

Assessing the stress effects of exogenously applied copper in tomato plant grown in organic farming

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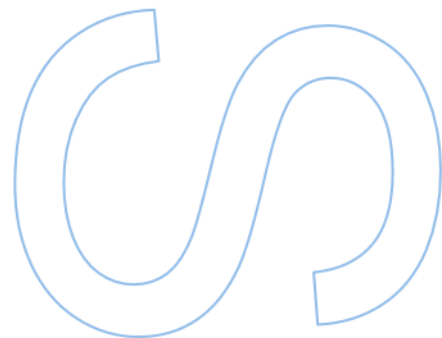
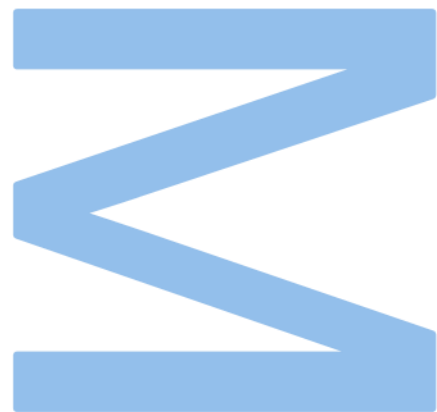
Master's degree in Functional Biology and Biotechnology of Plants
Biology Department
2022

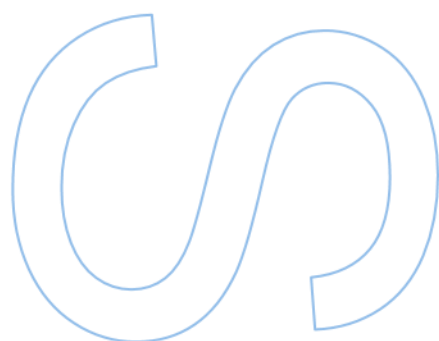
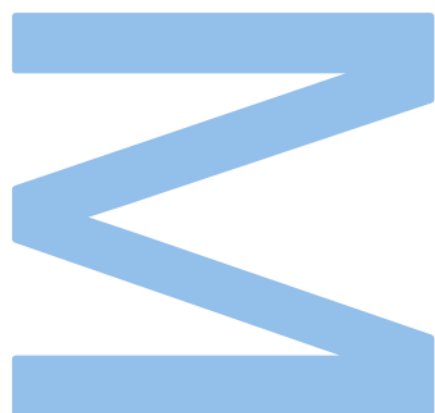
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Alexandre da Silva Alves

30/09/2022



Resumo

Apesar do cobre ser um micronutriente essencial, o seu uso intensivo na agricultura desde o final do século XIX, levou à acumulação desse metal pesado no solo. O principal fator para essa acumulação de cobre nos solos é o seu uso como agente antimicrobiano, especialmente por sistemas de agricultura biológica, pois esta é uma das melhores formas disponíveis de controlo de patogénicos. No passado, os agentes antimicrobianos à base de cobre eram amplamente e, muitas vezes, também incorretamente usados, sendo uma das causas para que alguns solos apresentem níveis extremamente altos de iões de cobre. Neste contexto, as plantas cultivadas nestes solos, como o tomateiro, podem sofrer fitotoxicidade, o que pode levar a um crescimento atrofiado e perdas gerais de produtividade. A qualidade e a segurança alimentar dos produtos agrícolas submetidos a elevados níveis de cobre pode também afetar. Na tentativa de compreender melhor os efeitos sobre os mecanismos responsáveis pela proteção ao stresse induzidos pelo cobre, este estudo concentrou-se em expandir o conhecimento em como *Solanum lycopersicum* L. responde à exposição ao cobre através do ambiente radicular contaminado e por pulverização com o fungicida calda Bordalesa (sulfato de cobre neutralizado com cal). Para este efeito, os padrões de expressão dos genes que codificam as metalotioninas (MT), as quais participam na homeostasia dos metais pesados da planta; a atividade enzimática de agentes responsáveis pelo metabolismo do azoto, como a glutamina sintetase (GS), a glutamato desidrogenase (GDH) e a nitrato redutase (NR); assim como a expressão dos genes que codificam as diferentes isoenzimas da GS, foram estudados. Além disso, a quantificação de cobre em tecidos vegetais também foi realizada, assim como uma avaliação dos efeitos adversos do metal no crescimento e rendimento da planta e em alguns aspetos relacionados com a homeostasia redox. Foi possível concluir que as condições usadas na agricultura biológica, no que diz respeito ao uso de cobre e à sua acumulação nesses solos, impõem um certo grau de stress oxidativo às plantas, com consequente diminuição da assimilação do azoto por mecanismos de regulação pós-transcrição. As plantas responderam com uma resposta antioxidante baseada na produção e regeneração de ascorbato e na produção de tióis proteicos. Os resultados obtidos demonstraram haver redistribuição do cobre pelo corpo da planta, sugerindo que as MT do tipo 1, podem ser as principais responsáveis pela translocação do cobre das folhas para as raízes, sendo necessários mais trabalhos neste assunto. Apesar desta redistribuição, os níveis de cobre acumulados nos frutos são inferiores às doses consideradas prejudiciais para o consumo humano.

Palavras-chave: Poluição do solo, Quantificação de Cu, *Solanum lycopersicum* L., Homeostasia de metais, Eficiência do uso do azoto, Metabolismo antioxidante.

Abstract

Although copper is an essential micronutrient, its intensive use in agriculture since the late 19th century has led to the accumulation of this heavy metal in soils. The main factor for this accumulation of copper in soils is its use as an antimicrobial agent, especially by organic farming systems, as this is one of the best available ways for controlling pathogens. In the past, copper-based antimicrobial agents were widely used in excess and often incorrectly, causing some soils to have extremely high levels of copper ions. As a result, plants grown in these soils, such as tomato, can suffer phytotoxicity, leading to stunted growth and general productivity losses. In an attempt to better understand the effects on the mechanisms responsible for copper-induced stress protection, this study focused on expanding the knowledge on how *Solanum lycopersicum* L. responds to copper exposure through the contaminated root environment and by spraying of Bordeaux mixture. For this purpose, expression patterns of genes encoding metallothioneins (MT), which participate in plant heavy metal homeostasis; the enzymatic activity of agents responsible for nitrogen metabolism, such as glutamine synthetase (GS), glutamate dehydrogenase (GDH) and nitrate reductase (NR), as well as the expression of genes encoding different GS isoenzymes, were studied. In addition, quantification of copper in plant tissues was also performed, as well as an evaluation of the adverse effects of the metal on plant growth and yield and in some aspects related to redox homeostasis. It was possible to conclude that the conditions used in organic farming, concerning the use of copper and its accumulation in these soils, impose a certain degree of oxidative stress on plants, with a consequent decrease in nitrogen assimilation due to post-transcriptional regulatory events. The plants responded with an antioxidant response based on the production and regeneration of ascorbate and the production of protein thiols. The results showed a redistribution of copper throughout the plant body, with type 1 MTs possibly being the main responsible for the translocation of copper from the leaves to the roots, with more work being needed in this subject. Despite this redistribution, the levels of copper accumulated in the fruits are not harmful to human consumption.

Keywords: Soil pollution, Cu Quantification, *Solanum lycopersicum* L., Metal homeostasis, Nitrogen use efficiency, Antioxidant metabolism.

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List of Abbreviations and Acronyms

·OH - Hydroxyl radical	NEM - N-ethylmaleimide
¹O₂ - Singlet oxygen	NH₄⁺ - Ammonium
APX - Ascorbate Peroxidase	NO₃⁻ - Nitrate
AsA - Ascorbate	NP - Non-protein bound thiols
BIP - Bipyridine	NR - Nitrate reductase
C - Control	NUE - Nitrogen Use Efficiency
Ct - Threshold cycle	O₂⁻ - Superoxide anion
DHA - Dehydroascorbate	PC - Phytochelatin
DHAR - Dehydroascorbate Reductase	PT - Protein bound thiols
DMSO - Dimethyl sulfoxide	RFU - Relative fluorescence units
DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid)	ROS - Reactive oxygen species
DTT - Dithiothreitol	RT - Room temperature
EDTA - Ethylenediaminetetraacetic acid	S - Contaminated Soil
EF1 - Elongation Factor 1	SN - Supernatant
GDH - Glutamate dehydrogenase	SOD - Superoxide dismutase
GOGAT - Glutamate Synthase	SP - Sprayed
GR - Glutathione Reductase	SSP - Contaminated Soil + Sprayed
GS - Glutamine synthetase	TCA - Trichloroacetic acid
GSH - Glutathione	TT - Total thiols
GSSG - Glutathione disulphide	ε - Coefficient of extinction
H₂O₂ - Hydrogen peroxide	
KI - Potassium iodide	
MDAR - Monodehydroascorbate Reductase	
MDHA - Monodehydroascorbate	
MT - Metallothionein	
N - Nitrogen	

1. Introduction

1.1. Tomato

Solanum lycopersicum L. belongs to the Solanaceae family, encompassing more than 3000 species of plants, many of which are of economic importance, namely potatoes, tobacco, capsicums, and eggplants, to name a few (Bergougnoux, 2014). Weese and Bohs (2007) argue, with this in mind, that *Solanum* may be the most economically important genus of the plant kingdom, not only since it contains numerous significant food crops but also because it contains many species that produce medicinal compounds.

Wild tomatoes are native to western South America, alongside the coast and the Andes. Tomatoes grow and are adapted to a wide variety of habitats, including low to high altitudes and arid to rainy climates. The wide range of ecological conditions may have contributed to the diversity seen in wild tomato (Peralta & Spooner, 2005; Spooner et al., 2005). Tomatoes were likely first introduced to Europe in the 16th century by Spanish conquistadores who brought them back from the Americas to their homeland. There they found some resistance to being consumed due to their resemblance to poisonous *Solanum* sp. such as mandrake and belladonna. As a result, it was relegated to decoration for the most part until the 17th century, when evidence of its use in European cuisine became more widespread, being incorporated into Italian cuisine in the late 17th or early 18th century (Bergougnoux, 2014; Gerszberg et al., 2015).

With its use in people's diets, tomatoes became an important source of various nutrients and antioxidants such as beta-carotene, vitamin C, flavonoids, and lycopene. This last one has been mainly associated with improving vascular function (Mozos et al., 2018) and some anti-cancer effects (Mirahmadi et al., 2020). Consequently, since these findings, the tomato has become a healthy food and has seen its consumption increase throughout the years (Gerszberg et al., 2015).

1.1.1. Gold Nugget tomato

Gold Nugget is a variety developed in the early 1980s by Dr. James Baggett, for home gardens that experience cooler climates, such as those found in western Oregon, USA. It is the resultant of a cross between Yellow Plum and Oregon Cherry, from which it inherited many of its characteristics such as its deep golden yellow colour, distinct sweet taste and its determinate and fast growth, needing approximately 70 days to bear ripe

fruit. Furthermore, Gold Nugget tomatoes are also vulnerable to *Phytophthora infestans* (Baggett & Kean, 1985), making this variety a prime candidate for copper applications when grown under organic farming systems. As such, due to all these characteristics, Gold Nugget was selected to be the focus of this work.

1.1.2 Economic importance of tomato

After their worldwide spread, tomatoes became an economically important crop, with their global production valued at 92.8 billion dollars in 2018 (FAOSTAT, 2020). Moreover, in 2020, 16.5 million tons of tomatoes were harvested in the EU, with Italy being the lead producer with 37.8%, followed by Spain with 26.1%. Portugal occupies the 3rd spot on the list, with 8.5% of the total production (Eurostat, 2021).

1.2. Organic Farming

Organic farming is a farm management and food production system that seeks to preserve natural resources and apply high production standards. This is a response to the growing demand for more sustainably produced food (European Commission, 2019).

Organic farming systems follow strict rules to be able to market their products as organic. Examples of some of these regulations in the European Union are the prohibition of GMOs, forbidding the use of ionizing radiation and, most relevant for this work, the restriction of artificial pesticides (European Commission, 2008). With this in mind, copper-based antimicrobial compounds become some of the most compelling chemicals that can be used, especially when treating fungal diseases (Lamichhane et al., 2018).

Furthermore, the EU's "Farm to Fork Strategy" aims to use at least 25% of agricultural land for organic farming by 2030. In 2019, the area used for organic farming was 13.8 million hectares corresponding to 8.5% of the total agricultural area. From 2012 to 2019, the organic farming area increased by almost 46% (Eurostat, 2020, 2021). This objective clashes with the recent push some countries made to ban the use of copper in agriculture due to its effects on the environment and the health of farm workers (La Torre et al., 2018).

1.3. Copper

Copper is an essential micronutrient required by all living organisms, it acts as a cofactor for many enzymes involved in respiration and electron transport (Sommer, 1931). It occurs in a wide range of foods and therefore, most diets contain between 1 and 2 mg of copper intake per day (Arena et al., 2018).

The primary inputs of copper in agricultural soils come from manure, sewage, fertilizers, and pesticides (Mantovi, 2003). It is this last one that is of main concern for this work.

Bordeaux mixture was the first copper-based antimicrobial compound, discovered by accident in 1885 by Pierre-Marie-Alexis Millardet, when he noticed that grapevines which had the mixture applied to them (to make the grapes unappealing to people and animals) were free of downy mildew. After testing this substance, he verified that it could be used to inexpensively control mildew diseases (Lamichhane et al., 2018).

The active principle in copper-based fungicides is the cupric ion (Cu^{2+}). It interacts with membrane proteins in a non-specific manner leading to alterations in membrane permeability. If dissolved in water, copper ions can enter fungi cells, such as oomycetes and bacteria. Copper ions in these cells affect multiple enzymatic processes, respiration and block spore germination (La Torre et al., 2018). Copper-based fungicides can only be used as protectants since they lack any curative or systemic activity, meaning that copper must be applied before the pathogen infects the plant.

1.3.1. Effects on soil

Since copper is applied as a spray, it persists in the plant's leaf, whereafter, through wind, rain, or irrigation, the copper has washed off the leaf surface and reaches the soil (La Torre et al., 2018).

Due to its metallic nature, copper doesn't degrade and remains in the environment after application (Eisler, 1998). Copper is not removed through leaching, run-off, or plant uptake and can persist in the soil for extended periods of time (Eisler, 1998; Flores-Vélez et al., 1996; Komárek et al., 2010; Lamichhane et al., 2018; MacKie et al., 2012; Torres & Johnson, 2001; Z. T. Xiong & Wang, 2005). Therefore, continuous use of copper-based fungicides and bactericides can quickly lead to high levels of copper accumulation in the soil.

Copper reacts with compounds in the soil, namely with organic matter and hydrated oxides of iron, manganese, and aluminium, causing it to become insoluble and rendering

further seepage into deeper parts of the soil impossible, causing the majority of contamination to occur in the surface and decreasing with depth (Ceccanti, 2004; Deluisa et al., 2008; Flores-Vélez et al., 1996; Leonardi et al., 2002).

It is not possible to extrapolate whether toxicity will be caused by the total quantity of copper, as this effect is influenced by how available it is to the plants. For instance, clay soils can retain higher amounts of copper without being toxic to the plant due to their composition that can adsorb and immobilize the metal, making it less available to the plants (Schiatti & Nutricato, 2006). In addition, soils rich in organic compounds are more humid and have a higher tendency to pull copper out of the soil solution, consequently reducing its availability for plants, and making it less phytotoxic (Martin, 2009).

1.3.2. Effects on plants

Copper phytotoxicity can be caused by many factors, such as applying soluble copper forms, often in excessive amounts and in acidic solutions, resulting in excess copper being available in the soil solution.

Elevated levels of copper in the soil cause plant stress and can have deleterious effects on crop yield and quality (Dumestre et al., 1999). Lešnik et al. (2011) studied some of the phytotoxic effects of copper in tomato. Toxicity manifests itself in plants in the form of chlorosis and necrosis in leaves, and as abnormal root morphology, causing stunted growth and development (Adrees et al., 2015). It influences biochemical and physiological processes such as respiration, photosynthesis, nutrient uptake, DNA and membrane integrity (Ovečka & Takáč, 2014; Qin et al., 2015). Copper causes oxidative stress by increasing the content of reactive oxygen species (ROS), such as superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) (Apel & Hirt, 2004; Miotto et al., 2014).

Heavy metals, such as copper, tend to build up in plant roots and poorly transfer to epigeous parts (Torres & Johnson, 2001). The effect copper has on the root systems hampers the absorption of nutrients, especially iron, in acidic soils (Lamichhane et al., 2018) as well as severely affecting nitrate (NO_3^-) uptake and thus hampering N metabolism (Hussain et al., 2020).

Toxicity on the plant's upper parts mainly manifests as chlorosis, darkening of the leaf surfaces, margin burning, and necrosis. It can cause value depreciation on fruits due to spots and lesions, leading to an overall loss of vigour or stunted growth (Dagostin et al., 2011; Lepp, 1981; Timmer, 1996; Woolhouse & Walker, 1981).

All these factors lead to the importance of proper maintenance and understanding of the effects of copper accumulation in soils caused by using copper-based antimicrobial agents in organic agriculture to avoid potential productivity losses.

1.3.2.1. Effects on Nitrogen use Efficiency

Since nitrogen is a major component of essential molecules such as amino acids, proteins, and hormones, to name a few examples, its essentiality cannot be understated. Xu and collaborators (2012) define that Nitrogen Use Efficiency (NUE) is primarily affected by Nitrogen uptake efficiency and Nitrogen assimilation efficiency. Therefore, it is necessary to understand the impacts of heavy metals on these two specific components, since reductions in any of these two can lead to decreases in NUE and thus to stunted growth, losses of yield and productivity.

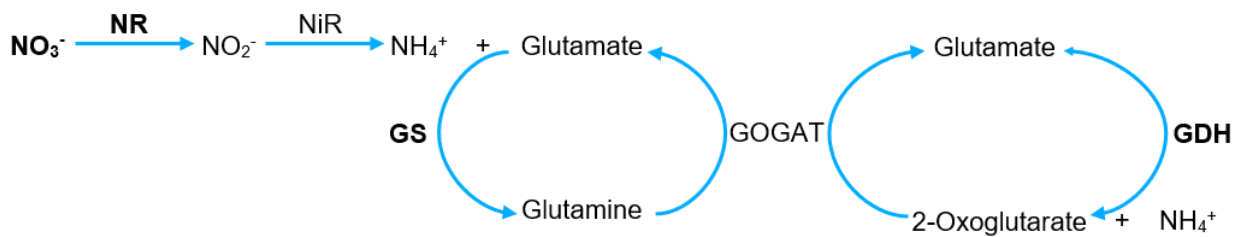


Figure 1. Simplified diagram of the nitrogen assimilation pathway with the analysed components highlighted in bold (Hussain et al. 2020; Lee et al. 1976).

The Nitrogen assimilation process begins with the absorption of NO_3^- which is the preferred source of N, where heavy metals present in the soil may inhibit nitrate uptake by the root cells (Hussain et al., 2020; Mcallister et al., 2012). Following the absorption of NO_3^- , in the first step, the assimilation process occurs, consisting of the reduction of nitrate into nitrite (NO_2^-) catalysed by nitrate reductase (NR; EC 1.7.1.1) (figure 1).

Nitrite is then reduced further by nitrite reductase (NiR; EC 1.7.7.1), converting it into ammonium (NH_4^+) which is then utilized by Glutamine Synthetase (GS; EC 6.3.1.2) together with Glutamate to produce Glutamine. This in turn, allows Glutamate Synthase (GOGAT; EC 1.4.7.1) to use the amine group in Glutamine to transfer it to the 2-Oxoglutarate molecule, thus regenerating the Glutamate from the beginning of the cycle and generating an additional molecule.

Glutamate Dehydrogenase's (GDH; EC 1.4.1.2) primary activity is that of deaminating Glutamate back into 2-Oxoglutarate and NH_4^+ , although in specific situations, such as when high levels of NH_4^+ are present, GDH can perform the reverse reaction, catalysing the amination of 2-Oxoglutarate into Glutamate as a way to reduce the excess NH_4^+ present that could lead to toxicity (Lee et al., 1976). Therefore, GDH is an enzyme that has a dual function in plants, depending on the cellular conditions. Normally, it plays the deaminating role of L-glutamate, giving rise to ammonia and oxoglutarate, thus providing ammonia to be assimilated by GS and oxoglutarate to be used in the carbon metabolism. Yet, when ammonia accumulates in the cell to high levels, GDH will catalyse the amination reaction, thus acting as an ammonia detoxification pathway (Taiz et al., 2015).

1.3.2.2. Oxidative stress response

As previously stated, excess heavy metals such as copper can cause oxidative stress by increasing the production or shifting the balance towards the accumulation of ROS. These, in turn, can cause damage to DNA and critical cellular components such as proteins and membranes (Mithöfer et al., 2004). Due to this, plants require ways of reducing these ROS into non-reactive molecules that aren't capable of causing damage. Such is the role of the Ascorbate-Glutathione pathway (figure 2), one of the mechanisms available to accomplish this task.

This process has the end goal of reducing the O_2^- ion into water, for that effect, superoxide dismutase (SOD; EC1.15.1.1) catalyses the reaction, converting it into Hydrogen Peroxide (H_2O_2) so that it can be further reduced by Ascorbate Peroxidase (APX; EC1.11.1.11) into water (H_2O). This is achieved with the reducing potential of the Ascorbate (ASA) molecule, which in turn becomes oxidized into Monodehydroascorbate (MDHA).

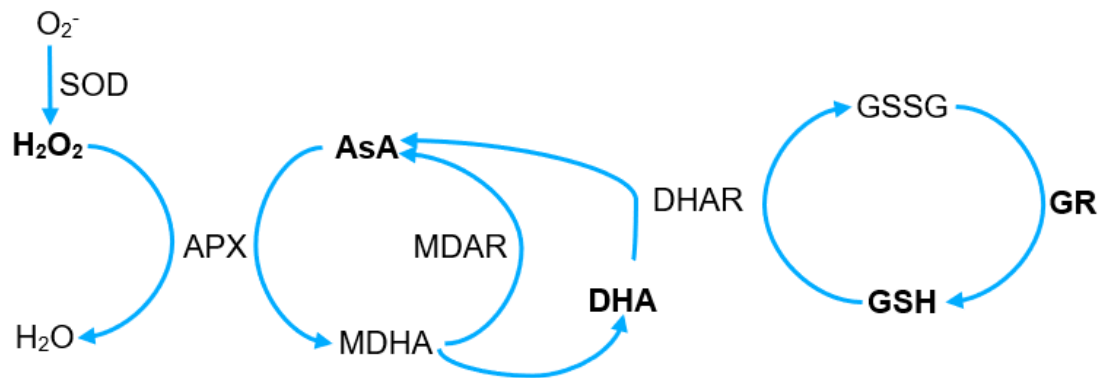


Figure 2. Simplified diagram of the Ascorbate-Glutathione assimilation pathway with the analysed components highlighted in bold (Mithöfer et al., 2004; Huang et al., 2013).

AsA can be regenerated in several different ways, MDHA can non-enzymatically generate AsA as well as Dehydroascorbate (DHA) (Huang et al., 2013), MDHA can be reduced into AsA by Monodehydroascorbate Reductase (MDAR; EC1.6.5.4)) and finally through the catalysis of DHA by Dehydroascorbate Reductase (DHAR; EC1.8.5.1) using Glutathione (GSH) as an electron donor, oxidizing it into Glutathione disulphide (GSSG), GSH is then generated by Glutathione Reductase (GR; EC1.6.4.2) (May et al., 1998).

1.3.2.3. Metal Chelation response

Plants can achieve metal chelation by using several chelators, including organic acids, such as citrate; GSH; phytochelatins; and metallothioneins. These chelators bind to the heavy metals in excess preventing them from reacting with other cellular components and reactions. In many cases, the heavy metal-chelator complex is transported elsewhere within the plant, thus further contributing to cellular detoxification (Cobbett & Goldsbrough, 2002).

1.3.2.3.1. Glutathione

Glutathione is an abundant low molecular weight thiol tripeptide involved in counteracting the toxic effects of xenobiotics, ROS and metal cations (Anjum et al., 2007, 2011; May et al., 1998). It is present in nearly all cellular compartments but with the highest concentrations found in the chloroplasts (Foyer, 2000; Noctor et al., 1998; Rennenberg & Brunold, 1994). It is a precursor of Phytochelatins (PC), which are enzymatically-synthesised heavy metal chelators, allowing plants to store these

elements in the vacuoles, granting them a degree of tolerance to heavy metal toxicity (Roychoudhury & Basu, 2012).

1.3.2.3.2. Metallothioneins

Metallothioneins (MTs) are proteins characterized by low molecular weight and high cystine content. Their capability to bind to metal ions makes them an important piece of the heavy metal detoxification mechanism (Cobbett & Goldsbrough, 2002; Kisa et al., 2017). Although they share many similarities with Phytochelatins, the enzymatic origin of PCs differentiates them from MTs, which are encoded by a small gene family. Metallothioneins can be divided into 4 types based on several characteristics, such as their isoelectric point, polarity and amino acid composition. Type 1 MTs possess Cys-Xaa-Cys motifs in both their N- and C terminal domains; type 2 MTs have Cys-Cys, Cys-Xaa-Cys and Cys-Xaa-Xaa-Cys motifs at the N-terminal domain and Cys-Xaa-Cys motifs in the C-terminal; type 3 are characterized by having four Cys in the N-terminal domain; and finally, type 4 MTs have four Cys in their N-terminal region.

Although the expression patterns of different MTs vary across species and tissues, some patterns still emerge. For instance, MT1 is generally expressed in roots and leaves, MT2 is mainly expressed in leaves and shoots, MT3 is primarily expressed in ripening fruits and finally MT4 is largely found in the developing seeds (Al-Shanfari & Abdullah, 2013; Kohler et al., 2004; M. Yang et al., 2015).

1.4. Objectives

As stated previously, the growing reliance on copper as a fungicide for organic farming can lead to the accumulation of this element in the soil. As such, it is necessary to ascertain its capacity to induce stress and toxicity in tomato plants through exposure to soils contaminated with copper by organic farming, as well as through spraying and a combination of these two situations. For this purpose, several assessments were carried out, such as:

- Verification if the exposure of this species to these situations could influence its growth and yield as a crop, as well as its safe consumption.
- Determination of copper's phytotoxic effects when *Solanum lycopersicum* L. grows in contaminated soils and is sprayed with Bordeaux mixture, its antioxidant response included.
- Quantification of the amount of copper that the plants accumulate in their tissues to assess whether there could be possible health concerns for the consumer.

- Determination of the relevance of the different MTs for copper homeostasis and tolerance and relate their expression patterns with the copper accumulation patterns found.
- Determination of the effects of the exposure of Cu in the NUE by determining the major N-assimilatory enzymes.

2. Materials and Methods

2.1. Plant material

Solanum lycopersicum L. seeds were surface-disinfected by immersing them in 70% ethanol for 10 min and then a 3 min washing in a solution of 20% (v/v) bleach and 0.02% Tween-20 with constant agitation, followed by repeated rinses in sterile distilled water until all the bleach solution was removed at which point the seeds were then dried and stored at 4 °C until further use.

2.2. Copper's effect on germination and growth

2.2.1. Germination assay

To ascertain copper's effects on tomato seed germination, a trial was conducted where *Solanum lycopersicum* L. seeds of the Micro-Tom and Gold Nugget varieties were subjected to Cu concentrations of 0.4, 8, 16, 24, 32 and 40 mg/L.

The surface-disinfected seeds (n=15) were distributed in Petri dishes containing Hoagland solution 0.5x, solidified with agar 0.625% (w/v) (Taiz et al., 2019), and enough copper sulphate pentahydrate to reach the desired amount of copper ions in solution. After this, the seeds were incubated for 2 days in a dark environment at 4 °C to allow stratification to synchronize germination. The Petri dishes were then transferred into a growth chamber (16 h light/ 8 h dark) at 25 °C and 120 $\mu\text{mol}/\text{m}^2/\text{s}$ photosynthetically active radiation for 2 weeks. Following this, the fresh weights, shoot and root lengths were measured and used to determine how much of an impact the copper ions had in germination and seedling development.

2.2.2. Growth trial

Tomato seeds (cv Gold Nugget) were germinated following the same procedures as in the germination trial. After 1 week in the growth chamber, they were transplanted to pots containing a mixture of expanded vermiculite:perlite (1:1). These plants were watered with Hoagland solution 0.5x for their first week and then following the first week, 1x Hoagland solution was used. Afterward, the plants were separated into four groups: The first group (SP) was only sprayed with Bordeaux mixture every 10 days, until a total of 5 applications was reached. The number of applications was calculated to achieve the maximum amount of copper allowed to be administered per hectare/year equal to 6 kg of Cu in organic farming programs. Two sets of plants were each watered with 2 L of

Hoagland solution containing copper sulphate pentahydrate with 8 mg/L copper ion concentration to raise the copper content of the soil (S), subsequently one of these groups was also sprayed with a solution of Bordeaux mixture every 10 days (SSP). Finally, the fourth and final group was the control (C). At the end of the treatment period leaflets from the 3rd, 4th and 5th leaves and root samples were harvested, frozen and stored at -80 °C until needed.

2.2.3. Moisture content determination

To estimate moisture content, samples of tissue from the shoots, roots and fruits of plants subjected to the different growth situations (C, S, SP and SSP) were collected and placed into paper envelopes and weighed using a high precision scale. These envelopes were then incubated at 60 °C to drive all water out of the tissue, being weighed periodically until their weight stabilized. Once this occurred the values were registered and the moisture content was calculated by taking the original fresh weight of the samples, subtracting the dry weight and expressing it in terms of a percentage of the total fresh weight.

2.3. Heavy metal quantification

Copper content in the plants' tissues was determined to assess the preferential organ for its accumulation. To do this, plant material from shoots, roots and fruits, that was dried in the previous step was ground to a fine powder and kept in a desiccator until the next step. After this, aliquots were subjected to an acid digestion with a HCl:HNO₃ (1:3) mixture to release the metal bound to any biological material. The digests were dissolved in a precise volume of water and then used in a Flame atomic absorption spectrometer with a suitable external standard solution (Perkin Elmer, AAnalyst 200 model, Shelton CT, according to manufacturer's instructions) (M. L. R. Martins, 2017).

Although more heavy metals were supposed to be analysed, due to a problem with acetylene, it was not possible to quantify them in time to be included in this report.

2.4. Biochemical analyses

2.4.1 Nitrate quantification

To quantify the nitrate present in the plant's leaf and root tissue, frozen aliquots of approximately 100 mg were homogenized with 1 mL of ddH₂O in a mortar and pestle with the aid of quartz sand. Following this, the samples were incubated at 100 °C for 15 min and then centrifuged at 15,000 *g* for another 15 min at RT after which the SN was collected into separate tubes. The colour reaction was carried out by mixing 100 µL of SN with 400 µL of salicylic acid 5% (w/v) in concentrated H₂SO₄, it was then vortexed briefly to mix thoroughly and then allowed to incubate at RT for 20 min. When this time was elapsed, this mixture was decanted into 15 mL tubes containing 9.5 mL of 2M NaOH solution. After allowing the mixture to cool back to room temperature, the absorbance was measured at 410 nm and with a calibration curve obtained with solutions of NO₃⁻ of known concentrations, the content of NO₃⁻ was calculated in mg/g f.w.

2.4.2. Nitrate Reductase and Glutamine synthetase quantification

2.4.2.1. Protein extraction and quantification

In order to obtain the protein extracts necessary to determine GS and NR activity of root and leaf tissue, aliquots of 300 mg were homogenized with a mortar and pestle over ice and chilled extraction buffer (750 µL for shoots and 500 µL for roots) comprised of 25 mM Tris-HCl (pH 6.4), 10 mM Magnesium Chloride (MgCl₂), 1 mM Dithiothreitol (DTT), 10% Glycerol, 0.05% Triton X-100 with quartz sand and 1% (w/v) Polyvinylpolypyrrolidone (PVPP). Following this, the homogenates were centrifuged at 15,000 *g* at 4 °C for 20 min and the supernatant (SN) was collected to a separate tube.

Following the preparation of the extracts, 75 µL of 1:20 diluted SN were added to new tubes containing 750 µL of Bradford solution, and the protein content was determined using the Bradford method (Bradford, 1976).

2.4.2.2. Nitrate Reductase quantification

The previously obtained protein extracts were used to determine NR activities by adding 100 µL to 900 µL of reaction mix composed of 25 mM Trizma-Base, 500 µM NADH, 10 µM FAD and 2 mM KNO₃, and measuring the variation in absorbance at 340 nm in intervals of 15 seconds during 1 min and 30 seconds using this variation, together with the coefficient of extinction (ϵ) of 6.22 mM⁻¹ cm⁻¹, to express NR activity in µmol/min/mg of protein. A blank was prepared using an extraction buffer instead of SN.

2.4.2.3. Glutamine Synthetase quantification

Using the extracts obtained in the previously explained step GS activity was quantified by reacting 50 μL of SN with 50 μL of 6.4% (w/v) Sodium arsenate (pH 6.4) and 400 μL of reaction mixture composed of 100 mM Trizma-Base, 125 mM L-glutamine, 157 mM Hydroxylamine, 0.26 mM Manganese chloride tetrahydrate and 1.25 μM Adenosine 5'-diphosphate sodium salt (ADP), (pH 6.4).

Therefore, three replicates were prepared from each SN, and a blank with the protein extract replaced with extraction buffer. All the reactions were then incubated at 30 °C for 30 min (shoots) and for 60 min (roots). After this time was elapsed, 500 μL of Stop solution (0.16 M Iron Chloride (FeCl_3), 0.25 M Trichloroacetic acid in 3.33% HCl) were added. The absorbances were measured at 500 nm, and the transferase activity of GS was expressed in $\mu\text{mol}/\text{min}/\text{mL}$ (M. Martins et al., 2021).

2.4.3. Glutamate dehydrogenase quantification

To quantify GDH present, it is first necessary to extract the proteins from the tissue, as such, frozen aliquots of root and leaf tissue weighting approximately 200 mg were homogenized over ice with the aid of quartz sand, PVPP and 400 μL extraction solution composed of 5% glycerol, 1 mM EDTA, 0.05% Triton x-100 and 40 mM Tris-HCl (pH=7.2), after this, the samples were centrifuged at 13,000 g for 10 min at 6 °C and the SN was transferred to new tubes and kept on ice.

For the GDH quantification, 25 μL of SN were added to tubes containing 75 μL ddH₂O and 900 μL of reaction mix containing 50 mM (NH_4)₂SO₄, 13 mM 2-Oxoglutarate, 0.25 mM NADH, 1 mM CaCl₂ and 100 mM Tris-HCl (pH=8), and after measuring the variation in absorbance at 340 nm in intervals of 15 seconds during 1 min. Using this variation, together with the coefficient of extinction (ϵ) 6,22 $\text{mM}^{-1} \text{cm}^{-1}$, GDH activity was expressed in $\mu\text{mol}/\text{min}/\text{mg}$ of protein. A blank was prepared using extraction buffer instead of SN.

2.4.4. Proline quantification

The method described by Bates et al. (1973) was utilized for quantifying proline levels. As such, 3% (w/v) sulfosalicylic acid was used to homogenize 200 mg of frozen aliquots of leaf and root tissue (1.5 mL for shoots and 1 mL for roots), in conjunction with quartz sand and over ice. After that, the SN was centrifuged at 500 g for 10 min, afterwards 200

μL of the centrifuged SN was mixed with 200 μL of glacial acetic acid and 200 μL of ninhydrin and then this mix was incubated for 1 h at 96 °C. Following this, the samples were briefly cooled on ice so that 1 mL of toluene was able to be added and vortexed for 15 seconds. The tubes were set at RT until the upper phase was formed, at which point, it was collected and together with a toluene blank, its absorbance measured at 520 nm.

Proline contents were determined via a standard curve created using known proline concentrations and the results were expressed as mg/g f.w..

2.4.5. Photosynthetic pigments quantification

To quantify the pigments present in leaf tissue of the plant, a method adapted from that developed by Bulda et al. (2008) was used, where it becomes possible to ascertain the content of β -carotene, lutein, chlorophyll *a* and *b*.

Therefore, the first step in this process was to extract the pigments into a solvent, for that effect, approximately 15 mg of leaf tissue were weighted and then added to 15 mL tubes, together with 10 mL of 80% acetone, this process was repeated 3 times for each group. Afterwards, the tubes were vortexed for 10 seconds and stored in darkness at 4 °C for 4 days, to allow for complete extraction of the pigments. After this time, the absorbances of the extracts were measured at 480, 495, 645 and 655 nm, then the amount of pigments was calculated and expressed as mg/g f.w..

2.4.6. Histochemical coloration of starch

To access cooper's impact on starch storage in tomato leaves, a histochemical coloration of the starch grains was carried out. For that effect, leaf discs of 1 cm diameter were collected from the terminal leaflet of the 5th emergent leaf. The discs were then boiled in 80% ethanol until bleached and then submerged in ddH₂O for 1 min. Subsequently the discs were incubated in a Lugol solution [2 mM iodide (I₂), 6 mM potassium iodide (KI)] for 30 min in the dark, afterwards, the discs were briefly washed in ddH₂O and then photographed.

2.4.7. Starch and Glucose quantification

2.4.7.1. Extracts preparation

Frozen aliquots of 50 mg of leaf tissue were homogenised with 10 mL of 80% ethanol in a mortar and pestle with the aid of quartz sand and then transferred to new 15 mL tubes. These tubes were then subjected to an incubation at 80 °C for 1 h and following this they

were subjected to a centrifugation at 5,000 *g* for 5 min at 6 °C and the SN, containing the soluble sugars, was separated from the pellet (containing starch) into identical tubes.

To measure starch content, it was first necessary to hydrolyse it into soluble sugars. To do so, the pellet obtained from the previous step was resuspended in 5 mL of 30% perchloric acid with manual and vortex agitation, after fully suspended, the tubes were incubated at 80 °C for 1 h and with constant agitation. Afterwards the tubes were centrifuged at 10,000 *g* for 10 min at 6 °C and the SN was collected.

2.4.7.2. Quantification

To carry out the quantification of the carbon content, 30 µL of the previously obtained SNs were mixed with 750 µL of 9.8 mM anthrone solution dissolved in 91% sulfuric acid, then incubated at 100 °C for 10 min and subsequently cooled in ice for 15 min. After cooling, the samples were centrifuged again at 10,000 *g* for 10 min at 6 °C and their absorbance was measured at 625 nm and registered, using 80% ethanol instead of SN as a blank. With the aid of a calibration curve created with known concentrations of glucose it was possible to ascertain both the contents of soluble sugars and starch in the tissue in mg/g f.w..

2.4.8. Hydrogen Peroxide Quantification

To quantify H₂O₂, 200 mg of frozen aliquots of root and leaf tissue were homogenized over ice with 1.2 mL of phosphate buffer 50 mM (pH=6.5) and quartz sand. Subsequently, the samples were centrifuged at 6,500 *g* for 20 min at 6 °C and the SN was collected. Afterwards, 500 µL of SN was added to 500 µL of TiSO₄ 0,1% (w/v) dissolved in H₂SO₄ 20% (w/v) and vortexed for 15 seconds and following this the solution was centrifuged again at 6,000 *g* for 15 min at 6 °C and then the SN was read at 410 nm. The H₂O₂ levels were quantified using the ϵ value of 0.28 µM⁻¹ cm⁻¹ and expressed as nmol/g f.w., using extraction buffer instead of SN as blank.

2.4.9. Ascorbate quantification

The quantification of ascorbate was carried out by following the methodology outlined by Gillespie and Ainsworth (2007). For this approach, frozen aliquots of 200 mg were homogenized in 6 % TCA (w/v) (1.5 mL for shoots and 1 mL for roots) at 4 °C, in a mortar and pestle over ice. Subsequently, the homogenates were centrifuged at 15,000 *g* for 10 min at 4 °C, and the SN was collected to new tubes. From this point forward, two sets of

reactions were prepared: one to quantify the total amount of ascorbate, and another to quantify the reduced ascorbate (AsA).

To quantify total ascorbate, 100 μL of SN were added to 50 μL of 75 mM PK buffer (pH 7.0) and 50 μL of 10 mM 1,4-dithiothreitol (DTT). The mixture was then briefly vortexed and incubated at RT for 10 min, and 50 μL of 0.5% (w/v) N-ethylmaleimide (NEM) were added.

Similarly, to determine AsA, 100 μL of SN were mixed with 50 μL of 75 mM PK buffer (pH=7.0) and 100 μL of ddH₂O, followed by 750 μL of reaction mixture containing 10% (w/v) TCA, 43% H₃PO₄, 4% (w/v) 2,2'-bipyridine (BIP) and 3% (w/v) FeCl₃.

All the samples were then incubated at 37 °C for 1 h, after which the absorbance was read at 525 nm and through a calibration curve obtained using known concentrations of Ascorbate it is possible to obtain the content of reduced and oxidized Ascorbate in $\mu\text{mol/g f. w.}$. The blanks were prepared using 6 % TCA (w/v) instead of SN.

2.4.10. Glutathione quantification

To quantify reduced glutathione, 100 mg of frozen aliquots of root and leaf tissue were homogenized in a mortar and pestle with 1 mL of 3% (w/v) sulfosalicylic acid, with the aid of quartz sand and over ice. The extracts were then incubated for 10 min in ice and subsequently centrifuged at 10,000 g for 10 min at 4 °C; the SNs were then collected to separate tubes.

Following this, 750 μL of reaction mix containing 400 μL of DTNB dissolved in DMSO (1.5 mg/mL), 14 mL of 1 mM EDTA [dissolved in 100 mM of phosphate buffer (pH=7)] were added to new tubes together with 200 μL of ddH₂O and 50 μL of the previously obtained SN and were left for 10 min to incubate at RT in the dark. Afterwards, the samples had their absorbance measured at 412 nm and with the aid of a calibration curve created with known concentrations of GSH, the content of reduced glutathione was expressed in nmole/g f.w.. A blank was prepared using 3% (w/v) sulfosalicylic acid instead of SN.

2.4.11. Thiol quantification

To quantify thiol content in plants, the method described by H. Zhang et al. (2009) was used. To do so, frozen aliquots of 300 mg were homogenized over ice while using quartz sand and extraction solution comprised of 20 mM EDTA and 20 mM AsA (1.9 mL for

shoots and 1.5 mL for roots). Following this, the homogenates were centrifuged at 12,000 *g* for 20 min at 4 °C and when the centrifugation was finished the SN was transferred into tubes to be used for total and non-protein thiol quantification.

To determine total thiol content, 100 µL of SN were mixed with 480 µL of 200mM Tris-HCl (ph=8.2) and 20 µL of 10mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and incubated for 15 min at RT and then the absorbance was measured at 412 nm. The total thiol content was expressed in mol/g f.w., using 13,600 mM⁻¹ cm⁻¹ as molar extinction coefficient.

To quantify non-protein thiols 250 µL of SN were added to 250 µL of 10% (w/v) sulfosalicylic acid and then incubated for 15 min at RT, with the samples then being centrifuged at 3,000 *g* for 15 min at 4 °C and the SN was collected to new tubes. Following this, 250 µL of SN were mixed with 237.5 µL of 400 nM Tris-HCl (pH=8.9) and 12.5 µL of 10 mM DNTB and left to incubate at RT for 5 min, at which point the absorbance measurements were performed as stated in the total thiol procedure and calculated the same way. To obtain the protein bound thiol content, the non-protein thiols were subtracted from the total thiol content. The blanks were prepared following the same procedure but using extraction solution instead of SN.

2.5. Gene expression analyses

2.5.1. RNA extraction

The RNA extraction was accomplished with NZYol (NZytech®, Portugal) in accordance with the instructions supplied by the manufacturer. As such, 50 mg of root and leaf tissue were homogenized in 1 ml of NZYol® and transferred into tubes to then be centrifuged at 12,000 *g* for 10 min at 4 °C. Following a 5 min incubation period at RT, 0.2 mL of chloroform were added to the SNs and shaken for 15 seconds to denature the proteins and other cell components. After another incubation for 3 min at RT the samples were centrifuged at 12,000 *g* for 15 min at 4 °C to separate it into 3 distinct phases: lower phenol-chloroform phase (where most DNA is located), an interphase (containing cell and protein debris), and an upper colourless aqueous phase (which contains the RNA). Using a micropipette, the aqueous phase was transferred to new tubes, at which point 0.5 mL of cold isopropanol was added and left for 10 min to precipitate the RNA, after which, the tubes were centrifuged at 12,000 *g* for 10 min at 4 °C and the SN discarded. The RNA pellet was then washed with 75% ethanol with the aid of vortex agitation and centrifuged again at 7,500 *g* for 5 min at 4 °C, the SN was once again discarded, and the

pellet was allowed to air dry to completion. Once this process was complete, the RNA was dissolved in RNase-free water.

Using a DS-11 Microvolume Abs Spectrophotometer from DeNovix® (DeNovix Inc., USA), RNA concentration was determined by measuring its absorbance at 260 nm and expressed in ng/μL. To ascertain the purity of the samples, the ratio between Abs_{260}/Abs_{280} was used to measure protein contamination while the Abs_{260}/Abs_{230} ratio reveals the extent of contamination caused by polysaccharides.

To verify the integrity of the extracted RNA, 300 ng of RNA from each sample was run in a 0.8 % (w/v) agarose gel electrophoresis, in 1x sodium boric acid (SB) buffer, at 250 V and non-limiting amperage, for 10 minutes.

2.5.2. Reverse transcription (*cDNA synthesis*)

Using the previously obtained RNA samples, reverse transcription reactions were performed to obtain cDNA using SuperScript™ IV VILO™ Master Mix kit and following the manufacturer's instructions (Invitrogen, USA). Firstly 2.5 μg of RNA were transferred into sterile, RNase-free tubes, then 4 μL of SuperScript™ IV VILO™ Master Mix (includes reverse transcriptase, dNTPs, oligo-dT and random primers), lastly RNase-free water was added to make up the final volume of 20 μL. The reaction was mixed and incubated at 25 °C for 10 min, to allow for primer annealing, following this the reverse transcription reaction took place at 50 °C for 30 min, afterwards the enzyme was inactivated by incubating the reaction mix at 85 °C for 5 min. The cDNAs were stored at -20 °C until future use.

2.5.3. Semi-quantitative polymerase chain reaction (*PCR*)

To ascertain the accumulation of mRNA coding for glutathione reductase, a semi-quantitative reverse-transcriptase PCR was utilized. The PCR conditions were optimised for previously design primers; the final conditions used are described in Table 1. The reactions were composed of 5 μL of 2x Taq Master Mix (Bioron®), 0.4 μL of 10 μM forward and reverse primers, 0.5 μL cDNA from each sample and sterile distilled water to make up the final volume of 10 μL and carried out on a MJ Mini thermocycler (Bio-Rad®).

To make sure that the variations observed were indeed due to differential gene expression, it was necessary to normalize the amount of cDNA that was used for the

samples. As such, the Elongation Factor 1 (*EF1*) housekeeping gene was used for this effect (Løvdaal & Lillo, 2009). The amplification products were loaded on to 2% (w/v) agarose gels prepared on sodium boric acid (SB) buffer and stained with Green safe (NZytech®, Portugal). GeneRuler 1 kb DNA Ladder - Thermo Fisher was used as a reference for accessing the size of the amplicons. The gels were then run at 300 V and non-limiting amperage, this process was repeated until volumes that give rise to uniform bands across all situations for *EF1* were determined.

Table 1. Gene-specific primers for GR and *EF1* and expected amplicon sizes produced by each primer pair for the performed semi-quantitative polymerase chain reactions.

Gene	Primer	Sequence	Amplicon size (bp)
GR	Forward	5' AAAGACCGAGGAGATTGTACG 3'	322
	Reverse	5' CATTCTCGCCATATAGAAGC 3'	
<i>EF1</i>	Forward	5' GGAACCTTGAGAAGGAGCCTAAG 3'	158
	Reverse	5' CAACACCAACAGCAACAGTCT 3'	

2.5.4. Expression of MT and GS family genes by Real-Time (q) PCR

The accumulation of mRNA of MT family genes (MT 1, 2, 3 and 4) and GS family genes (GS 1.1, 1.2, 1.3 and 2) was carried out by real-time reverse-transcription PCR with specific primers determined during previous work by (M. L. R. Martins, 2017) and (M. Martins et al., 2021) that can be found in table 2.

Table 2. MT and GS gene-specific primers, respective melting temperature (T_m) and expected amplicon sizes produced by each primer pair for the performed RT-qPCR reactions.

Gene	Primer	Sequence	T _m (°C)	Amplicon size (bp)
MT1	Forward	5' GGAGGAAGCTGTAATTGTGG 3'	57	166
	Reverse	5' CCCCTTCTGTAGCTTTCTC 3'	58	
MT2	Forward	5' GCTGTGGAGGATGTGGTATG 3'	59	117
	Reverse	5' CCTTCTCCAGCTGCTTTCTC 3'	59	
MT3	Forward	5' GGAAGGAGAGCCAATACGAC 3'	59	78
	Reverse	5' TGTTCTTCTGCTCCAACGTC 3'	59	
MT4	Forward	5' ATGAGAGGTGTGGTTGCCTT 3'	59	180
	Reverse	5' CCGCACTTGCAGTTAGACTT 3'	58	
GS1.1	Forward	5' CGGAGAAAGGAAAGGGATAC 3'	70	178
	Reverse	5' CATGTGAGGAAAGGGCTGTTAAG 3'	68	
GS1.2	Forward	5' GGTGCTGGAGTTGTTGTTTC 3'	58	191
	Reverse	5' CGTTCATTGCCTTCACCATA 3'	58	
GS1.3	Forward	5' CTGGTGATGAAGTGTGGGTAG 3'	64	164
	Reverse	5' TTTCATAGCCTCCGTCTTCC 3'	60	
GS2	Forward	5' TCTCTCACTCCTAACACAAC 3'	68	189
	Reverse	5' GCGTGTCTTTTCTGCTTC 3'	62	

The qPCR reactions for leaf and root tissue were carried out with the EF1 gene as an internal control. All assays were performed with SsoAdvanced™ Universal SYBR® Green supermix (Bio-Rad Laboratories, Inc.) in a 10-µL reaction final volume with both gene-specific 0.5 µM primer concentration, 1 µL of 1/10 diluted cDNA. The reactions were then run in a CFX384 Touch™ real-time PCR detection system (Bio-Rad) with the following parameters for each reaction group: Group 1 (MT1, MT2, MT3, MT4 and GS1.2), 2 minutes at 95 °C and 40 cycles alternating between 5 seconds at 95 °C and 30 seconds at 58 °C. Group 2 (GS1.1, GS1.3 and GS2), 2 minutes at 95 °C and 40 cycles alternating between 5 seconds at 95 °C and 5 seconds at 60 °C. Raw RFU (relative fluorescence units) data and Ct (threshold cycle) values were analysed in CFX Manager™ software (Bio-Rad) to obtain the relative normalized expression ($\Delta\Delta C_t$) of the targeted genes in relation to the control situation and normalized to the reference gene (EF1) (Løvdaal & Lillo, 2009) and calculated according to the method $2^{-\Delta\Delta C_t}$ (Livak & Schmittgen, 2001).

2.6 – Statistical analysis

For every parameter, at least three biological replicates ($n \geq 3$), with at least three technical replicates were used per assay. The results were expressed as mean $\pm$ standard deviation (SD). Significant differences were monitored through a one-way ANOVA followed by Dunnett multiple comparison tests. These analyses were performed using Prism® 8 (GraphPad Software Inc., USA), considering the statistically significant differences at $p \leq 0.05$.

3. Results

3.1. Micro-Tom germination assay

The presence of copper ions in the growth media doesn't significantly impact the germination rate of tomato. On the contrary, the seedling's shoot, root length, and fresh weight showed statistically significant decreases in all copper concentrations tested (figure 3).

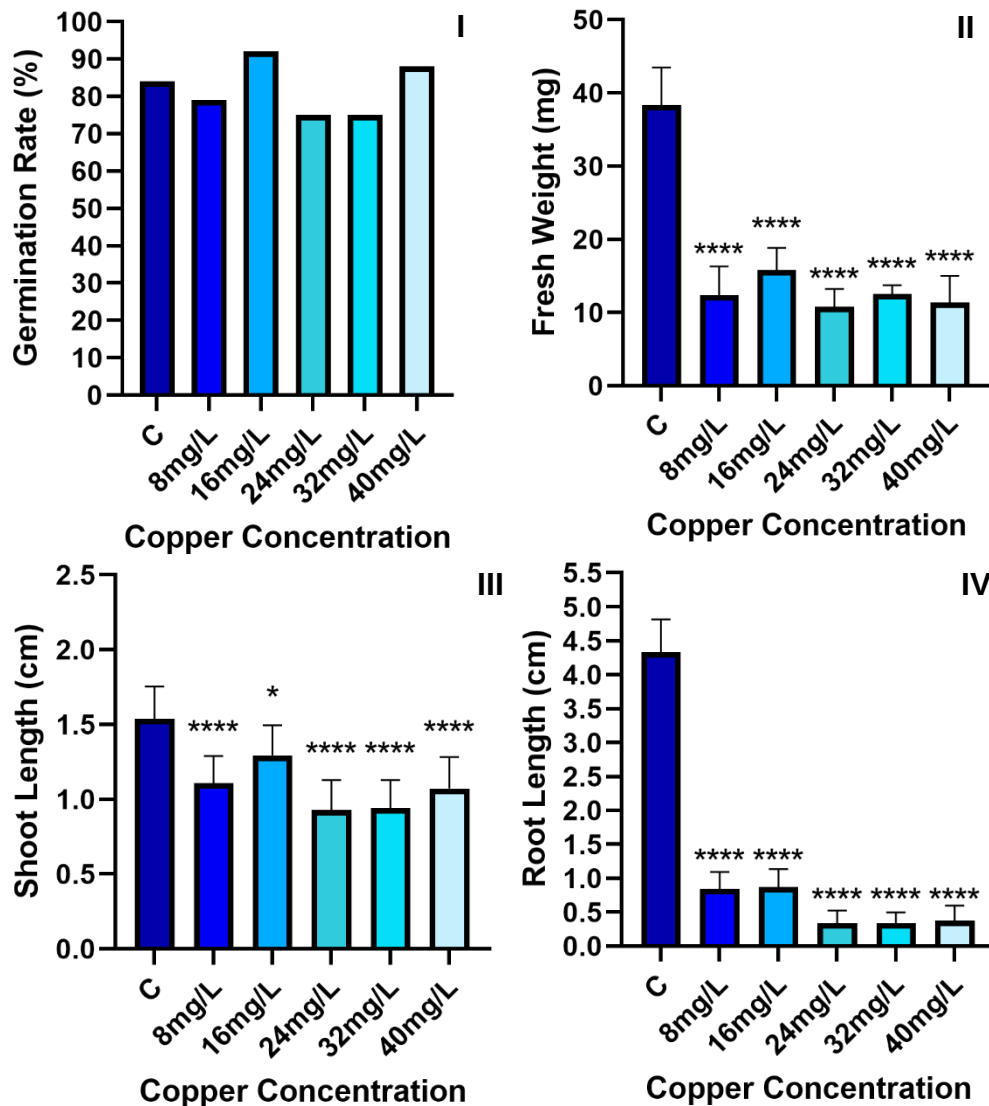


Figure 3. Germination rate (I), Fresh weight (II), Shoot length (III) and Root length (IV) of *S. lycopersicum* (cv Micro-Tom) seedlings grown in nutritive media supplemented with increasing Cu ion concentrations from 0.4 mg/L to 40 mg/L. Values presented are mean $\pm$ SD. * above bars represent statistically significant differences from the control at * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

The seeds germinating in media with 24 mg/L of copper ions showed a decrease of 39% in size, followed closely by 32 mg/L with a 38% reduction in shoot length. The highest

concentration tested exhibited a 30% decrease in length, while the lowest showed a 27% reduction. Finally, the seeds germinating in 16 mg/L of copper in media showed the lowest decrease of all tested concentrations, with the length of the shoots decreasing by 15%.

The presence of copper significantly affected the development of the seedlings' roots. The higher concentrations of copper ions (24, 32 and 40 mg/L) showed reductions of approximately 92%, with the two remaining ones (8 and 16 mg/L) exhibiting reductions of roughly 80%.

Copper's effect was also apparent in the seedlings fresh weight, where all the concentrations exhibited significant reductions. Plants grown in 8 and 32 mg/L of copper ions present in their growth media both showed a decrease of 67% in their fresh weight, with plants grown in 24 and 40 mg/L displaying an approximate 71% decrease in size. Lastly, the 16 mg/L concentration showed a 58% reduction in the root's size, the smallest one among all tested concentrations. Finally, the average germination rate across all tested concentrations was 82%, with no significant variations between treatments.

3.2. Gold Nugget germination assay

Gold nugget seedlings were significantly affected by the copper's presence within the growth media, with the length of the shoot decreasing with the increase of copper ions present (figure 4). The lowest concentration showed a 40% reduction in shoot size, which increased to 44% when the concentration of copper was doubled. Further increasing the presence of copper in the media to 24 mg/L resulted in another decrease in length, this time by 55%, followed closely by the next concentration (32 mg/L) with a 57% shortening of the shoot. Finally, the highest concentration tested showed the largest reduction in size of 65%.

The tendency for length to decrease with the increase of copper in the growth media was also verified with the root length in the lowest concentration, having a reduction of 47%. Copper's effect continued to manifest itself in the 16 mg/L concentration, with a 67% decrease being observed, this result was similar to that of 24 mg/L exhibiting a 71% reduction in overall root length. The second largest decrease in root length was found in the 32 mg/L concentration, corresponding to 80%. Lastly, the highest tested concentration showed a 90% reduction in root size.

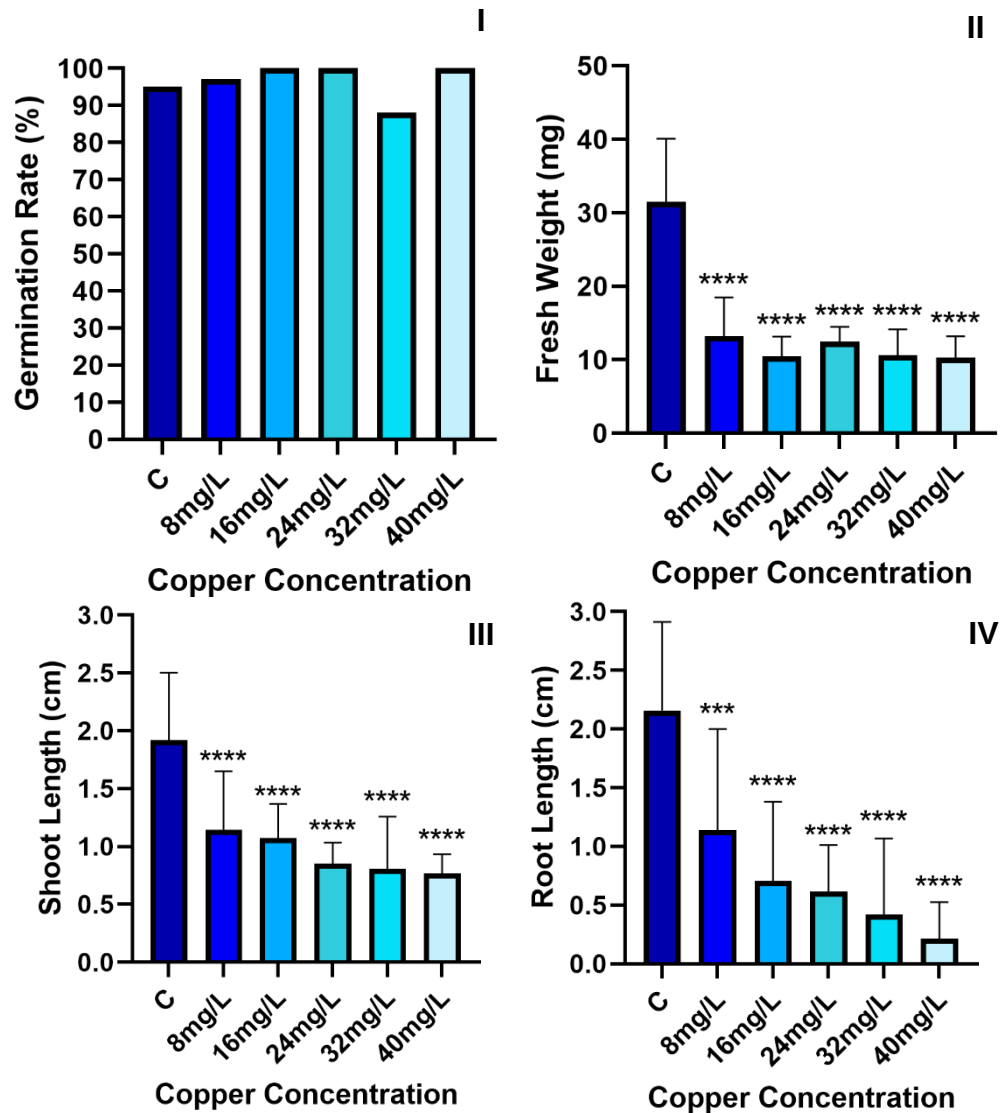


Figure 4. Germination rate (I), Fresh weight (II), Shoot length (III) and Root length (IV) of *S. lycopersicum* (cv Gold Nugget) seedlings grown in nutritive media supplemented with increasing Cu ion concentrations from 0.4 mg/L to 40 mg/L. Values presented are mean $\pm$ SD. * above bars represent statistically significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Gold nugget seedlings' fresh weights were significantly affected by the presence of copper ions, with all tested concentrations yielding similar decreases in weight. The largest reductions were observed in the 16, 32 and 40 mg/L concentrations corresponding to approximately 67%. The remaining concentrations (8 and 24 mg/L) also showed similar results, with around 60% reductions. Lastly, the germination rate across all tested cases averaged 97%, with no significant variations between treatments.

3.3. Moisture content determination

The results regarding the moisture content are depicted in figure 5.

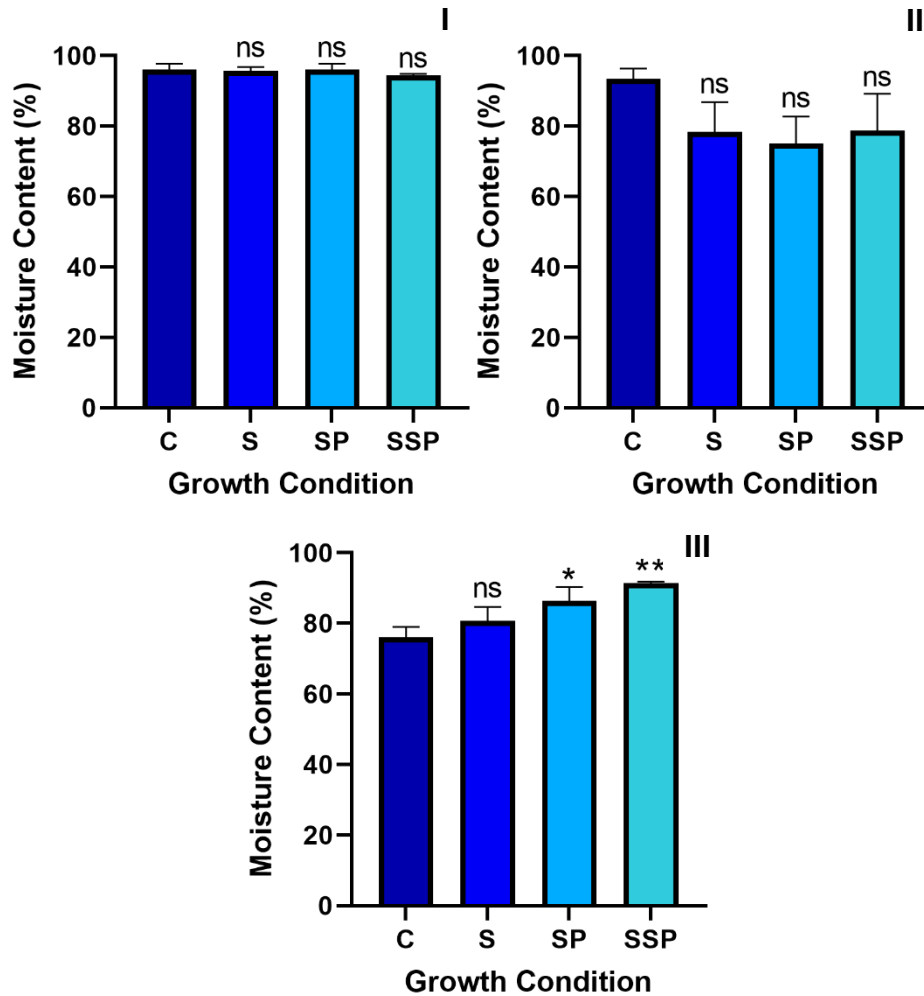


Figure 5. Moisture content in shoots (I), roots (II) and fruits (III) of *S. lycopersicum* (cv Gold Nugget) plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Copper appears to have had little effect on the relative water content of both leaf and root tissue of all tested situations, with no statistically significant changes being observed. However, this was not the case in the fruits, where both treatments in which the plants were sprayed with copper fungicides (SP) and those subjected to combined exposure (SPP) exhibited increases in RWC corresponding to 13% for SP and 20% for SSP.

3.4. Heavy metal quantification

Copper accumulation was evident in both shoot and root tissue, with shoots being greatly impacted, especially in growth conditions where the tissue was exposed to the contaminant through spraying, with SP registering an 11-fold (1029%) increase in copper content and SSP a 7-fold (693%) uptick. Finally, the plants that grew on contaminated soils exhibited the lowest accumulation of copper in the shoots, with a 47% increase (figure 6).

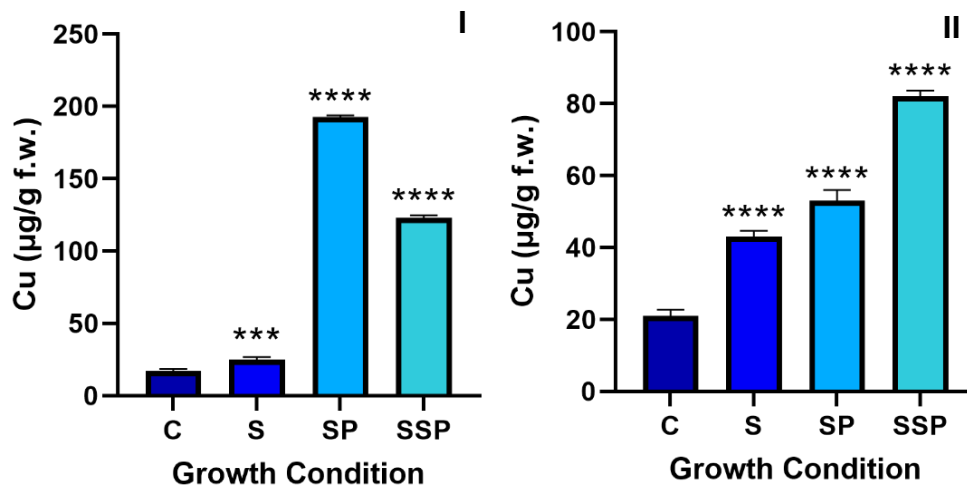


Figure 6. Copper ion content in shoots (I) and roots (II) of *S. lycopersicum* (cv Gold Nugget) plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Regarding root tissue, all growth conditions showed significant increases in copper content. The S group plants registered the smallest one, corresponding to 104%, followed by the plants sprayed with copper-based fungicides (SP), exhibiting a 152% uptick and, finally, the growth condition that accumulated the most copper, SSP, with an increase of 290%.

In fruits, all determined values for copper content in all tested situations were under 11 µg/g of dry plant material, this value being the lower limit of sensitivity of the technique used. As such, it was not possible to determine the exact amount of copper present.

3.5. Nitrate quantification

Plants' leaves exposed to copper through spraying (SP) and combined exposure (SSP) did not present statistically significant changes in nitrate content. This was not the case for the soil treatment (S), which showed an increase in nitrate levels of 38% (figure 7).

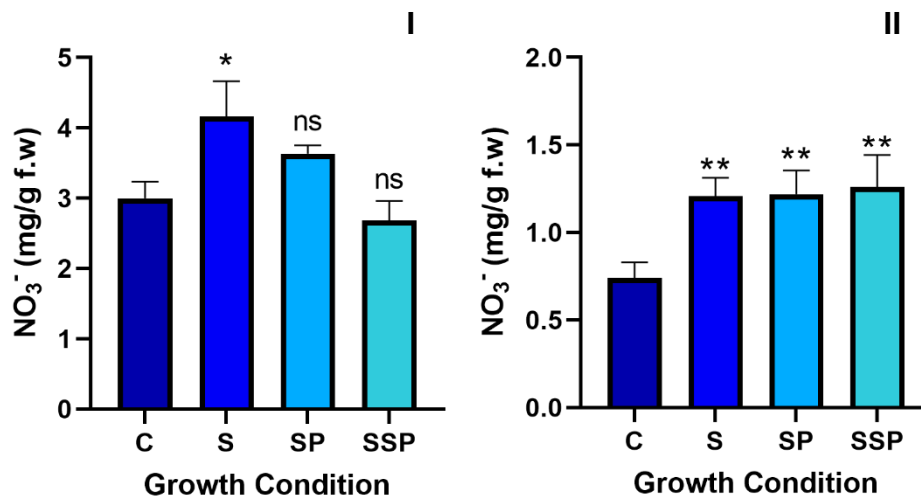


Figure 7. Nitrate (NO_3^-) content in leaves (I) and roots (II) of *S. lycopersicum* (cv Gold Nugget) plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In roots, all tested copper growth conditions revealed statistically significant results. Plants that grew on soil contaminated with copper (S) showed a 63% increase in nitrate content, with sprayed plants (SP) closely matching this result with a 64% uplift. Finally, the combined exposure group (SSP) showed a slightly higher increase of 70%.

3.6. Nitrate reductase activity

In both leaf and root tissue, all tested growth conditions showed no statistical differences in Nitrate Reductase activity (figure 8).

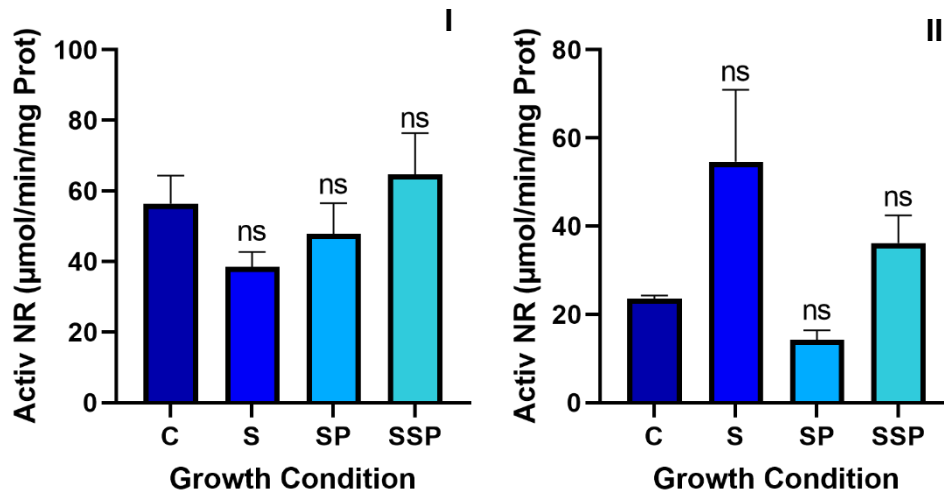


Figure 8. Nitrate Reductase (NR) activity in leaves (I) and roots (II) of *S. lycopersicum* (cv Gold Nugget) plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Despite the 131% increase observed in S plants roots' the high SD value causes the result to not be statistically significant and as such it cannot be taken into consideration.

3.7. Glutamine Synthetase activity

Copper significantly influenced GS activity in leaves with significant reductions across all treatments (figure 9).

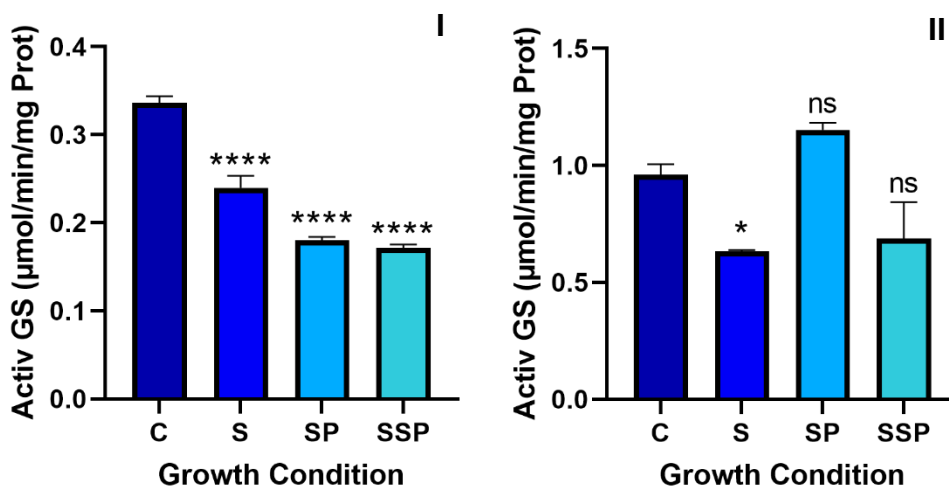


Figure 9. Glutamine Synthetase (GS) activity in leaves (I) and roots (II) of *S. lycopersicum* (cv Gold Nugget) plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent statistically significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In leaves, the SSP group was most affected by copper, showing a 49% decrease in GS activity, closely matched by the SP group, registering a 46% decrease. Finally, the least impacted were the plants exposed to copper through contaminated soil (S), displaying a 28% decrease in the enzyme's activity.

In roots, the results were similar in the S group, with plants again showing a 34% decrease in activity. The two other tested growth conditions showed no statistically significant changes.

3.8. Glutamate dehydrogenase activity

Plants' roots sprayed with copper-based fungicides did not show statistically significant changes among treatments. Although decreases in root GDH activity were observed with the SSP group, registering a 60% decrease and plants grown in copper contaminated soils (S) exhibited a 33% reduction in GDH activity (figure 10).

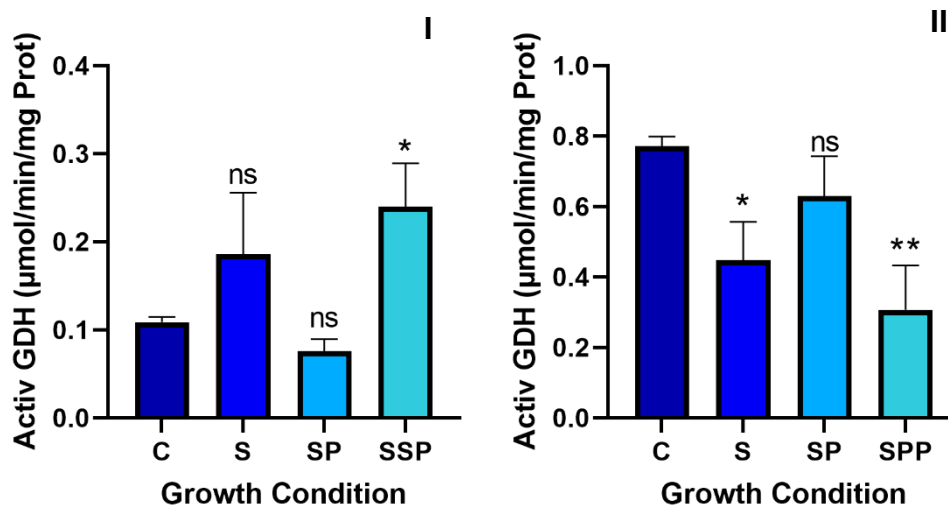


Figure 10. Glutamate dehydrogenase (GDH) activity in leaves (I) and roots (II) of *S. lycopersicum* (cv Gold Nugget) plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In leaf tissue, only the combined copper exposure group plants (SSP) showed a significant 120% increase in GDH activity, and no impact on GDH was found in the other tested modalities of copper exposure.

3.9. Proline levels

Proline content in leaves was affected by copper's presence, with significant decreases observed in leaf tissue in all tested conditions (figure 11).

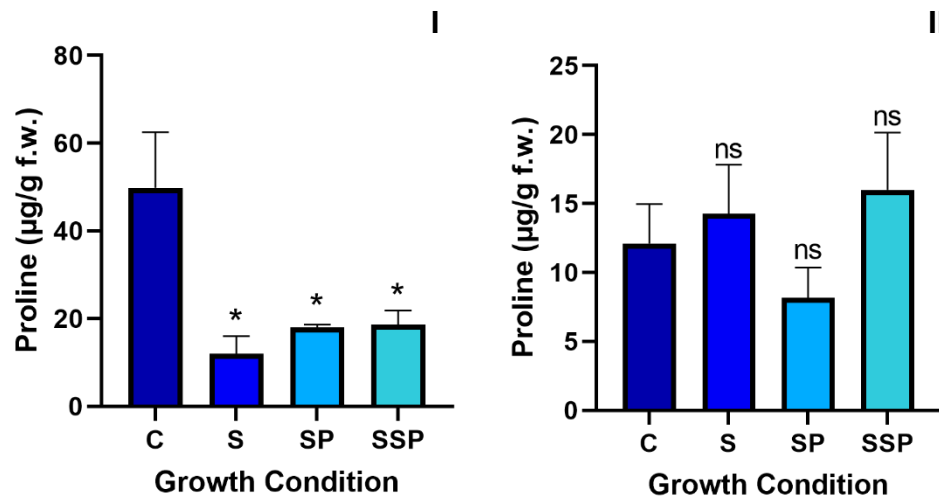


Figure 11. Proline content in leaves (I) and roots (II) of *S. lycopersicum* (cv Gold Nugget) plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The most significant decrease observed was detected in S plants, corresponding to 76%, plants sprayed with copper-based fungicides (SP) showed a smaller decrease of 63%. Finally, SSP group plants exhibited the smallest reduction in proline content equal to 48%.

No significant variations were gleaned from the tests performed on roots.

3.10. Photosynthetic pigments levels

Plants exposed to copper suffered an impact on pigment content present in leaf tissue. In respect to Chlorophyll A and B, plants grown on copper contaminated soils (S) and those exposed to copper through spraying (SP) showed no significant changes. However, plants that suffered combined exposure to Cu ions (SPP) showed a 47% decrease in Chlorophyll content (figure 12).

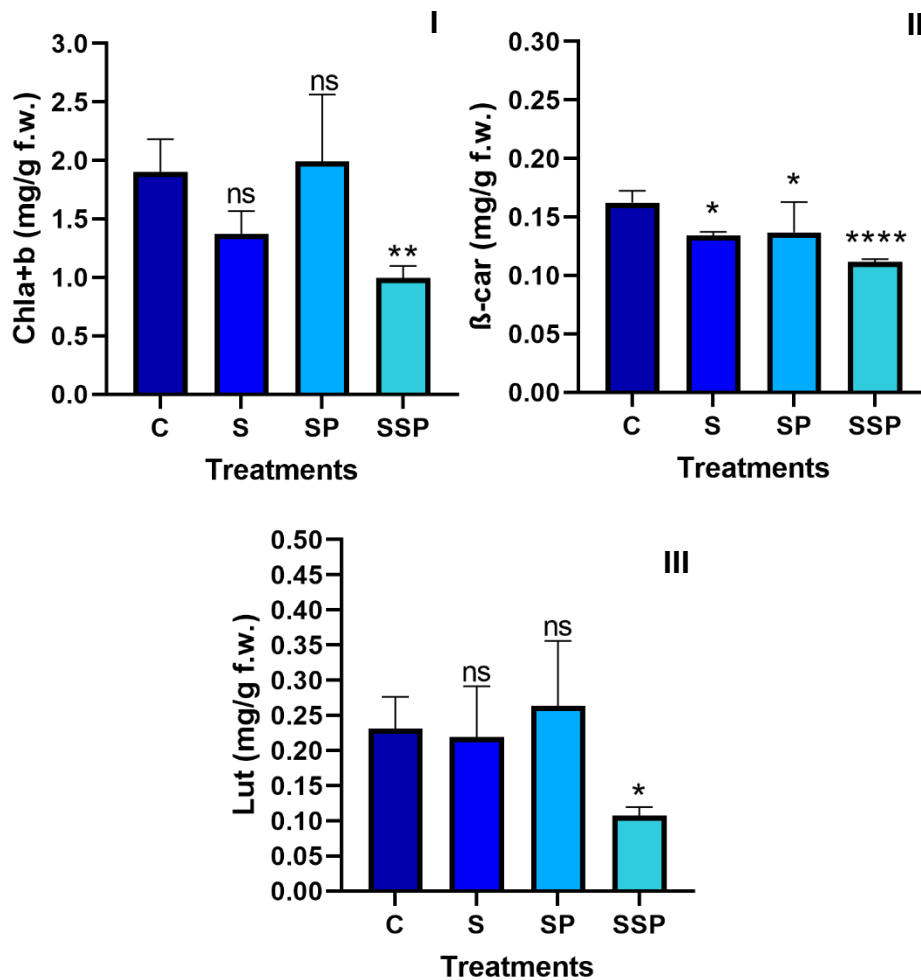


Figure 12. Photosynthetic pigment contents, (I) Chlorophyll A+B, (II) B-carotene and (III) Lutein in leaves of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Regarding β -carotene content, all tested growth conditions led to significant decreases, with SP group plants exhibiting a 15% decrease in β -carotene content, followed by the spraying group with a 17% reduction, and SSP plants exhibiting a 31% decrease.

Lastly, lutein levels only showed significant statistical changes in plants that experienced combined exposure (SSP), with a 53% decrease in lutein content.

3.11. Histochemical coloration of starch

The histochemical coloration of the leaf discs reveals no observable differences in starch content across all tested growth conditions (figure 13).

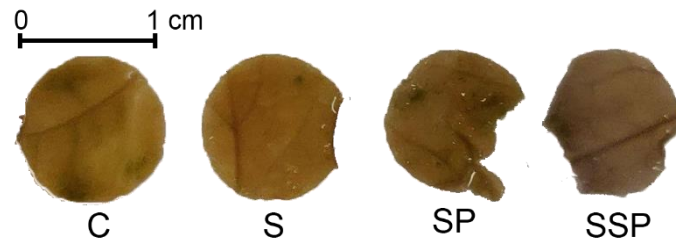


Figure 13. Typical results obtained for the lugol staining of starch in leaf disks of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP).

3.12. Starch and Glucose levels

Regarding starch and soluble sugar content of the leaves, no statistically significant changes were determined to have occurred in any of the tested growth conditions (figure 14).

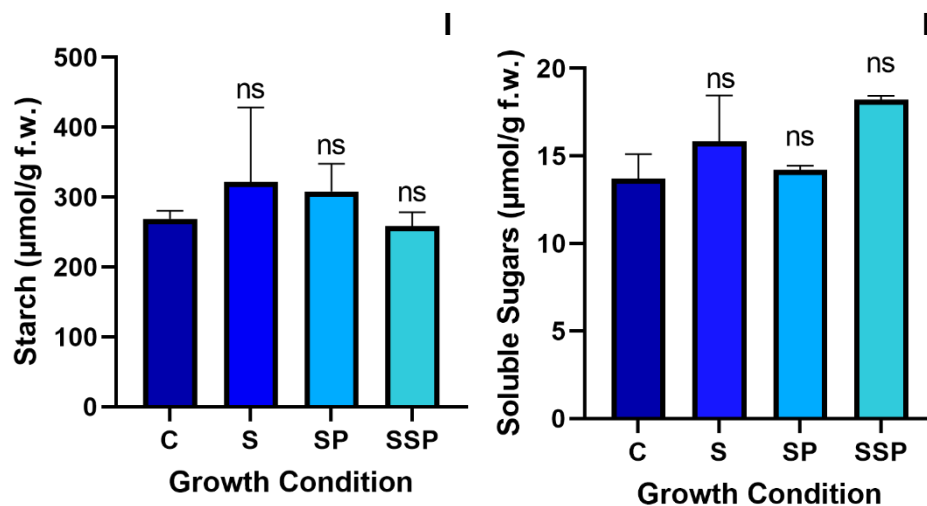


Figure 14. Starch (I) and soluble sugar content (II) in leaves of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.13. Hydrogen Peroxide levels

For hydrogen peroxide content, leaf tissue showed no statistically significant changes in any treatment performed. It contrasts with the root tissue samples, which showed

significant differences in all tested growth conditions. The SP treatment exhibited the lowest increase in H_2O_2 of 13%, followed by the S treatment with a rise in H_2O_2 levels of 36% for and 43% for the SSP plants (figure 15).

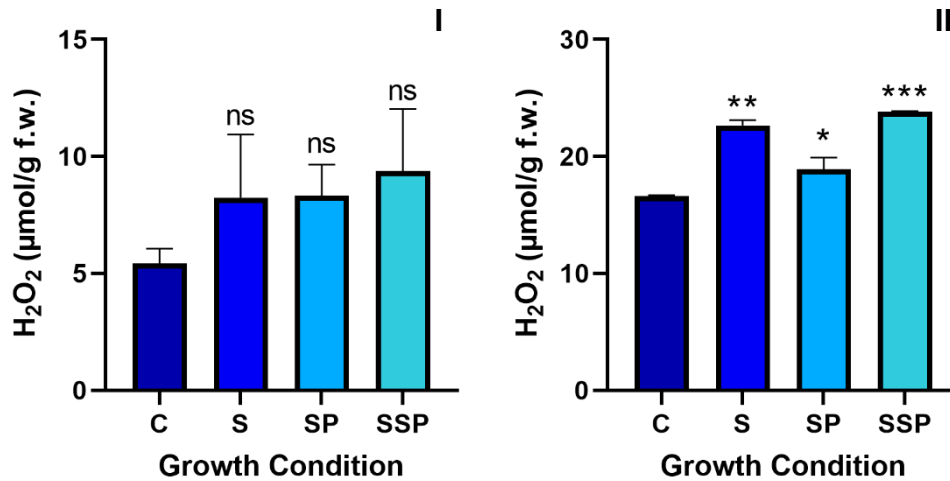


Figure 15. Hydrogen Peroxide (H_2O_2) content in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.14. Ascorbate quantification

3.14.1. Total ascorbate quantification

Leaves showed significant changes in total ascorbate content when exposed to copper through spraying (S), registering an increase of 70% followed by SSP group plants with a 64% increase in total ascorbate (figure 16).

Roots exhibited similar results to those observed in leaves, with S plants showing no significant changes in total ascorbate content, SP plants showing an increase of 30% and the combined exposure plants exhibiting a 43% increase.

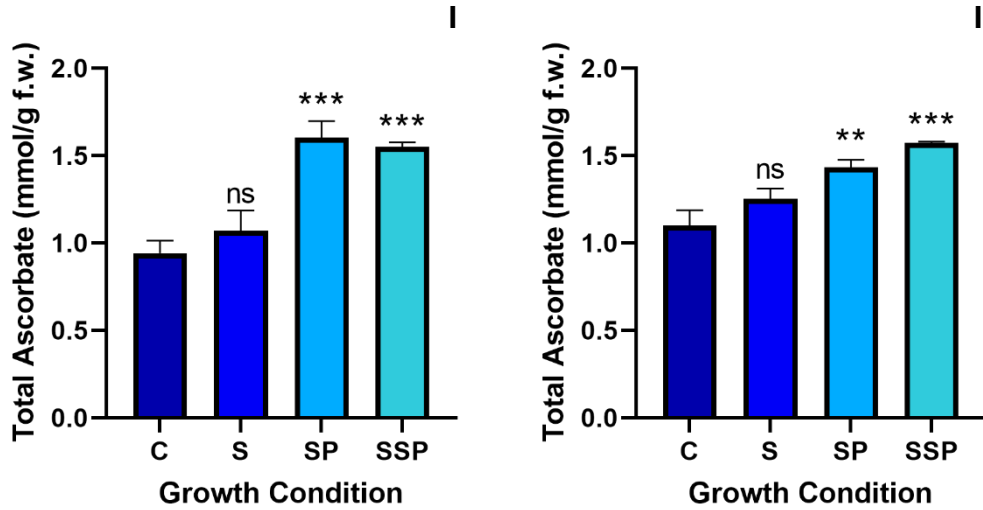


Figure 16. Total ascorbate (TA) content in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.14.2. Reduced ascorbate quantification

Copper exposure significantly increased (except SP in leaves) in the reduced ascorbate levels. Still, its impacts across modalities tested differed for leaves or roots (figure 17).

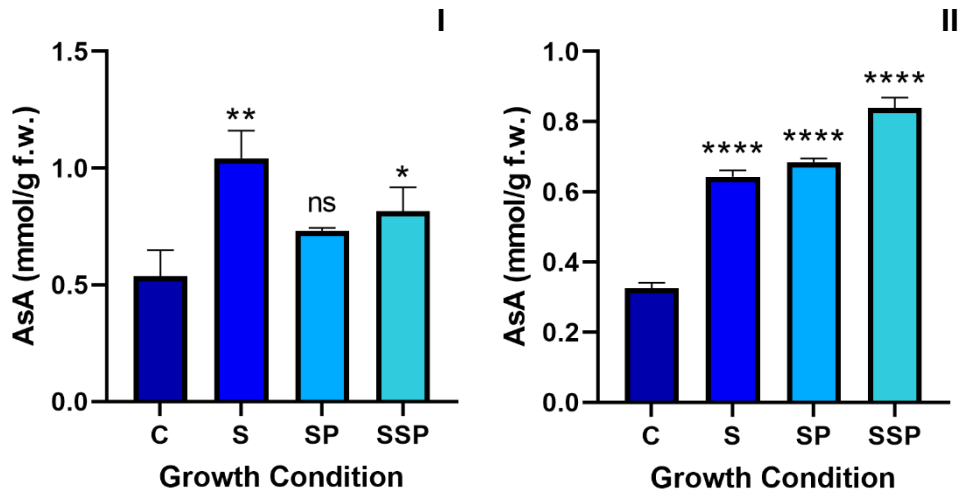


Figure 17. Reduced ascorbate (AsA) content in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Roots were the most impacted with the SSP treatment showing the greatest increase corresponding to 157%. Plants sprayed with copper fungicides (SP) and plants that grew on soils contaminated with copper (S) also revealed increases in ASA content, 110% for SP and 97% for SSP.

For leaf tissue, there were no statistically significant changes in SP group plants, although S treated plants showed a 93% increase and the SSP group exhibited a 51% increase in ascorbate levels.

3.14.3. Oxidized ascorbate

The ratio between reduced ascorbate and total in leaf tissue did not change in SP and SSP plants, but S group plants showed a 65% increase in this ratio (figure 18).

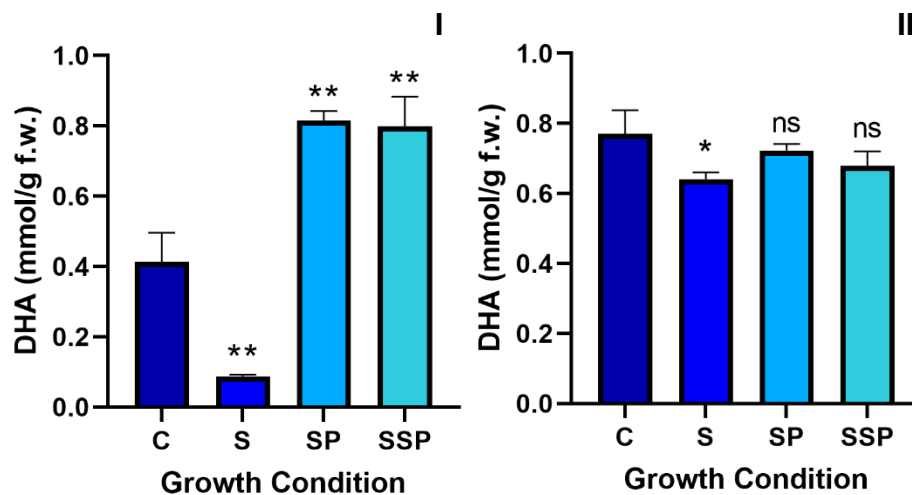


Figure 18. Oxidized ascorbate (DHA) content in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In roots, the ratio increased for all tested growth conditions, with SSP exhibiting the highest one, corresponding to 89%, followed by the group of plants that were sprayed with copper fungicides (SP) with a 65% increase and by S plants, that showed a 62% increase in this ratio.

3.14.4. AsA/DHA

The ratio between reduced and oxidized ascorbate was impacted by copper with roots exhibiting an 122% increase in S plants, followed closely by the SP group plants that showed a 129% increase and finally the in combined exposure group (SPP), presented the most significant increase was observed, corresponding to 208% (figure 19).

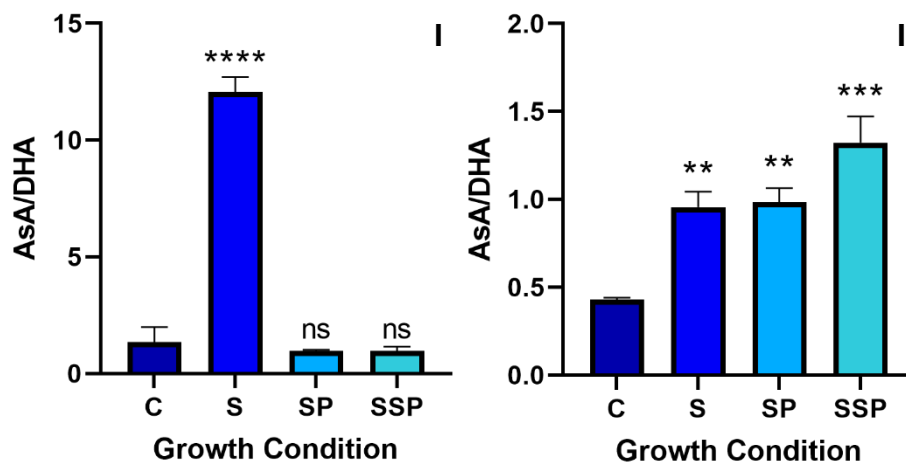


Figure 19. Ratio between reduced ascorbate (AsA) and oxidized ascorbate (DHA) in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SPP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In leaves, only the plants that grew on copper-contaminated medium (S) showed an 8-fold (797%) significant change in the ratio between reduced and oxidized ascorbate. No differences were detected for the other treatments.

3.14.5. AsA/TA

In leaves, the ratio between reduced ascorbate and total did not change in SP and SSP plants. Contrary to this, the S group plants showed a 65% increase in the ratio (figure 20).

In roots, this ratio increased for all tested growth conditions, with SSP exhibiting the highest one, corresponding to 89%, followed by the group of plants that were sprayed with copper (SP), with a 65% increase, and by S plants, that showed a 62% increase.

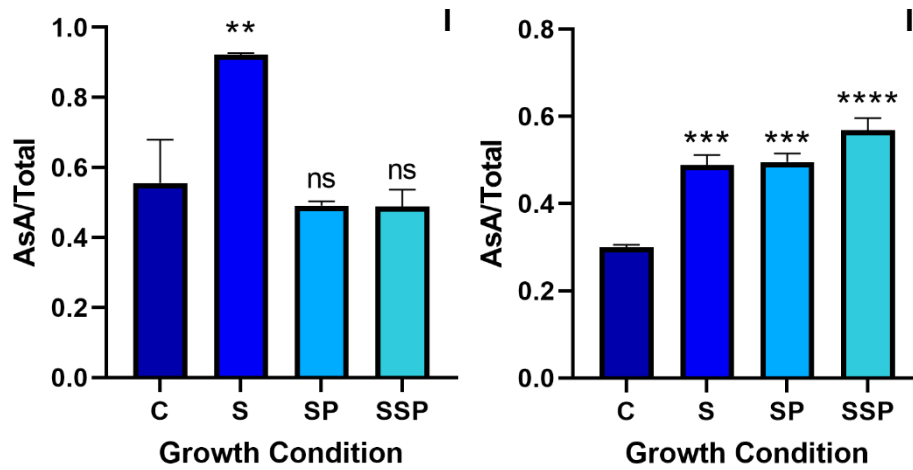


Figure 20. Ratio between reduced ascorbate (AsA) and total ascorbate (TA) in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.14.6. DHA/TA

A similar result to the previous one was seen regarding the ratio between oxidized ascorbate and total. Again, only the S group plants showed a significant change in leaves, corresponding to a 72% decrease (figure 21).

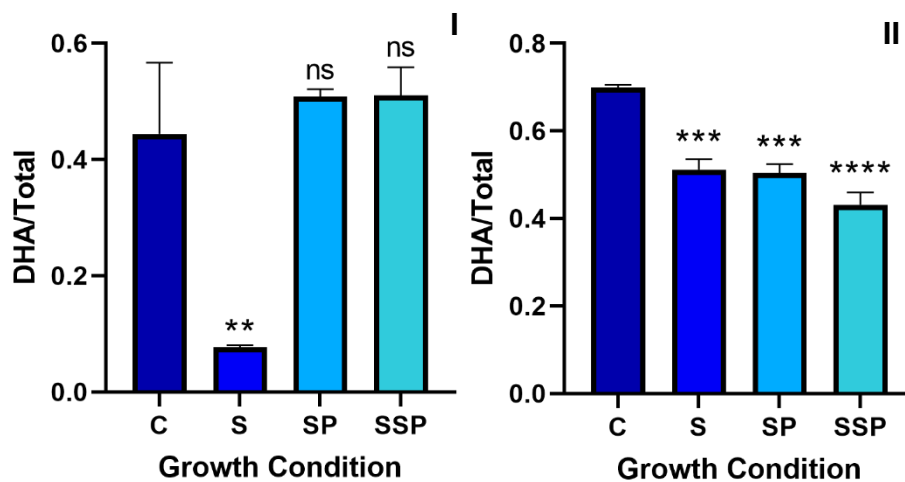


Figure 21. Ratio between oxidized ascorbate (DHA) and total ascorbate (TA) in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In roots, the ratio decreased in every tested condition, especially in the plants that suffered combined exposure to copper (SPP), registering the most significant decrease of 38%. The other tested conditions exhibited similar decreases of approximately 27%.

3.15. Glutathione levels

In leaves, GSH content showed no statistically significant changes. Furthermore, even though root samples were tested with increased amounts of tissue, no GSH was detected in these tissues (figure 22).

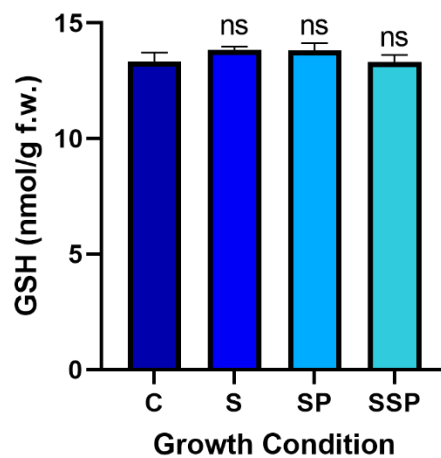


Figure 22. Glutathione (GSH) content in leaves of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.16. Thiol quantification

3.16.1. Total thiol levels

Total thiol content showed no statistically significant changes in the roots of tested plants. This result was also mirrored in leaves in SP and SSP group plants, although significant increases were observed in S group plants, registering a 57% rise in total thiol content (figure 23).

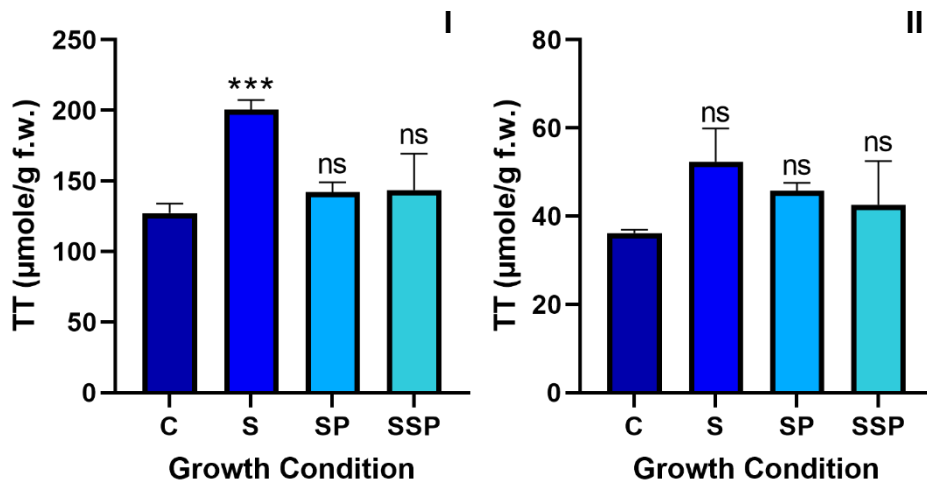


Figure 23. Total thiol (TT) content in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.16.2. Non-protein bound thiol quantification

In leaves, non-protein bound thiols exhibited significant decreases across all tested growth conditions, with plants grown in copper contaminated medium (S) showing the greatest decrease of 54%, followed closely by SP plants, displaying a 53% reduction in this parameter: Lastly, the SSP group showed the smallest decrease, equal to 50% (figure 24).

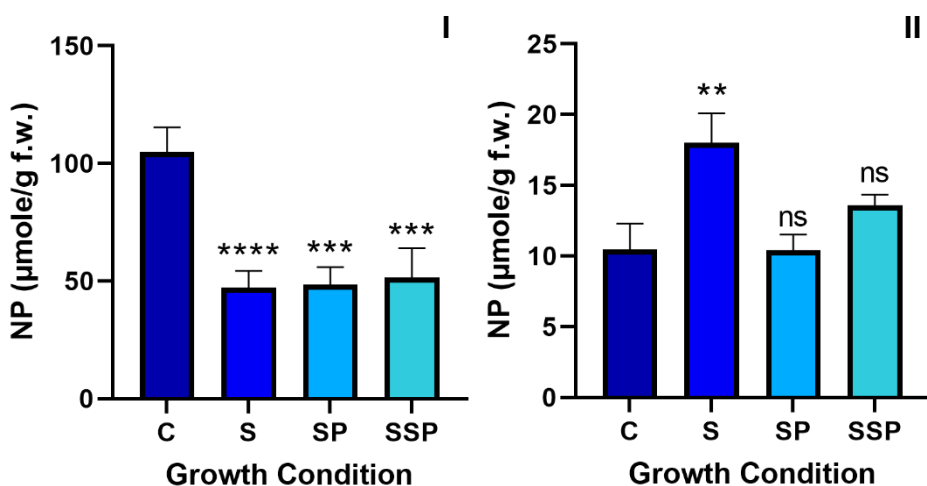


Figure 24. Non-protein bound thiol (NP) content in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In roots, SP and SSP group plants showed no statistically significant changes, while S plants exhibited an increase of 71%.

3.16.3. Protein bound thiol quantification

All tested growth conditions exhibited statistically significant increases in protein thiols (figure 25). In leaves, plants exposed to copper through medium contamination (S) showed the most significant increase, corresponding to 257%, followed by SP plants, with an 153% increase, and finally, the SSP plants, with an 140% increase.

In roots, plants of the S group exhibited the largest increase in protein thiol content of 136%, followed by SP plants, with 80% and finally SSP group plants with a 27% increase.

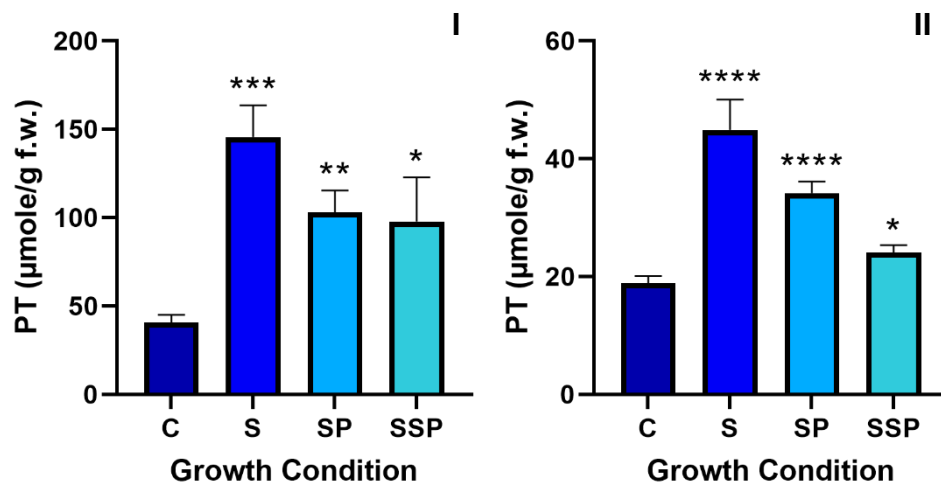


Figure 25. Protein bound thiol (PT) content in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.16.4. NP/PT

Roots exhibited no statistically significant results regarding the ratio between non-protein thiols and protein thiols. However, leaves showed decreases in both S and SP growth conditions corresponding to 55% and 35%, respectively (figure 26).

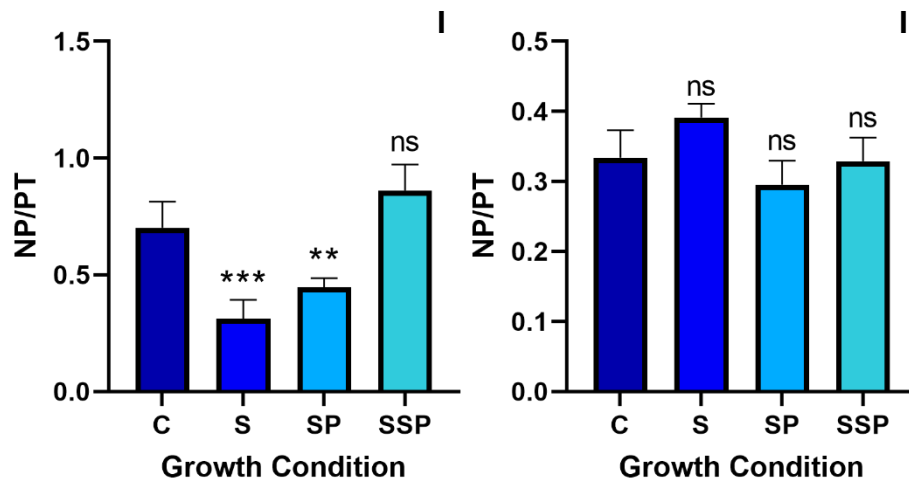


Figure 26. Ratio between and non-protein bound thiols (NP) and protein bound thiols (PT) in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.16.5. PT/TT

The ratio between PT and total thiols in leaves showed no statistically significant differences. This result was also observed for roots from both S and SP growth conditions, while SSP roots exhibited a 13% decrease in this ratio (figure 27).

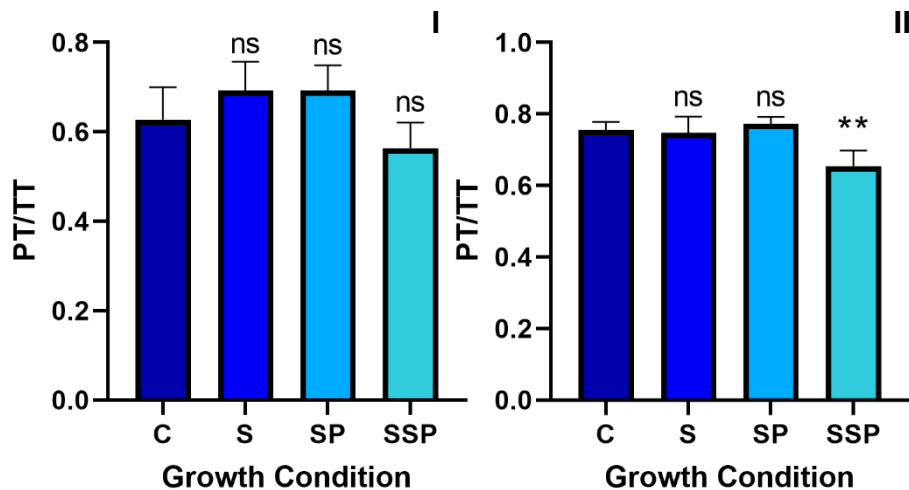


Figure 27. Ratio between protein bound thiols (PT) and total thiols (TT) in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.16.6. NP/TT

The ratio between non-protein and total thiol decreased significantly in leaves, with S and SP plants both exhibiting reductions of approximately 28%, while the combined exposure group (SSP) showed no statistically significant changes (figure 28).

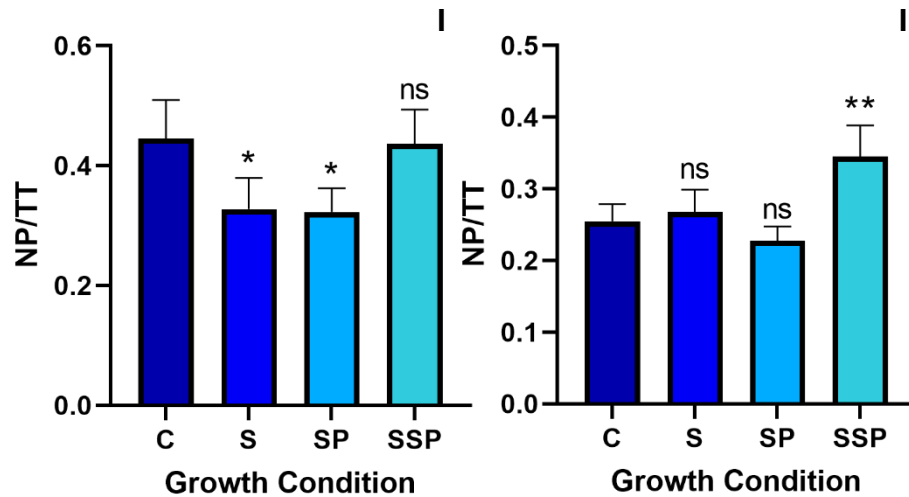


Figure 28. Ratio between non-protein bound thiols (NP) and total thiols (TT) in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In roots, only the combined exposure group (SSP) showed a significant increase, corresponding to 35%.

3.17. Semi-quantitative polymerase chain reaction (PCR) for GR

In leaves it was possible to observe that spraying the plants with copper (SP) decreased the expression of the GR-encoding gene (figure 29). The same was verified in the combined exposure group (SSP) with a much more significant reduction in the expression. However, S plants appear to show higher expression of this gene than in the control.

Regarding roots, expression appears to have increased across all tested growth conditions, with SSP showing the highest increase.

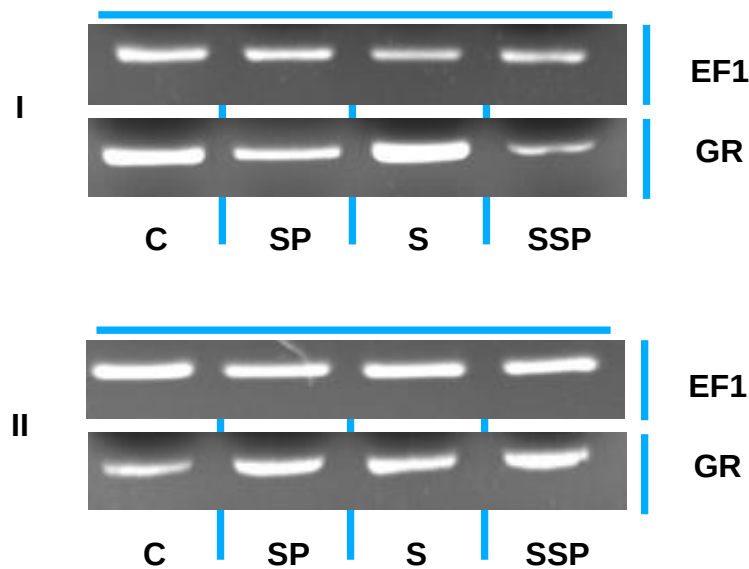


Figure 29. Typical results for the GR semi-quantitative RT-PCR analysis by 1% (w/v) agarose gel electrophoresis in leaves (I) and roots (II) of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). EF1 stands for Elongation Factor 1; Gr stands for Glutathione Reductase.

3.18. Expression of MT family genes

Copper exposure influenced mRNA accumulation of the MT-encoding genes in leaves, with all of the tested growth conditions showing increases in expression of the studied genes (figure 30).

For MT 1, all tested growth conditions exhibited increases in gene expression, with the largest one being found in SSP plants corresponding to a 15-fold (1494%) uplift, the second largest one was in the spraying group (SP) where a 11-fold (1089%) increase occurred, and lastly in S plants there was a 42% increase. The MT 2 gene did not show any significant changes in expression in leaves of plants exposed to copper through spraying (SP). However, S and SSP did exhibit significant increases, with the former being 7-fold (643%) and the latter 11-fold (1020%).

This trend continued in MT 3, with the leaves from the combined exposure plants (SSP) showing a massive 135-fold (13445%) increase in gene expression. The SP group also showed a significant 21-fold (2029%) increase, followed by S plants that exhibited a 2-fold (163%) upregulation of gene expression.

Lastly, despite increased expression in MT 4, statistical analysis revealed these changes were not significant.

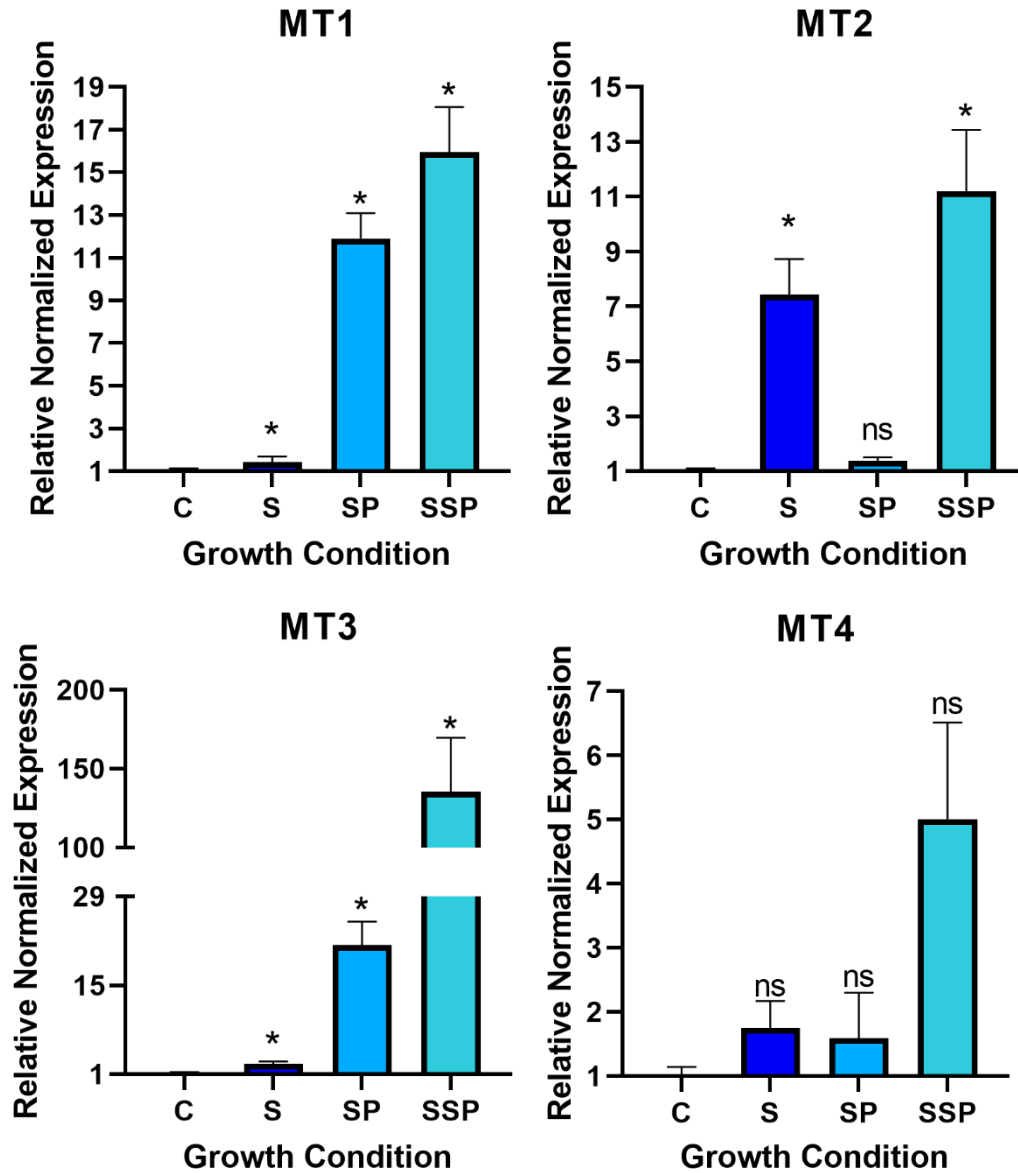


Figure 30. Expression profile of MT family genes in leaves of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$

In roots, MT family genes transcripts were clearly impacted by the exposure to copper, in MT 1, all tested growth conditions exhibited reductions in mRNA accumulation. S and SSP plants both showed similar decreases, 59% and 61%, respectively, while the SP group displayed a lower reduction of 40% (figure 31).

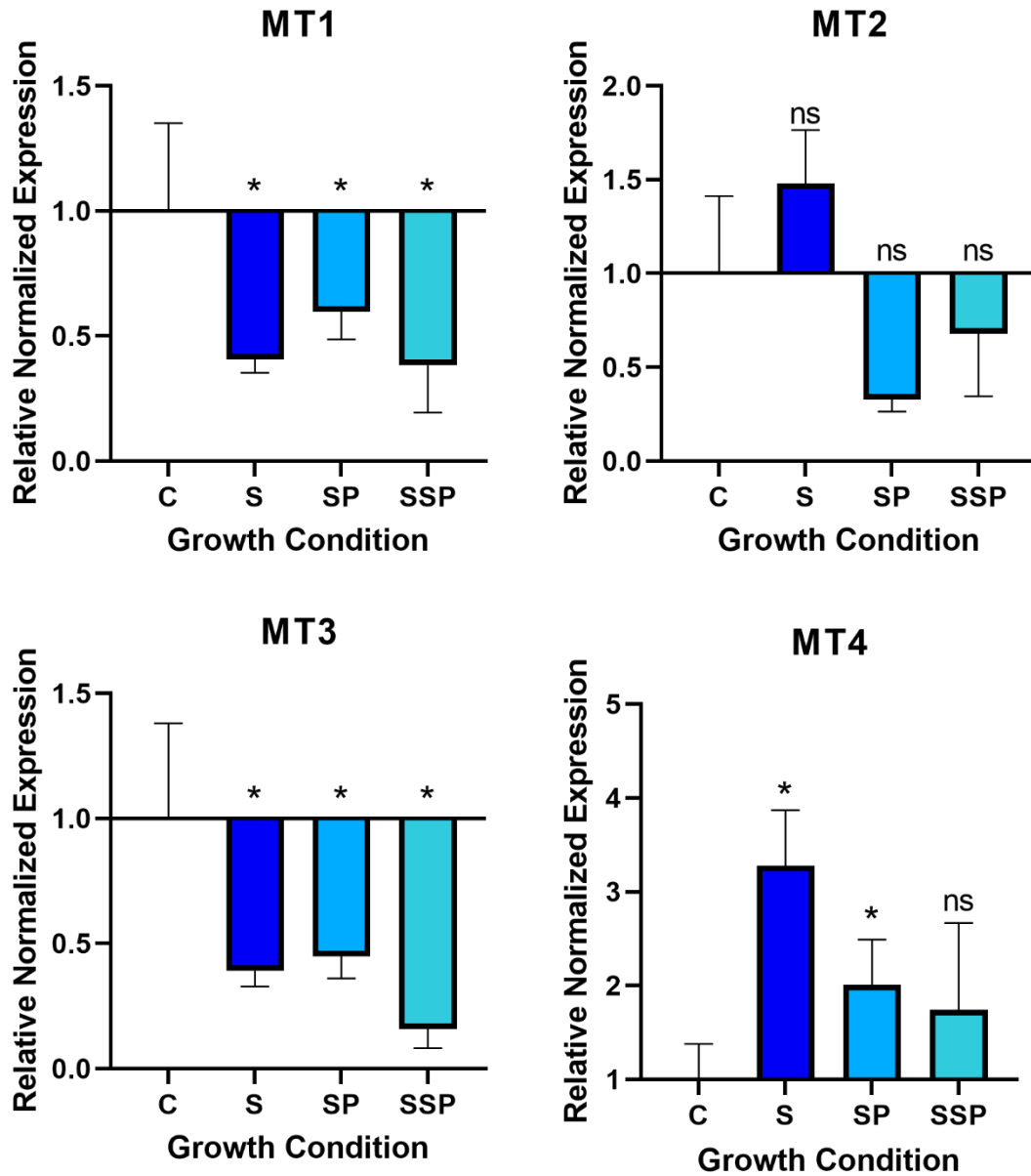


Figure 31. Expression profile of MT family genes in roots of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$

MT 2 did not exhibit any statistically significant changes in expression, while MT 3 expression was also negatively impacted, with the combined exposure group showing the largest decrease of 84%, followed by the decreases in S group plants with a 60% decrease and finally by SP plants exhibiting a 55% reduction.

Finally, the MT 4 gene showed no significant changes in expression in the SSP group. However this was not the case for the remaining growth conditions, with the plants that grew on contaminated medium (S) displaying a 3-fold (228%) increase and SP plants exhibiting a 2-fold (100%) increase in expression.

3.19. Expression of GS family genes

Copper's effect in the expression of GS family gene members in leaves was quite pronounced, with all tested genes showing statistical significant changes on all tested growth conditions.

Starting with GS 1.1, the S group plants showed a 24-fold (2279%) increase in this gene's expression, while both SP and SSP plants exhibited a 4-fold (317%) upregulation.

The GS 1.2 gene's expression was negatively affected by copper ions in all tested growth conditions, leading to a decrease in expression. Tomato plants that grew on contaminated medium (S) showed a 42% decrease in expression, with SP plants displaying a 55% downregulation, and the SSP group exhibited a 60% decline.

For GS 1.3 transcript levels, S group plants had an 8-fold (690%) increase in expression, plants that were sprayed with copper (SP) showed a 4-fold increase in expression, but SSP plants suffered a 21% decrease.

Finally, GS 2 expression in leaves seems to have been stimulated by the plants' exposure to copper with all tested growth conditions registering increases in expression, the highest being in the co exposure group (SSP) where it increased 7-fold (611%), followed by SP plants with a 5-fold (415%) increase and lastly the copper contaminated medium (S) plants showing the lowest increase of 74%.

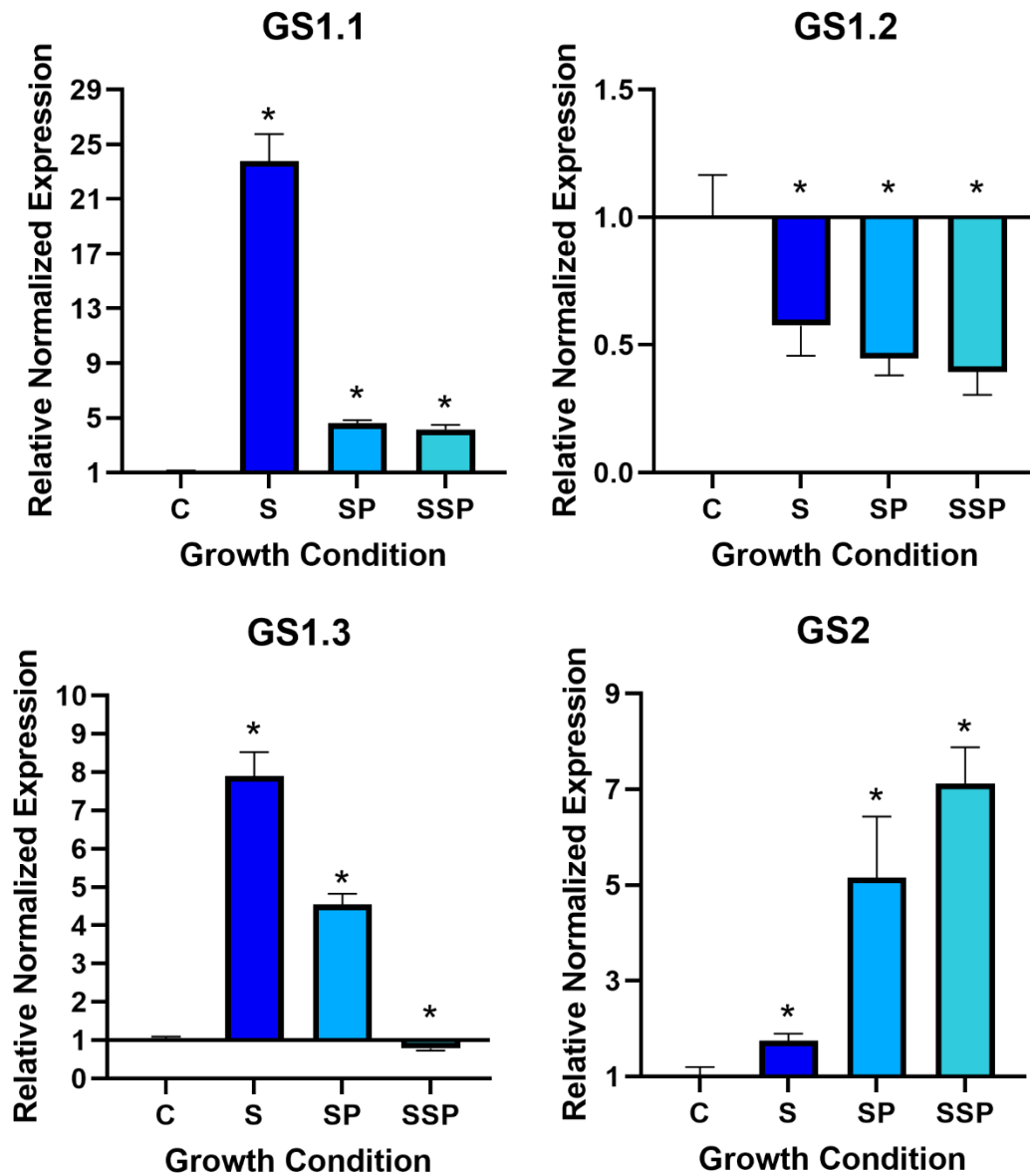


Figure 32. Expression profile of GS family genes in leaves of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$

The GS gene family showed a general decrease in expression in roots, especially GS 1.1, which exhibited the largest decreases with a 57% decline in plants grown in copper contaminated medium (S), an 84% decrease in expression in SP plants and there were no statistically significant changes in SSP group plants (figure 33).

For GS 1.2, this tendency continued, with plants sprayed with copper (S) having the smallest decrease of 33%, followed by the S group with a 39% decrease in gene expression and the biggest decrease being in SSP plants with 59%.

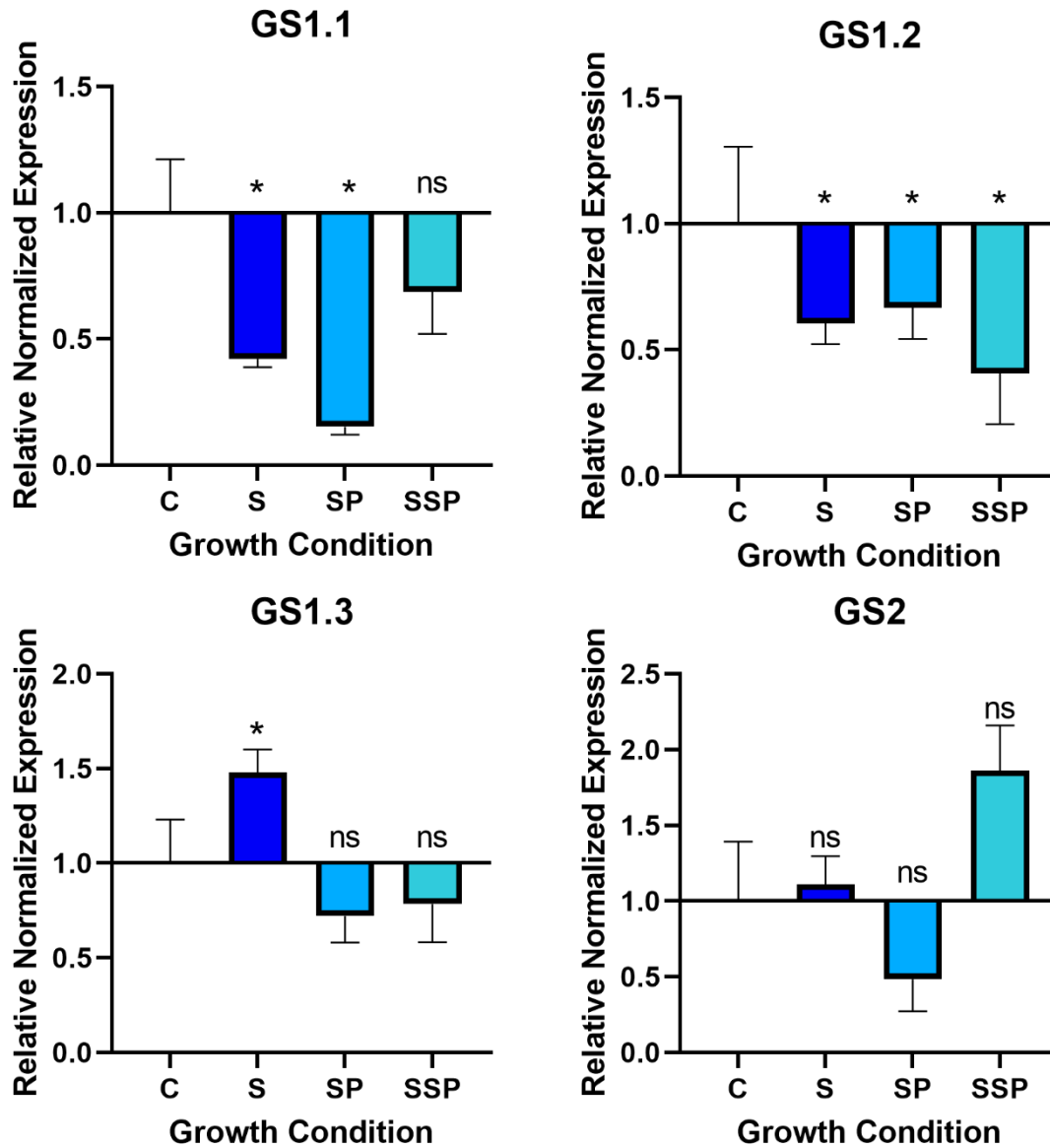


Figure 33. Expression profile of GS family genes in roots of *S. lycopersicum* plants grown in nutrient medium (C), nutrient medium supplemented 8 mg/L Cu ions (S), nutrient medium and sprayed with the equivalent of 6 kg/ha/year of Cu (SP) and nutrient medium supplemented 8 mg/L Cu ions and sprayed with the equivalent of 6 kg/ha/year of Cu (SSP). Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$

Copper seemed to affect gene expression of GS1.3 in S group plants' roots, which exhibited a 48% increase, although both SP and SSP plants did not display any significant changes in expression.

For GS 2, none of the growth conditions lead to significant changes in gene expression in roots.

4. Discussion

4.1. Copper's effect on the germination and growth of *S. lycopersicum*

The exposure to copper did not affect seed germination in both tested varieties. Although it seems to impact the development of the seedling in a dose-dependent manner. Germination was not impaired when exposed to copper ion concentrations ranging from 0.4 mg/L to 40 mg/L. This goes against the findings of Baruah et al. (2019), which report that tomato plants exposed to concentrations of CuSO_4 ranging from 20 mg/L to 220 mg/L, showed decreases across all concentrations, and the largest one in 175 mg/L corresponding to a 51% decrease in germination rate. Baruah et al. (2019) hypothesize this negative impact may be due to the higher permeability of the embryo cover to copper; it is then possible that the seeds tested in this work are less permeable to copper ions and thus less susceptible to its effects. Seneviratne et al., (2019) also stated that copper appeared to impact the release of nitrogen from the seed's storage, thus suggesting that the tested varieties in this work may be resistant to copper's deleterious effects.

Regarding the effects of exogenously applied copper on the development of the seedling, it is well known that roots are the most affected tissue by copper (Migocka & Malas, 2018), with their lengths having decreased in a dose-dependent manner in both tested varieties, but more substantially in Micro-Tom, with an 80% reduction in length compared to Gold Nugget's 47% in 8 mg/L of Cu. These decreases can be explained by the fact that the roots are the plants' first point of contact with the copper ions and because roots are the preferential storage location for copper (Salem et al., 2016). This reasoning also explains the lower inhibition of the shoot's growth, with the Micro-Tom variety showing the higher decreases in root length but then exhibiting lower decreases in shoot length when compared with Gold Nugget, possibly due to higher amounts of copper being translocated from the roots to shoots in the latter, as proposed (Migocka & Malas, 2018), and as was the case in this study with fully-grown plants, later discussed.

The seedling's fresh weight was reduced in both tested varieties across all tested concentrations. This result was also verified by al Khateeb & Al-Qwasemeh, (2014) in tomato and by (Adrees et al., 2015) in wheat, showing that exposure to copper during seed germination and seedling early development affects both root and shoot elongation and it is reflected in a decline of fresh biomass.

Although both varieties showed decreases in the tested parameters, Micro-Tom, in particular, was more impacted by copper's toxic effects than Gold Nugget. Therefore, it was decided to move forward with Gold Nugget in this work.

4.2. Copper accumulation and transport

Tomato plants can accumulate heavy metals, with copper being no exception. Due to copper's ability to be transported from tissue to tissue (Adrees et al., 2015), it becomes necessary to quantify to better understand how it is translocated within the plant and how it may affect locally the organ's physiology.

The high levels of copper present in the roots of plants that were only exposed to copper through foliar applications give credence to the fact that copper was transported from the shoots to the roots. This phenomenon could be explained by copper being transported in the phloem by Metallothioneins (Guo et al., 2008; Verbruggen et al., 2009) and in fact this correlates with the increased expression in leaves of MT 1 and MT 3 in sprayed plants (SP) and of MT 1, MT 2 and MT 3 in combined exposure (SSP) situations, with downregulation of MT 1 and MT 3 gene expression in roots, maybe as a response to their transport from the leaves and accumulation in roots. It is also worth noting that it is well known that MT participates in the homeostasis of heavy metals, copper included. Especially in roots, MT 1 is known to be involved in chelating excess copper (Guo et al., 2008), which is in accordance with the results obtained in this work.

Although shoot-to-root transport can be inferred from the data obtained, root-to-shoot transport of copper may also be occurring. The higher copper content in the leaves from the S treatment, when compared to the control, indicates Cu being transported from the roots to the leaves, as already reported (Adrees et al., 2015). In this treatment, the leaves were not exposed to exogenous copper. Therefore, a plausible explanation for its higher content in leaves is copper being translocated after following its adsorption to MT 4, as these were the only MT-related genes who suffered an increased expression in roots with this treatment.

The elevated copper content in the SP and SSP treatments' leaf samples can be explained by the fact that the plant shoots were not washed at harvest, as no copper was visible. Nevertheless, the lower content of copper in SSP shoots can again be explained by copper being transported from the leaf to other organs, such as roots. The fact that copper wasn't present in detectable amounts in fruits demonstrates that these were

protected by the rest of the plant, possibly as a strategy to protect the future generations (seeds) from being affected by copper toxicity.

4.3. Copper's effect on photosynthesis and carbon assimilation

Copper's effect on chlorophyll and lutein seems limited to combined exposure (SSP) plants, with β -carotene being affected in all tested growth conditions. The reduction in chlorophyll and lutein content could be attributed to decreases in pigment synthesis (van Assche & Clijsters, 1990) or by the magnesium present in the chlorophyll molecules being substituted by copper (Prasad & Strzałka, 1999).

β -carotene's reduction could also be explained by it being oxidized by ROS due to its ability to act as an antioxidant (Dietz et al., 1999). These reductions in photosynthetic pigments are a well-known consequence of heavy metal stress in plants with reductions in chlorophyll and carotenoid content after *Solanum lycopersicum* L. (Abid et al., 2017) and *Solanum nigrum* L. (Wan et al., 2012) were exposed to cadmium. Furthermore, a reduction in lutein content was reported when *Carex vulpina* was exposed to cadmium (Haisel et al., 2019).

The histochemical starch coloration did not show any changes with the conducted treatments. This result is further backed up by the starch quantification, which also didn't have any significant changes in starch content. Furthermore, the sugar content showed no significant changes as well. These results indicate that copper had no effect on carbon assimilation and starch accumulation in leaf tissue, despite the changes observed for the SSP's photosynthetic pigments, suggesting that all treated plants could protect their photosynthesis. Interestingly, other reports revealed that exposure to copper lead to increased starch and sugar contents in cucumber leaves (Alaoui-Sossé et al., 2004; Vinit-Dunand et al., 2002) which may suggest that copper exposure may trigger a response mechanism that protects photosynthesis (Vinit-Dunand et al., 2002).

4.4. Copper's effect on nitrogen use efficiency

Plants utilize many different molecules as nitrogen sources, such as nitrate, nitrite, ammonium, and amino acids (McAllister et al., 2012). These molecules are absorbed, stored and converted into necessary compounds for the organism's normal function. As such, it is necessary to understand the nitrogen use efficiency and if plant productivity can be impaired in plants grown under the conditions used in organic farming.

Nitrate is plants' preferred nitrogen source (Good et al., 2004). Its increase in all tested growth conditions in roots and S plants' leaves indicates that nitrate is accumulated in these tissues. The nitrate accumulation in roots is in accordance with what was verified in *Luffa cylindrica* roots (Zhang et al., 2014), where copper induced nitrate accumulation in the lower tested concentrations. Despite this, copper generally leads to lower nitrate uptake and accumulation in plants (Hippler et al., 2018; Huo et al., 2020).

The absorbed nitrate is then converted to nitrite by Nitrate reductase (NR). Because the activity of this enzyme did not show any statistically significant changes with any treatment, it is possible to assert that copper did not impact the function of NR under these conditions. Burzyński & Burzyński (2014) stated that nitrate supply regulates NR activity and since an increase in nitrate was verified, a non-variation in NR activity would go against this. Moreover, the results depicted in this report are contrary to what Z.-T. Xiong et al. (2006) obtained, who suggested that Cu directly affects the enzyme by attaching itself to the SH groups. Vajpayee et al. (2000) also affirmed that reductions in NR activity in plants under chromium stress were due to lower chlorophyll production, which led to lower photosynthesis and, thus, a lower supply of photosynthates.

A decrease in Glutamine Synthase activity was observed in leaves from all tested growth conditions, as well as in roots of plants that were exposed to copper in their rhizosphere (S), even though gene expression of GS family genes increased in leaves, suggesting the existence of post-transcriptional regulatory events. The expression in roots was generally reduced, but only those from the S group suffered a decreased activity, also pointing to post-transcriptional regulatory events. A reason for this reduction in activity may be due to copper directly inhibiting GS by interfering with the SH groups of the enzymes, or by decreasing the activity of GOGAT which in turn can cause GS activity to also decrease since their activities are concerted (Hussain et al., 2020). This reduction in activity as a consequence of copper stress is corroborated by several other authors, such as in rice (Chi Yu et al., 2005; Mifflin et al., 1980) and in cucumber (Burzyński & Buczek, 1998), as well as in other heavy metal stress situations (Chaffei et al., 2004; Jha & Dubey, 2004).

GDH increased in SSP leaves and decreased in S and SSP roots. It has been described that GDH activity is highly dependent on the species, cultivar, tissue and duration of exposure to heavy metals (Mifflin & Habash, 2002). The increase in activity observed in leaves that were affected by combined exposure to copper (SSP) may be due to the GDH aminating activity to compensate for the lower GS activity. Similar increases in GDH activity were observed in bean (Gouia et al., 2000) and maize under Cd stress

(Boussama et al., 1999). Nevertheless, the decrease in activity in roots is in accordance with what was observed in pea under lead stress (Mittal & Sawhney, 1990). These results should be supplemented with a western blot for GDH, plus the quantification of ammonium in the analysed tissues, to better understand if GDH is performing amination or deamination reactions.

Proline content was reduced in leaves across all conditions, although no changes were observed in roots. These results go against what is expected of plants under heavy metal stress, as proline levels tend to increase in these situations (Szabados & Saviouré, 2010). For example, proline increased in *Brassica* under cadmium stress (Singh & Tewari, 2003) and lead stress in wheat (Y. Yang et al., 2011). These increases usually translate into higher heavy metal tolerances, possibly due to proline's role in osmoregulation and as a free radical scavenger (Delauney & Verma, 1993; Hare & Cress, 1997). A possible explanation for the decrease in proline content observed is given by Brugière et al. (1995), who concluded that a decrease in GS activity in the phloem leads to a decrease in proline in the tissues due to less available glutamate. Considering this, leaves, which registered the highest decreases in GS activity, had a substantial decrease in proline content.

4.5. Copper's effect on the Ascorbate-Glutathione pathway

Roots showed increased H_2O_2 , revealing an increase in ROS in this organ.

The Ascorbate-Glutathione pathway is a key mechanism in dealing with ROS; its changes indicate possible oxidative stress. Total ascorbate content increased in all tested growth conditions, with significant changes in plants sprayed with copper (SP) and those that suffered combined exposure (SSP) in both shoots and roots, indicating that foliar the application of copper was responsible for an increase in ascorbate production.

This increase in total ascorbate is mostly due to the rise in AsA, a conclusion that is reinforced by the increases in AsA/TA ratios, with all tested conditions showing increases in roots and S and SSP following this trend in leaves.

Oxidized ascorbate (DHA) content was reduced very slightly in roots, with only S plants showing significant changes, however in leaves, SP and SSP plants showed increases while the content of DHA in S plants decreased. This decrease that was observed in S leaves and all the root samples is indicative of AsA being regenerated from DHA, while

SP and SSP leaves show signs of a much higher oxidative stress due to their increases in oxidized and reduced ascorbate contents.

The increases in the AsA/DHA ratio observed in S leaves and all root samples can be attributed to the plant's response to oxidative stress, since such a high ratio may be the key to efficiently protecting tissues from the accumulation of ROS caused by abiotic stress (Szalai et al., 2009).

These changes in the ascorbate cycle are in line with what was observed in tomato plants that suffered heavy metal stress (Ullah Qadir et al., 2022), as well as by roots and leaves of *Phaseolus vulgaris* exposed to copper (Cuypers et al., 2000; Gupta et al., 1999).

Glutathione's (GSH) role in this pathway is of an electron donor in reducing DHA back into AsA by DHAR, becoming oxidized and then regenerating again into GSH by GR (Latowski et al., 2010). Even though GSH did not show any significant changes in its contents in leaves, the changes in the expression of GR appear to correlate with the DHA content in leaf tissue, as SP and SSP plants show decreases in expression, thus explaining the increase in DHA that was observed in these group's leaves. This also explains the reduction in DHA in S leaves due to the increase in GR gene expression. Lastly, the increase in GR expression observed in roots is in line with the results obtained in tomato plants under salt stress (Shalata & Neumann, 2001).

4.6. Copper's effect on thiol compounds

Copper's presence in the plant tissues affected thiol compounds and their associated ratios. In leaves, protein thiols increased when exposed to copper, while non-protein (NP) thiols decreased upon such exposure. Additionally, total thiol content only increased in S plants, with the rest of the tested situations showing no changes, thus indicating an increased production of protein thiols (PT) in this growth condition. The general decrease observed in NP/PT ratios in leaves indicate a possible reliance on thiol proteins, such as Thioredoxins (TRX) and Glutaredoxins (GRX), to combat oxidative stress. Furthermore, non-protein thiols decreased while GSH levels remained stable, suggesting that other non-protein thiols are being consumed or underproduced due to metal-imposed stress.

In roots, total thiol content showed a non-significant increase in all tested situations, with only the S treatment plants exhibiting an increase in non-protein thiols; protein thiols increasing across all tested growth conditions. However, these results did not change the NP/PT ratio for roots.

These results are contradictory with those published by other authors studying plants under metal stress. For example, in *Solanum nigrum* L., cadmium caused an increase in both protein and non-protein thiols (Deng et al., 2010), while a decrease in protein thiols and an increase in non-protein plants when *Oryza sativa* L. was exposed to nickel were observed (Maheshwari & Dubey, 2009).

5. Conclusion

This research provided novel information on the effects on *Solanum lycopersicum* L. of exposure to copper through spraying, as well as combined exposure to copper-based fungicides and copper contaminated rhizosphere. This field of study is yet mostly unexplored and of high interest due to humanity's growing reliance on organic farming and its associated use of copper products for food production.

The data gathered shows that even small amounts of copper in the rhizosphere and spraying of copper, below those allowed in organic farming programs, can lead to stress responses in tomato, with alterations in AOX mechanisms and a decrease in the nitrogen assimilation process. These results validate the current legislation to try and slow the build-up of copper in agricultural soils. As the tested concentrations are well below many of those found in farms that have suffered copper usage for more than 100 years, where non-resistant varieties of food crops would experience losses in productivity. Due to the variance observed in the response of different species and varieties of crops to copper and other heavy metals in stress or toxicity situations, it becomes necessary to test each species for the specific effect of these metals in the plants. Further research is especially required for the stress and toxic effects of foliar exposure to copper on other food crops that are staples in organic production systems such as lettuce, corn, grapes, and strawberries.

6. References

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