* by 24% and 17% compared with its initial control value at optimal temperature (25°C day/20°C night), respectively for the evaluations performed during chilling and the rewarming (Figure 1A and 1B).

*Coffea canephora* showed as the main leaf damage in the leaves a yellowish-green color during and after the cold stress (Figure 1C). Although the *C. arabica* did not show high damage by cold, it was noticed slight darkened damage in the leaves after two days of exposure to chilling stress (Figure 1C).

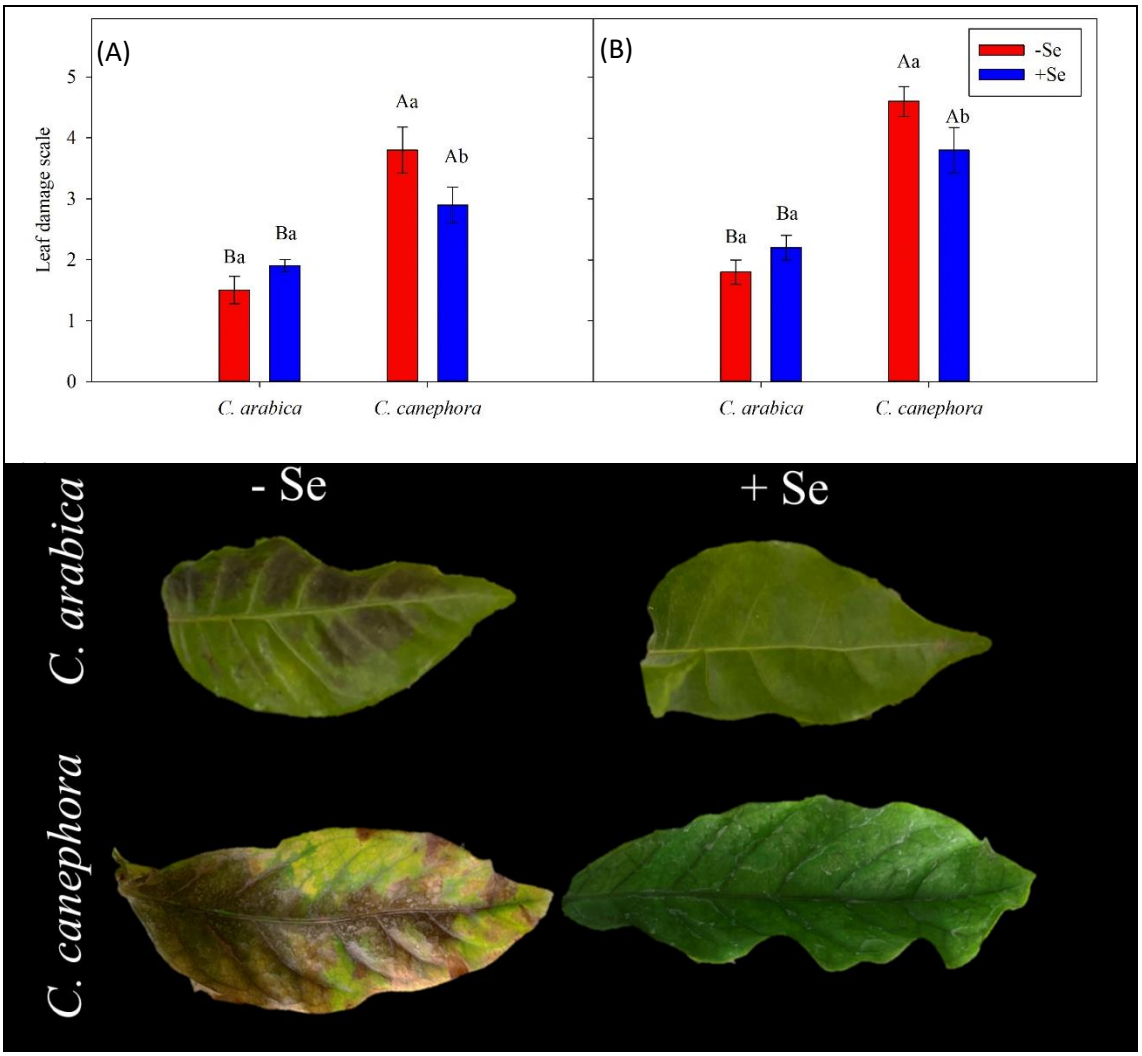


Figure 1: Leaf visual damage in coffee species exposed to chilling stress and two conditions of Se foliar supply after two days of exposure to low-temperature. Visual damage scale during (A) and after stress (B) according to Manetti Filho e Caramori (1986). (C) Visual damage in coffee after low-temperature stress. Mean values followed by different lowercase letters within Se supply conditions (with selenium and without Se) in each genotype and different uppercase letters indicate significant differences within each genotype (*C. arabica* and *C. canephora*) in each Se supply condition are significantly different ($p<0.05$, $n=5$) by Tukey multiple comparison test. Vertical bars represent the standard error.

### 3.2 Analysis of selenium, sulfur, and nitrogen

Leaf Se content ranged from 0.18 mg $kg^{-1}$ DW (control treatment) to 2.13 mg $kg^{-1}$ DW (after chilling stress) in the *C. arabica* and 0.18 mg $kg^{-1}$ DW (control treatment) to 1.81 mg $kg^{-1}$ DW (after chilling stress) in the *C. canephora*. There was no statistical difference between the species (Figure 2).

In the present study, Se foliar supply increased the N content in the leaves before plants were submitted to chilling stress, but N content was reduced in the low-temperature condition by Se application (Figure 2).

The leaf S content was affected by species and Se supply in all the evaluation times ($p < 0.05$). The S content in *C. canephora* was significantly higher than in *C. arabica*. Selenium foliar supply promoted 9% higher S content in leaves on the evaluation performed before the cold, but Se supply reduced the S content in the leaves during and after stress (Figure 2). The S content decreased 10.5% and 10.7%, respectively during and after chilling stress by Se application.

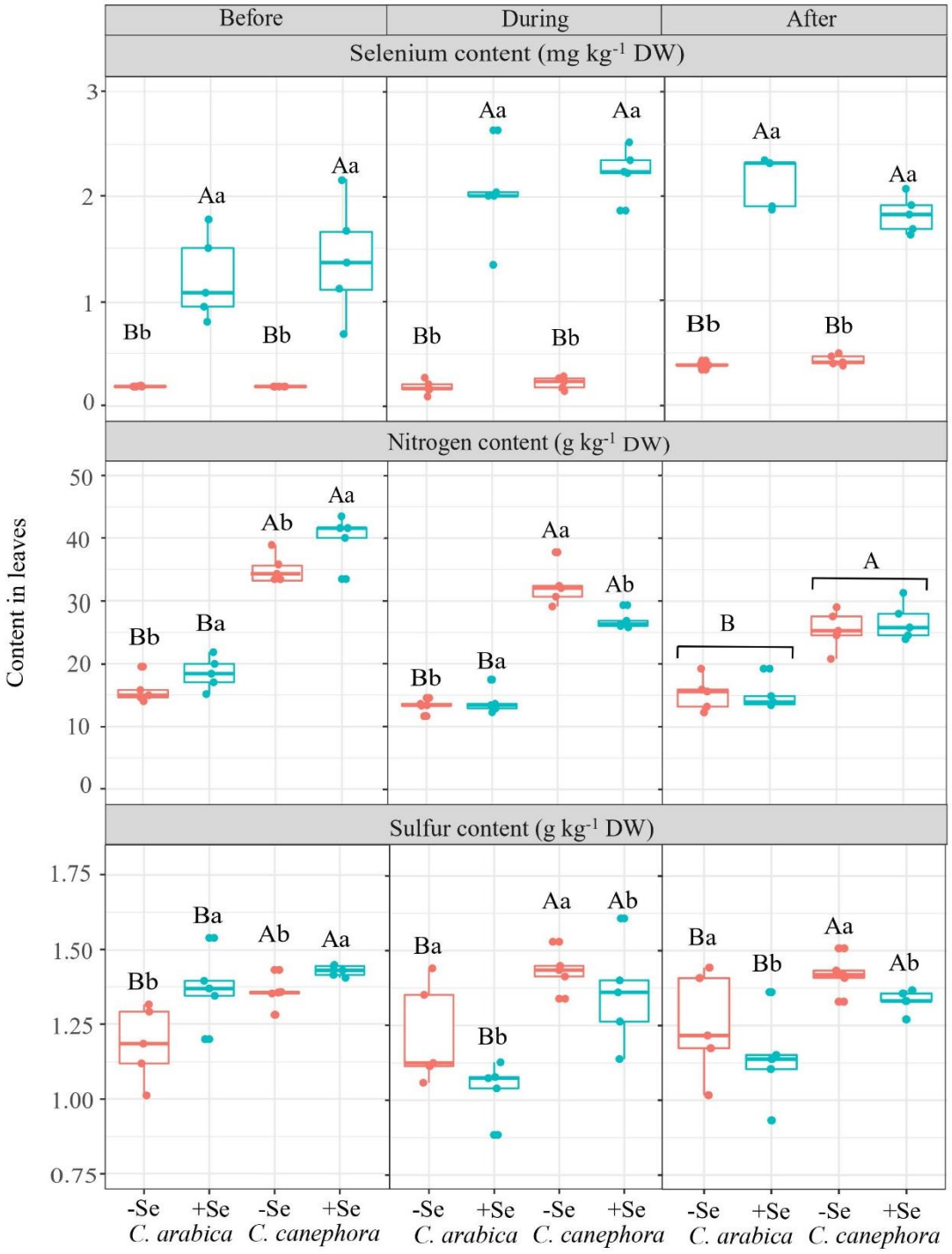


Figure 2: Effects of Se foliar application and temperature condition on Se, S, and N content in leaves of *C. arabica* and *C. canephora*. Mean values followed by different lowercase letters within Se supply conditions (-Se or +Se) in each genotype are significantly different ($p<0.05$, $n=5$) by Tukey multiple comparison test as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*).

### 3.3 Antioxidant enzymes (SOD, CAT, APX, GR)

The average values of antioxidant enzyme activity (GR, SOD, CAT, and APX), as well as the hydrogen peroxide ($H_2O_2$) and lipid peroxidation (MDA), assessed in the species treated and non-treated with foliar Se are presented in Figure 3 and Table S1.

Chilling stress promoted an increase of 44 % in GR activity in non-treated plants with Se, but these plants were unable to keep high GR activity during the rewarming condition and the GR activity was reduced by 97 % (Figure 3, Table S1). On the other hand, Se supply was responsible for statically increasing the GR after the chilling stress compared with non-treated plants with Se.

The SOD activity was notably increased during chilling stress compared with optimal temperature conditions. After stress, SOD was affected by the interaction of the two factors (Species × Se supply). Foliar supply promoted 23.5 % higher SOD activity in *C. arabica* (Figure 3, Table S1). The same effect was not shown in the *C. canephora*.

Foliar supply of Se promoted 50% less CAT activity in *C. canephora* than the same non-treated species during chilling stress. Moreover, Se foliar supply increased CAT activity before and after the stress, independently of the species ($p < 0.05$). APX activity was not influenced by Se application and was affected by the species in which the *C. arabica* showed higher activity regardless during and after chilling (Figure 3, Table S1).

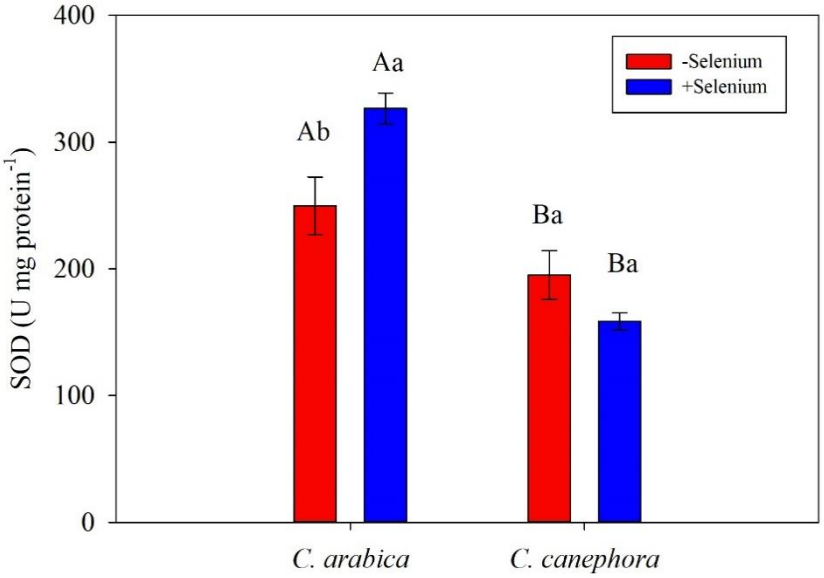


Figure 3. Effects of Se foliar application on SOD activity in leaves of *C. arabica* and *C. canephora* during the rewarming. Mean values followed by different lowercase letters within Se supply conditions (-Se or +Se) in each genotype are significantly different ($p<0.05$, $n=5$) by Tukey multiple comparison test as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*).

### 3.4 Hydrogen peroxide ($H_2O_2$) and lipid peroxidation (MDA)

Levels of malonaldehyde (MDA) and $H_2O_2$ were not influenced by the presence of Se and species (Figure 3, Table S1). The stress increased the MDA content by 7.7% and 35.6% in the *C. arabica* and *C. canephora* compared with the respective genotype before the stress. After the stress, MDA increased by 58.3% and 38%, respectively for *C. arabica* and *C. canephora* compared with the same species before stress. This supports the hypothesis that *C. canephora* has less ability to tolerate low-temperatures than *C. arabica* because the MDA content increased promptly after the plants were submitted to chilling stress. On the other hand, MDA content in the *C. arabica* showed subtle adjustment during the stress but increased abruptly from 36.2 to 53.2 nmol $g^{-1}$ $FW^{-1}$ during the rewarming.

### 3.5 Carbohydrates, total protein (Prt), amino acids (AA)

The Suc content in leaves was affected by the species in all periods of evaluation and *C. arabica* had higher Suc content than *C. canephora*. In addition, *C. arabica* showed less impact from chilling stress on Suc (Table S2).

*C. canephora* showed less ability to maintain the initial content of Suc and RS after the exposure to low-temperature than *C. arabica*. The reduction of Suc and RS in the *C. canephora* was 22.7% and 25.7%, respectively. During rewarming, the *C. canephora* plants were unable to increase the Suc and RS content as *C. arabica*, showing a reduction of 45.2% and 44.2% compared with the plants before the stress. The *C. arabica* plants also showed a subtle reduction in Suc when exposed to chilling stress, but it was less pronounced than in *C. canephora*. Meanwhile, the *C. arabica* plants reduced the RS content in the leaves during the stress, but its content was increased by 8.8 % in the rewarming period.

The Se foliar application promoted lower starch content in the plants before and during stress, but its supply modulated the starch content after the plants were subjected to chilling stress, which led to an increase of ~ 30.7% in the starch when compared with plants that did not receive Se foliar application (Figure 4A). In addition, Pearson's correlation analysis showed a positive correlation ($p < 0.05$) of Se and starch in both species after the chilling stress - $R2 = 0.92$ and $R2 = 0.68$ respectively to *C. arabica* and *C. canephora* (Figures S5 and S6). There were significant differences ($p < 0.05$) between Se supply in the TSS content before and after the stress. The TSS content in foliar tissue from Se supplied plants was 18% lower than in those that did not receive Se supply (Figure 4). Foliar supply of reduced the TSS content before stress. In contrast, Se supply increased the TSS content after the chilling stress in both species. After the stress, TSS showed a correlation with Se content in the leaves according to PCAs (Figure 4E, F). This behavior is also supported by a significant correlation ($p < 0.05$) to Se content in leaves in both species according to Pearson's correlation analysis (Figures S5 and S6).

The application of Se improved the Prt content in *C. arabica* leaves before the plants were submitted to chilling stress, but this effect was not noticed during the chilling stress and the rewarming period. Despite this, Prt was higher in *C. canephora* than in *C. arabica* during all growth temperature conditions. Similarly, the AA content was higher in *C. canephora* than in *C. arabica*, where the AA content was not influenced by Se application before and after the chilling stress.

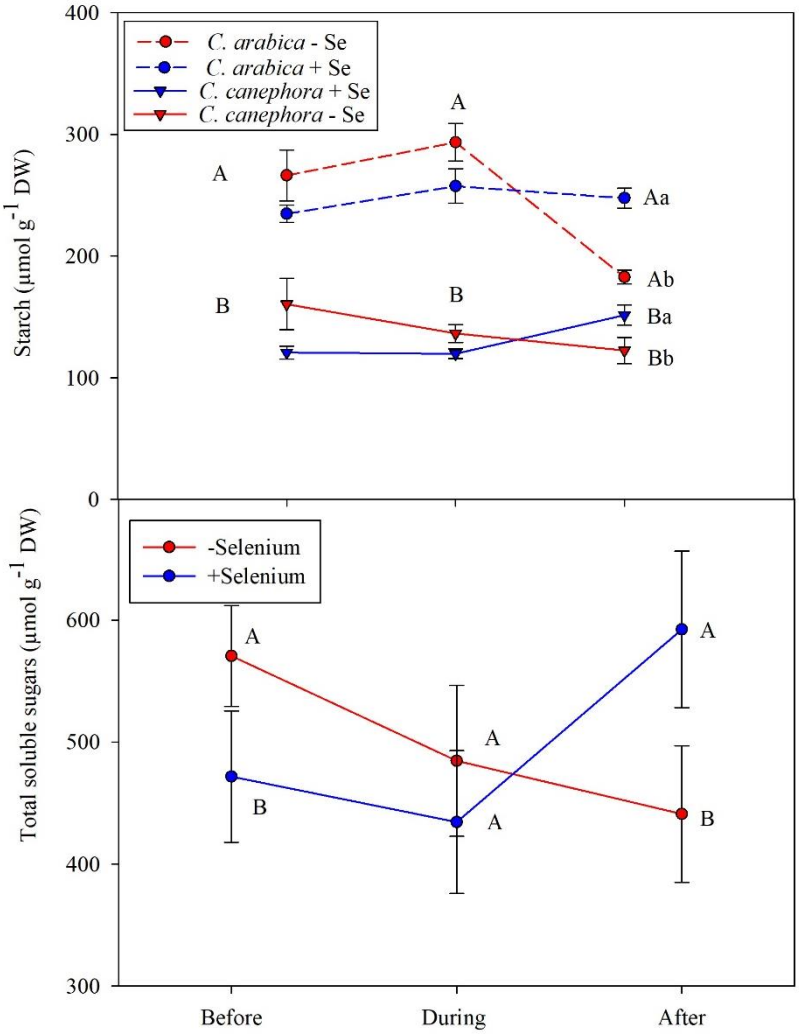


Figure 4. Effects of Se foliar application and temperature conditions on Starch (A) and TSS (B) content in leaves of *C. arabica* and *C. canephora*. The TSS content was obtained by the average of both species. Mean values followed by different lowercase letters within Se supply conditions (-Se or +Se) in each genotype are significantly different ($p<0.05$, $n=5$) by Tukey multiple comparison test as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*).

### 3.6 Proline

The Pro content was affected by species before and during chilling stress, in which *C. canephora* has shown notably higher content than *C. arabica*. Nevertheless, Pro content in *C. canephora* during the stress was reduced by 44 % after the stress, showing that the low-temperature can exert great influence on the Pro content in stress conditions. Despite the lower initial Pro content in the *C. arabica*, this genotype was able to increase significantly the content in the rewarming, which was potentialized by the Se application. Selenium

application increased 20.4% and 133% of the Pro content, respectively to *C. arabica* and *C. canephora* without Se application (Figure 5).

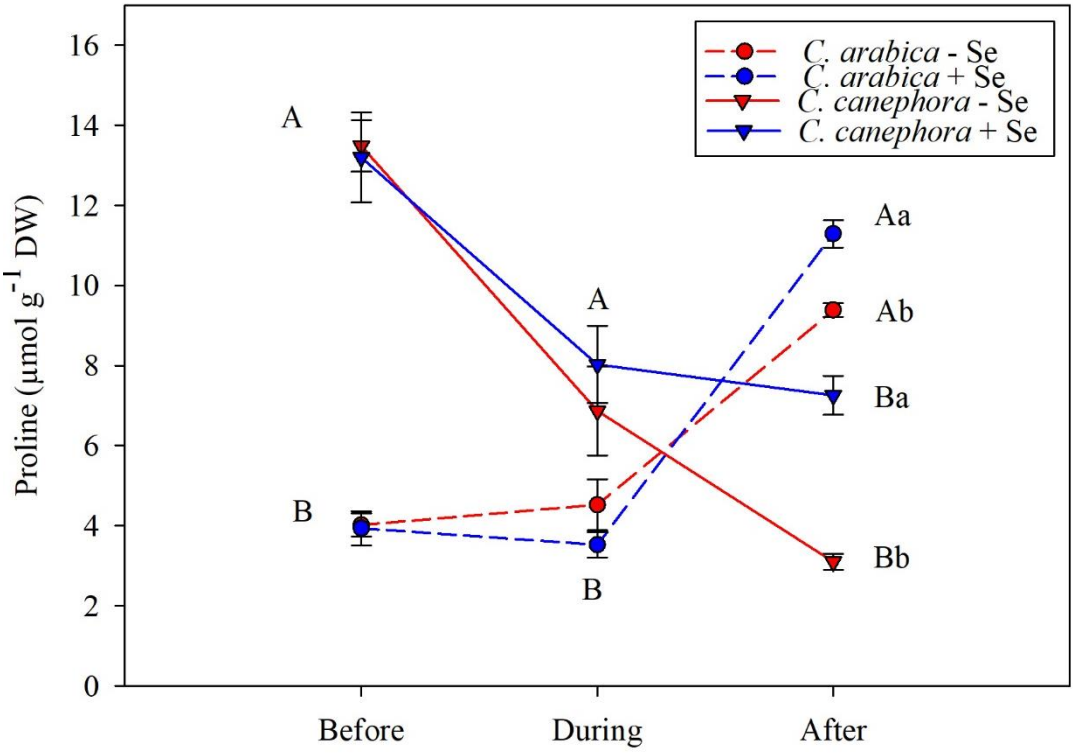


Figure 5. Effects of Se foliar application and temperature conditions on Pro content in leaves of *C. arabica* and *C. canephora* during the rewarming. Mean values followed by different lowercase letters within Se supply conditions (-Se or +Se) in each genotype are significantly different ($p<0.05$, $n=5$) by Tukey multiple comparison test as well as different uppercase letters that indicate significant differences in species (*C. arabica* and *C. canephora*).

### 3.7 Principal component analysis (PCA)

The principal component analysis (PCA) showed that the relations between the analyzed parameters and Se content in leaves vary in function of specie and temperature condition (before, during, and after chilling stress). Overall, it is possible to find two groups enclosed in the ellipses, which are composed of samples supplied with Se at all evaluation times (Figure 6).

Before stress in *C. arabica*, the application of Se promoted the higher contents of Se, and this Se had a positive relationship with CAT and the content of S and a negative relation with AA (Figure 6). During stress, the positive relationship between Se content and CAT was maintained, with CAT having also a positive correlation with Prt. After chilling stress, the relationship between CAT and Se content was not maintained. Selenium content was increased by its application and had a positive correlation with Pro, TSS, Sta, GR, and SOD.

To *C. canephora* after chilling, the PCA showed a positive and significant correlation ($p < 0.05$) of Se with CAT, Sta, Suc, TSS, and Pro, which was supported by the correlation matrix (Figure S6).

A negative relationship was observed between S and TSS content. During the low-temperature stress, the Se content showed a negative relationship with the content of N, AA, Sta, CAT, and GR. After the stress in *C. canephora*, Se content had a positive relationship with Pro, TSS, Sta, Suc, and CAT.

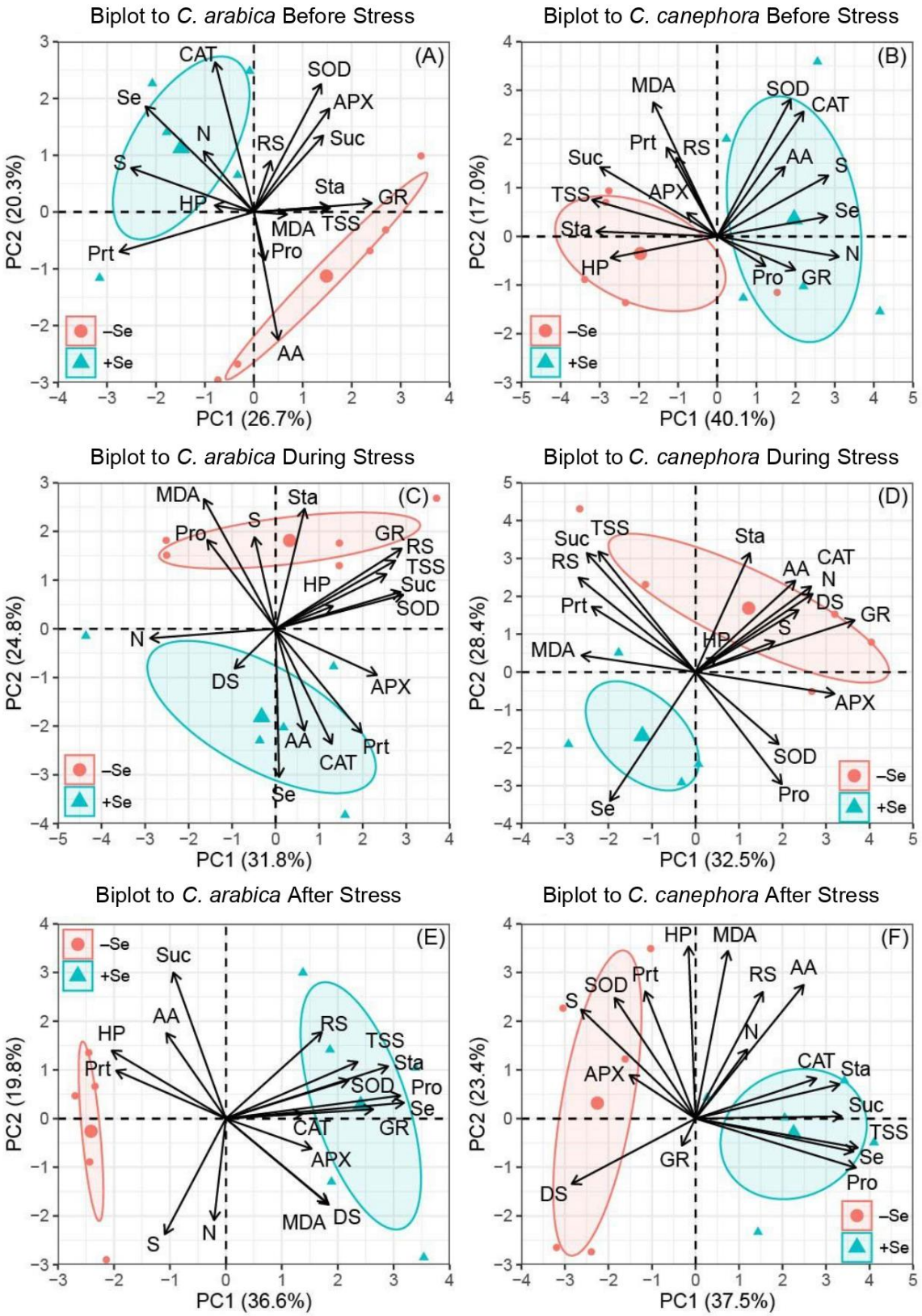


Figure 6. PCAs biplot representation of leaves composition data before, during, and after chilling stress in two species. Figures 6A, 6C, and 6E represent *C. arabica* before, during, and after stress, respectively; Figures 4, 4D, and 4F represent *C. canephora* before, during, and after stress, respectively.

# 4 Discussion

The stress promoted by chilling negatively impacted plant development and caused significant damage to the leaves. The injuries to the leaves from chilling stress resulted from an inhibition of the photosynthetic process, stomatal closure, and low availability of CO2, hence the plants have limited fixation that leads them to produce and accumulate reactive oxygen species (ROS) (Larcher, 1985; Oliveira et al., 2002). Partelli et al., (2009) showed that increased ROS promotes lipidic peroxidation and loss of membrane selectivity, then, coffee plants submitted to low-temperature have shown chlorophyll loss and leaf tissue degradation, reflecting in injuries in the leaves. These damages were also observed in this trial (Figure 1). Lipid peroxidation is a good indicator of ROS activity on cell damage, mainly because oxidative stress causes the peroxidation of unsaturated fatty acids, whereas increasing MDA concentration (Farooq et al., 2019).

Exogenous Se supply has been related to reduced ROS - such as $H_2O_2$ - and lipidic peroxidation (MDA) under stress conditions (Jóźwiak and Politycka, 2019; Silva et al., 2020; Mateus et al., 2021). However, this behavior was not seen in this trial with coffee (Table S1). Despite this, Se supply significantly reduced injuries to leaf tissue of *C. canephora* plants during and after the stress (Figure 1), suggesting that the negative effects of chilling stress are mitigated by pathways other than ROS scavenging. The higher damage in the *C. canephora* leaves compared with *C. arabica* suggests that each genotype might act distinctly when submitted to stress in the triggering of metabolic responses to temperature changes (Petek et al., 2005; Fortunato et al., 2010; Damatta et al., 2018), including different responses to Se application. These results are explained by the allopolyploidy of *C. arabica*, which promotes an evolutionary advantage in having additional genetic materials that attribute greater plasticity in coping with environmental variations compared with its parentals - in this case, *C. canephora* and *C. eugenioides*. In other words, the allopolyploidy of *C. arabica* makes this species able to up and down-regulate certain genes responsible to keep the homeostasis during low-temperatures, as reported by Bardil et al. (2011), or even at higher temperatures (Oliveira et al, 2020).

Although, the effect of Se on the improvement of antioxidant enzyme activity during chilling stress has been supported by some authors (Chu et al., 2010; Abbas, 2012). Thereby, the abrupt temperature change might hinder species' abilities to adapt to novel conditions (Damatta et al., 2018). In addition, the antioxidant enzymes are highly dependent on protein

functions and low-temperatures can lead the proteins to reduce their activity and reduce cellular fluidity (Maksimov et al., 2017).

The increase in the S content in the coffee leaves before the chilling stress probably occurred due to its intimate relation with Se metabolism in plants. Currently, some studies have shown that the high-affinity sulfate transporters involved in sulfate uptake and translocation throughout plant tissues may be utilized by selenate ($Na_2SeO4$) as well (Sors et al., 2005; White, 2018). At this point, low content of Se can improve S uptake by mimicking S deficiency to activate specific sulfate transporter expression and stimulate S uptake, resulting in the selenate-induced S accumulation (Boldrin et al., 2016).

The higher S content in leaves led to an increase in the Prt before stress, which was supported by significative Pearson's correlation ($R2=0.80$, $p < 0.05$) in the *C. arabica* (Figure S1). Sulfur is a structural constituent of certain amino acids (e.g., methionine; Met and cysteine; Cys) and coenzymes, as well as in prosthetic groups such as ferredoxin, essential composts for plants to survive in unfavorable conditions (Saleem et al., 2021).

In addition, S composes the amylase molecule through Cys. Since Cys compose the amylase, this amino acid can increase amylase activity aiming to face the stress. Then, most of the stored source of carbohydrates is degraded by amylase and the product is then supplied to the plants for energy and carbon for growth (Thalmann and Santelia, 2017). Meanwhile, Se supply can also stimulate amylase by the same mechanisms indorsed by S, since they share the same primary metabolism in plants and Se can be incorporated in Cys, giving rise to Se-Cys (Jacob et al., 2003; White, 2018).

The reduction of S content during and after chilling stress by Se supply could be connected with the potential changes in energetic metabolism of plants under severe stress, which cause its remobilization from leaves to storage parts, such as roots and stems. The storage of nutrients may be an effective alternative for sustaining plant growth and plays a key role in energy saving during the rewarming condition (Etienne et al., 2018).

The higher content of Se in leaves and the remobilization of S from shoot to roots probably are correlated with de TSS, starch, AA, and Pro content during the rewarming (Table S2; Figure 4). It can be suggested based on data that to keep the carbohydrate demand for growth under low-temperature, Se can help plants to remobilize the S from leaves after the stress.

In this way, Se application helped the plants to maintain the starch content during the rewarming, since Se increased starch content by 12% compared with the same treatment before the chilling stress (Figure 4; Table S2). On the other hand, non-Se treated plants

reduced the starch content by 28%. These results show that foliar Se can not only reduce the starch breakdown but also increase the content after the low-temperature stress compared with those that do not receive foliar Se.

Provided that the effect of chilling stress includes impairment of photosynthesis, Se supply in plants cause increases in the structure and functionality of the photosynthetic apparatus, allowing the plants to maintain higher net photosynthesis during stress condition (Lara et al., 2019; Souza et al., 2019). At this point, transitory starch is synthesized in the leaves directly from photosynthates during the day and can be degraded the following night to sustain metabolism, energy production, and biosynthesis in the absence of photosynthesis (Pfister and Zeeman, 2016). According to Stein and Granot, (2019) and Ribeiro et al., (2022), starch not only acts in the energetic metabolism, but also as promoting rapid stomatal opening, making osmoprotectants, cryoprotectants, scavengers of free radicals and signals, and reverting embolized vessels. Besides, its cleavage products are available for many metabolic pathways, including the synthesis of complex carbohydrates.

According to PC1, during the rewarming, the effect of Se on Suc was positive in *C. canephora*, but negative in *C. arabica*. Moreover, Suc was found on the opposite side of DS in PCA1 (Figure 6F) and also significantly negative according to Pearson's correlation ($R2 > -0.74$) (Figure S6). In addition to higher Suc, Se application also promoted higher TSS, total amino acids (AA), and Pro content in leaves, regardless of genotype during the rewarming (Table S2). These results evidence that, although *C. canephora* plants were not able to maintain their full development during the stress, Se supply can impair plant metabolism after the low-temperature stress, which results in less damage to *C. canephora* plants.

Proline content was affected by the species before and during low-temperature stress and *C. canephora* showed higher content than *C. arabica*. Nevertheless, the *C. canephora* reduced the content of Pro by 43% when submitted to low-temperature, and 60% during the rewarming. Meanwhile, the Pro content in *C. arabica* maintained the same status during chilling stress but increased by 15% compared with Pro content before stress. Although Se affected positively the Pro content in both species, it is remarkably in *C. canephora* (135%) when compared with *C. arabica* (20%).

The considerable depletion of Pro content in *C. canephora* showed that this specie had less ability to survive during the stress. In contrast, *C. arabica* was able to modulate the content of Pro to protect the cellular structures and reduce the production of ROS. It is also supported by the allopolyploidy of *C. arabica*, in which these plants are able to activate different genes to induce the production of Pro in the rewarming and downregulate its content

in the *C. canephora*. Moreover, the regulation of these genes can also be dependent on the external stimulus, which was remarkably changed by temperature and/or Se supply (Bardil et al., 2011; Krishnan et al., 2009; Ni et al., 2009).

As a result of chilling stress, the plants are submitted to osmotic constrictions due to the reduced uptake of water. Then, the soil water potential progressively decreases, hampering and eventually halting the gradient of water flow from roots to apical shoot. The resulting osmotic stress may cause stomatal closure, reduced photosynthesis rate, growth inhibition, and ROS accumulation (Trovato et al., 2008). A response to osmotic stress widespread in plants consists in the accumulation of compatible osmolytes – such as Pro - which are thought to protect cells against stress damage.

The catabolism of Pro occurs in the mitochondria and it is connected to oxidative respiration and administers energy to resume growth after stress. During energy-depleted, Pro might be oxidated to glutamate by flavin-dependent proline-dehydrogenase (PRODH) and NAD+-dependent P5C dehydrogenase (P5CDH), two enzymes found in the mitochondria (Liang et al., 2013; Qamar et al., 2015; Zhang and Becker, 2015). Thus, the oxidation of Pro contributes to mitochondrial metabolism and ATP production by providing carbon skeletons and saving extreme energy depletion (Hildebrandt et al., 2015). The Pro behavior in this trial is supported by its negative correlation with TSS, and Suc during the stress with *C. canephora*, which showed $R2 = -0.77$ and $R2 = -0.79$, respectively ($p < 0.05$) (Figure S4). In this case, the *C. canephora* reduced the Pro content during the stress to maintain the carbohydrates contents as an energetic source, avoiding carbohydrate starvation.

The PCA showed that Se supply responses vary not only in the species but also in different temperature conditions. It is also important to highlight that none of the analyzed variables showed a positive correlation with Se content in leaves during stress with *C. canephora* plants according to PCA1 (28.4%) and PCA2 (32.5%) (Figure 4D). Figure 4D shows that the variables analyzed presented a neutral or negative correlation with Se content. The absence of positive correlation during the stress is probably due to metabolic dysfunctions in *C. canephora* during low-temperature, which resulted in higher injuries in the leaves.

Plant cells can sense chilling stress through low-temperature-induced changes in membrane fluidity, protein, nucleic acid conformation, and/or metabolite concentration (a specific metabolite or redox status) (Chinnusamy et al., 2007). Low-temperature can inhibit the activities of some antioxidant enzymes (e.g., GR) that protect plants against ROS. The reduction of GR during the low-temperature was not observed in the treatment of *C.*

*canephora* without Se application. In this treatment, the GR increased 78% during the chilling stress compared with the same treatment before the stress (Table S1). However, after the chilling stress, Se application promoted three times more GR activity in plants when compared with those that did not receive Se. In other words, plants without Se were unable to maintain the GR activity after chilling stress.

## 5 Conclusion

Our findings showed a considerable depletion of plant metabolism at low-temperature in both of the species studied, resulting in leaf damage and lipidic peroxidation (MDA), notably higher in *C. canephora*. The cold makes plants unable to trigger metabolic responses during the stress, reducing the content of carbohydrates and AA. Despite this, foliar Se application improved plants' odds of survival and reduced the leaf's injuries largely through enhancement in increasing the content of carbohydrates (TSS, starch, and Suc) and AA in the rewarming. All these compounds might also work as cryoprotective substances toward cold-sensitive enzymes, avoiding high membrane rigidity and also maintaining the membrane structure. Therefore, the application of Se at lower levels could be suggested as an important strategy for improving coffee development during cold, helping the plants to recover from the low-temperature stress. New trials focused on the impact of Se on gene expression and associated thermotolerance should be conducted in order to elucidate the role of this beneficial element on plant metabolism aiming at clarifying these results.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

G.F.S, M.A.S., R.R.O., A.C.J., and L.G.G.G designed the research. G.F.S., M.A.S., G.A.Z van O, and G.G.A.Z van O. conducted the experiments and chemical analyses. G.F.S, M.A.S, and E.G. de M. analyzed the data. G.F.S. and M.A.S. wrote the original draft; R.R.O., G. L., P.H.B., D.A., A.C.J, and L.R.G.G. wrote the final text and approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ fpls.2022.1000430/full#supplementary-material

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## Supplementary material

Table 1. Effects of Se and temperature conditions on superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC:1.11.1.6), ascorbate peroxidase (APX, EC:1.11.1.11), glutathione reductase (GR, EC:1.6.4.2), lipidic peroxidation (MDA), and hydrogen peroxide ($H_2O_2$). Mean values are significantly different ($p<0.05$, n=5) by Tukey multiple comparison test.

| GR (nmol $g^{-1}$ $min^{-1}$ g $protein^{-1}$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Before | | | During | | | After | | |
| | -Se | +Se | Mean | -Se | +Se | Mean | -Se | +Se | Mean |
| *C. arabica* | 2.1ns | 1.3ns | 1.7ns | 3.1ns | 1.3ns | 2.2ns | 0.1ns | 0.4ns | 0.3ns |
| *C. canephora* | 1.8ns | 1.8ns | 1.8ns | 3.6ns | 0.5ns | 2.1ns | 0.1ns | 0.2ns | 0.2ns |
| Mean | 1.9ns | 1.56ns | | 3.4A | 0.9B | | 0.1B | 0.3A | |
| SOD (U mg $protein^{-1}$) | | | | | | | | | |
| *C. arabica* | 273.3ns | 295.0ns | 284.1ns | 672.4ns | 433.2ns | 552.8a | 249.8Ba | 326.4Aa | 288.1a |
| *C. canephora* | 289.9ns | 440.8ns | 365.3ns | 684.2ns | 794.6ns | 739.4b | 195.2Ab | 158.6Ab | 176.9b |
| Mean | 281.6ns | 367.9ns | | 678.3ns | 613.9ns | | 222.5ns | 242.5ns | |
| CAT (nmol $H_2O_2$ $g^{-1}$ MF $min^{-1}$ g $protein^{-1}$) | | | | | | | | | |
| *C. arabica* | 0.9ns | 1.4ns | 1.1a | 1.2Aa | 1.7Aa | 1.5ns | 0.9ns | 1.1ns | 1.0a |
| *C. canephora* | 0.6ns | 1.3ns | 0.9b | 1.6Aa | 0.8Bb | 1.2ns | 0.3ns | 0.6ns | 0.5b |
| Mean | 0.9B | 1.3A | | 1.4ns | 1.3ns | | 0.6B | 0.9A | |
| APX (nmol AsA $min^{-1}$ g $protein^{-1}$) | | | | | | | | | |
| *C. arabica* | 6.9ns | 7.5ns | 7.2ns | 10.7ns | 10.8ns | 10.8a | 10.4ns | 12.3ns | 11.3a |
| *C. canephora* | 7.0ns | 5.3ns | 6.1ns | 6.5ns | 4.0ns | 5.2b | 4.3ns | 3.6ns | 4.0b |
| Mean | 6.9ns | 6.4ns | | 8.6ns | 7.4ns | | 7.4ns | 8.0ns | |
| MDA (nmol g $FW^{-1}$) | | | | | | | | | |
| *C. arabica* | 33.8ns | 33.5ns | 33.6ns | 40.6ns | 31.7ns | 36.2ns | 43.9ns | 62.5ns | 53.2ns |
| *C. canephora* | 34.3ns | 32.4ns | 33.7ns | 44.3ns | 47.0ns | 45.7ns | 45.9ns | 47.0ns | 46.5ns |
| Mean | 34.1ns | 32.9ns | | 42.5ns | 39.3ns | | 44.9ns | 54.8ns | |
| $H_2O_2$ (µmol $H_2O_2$ g $FW^{-1}$) | | | | | | | | | |
| *C. arabica* | 5.3ns | 5.4ns | 5.4a | 5.1ns | 5.2ns | 5.1a | 4.7ns | 3.7ns | 4.2a |
| *C. canephora* | 2.1ns | 1.7ns | 1.9b | 2.8ns | 2.3ns | 2.6b | 2.3ns | 2.2ns | 2.2b |
| Mean | 3.7ns | 3.5ns | | 4.0ns | 3.8ns | | 3.5ns | 3.0ns | |

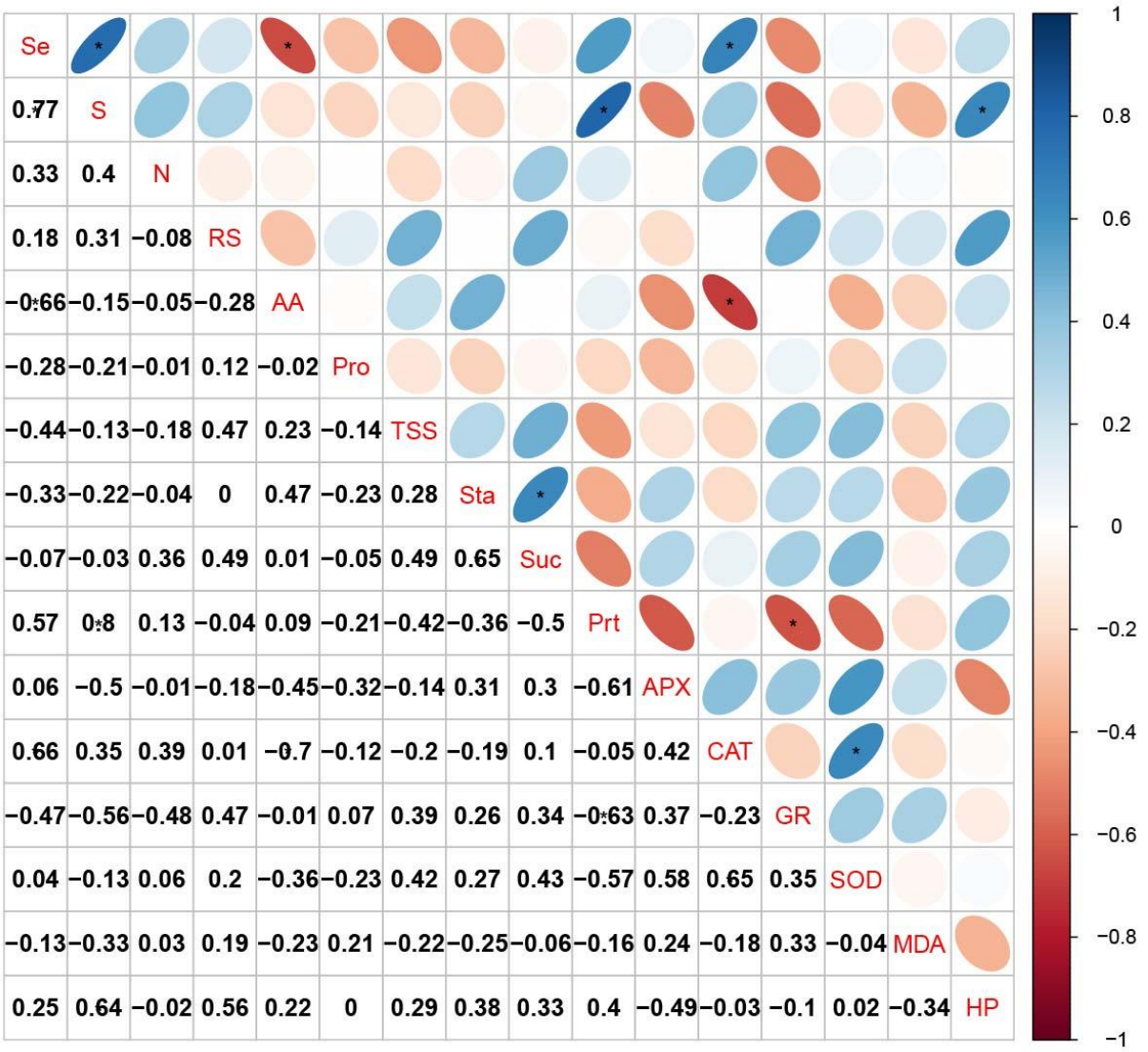
Mean values followed by different lowercase letters within Se supply conditions (-Se or +Se) in each genotype are significantly different ($p<0.05$, n=5) by Tukey multiple comparison test as well as different uppercase letters that indicate significant differences in genotypes (*C. arabica* and *C. canephora* ). ns = non-significance by Tukey multiple comparison test ($p<0.05$, n=5).

Supplementary Table 2. Effects of Se and temperature conditions on sucrose, reducing sugars (RS), total soluble sugars (TSS), starch, protein, and total amino acids (AA).

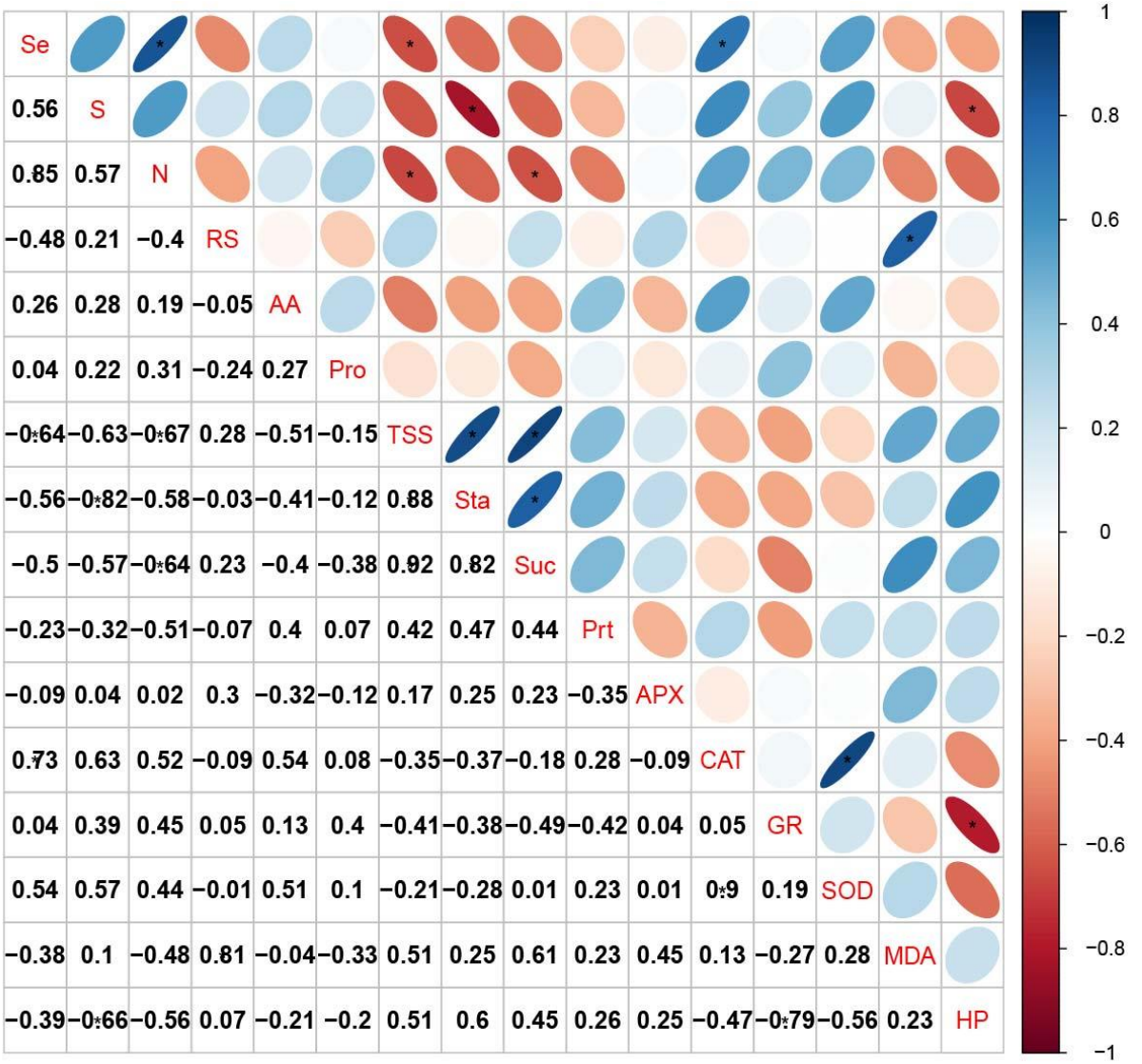
| Sucrose (µmol of glucose $g^{-1}$ DW) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Before | | | During | | | After | | |
| | -Se | +Se | Mean | -Se | +Se | Mean | -Se | +Se | Mean |
| *C. arabica* | 325.3ns | 315.1ns | 320.2a | 287.7ns | 281.8ns | 289.8a | 249.2Aa | 246.7Aa | 247.9a |
| *C. canephora* | 205.8ns | 152.5ns | 179.2b | 145.1ns | 121.3ns | 133.2b | 85.5Bb | 110.31Ab | 97.9b |
| | 265.5ns | 233.8ns | | 221.4ns | 201.6ns | | 167.3ns | 178.5ns | |
| RS (µmol of glucose $g^{-1}$ DW) | | | | | | | | | |
| *C. arabica* | 490.1ns | 483.83ns | 487.0b | 441.1ns | 393.8ns | 417.4ns | 478.9a | 562.66a | 520.8ns |
| *C. canephora* | 693.82ns | 648.22ns | 671.5a | 385.3ns | 381.1ns | 383.2ns | 359.8a | 389.0b | 374.5ns |
| Mean | 592.0ns | 566.5ns | | 413.2ns | 387.5ns | | 419.3ns | 475.9ns | |
| TSS (µmol of glucose $g^{-1}$ DW) | | | | | | | | | |
| *C. arabica* | 683.8ns | 619.5ns | 651.7a | 644.7ns | 602.77ns | 623.7a | 608.2ns | 764.5ns | 686.4a |
| *C. canephora* | 457.3ns | 323.8ns | 390.5b | 324.5ns | 266.1ns | 295.3b | 273.8ns | 420.1ns | 346.9b |
| Mean | 570.5A | 471.7B | | 484.6ns | 434.4ns | | 441.0B | 592.3A | |
| Starch (µmol of glucose $g^{-1}$ DW) | | | | | | | | | |
| *C. arabica* | 266.2ns | 234.8.5ns | 250.5a | 293.5ns | 257.5ns | 275.5a | 182.8Ba | 247.6Aa | 215.2a |
| *C. canephora* | 160.5ns | 120.8ns | 140.6b | 136.4s | 119.7ns | 128.1b | 122.4Bb | 151.4Ab | 136.9b |
| Mean | 213.4A | 177.8B | | 214.9A | 188.6B | | 152.6B | 199.5A | |
| Protein (µg protein $g^{-1}$ DW) | | | | | | | | | |
| *C. arabica* | 26.4Bb | 31.05Ab | 28.7b | 21.5ns | 23.0ns | 22.3b | 30.6ns | 26.8ns | 28.7b |
| *C. canephora* | 44.2Aa | 42.2Aa | 43.2a | 37.5ns | 36.3ns | 36.9a | 34.7ns | 33.3ns | 34.0a |
| Mean | 35.3ns | 36.6ns | | 29.5ns | 29.6ns | | 32.7ns | 30.1ns | |
| Proline (µmol proline $g^{-1}$ DW) | | | | | | | | | |
| *C. arabica* | 4.0ns | 3.9ns | 4.0b | 4.5ns | 3.5ns | 4.0b | 9.4Ba | 11.3Aa | 10.3a |
| *C. canephora* | 13.5ns | 13.2ns | 13.3a | 6.9ns | 8.0ns | 7.5a | 3.1Bb | 7.3Ab | 5.2b |
| Mean | 8.8ns | 8.6ns | | 5.7ns | 5.8ns | | 6.3B | 9.3A | |

Mean values followed by different lowercase letters within Se supply conditions (-Se or +Se) in each genotype are significantly different ($p<0.05$, n=5) by Tukey multiple comparison test as well as different uppercase letters that indicate significant differences in genotypes (*C. arabica* and *C. canephora* ). ns = non-significance by Tukey multiple comparison test ($p<0.05$, n=5).

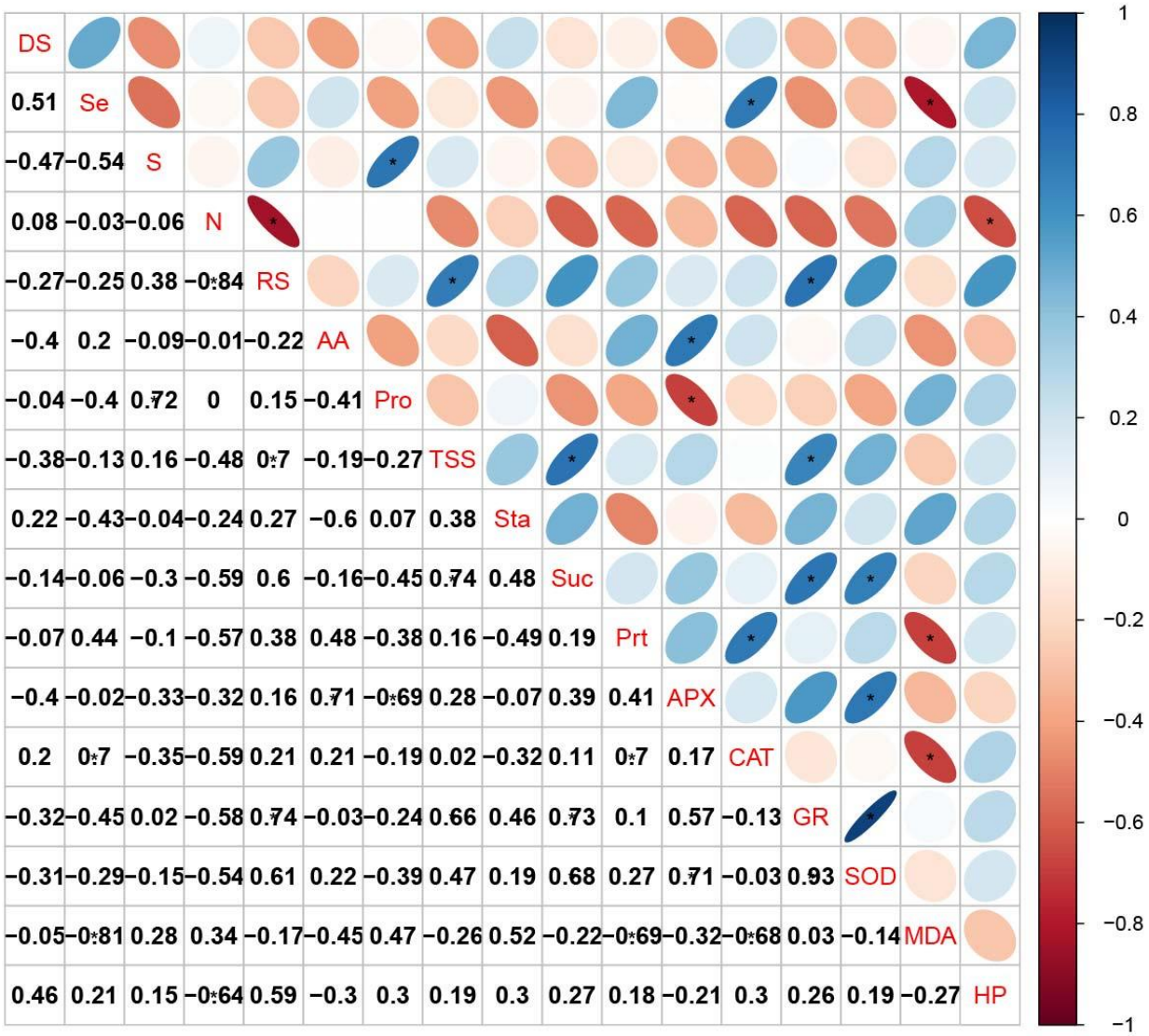
Supplementary Figure 1. Correlation matrix showing Pearson's correlation of physiological, biochemical, and nutritional parameters of *Coffea arabica* seedlings before the cold shock. Se-Selenium content; S-sulfur content; N-nitrogen content; RS-reducing sugars; AA-total amino acids; Pro-proline; TSS-total soluble sugars; Sta-starch; Suc-sucrose; Prt-protein; APX-ascorbate peroxidase; CAT-catalase; GR-glutathione reductase; SOD-superoxide dismutase; MDA-lipidic peroxidation; HP- hydrogen peroxide.



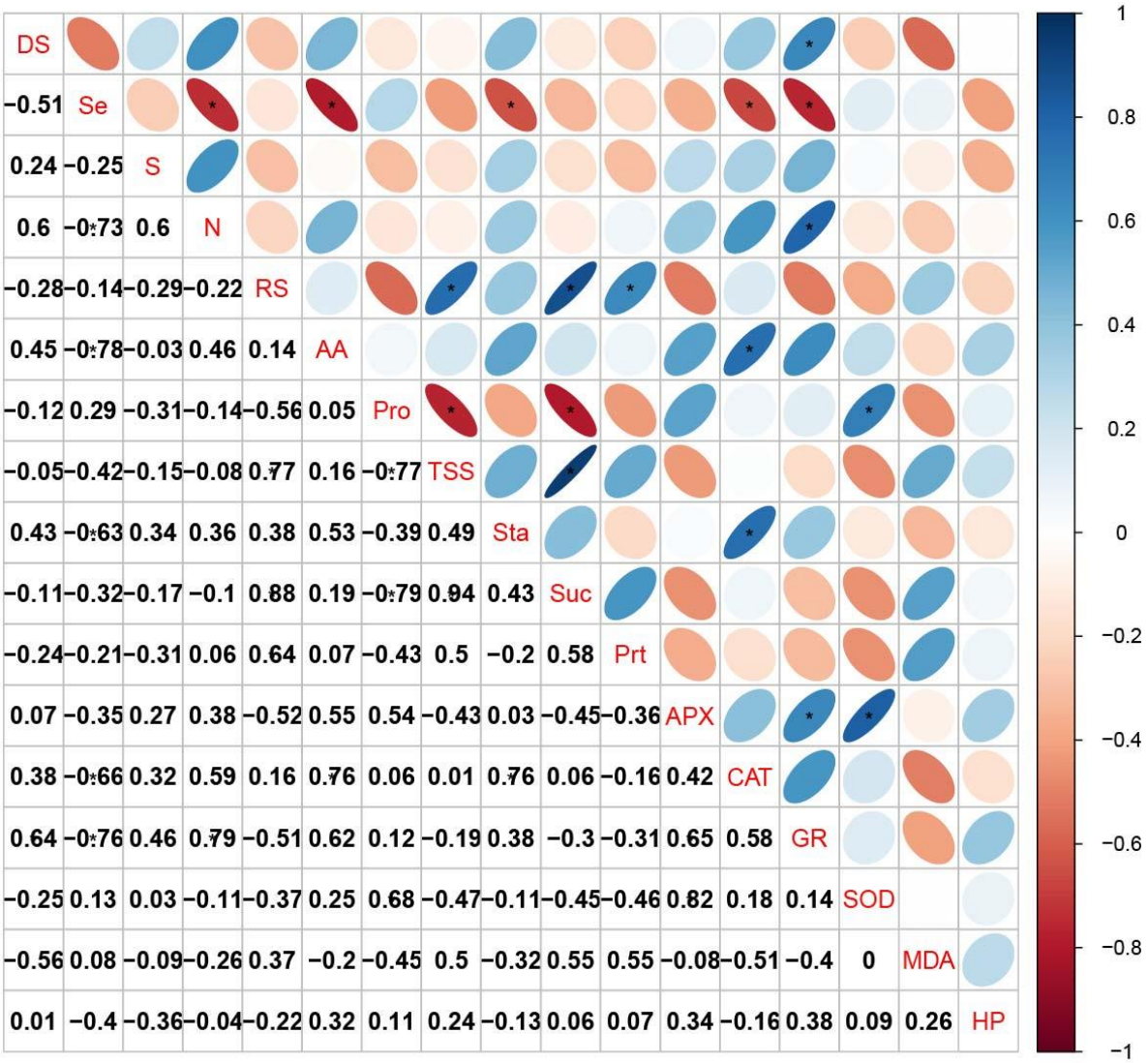
Supplementary Figure 2. Correlation matrix showing Pearson's correlation of physiological, biochemical, and nutritional parameters of *Coffea canephora* seedlings before the cold shock. Se-Selenium content; S-sulfur content; N-nitrogen content; RS-reducing sugars; AA-total amino acids; Pro-proline; TSS-total soluble sugars; Sta-starch; Suc-sucrose; Prt-protein; APX-ascorbate peroxidase; CAT-catalase; GR-glutathione reductase; SOD-superoxide dismutase; MDA-lipidic peroxidation; HP- hydrogen peroxide.



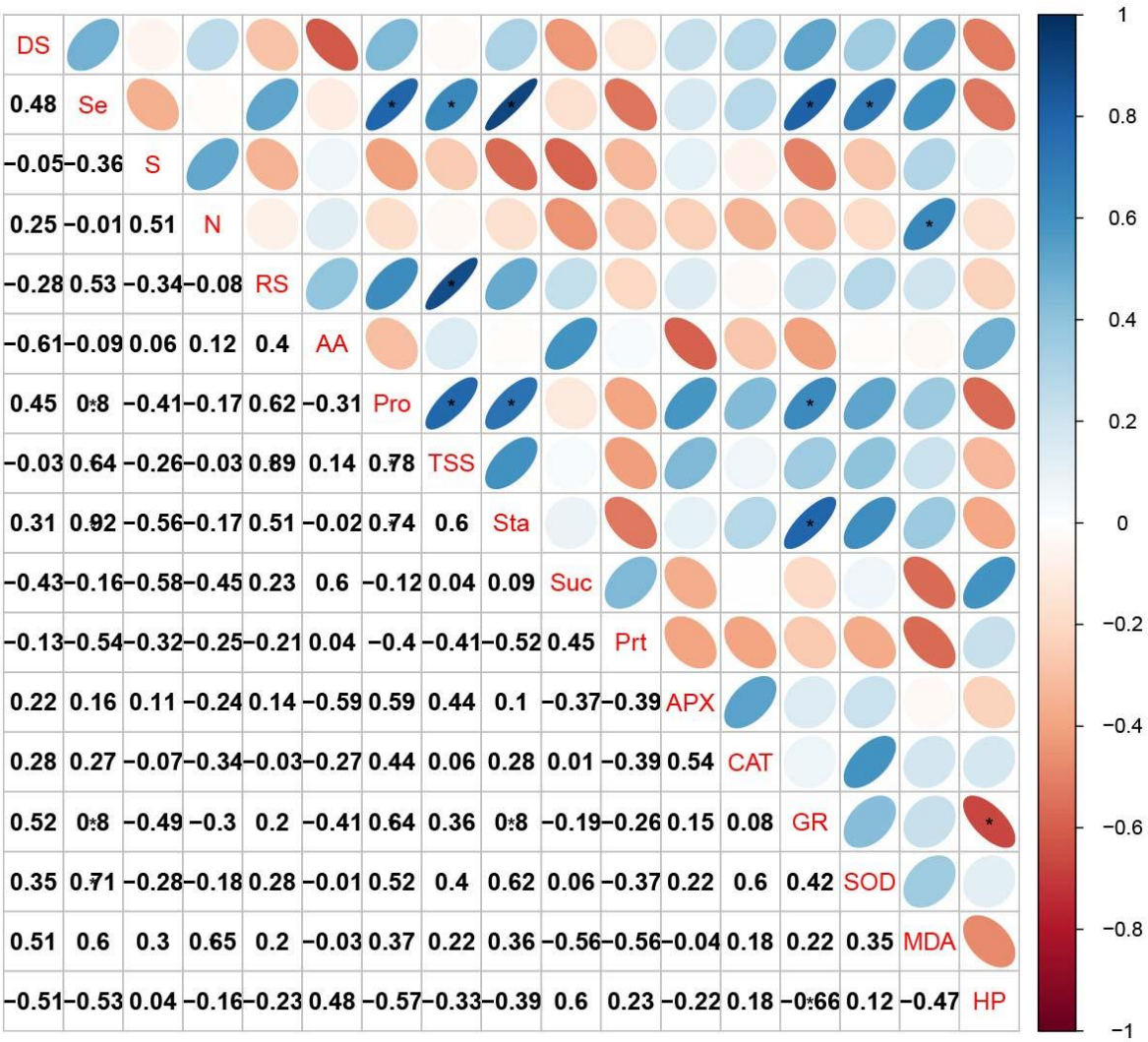
Supplementary Figure 3. Correlation matrix showing Pearson's correlation of physiological, biochemical, and nutritional parameters of *Coffea arabica* seedlings during the cold shock. DS=damage scale; Se-Selenium content; S-sulfur content; N-nitrogen content; RS-reducing sugars; AA-total amino acids; Pro-proline; TSS-total soluble sugars; Sta-starch; Suc-sucrose; Prt-protein; APX-ascorbate peroxidase; CAT-catalase; GR-glutathione reductase; SOD-superoxide dismutase; MDA-lipidic peroxidation; HP- hydrogen peroxide.



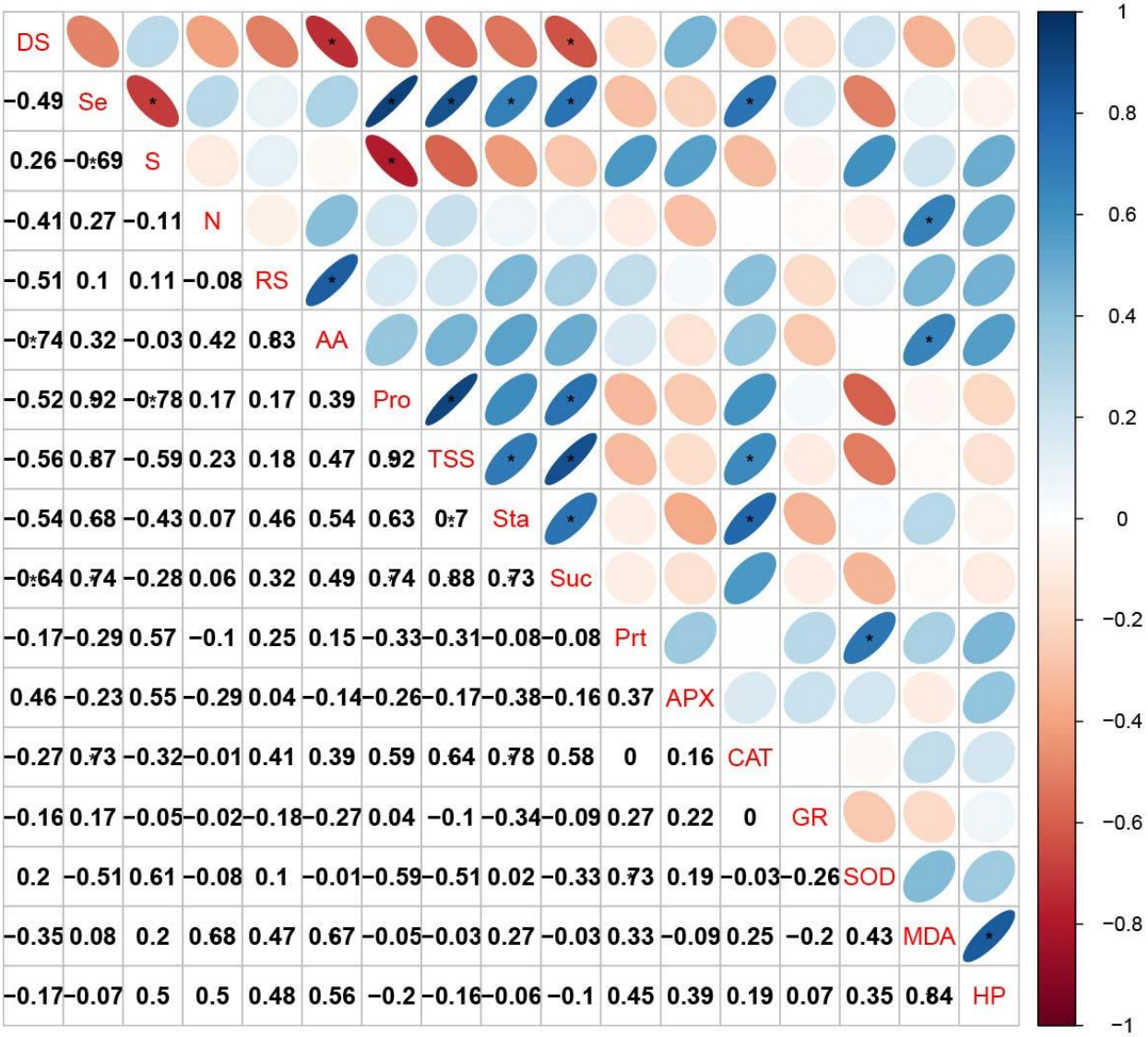
Supplementary Figure 4. Correlation matrix showing Pearson's correlation of physiological, biochemical, and nutritional parameters of *Coffea canephora* seedlings during the cold shock. DS=damage scale; Se-Selenium content; S-sulfur content; N-nitrogen content; RS-reducing sugars; AA-total amino acids; Pro-proline; TSS-total soluble sugars; Sta-starch; Suc-sucrose; Prt-protein; APX-ascorbate peroxidase; CAT-catalase; GR-glutathione reductase; SOD-superoxide dismutase; MDA-lipidic peroxidation; HP- hydrogen peroxide.



Supplementary Figure 5. Correlation matrix showing Pearson's correlation of physiological, biochemical, and nutritional parameters of *Coffea arabica* seedlings after the cold shock. DS=damage scale; Se-Selenium content; S-sulfur content; N-nitrogen content; RS-reducing sugars; AA-total amino acids; Pro-proline; TSS-total soluble sugars; Sta-starch; Suc-sucrose; Prt-protein; APX-ascorbate peroxidase; CAT-catalase; GR-glutathione reductase; SOD-superoxide dismutase; MDA-lipidic peroxidation; HP- hydrogen peroxide.



Supplementary Figure 6. Correlation matrix showing Pearson's correlation of physiological, biochemical, and nutritional parameters of *Coffea canephora* seedlings after the cold shock. DS=damage scale; Se-Selenium content; S-sulfur content; N-nitrogen content; RS-reducing sugars; AA-total amino acids; Pro-proline; TSS-total soluble sugars; Sta-starch; Suc-sucrose; Prt-protein; APX-ascorbate peroxidase; CAT-catalase; GR-glutathione reductase; SOD-superoxide dismutase; MDA-lipidic peroxidation; HP- hydrogen peroxide.



**MANUSCRIPT 2: Foliar selenium application to reduce the induced-drought stress effects in coffee seedlings: induced priming or alleviation effect?**

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**Abstract:** This study aimed to investigate the role of Se supply in improving osmotic stress tolerance in coffee seedlings while also evaluating the best timing for Se application. Five times of Se foliar application were assessed during induced osmotic stress with PEG-6000 using the day of imposing stress as a default, plus two control treatments: with osmotic stress and without Se, and without osmotic stress and Se. Results demonstrated that osmotic stress (OS) promoted mild stress in the coffee plants (ψw from − 1.5MPa to −2.5 MPa). Control plants under stress showed seven and five times lower activity of the enzymes GR and SOD compared with the non-stressed ones, and OS was found to further induce starch degradation, which was potentialized by the Se foliar supply. The seedlings that received foliar Se application 8 days before the stress exhibited higher CAT, APX, and SOD than the absolute control (−OS-Se)—771.1%, 356.3%, and 266.5% higher, respectively. In conclusion, previous Se foliar spray is more effective than the Se supply after OS to overcome the adverse condition. On the other hand, the post-stress application seems to impose extra stress on the plants, leading them to reduce their water potential.

**Keywords:** beneficial elements; oxidative stress; tropical agriculture; coffee belt; osmotic potential

## 1 Introduction

Atmospheric carbon dioxide (CO2) has increased over the past seven decades. It is correlated with gradual and systematic modifications in average climate conditions, such as temperature and precipitation variance [1]. Indeed, such extreme events (e.g., heat waves, floods, and severe drought seasons) expose the remarkable vulnerability of agricultural systems [2,3].

These environmental changes have modified temperature and rain patterns worldwide, making coffee cultivation uncertain in commonly cultivated areas [4,5]. Coffee is a crop sensitive to precipitation variability, and rainfall instability can lead to high losses in coffee production. Arabica coffee requires between 1000 and 2700 mm of annual precipitation and from one to three months of dry season annually [6]. Due to its temperature and humidity demands, coffee cultivation is limited to the intertropical region, commonly called the coffee belt [7].

The plant side effects of the lack of water in the crop system include drought stress [8]. Drought stress imposes osmotic stress (OS) due to the lack of water in the plant tissue. OS promotes changes in plants' physiological, morphological, ecological, biochemical, and molecular traits [9,10]. Water deficit directly affects crops' growth, development, and yield [11]. As an immediate response to OS, the stomata close, which constrains the transpiration flow and the $CO_2$ fixation. These responses vigorously reduce the photosynthetic rates and hence the production of photoassimilates [12]. The impact of OS on coffee plants reflects negatively in the harvest in progress and future ones [13].

Plant mineral nutrition is considered a strategy to reduce the adverse effects of OS. Selenium (Se) is one of the promising approaches to fight the metabolic responses in plants under this type of adverse condition [14,15,16]. Selenium is not a plant nutrient, but several studies have reported its beneficial effects, mainly under stress conditions (e.g., salinity, chilling stress, metals accumulation, and drought stress) [11,17,18,19]. The extensive antioxidant capacity of Se arises from its ability to enhance selenoproteins, like glutathione peroxidase. These selenoproteins play a crucial role in counteracting reactive oxygen species (ROS) generated during plant osmotic imbalance in challenging conditions. Thus, using selenium as an osmoprotective strategy may effectively alleviate the detrimental impact of abiotic stresses [20,21].

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As a result of Se application in plants, some authors have noticed an increase in shoot and root biomass and better plant development [25], as well as improved regulation in the status of water, and higher antioxidant apparatus activation in water-stressed crops [26]. Sousa et al. [19] found that Se can modulate nutrient uptake, carbohydrate breakdown, and enzymatic activity in coffee plants after low-temperature stress, helping the plants to overcome adverse conditions. Assessing the effect of foliar Se supply in coffee plants cultivated in field conditions, Mateus et al. [27] found that Se can protect the photosynthetic pigments and increase coffee bean yield. Moreover, Luo et al. [28] showed that Se increased photosynthetic parameters during OS in rice. Also, the same authors found that Se can promote a higher transcript level of antioxidant-related genes. However, Se concentrations in soils vary widely in the earth's crust. Selenium is an element that has several physiological and biochemical characteristics, such as the mitigation of different types of abiotic stress.

Selenium content in plant tissue is driven mainly by the soil Se content and the chemical interactions that this element undergoes in soils [29]. Tropical soils are generally considered Se-poor environments, i.e., have $\leq$0.5 mg $kg^{-1}$ Se [30], and the average Se concentration in soils worldwide is relatively low (~0.4 mg $kg^{-1}$) [31]. Indeed, researchers have found Se deficiency in soils across various countries, including Brazil. Gabos et al. [32] found that Se content in soils from the São Paulo State in Brazil ranges from <0.08 to 1.61 mg $kg^{-1}$, with a mean of 0.19 mg $kg^{-1}$.

Studies determining the most effective time to apply Se for achieving OS mitigation have previously been poorly investigated in the literature. Yet, plant supplementation using Se before stress has been responsible for triggering metabolic responses in plants, inducing a priming effect [33]. Priming effects were first used to describe the application technique of nutrient and/or plant biostimulants in seeds to increase their vigor during germination [34]. However, applying biostimulants, such as Se, has been considered a resistance inducer strategy in plants and can be thought as a promising strategy for crop production in response to future climate changes [35,36,37].

In this paper, the foliar application of such biostimulant element is also called “priming” due to the preparation effect that it can promote in the plants and its implication on metabolic responses before the stress [38]. However, exogenous Se applied post-stress can also be used as a last resource to alleviate the side effects of the lack of water in plants, but the effects of Se on these conditions need to be clarified. Thus, this study aimed to investigate the role of the Se supply in improving OS tolerance in coffee seedlings while also assessing the best time for Se application.

## 2 Materials and Methods

### 2.1 Plant Materials and Study Site

The experiment was carried out in a greenhouse using arabica coffee seedlings (*Coffea arabica* cv. Catuaí), one of Brazil's most traditional species. The cv. Catuaí is well known for its high beverage quality, good plant health, and high yield [66,67]. The plants used in the trial were at the age of 5-6 fully expanded leaves and were previously selected to keep uniformity and high health. The plants were provided by the National Institute of Science and Technology of Coffee (INCT Café).

The seedlings were produced in 1 L plant grow bags filled with subsoil + cattle manure at a ratio of 3:1, with 5 g of single superphosphate being added to each kilogram of the mixture. After the seedlings reached 5–6 fully expanded leaves, they were acclimated in a greenhouse at the Soil Science Department at the Federal University of Lavras (UFLA), located in Lavras, state of Minas Gerais, for 20 days. The greenhouse temperature was 25/15 ˚C day/night, and the relative humidity was 50/85% day/night. Irrigation was conducted daily with 80 mL of deionized water.

After the acclimation, the substrate was removed from each root system, and the plants were transferred into 1 L black plastic pots with nutritive solution [90]. The nutritive solution was composed of the following: 2 mM $NH_4H_2PO_4$, 6 mM $KNO_3$, 4 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 50 µM $H_3BO_3$, 10 µM $MnCl_2{\cdot}4H_2O$, 7.6 µM $ZnSO_4{\cdot}7H_2O$, 8 µM $CuSO_4{\cdot}5H_2O$, 0.40 µM $Na_2MoO_4$, 0.10 mM NaCl, 90 µM NaEDTA, and 89 µM $FeSO_4{\cdot}7H_2O$, as described by Kane et al. [91]. All plants underwent an acclimatization process for two weeks by applying a 20% and 40% ionic strength, respectively, for each week. After that, the plants were randomly selected to compose the treatments. In accordance with Salgado et al. [92], the plants were kept at 40% of the ionic strength until the end of the

trial. A polystyrene layer was used on top of the pots filled with nutritive solution to avoid algae growth into the nutrient solution. In addition, we used a system composed of an air compressor pump and clear PVC flexible tubing to keep the nutrient solution oxygenating during the experiment.

## 2.2 Experimental design and treatments

The experimental design was composed of a randomized block with seven treatments and four replicates. The treatments consisted of Se application through foliar supply on five different days compared with the day of induced osmotic stress to establish the best day to apply Se in coffee plants under induced OS. The treatments were: (i) eight days before induced osmotic stress (−8 BOS); (ii) four days before induced osmotic stress (−4 BOS); (iii) the same day of the induced osmotic stress (0 OS); (iv) four days after induced osmotic stress (+4 AOS); and (v) eight days after induced osmotic stress (+8 AOS). Two control treatments were also included: (vi) induced osmotic stress without Se (+OS-Se); and (vii) without stress and Se. Osmotic stress was induced using polyethylene glycol (PEG-6000). To induce the priming effect of Se foliar application and the alleviating effect, the treatments consisting of Se foliar application before the osmotic stress were called “priming treatments”, whereas plants treated after the osmotic stress were named “alleviated treatments”.

## 2.3 Application of foliar treatments

The foliar Se application was performed according to Sousa et al. [19]. In brief, the plants with Se application were sprayed with 5 mL of Se solution at 80 mg $L^{-1}$ Se + 0.5% v/v of mineral oil, and the remaining plants were sprayed with a mineral oil solution at the rate of 0.5% v/v. On the day of application, the plants were moved to the outer part of the greenhouse to avoid contaminating the remaining plants. The Se source was Na2SeO4-Sigma Aldrich, 98.9%.

## 2.4 Osmotic stress imposition and leaf water status

Polyethylene glycol with a molecular weight of 6000 (PEG-6000) was added to the nutritive solution according to Villela et al. [93] to induce the osmotic stress of −0.8 MPa. For

this, 261.95 g $L^{-1}$ of PEG-6000 was added into each plastic pot containing the nutritive solution in the respective treatment with stress. The osmotic potential was used based on previous tests with coffee considering the osmotic potential of −0.1; −0.2; −0.4; −0.6; −0.8; and −1.0 MPa. The osmotic potential of −0.8 MPa promoted a leaf water potential (Ψw) between −1.5–−2.5 MPa, considered moderate stress to the coffee plants [94]. The determination of the Ψw in each leaf was carried out with a Scholander pressure chamber (model 1000, PMS Instruments, Albany, NY, USA) [95] to confirm the leaf turgor on the day of sample collection and chlorophyll fluorescence parameter evaluation (Supplementary Figure S1). The parameters elasticity, osmotic potential, relative water content, and turgor loss point were derived from PV curves, according to Tyree and Hammel [96].

## 2.5 Leaf sample collection and preparation

All treatments' leaf sample collection and photosynthetic parameters were performed seven days after the last Se foliar application (+8 AOS). The second fully expanded pair of leaves from top to bottom was used to perform the non-invasive analysis of the photosynthetic parameters (MultispeQ®) [97]. After the measurement, the leaves were collected and immediately snap frozen in liquid nitrogen, individually macerated in liquid nitrogen, homogenized in a cooled mortar using 0.1 g of the antioxidant polyvinylpyrrolidone (PVPP), and stored at −80 ◦C. The frozen samples were used to determine the analyses of lipid peroxidation (MDA content), hydrogen peroxide (H2O2), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX).

The third and fourth fully expanded pairs of leaves from top to bottom were collected and washed three times with distilled water. All samples were dried at 65 ◦C for 72 h and were subjected to grinding in the Willey grinder. Ground samples were labeled and kept in air-tight plastic containers until they were used to quantify Se content, carbohydrates, protein, total free amino acids, and proline.

## 2.6 Determination of Examined Parameters

### 2.6.1 Selenium content in leaves and detection limit (LOD and LOQ)

The Se content in the leaves was performed according to the USEPA 3051A protocol (U.S. Environmental Protection Agency—USEPA) with modifications [98]. Briefly, 0.5 g of

dried leaf samples was digested with 5 mL of $HNO_3$ in a microwave (Mars 5, CEM Corporation, Matthews, NC, USA). To avoid foaming and splashing, the vessels were kept in a cool room with a controlled temperature for 30 min after the end of the digestion program and opened carefully, and the volume was made up to 50 mL with water. A blank and a certified reference material for Se (white clover, BCR402-IRMM) were included in each batch of samples. The Se content in the leaves was measured using GFAAS (graphite furnace atomic absorption spectrometry), atomic absorption spectrometry with Zeeman background correction, and an EDL lamp for Se; Analyst™ 800 AAS, Perkin Elmer. The detection and quantification limits (LOD and LOQ) were determined according to Silva Junior et al. [99]. The LOD and LOQ for Se were 2.49 and 8.32 µg $kg^{-1}$, respectively. The Se recovery rate in the reference material was 96.7% ± 1.28.

### 2.6.2 Carbohydrates, Total Protein, and Total Free Amino Acids

The extraction of carbohydrates (starch, sucrose, and reducing sugars), total free amino acids, and proteins was based on Zanandrea et al. [100]. Dried samples were weighed (0.2 g) and mixed with 5 mL of 100 mM potassium phosphate buffer (pH 7.0) and then warmed in a water bath at 40 ◦C for 30 min. The solution was centrifuged at 10,000× g for 20 min and the supernatant was collected. This procedure was performed twice, and the supernatant was combined, totaling 10 mL. The first supernatant sample was used to quantify the carbohydrates and total free amino acids. The pellet was resuspended and used for starch extraction, mixing 8 mL of potassium acetate buffer (200 mM; pH 4.8) and 2 mL of amyloglucosidase (1 mg $mL^{-1}$; 16 units of enzyme).

The contents of starch and sucrose were determined using the anthrone method as follows: 30 µL of the supernatant was mixed with 2 mL of the ice-cold anthrone reagent (0.84 g of anthrone in 1 L of 63% sulfuric acid), and the mixture was heated in a boiling water bath for 3 min and cooled in ice. Absorbance was measured at 620 nm [101]. Reducing sugars were quantified using the 3,5-dinitrosalicylic acid (DNS) method as follows: 150 µL of the supernatant was mixed with 0.5 mL of DNS solution (2.50 g of DNS in 50 mL of NaOH 2 M solution, 125 mL of distilled water, and 75 g of potassium sodium tartrate were heated using a water bath until completely dissolving and then diluted to 100 mL with distilled water) and 0.6 mL of distilled water. The mixture was heated in a boiling water bath for 5 min and water cooled. Absorbance was measured at 530 nm [102].

Total free amino acids were analyzed according to the ninhydrin method (0.2 mL of ninhydrin—5% w/w—in ethylene glycol monoethyl ether). For these measurements, 30 µL of the supernatant was mixed with 0.2 M citrate (pH 5.0), and 5% ninhydrin, 2% potassium cyanide, and 60% ethanol were added to the samples. Reactions were assessed using a spectrophotometer at 570 nm, and the results were compared with a standard curve of 0.1 µmol $mL^{-1}$ glycine [103]. The protein content was determined using the Bradford assay as described by Bradford [104], with BSA applied as a protein standard. The analyses were carried out in duplicate and were measured using an Epoch® Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA).

### 2.6.3 Proline

Proline content was estimated using the method described by Bates et al. [105]. Dried leaf samples (0.2 g) were weighed and macerated with 3% sulfosalicylic acid and heated in a water bath for 60 min at room temperature. After that, the samples were centrifuged at 10,000× g for 30 min. The supernatant (0.1 mL) was then mixed with 2 mL of acid ninhydrin (2.5g of ninhydrin in 40mL of phosphoric acid and 60mL of acetic acid) and determined using a colorimetric method (520 nm).

### 2.6.4 Antioxidant enzymes (SOD, CAT, APX, GR)

The extraction of antioxidant enzymes was based on Biemelt et al. [106]. Frozen leaf samples were weighted (0.2 g) and mixed with 1.5 mL of potassium phosphate buffer solution (0.1 mol $L^{-1}$, pH 7.8 + 0.1 mol $L^{-1}$ EDTA, pH 7.0, 0.01 mol $L^{-1}$ ascorbic acid, and 22 mg of PVPP). The solution was centrifuged at 13,000× g for 10 min at 4 °C. The enzymatic analyses' quality assurance and quality control were warranted using two blanks in each reading plate and operating the samples at 0–4 °C. In addition, the enzyme extraction was performed on the day of the analysis to avoid the oxidation of the enzyme extract. The analyses were carried out in triplicate and were measured using an Epoch® Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, United States). The supernatant was used to quantify the activity of superoxide dismutase (SOD, EC: 1.15.1.1), catalase (CAT, EC: 1.11.1.6), ascorbate peroxidase (APX, EC: 1.11.1.11), and glutathione reductase (GR, EC: 1.8.1.7).

The assay on SOD activity was performed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium at 560 nm [107]. Catalase (CAT) activity was assayed through measuring the rate of decomposition of $H_2O_2$ at 240 nm [108]. Ascorbate peroxidase (APX) was determined by reducing ascorbate at 290 nm [109]. Glutathione reductase (GR) was assayed according to the methodology proposed by Schaedle and Bassham [110] and adapted by García-Limones et al. [111].

### 2.6.5 Hydrogen Peroxide and Lipid Peroxidation (Malondialdehyde)

The frozen leaf tissue (0.2 g) was homogenized in 5 mL of trichloroacetic acid (TCA), and centrifuged at 12,000× g for 15 min at 4 °C. The supernatant was collected to quantify the hydrogen peroxide according to Velikova [112] with modifications [113]. Lipid peroxidation analysis was assayed from the content of malondialdehyde (MDA) using thiobarbituric acid (TBAR) according to Buege and Aust [114] and Silva et al. [75].

### 2.6.6 Chlorophyll fluorescence parameters (MultispeQ®)

The electron transport and electrochromic shift parameters were measured with the handheld unit MultispeQ® using the PhotosynQ web application (https://photosynq.org; accessed on 15 April 2021) according to Kuhlgert et al. [97]. The following parameters were measured: total electrochromic shift (ECSt); linear electron flow (LEF); total nonphotochemical quenching (NPQt); quantum yield of photosystem II (Phi2); quantum yield of non-regulated energy loss in PSII (PhiNO), quantum yield of regulated non-photochemical quenching in PSII (PhiNPQ), and a fraction of PSII centers which are in the open state (qL).

## 2.7 Statistical analysis and PCA

Generalized linear models (GLMs) were constructed to compare the treatments tested to each variable studied. The GLMs were used due to the non-uniformity of the residues of certain variables. After building the models, a Chi-squared test was performed to determine the differences that existed between treatments studied with the ANOVA function [115], complemented with multiple comparisons with the “ghlt function” [116]. The comparisons were carried out as follows: (I) Each treatment with water deficit was compared with the treatment without water deficit, and (II) comparison involved the different strategies of Se application compared with the cultivation with a water deficit. In addition, principal

component analysis (PCA) was performed to determine the relationships among several variables. The variables were selected according to the main effects observed in the univariate analysis, and to increase the explained variance in the PCA. All statistical analyses were performed with the R software [117] using the base, stats, nlme, multcomp, FactoMineR, and factoextra packages [118–120].

## 3 Results

### 3.1 Analysis of Se content

Selenium content in leaves was significantly increased by foliar application. There was a statistical difference observed between all the treatments with Se application and the controls without Se supply (Figure 1). The Se content in the control treatments was 0.37 and 0.38 mg $kg^{-1}$ DW for the treatments with stressed (+OS-Se) and non-stressed plants (−OS−Se), respectively. In contrast, the average Se content in the remaining treatments' leaves was 1.95 mg $kg^{-1}$ DW. The highest leaf Se content was found in the −4BOS treatment, i.e., 3.22 mg $g^{-1}$ DW, which corresponded to eight times the content analyzed in the control treatments.

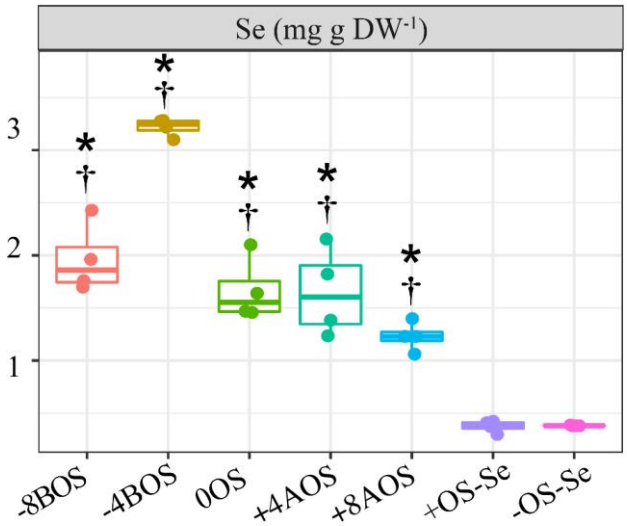


**Figure 1:** Leaf Se content as a result of Se application in Coffea arabica cv. Catuaí seedlings under osmotic stress induced with PEG-6000. The values displayed are the distribution of four replicates. Asterisks refer to the significant difference when comparing all treatments with non-stressed plants without Se supply (−OS-Se) ($p < 0.05$). Dagger refers to the significant difference when comparing all treatments with stressed plants without Se supply (+OS-Se) ($p < 0.05$). Treatments: −8BOS—

application of Se 8 days before stress (stressed plants); −4BOS—application of Se 4 days before stress (stressed plants); 0OS—application of Se on the day of stress occurrence (stressed plants); +4AOS—application of Se 4 days after stress (stressed plants); +8AOS—application of Se 8 days after stress (stressed plants); +OS-Se—without Se (stressed plants); and −OS-Se—without Se (non-stressed plants).

### 3.2 $H_2O_2$, MDA, and antioxidant enzymes (SOD, CAT, APX, GR)

There was no marked trend of OS on $H_2O_2$ and MDA, even when the control with stressed treatment was compared with the non-stressed one (Figure 2). In the $H_2O_2$ assays, even if OS is considered one of the main triggering agents of reactive oxygen species (ROS), there was no statistically significant difference observed between the treatments. On the other hand, the treatments with Se application −4BOS and +4AOS promoted higher values of MDA content, indicating that these treatments induced lipid peroxidation in the leaves.

When the control treatments were compared, OS significantly reduced the activity of GR and SOD, but did not affect the activities of APX and CAT (Figure 2), i.e., there was no significant difference observed between the +OS-Se and −OS-Se treatments.

The Se application 8 days before the plants were submitted to OS (−8BOS) promoted higher APX, CAT, and SOD activity levels than the treatment +OS-Se. The Se application at −8BOS increased the activity of these enzymes in the order of 356.3%, 228.5%, 771.1%, and 266.5% compared with +OS-Se for APX, CAT, and SOD, respectively. A reduction in the GR enzyme was noticed in the treatments 0OS; +4AOS; +8AOS; and +OS-Se compared with the treatment −OS-Se. Plants that were pre-treated with Se (−8 and −4BOS) displayed a higher GR content compared to the plants that had only received OS and no Se application. Furthermore, the levels of GR activity detected in the −8 and −4BOS treatments were found to be equivalent to those found in the non-stressed plants. (Figure 2).

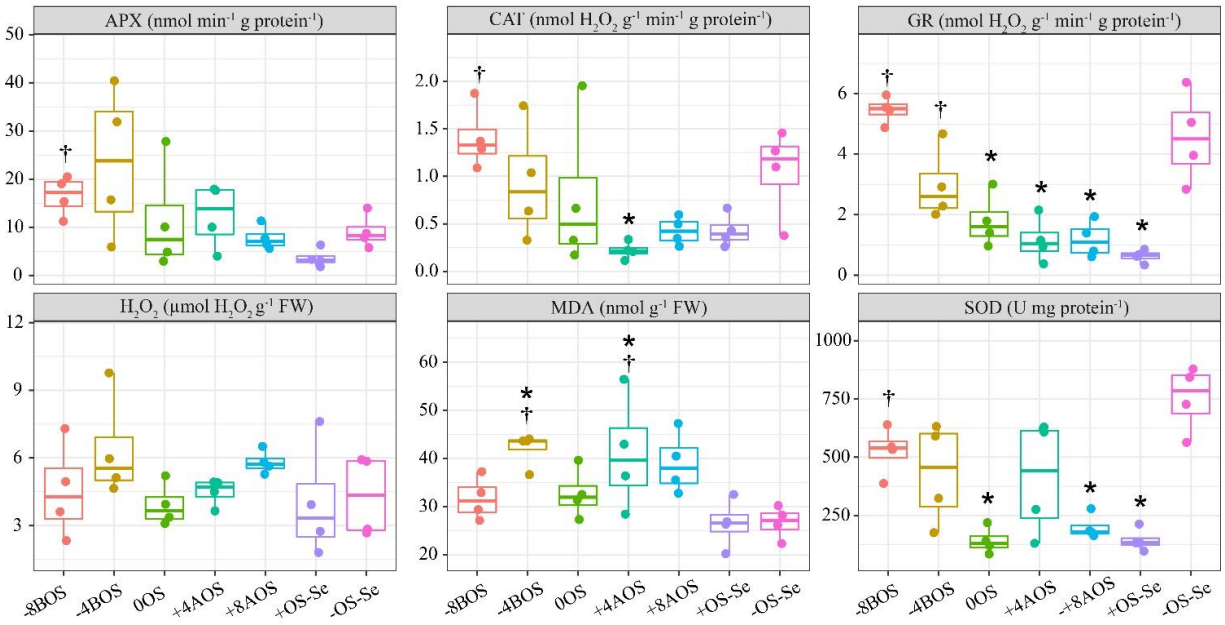


**Figure 2:** Hydrogen peroxide ($H_2O_2$), and lipid peroxidation (MDA) content, and activity of leaf antioxidant enzymes as a result of Se application in *Coffea arabica* cv. Catuaí seedlings under osmotic stress induced with PEG-6000. The values displayed are the distribution of four replicates. Asterisks refer to the significant difference when comparing all treatments with non-stressed plants without Se supply (−OS-Se) ($p < 0.05$). Dagger refers to the significant difference when comparing all treatments with stressed plants without Se supply (+OS-Se) ($p < 0.05$). Treatments: −8BOS—application of Se 8 days before stress (stressed plants); −4BOS—application of Se 4 days before stress (stressed plants); 0OS—application of Se on the day of stress occurrence (stressed plants); +4AOS—application of Se 4 days after stress (stressed plants); +8AOS—application of Se 8 days after stress (stressed plants); +OS-Se—without Se (stressed plants); and −OS-Se—without Se (non-stressed plants).

### 3.3 Carbohydrates, Protein, Amino Acids, Proline

Regardless of the Se supply and OS, the total free amino acids, reducing sugars, and sucrose content were unaffected (Figure 3). On the other hand, Se foliar supply increased, to some extent, the proline and protein content. The proline content obtained with the application of Se at −4BOS and +8AOS was significantly higher than that observed for the stressed plants without Se (+OS-Se). Hence, the Se supplementation could be seen as a strategy to increase these compounds in coffee leaves under OS.

On the other hand, the imposed OS affected the starch content, with all the treatments submitted to the stress showing lower starch content compared with the absolute control treatment (−OS-Se). However, all the treatments with Se application promoted lower starch content than the positive control treatment (+OS-Se), except for the treatment with the application on the day on which the stress was imposed (0OS) (Figure 3). Such results indicate that OS can reduce the starch content, but the Se supply can impose a lower starch content than that detected in plants subjected to OS without Se.

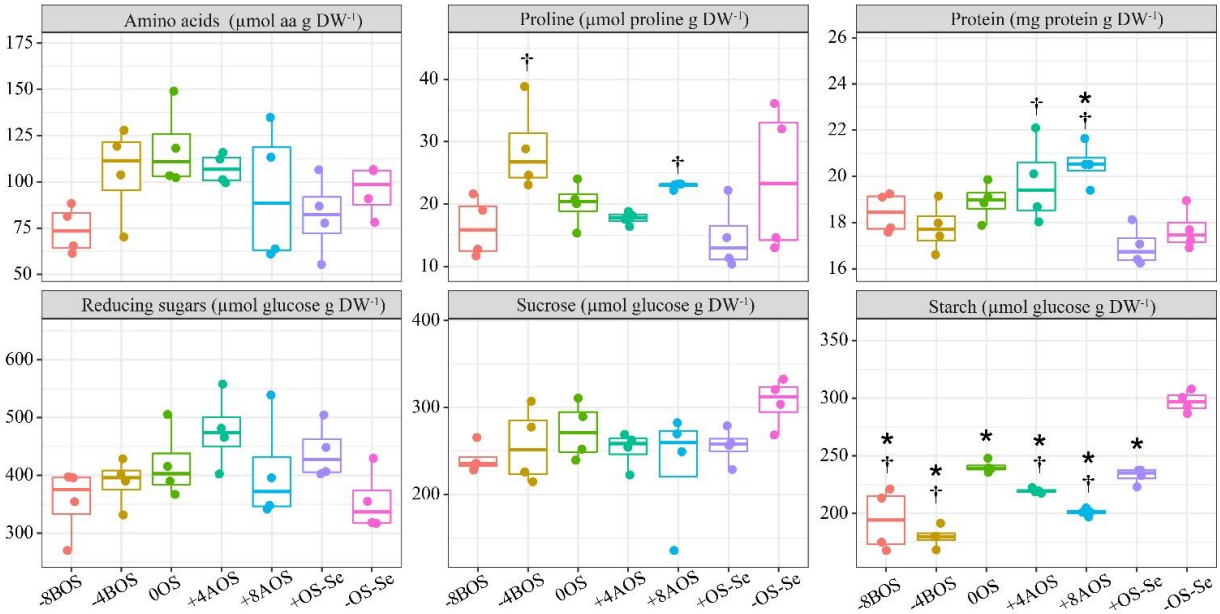


**Figure 3:** Total free amino acids (AA), proline (Pro), carbohydrates, and protein (Prt) as a result of Se application in *Coffea arabica* cv. Catuaí seedlings under osmotic stress induced with PEG-6000. The values displayed are the distribution of four replicates. Asterisks refer to the significant difference when comparing all treatments with non-stressed plants without Se supply (−OS-Se) ($p < 0.05$). Dagger refers to the significant difference when comparing all treatments with stressed plants without Se supply (+OS-Se) ($p < 0.05$). Treatments: −8BOS—application of Se 8 days before stress (stressed plants); −4BOS—application of Se 4 days before stress (stressed plants); 0OS—application of Se on the day of stress occurrence (stressed plants); +4AOS—application of Se 4 days after stress (stressed plants); +8AOS—application of Se 8 days after stress (stressed plants); +OS-Se—without Se (stressed plants); and −OS-Se—without Se (non-stressed plants).

### 3.4 Chlorophyll Fluorescence parameters (MultispeQ®)

The graph of chlorophyll was inserted as supplementary data (Supplementary Data, Figure S2). There was no statistically significant difference observed regarding the ECSt, Phi2, PhiNO, PhiNPQ, and qL. The supply of selenium on the same day the plants were submitted to the OS (0OS) and 8 days after the plants were submitted to the OS increased the LEF—linear electron flux—compared with the treatment without Se supply and OS (Supplementary Data, Figure S2). The Se application at +4AOS promoted the highest NPQt, showing that, in a certain way, Se can act to quench the excess of light energy.

### 3.5 Principal component analysis (PCA)

The variables reducing sugars, total free amino acids, sucrose, ECSt, Phi2, PhiNO, PhiNPQ, and qL were excluded in the PCA analysis as they all exhibited a low effect of the treatments, as shown in the univariate analysis. Furthermore, the addition of these variables to

the PCA reduced the explanation of the variables to 43.1%. The contribution of the selected variables is shown in the Supplementary Data (Table S1).

Results of the PCA are shown in Figure 4. The PCA explained 64.8% of the data variance, with the first axis (PC1) explaining 45.0%, and the second axis (PC2) 19.8%. The PC1 was affected mainly by APX, SOD, and starch, while the values of GR, CAT, MDA, proline, and protein were explained by the PC2 (Supplementary Table S1). The Se content in leaves showed a significant correlation with APX and SOD, but a low correlation with starch (Figure 4). This behavior was also noticed in the correlation matrix (Supplementary Table S1), in which Se and APX showed a positive and statistically significant correlation ($R^2$ = 0.60, $p < 0.05$) and a negative and statistically significant correlation with starch ($R^2 = -0.69$, $p < 0.05$) (Supplementary Figure S4).

The biplot correlation clusters clearly distinguished the treatments and their respective correlations (Figure 4). The treatment −8BOS showed a clear correlation with GR and CAT, corroborating the previously shown results (Figure 2). The biplot correlation clusters also revealed a strong correlation of Se, APX, and SOD with the treatment related to the previous Se application 4 days before the stress (−4BOS).

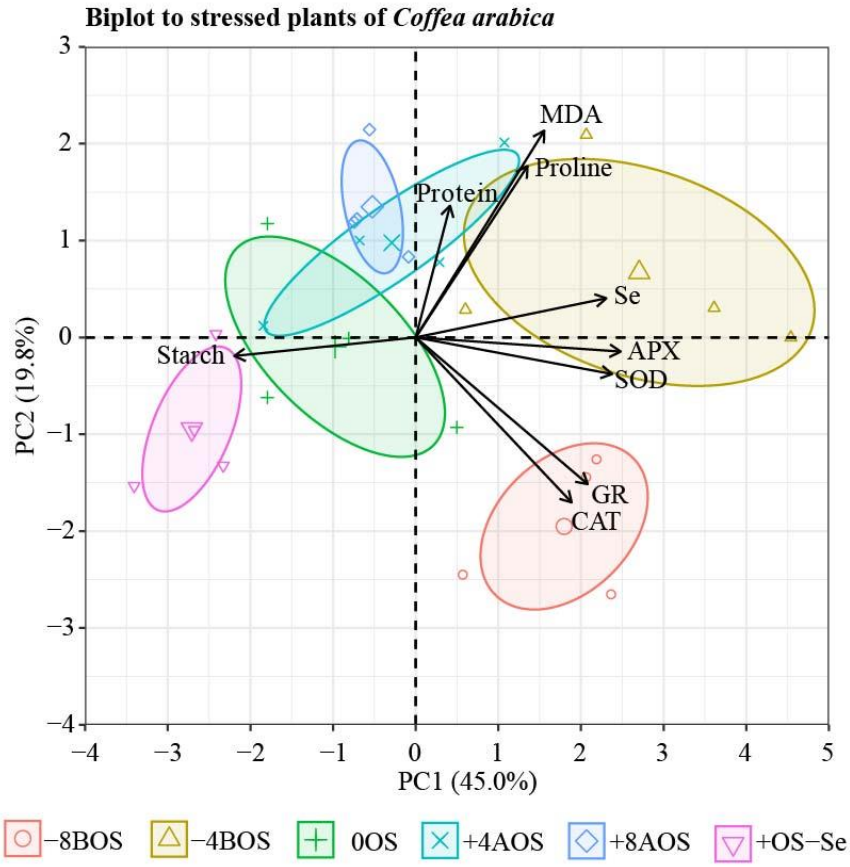

**Figure 4:** Principal component analysis (PCA) of leaf compounds and Se content in leaves. The leaf attributes included were leaf Se content (Se); ascorbate peroxidase (APX); superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR); proline; protein; lipid peroxidation (MDA), and starch. Arrows represent the contribution of leaf compounds on the principal component axes. Treatments: −8BOS—application of Se 8 days before stress (stressed plants); −4BOS—application of Se 4 days before stress (stressed plants); 0OS—application of Se on the day of stress occurrence (stressed plants); +4AOS—application of Se 4 days after stress (stressed plants); +8AOS—application of Se 8 days after stress (stressed plants); +OS-Se—without Se (stressed plants); and −OS-Se—without Se (non-stressed plants).

## 4 Discussion

Osmotic stress encompasses stress-induced decreasing water potential (Ψw) in plant cells [39]. Considering that the water flow moves towards the lowest Ψw, if the stress continues, the leaves start to lose water, reflecting in the Ψw in the leaf [40]. As a typical response to low water potential, the leaves of the coffee plants in this trial started to become wilted and flabby 5 days after the imposed stress, leading to leaf prostration due to the turgor loss during abiotic stress. Osmotic stress tolerance involves the maintenance of the plant's water status and, hence, cell turgor. This condition may be achieved through stomatal regulation, decreasing transpiration loss or osmotic adjustment with the accumulation of osmoprotective substances, such as proline, glycine betaine, soluble proteins, and sugars, which help plants conserve their water status [41].

The results of Ψw (Supplementary Data, Figure S1) showed that all plants treated with PEG-6000 suffered from OS. The effect of OS is also illustrated in Figure 5. OS imposed mild stress in the treatments −8BOS, −4BOS, 0OS, and +OS-Se (Ψw from −1.5 to −2.5MPa). Meanwhile, the treatments +4AOS and +8AOS were subjected to severe stress (Ψw > −2.5 MPa) (Supplementary Data, Figures S1 and S3). According to Suma [42], non-susceptible plants can keep a minor reduction in Ψw (6.9%) compared with a higher reduction (14.4%) in susceptible genotypes of finger millet. Then, after the plants were submitted to the stress, the Se application may have acted as a stressor in coffee plants, leading those plants to higher water potential loss and potentializing the OS response.


**Figure 5:** *Coffea arabica* cv. Catuaí seedlings under osmotic stress induced by PEG-6000 and Se foliar application. Treatments: -8BOS - Application of Se 8 days before stress (Stressed plants); -4BOS - Application of Se 4 days before stress (Stressed plants); 0OS - Application of Se in stress (Stressed plants); +4AOS - Application of Se 4 days after stress (Stressed plants); +8AOS - Application of Se 8 days after stress (Stressed plants); +OS-Se - Without Se (Stressed plants); -OS-Se - Without Se (Non-stressed plants).

Plants treated with Se at all times had a higher relative water content (RWC) than the controls, including at the turgor loss point ($RWC_{tlp}$), whereas the turgor loss point ($\pi_{tlp}$) was less negative in all the same plants. The $\pi_{tlp}$ indicates the cell water potential inducing turgor pressure loss, which is crucial to maintain gas exchange and plant growth. Plants with a low $\pi_{tlp}$ tend to maintain stomatal and hydraulic conductance, photosynthetic efficiency, and growth at a lower external water potential [43]. This parameter is thus correlated with the ability to tolerate stress rather than avoid it.

Although it is considered that a more negative $\pi_{tlp}$ improves drought tolerance, as described above, it is also suggested that a less negative $\pi_{tlp}$ may be helpful, as it enables leaves to lose turgor quickly and close their stomata, and thereby maintain a high $RWC_{tlp}$ [44]. This response pattern was observed in our study. Plants treated with Se showed a 20% higher $RWC_{tlp}$ than untreated plants. According to DaMatta and Ramalho [7], coffee leaves usually have a high $RWC_{tlp}$, regardless of water availability, to avoid stress rather than tolerate it. For the authors, this seemed to be more related to stomatal regulation and gas exchange maintenance than turgor. We suggest that in our experiment, Se helped the stomatal regulation in coffee plants under osmotic stress as a strategy to decrease transpiration rates. Similar results were related to yellow sweet clover under OS and Se addition [45].

A high $RWC_{tlp}$ formed despite very low water potential is generally correlated with osmotic adjustment. However, our study did not observe an increase in the concentration of

proline or soluble sugars as a standard response to stress or Se application (Figure 3). Furthermore, it has been reported for coffee leaves that the accumulation of proline and other solutes does not always correlate well with OS tolerance [7]. In our study, the application of Se 4 days before and 8 days after stress (−4BOS and +8AOS) seemed to have stimulated an osmotic adjustment due to the combination of a very low water potential, high relative water content, and proline accumulation concerning the controls (with and without stress). The high $RWC_{tlp}$ in all treatments with Se application can be better explained by stomatal regulation, as mentioned before.

Stomatal closure in response to stress might limit $CO_2$ absorption by the leaves. In our study, photosystem II efficiency showed no change in response to stress or Se (Supplementary Data, Figure S2). Associated with the fact that the plants did not show a reduction in growth, we can conclude that there was no photochemical limitation in photosynthesis. In line with this, we also observed no alteration in soluble sugars or sucrose in response to stress, suggesting no significant chemical limitations (Figure 3). Only starch was reduced in response to stress and Se application.

In photosynthetic cells, starch is mostly synthesized using a fraction of the $CO_2$-fixed carbon and temporarily stored in the chloroplast called “transitory”. The transitory starch is usually synthesized during the day and consumed at night to provide a constant flow of carbon and energy without photosynthesis [46]. Starch is considered the major carbohydrate storage in plants [47]. In stressful conditions, starch represents a pool of energy that can induce metabolic responses and help plants overcome harmful circumstances. It can be broken down into low molecular weight compounds. Starch degradation can be stimulated in response to osmotic stress to promote osmotic adjustment, which might explain the response to treatments in which OS was imposed. In addition to this, a noteworthy factor is that abscisic acid (ABA) biosynthesis is the primary signal for starch degradation in response to osmotic stress [48].

An improvement of carbohydrate metabolism and water status caused by Se application has been found by Rady et al. [22] in tomato plants. According to these authors, Se has been correlated with elevated activity levels of the antioxidative defense system components — both enzymatic and non-enzymatic — under an insufficient water supply. Furthermore, increased levels of osmoprotectants have been associated with a higher cellular relative water content and membrane stability index, resulting in reduced electrolyte leakage, lipid peroxidation, and oxidative stress biomarkers.

In the extensive literature survey conducted by Thalmann et al. [47], the authors discovered that in 23 of the 36 studies considered, leaf starch content was said to decrease in response to abiotic stress, regardless of the species assessed. This result highlights the importance of starch in providing energy to deal with abiotic stresses. Then, the starch catabolism displaces carbons to produce osmoprotectants that induce osmotic adjustments and stabilize proteins [49,50], and also promotes signals that induce stress responses [47]. Our findings are in line with the research conducted by Lee et al. [51], who also observed a notable reduction in starch content in white clover leaves when exposed to OS. This reduction in starch content has been believed to be part of the adaptation mechanism that enables rice plants to carry out basal metabolism, thereby countering the changes induced by OS in photosynthesis.

The fact that Se application caused a more substantial reduction in starch content in coffee leaves under OS led us to the hypothesis that reduced starch accumulation during OS may be a plant strategy to maintain the flow of carbon and energy availability for growth during the harmful condition (Figures 3 and 4) [52]. This assumption is supported by Malik et al. [53], who showed that the presence of Se stimulates a significant rise in α-amylase and β-amylase activity in mungbean, ultimately leading to the hydrolysis of starch.

The higher Se content in the plants supplied with Se was expected, since Se supplementation in coffee plants via foliar application (and other plant species) has been studied in the literature [19,27]. Selenium can be supplied via seed, soil, and foliar application routes [54,55]. However, when applied at the same rate, foliar applications have been considered the most efficient way to increase Se content in plant tissue [54,56,57]. Since an active chemical chain builds the Se assimilation pathway, the addition of Se to stressed plants (+4AOS and +8AOS) possibly consumed the energy used to trigger metabolic responses that was supposed to be used to overcome the stress, making the plants unable to keep the Ψw at higher levels in the leaves.

Despite the beneficial effects of Se having been detailed in the literature, it can be toxic depending on the tissue levels and plant health condition [58,59]. Due to the chemical similarity of Se and S, selenate ($SeO_4^{-}$) is transported into the plants through sulfate transporters [21,60]. Since it is inside the plant cell, it is metabolized in the plastids via the sulfur assimilation pathway to selenocysteine (SeCys) or selenomethionine (SeMet) [61,62]. Se-$SeO_4^{-}$ is first assimilated by an active form via the enzyme adenosine triphosphate sulfurylase (APS) and APS-reductase (APR). Adenosine triphosphate sulfurylase binds selenate with adenosine triphosphate (ATP) to form adenosine 5′-phosphoselenate (APSe).

After that, APSe is reduced to selenite by APR [20,21,63]. Selenite is then converted into SeCys and available to be converted into other organic compounds — like SeMet and proteins — or stocked in the vacuoles [64]. Notably, these Se-amino acids serve as precursors of ethylene, and the production of this phytohormone is enhanced under stress conditions collaborating with stomatal closure [45,65].

Excess organic Se, such as SeMet and SeCys, might cause toxicity to plant cells by forming malformed selenoproteins due to the replacement of Cys/Met with SeCys and SeMet in the peptide chain. Changing between Cys and SeCys changes the cellular protein's structure by replacing the disulfide bond with a diselenide bond, which affects the peptide chains redox potentials [66]. Protein function might be compromised if the organic selenocompounds are non-specifically integrated into proteins in place of their sulfur (S) equivalents. This condition might trigger the plants' negative responses and osmotic imbalances [67–69]. This result is also supported by the protein content in the leaves of the treatments +4AOS and +8AOS, in which the protein content was higher than in the stressed plants without Se supply (+OS-Se) (Figure 3).

Several studies have shown the positive effect of Se on increasing antioxidant enzyme activities [27,70]. This wide antioxidant capacity is due to the promotion of the selenoproteins and the enzyme cofactor role. These compounds enhance the antioxidant enzymes, such as glutathione peroxidase (GPX) and glutathione reductase (GR), which combat ROS during plant osmotic imbalance under stressful situations. The positive correlation between Se and GPX has been described and implicated in the presence of Se-dependent GPX [71,72]. It may be an osmoprotective strategy to mitigate the harmful effects of abiotic stresses, such as drought [18,26], salinity [73], heavy metals [74], and low temperature [19].

Indeed, in this trial, GR was the enzyme that better responded to the application of Se, and only the treatment −8BOS was able to increase the content of APX, CAT, SOD, and GR at the same time. This result shows that prior Se supply is the best way to induce antioxidant activity to trigger metabolic responses to ROS while also stimulating priming responses against the upcoming oxidative stress. These results corroborated those of Silva et al. [75], who also found that Se foliar application can provide an enhanced antioxidant metabolism by increasing superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activity.

The major members of the ROS family include free radicals, like $O^{\bullet -2}$, OH•, and non-radicals, like $H_2O_2$ and $O_2$, and they are continuously produced at basal levels under favorable conditions. Under this condition, their potential to cause harm is neutralized through various

antioxidant mechanisms that scavenge them [76]. However, ROS can be produced in excess when plants suffer from long-term stress, promoting serious damage to the cells by inhibiting proteins, DNA synthesis, and other metabolic pathways [77].

In the ROS detoxification process, SOD is considered the first line of defense because it is responsible for converting the superoxide radical ($O^{2-}$) into hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$) and thus reduces the risk of hydroxyl radical formation. As a second pathway to scavenge ROS in the cell, CAT catalyzes the dismutation of $H_2O_2$ into $H_2O$ and $O_2$; meanwhile, APX and GR also help to scavenge the $H_2O_2$ into $H_2O$ using ascorbic acid (AA) and glutathione as a reducing agent [76,77].

The improvement of the enzymatic antioxidant system has been responsible for mitigating different abiotic stresses. For example, heavy metal exposure tends to induce the production of excessive ROS, which interact with macromolecules, such as DNA, proteins, and lipids, leading to a series of vicious processes together. These changes can alter cellular redox equilibrium and redox homeostasis [78]. A moderate exposure to lead (Pb) increased leaf SOD (251%), CAT (60%), and APX (537%) compared with the control [79]. The authors attributed this result to tentative plant metabolic changes to trigger key antioxidant enzyme responses to resist oxidative damage.

In a vast literature review, Rajput et al. [80] pointed that the transgenic overexpression of different genes might improve the enzymatic activity in plants and increase their stress tolerance to adverse conditions. According to these authors, a specific gene from *Sedum alfredii* is responsible for increasing Cu/Zn-SOD activity, conferring Cd tolerance in *Arabidopsis* [81]. In another study, the gene SiCSD from *Saussurea involucrate* increased drought and cold tolerance in transgenic tobacco by promoting higher activities of SOD, CAT, and APX [82]. Overexpression of the ascorbate peroxidase gene (AgAPX1) from *Apium graveolens* enhanced ascorbate content, antioxidant capacity, and drought resistance in transgenic *Arabidopsis* [83].

Other non-enzymatic pathways have also been responsible for mitigating abiotic stresses by increasing the antioxidant system. Amino acids, proline, carbohydrates, and certain fungicide responses in plants have been attributed to stress alleviation in plants. In *Arabidopsis thaliana* leaves under drought stress, Sperdouli and Moustakas [84] reported the buildup and interaction of proline, anthocyanins, and soluble sugars retaining a strong antioxidant defense. In response to osmotic stress, soluble carbohydrates are synthesized, acting as osmoprotectants that stabilize cellular membranes and sustain turgor, avoiding

overstress by ROS [85]. Proline operates as a non-enzymatic antioxidant by obtaining OH· through the H- on its amine group and is further decarboxylated [86].

Additionally, a recent study showed the effect of fungicides acting as a non-enzymatic antioxidant in lettuce [87]. The authors showed that the fungicide named fluazinam and its mixtures induced diversified changes in plant defense to increase ROS scavenging in lettuce. In this trial, the processes of fungicide degradation induced the activation of antioxidant enzymes (CAT, POD, and SOD), also inducing an antioxidant response in the plants.

The effects of Se on the antioxidant system of plants under abiotic stresses have been extensively explored as the primary regulator of plant growth and yield under these conditions [59]. This condition was evident in our study, in which most of the found results can be explained by factors related to the antioxidant metabolism of the plant (Figure 4). What has also been well discussed is how, and to what extent, different doses of Se in other species, plant organs, and developmental stages affect plant metabolism.

## 5 Conclusions

OS induced with PEG-6000, imposed significant stress on the *Coffea arabica* cv. Catuaí, promoting an imbalance in the water relationship. At the same time, OS reduced the GR and SOD activity compared with the control treatment. Selenium foliar supply revealed great potential for reducing the adverse effects of OS as a priming strategy 8 days before stress, improving the water relations, increasing the enzymatic activity (GR, SOD, and CAT), and potentiating the starch degradation under stress conditions. These findings also assist decision makers in how to deal with a foreseen drought in coffee plantations, where the earlier administration of foliar Se aids in the setting up of metabolic reactions that help the plants to combat the stress caused by a shortage of water. On the other hand, the post-stress application seems to impose extra stress on the plants, leading them to reduce their water potential. These results might support new nutritional strategies to induce stress responses in plants, leading to better plant development and sustainable crop production. To elucidate the role of Se on triggering metabolic responses in plants under OS, we suggest that other studies should be conducted to assess: (i) combined Se application with other nutrients; (ii) genetic assays; (iii) cross-species testing; and, (iv) long-term effects of Se in plants.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12173026/s1, Figure S1: Water potential (WP)

of coffee leaves as a result of Se application in *C. arabica* cv. Catuai seedlings under osmotic stress induced by PEG-6000. The values displayed are the distribution of four replicates. Treatments: -8BOS – application of Se 8 days before stress (stressed plants); -4BOS - application of Se 4 days before stress (stressed plants); 0OS - application of Se on the day of stress occurrence (stressed plants); +4AOS - application of Se 4 days after stress (stressed plants); +8AOS - application of Se 8 days after stress (stressed plants); +OS-Se - without Se (stressed plants); -OS-Se - without Se (non-stressed plants); Figure S2: Total electrochromic shift (ECSt), linear electron flow (LEF), total non-photochemical quenching (NPQt), quantum yield of photosystem II (Phi2), quantum yield of non-regulated energy loss (PhiNO), quantum yield of regulated non-photochemical energy loss in PSII (PhiNPQ), and fraction of PSII centers which are in the open state (qL) as a result of Se application in *C. arabica* cv. Catuai seedlings under osmotic stress induced by PEG-6000. The values displayed are the distribution of four replicates. Asterisks refer to the significant difference when comparing all treatments with non-stressed plants without Se supply (-OS-Se) ($p < 0.05$). Dagger refers to the significant difference when comparing all treatments with stressed plants without Se supply (+OS-Se) ($p < 0.05$). Treatments: -8BOS - application of Se 8 days before stress (stressed plants); -4BOS - application of Se 4 days before stress (stressed plants); 0OS - application of Se on the day of stress occurrence (stressed plants); +4AOS - application of Se 4 days after stress (stressed plants); +8AOS - application of Se 8 days after stress (stressed plants); +OS-Se - without Se (stressed plants); -OS-Se - without Se (non-stressed plants); Figure S3: Elasticity (E), osmotic potential (Osm), relative water content at turgor loss point ($RWC_{TLP}$), and turgor loss point (TLP) of coffee leaves as a result of Se application in *C. arabica* cv. Catuai seedlings under osmotic stress induced by PEG-6000. The values displayed are the distribution of four replicates. Treatments: -8BOS - application of Se 8 days before stress (stressed plants); -4BOS - application of Se 4 days before stress (stressed plants); 0OS - application of Se on the day of stress occurrence (stressed plants); +4AOS - application of Se 4 days after stress (stressed plants); +8AOS - application of Se 8 days after stress (stressed plants); +OS-Se - without Se (stressed plants); -OS-Se - without Se (non-stressed plants); Table S1: Contributions on the first two PC axes of i) all variables and ii) reduced number of variables; Figure S4: Correlation matrix showing Pearson's correlation of physiological, biochemical, and nutritional parameters of *Coffea arabica* seedlings. The leaf attributes were leaf Se content (Se); ascorbate peroxidase (APX); superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR); proline; protein; lipid peroxidation (MDA), and starch.

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**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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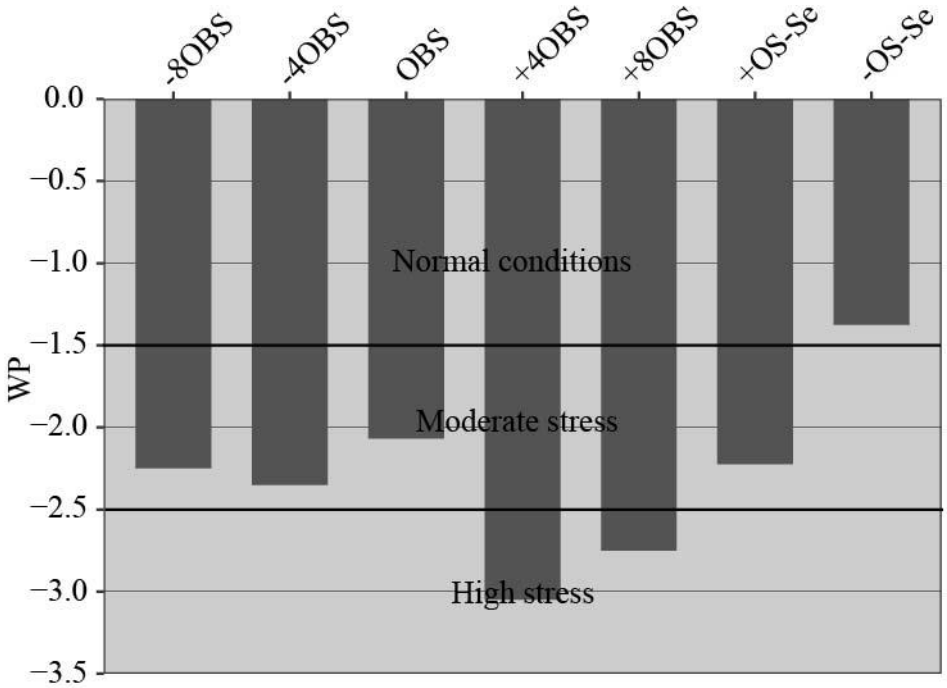
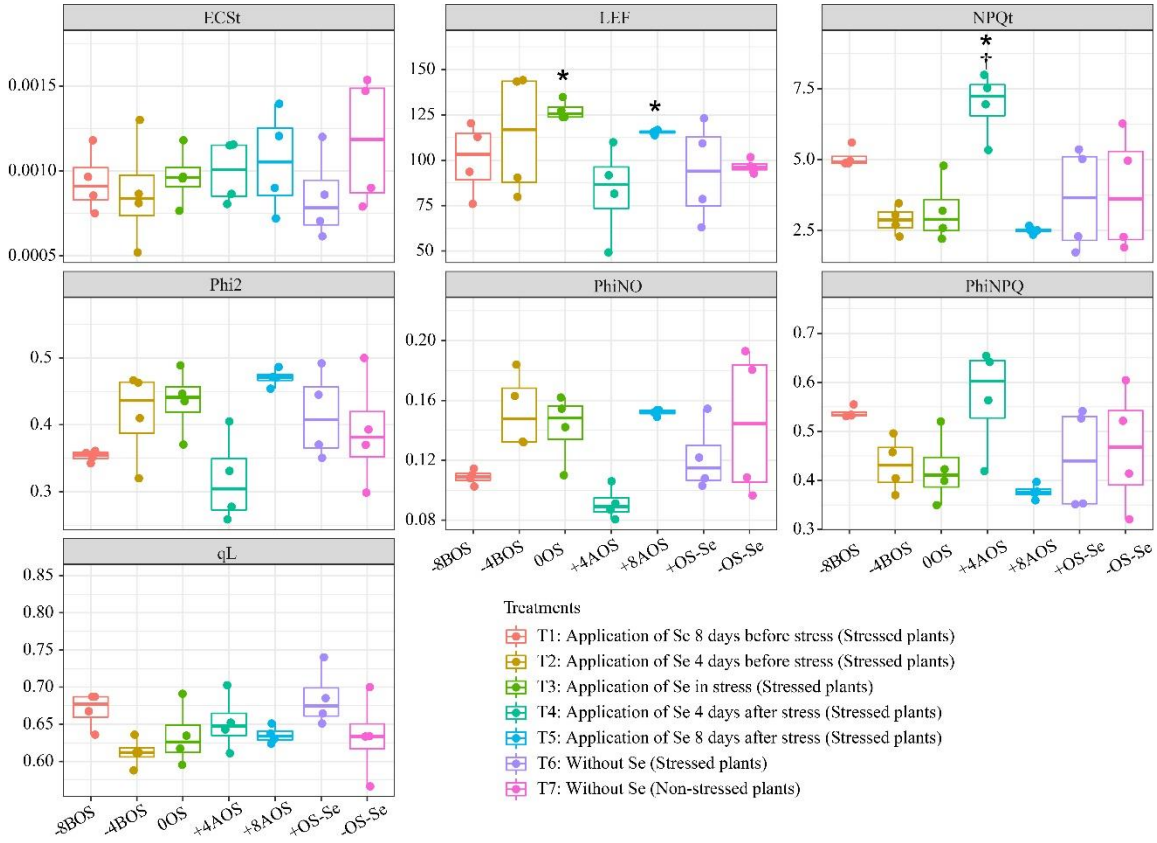
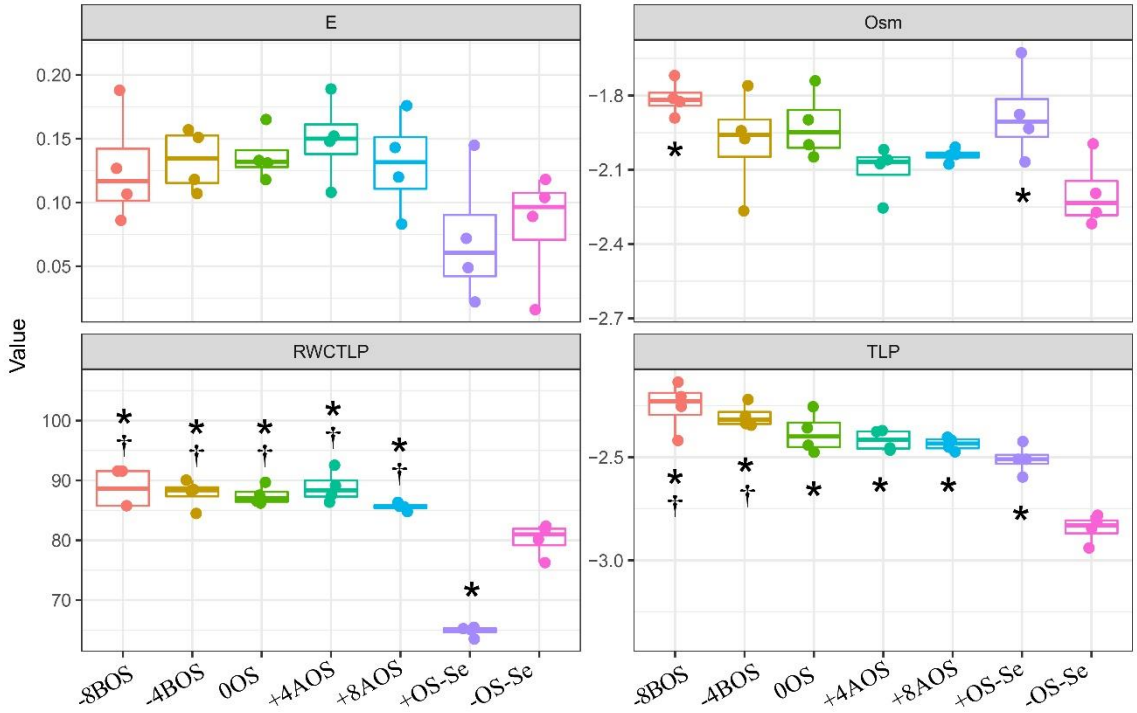
**Supplementary material**




**Supplementary figure 1:** Water potential of coffee leaves as a result of Se application in *C. arabica* cv. Catuai seedlings under osmotic stress induced by PEG-6000. The values displayed are the distribution of four replicates. Treatments: -8BOS - Application of Se 8 days before stress (Stressed plants); -4BOS - Application of Se 4 days before stress (Stressed plants); 0OS - Application of Se in stress (Stressed plants); +4AOS - Application of Se 4 days after stress (Stressed plants); +8AOS - Application of Se 8 days after stress (Stressed plants); +OS-Se - Without Se (Stressed plants); -OS-Se - Without Se (Non-stressed plants).




**Supplementary figure 2:** Total electrochromic shift (ECSt), total flow of electrons (LEF), total flow of electrons (NPQt), quantum yield of PSII (Phi2), quantum yield of non-regulated energy loss in PSII (PhiNO), quantum yield of regulated non-photochemical energy loss in PSII (PhiNPQ), and fraction of PSII centers which are in the open state (qL) as a result of Se application in *C. arabica* cv. Catuai seedlings under osmotic stress induced by PEG-6000. The values displayed are the distribution of four replicates. Asterisks refer to the significant difference when comparing all treatments with non-stressed plants without Se supply (-OS-Se) ($p < 0.05$). Dagger refers to the significant difference when comparing all treatments with stressed plants without Se supply (+OS-Se) ($p < 0.05$). Treatments: -8BOS - Application of Se 8 days before stress (Stressed plants); -4BOS - Application of Se 4 days before stress (Stressed plants); 0OS - Application of Se in stress (Stressed plants); +4AOS - Application of Se 4 days after stress (Stressed plants); +8AOS - Application of Se 8 days after stress (Stressed plants); +OS-Se - Without Se (Stressed plants); -OS-Se - Without Se (Non-stressed plants).




**Supplementary figure 3:** Elasticity (E), Osmotic potential (Osm), Relative water content at turgor loss point ($RWC_{TLP}$), and turgor loss point (TLP) of coffee leaves as a result of Se application in *C. arabica* cv. Catuai seedlings under osmotic stress induced by PEG-6000. The values displayed are the distribution of four replicates. Treatments: -8BOS - Application of Se 8 days before stress (Stressed plants); -4BOS - Application of Se 4 days before stress (Stressed plants); 0OS - Application of Se in stress (Stressed plants); +4AOS - Application of Se 4 days after stress (Stressed plants); +8AOS - Application of Se 8 days after stress (Stressed plants); +OS-Se - Without Se (Stressed plants); -OS-Se - Without Se (Non-stressed plants).

| Variables | i) Contributions of all variables | | | | Variables | ii) Contributions of reduced number of variables | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PC1 | PC2 | PC3 | PC4 | | PC1 | PC2 | PC3 | PC4 |
| PL | 7.335 | 0.739 | 10.392 | 7.945 | PL | 7.064 | 30.181 | 4.701 | 3.123 |
| PH | 9.152 | 0.327 | 1.796 | 1.335 | PH | - | - | - | - |
| APX | 8.232 | 5.634 | 3.123 | 4.584 | APX | 17.959 | 0.142 | 0.501 | 4.211 |
| CAT | 3.630 | 2.789 | 18.581 | 0.003 | CAT | 10.394 | 19.244 | 0.948 | 23.059 |
| GR | 3.028 | 7.442 | 8.588 | 4.391 | GR | 12.638 | 15.202 | 0.529 | 0.233 |
| SOD | 3.646 | 14.082 | 0.767 | 0.082 | SOD | 16.491 | 0.950 | 7.555 | 22.579 |
| RS | 2.700 | 0.267 | 0.016 | 21.803 | RS | - | - | - | - |
| AA | 1.567 | 0.003 | 10.418 | 5.192 | AA | - | - | - | - |
| Pro | 11.854 | 0.405 | 8.790 | 0.526 | Pro | 5.343 | 20.702 | 22.985 | 6.861 |
| TSS | 3.719 | 0.001 | 9.315 | 13.493 | TSS | - | - | - | - |
| Starch | 9.582 | 1.860 | 1.278 | 2.334 | Starch | 14.069 | 0.245 | 2.407 | 20.424 |
| Prt | 0.432 | 0.007 | 3.170 | 10.096 | Prt | 0.505 | 12.252 | 48.321 | 13.277 |
| Sac | 0.092 | 0.051 | 6.206 | 18.417 | Sac | - | - | - | - |
| Se | 11.420 | 1.475 | 0.534 | 0.403 | Se | 15.536 | 1.081 | 12.053 | 6.231 |
| ECSt | 0.132 | 0.196 | 0.536 | 1.079 | ECSt | - | - | - | - |
| LEF | 3.474 | 4.826 | 3.183 | 4.346 | LEF | - | - | - | - |
| NPQt | 3.443 | 13.771 | 4.769 | 0.419 | NPQt | - | - | - | - |
| Phi2 | 1.225 | 15.843 | 1.842 | 0.002 | Phi2 | - | - | - | - |
| PhiNO | 5.915 | 10.830 | 3.444 | 0.195 | PhiNO | - | - | - | - |
| PhiNPQ | 1.193 | 16.279 | 3.247 | 0.246 | PhiNPQ | - | - | - | - |
| qL | 8.229 | 3.174 | 0.003 | 3.108 | qL | - | - | - | - |

**Supplementary table 1**: Contributions on the first two PC axes of i) all variables and ii) reduced number of variables.

## GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

During the winter of 2019, an unforeseen cold wave hit coffee crops in the Southeast region of Brazil, leading growers to be cautious in managing this crop. Although the cold damage at that time was not severe enough to cause a significant decline in coffee production, it raised concerns. In 2020, a severe frost wave hit South America, including the coffee-growing regions in Brazil. These consecutive events prompted us to assess selenium application in coffee plants under low-temperature stress, which resulted in the first paper of this thesis (Manuscript I - Selenium enhances chilling stress tolerance in coffee species by modulating nutrient, carbohydrates, and amino acid content).

In addition to the cold waves, the coffee crop also experienced low precipitation in the commonly cultivated areas of Brazil in 2019, 2020, and 2021, resulting in a decline in yield. In this context, we chose to investigate the effect of selenium on coffee plants under induced drought stress, which led to the second paper of this thesis (Manuscript II - Foliar selenium application to mitigate the effects of induced drought stress in coffee seedlings: induced priming or alleviation effect?). According to the National Food Supply Company (CONAB), coffee production in Brazil in 2022 was 120,000 tons lower than the forecasted amount in May 2021 due to the cold waves and drought of 2021.

Tremendous progress has been made toward understanding the biochemical effects of Se in coffee plants under abiotic stresses. Plant responses to Se foliar supply are addressed in the following ways: i) changes in enzymatic activity, ii) carbohydrate production and breakdown, iii) nutrient compartmentalization, and iv) optimization of water relations under adverse conditions. In this study, we highlight the effects of Se in coffee plants under low-temperature stress, where its supply reduced the visual damage effects in *C. canephora* plants. Selenium-nourished plants showed a stronger response when the temperature returned to optimal conditions (rewarming), increasing the content of important energy and osmoprotector compounds. Moreover, Se supply also played a key role in mitigating the effects of drought-induced stress by PEG-6000. The benefits of Se were noticed when the application occurred prior to the stress, demonstrating that Se has a priming effect on enhancing antioxidant enzymatic activity and improving leaf water relations. On the other hand, stressed plants responded negatively to Se application, resulting in a less negative water potential, a leaf response indicating reduced ability to overcome stress. In conclusion, earlier Se application provided reliable protection for coffee plants under stress by activating both antioxidant and non-antioxidant defense mechanisms. These results might support new

nutritional strategies to induce stress responses in plants, leading to better plant development and sustainable crop production, such as previous Se application in coffee plants and new studies accessing the effect of Se application in field conditions. To elucidate the role of Se in triggering metabolic responses in plants under abiotic stress, the author suggests that other studies should be conducted to access: i) Combined Se application with other nutrients related to anti-stress pathways like Zn, Cu, Mn, Mg, and Fe; ii) Genetic assays to elucidate the effects of Se on gene expression; iii) Cross-species testing; and, iv) Long-term effects of Se in coffee plantation.