

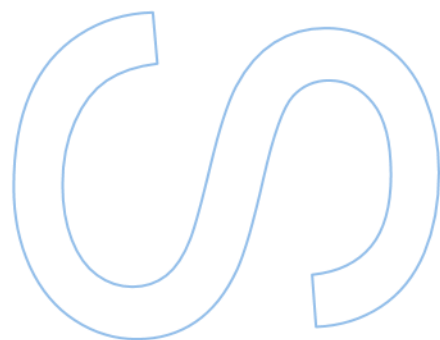
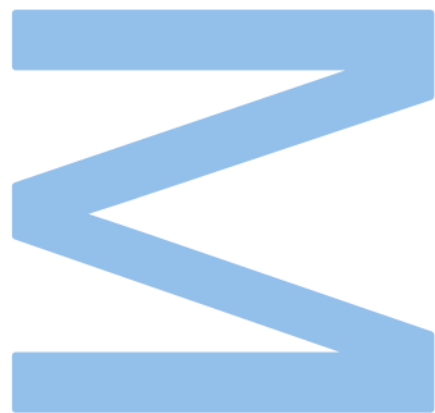
Phytomining of copper metal. Are tomato plants a good choice?

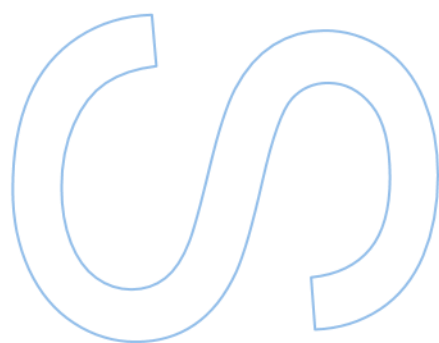
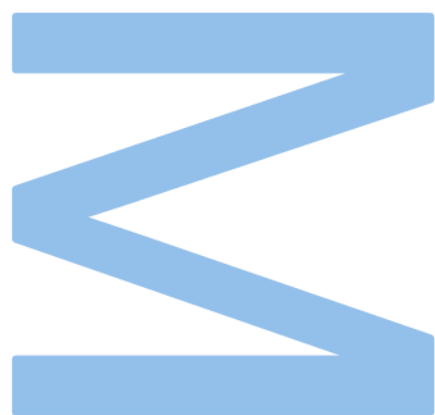
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Mestrado em Ecologia e Ambiente
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Resumo

A poluição do solo por metais pesados tem sido uma preocupação crescente para o bem-estar dos ecossistemas, bem como a saúde humana. A exploração mineral e industrial teve um aumento exponencial com a revolução industrial culminando nos níveis de contaminação ambiental que hoje são evidentes. Muitas técnicas foram desenvolvidas para amenizar o impacto na biosfera. Um método menos dispendioso foi desenvolvido, a fitorremediação, com consequente criação da fito-mineração, em que, são extraídos metais e aproveitados para uso futuro. O cobre (Cu), é um metal de transição, sendo um dos muitos metais explorados pelas indústrias e um micronutriente essencial às plantas. Desempenha um papel como cofator de inúmeras enzimas e recetores e servindo em reações de transferência de eletrões. No entanto, a sua concentração nas plantas deve ser bem controlada, para evitar stress oxidativo e limitação do crescimento. Uma vez que os tomateiros (*Solanum lycopersicum* L.) são utilizadas na indústria agrícola, os efeitos do Cu no desenvolvimento da planta foram testados, assim como as suas potencialidades de fitorremediação e fito-mineração. A taxa de germinação e as análises de comprimento e massa fresca na parte aérea e raízes das plântulas de duas variedades de *S. lycopersicum* (Golden Nugget e Micro-Tom) determinaram a Golden Nugget como a mais resistente à exposição ao Cu. As concentrações de 8 mg L⁻¹, 16 mg L⁻¹, 24 mg L⁻¹, 32 mg L⁻¹ e 40 mg L⁻¹ de Cu foram testadas sendo a primeira selecionada para os ensaios seguintes. O ensaio com plantas durante cinco semanas não revelou alterações significativas na massa fresca da parte aérea e raízes, mas uma diminuição do teor de água nas raízes, com aumento do teor de Cu nos dois órgãos. Nenhuma concentração perceptível de Cu foi detetada nos frutos, sugerindo as raízes como o órgão preferencial para a acumulação deste. Foi registada uma diminuição de pigmentos fotossintéticos, mas não foram detetadas alterações nos teores de amido e açúcares solúveis, evidenciando nenhuma alteração na capacidade fotossintética. A avaliação dos marcadores de stress demonstrou um baixo estado de stress oxidativo, com aumentos nos níveis de H₂O₂ e prolina apenas nas raízes, ocorrendo o contrário na parte aérea. O alívio deste stress foi realizado principalmente por AsA e Tióis. As atividades da Nitrato redutase (NR), apenas na parte aérea, e da Glutamina sintetase (GS) foram afetadas, apesar do aumento do nitrato em ambos os órgãos. O aumento da atividade da Glutamato desidrogenase (GDH) foi detetado apenas nas raízes. Houve um aumento da expressão de MTs na parte aérea e de *MT4* nas raízes. Na família de genes da GS, apenas na *GS1.2* na parte aérea e *GS1.1* nas raízes foi detetada uma diminuição. Esta pesquisa forneceu informações sobre o alívio do stress oxidativo e a quelatação de Cu, revelando que, nas concentrações atuais, a

fitorremediação é possível, e nenhum dano à saúde humana está presente no consumo dos frutos. No entanto, a aplicação de fito-mineração pode ser alcançada, embora análises de eficiência devam ser realizadas para avaliar essa hipótese no campo.

Palavras-chave

Assimilação de Nitrogénio, Fitorremediação, Fito-mineração, *Solanum lycopersicum* L., contaminação por metais pesados, stress oxidativo, planta do tomateiro.

Abstract

Soil pollution by heavy metals has been a growing concern for the well-being of ecosystems as well as human health. Mineral and industrial exploration had an exponential increase with the industrial revolution, culminating in the levels of environmental contamination that are evident today. Many techniques have been developed to mitigate the impact on the biosphere. A less expensive method was developed, phytoremediation, with the consequent creation of phytomining, in which metals are extracted and used in the future. Copper (Cu) is a transition metal, being one of the many metals exploited by industries and an essential micronutrient for plants. It plays a role as a cofactor in numerous enzymes and in electron transfer reactions. However, its concentration in plants must be well controlled to avoid oxidative stress and limit growth. Since tomato plants (*Solanum lycopersicum* L.) are used in the agricultural industry, the effects of Cu on plant development were tested, also to evaluate its phytoremediation and phytomining potential. Germination rate and length and fresh mass analyses in shoots and roots of seedlings of two varieties of *S. lycopersicum* (Golden Nugget and Micro-Tom) determined Golden Nugget as the most resistant to Cu exposure. The concentrations of 8 mg L⁻¹.16 mg L⁻¹.24 mg L⁻¹.32 mg L⁻¹ and 40 mg L⁻¹ of Cu were tested, the first being selected for subsequent tests. The test with plants for five weeks did not reveal significant changes in the fresh mass of shoots and roots, but a decrease in the water content in the roots, with an increase in the content of Cu in both organs. No perceptible Cu concentration was detected in the fruits, being the roots the preferred organ for Cu accumulation. A decrease in photosynthetic pigments was recorded, but no changes were detected in the contents of starch and soluble sugars, showing no changes in photosynthetic capacity. The evaluation of stress markers showed a low state of oxidative stress, with an increase in H₂O₂ and proline levels only in the roots, the opposite occurring in the shoot. The relief of this stress was carried out mainly by AsA and Thiols. Nitrate reductase (NR), only in shoots, and Glutamine synthetase (GS) activities, were affected despite the increase in nitrate in both organs. The increase in Glutamate dehydrogenase (GDH) activity was detected only in the roots. There was an increase in the expression of all MTs in the leaves and of *MT4* in the roots. In the GS gene family, only *GS1.2* in the leaves and *GS1.1* in the roots was detected a decrease. This research provided information on oxidative stress relief and Cu chelation, revealing that at current concentrations, phytoremediation is possible, and no harm to human health is present in fruit consumption. However, the application of phytomining can be achieved, although efficiency analyses must be carried out to evaluate this hypothesis in the field.

Keywords

Nitrogen assimilation, Phytoremediation, Phytomining, *Solanum lycopersicum* L., heavy metal contamination, oxidative stress, tomato plants.

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Abbreviations and Acronyms

5H₂O - Copper sulphate	GSH – glutathione
•OH – hydroxyl radical	GSS – glutathione synthetase
aa - amino acid	GSSG – oxidized glutathione
Abs - absorbance	H₂O₂ – hydrogen peroxide
AOX - antioxidant	HO₂⁻ - hydroperoxyl radical
APX – ascorbate peroxidase	LPO - lipid peroxidation
AsA – ascorbate	MDHA - monodehydroascorbato
C - control	MDHAR -monodehydroascorbato reductase
CAT – catalase	NADPH - nicotinamide adenine
cDNA – complementary DNA	Dinucleotide phosphate
Chl - chlorophyll	O₂ - molecular oxygen
Cu - Copper	O₂⁻ - superoxide anion
cv. - cultivar	PCR - polymerase chain reaction
DHA - dehydroascorbate	PCS - phytochelatins synthase
DHAR – dehydroascorbate reductase	Pro - proline
d. w. - dry weight	PVPP – polyvinylpolypyrrolidone
ddH₂O - distilled and deionized water	ROS – reactive oxygen species
DTNB - 5,5'-dithiobis(2-nitrobenzoic acid)	RT – room temperature
DTT – dithiothreitol	RT-PCR – reverse transcriptase PCR
EDTA - ethylenediaminetetraacetic acid	SB – sodium boric acid
EF1 - Elongation Factor 1	SD – standard deviation
Fe – iron	SN – supernatant
fw – fresh weight	SOD – superoxide dismutase
GR – glutathione reductase	β-car – β-carotene
GR – Glutathione reductase	γ-ECS – γ-glutamyl-cysteinyl synthetase
Grx - glutaredoxin	ε – molar extinction coefficient

1. Introduction

1.1. Phytoremediation

In soil, pollution mainly refers to toxic substances that exceed the bearing capacity and change the soil health thus affecting crop and animal production as well as drinking and groundwater which, at the end, affect human health and well-being (Golui et al., 2019). The main causes of soil pollution are heavy metals, sewage and industrial urban waste, including large amounts of fertilizer and pesticides applied to farmland (Chen et al., 2015). The traditional methods to deal with soil include heat, physical, chemical, and biological treatments (Dave & Ghaly, 2011; Ndimele, 2010). Unfortunately, these methods are not only costly but also have significant side effects on the ecosystem and therefore, phytoremediation is recommended as a more effective, economic and environmental remediation strategy (Si-Zhong et al., 2009; Walls, 2010).

Phytoremediation is a plant-based approach, which involves the use of plants to extract and remove elemental pollutants or lower their bioavailability in soil (Berti & Cunningham, 2000). This can be achieved by a variety of methods such as phytoextraction, phytovolatilization, phytodegradation, phytostabilization, rhizofiltration and rhizodegradation (Pandey et al., 2021). These methods, like phytoextraction consist in the extraction and accumulation in aerial tissue for posterior combustion (Berti & Cunningham, 2000). Phytovolatilization consists in the uptake, transport, and transpiration of a volatile contaminant by a plant generally through the plant leaves, from where the modified contaminants or wastes are evaporated and released to the atmosphere (Doty et al., 2007). Phytodegradation is the breakdown of pollutants through metabolic processes within the plants by degradation into simpler molecules through the enzymatic activity, which are further used as building blocks in the plant tissues to help the plant achieve faster growth (Feng et al., 2017). Phytostabilization makes use of certain plant species to immobilize contaminants in the soil and groundwater by absorption and accumulation in roots (Ahsan et al., 2018). Lastly, the other remaining methods, rhizofiltration and rhizodegradation, have a predominant focus in contaminant containment and degradation in the root tissue leading to the decrease in the possible interaction in the environment where these contaminants are present (Abdullah et al., 2020). Absorption of ionic compounds in the soil by plants can occur even at low concentrations through their root system. Plants expand their root system into the soil

matrix and create a rhizosphere ecosystem to accumulate heavy metals and consequently, modulate their bioavailability, thereby reclaiming the polluted soil and stabilizing soil fertility (Ali et al., 2013). Some advantages of phytoremediation include: economically achievability, in a sense that phytoremediation can be achieved by low-cost solar energy; environment friendly, because its application reduces exposure of the pollutants to the environment and ecosystem; applicability, it can be applied over a large-scale field and can easily be disposed; it prevents erosion and metal leaching through stabilizing heavy metals, reducing the risk of spreading of contaminants; and soil fertility improvement by releasing various organic matters to the soil (Aken et al., 2010; Jacob et al., 2018). Phytoremediation of soil organic pollutants differs among polluted sites and therefore requires different selection of plants. Because plants transform organic pollutants into non-toxic substances, there is no need to deal with potential waste.

However, disadvantages of this method still exist in a sense that plants must be chosen based on metal resistant, drought-resistant, and growth cycle (Tőzsér et al., 2017). Another negative aspect derives from the common extreme soil conditions where heavy metal elevated concentrations are present and many plants are not suitable for survival (Arreghini et al., 2017). Bolan et al. (2011) demonstrated that phytostabilization, the reduction of bioavailability of contaminants, may be boosted via soil amendments that immobilize metal(loid)s associated with plants that are having a high tolerance to contaminants and low-fertility soils or tailings.

With the development of industrialization and urbanization, the abundance of heavy metals in the environment has increased enormously during the past decades. (Suman et al., 2018; Ashraf et al., 2019). Heavy metals are a group of metallic chemical elements that have relatively high densities, atomic weights, and atomic numbers. The common heavy metals/metalloids include cadmium (Cd), mercury (Hg), lead (Pb), arsenic (As), zinc (Zn), copper (Cu), nickel (Ni), and chromium (Cr).

Due to the continuous emission of industrial production activities, there is a continuous increase in metal soil pollution (Dolezalova Weissmannova et al., 2019). Heavy metals in the soil also enter the food chain causing significant health problems in humans (Pizarro et al., 2016). Analysis of metal pollution of soil and plants show that the metal accumulation in different weeds was different (Singh et al., 2016). By analysing their metal mobility and accumulation potential, weed plants are proved to be an efficient and eco-friendly method for toxic metal phytoremediation application techniques (X. Wang et al., 2018). The same authors found that planting *Brassica napus* increased the

abundance and diversity of soil microorganisms, thereby reducing the harmful effect of Cd on soil. In metal polluted soil, the growth of metal tolerant plants promotes microorganisms to maintain soil fertility and plant growth (Kumar et al., 2019). Thus, revealing the importance that careful application of suitable heavy metal tolerant plants and plant growth promoting rhizobia, including symbiotic nitrogen fixing organisms, can improve the efficiency of plant restoration (Khan et al., 2009), resulting in different plants having different capacities in heavy metals removal, being necessary more research to improve the efficiency of phytoremediation such as, for example, genetic manipulation. Simultaneously, remediation measures are vital to prevent heavy metals from entering into terrestrial, atmospheric, and aquatic environments, and relieve the contaminated land (Hasan et al., 2019).

1.2. Phytomining

Phytomining is a technique that recovers metals from contaminated soils for economic benefits based on growing, harvesting, and processing metal hyperaccumulator plant species (Brooks, 1994); it involves the production of a “crop” of metals by growing high biomass hyperaccumulators that accumulate high concentrations of metals (Robinson et al., 1999) proceeded by biomass combustion making this technique a cost-effective and environmentally friendly option for obtaining valuable metals (Anderson et al., 1999). Conventional mineral exploration targets high ore concentrations leaving behind zones where these techniques aren’t economically feasible, but possible with less expensive, invasive and more environmental friendly methods like phytomining (Sheoran et al., 2013). Thus, this method seems to be an environmentally friendly and sustainable method for the commercial recovery and smelting of economically valuable metals from soils. There are several cases of plants showing high tolerance and resistance to metals that can accumulate high concentrations of metals in aboveground tissues (Anderson, 2003; Cosio et al., 2004; Verbruggen et al., 2009). This process can be possible by the reduction of the toxic effect of metals by chelation and compartmenting (Kumar Yadav et al., 2018).

In comparison to conventional mining technologies, phytomining has several advantages. The two most important advantages are the fact that phytomining offers the

option to exploit ores that are not economic for conventional mining approaches (Sheoran et al., 2009) and the low cost of operation (Robinson et al., 2003). Furthermore, the use of cheap solar energy for creating a “bio-ore”, low energy inputs for melting, less SO emissions due to bio-ore being sulphur-free, the improvement of site quality and other synergy effects with related industries as a consequence of biomass growth, e.g., generation of bioenergy (combustion, fermentation, generation of heat and biofuels), and the public acceptance as a “green” technology (Ali et al., 2013; Harris et al., 2009) makes this technique even more attractive. Robinson et al. (2009) emphasized the positive effects of phytomining in the remediation of sites polluted and degraded due to mining and metallurgical processes, tailings, dumps, etc. The reduction of erosion (wind, surface runoff) and leaching of toxic substances to groundwater will also improve water quality (Saxena et al., 2019).

However, there are also severe limitations and restrictions negatively affecting the general applicability and success of phytomining. The most important limitation is due to the plants’ shallow rooting system which allows for minerals close to the surface only to be extracted via this “green” technology (Hunt et al., 2014). Furthermore, adverse site conditions, e.g., poor physical and chemical properties of soils on contaminated sites, usually limit plant growth severely; most stressful soil factors are the low content of nutrients and soil organic matter, and high soil acidity leading to solubilisation of heavy metals (Hunt et al., 2014; Koptsik, 2014), making phytomining being more suitable in tropical and subtropical climates. Phytomining is a long-term process with a much larger demand for area per unit of valuable elements compared to conventional mining and therefore may cause huge environmental disturbances in case, e.g., of clearing of natural vegetation for phytomining. Even if the total concentration of target elements in the soil may be high, their availability to the plants is often too low for effective uptake (Heilmeier & Wiche, 2020). The type of heavy metal extracted is in itself, a disadvantage, since only certain metals, as platinum, gold, trace metals (e.g., arsenic and selenium) have a positive net cost of the phytomining extraction (Pilon-Smits, 2005), with only a feasible application when concentrations of these elements are too low for economic activities applying conventional mining technologies, having the possible application in zones such as subeconomic ore bodies, mineral wastes (mine tailings), metal-bearing, or polluted soils, e.g., in (post-)mining areas (Naila et al., 2019; Sheoran et al., 2009).

1.3. Copper role in plants and its effects as a pollutant.

In plants, the transition metal copper (Cu) is a cofactor for plastocyanin, copper/zinc superoxide dismutase (Cu/ZnSOD), cytochrome-c oxidase, the ethylene receptors and the apoplastic oxidases: ascorbate oxidase, diamine oxidase and polyphenol oxidase (Clarkson & Hanson, 1980). In the environment of the cell, Cu can exist in two oxidation states, Cu^{2+} and Cu^+ . Therefore, Cu is redox-active and can be used in electron-transfer reactions when bond to proteins. However, this activity also promotes the formation of reactive oxygen radicals and therefore the cellular concentration of Cu should be tightly controlled. Copper is classified as a soft metal and prefers to bind with soft ligands. For example, Cu^{2+} is often found to be bound to nitrogen in histidine side chains, whereas Cu^+ prefers interaction with the sulphur in cysteine or methionine. Their role as electron transport components in most redox enzymes is based on their ability to change their valence more easily as compared to other metals found in cells (Berg, 1994). Cu along with Fe are the most abundant transition metals in living systems (Outten and Theil, 2009; Robinson and Winge, 2010). Within the plant cell, Cu is required in at least six locations: the cytosol, the endoplasmic reticulum (ER), the mitochondrial inner membrane, the chloroplast stroma, the thylakoid lumen and apoplast (Clarkson & Hanson, 1980).

Experiments with tomato established Cu as an essential nutrient for plants (Arnon & Stout, 1939), playing roles in photosynthesis, respiration, perception of ethylene, reactive oxygen metabolism and cell wall remodelling. In addition to its role as a stable cofactor, Cu may also play a role in thylakoid grana stacking. There is a possible indirect role for Cu in nitrogen assimilation and abscisic acid (ABA) biosynthesis, which are functions of molybdenum (Mo) cofactor-requiring enzymes. While determining the structure of CNX1, an enzyme that functions in Mo cofactor synthesis, it was found that a Cu ion temporarily occupies the site for Mo insertion in the bound molybdopterin substrate (Kuper et al., 2004).

Adequate Cu levels in the shoots in plants range from 2 to 50 $\mu\text{g g}^{-1}$ DW (ppm) with optimal concentrations of 6 $\mu\text{g g}^{-1}$. However, great variability in Cu contained in plants is present depending on the plant species and Cu feeding status (Cohu & Pilon, 2007). Typically, symptoms of deficiency start when Cu decreases below 5 $\mu\text{g g}^{-1}$ DW in vegetative tissues, while toxicity levels are observed above 20 $\mu\text{g g}^{-1}$ DW or higher in the same tissue (Clarkson & Hanson, 1980), with toxicity symptoms being: decreased growth rate, distortion or whitening (chlorosis) of young leaves, curling of leaf margins and

damage to the apical meristem, as well as a decrease in fruit formation. A secondary effect of Cu deficiency can be insufficient water transport caused by a decrease in cell wall formation and lignification in several tissues, including xylem (Clarkson & Hanson, 1980).

Copper toxicity thresholds vary greatly between species of plants and affect tissues differently depending on metabolic requirements. Excess Cu concentrations in the soil tend to decrease root growth before shoot growth because of preferential Cu accumulation in that organ (Alaoui-Sossé et al., 2004; Navari-Izzo et al., 2006). Copper toxicity can also reduce Fe uptake, even to the point of deficiency, depending on the form of Fe available in the soil (Clarkson & Hanson, 1980). In the shoot, the thylakoid membrane in the chloroplast, especially photosystem II (PSII), is a primary target of Cu toxicity (Bernal et al., 2006; Yruela et al., 1996). Both PSII and chlorophyll are major targets for Cu toxicity in aquatic algae (Kuper et al., 2004).

Copper concentrations range from 3 to 110 ppm with an average abundance of 55 ppm in the earth's crust (C.Mistra, 2000). Copper is comparable in average abundance to zinc (Zn, 70 ppm) but is rare when compared with Fe (50 000 ppm), aluminium (Al, 81 000 ppm) and Magnesium (Mg, 950 ppm). The bioavailability of Cu to plants is dependent on the soil type. Copper, especially as Cu(II), has a high affinity to bind to organic matter and therefore, organic soils are more likely to be Cu-deficient (Clarkson & Hanson, 1980). Even though Cu tends to bind to agar, it is much more bioavailable in agar media when compared to soils. Copper is even more bioavailable in hydroponic growth media. For example, *Arabidopsis* plants grown on agar medium with Cu below 0.05 μM (0.0032 ppm) start to become deficient (Abdel-Ghany et al., 2005) whereas toxicity occurs above 20 μM (1.27 ppm) in agar media (Murphy & Taiz, 1995).

Copper production is a multi-stage process involving: mining, crushing, milling, ore concentration, smelting, refining and waste management, generally resulting in products contaminated with toxics such as As, Cd, Hg and Pb (Timofeev et al., 2018). Pollutants emitted by these processes pose an increasing environmental and biodiversity risk, animals and humans are among the last recipients of this toxic substances in the trophic chain. Areas around mineral extraction and production show an increased heavy metal concentration in the soil (Rucandio et al., 2011), however, even at great distances, the presence of this metal in the root system of plants indicates that mineral run-off can have a long last impact not only in the immediate vicinity of industrial complexes but to the surrounding ecosystems (Sawidis et al., 2011). In turn, these same areas become improper to agricultural exploration (Nujkić et al., 2016).

1.4. Oxidative stress

Nearly all environmental and biotic stresses (salinity, drought, extreme temperatures, metal toxicity, high pesticide doses and pathogen infections) trigger a generalised stress response called an oxidative stress which can damage cell components and cause their dysfunction (Choudhury et al., 2013). This is induced by an overproduction and/or accumulation of molecules containing activated oxygen called “reactive oxygen species” (ROS). The reasons causing an oxidative stress mainly include an imbalance between ROS generation and detoxification due to disturbance of the “normal” cell physiology, and ROS biosynthesis *de novo* as a constituent part of stress signalling and immunity response needed for defence and adaptation. These mechanisms co-exist, because stress factors directly producing ROS additionally stimulate ROS generation by NADPH oxidases and peroxidases (Ranieri et al., 2003; Rao et al., 1996). The term ROS incorporates substances containing one or more activated atoms of oxygen but are not necessarily radicals. Free radicals are any chemical species that exist independently and contain unpaired electron(s). Some free radicals do not have oxygen atoms. Both ROS and free radicals promote oxidative stress through oxidation of cell compounds. Oxidative stress is therefore associated with severe and long-term redox (reduction/oxidation) imbalance due to the lack of electrons (Mhamdi & Van Breusegem, 2018).

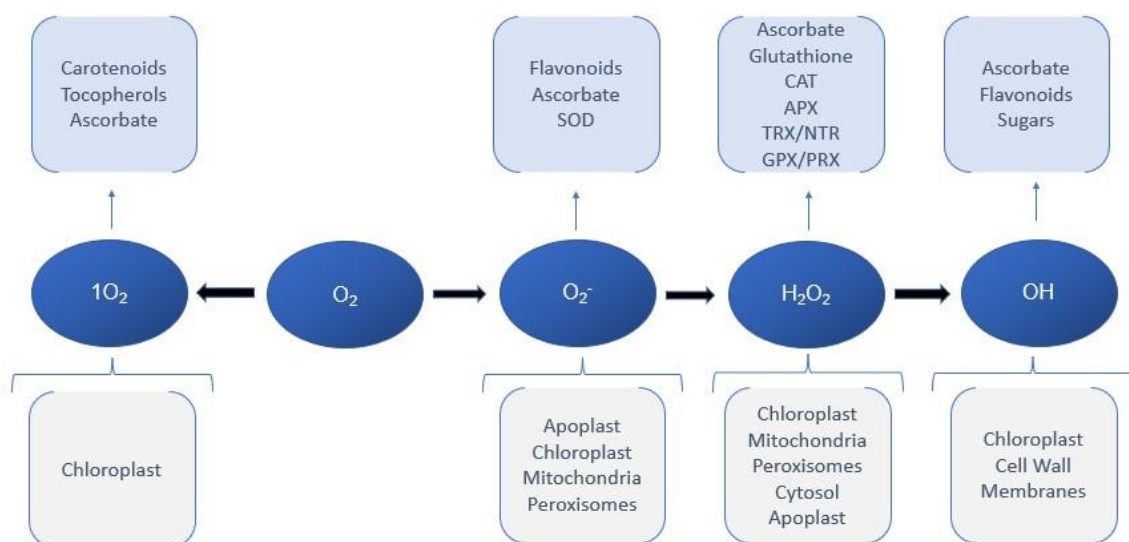


Figure 1: **An outline of the major ROS production sites and processing pathways in plants.** ROS species are represented in the middle. Shapes at the top represent ROS processing pathways and bottom shape indicate ROS generation sites. Adapted from (Mhamdi & Van Breusegem, 2018).

Triplet oxygen (O_2) can lose its “spin restriction” by accepting a single electron, for example, due to the “leak” of electrons in plant electron transport chains or the functioning of NADPH oxidase (figure 1). This leads to formation of O_2^- which is more reactive than O_2 , defined as superoxide anion radical. It has a role in several reactions, but the prevalent will be the reaction with H^+ giving hydroperoxyl radical HO_2 . The latter molecule is more reactive, more stable, and hypothetically permeable through biological membranes. Two molecules of HO_2 react and give O_2 and H_2O_2 . The ratio O_2^-/HO_2 increases with several ratios dependent on reaction pH. It can reduce other radicals and ROS ($Fe^{2+/3+}$, $Cu^{2+/3+}$, NO, phenoxyl radical, Fe–S clusters, etc.), leading to the formation of strong oxidants (Sawyer & Gibian, 1979). It reduces Fe and Cu cations, followed by interaction with H_2O_2 to produce OH (Pryor & Squadrito, 1995).

Singlet O_2 is detoxified rapidly by beta-carotene in the PSII reaction centre, water, tocopherol, reduced plastoquinone or flavonoids (Asada, 2006; Beat B. Fischer, 2013; Kruk & Trebst, 2008; Schweitzer & Schmidt, 2003). It is believed that over-production of singlet oxygen takes place under stress conditions (Asada, 2006; Beat B. Fischer, 2013) leading to oxidative injuries, induction of programmed cell death and retrograde signalling (Beat B. Fischer, 2013; Møller et al., 2007). In animal cells, singlet O_2 generation leads to inhibition of the mitochondrial inner membrane K^+ influx channels which regulate mitochondrial volume (Duprat et al., 1995; Fornazari et al., 2008), resulting in cytochrome c release and transporting superoxide anion to the cytosol. Whether similar reactions take place in plants is unknown. The involvement of singlet O_2 in ion channel activation could be through H_2O_2 , which can accumulate after singlet oxygen detoxification in organelles and diffuse to the cytosol where it activates Ca^{2+} channels and triggers signalling cascades, for example sending ROS/ Ca^{2+} messages to the nucleus. The latter is called retrograde signalling and might play an essential role in the regulation of organelle protein biosynthesis under high light and probably other environmental stresses (Chang et al., 2009; Fernandez & Strand, 2008; Karpínski et al., 2013; Kim & Apel, 2013).

Hydrogen peroxide (H_2O_2) is the most stable ROS with essential physiological functions (Foyer, 2009). H_2O_2 is a weak acid without unpaired electrons (“non-radical”), and it is a stable molecule as compared to superoxide, hydroxyl, and singlet oxygen. H_2O_2 is a weak oxidant and cannot chemically modify DNA, amino acids, or lipids. It may directly interact with SH- groups but its main ‘target’ is transition metal binding sites converting it to OH (Fry, 2004). H_2O_2 has been reported to deactivate some enzymes, for example fructose biphosphate; however, this may be caused by residues of transition

metals in experimental solutions. Most proteins withstand 100 mM H_2O_2 in transition metal-free solutions (Halliwell, 2015).

The hydroxyl radical ($\text{OH}^\cdot$) is a prime cause of oxidative damage to proteins and nucleic acids as well as lipid peroxidation during oxidative stress, therefore being directly involved in oxidative stress signalling (Demidchik et al., 2003). For example, this ROS induces the greatest activation of Ca^{2+} and K^+ channels amongst all known ROS and free radicals, leading to Ca^{2+} influx and K^+ efflux immediately after application of a stress factor (Demidchik, 2010). $\text{OH}^\cdot$ can be produced by homolytic bond fission of H_2O , this involving significant input of energy (Halliwell, 2015). The generation of $\text{OH}^\cdot$ from H_2O_2 has a lower energy threshold thought, therefore, under some natural conditions, $\text{OH}^\cdot$ can probably be directly generated from H_2O_2 and hydroperoxides by sunlight (Jain et al., 2004).

1.5. Antioxidant mechanism

Plants have several strategies to withstand an oxidative stress (Gill & Tuteja, 2010). Firstly, is an activation and *de novo* synthesis of antioxidant enzymes and non-enzymatic substances directly scavenging (removing) ROS and free radicals (figure 2). Non-enzymatic antioxidants are non-specific to different ROS and comprise ascorbic acid, glutathione (GSH), proline, polyamines, betaine, carotenes, some flavonoids, and α -tocopherol. Major enzymatic antioxidants, which show high affinity to specific ROS, include cytosolic Cu-ZnSOD, mitochondrial Mn-SOD, chloroplast Fe-SOD, catalases, peroxidases, peroxiredoxins, thioredoxins and glutaredoxins (Demidchik, 2015).

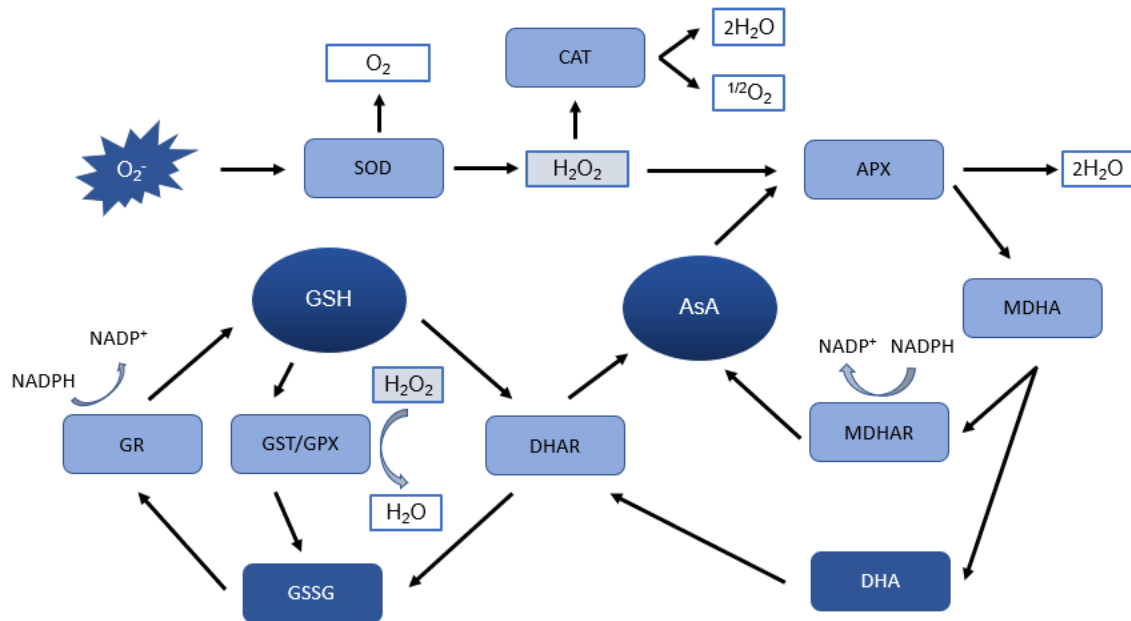


Figure 2: **Simplification of the antioxidant defence mechanisms in plants**; Darker blue round and rectangle shapes represent non-enzymatic antioxidants in reduced and oxidized forms respectively. Lighter blue represents enzymatic antioxidants. Adapted from (Hasanuzzaman et al., 2020).

Firstly, Superoxide dismutase (SOD) catalyses the dismutation of O_2^- to both O_2 to H_2O_2 the latter being scavenged by catalase (CAT) and ascorbate peroxidase (APX), resulting in the formation of H_2O (Pandhair & Sekhon, 2006). Glutathione (reduced form: GSH; oxidized form: GSSG) is one of the major low molecular weight soluble antioxidants in $NADP^+$ -containing plant cells (Foyer & Noctor, 2011). GSH is a low-molecular-weight thiol tripeptide constituted by glutamate (Glu), cysteine (Cys) and glycine (Gly), with cysteine at its active site. The presence of a sulfhydryl group (-SH) makes GSH a powerful reducing agent in cells. Simultaneously, there is a distinctive c-peptide bond between glutamic and cysteine, and it can protect GSH from hydrolysis by peptidases, thus providing a significant stability. The special chemical structure of GSH makes it an extremely important physiological function in plants. Plants use GSH for the metabolic detoxification of a range of xenobiotics, herbicides, air pollutants and heavy metals (Ilyas & Rehman, 2015). GSH also plays a key role in the tolerance of plants to other abiotic stresses including salt tolerance, drought stress, chilling damage as well as plant development by being involved directly or indirectly in ROS metabolism (Diaz-Vivancos et al., 2015; Gómez et al., 2004; Mittova et al., 2003; Parisy et al., 2007; Vernoux et al., 2000).

It is well established that the ascorbate–glutathione (ASA–GSH) pathway plays a vital role in the control of ROS concentrations (Naser A. Anjum et al., 2012). The main role of GSH in plant resistance to oxidative stress is the reduction of dehydroascorbate

(DHA) through the ASA–GSH cycle (López-Vidal et al., 2016). In this pathway, GSH exists as a recycling intermediate of H_2O_2 reduction, while efficient recycling of GSH is accomplished by Glutathione Reductase (GR; EC 1.6.4.2). Under oxidative stress conditions, ROS, such as H_2O_2 , are reduced by the ASA–GSH cycle, with GSH being oxidized to GSSG; under normal physiological conditions, GSSG can be reduced by GR to GSH in the presence of NADPH, thus forming an oxidation–reduction cycle (Lu, 2009). When the reduction ability of the cell is insufficient, the intracellular GSH/GSSG ratio decreases, and this redox state can activate many redox sensitive transcription factors and enzymes. Therefore, intracellular GSSG concentration has been used as an indicator of oxidative stress, while the GSH/ GSSG ratio reflects intracellular redox state. GSH may be involved in the stress responsive gene expression (Mulcahy and Gipp 1995). Simultaneously, H_2O_2 scavenging via oxidation of ascorbate is achieved by ascorbate peroxidase (APX) resulting in the formation of monodehydroascorbate reductase (MDHAR), being followed by its rapid conversion to dehydroascorbate (DHA) and back to ascorbate by the action of dehydroascorbate reductase (DHAR) (Anjum et al., 2010).

Other important lines of defence include substances that decrease the catalytic activity of transition metals, such as metallothioneins (small Cys-rich proteins), phytochelatins (oligomers of glutathione), pectins and other cell wall polysaccharides and structural proteins (Cobbett, 2002; Fry, 2004; V. H. Hassinen et al., 2011; Zagorchev et al., 2013). Plants can synthesise protein isoforms and lipids which are less sensitive to oxidation, requiring an upstream signalling step to activate specific genetic and metabolic pathways (Myouga et al., 2008; Wang et al., 2008). The majority of plants protect living tissues from stresses by the layers of dead cells, which die rapidly by programmed ROS-induced mechanisms (Demidchik et al., 2014). These provide a shield against infection or aggressive agents activate biosynthesis systems for reparation of damaged components (Møller et al., 2007; Yoshiyama et al., 2013).

1.6. Metallothioneins

Metallothioneins (MTs) are low-molecular-weight (4–8 kDa), heat stable, intracellular, and cysteine (Cys)-rich polypeptides, which have a strong affinity for heavy metals found in plants, animals, cyanobacteria, and fungi. In terms of structural characteristics (protein folding), most MTs are flexible and dynamic as they often contain

small amino acids. Due to the scarcity of amino acids with structure-inducing or stabilizing properties, MTs can have ordered structures only in the presence of metal ions, but otherwise adopt mostly disordered random coil conformations. Structure of pMTs, their dissociation energies, and their binding capacity with different metal ions have been reported so far (Freisinger, 2009). Metallothioneins are thought to participate in various metal tolerance or homeostasis processes since they can bind to metal ions (e.g., zinc (Zn), copper (Cu), and cadmium (Cd)) through the abundant thiol groups found in their Cys residues (V. Hassinen et al., 2011). Metallothioneins in plants defend against heavy metal-induced stress through different mechanisms such as scavenging of reactive oxygen species (ROS), sequestration, regulation of metalloenzymes, activation of transcription genes, and metabolism of metallodrugs under stress conditions (Hassinen et al., 2009).

Based on the arrangements and numbers of Cys residues, MTs have been categorized into four types (MT1–MT4) (Freisinger, 2008). Type 1 MT genes are commonly expressed in both roots and leaves, while type 2 MT genes are expressed predominantly in developing seeds, leaves, and stems (Waters et al., 2005). Type 3 MTs are more abundantly expressed in leaves and ripening fruits, while type 4 MTs are not only expressed in seeds but are also expressed in vegetative tissues and reproductive organs (Leszczyszyn et al., 2013). During plant ontogeny, each type displays a unique temporal expression pattern in different tissues and potentially performs different functions. Various genes from different plants such as *Arabidopsis*, soybean, tomato, rice, and wheat have been reported encoding MTs and the evidence suggests that the MTs participate in key physiological processes, including embryo germination and response to abiotic stresses (Yang et al., 2015).

Primary structures of MTs are rich in different conserved motifs, which have the potential to bind with a number of mono- and divalent metal ions such as Zn(II), Cu(I), and Cd(II), and enable MTs to participate in the homeostasis of essential metal as well as in metal detoxification (Blindauer & Leszczyszyn, 2010). In addition, the MT thiol groups can have a strong antioxidant ability so that MTs protect against oxidative stress (V. Hassinen et al., 2011). Metallothioneins have also been reported to participate in other activities (cellular processes) in the plants, including DNA damage repair, cell growth and proliferation regulation, reactive oxygen species scavenging and sequestration (Grennan, 2011), transcriptional gene activation and metalloenzyme activity regulation (Bratić et al., 2009), root development, and fruit ripening and suberization (Yuan et al., 2008). In addition, the MT functions described above in plants

have been confirmed using different techniques, including the characterization of MT-metal complexes, tissue-specific and metal-regulated expression, and knock-down and over-expresser lines (Yuan et al., 2008). Several MT gene expression assays were already conducted with results indicating MT2 gene up-regulation under copper and zinc stress (Bolukbasi & Karakas, 2019; Talukder, 2021) and cadmium (Bölükbaşı, 2021), indicating a concentration threshold metal type dependent were regulation inverts the expression of MT revealing a induced metal toxicity tolerance interval.

1.7. Nitrogen metabolism

Plants need larger quantities of the macro element N compared to other essential elements. Most plants absorb N soluble forms such as ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), amino acids and urea that are usually available in the soil solution. In aerobic and well-drained soils, NO_3^- is the major form of inorganic N and plants take up NO_3^- preferentially. Nitrate reductase (NR, EC 1.6.6.1) is the first enzyme in NO_3^- assimilation in plants. NR reduces NO_3^- into NO_2^- catalysed by NAD(P)H in the cytosol of the cells. NR is NO_3^- -inducible, and there is a close relationship between NR activity and NO_3^- concentration in plants. Nitrite is then converted by Nitrite Reductase (NiR, EC 1.6.6.4) to ammonium, which is the inorganic nitrogen form amenable to be incorporated into organic carbon skeletons by plants (Pinto et al, 2014).

In higher plants, glutamine synthetase (GS, EC 6.3.1.2) is the key enzyme involved in the assimilation of inorganic nitrogen into organic forms. GS catalyses the ATP-dependent condensation of ammonium with glutamate to yield glutamine, which then provides nitrogen groups, either directly or via glutamate, for the biosynthesis of all nitrogenous compounds in the plant. Two groups of GS isoenzymes, plastidic (GS2) and cytosolic (GS1), have been identified in higher plants. In the majority of higher plants GS2 is predominant in most chlorophyllous tissues and is localized in the chloroplast stroma, being encoded by a nuclear gene; GS1 is the predominant isoenzyme present in non-photosynthetic tissues. Immunocytochemical studies demonstrated that GS1 protein is localized in the vascular tissue, in which a high proportion of the protein is concentrated in the phloem companion cells. In most plant species GS1 is encoded by a multigene family (Teixeira et al, 2005).

Glutamate dehydrogenase (GDH; EC 1.4.1.2) is an ambivalent enzyme that catalyses the deamination of glutamate to produce 2-Oxoglutarate (2-OG) and NH_4^+ , with

the production of reducing power, or originates glutamate from NH_4^+ and 2-OG, using NADH or NADPH as coenzyme (amination reaction), depending on the cellular conditions that favour one or the other reaction. GS and GDH may be differentially influenced by metals at the gene expression, protein, and activity levels (Martins et al, 2021).

1.8. *Solanum lycopersicum* L. as a model species

Tomato (*Solanum lycopersicum* L.) is one of the best studied cultivated dicotyledonous plants (Bai & Lindhout, 2007). Its domestication has mainly experienced two stages, represented by *S. pimpinellifolium* and *S. lycopersicum* var. *cerasiforme* as the ancestors, while persistent breeding of modern cultivars mainly concerns the combinations of various traits to meet with market requirements (Bai & Lindhout, 2007; Lin et al., 2014). As one of the common vegetables originated from Central and South America, tomato is favoured by numerous consumers for its delicious taste and high nutritional value, and have thus become one of the most economically important crops since the 16th century (Bai & Lindhout, 2007).

Tomato is a member of the Solanaceae family, which includes several other economically important crops such as potato, pepper (*Capsicum annuum* L.), and eggplant (*S. melongena* L.), representing one of the most valuable plant families for vegetable and fruit crops (Quinet et al., 2019). The nutritional importance of tomatoes is largely explained by their various health-promoting compounds, including vitamins, carotenoids, and phenolic compounds (Li et al., 2018). These bioactive compounds have a wide range of physiological properties, including anti-inflammatory, anti-allergenic, antimicrobial, vasodilatory, antithrombotic, cardio-protective, and antioxidant effects (Raiola et al., 2014). Tomatoes are rich in carotenoids, representing the main source of lycopene in the human diet (Viuda-Martos et al., 2014).

Portugal was the 7th world producer of raw tomatoes for processing with 4% of world production and the 3rd producer within the European Union (EU), with 14% of the EU production. Portuguese production of tomatoes for processing increased from 732 thousand tons in 1993 (95) to 1,285 thousand tons in 2013, representing a 2.0% higher annual average growth rate than the growth in world tomato production, which was 2.5 % per year over the same period (WTPC, several years). The Alentejo region accounts

for 83% of the national production, followed by the Lisbon and Tagus Valley region with 17% (INE, 2016).

S. lycopersicum has become a studied model for fruit ripening regulation and development biology of climacteric fruits (Chen et al., 2020), and several abiotic stresses like low and high light responses (Wang et al., 2022), salinity (Sholi, 2012), cold stress (Miura et al., 2012), drought (Gong et al., 2010) and heavy metal stress (Alamer et al., 2022). However, heavy metal exposure studies performed in *S. lycopersicum* Golden nugget cultivar, present in this dissertation, are hardly any comparing with its Micro-Tom cultivar. Literature analysis indicates generally fruit quality, plant metabolism and relative total phenolics studies in the *Solanum* family (Al-Daej, 2009; Lange, 2013), becoming of increasing importance the study of this particular tomato cultivar under heavy metal stress to allow an assessment of its phytomining in heavy metal contaminated soils potential.

1.9. Objectives

Because the automotive and electronics industries are on the rise since the beginning of the last century, higher contaminations by heavy metals continue to pose a serious and increasing threat to ecosystems' balance and biodiversity preservation. With this, soil cleaning techniques are, in some cases, employed to alleviate the contamination degree of the immediate surroundings of exploration sites. However, in many cases, these techniques include the usage of expensive compounds accompanied by large energy expenditure. The applications of phytoremediation and, for the purposes of this dissertation, phytomining, pose a serious advantage in a sense that, with the right combination, through site analysis, plant species, contaminant concentration present and soil conditions, this technique can be applied, and the materials extracted for purification and future use. In this dissertation the potential use of tomato plant *S. lycopersicum* for copper phytomining application was performed by analysing copper content in shoots, roots and fruits in 5-week-old plants and an insight on the possibility of toxicity by copper exposure on human consumption. Simultaneously, since this heavy metal is an essential micronutrient in plants, biometry, photosynthetic pigments content, leaf starch and sugar accumulation, oxidative stress status and transcript accumulation of genes involved in plants Cu detoxification and nitrogen metabolism mechanisms were

performed. By the end of this study, the potential for this plant usage in Cu phytomining was clarified, as well as the effects of that heavy metal on the plant's development.

2. Materials and Methods

2.1. *Solanum lycopersicum* L. seeds.

In this study, *S. lycopersicum* L. seeds were disinfected with a 10-minute immersion in 70 % ethanol and a 3-minute immersion in a solution containing 20 % commercial bleach and 0.02 % Tween-20, with constant agitation. Afterwards, the seeds were repeatedly washed with sterile distilled and deionized water, dried and stored at 4°C for future use.

2.2. Germination assay.

Following disinfection, cv Golden nugget and cv Micro-Tom seeds were distributed in sterile Petri dishes (10 cm diameter) containing a solidified medium consisting of 0.5X Hoagland solution (Taiz & Zeiger, 2010) supplemented with the respective copper sulphate (CuSO_4) concentrations (0.4 mg L⁻¹, 8 mg L⁻¹, 16 mg L⁻¹, 24 mg L⁻¹, 32 mg L⁻¹ and 40 mg L⁻¹) and 0.625% (w/v) agar. Afterwards, these Petri dishes were stored at 4°C during 48h to break seed dormancy and synchronize germination, and later placed in a growth chamber with controlled optimized conditions (16 h light/8 h dark; 25 °C) and 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation for 2 weeks. Following this period, the germination rate in each Petri dish was registered and the grown plantlets were evaluated regarding their shoot and root size and total fresh weight. This assay permitted the selection of the appropriate cultivar to be submitted to the trials to be described.

2.3. Growth trial.

After concentrations optimization, seeds were grown under the same conditions described for the control in the germination assay. After 1 week in the growth chamber, seedlings were transferred to individual pots filled with a 1:1 mixture of expanded vermiculite and perlite and separated in experimental conditions accordingly. Trays, each containing 4 pots with 2 plants each were moved to the growth chamber and left to grown under controlled optimized conditions (16h light/8 h dark; 25°C) and $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation. For the first week, to acclimatize plantlets to the new conditions, every tray was irrigated only with 0.5X Hoagland solution, while for the following 5 weeks one tray was supplied with copper sulphate ($.5\text{H}_2\text{O}$) at the concentration of 8 mg L^{-1} diluted in 1X Hoagland solution; with applications being done to maintain copper soil concentration and nutritive medium renovation when necessary. For the control group plants, the same procedure was conducted but without additional copper sulphate application besides that common in the Hoagland solution.

After the final copper application was completed, the grown plants from both treatments were removed from the pots and their roots washed with tap and deionized water. Finally, shoots and roots were separated and the length and fresh weight determinations of both portions of the plant were conducted. Terminal leaflets from the 3rd to the 5th leaf counting from the apex, as well as roots, were harvested from all plants and stored at -80°C. Afterwards, plants were left to dry at 65°C in an oven until weight stabilization monitored by weekly measurements for the posterior determination of the hydric content. Duplicates of each tray were separated for plant growth and their respective treatments maintained until fruit development was observed and fruit maturity achieved. Subsequently, random mature fruits were selected for fresh weight and posterior essential metal content evaluation and left to dry at 65°C in an oven until weight stabilization.

2.4. Copper metal quantification.

The levels of copper in fruits, shoots and roots of plants exposed to the two treatments were quantified to assess the preferential organ for copper accumulation. The collected plant parts (processed as described above) were dried at 65 °C until constant weight was recorded. The material was ground to a fine powder and kept in a desiccator until required. Then, aliquots were subjected to an acid digestion with a HCl: HNO₃ (1:3) mixture to release the metals bound to proteins or other biological structures. The resultant digests were dissolved in a specific volume of water. The quantification of copper content was accomplished using a Flame atomic absorption spectrometer, based on a suitable external standard solution (Perkin Elmer, AAnalyst 200 model, Shelton CT, according to manufacturer's instructions).

2.5. Leaf physiology parameters.

2.5.1. Leaf Water Content (LWC).

For estimating the leaf water content (LWC), leaf fresh mass was extracted from the 3rd emergent leaf, lateral foliole (1 leaf per pot), and contained in aluminium paper, leaving an opening to promote air flow. Afterwards, the bulk leaves were weighted in a high precision scale before and after being dried at 65°C until complete dehydration. The LWC was determined by calculating the difference between the fresh and dry weight and relative LWC was expressed in terms of percentage of the total fresh weight (f. w.).

2.5.2. Photosynthetic pigments evaluation.

The quantification of total photosynthetic pigments was performed according to (Tosin et al., 2021). A determined mass was collected for each treatment, as described above but from the terminal leaflet. Samples were emerged in 5 mL of 80 % acetone and stored at 4 °C for approximately 24 h, until a complete bleaching of the material was observed. Afterwards, extracts were centrifuged for 10 min at 2,000 g and the supernatant (SN) transferred to another tube, where the volume was adjusted to 5 mL

with 80 % acetone, before measuring the absorbances at 480 nm (Abs_{480}), 495 nm (Abs_{495}), 645 nm (Abs_{645}) and 655 nm (Abs_{655}). Following (Bulda et al., 2008), equations (1) and (2), relative to Abs_{480} and Abs_{495} , were adopted to correct the influence of chlorophylls (Chl) and the remaining pigments in the extract's absorbances. In these equations, Abs_{480}^0 and Abs_{495}^0 represent the original values for the absorbance at 480 and 495 nm.

$$(1) Abs_{480} = Abs_{480}^0 - 0.566 \times Abs_{645} + 0.121 \times Abs_{655}$$

$$(2) Abs_{495} = Abs_{495}^0 - 0.112 \times Abs_{645} - 0.0036 \times Abs_{655}$$

The following equations, present in the same study, were then used to obtain the concentrations of each pigment (expressed in $mg\ L^{-1}$) [(3) chlorophyll a (Chla), (4) chlorophyll b (Chlb), (5) β -carotene (β -car), (6) lutein (Lut)]:

$$(3) Chla = 19.00 \times Abs_{655} - 7.61 \times Abs_{645}$$

$$(4) Chlb = 21.45 \times Abs_{645} - 5.92 \times Abs_{655}$$

$$(5) \beta\text{-car} = 17.16 \times Abs_{495} - 3.96 \times Abs_{480}$$

$$(6) Lut = 11.51 \times Abs_{480} - 20.61 \times Abs_{495}$$

The content of each pigment studied was expressed in $mg\ g^{-1}\ f.w.$

2.5.3. Histochemical coloration of starch and starch and glucose quantification.

To assess whether copper had any influence on starch storage of tomato leaves, a histochemical coloration of the starch grains was performed. First, leaf discs of 1 cm diameter were collected from the terminal leaflet of the 4th emergent leaf, four disks per treatment, each from a different plant. Afterwards, the discs were boiled in 80 % ethanol, to allow leaf bleaching, and submerged in ddH₂O water for 1 min, to allow tissue rehydration. Subsequently, the discs were incubated in a Lugol solution (2 mM iodide (I_2), 6 mM potassium iodide (KI)) for 30 min in the dark, to stain the starch present in the

tissues. Finally, the samples were momentarily distained in distilled water and the results were visualized and captured with a personal photographic camera.

To measure the amount of carbon being assimilated by the plant, the quantification of starch and soluble sugars, which are both final bio-products of two biosynthesis pathways originating from the distribution of the triosephosphate produced in the Calvin cycle, was conducted. Frozen leaf aliquots of about 50 mg were homogenized in 10 mL ethanol 80% and incubated for 60 minutes at 80°C being followed by centrifugation at 5000 g for 5 minutes at 6°C, resulting in the separation of the starch (sediment) and soluble sugars (supernatant I) being the latter transferred to tubes for subsequent quantification. For starch quantification, the sediment was resuspended in 5 mL perchloric acid 30% until homogeneity was achieved. Afterwards, the solution was incubated for 60 minutes at 60°C with constant agitation followed by vortex mix and centrifugation at 1,000 g for 10 minutes at 6°C. The resulting supernatant was used for starch quantification (supernatant II). Afterwards, 30 µL of both supernatants (I) and (II) were transfer to new tubes and mixed with 750 µL of anthrone solution (anthrone dissolved in a solution of 1:20 ratio of H₂O and concentrated sulfuric acid (H₂SO₄)). For the blanks, instead the supernatant, ethanol 80% was used. Finally, the solutions were maintained at 100°C for 10 minutes followed by a period of 10 minutes on ice a then centrifuged at 10,000 g for 10 minutes at 6°C and the supernatant used to register absorbance values at 625 nm. Starch and soluble sugars were estimated via a standard curve of increasing glucose concentrations and expressed in mg g⁻¹ of fresh weight.

2.6. Biochemical parameters analysis.

2.6.1. Hydrogen peroxide quantification (H₂O₂).

Frozen samples of leaflets and roots (200 mg) were ground in a chilled mortar with 1.2 mL of 50 mM phosphate buffer (pH 6.5) and quartz sand, and then centrifuged at 6,000 g, 4 °C for 25 minutes, for supernatant (SN) recovery. A volume of 500 µL of supernatant of each growth situation reacted with 500 µL of 0.1 % (w/v) TiSO₄ in 20 % (w/v) H₂SO₄. A blank was prepared in which the supernatant was substituted by 50 mM phosphate buffer (pH 6.5). After a new centrifugation at 6,000 g, 4° C for 15 minutes, absorbances were measured at 410 nm. Hydrogen peroxide content was measured

applying $\epsilon = 280 \text{ mM}^{-1} \text{ cm}^{-1}$ and the results were expressed in $\mu\text{mol g}^{-1} \text{ F.W.}$ (Jana and Choudhuri, 1982).

2.6.2. Proline quantification.

Leaflets and roots samples of 200 mg were grounded in 1.5 mL of 3 % (w/v) sulfosalicylic acid with the aid of quartz sand and then centrifuged at 500 *g* for 10 minutes at room temperature. Subsequently, 200 μL of SN were mixed with 200 μL of glacial acetic acid and 200 μL of acid ninhydrin, followed by an incubation at 96 °C for one hour. After briefly cooling on ice, 1 mL of toluene was added to each sample and the mixture was vortexed for 15 seconds to separate a red upper phase (organic) from a whiteish lower phase (aqueous). The upper one was retrieved, and its absorbance read at 520 nm. Toluene was used as the blank. Proline concentration was estimated via a standard curve designed with increasing known Pro concentrations and results were expressed as $\mu\text{g g}^{-1} \text{ f. w.}$ (Bates et al., 1973).

2.6.3. Reduced glutathione quantification (GSH).

Leaflets and roots samples of 200 mg were grounded in 1.5 mL of 3 % (w/v) sulfosalicylic acid with quartz sand and then centrifuged at 500 *g* for 10 minutes at room temperature. Afterwards, 50 μL of Supernatant were added to 200 μL of H_2O and 750 μL of a reaction mixture containing 100 mM PK buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 M 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's Reagent). Tubes were briefly vortexed and incubated at room temperature, in darkness, for 10 min. Afterwards, the Absorbance was read at 412 nm and the reduced GSH levels were calculated according to a standard curve, obtained with solutions of known reduced GSH concentrations. The results were expressed in $\mu\text{mol g}^{-1} \text{ f. w.}$ (Soares et al., 2019).

2.6.4. Total and non-protein thiols quantification.

Frozen aliquots of 300 mg were homogenized in ice utilizing quartz sand and an extraction solution of 20 mM EDTA and 20 mM AsA (1.9 mL for leaflets and 1.5 mL for roots). Subsequently, the homogenates were centrifuged at 12,000 *g* for 20 mins at 4 °C. The SNs were then recovered and utilized for total and non-protein thiols quantification. To determine total thiol content, 100 μ L of SN were mixed with 480 μ L of Tris-HCl (200 mM, pH 8.2) and 20 μ L of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 10 mM) and the solution was incubated for 15 minutes at room temperature. Total thiols were expressed in mol g^{-1} f.w., using 13,600 $M^{-1} cm^{-1}$ as molar extinction coefficient. Measurements of Abs₄₁₂ nm were accomplished. For the quantification of non-protein thiols, 250 μ L of SN were added to 250 μ L of 10% (w/v) sulfosalicylic acid and then incubated at room temperature for 15 minutes, followed by a 15-minute centrifugation (3,000 *g*, 4 °C). Two hundred and fifty μ L of this SN were then mixed with 237.5 μ L of Tris-HCl (400 nM, pH 8.9) and 12.5 μ L of 10 mM DNTB and left at room temperature to develop colour for 5 minutes. Lastly, non-protein thiol content was calculated applying the same molar extinction coefficient, wavelength, and units as before. Protein-bound thiol levels were then determined by subtracting the non-protein thiols to the total thiol content (Zhang et al., 2009).

2.6.5. Ascorbate-reduced (AsA) and oxidized (dehydroascorbate – DHA) forms quantification.

For this assay, frozen aliquots of approximately 200 mg were homogenized in 6 % TCA (w/v) (1.5 mL for leaflets and 1 mL for roots) in a chilled mortar with quartz sand at 4 °C. Afterwards, homogenates were centrifuged at 15,000 *g* for 10 min at 4 °C, and the SN was collected to separate tubes. At this moment, two separate sets of reactions were assembled, one to quantify the levels of total ascorbate and a second one to quantify reduced ascorbate (AsA) levels. For total ascorbate quantification, 100 μ L of SN were added to 50 μ L of 75 mM PK buffer (pH 7.0) and 50 μ L of 10 mM 1,4-dithiothreitol (DTT). DTT reduces DHA, turning all ascorbate present in the sample to AsA. This mixture was briefly vortexed and incubated at RT for 10 min. Subsequently, 50 μ L of 0.5 % (w/v) N-ethylmaleimide (NEM) were added to remove excess active DTT, by blocking its sulfhydryl groups.

To assess the levels of AsA, 100 μL of SN were mixed with 50 μL of 75 mM PK buffer (pH 7.0) and 100 μL of ddH₂O (for DTT and NEM levels that had been added to the total ascorbate quantification test tubes). Then, 750 μL of a reaction mixture containing 10 % (w/v) TCA, 43 % H₃PO₄, 4 % (w/v) 2,2'-bipyridine (BIP) and 3 % (w/v) FeCl₃ were added to all tubes for total ascorbate and AsA quantification. All samples were incubated at 37 °C for 1 h. In this period, AsA reduced the Fe³⁺ ions from FeCl₃ to Fe²⁺, which subsequently reacted with BIP, forming a complex with a characteristic Abs peak at 525 nm. This allows for an indirect AsA quantification. After incubation, the Abs were recorded at 525 nm (Fe²⁺-BIP complex Abs peak). The concentrations of total and reduced AsA were calculated from a calibration curve previously prepared with solutions of known AsA concentration. The levels of oxidized ascorbate were estimated by subtracting the reduced portion from the total ascorbate pool. Results were expressed in $\mu\text{mol AsA (or DHA) g}^{-1}$ f. w.. (Gillespie & Ainsworth, 2007).

2.6.6. Glutamate Dehydrogenase activity quantification.

For protein extraction, frozen aliquots of roughly 200 mg were ground in a chilled mortar with the aid of some quartz sand and PVPP in 400 μL of buffer solution containing 40 mM Tris-HCl, pH 7.2, 1 mM EDTA and 5% glycerol. Triton X-100 was added to a final concentration of 0.05% for organelle and membrane disruption and soon after centrifuged at 13,000 g for 10 minutes at 6°C followed by incubation on ice for 30 minutes. Subsequent, the supernatants (SN) were transferred to new tubes and two sets of reaction were assemble, for protein quantification by the Bradford method (Bradford, 1976) and glutamate dehydrogenase quantification (Turano et al., 1996). Afterwards, 75 μL of 1:20 dilution of the supernatant was used with 750 μL of Bradford reagent and Abs at 595 nm measured. Finally, for GDH activity quantification, of the amination reaction, 900 μL of reaction mix containing 100 mM (NH₄)₂SO₄, 13 mM α -ketoglutarate (2-oxoglutarate), 0.25 mM NADH and 1 mM CaCl₂ in 100 mM Tris-HCl pH 8.0 with 25 μL of supernatant and 75 μL of ddH₂O were used to measure Abs₃₄₀ for 1 minute in 5 seconds intervals. For the blank, extraction buffer was used. Levels of GDH activity were obtain using the extinction the coefficient of $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$, being expressed in $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ protein.

2.6.7. Glutamine synthetase activity quantification.

Fresh samples of around 300 mg were grounded in chilled mortar with quartz sand and extraction buffer (1.5 mL for leaflets and 2.0 mL for roots) containing 25 mM Tris-HCl (pH 6.4), 10 mM MgCl_2 , 1mM DTT, 0.05% Triton X-100, 10% glycerol and 2% (w/v) PVPP, followed by centrifugation at 15,000 g for 15 minutes at 6 °C. The enzymatic reaction started by mixing 400 μL of reaction mixture composed of 100 mM Trizma base, 125 mM L-glutamine, 157 mM Hydroxylamine, 1.26 mM Manganese (II) chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) and 25 μM Adenosine 5'-diphosphate sodium salt (ADP), pH=6.4 with 50 μL of protein extracts and 50 μL of 6.4% (w/v) sodium arsenate (pH 6.4). A total of three reactions were prepared for each protein extract. Simultaneously, a blank was produced in which the protein extract was replaced by extraction buffer. All the reactions were incubated at 30° C for 20 and 30 minutes, for leaflets or roots, respectively. Finally, 500 μL of Stop solution (0.16 M Iron (III) chloride (FeCl_3), 0.25 M TCA in 37% HCl), as added and absorbances were recorded at 500nm. Protein quantification as determined by Bradford method (Bradford, 1976). Glutamine synthetase activity was expressed in $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein.

2.6.8. Nitrate reductase (NR) activity quantification.

Fresh samples of about 300 mg to roots and leaflets, were homogenised in extraction buffer (50mM HEPES-KOH (pH=7.8), 1mM Phenylmethylsulphonyl fluoride (PMSF), 10mM DTT and 10mM MgCl_2 and 5% (w/v) PVPP in ice cold conditions and transferred to tubes and centrifuged at 15,000 g for 20 minutes at 6°C. The supernatant (SN) was recovered to new tubes and kept on ice. Afterwards, for the protein content quantification, 75 μL of diluted 1:10 supernatant was mixed with 750 μL of Bradford reagent and kept in darkness for 15 minutes followed by absorbance reading at 595 nm. Simultaneously, for NR activity determination 100 μL of SN was mixed with 900 μL of reaction mixture (25 mM HEPES-KOH (pH=7.8), 500 μM β -Nicotinamide adenine dinucleotide hydrate (NADH), 10 μM FAD and 2mM KNO_3) and absorbance reading was verified at 340 nm for 1 minute and 30 seconds in 20 seconds intervals. A blank was produced in which the protein extract was replaced by extraction buffer. Activity was recorded by applying the extinction coefficient (ϵ) of $6.22 \text{ mM}^{-1} \text{cm}^{-1}$ and expressed in $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein.

2.6.9. Colorimetric quantification of nitrate ions (NO_3^-).

Frozen aliquots of leaflets and roots with 100 mg were, with an aid of a mortar with quartz sand, homogenised in ddH₂O and transferred to tubes and maintained at 100 °C for 15 minutes. Afterwards, samples were centrifuged at 15,000 g for 15 minutes at room temperature and the supernatant (SN) transferred to new tubes. Subsequently, 0.1 mL of SN were added to 0.4 mL of 5 % (w/v) salicylic acid dissolved in 95% H₂SO₄ and vigorously vortex and incubated 20 minutes at room temperature. Finally, everything was transferred to new tubes containing 9.5 mL of NaOH at 2M and Abs_{410 nm} was measured. For the blank, ddH₂O was used. Nitrate ion concentration was calculated according to a standard curve, obtained with solutions of known NO_3^- concentrations (10-500 mg L⁻¹). Results were expressed in mg NO_3^- g⁻¹ f. w.

2.7. Analysis of molecular parameters.

2.7.1. RNA extraction and quantification.

The extraction of total RNA from plant tissues was attained with NZYol (Nzytech®, Portugal) according to the supplier's instructions. Frozen leaflets and roots aliquots of about 50-100 mg were grounded in 1 mL of NZYol in 1.5 mL tubes and then centrifuged at 12,000 g for 10 minutes at 8 °C. Afterwards, the retrieved supernatants were incubated for 5 minutes at room temperature, followed by the addition of 0.2 mL of chloroform and 15 seconds of vigorous shaking to successfully denature proteins and other cell constituents. The mixture was incubated at room temperature for 3 minutes and centrifuged at 12,000 g for 15 minutes at 8 °C to with the goal of obtaining three dissimilar phases: a phenol-chloroform phase (where DNA is situated), an interphase (containing cell and protein debris), and an upper colourless aqueous phase (which comprises of RNA). The aqueous phase was smoothly transferred to a new tube, at which it was added 0.5 mL of chilled isopropanol and left at room temperature for 10 minutes for RNA precipitation. The samples were once again centrifuged at 12,000 g for 10 minutes at 8 °C and the supernatant removed. The pellet containing the RNA was washed with 75 % ethanol with the aid of a vortex and centrifuged at 7,500 g for 5 minutes

at 8 °C followed by air-dried in ice to allow total ethanol evaporation and the resuspended in RNase-free water.

Potential DNA contaminations were removed by DNase treatment with NZY DNase I (Nzytech, Portugal), according to the manufacturer's instructions. RNA quantification and purity evaluation were spectrophotometrically assessed by 260, 280 and 230 nm measurements, using a μ Drop Plate (Thermo Fisher Scientific, Vantaa, Finland) and a Multiskan GO microplate spectrophotometer (Thermo Scientific). The ratios Abs_{260}/Abs_{280} and Abs_{260}/Abs_{230} were calculated to deduce RNA purity. Pure RNA sample presents an A_{260}/A_{280} ratio of 2.0 ± 0.1 and a Abs_{260}/Abs_{230} between 2.0-2.4. Values outside of the specified values may indicate protein and phenolic contamination respectively. The quality of RNA was evaluated by separating 300 ng RNA in a 1 % (w/v) agarose gel electrophoresis in 1X sodium boric acid (SB) at 250V and non-limiting amperage for 10 minutes, to detect possible RNA degradation and genomic DNA contamination. Samples containing the selected parameters were stored for future use at -80°C.

2.7.2. Reverse transcription (RT) - cDNA synthesis.

To generate complementary DNA (cDNA) from the previously extracted mRNA, RT reactions were conducted utilizing the SuperScript™ IV VILO™ Master Mix kit, following the supplied instructions (Invitrogen, USA). Initially, a tube was prepared by mixing 2.5 μ g of template RNA and nuclease-free water to a final volume of 10 μ L. After a quick mix, the prepared tubes were incubated for 2 minutes at 37 °C, briefly centrifuged and placed on ice. Then, 4 μ L of SuperScript™ IV VILO™ Master Mix and 6 μ L of nuclease-free water were added to the tubes containing the 10 μ L mix. Primers were annealed with a gentle mix and 10-minute incubation at 25 °C and the RT reaction occurred in a 20-minute incubation at 50 °C. To heat-inactivate the enzyme, tubes were incubated at 85 °C for 5 minutes and then stored at -20 °C until further use.

2.7.3. Semi-quantitative polymerase chain reaction (PCR).

To assess the differences in the accumulation of the mRNAs coding for several enzymes involved in the GSH metabolism (PCS, GR, γ -ECS), after the treatment with Cu, semi-quantitative reverse-transcriptase PCRs (RT-PCRs) were performed. Each reaction consisted in 5 μ L of 2x Taq Master Mix (Bioron®), 0.4 μ L of 10 μ M forward and

reverse primers, 0.5 µL cDNA obtained for each treatment and PCR water to a final volume of 10 µL. The reactions were performed in a MJ Mini thermocycler (Bio-Rad®) and the PCR conditions, as well as the primers' sequences and amplicon sizes, are depicted in Table 1. Afterwards, the amplification products were loaded on 1 % (w/v) agarose gels, following the same electrophoresis conditions previously described. To guarantee that an equal amount of cDNA was loaded for all samples, the *Elongation Factor 1 (EF1)*, a housekeeping gene, was used as a reference to determine the volume of all treatments' amplicons to load (Løvdaal & Lillo, 2009) and thus, allowing to observe differences in gene expression. For this reason, *EF1* amplicons for each treatment were loaded and the volumes which resulted in similar bands were utilized in the ensuing comparative quantifications. Gene Ruler 50 bp DNA Ladder (Thermo Fisher Scientific®) was used to verify the size of the amplified fragments. All images were captured and treated with GenoSmart2 (VWR, USA)

Table 1: Specific primers for *EF1*, *MT1*, *MT2*, *MT3*, *MT4*, *GS* (1.1,1.2,1.3 and 2), *PCS*, *GR* and γ -*ECS* used in PCR reactions, with respective expected amplicon sizes and programs used. F – Forward primer, R – Reverse primer

Gene	Primer sequence	Amplicon Size (bp)	Thermocycler program
<i>EF1</i>	F: 5' GGAAGCTTGAGAAGGAGCCTAAG 3' R: 5' CAACACCAACAGCAACAGTCT 3'	158	Lid 110 °C, 94 °C – 2 ' 29 cycles of: 94 °C – 30 ", 60 °C – 30 ", 72 °C – 45 ", 72 °C – 5 '
<i>MT1</i>	F: 5' GGAGGAAGCTGTAATTGTGG 3' R: 5' CCCCTTCTGTAGCTTTCTC 3'	166	
<i>MT2</i>	F: 5' GCTGTGGAGGATGTGGTATG 3' R: 5' CCTTCTCCAGCTGCTTTCTC 3'	117	Lid 110 °C 94 °C – 2 ' 29 cycles of: 95 °C – 5', 60 °C – 30 ", 72 °C – 45 " 72 °C – 5 '
<i>MT3</i>	F: 5' GGAAGGAGAGCCAATACGAC 3' R: 5' TGTTCCTTCTGCTCCAACGTC 3'	78	
<i>MT4</i>	F: 5' ATGAGAGGTGTGGTTGTCCTT 3' R: 5' CCGCACTTGCACTTAGACTT 3'	180	
<i>GS1.2</i>	F: 5' GGTGCTGGAGTTGTTGTTTC 3' R: 5' CGTTCATTGCCTTCACCATA 3'	191	
<i>GS1.1</i>	F: 5' CGGAGAAAGGAAAGGGATAC 3' R: 5' CATGTGAGGAAAGGGCTGTTAAG 3'	178	Lid 110 °C 94 °C – 2 ' 39 cycles of: 95 °C – 5', 58 °C – 30 ", 72 °C – 45 " 72 °C – 5 '
<i>GS1.3</i>	F: 5' CTGGTGATGAAGTGTGGGTAG 3' R: 5' TTTCATAGCCTCCGTCTTCC 3'	164	
<i>GS2</i>	F: 5' TCTCTCACTCCTAACACAAC 3' R: 5' GCGTGTCTTTTCTGCTTC 3'	189	
<i>PCS</i>	F: 5' CAGAATGGAACAATGGAAGG 3' R: 5' GCAAATAAAAGGGAGGTG 3'	570	Lid 110 °C 94 °C – 2 ' 29 cycles of: 94 °C – 30 ", 53 °C – 30 ", 72 °C – 45 " 72 °C – 5 '
<i>GR</i>	F: 5' AAAGACCGAGGAGATTGTACG 3' R :5' CATTCTCGCCATATAGAAGC 3'	322	Lid 110 °C 94 °C – 2 ' 29 cycles of: 94 °C – 30 ", 57 °C – 30 ", 72 °C – 45 " 72 °C – 5 '
γ-<i>ECS</i>	F: 5' GAAACAGGGAAAGCAAAGC 3' R: 5' CATCAGCACCTCTCATTTC 3'	322	Lid 110 °C, 94 °C – 2 ' 29 cycles of: 94 °C – 30 ", 51 °C – 30 ", 72 °C – 45 ", 72 °C – 5 '

2.7.4. Real-time RT-qPCR.

The qPCR reactions for leaves and roots of each treatment of MT- and GS-encoding genes were carried out in four technical replicates, with *EF1* as 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 final primers concentration, 1 μL of 1/10 diluted cDNA. Then, the reactions were run in a CFX384 Touch™ real-time PCR detection system (Bio-Rad) with the following cycling parameters: 2 minutes at 98°C and 40 cycles alternating between 2 seconds at 98 °C and 5 seconds at 58 °C.

Pertain to the RT-qPCR assay, the resulting data was normalized by the $2^{(-\Delta\Delta Ct)}$ formula (Livak & Schmittgen, 2001), using *EF1* as reference genes. This analysis was achieved with CFX Maestro Software (Bio-Rad®).

2.8. 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 examined through multiple unpaired t tests. These analyses were performed using Prism® 9 (GraphPad Software Inc., USA), considering significant the differences at $p \leq 0.05$.

3.Results

3.1. Effects of copper concentrations on tomato plants biometry.

3.1.1. Effects of increasing copper concentrations on tomato plant seed germination and determination of Cu levels to be used in the following trials.

3.1.1.1. Golden nugget seedlings' biometry.

With the objective of analysing the effect of increasing copper concentrations in tomato plants seedlings for subsequent growth trials, the seedlings were germinated on a petri-dish containing a agarose medium with different copper concentrations (0.4 mg L⁻¹, 8 mg L⁻¹, 16 mg L⁻¹, 24 mg L⁻¹, 32 mg L⁻¹ and 40 mg L⁻¹), being the 0.4 mg L⁻¹ the concentration of this metal, in the nutrient mixture of, the control group. Data for the germination rate indicates no impact, as these don't present statistical difference (figure 3A). As for the shoot size (figure 3B) and root size (figure 3C), there is evidence of negative impacts with increasing copper concentration. Shoot size shows statistical differences in all non-control concentration except for 8 mg L⁻¹ with a decrease in shoot size of 26.5%, 48.8%, 46.9% and 57.2% in the 16 mg L⁻¹ to 40 mg L⁻¹ copper concentrations respectively. Root size showed a significant continuing decrease in all concentrations with 36.5% for 8 mg L⁻¹, 79.5% for 16 mg L⁻¹, 81.3% for 24 mg L⁻¹, 92.1% for 32 mg L⁻¹ and 94.4% for 40 mg L⁻¹. Similar results were observed in the total seedling's fresh weight, albeit a smaller decrease, of 54.3%, 65.3%, 64.4%, 73.1% and 73.6% in the respective copper concentrations. Results obtained from this trial allowed for the selection of the copper concentration to be used in the following experiments, as the concentration with fewer impacts (8 mg L⁻¹) in seed development is indicative of the possibility of future plant development despite metal excess exposure.

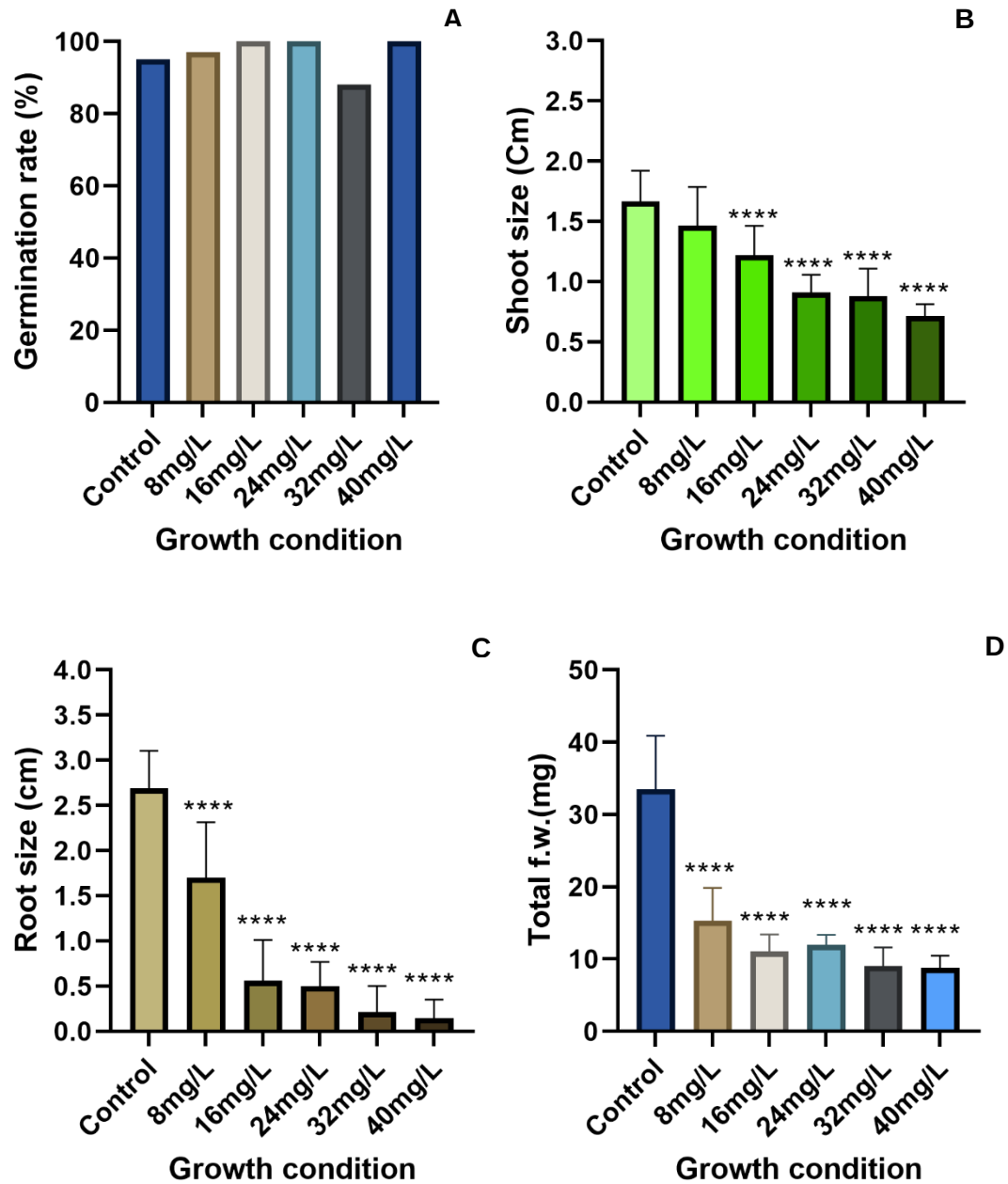


Figure 3: Germination rate (A), shoot (B) and root (C) size and total fresh weight (D) of *S. lycopersicum* seedlings (cv Golden nugget) grown in nutrient medium supplemented with increasing copper concentrations. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.1.1.2. Micro-Tom seed cultivar biometry.

Results for cv Micro-Tom, relative to shoot size (figure 4A), statistical differences were observed, however, 16 mg L⁻¹ copper concentration, showed a smaller decrease in shoot length of 15.6%. The remaining growth conditions revealed a similar response in shoot length by 28.1% with 8 mg L⁻¹, 39.8% with 24 mg L⁻¹, 38.5% with 32 mg L⁻¹ and 30% with 40 mg L⁻¹ copper. For root length, all growth conditions revealed significant differences. For the first copper concentrations, 8 mg L⁻¹ and 16 mg L⁻¹, there was a smaller decrease in root size (80.6% and 79.9% respectively), comparing with the following 24 mg L⁻¹, 32 mg L⁻¹ and 40 mg L⁻¹ growth conditions where decreases values reached 92% in the two first, and 91.4% in the latter (figure 4C). Total fresh weight results revealed, similarly to the previously testes parameters, a significant difference in all growth conditions assessed, although, exhibiting more similarities with shoot size values. A smaller decrease was observed in the 16 mg L⁻¹ growth condition with 58.7%. Equivalent results were present in 8 mg L⁻¹ and 32 mg L⁻¹ of about 67% decrease with the greater values being present in the remaining condition with 72% and 70% for 24 mg L⁻¹ and 40 mg L⁻¹ respectively (figure 4D).

By direct analysis of both seed germination and seedlings biometry assays, better results were obtained for the Golden nugget variety (figure 4). All tested parameters in this variety suggest better resistance to copper by indicating a lesser decrease in shoot and root size and total fresh weight of the seedlings (figure 4B and 4C). Germination rates, compared between cultivars, show similar values. Data pretending to the 8 mg L⁻¹ suggests a lesser impact in the tested parameters indicating the concentration to be used in the following trials. Consequently, for the assays to be described, the Golden nugget cultivar tomato plants were used.

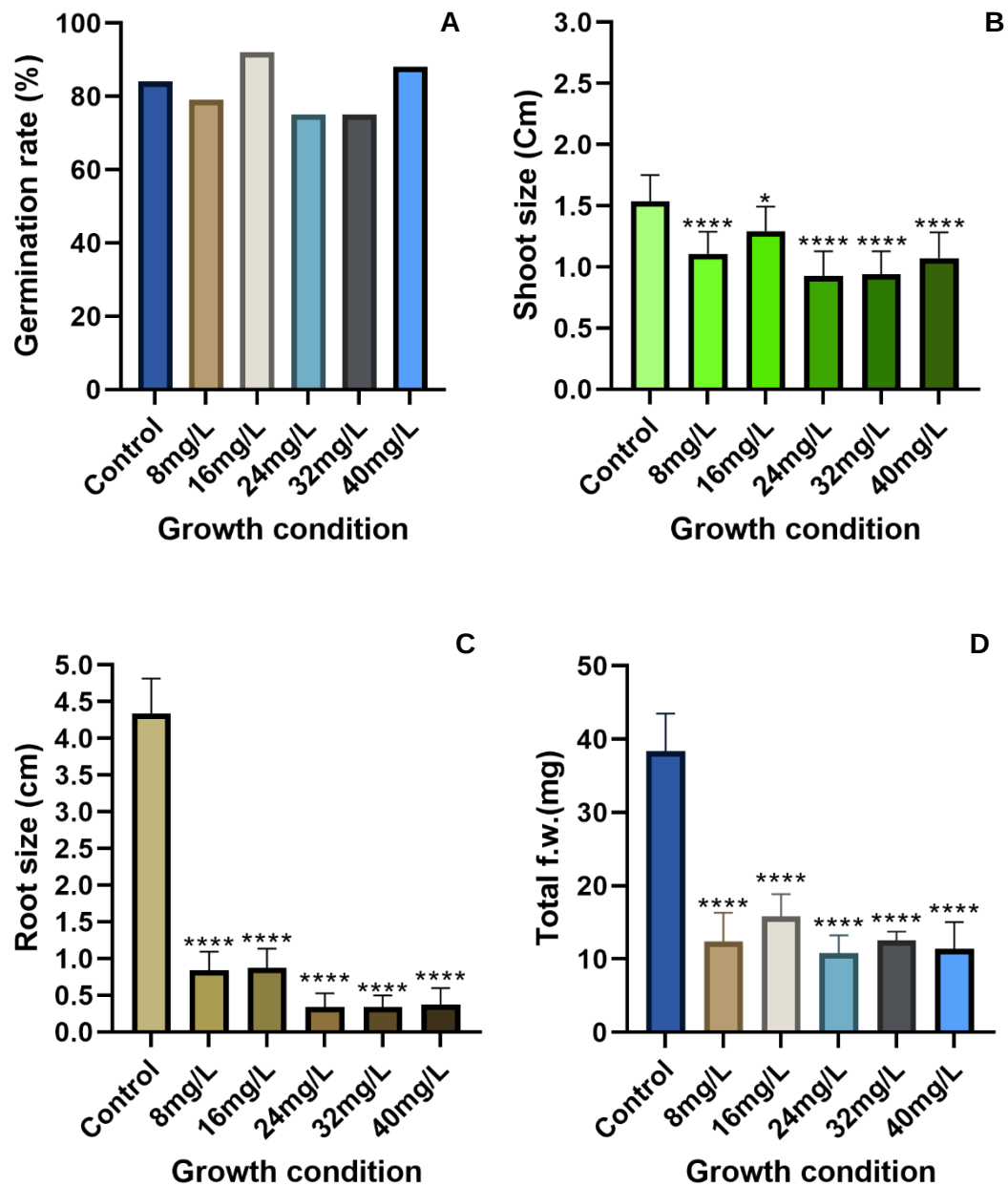


Figure 4: Germination rate (A), shoot (B) and root (C) size and total fresh weight (D) of *S. lycopersicum* seedlings (cv Micro tom) grown in nutrient medium supplemented with increasing copper concentrations. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.1.2. Growth trial.

Biometrical measures in shoot and root biomass (figure 5A), indicate that no significant changes were detected in shoots and roots. Regarding water content percentage, results show similar levels in shoots to the control group, however, significant changes were registered in root water content with a 16% decrease, with no changes being detected in the fruits (figure 5B).

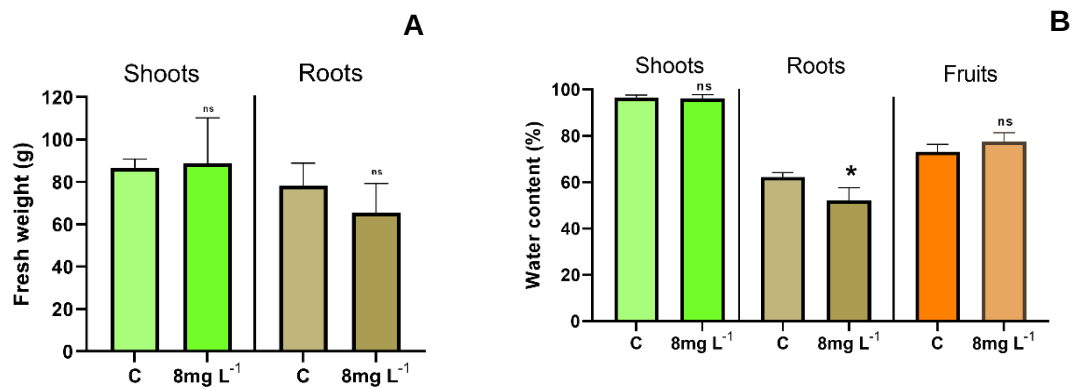


Figure 5: Fresh weigh (A) and water content percentage (B) of *S. lycopersicum* shoots and roots of 5 weeks old plants grown in nutrient medium supplemented with increasing copper concentrations. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The value ns represent no significance ($p > 0.05$).

Regarding flowering stage and fruit maturity time, the exposure to the present copper concentration had no negative impacts to the plant. Both tested groups reached flowering stage by the same time well as in fruit development and number. No morphological changes were also detected in fruits.

3.1.3. Copper metal content quantification in tomato plants.

To assess the organ preference for copper accumulation, and thus evaluate the viability of *S. lycopersicum* fruits for human consumption, shoots, roots, and fruits samples were analysed. Results indicate significant differences in copper accumulation in the shoots and roots, indicating an 47.05% increase in the first case and, in the later, revealing higher levels of copper metal with an increase by 2-fold (figure 6). Results from copper concentrations in the fruits revealed an undetectable amount by the method performed in this essay indicating values lower than $11 \mu\text{g g}^{-1}$ which is the detection limit for the used assay, thus limiting the capacity to assess the exact amount of Cu present in this organ.

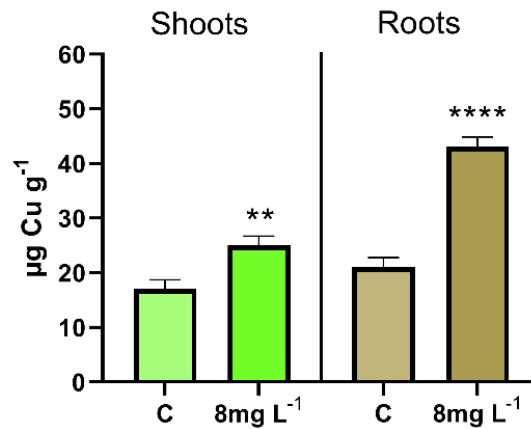


Figure 6: Copper concentration in $\mu\text{g g}^{-1}$ in *S. lycopersicum* shoots and roots of 5 weeks old plants grown in nutrient medium supplemented with increasing copper concentrations. No copper was detected in the fruits. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2. Effects of Cu on leaf physiology.

3.2.1. Photosynthetic pigments.

Regarding pigments' concentrations (figure 7), results show significant differences in the total chlorophyll (Chl_{a+b}), and β -Carotene with a decrease of 27.8% and 18.7%, respectively. Despite results suggesting a decrease in Lutein concentration, no statistical differences were recorded.

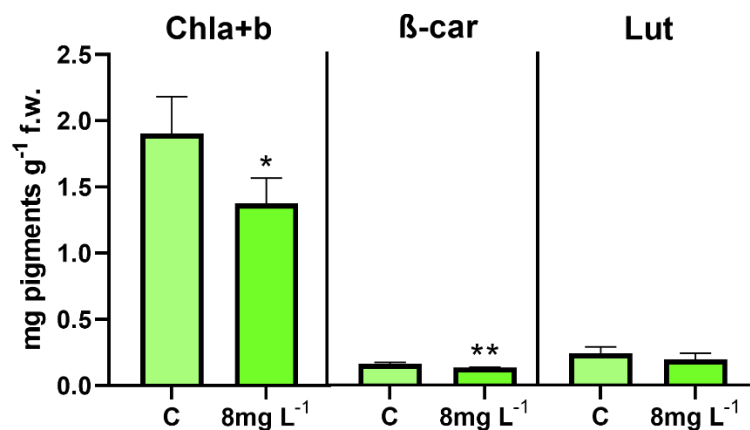


Figure 7: Pigments contents expressed in mg per g of fresh weight in leaf samples of 5 weeks old *S. lycopersicum* plants, grown in nutrient medium supplemented with increasing copper concentrations. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Chl_{a+b} : total chlorophyll; β car: β -Carotene; Lut: Lutein.

3.2.2. Histochemical coloration of starch.

Histochemical coloration results indicate no apparent changes in starch content in the copper concentration tested (figure 6), since there was no variation in colour content between both growth conditions.

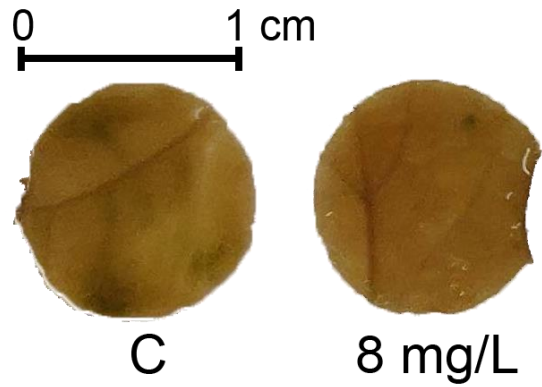


Figure 8: Typical results depicting the lugol staining of starch in leaf disks of *S. lycopersicum* plants grown for 5 weeks in vermiculite: perlite (1:1) watered with nutrient medium (C) or supplemented 8 mg L⁻¹ copper concentrations.

3.2.3. Starch and soluble sugars accumulation.

Results of starch quantification, achieved by perchloric acid digestion, and soluble sugars (glucose, sucrose, and fructose) indicate no statistical differences (figure 9), however, a tendency for a small increase was detected, in both parameters assessed.

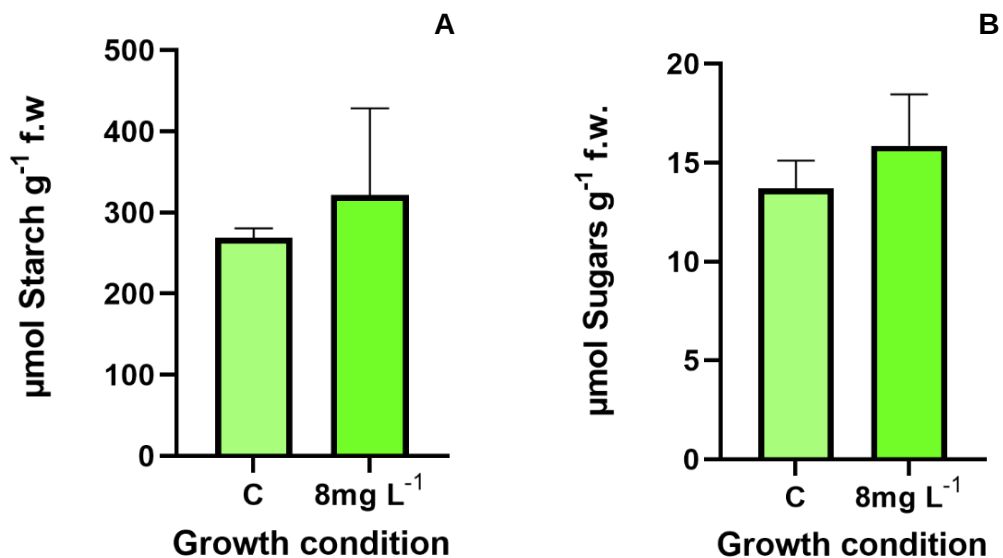


Figure 9: Glucose (Starch) (A) and Soluble sugars (B) concentrations, expressed in μmol per g of fresh weight in leaf samples of 5 weeks old *S. lycopersicum* plants, grown in vermiculite: perlite (1:1) watered with nutrient medium (C) or supplemented with 8 mg L⁻¹ copper. Values presented are mean ± SD.

3.3. Effects of Cu on various stress indicators.

3.3.1. Quantification of Hydrogen peroxide (H₂O₂).

Results reveal no significant differences in H₂O₂ concentration (figure 8) in the leaves despite suggesting a slight increase with the exposure to Cu. Contrary, in the roots, significant differences with an increase of 36.6% in hydrogen peroxide concentration were obtained.

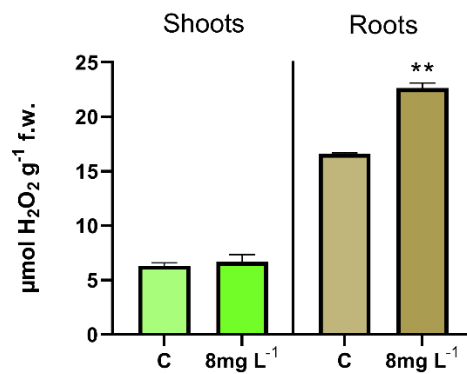


Figure 10: Hydrogen peroxide (H₂O₂) concentration expressed in μmol per g of fresh weight in leaves and roots samples of 5 weeks old *S. lycopersicum* plants, grown in nutrient medium (C), or supplemented with 8 mg L⁻¹ copper. Values presented are mean ± SD. * above bars represent significant differences from the control at * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

3.3.2. Quantification of Proline and reduced glutathione (GSH).

Results indicate no significant differences in both proline levels and GSH in the roots. However, proline levels in the leaves suffered a decreased by 76.80% in concentration opposite to what was observed in the roots. Despite reduced glutathione (GSH) assay was conducted in the roots, its levels were not detected due to the reduced sensibility, of the method, and therefore, are not represented in the following figure 11.

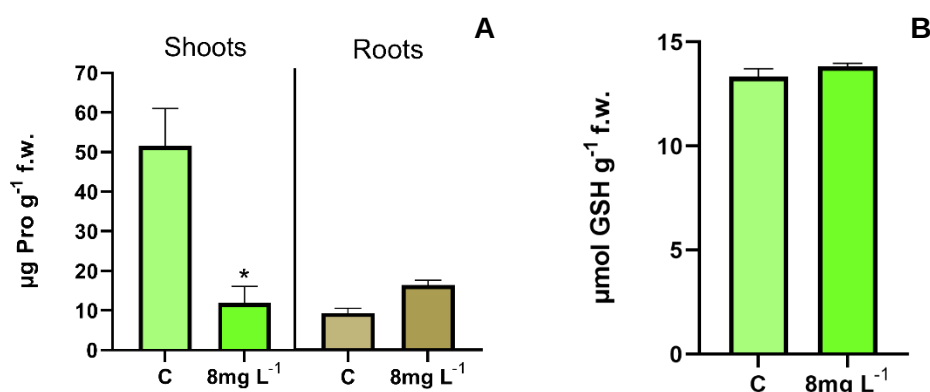


Figure 11: Hydrogen peroxide (H₂O₂) concentration expressed in µg per g of fresh weight in shoots and roots (A) and reduced glutathione (GSH) expressed in µmol per gram of fresh weight in shoots (B), in samples of 5 weeks-old *S. lycopersicum* L. plants, grown in nutrient medium (C), or supplemented with 8 mg L⁻¹ copper. Values presented are mean ± SD. * above bars represent significant differences from the control at * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

3.3.3. Quantification of reduced (AsA) and oxidized (dehydroascorbate – DHA) ascorbate forms.

Results indicate no significant differences in total ascorbate (figure 12A) in both leaves and roots. For the reduced (AsA) values (figure 12B), significant differences were present with a 96.2% increase in the leaves and a 100% increase in the roots. Opposite results were detected for the oxidized (DHA) values (figure 12C) revealing a significant decrease in both leaves and roots, albeit more pronounce in the leaves, of 80% and 16.8% respectively. For the total/AsA ration (figure 12D) and DHA/total ratio (figure 12E), significant differences were detected. For the first, an increase in both shoots and roots of 67.27% and 60% respectively was revealed. For the latter, results indicate a decrease in 84% in leaves and 26% in roots. Lastly, for AsA/DHA ratio (figure 12F), no significant differences in both leaves and roots were detected.

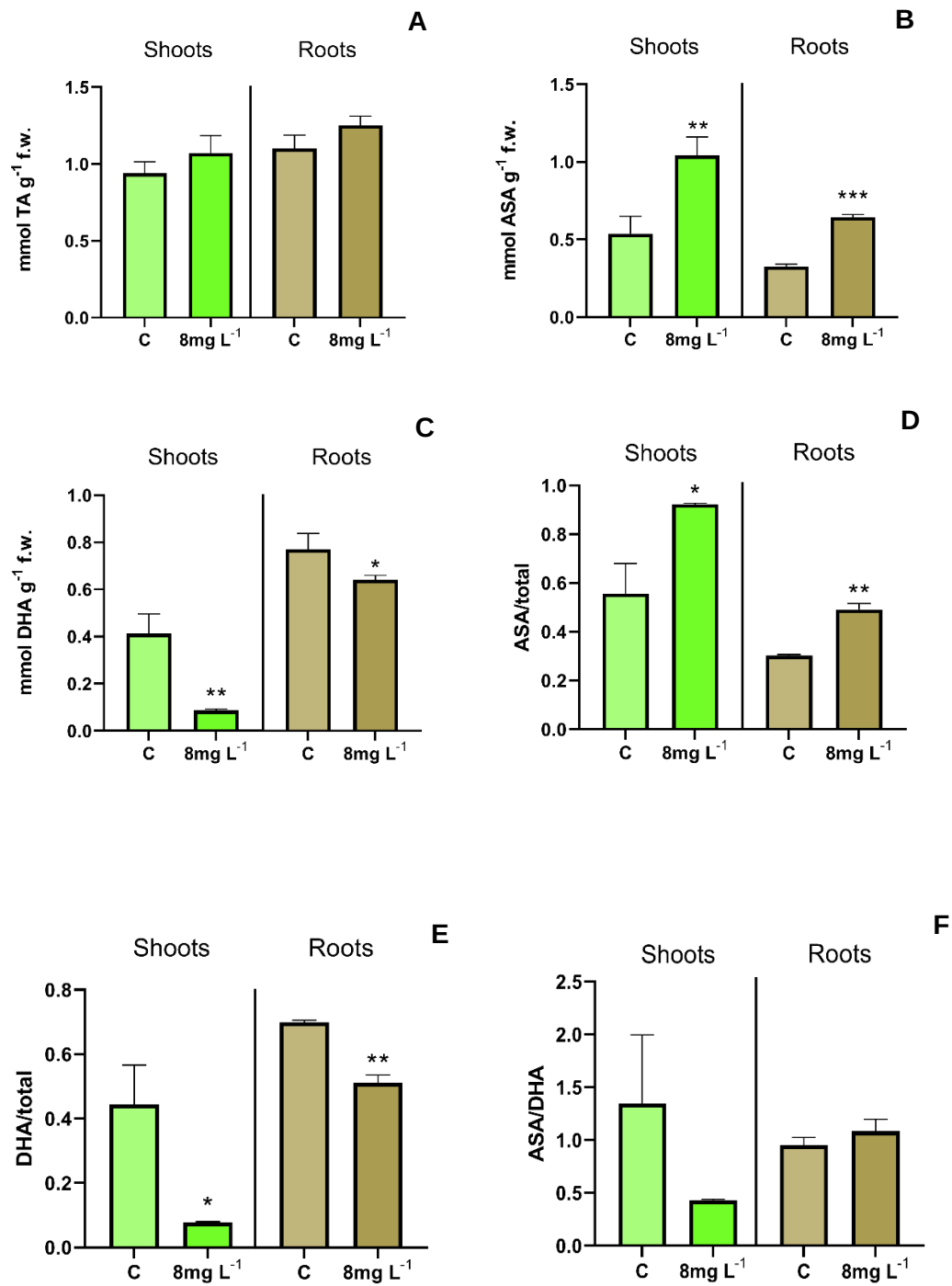


Figure 12: Total ascorbate (A), reduced (B), oxidized (C), AsA/total ratio (D), DHA/total ratio (E), and AsA/DHA ratio(F) concentration expressed in mmol per g of fresh weight in leaves and roots samples of 5 weeks old *S. lycopersicum* plants, grown in nutrient medium C), or supplemented with 8 mg L⁻¹ copper. 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.

3.3.4. Total and non-protein thiols.

Results indicate, firstly, for total the thiol content (figure 13A), in both leaves and roots, significant differences with documented increases of 80.3% for the first, and 85.1% for the latter.

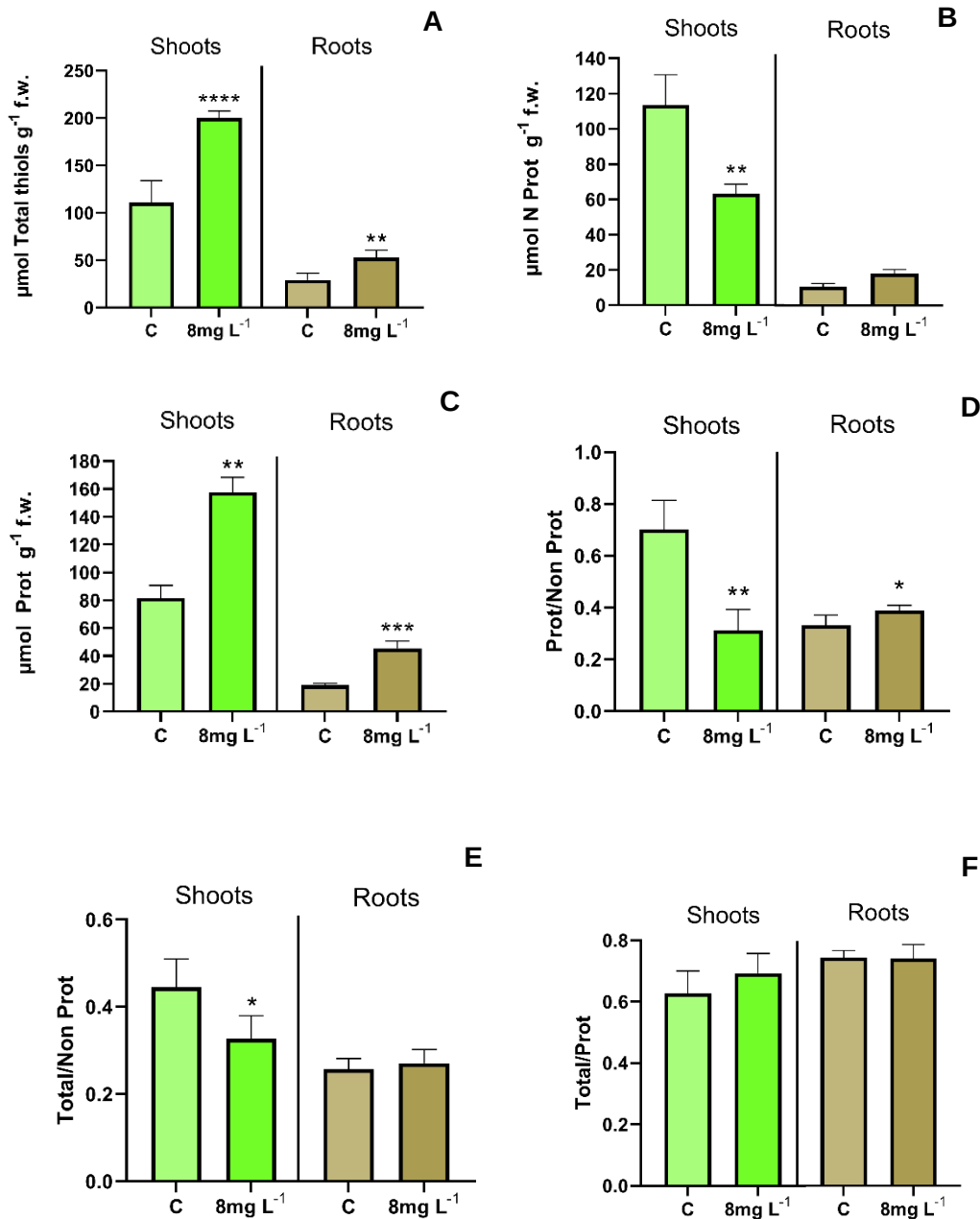


Figure 13: Total (A), Non-protein (B), Protein (C), total/protein ration (D), Total/non-protein ration (E) and Protein/non-protein ratio (F) thiols concentrations expressed in μmol per gram of fresh weight in leaves and roots samples of 5 weeks-old *S. lycopersicum* plants, grown in nutrient medium C), or supplemented with 8 mg L⁻¹ copper. Values presented are mean ± SD. * above bars represent significant differences from the control at * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Data obtained for non-protein thiols (figure 13B) denotes only significant differences in the leaves with a recorded decrease by 44.2% and no significant differences in the roots. For protein-thiols data (figure 13C), significant differences were present indicating a concentration increase of 92.89% and 137% in leaves and roots, respectively. From the three ratios of thiol concentration evaluated, only two of them revealed significant difference. For total/protein thiols (figure 13E), significant differences were only present in the shoots, indicating a decrease by 27.2%. Data recorded for total/non protein (figure 13D) showed no significant differences for both leaves and roots the opposite being observed in the protein/ non- protein thiols ratio (figure 13F), where a decrease by 55.7% in leaves and an increase of 18.2% in the roots was documented.

3.4. Nitrogen regulation levels under copper stress.

3.4.1. Quantification of nitrate ions (NO_3^-).

As figure 14 illustrates, it is possible to observe a significant increase of nitrate ions (NO_3^-) in both in leaves and in roots with values of 39.13% and 26.08% respectively.

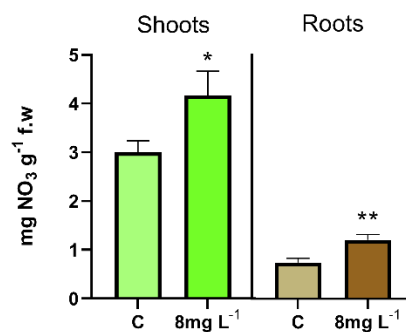


Figure 14: Nitrate ions (NO_3^-) concentration expressed in mg per g of fresh weight in leaves and roots samples of 5 weeks old *S. lycopersicum* plants, grown in nutrient medium C), or supplemented with 8 mg L^{-1} copper. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4.2. Nitrate reductase (NR) and Glutamine synthetase (GS) activity quantification.

Nitrate reductase (NR) results indicate significant differences only in the leaves showing a decrease in NR activity by 31.8% (figure 15A). For Glutamine synthetase (GS) activity levels, similarly, significant differences were detected in both leaves and roots with a 30.3% decrease in activity in the first and 34.3% in the latter (figure 15B).

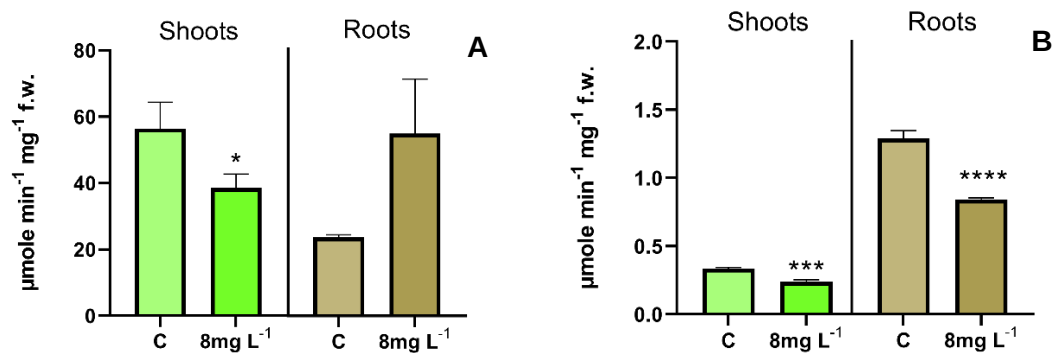


Figure 15: Nitrate reductase (A) and Glutamine synthetase (B) activities expressed in μmol per g of fresh weight in leaves and roots samples of 5 weeks old *S. lycopersicum* plants, grown in nutrient medium (C), or supplemented with 8 mg L⁻¹ copper. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4.3. Quantification of GDH.

Results for Glutamate Dehydrogenase activity indicate significant changes with increases by 3.2-fold, while in the roots, a decrease by 0.6-fold was detected (figure 16).

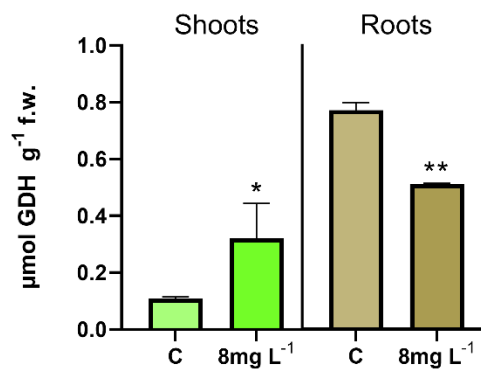


Figure 16: Glutamate Dehydrogenase (GDH) activity expressed in μmol per minute per mg of protein in leaves and roots samples of 5 weeks old *S. lycopersicum* plants, grown in nutrient medium (C), or supplemented with 8 mg L^{-1} copper. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5. Effects of Cu on transcript accumulation patterns.

3.5.1. Semi-quantitative quantification of gene expression.

Results for *GR* gene expression by semi-quantitative RT-PCR analysis (figure 15) indicate a slight decrease in the leaves with a more pronounced increase being observed in the roots. Both PCS and γ -ECS gene expression PCRs were conducted, however, at the present date, no data can be displayed, due to lack of time to perform the analysis.

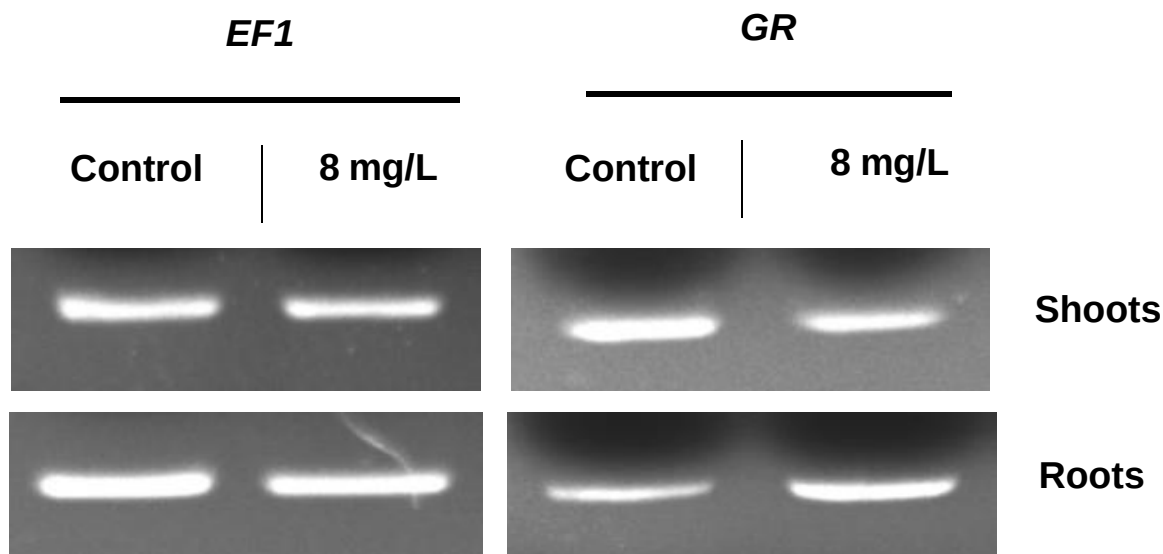


Figure 17: Typical results for *EF1* and *GR* semi-quantitative RT-PCR analysis by 1% (w/v) agarose gel electrophoresis in leaves and roots samples of 5 weeks old *S. lycopersicum* plants, grown in nutrient medium (Control), or supplemented with 8 mg L⁻¹ copper.

3.5.2. Effects of increasing copper concentrations on the transcript accumulation of MT-s and GS-encoding genes.

Results for both *MTs* and *GSs* gene family members were indicated as a value of 1 for the control group, thus allowing comparison by analysing x-fold changes as an effect of the exposure to Cu. For the *MT1* encoding gene, an increase in expression by 2.5-fold was detected in the leaves, with the opposite being detected in the roots with a decrease by 0.6-fold. Regarding *MT2* gene expression, increases in both organs were detected despite being only significant in the leaves by 10.8-fold. Expression *MT3* and *MT4* genes showed significant changes in leaves and roots with an up-regulation of *MT3* in the leaves by 2.9-fold and a decrease by 0.6-fold in the roots. *MT4* gene expression was up regulated in both leaves and roots by 1.9-fold and 3.2-fold respectively (figure 18).

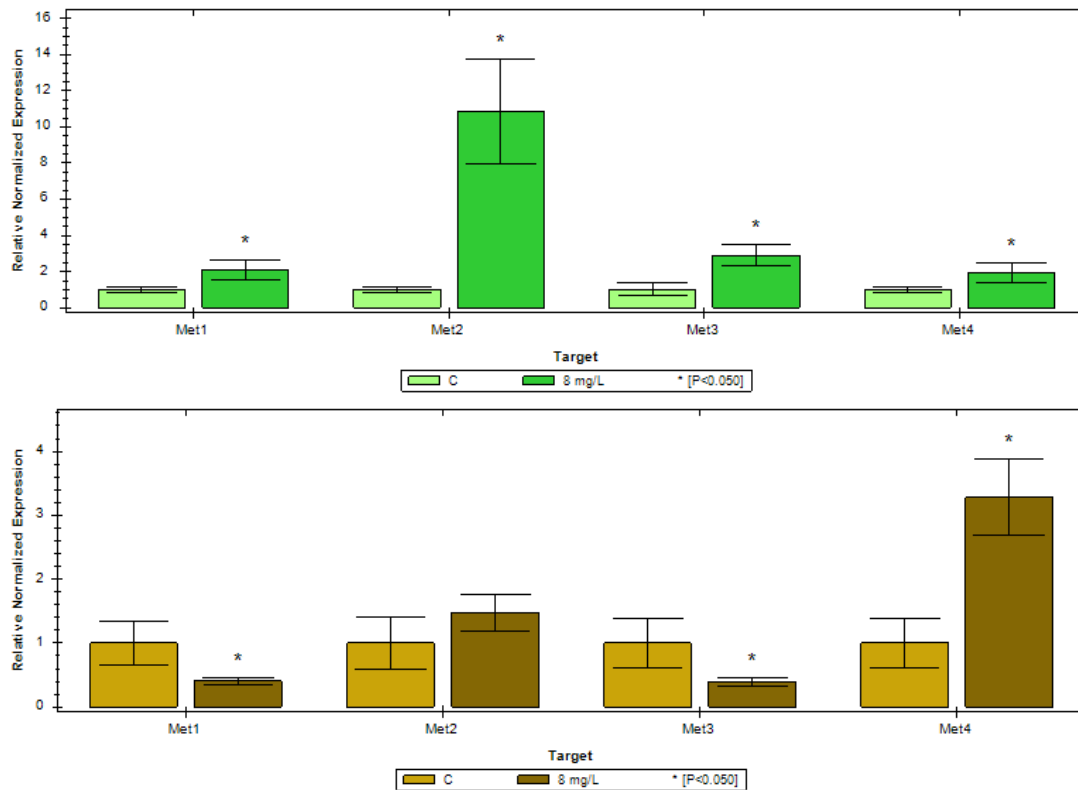


Figure 18: Relative normalized expression of MT1-, MT2-, MT3-, and MT4-encoding genes in leaves and roots samples of 5 weeks old *S. lycopersicum* grown in nutrient medium (Control), or supplemented with 8 mg L⁻¹ copper. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Analysis of *GS1.1* gene expression indicates it increases by 23.7-fold in the shoots and decreased by 0.3-fold in the roots. For *GS1.2*, a down-expression by 0.4-fold was detected in leaves and, despite a slight increase, no significant changes in the roots. The opposite can be observed in *GS2*, where expression increased by 1.7-fold and a with a non-significant decrease in the roots. Lastly, *GS1.3* results indicate an increase in expression in both shoots and roots by 7.8-fold and 1.24-fold respectively (figure 17).

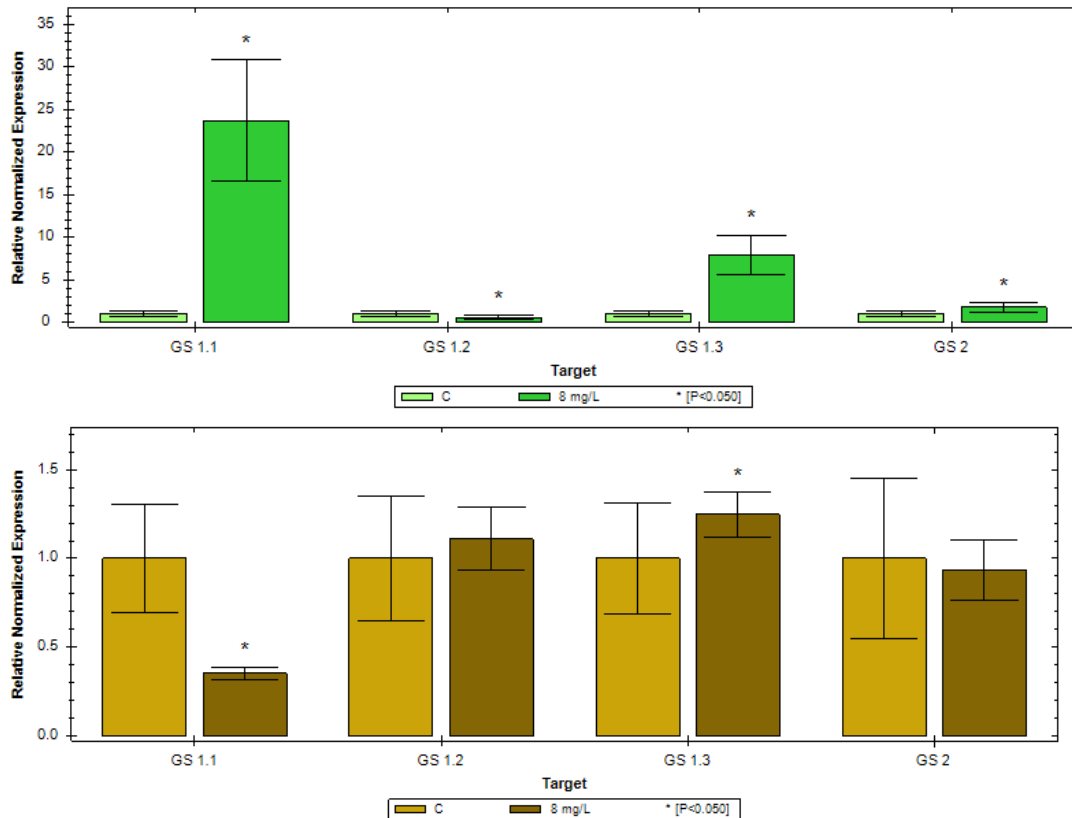


Figure 19: Relative normalized expression of GS1.1-, GS1.2, GS1.3- and GS2-encoding genes in leaves and roots samples of 5 weeks old *S. lycopersicum* subjected to increasing copper concentrations. Values presented are mean $\pm$ SD. * above bars represent significant differences from the control at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

4.1. Copper effects on tomato seed germination and plant biometry.

With the progress of human technology and consequent rising in industrial growth, the impacts in the biosphere became a growing concern, with special importance for this study, the possible effects of soil contamination by heavy metals, and its impacts in homeostasis with its bioaccumulation (R. Wang et al., 2018). Particularly, copper metal compounds production represents one of the many sources of soil pollution as its production contains many processes (mining, metallurgic milling and refining) that results in harmful effects for the healthy development of ecosystems (Rehman et al., 2019). Therefore, it is imperative to study the effects in plant development and yield throughout their lifecycle. Firstly, the effects of increasing copper concentration in seed germination

and seedling biometry were performed in two cultivars of *S. lycopersicum*, since this is a more sensitive stage of the plants development to both chemical and physical conditions (Seneviratne et al., 2019), thus allowing a better understanding of the initial plant growth under this metal toxicity condition.

Regarding the germination essay, in the Golden nugget cultivar, germination rates indicate no significant alteration with copper exposure, the same being observed for the Micro-Tom. Interestingly, studies indicate substantial increases and decreases in germination rates in other plant species like *Oryza sativa* (Ahsan et al., 2007), *Zea mays* (Bashmakov et al., 2005), *Triticum aestivum* (Singh et al., 2007) and many others. However, the absence of elevated germination abortions could be a result from the resistance added by the seed coat (Adrees et al., 2015) conferring enough protection to the copper concentrations tested. A dose-dependent response is apparent in both shoots and roots length and fresh weight. Copper metal exposure results in a decreased shoot and root length, aligning with results observed in other heavy metal germination assays like cadmium (Cd) and lead (Pb) (Ait Elallem et al., 2022), however, greater decreases were observed in the roots, possibly because this organ is the first to be in contact with potential contaminant and being the point of entry by any soil-related compounds (Pan & Chu, 2016), resulting in the greater inhibition of root growth caused by both reduced cell division and elongation (Sresty & Rao, 1999). Fresh weight decreases result from both shoots and roots reduced biomasses upon Cu exposure. Consequently, the lower copper concentration (8 mg L^{-1}) was chosen to carry on with this study. With this, despite evidence indicated a significant impact in the analysed parameters, it is revealed that growth can be achieved even in the tested stress condition, being this of importance and relevance for copper metal soil phytoremediation applications.

Following germination trials and copper concentration selected for the growth trials, exposure of plants to the selected Cu concentration was conducted for five weeks with the aim to understand its effects on plants biometry, as well as on several biochemical and molecular parameters. At this Cu concentrations, fresh weight for both shoots and roots did not impose enough changes to be considered relevant as having a possible toxicity effect. These results indicate that nutrient uptake and consequent growth did not seem to be affected. Generally, no considerably negative biomass alterations could be detected, an indication that no toxicity regime was present, since it is documented that high levels of heavy metals can result in the decrease in both shoot and root biomass (Athar & Ahmad, 2002; John et al., 2009). Another possibility, since Cu is a micronutrient necessary for plant growth (Nazir et al., 2019), there is likelihood

that the chosen concentration could be well within the parameters favourable for plant development (Souza et al., 2014).

However, despite the lack of negative impacts in both shoots and roots fresh weights, a decrease in root water content was detected. Since the root system is the main entry point of nutrients, and most importantly, water, the ability for the uptake of both compounds could be affected by, in an abiotic stress environment, the formation of excess ROS resulting in the reduction of the hydraulic conductivity (Rucińska-Sobkowiak, 2016) and alteration in root morphology (Marques et al., 2019), thus resulting in a decrease in root water content despite minimal differences in its biomass. Lack of significant alterations of this parameter in both shoots and fruits may indicate that 8 mg L⁻¹ of copper was not enough to induce alterations in the cell membrane, water transport and conductivity in these organs (Asati et al., 2016), as in cell expansion (Shah et al., 2010). Different heavy metals paired with different concentrations and testes in different plant species tend to lead to opposite results (Costa & Morel, 1994).

The copper quantification analysis performed indicated the roots as the preferential organ for this metal accumulation. Elevated levels in the shoots go in line with other observed results in a sense that, upon copper absorption by the roots, a member of the heavy metal P-type ATPase OSHMA5 could primarily being expressed promoting Cu transport through the xylem to the shoots, as assays performed with inactive ATPase's mutants indicate the opposite results (Deng et al., 2013). Simultaneously, since Cu can be in both reduced and oxidized states, during long-distant transport, following oxidation, chelation is performed by a group of small cysteine-rich proteins separated in four types (MT1-MT4) enabling its transport to the shoots (Migocka & Malas, 2018). Results for mRNA expression for these genes was performed in this study and an increased expression in both shoots and roots was detected primarily in for the *MT2* gene, with similar results being obtained for *A.thaliana* (Guo et al., 2003), reinforcing the results obtained for copper quantification in these organs.

However, higher levels in the roots could indicate an accumulation of copper in sites that prevent full transport to the shoots. Since copper ion contains a positive charge, negative binding sites like cell walls and cell membranes could prevent the passage into the cytosol thus preventing transportation (Iwasaki et al., 1990). For fruit copper accumulation, the design experiment was not sensitive enough to detect the exact quantity present since that was only from 11 µg for gram of biomass on. However, this confirms the findings of other researchers (Allinson & Dzialo, 1981; Barman & Lal, 1994; Barman et al., 2000), where, generally, lower heavy metal concentrations are detected

in the fruits suggesting, within species and dose-dependent exposure, that a safe consumption can be achieved (Singh et al., 2012).

4.2. Copper effects in leaf's biochemistry of tomato plants.

Inhibition of photosynthesis by heavy metals in plants is well known (MacFarlane & Burchett, 2001), with reduction on photosynthetic pigments, especially by Cu exposure, like chlorophylls *a* and *b* and carotenoids, like β -Carotene (Van Assche & Clijsters, 1990), with the same, by some degree, results being recorded for other heavy metals like Lead (Krzyszowska, 2011) and Zinc (Krupa et al., 1996). The same can be confirmed with the results obtained in this study, that indicate decreases in both total chlorophyll (*a+b*), and β -Carotene in the leaves. This results may indicate an inhibition of enzymes involved in chlorophyll biosynthesis as well as heavy metal substitution of the central magnesium ion, preventing photosynthetic light harvest resulting in chlorophyll decay and subsequent photosynthetic activity (Prasad & Strzałka, 1999), inhibition caused by, in this case, copper accumulation in the shoots. However, despite this metal being essential to plant development (Baker & Walker, 1990), the concentration used surpasses the threshold in which the concentration turns from beneficial to harmful.

As for Lutein lack of significant results, and since β -Carotene lessened in concentration, this could suggest an impact through the biosynthesis process, in a sense that both carotenoids differ in their synthesis pathways (Flemotomou et al., 2011), and thus, vary in effects when heavy metals are present.

As for starch and soluble sugars concentration in the leaves, despite no significant changes were detected, some degree of increase was present. Indeed, accumulation of starch, under excess copper concentrations, has well been documented in other plant species like in *C. sativus* (Vinit-Dunand et al., 2002) and *Arabidopsis thaliana* (Wójcik & Tukiendorf, 2003). These results could not be caused by an inhibition of photosynthesis (Moya et al., 1993), since this results would suggest a low CO₂ fixation, and thus, a decrease in starch accumulation. Interestingly, results obtained by Vinit-Dunand et al.(2002) suggest that low photosynthetic levels could be an indication of an altered source-sink relationship, with greater impact than excessive copper concentrations. It is postulated that growth inhibition, which wasn't observed in this study, decreases carbohydrate export from the leaves leading to both starch and soluble sugars accumulation. Furthermore, this accumulation could be linked to non-variation in phloem

loading and capacity of transport of assimilates to the sink organs (roots) from the source (leaves), as a fall in the rate of its utilization (Alaoui-Sossé et al., 2004). Another hypothesis, presented by Greger et al. (1991), is that the decrease in utilization of carbohydrates for growth, in heavy metal toxicity conditions, is more pronounced than the decrease in CO₂ fixation.

4.3. Copper effects on oxidative stress markers in tomato plants.

To prevent ROS damage, plant cells are equipped with antioxidant systems consisting of peroxidases (POXs), superoxide dismutases (SODs) and catalase (CAT) and other regenerating antioxidants like glutathione, glutathione reductase (GR) and both reduced and oxidized ascorbates (Smirnoff, 1993), being the shift in balance between these two systems the definition for oxidative stress (Drazkiewicz et al., 2004). The origins of this balance shifts could be the changes in production and scavenging of these systems, which could result in H₂O₂ accumulation (Veal et al., 2007).

The results obtained in this study indicated that only in the roots were detected differences with an increase in hydrogen peroxide levels. One possibility for this could be the prevention of its accumulation by both CAT and APX. The latter is an antioxidant enzyme that uses ascorbate (AsA) as a reductant in the first steps of the ascorbate/glutathione cycle, being considered as the most important peroxidase in H₂O₂ detoxification (Noctor & Foyer, 1998). Since the content of reduced ascorbate in the roots is bigger than its oxidized counterpart (DHA), this could suggest that a APX activity could be compromised or altered leading to an increase in H₂O₂ content in the roots. The lack of alteration levels in the leaves suggests that the rate of production and scavenging was not affected resulting in the same levels as the control group.

However, these results cannot be fully understood only with the metabolic pathways described above. Reduced GSH is a water-soluble and ubiquitously distributed low molecular weight antioxidant present in all cellular compartments (Noctor et al., 2002), playing a central role in numerous physiological processes involving sulphate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics, redox balance, expression of stress-responsive genes, and scavenging of ROS produced during cellular metabolism or oxidative stress (Naser A Anjum et al., 2012). As such, this antioxidant is capable of detoxifying hydrogen peroxide in a sense that is intrinsic connected to the ascorbate-glutathione cycle (Gill & Tuteja, 2010). Results

obtained in this study revealed no changes of GSH levels in the leaves, and no detectable quantities, by the method used, in the roots. Since the same lack of significant differences is observed as previously stated in the hydrogen peroxide content in the shoots, this could indicate normal levels of GSH conversion to its oxidized form (GSSG). The ratio between GSH and GSSG, as APX activity is known to strengthen the role of GSH as a rate-limiting factor for ascorbate-glutathione cycle (Kuźniak & Skłodowska, 2001), would provide a better insight in complex detoxification of H_2O_2 in leaves.

AsA is major non-enzymatic antioxidant that plays comprehensive roles in plant metabolism under both normal and stress conditions. It scavenges ROS directly and constitutes the essential component of an antioxidant defence system being well established that not only AsA concentration, but also its redox ratios are important determinants of plant survival under stress conditions (Gill & Tuteja, 2010). Results for total ascorbate suggest no alterations to the biosynthesis process. However, differences are detected in both reduced and oxidized forms, AsA and DHA respectively, with increases in the first and decreases in the latter, with this being observed simultaneously in leaves and roots. Since H_2O_2 levels remain unaltered when compared with the control group, the increase found in AsA could indicate that both monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) convert MDHA, an oxidized intermediate, and DHA at a faster rate than AsA and GSH are generated (Hasanuzzaman et al., 2012). The change of ratios between AsA and DHA observed support this claim, since there is an increase in Total/AsA ratio and decrease in the Total/DHA ratio.

Proline is a five-carbon amino acid involved in osmotic balance during stress and is also essential in scavenging free radicals, stabilization of macromolecules, and signalling mechanism pathways (Hayat et al., 2012), being regarded as a stress metabolite (Mostofa & Fujita, 2013). Many plants have been shown to accumulate proline when exposed to heavy metal stress (Bassi & Sharma, 1993). Results indicated a decrease, despite non-significant, in the leaves and a slight rise in the roots. This opposes to what was observed in other studies with copper (Ku et al., 2012). The lack of proline in both organs may result from the defect in its synthesis or its degradation, since the levels of hydrogen peroxide (H_2O_2) remain relative constant, being this an important trigger for proline biosynthesis (Ben Rejeb et al., 2015). The proline biosynthetic pathway gene group (PCS) could potentially offer an insight in the lack of proline accumulation in the copper concentration tested, and possibly identify a relation between the low levels observed and a possible down-regulation of this genes (Siddique et al., 2018). However,

at the present time of this dissertation evaluation, no data could be further collected. On the other hand, proline can act as an osmotic protectant, ROS quencher and heavy metal chelator, by the formation of phytochelatins (Farago & WA, 1979). Studies performed by Sharma et al. (1998) indicate the possibility that proline could have a protective effect in the activity of glucose-6-phosphate dehydrogenase and nitrate reductase via the formation of a proline-metal complex thus resulting in the low free proline observed in the analysed organs. Moreover, levels of proline in both organs could be linked to the low GS activity observed since this enzyme is responsible for the synthesis of several osmoprotectants, in particular both proline and polyamines (James et al., 2018).

There is evidence that a number of non-protein (NPT) and protein thiols (PT), collectively with a network of sulphur-containing molecules and associated compounds, also fundamentally contribute to plant stress tolerance (Meyer & Hell, 2005) in the sense that they detoxify ROS at the site of its production (Baier et al., 2005). The first thiol group, the non-thiols, are composed by the sulphur-containing amino acids, cysteine (Cys), and methionine (Met), and the low molecular weight thiol glutathione, in its reduced and oxidized form, GSH and GSSG, respectively. And the last group, the protein thiols, are composed by thioredoxins (TRX) and glutaredoxins (GRX). Thioredoxins are reduced by different reductases depending on the cell compartment being NADPH-dependent (Lemaire et al., 2007), while GRX are directly reduced by GSH to produce GSSG (Zagorchev et al., 2013), being the GRX among the most potent protein-based protection mechanisms (Meyer et al., 2012).

Results recorded indicated an increase in total thiols content in both leaves and roots, indicating that an oxidative stress environment was present with the tested copper concentration, aligning to what is detected in the literature, where thiols levels increases are a well recorded effect of heavy metal stress (Hou et al., 2019). However, a decrease in non-protein thiols and an increase in protein thiols is observed, especially in the leaves. Since some NPT can have metal chelating properties (Li et al., 2011), they could lead to NPT increase in the roots, but the opposite is observed. So, the detected decrease could be caused by changes in its biosynthesis, since heavy metal treatments are known to increase and decrease NPT synthesis in optimal and supra-optimal concentrations (Olness et al., 2000). Protein thiols increases could be indicative that dependency on GSH concentration and GR activity, as suggested by (Lemaire et al.), however, only a GR expression increase could justify the rise in PT since GRX levels indicated no changes in the leaves, leaving the TRX content as the main source of protein-thiols in the plants.

4.4. Cooper effects on nitrogen metabolism response in tomato plants.

Plants can utilise a selection of nitrogen (N) composites in the soil, including nitrate, ammonium, nitrite, urea, amino acids, and proteins, and all of these potential N sources have significant differences in their reduction level. In this process, the enzyme Nitrate reductase, is responsible, as the name implies, for the reduction of nitrate to nitrite, with the latter being further reduced by nitrite reductase (NiR) to ammonia (Srivastava, 1980), which is the ultimate form of inorganic nitrogen available to the plant and is incorporated into an organic molecule, 2-oxoglutarate, by the combined action of the two enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). Both glutamine and glutamate which represent the two products of this metabolic process are further used as amino group donors to other amino acids utilised for protein synthesis and to nucleotides used as basic molecules for RNA and DNA synthesis (Hirel & Lea, 2001). Another means for incorporation of ammonia into glutamate is achieved by the action of the enzyme glutamine dehydrogenase (GDH) involving the reductive amination of 2-oxoglutarate using NAD or NAD(P) as coenzyme (Eprintsev et al., 2021).

Results for nitrate (NO_3^-) concentration indicate a considerable increase in both leaves and roots. These results highlight the behaviour observed in the increase of NR activity (Huo et al., 2020). The same results were present, in both shoots and roots, in *Elsholtzia haichowensis* (Huo et al., 2020) and in *Luffa cylindrica* (Zhang et al., 2014) to copper exposure. However, a decrease in NR activity was only detected in the leaves, with an increase, despite non-significant, in the roots. The high nitrate levels would allow for an increased NR activity. One possibility for the low NR activity in the leaves could refer to the fact that the $-\text{SH}$ group in the active sites of NR is sensitive to oxidative environments and can bind toxic metals (Singh et al., 2019; Xiong et al., 2006), and heavy metal stress may decrease the NR activity by displacing the essential molybdenum cofactor of NR (Singh et al., 2019). In addition, ROS induced by excess Cu may have indirect effects on NR and decrease its activity (Zhang et al., 2010).

Glutamine synthetase (GS) levels decreased in both leaves and roots and an increase in GDH activity levels was only observed in the shoots. This reduction in GS activity was also present in high Cu levels in other assays, such as *Elsholtzia haichowensis*, *Vitis vinifera* and cucumber (Burzyński & Buczek, 1997; Li et al., 2007; Zhang et al., 2014), and GSH levels paired with GDH are also reported under heavy

metal contamination (Gajewska & Skłodowska, 2009; Jha & Dubey, 2004; Kwinta & Cal, 2005). Low GS activity, suggested by Gajewska and Skłodowska (2009), could be caused by alterations in the oxidative metabolism of plants (Balestrasse et al., 2001). As metal toxicity leads to the overproduction of ROS in a plant cell, thus the decrease in both GS and GOGAT activities might be related, at least partly, to oxidative destruction of these enzymes. As for GDH levels, the increase in the leaves could be linked to the low GS activity observed in the same organ, since the latter is not sufficient to detoxify the excess NH_4^+ and thus higher amination reactions are required resulting in the increased activities detected (Gajewska & Skłodowska, 2009). Furthermore, the decrease detected in the roots may be result from the decrease in NR activity in the same organ. Kwinta & Kolik (2006) suggested that the low NR activity, and subsequent decrease in nitrite ions, could lead to an ammonia generation compensation by GDH for glutamine synthesis.

4.5. Role of specific GSs and MTs in the response of tomato plants to copper.

Metallothioneins (MT), with their low molecular weight, are distinguished by being rich in cysteine amino acids and being devoid of aromatic amino acids. MTs bind easily to heavy metals with high affinity because of their high cysteine content, so they are thought to play a protective role in the cells and organelles exposed to heavy metals (Brewer & Marcus, 2007; Shi & Chance, 2008). Therefore, they provide insight, as a biological indicator, in the assessment of the stress in organisms, and monitoring of various metal pollutants (Canpolat & Lynes, 2001; Dabrio et al., 2002).

Gene expression of *MT1* was up regulated in the leaves and the opposite in the roots. This results contradict to what was expected, since the gene expression of this type of MT was previously described as being higher in roots than shoots (Cobbett & Goldsbrough, 2002), or upregulated in both shoots and roots, albeit with cadmium (Kısa et al., 2016), and zinc (Castiglione et al., 2007). The present results could be an indication that the other MT forms may compensate the decrease of *MT1*-related transcripts levels in roots, the organ where most copper accumulation was detected. As for *MT2* gene expression, up-regulations were detected in both organs, although non-significant in roots. Same observations were recorded under copper stress in *Solanum nigrum* L. (Fidalgo et al., 2013), and for other heavy metals and plant species

like *Avicennia germinans* (Gonzalez-Mendoza et al., 2007), *Arachis hypogaea* (Quan et al., 2007), exposed to cadmium. Greater levels of *MT2* expression in the leaves could indicate a translocation from this organ to the roots, where higher Cu concentrations were detected.

Both *MT3* and *MT4* expression were not expected, both forms are found in fleshy fruits and developing seeds, respectively (Ferraz et al., 2012). Nonetheless, up-regulation was detected in both leaves and roots, with exception of the decreased expression of *MT3* in the roots. For copper and other heavy metals, like Fe, in *Populus alba*, decreases were detected in roots for *MT3* expression (Kohler et al., 2004), indicating the possibility of *MT3* gene expression contributing to Cu homeostasis in *S. lycopersicum* L leaves. As for *MT4* gene expression, the detected up-regulation was a curious result, evidencing the participation of *MT4* in the defence of leaves and roots against the excess copper, probably chelating it, or even exporting it from the leaves to the roots. Gritich et al (1998) noticed that Fe, Mn, and Cu did not induce *MT4* gene expression, while Zn induced it in tomato. These transcript results are associated with their physiological functions in plants (Huang & Wang, 2009), and the response depends on the developmental stage, tissue type, heavy metal concentration and uptake, and hormonal status of the tissue (Castiglione et al., 2007).

Since PCs (Phytochelatins) key related genes expression assay as performed, but at present time, no data could be displayed, no conclusive data could be taken, however, since copper is detected in both organs, both chelation and transport agents (PCs and MTs) could be also parallelly engaged.

GS is categorized into two groups: (1) the cytosol-localized GS1 group, and (2) the GS2 group, localized mainly in the chloroplasts (Swarbreck et al., 2011). Three isozymes were identified for GS1 (GS1.1,1.2,1.3) and one for GS2 in *S. lycopersicum* (Liu et al., 2016). GS2 assimilates the ammonium derived from photorespiration (Wallsgrave et al., 1987), whereas GS1 isozymes assimilate non-photorespiratory ammonium (Tobin & Yamaya, 2001). This could account for the low rise in GS2 in the shoots and decrease, despite non-significant, in the roots. Since photosynthetic pigments results showed a decrease in concentration, therefore, correlating to lower photosynthetic activity, it could result in low ammonium derived from the photorespiration (Konishi et al., 2017), explaining the expression detected in GS2 in both organs.

As for the GS1-encoding genes, a decrease in expression in *GS1.2* in the leaves may be a consequence of the low NR levels detected in the same organ and consequently, a response to the low ammonium levels present. Guam et al (2015) proposed that this specific isozyme could be responsible for ammonium relieve in both

organs and, therefore, the expression levels observed could be a reflection of the ammonium levels present. Previous studies on the physiological functions of GS1 isozymes in *Arabidopsis* using reverse-genetic approaches by the same author and Lothier et al (2011) further cement this hypothesis. Furthermore, the apparent increase in root GS1.2, according to Hachiya et al (2021), could indicate a barrier effect preventing the shoot-to-root transport of ammonium, thus avoiding assimilation by shoot GS2.

The higher increase observed in GS1.1 could be explained by this isozyme not accounting for the major GS isozyme pool, and thus, GS activity remaining low, as knockout experiments in GS1;2 and GS1;3 performed by Hachiya et al (2021) seem to imply. Their results were accompanied by a decrease in 35% of the GS activity in the shoots, aligning with the data here obtained for both previously mentioned isozymes. However, this tendency, alas reversed, can be detected in the roots, but cannot be explained by the same logic. As referred above, increased nitrate reductase levels, despite non-significant, and consequently higher ammonium levels, could induce GS gene expression, however the opposite was detected in the enzyme activity. GS isozymes performed differently in the different crops, owing to their differential kinetic and expression profile (Gao et al., 2020), emphasizing the need to access in more detail the GS family gene behavioural expression under similar copper exposure conditions.

5. Conclusions

This study demonstrated that *S. lycopersicum* Golden nugget cultivar was the least affected variety by copper exposure when compared to Micro-Tom: germination was not affected but both shoot and root length of seedlings were severely affected consequently affecting fresh weight. However, 5 weeks old plants treated with 8 mg L⁻¹ of copper did not reveal changes in fresh weight besides high copper accumulation in both organs was detected, suggesting a metal tolerance at this concentration. Copper content in the fruits was analysed, and lower concentrations than the method's sensitivity were in order. These results reveal that fruits of plants exposed to this specific concentration pose no threat to human health upon consumption.

As for leaf physiology, a decrease in photosynthetic pigments and in the antioxidant β -car indicate an oxidative stress status at this concentration, but no differences were detected in starch and sugar accumulation, suggesting that photosynthesis was not severely affected. Hydrogen peroxide levels confirm the low

oxidative stress since no alteration were detected in the leaves, paired with normal GSH concentrations and GR regulation. The opposite was detected in roots, however, with the current method, no GSH root concentration could be presented.

Differences in stress responses between both organs could originated from the different concentration of copper present. Proline levels decreased in leaves, indicating that no strong oxidative stress seem to be present, but an antioxidant response based on AsA and thiols seems to be the preferred method for ROS protection. Despite de low oxidative stress status presented, both NR and GS activity were diminished (with exception in the roots for NR). Nitrate accumulation in the roots paired with the apparent increase in NR activity indicates that the rate of Nitrogen uptake could occur at a faster rate that its reduction to nitrite. Interestingly, GS up-regulation was detected, and GDH activity increased in the leaves with the opposite happening in the roots, indicating that copper affects its enzymatic activity. Finally, metallothionines, contrary to what other investigations found, presented more gene regulation events in the leaves than in the roots.

Further studies with the present and additional copper concentrations together with more ROS stress markers, on an enzymatic and expression levels, need to be conducted to better understand the process of this heavy metal oxidative stress response and tolerance mechanisms.

6. Future work

This study provided an insight on the possible use of tomato for copper phytomining and post fruit consumption as Cu concentration effects on plants development and detoxification mechanisms were studied. However, some results indicated that further hypothesis can, in the future, be explored.

- Starch and sugar accumulation quantification was performed only in the leaves. The same quantification could be applied in the roots to better indicate root accumulation status of both sugars.
- Essential nutrients quantification, parallelly to copper, was performed, but at the time of this dissertation preparation, no data could be presented. Future data analysis could provide insight if significant differences are detected on nutrient uptake and translocation upon copper exposure.
- Evaluation of lipidic peroxidation and soluble proteins to further ascertain the degree of oxidative stress;
- Evaluate more enzymatic components of the ROS system: APX, CAT, GR and SOD to better understand their role in the plant defence and tolerance to copper exposure;
- Assessment of ROS-related genes expression for a better comprehension of their regulation and participation in the response of tomato plants to copper exposure.

7. References

- Abdel-Ghany, S. E., Burkhead, J. L., Gogolin, K. A., Andrés-Colás, N., Bodecker, J. R., Puig, S., Peñarrubia, L., & Pilon, M. (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. *FEBS Letters*, 579(11), 2307-2312. <https://doi.org/https://doi.org/10.1016/j.febslet.2005.03.025>
- Abdullah, S. R. S., Al-Baldawi, I. A., Almansoory, A. F., Purwanti, I. F., Al-Sbani, N. H., & Sharuddin, S. S. N. (2020). Plant-assisted remediation of hydrocarbons in water and soil: Application, mechanisms, challenges and opportunities. *Chemosphere*, 247, 125932. <https://doi.org/https://doi.org/10.1016/j.chemosphere.2020.125932>
- Adrees, M., Ali, S., Rizwan, M., Ibrahim, M., Abbas, F., Farid, M., Zia-ur-Rehman, M., Irshad, M. K., & Bharwana, S. A. (2015). The effect of excess copper on growth and physiology of important food crops: a review. *Environmental Science and Pollution Research*, 22(11), 8148-8162. <https://doi.org/https://doi.org/10.1007/s11356-015-4496-5>
- Ahsan, M. T., Najam-ul-haq, M., Saeed, A., Mustafa, T., & Afzal, M. (2018). Augmentation with potential endophytes enhances phytostabilization of Cr in contaminated soil. *Environmental Science and Pollution Research*, 25(7), 7021-7032. <https://doi.org/10.1007/s11356-017-0987-x>
- Ahsan, N., Lee, D.-G., Lee, S.-H., Kang, K. Y., Lee, J. J., Kim, P. J., Yoon, H.-S., Kim, J.-S., & Lee, B.-H. (2007). Excess copper induced physiological and proteomic changes in germinating rice seeds. *Chemosphere*, 67(6), 1182-1193. <https://doi.org/https://doi.org/10.1016/j.chemosphere.2006.10.075>
- Ait Elallem, K., Ben Bakrim, W., Ennoury, A., Metougui, M. L., Yasri, A., & Boularbah, A. (2022). Germination Parameters and Responses of Antioxidant Enzymatic Activities of Two Medicinal Plants (*Peganum harmala* L. and *Origanum majorana* L.) Under Heavy Metal Stress. *Journal of Soil Science and Plant Nutrition*, 1-16. <https://doi.org/https://doi.org/10.1007/s42729-022-00943-4>
- Aken, B. V., Correa, P. A., & Schnoor, J. L. (2010). Phytoremediation of Polychlorinated Biphenyls: New Trends and Promises. *Environmental Science & Technology*, 44(8), 2767-2776. <https://doi.org/10.1021/es902514d>
- Al-Daej, M. I. (2009). *Relative total phenolics in potato (Solanum tuberosum L.) progeny from 15 families*. Colorado State University. <https://doi.org/https://www.proquest.com/openview/250d3aa0ab1c0cd3ffb9487ca547abd/1?pq-origsite=gscholar&cbl=18750>
- Alamer, K. H., Attia, H., Alrobaie, H. S., & Hamad, A. A. (2022). Biocompatibility of *Solanum lycopersicum* and *Solanum melongena* which developed in heavy metal polluted soils. *South African Journal of Botany*, 147, 24-34. <https://doi.org/https://doi.org/10.1016/j.sajb.2021.12.033>
- Alaoui-Sossé, B., Genet, P., Vinit-Dunand, F., Toussaint, M.-L., Epron, D., & Badot, P.-M. (2004). Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. *Plant Science*, 166(5), 1213-1218. <https://doi.org/https://doi.org/10.1016/j.plantsci.2003.12.032>
- Ali, H., Khan, E., & Sajad, M. A. (2013). Phytoremediation of heavy metals—concepts and applications. *Chemosphere*, 91(7), 869-881. <https://doi.org/https://doi.org/10.1016/j.chemosphere.2013.01.075>
- Allinson, D., & Dzialo, C. (1981). The influence of lead, cadmium, and nickel on the growth of ryegrass and oats. *Plant and soil*, 62(1), 81-89. <https://doi.org/https://doi.org/10.1007/BF02205027>

- Anderson, C. W. N. (2003). Gold phytomining. Novel developments in a plant-based mining system. <https://doi.org/https://kiwiscience.com/downloads/Van2003.pdf>
- Anderson, C. W. N., Brooks, R. R., Chiarucci, A., LaCoste, C. J., Leblanc, M., Robinson, B. H., Simcock, R., & Stewart, R. B. (1999). Phytomining for nickel, thallium and gold. *Journal of Geochemical Exploration*, 67(1), 407-415. [https://doi.org/https://doi.org/10.1016/S0375-6742\(99\)00055-2](https://doi.org/https://doi.org/10.1016/S0375-6742(99)00055-2)
- Anjum, N. A., Ahmad, I., Mohmood, I., Pacheco, M., Duarte, A. C., Pereira, E., Umar, S., Ahmad, A., Khan, N. A., & Iqbal, M. (2012). Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review. *Environmental and Experimental Botany*, 75, 307-324. <https://doi.org/https://doi.org/10.1016/j.envexpbot.2011.07.002>
- Anjum, N. A., Ahmad, I., Mohmood, I., Pacheco, M., Duarte, A. C., Pereira, E., Umar, S., Ahmad, A., Khan, N. A., Iqbal, M., & Prasad, M. N. V. (2012). Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—A review. *Environmental and Experimental Botany*, 75, 307-324. <https://doi.org/https://doi.org/10.1016/j.envexpbot.2011.07.002>
- Anjum, N. A., Umar, S., & Chan, M.-T. (2010). *Ascorbate-glutathione pathway and stress tolerance in plants*. Springer Science & Business Media. <https://doi.org/https://doi.org/10.1007/978-90-481-9404-9>
- Arnon, D. I., & Stout, P. R. (1939). THE ESSENTIALITY OF CERTAIN ELEMENTS IN MINUTE QUANTITY FOR PLANTS WITH SPECIAL REFERENCE TO COPPER. *Plant Physiol*, 14(2), 371-375. <https://doi.org/10.1104/pp.14.2.371>
- Arreghini, S., de Cabo, L., Serafini, R., & de Iorio, A. F. (2017). Effect of the combined addition of Zn and Pb on partitioning in sediments and their accumulation by the emergent macrophyte *Schoenoplectus californicus*. *Environmental Science and Pollution Research*, 24(9), 8098-8107. <https://doi.org/https://doi.org/10.1007/s11356-017-8478-7>
- Asada, K. (2006). Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions. *Plant Physiology*, 141(2), 391-396. <https://doi.org/10.1104/pp.106.082040>
- Asati, A., Pichhode, M., & Nikhil, K. (2016). Effect of heavy metals on plants: an overview. *International Journal of Application or Innovation in Engineering & Management*, 5(3), 56-66. <https://doi.org/10.13140/RG.2.2.27583.87204>
- Athar, R., & Ahmad, M. (2002). Heavy metal toxicity: effect on plant growth and metal uptake by wheat, and on free living *Azotobacter*. *Water, Air, and Soil Pollution*, 138(1), 165-180. <https://doi.org/https://doi.org/10.1023/A:1015594815016>
- Bai, Y., & Lindhout, P. (2007). Domestication and breeding of tomatoes: what have we gained and what can we gain in the future? *Annals of Botany*, 100(5), 1085-1094. <https://doi.org/https://doi.org/10.1093/aob/mcm150>
- Baier, M., Kandlbinder, A., Gollack, D., & DIETZ, K. J. (2005). Oxidative stress and ozone: perception, signalling and response. *Plant, Cell & Environment*, 28(8), 1012-1020. <https://doi.org/https://doi.org/10.1111/j.1365-3040.2005.01326.x>
- Baker, A. J., & Walker, P. L. (1990). Ecophysiology of metal uptake by tolerant plants. *Heavy metal tolerance in plants: evolutionary aspects.*, 155-177. <https://doi.org/https://www.cabdirect.org/cabdirect/abstract/19900734154>
- Balestrasse, K. B., Gardey, L., Gallego, S. M., & Tomaro, M. L. (2001). Response of antioxidant defence system in soybean nodules and roots subjected to cadmium stress. *Functional Plant Biology*, 28(6), 497-504. <https://doi.org/https://doi.org/10.1071/PP00158>
- Barman, S., & Lal, M. (1994). Accumulation of heavy metals(Zn, Cu, Cd and Pb) in soil and cultivated vegetables and weeds grown in industrially polluted fields. *Journal of Environmental Biology*, 15(2), 107-115.
- Barman, S., Sahu, R., Bhargava, S., & Chatterjee, C. (2000). Distribution of heavy metals in wheat, mustard, and weed grown in field irrigated with industrial effluents.

- Bulletin of Environmental Contamination and Toxicology*, 64(4), 489-496.
<https://doi.org/10.1007/s001280000030>
- Bashmakov, D. I., Lukatkin, A. S., Revin, V. V., Duchovskis, P., Brazaitytė, A., & Baranauskis, K. (2005). Growth of maize seedlings affected by different concentrations of heavy metals. *Ekologija*, 3, 22-27.
- Bassi, R., & Sharma, S. S. (1993). Changes in proline content accompanying the uptake of zinc and copper by *Lemna minor*. *Annals of Botany*, 72(2), 151-154.
<https://doi.org/https://doi.org/10.1006/anbo.1993.1093>
- Bates, L. S., Waldren, R. P., & Teare, I. (1973). Rapid determination of free proline for water-stress studies. *Plant and soil*, 39(1), 205-207.
<https://doi.org/https://doi.org/10.1007/BF00018060>
- Beat B. Fischer, E. v. H., Anja Krieger-Liszky. (2013). Production, Detection, and Signaling of Singlet Oxygen in Photosynthetic Organisms. *Antioxidants & redox signaling*, 18(16), 2145-2162. <https://doi.org/10.1089/ars.2012.5124>
- Ben Rejeb, K., Lefebvre-De Vos, D., Le Disquet, I., Leprince, A. S., Bordenave, M., Maldiney, R., Jdey, A., Abdely, C., & Savouré, A. (2015). Hydrogen peroxide produced by NADPH oxidases increases proline accumulation during salt or mannitol stress in *A. rabidopsis thaliana*. *New Phytologist*, 208(4), 1138-1148.
<https://doi.org/https://doi.org/10.1111/nph.13550>
- Berg, J. (1994). *Principles of bioinorganic chemistry*.
<https://doi.org/https://resolver.caltech.edu/CaltechBOOK:1994.002>
- Bernal, M., Ramiro, M. V., Cases, R., Picorel, R., & Yruela, I. (2006). Excess copper effect on growth, chloroplast ultrastructure, oxygen-evolution activity and chlorophyll fluorescence in *Glycine max* cell suspensions. *Physiologia Plantarum*, 127(2), 312-325. <https://doi.org/https://doi.org/10.1111/j.1399-3054.2006.00641.x>
- Berti, W. R., & Cunningham, S. D. (2000). Phytostabilization of metals. *Phytoremediation of toxic metals: using plants to clean up the environment*. Wiley, New York, 71-88. <https://doi.org/https://doi.org/10.1515/REVEH.2010.25.2.135>
- Blindauer, C. A., & Leszczyszyn, O. I. (2010). Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more. *Natural product reports*, 27(5), 720-741. <https://doi.org/https://doi.org/10.1039/B906685N>
- Bolan, N. S., Park, J. H., Robinson, B., Naidu, R., & Huh, K. Y. (2011). Phytostabilization: a green approach to contaminant containment. *Advances in agronomy*, 112, 145-204. <https://doi.org/https://doi.org/10.1016/B978-0-12-385538-1.00004-4>
- Bölükbaşı, E. (2021). Expression analysis of Some Stress-Related Genes Induced by Cadmium on Tomato (*Solanum lycopersicum* L.) Plants. *Hittite Journal of Science and Engineering*. <https://doi.org/10.17350/hjse19030000247>
- Bolukbasi, E., & Karakas, M. (2019). Effect of Zinc Heavy Metal on Stress-Related Genes in Tomato (*Solanum lycopersicum* L.) Plants. *International Journal of Environment, Agriculture and Biotechnology*, 4(4), 1211-1216.
<https://doi.org/10.22161/ijeab.4448>
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254. [https://doi.org/https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/https://doi.org/10.1016/0003-2697(76)90527-3)
- Bratić, A. M., Majić, D. B., Samardžić, J. T., & Maksimović, V. R. (2009). Functional analysis of the buckwheat metallothionein promoter: tissue specificity pattern and up-regulation under complex stress stimuli. *Journal of plant physiology*, 166(9), 996-1000. <https://doi.org/https://doi.org/10.1016/j.jplph.2008.12.002>
- Brewer, T. M., & Marcus, R. K. (2007). Separation and Determination of Iron-Containing Proteins via Liquid Chromatography– Particle Beam/Hollow Cathode– Optical Emission Spectroscopy. *Analytical chemistry*, 79(6), 2402-2411.
<https://doi.org/doi:10.1021/ac061516p>

- Brooks, R. R. (1994). Plants that Hyperaccumulate Heavy Metals. In *Plants and the Chemical Elements* (pp. 87-105). <https://doi.org/https://doi.org/10.1002/9783527615919.ch4>
- Bulda, O., Rassadina, V., Alekseichuk, H., & Laman, N. (2008). Spectrophotometric measurement of carotenes, xanthophylls, and chlorophylls in extracts from plant seeds. *Russian Journal of Plant Physiology*, 55(4), 544-551. <https://doi.org/https://doi.org/10.1134/S1021443708040171>
- Burzyński, M., & Buczek, J. (1997). The effect of Cu²⁺ on uptake and assimilation of ammonium by cucumber seedlings. *Acta Physiologiae Plantarum*, 19(1), 3-8. <https://doi.org/https://doi.org/10.1007/s11738-997-0015-8>
- C.Mistra, K. (2000). *Understanding Mineral Deposits* (S. B. Media., Ed.). Springer. <https://doi.org/https://doi.org/10.1007/978-94-011-3925-0>
- Canpolat, E., & Lynes, M. A. (2001). In vivo manipulation of endogenous metallothionein with a monoclonal antibody enhances a T-dependent humoral immune response. *Toxicological Sciences*, 62(1), 61-70. <https://doi.org/https://doi.org/10.1093/toxsci/62.1.61>
- Castiglione, S., Franchin, C., Fossati, T., Lingua, G., Torrigiani, P., & Biondi, S. (2007). High zinc concentrations reduce rooting capacity and alter metallothionein gene expression in white poplar (*Populus alba* L. cv. Villafranca). *Chemosphere*, 67(6), 1117-1126. <https://doi.org/https://doi.org/10.1016/j.chemosphere.2006.11.039>
- Chang, C. C. C., Ślesak, I., Jordá, L. a., Sotnikov, A., Melzer, M., Miszalski, Z., Mullineaux, P. M., Parker, J. E., Karpińska, B., & Karpiński, S. (2009). Arabidopsis Chloroplastic Glutathione Peroxidases Play a Role in Cross Talk between Photooxidative Stress and Immune Responses *Plant Physiology*, 150(2), 670-683. <https://doi.org/10.1104/pp.109.135566>
- Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., & Zhang, J. (2015). Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: applications, microbes and future research needs. *Biotechnology advances*, 33(6), 745-755. <https://doi.org/https://doi.org/10.1016/j.biotechadv.2015.05.003>
- Chen, T., Qin, G., & Tian, S. (2020). Regulatory network of fruit ripening: current understanding and future challenges. *New Phytologist*, 228(4), 1219-1226. <https://doi.org/https://doi.org/10.1111/nph.16822>
- Choudhury, S., Panda, P., Sahoo, L., & Panda, S. K. (2013). Reactive oxygen species signaling in plants under abiotic stress. *Plant Signaling & Behavior*, 8(4), e23681. <https://doi.org/https://doi.org/10.4161/psb.23681>
- Clarkson, D. T., & Hanson, J. B. (1980). The Mineral Nutrition of Higher Plants. *Annual Review of Plant Physiology*, 31(1), 239-298. <https://doi.org/10.1146/annurev.pp.31.060180.001323>
- Cobbett, C. (2002). Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual review of plant biology*, v. 53, pp. 159-182-2002 v.2053. <https://doi.org/10.1146/annurev.arplant.53.100301.135154>
- Cobbett, C., & Goldsbrough, P. (2002). Phytochelatins AND Metallothioneins: Roles. *Annu. Rev. Plant Biol*, 53, 159-182.
- Cohu, C. M., & Pilon, M. (2007). Regulation of superoxide dismutase expression by copper availability. *Physiologia Plantarum*, 129(4), 747-755. <https://doi.org/10.1111/j.1399-3054.2007.00879.x>
- Cosio, C., Martinoia, E., & Keller, C. (2004). Hyperaccumulation of cadmium and zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. *Plant Physiol*, 134(2), 716-725. <https://doi.org/10.1104/pp.103.031948>
- Costa, G., & Morel, J.-L. (1994). Water relations, gas exchange and amino acid content in Cd-treated lettuce. *Plant physiology and biochemistry (Paris)*, 32(4), 561-570. <https://doi.org/https://pascal-francis.inist.fr/>

- Dabrio, M., Rodriguez, A. R., Bordin, G., Bebianno, M. J., De Ley, M., Sestakova, I., Vasak, M., & Nordberg, M. (2002). Recent developments in quantification methods for metallothionein. *J Inorg Biochem*, 88(2), 123-134. [https://doi.org/10.1016/S0162-0134\(01\)00374-9](https://doi.org/10.1016/S0162-0134(01)00374-9)
- Dave, D., & Ghaly, A. E. (2011). Remediation technologies for marine oil spills: A critical review and comparative analysis. *American Journal of Environmental Sciences*, 7(5), 423.
- Demidchik, V. (2010). Reactive Oxygen Species, Oxidative Stress and Plant Ion Channels. In *Ion Channels and Plant Stress Responses* (pp. 207-232). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-10494-7_11
- Demidchik, V. (2015). Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. *Environmental and Experimental Botany*, 109, 212-228. <https://doi.org/10.1016/j.envexpbot.2014.06.021>
- Demidchik, V., Shabala, S. N., Coutts, K. B., Tester, M. A., & Davies, J. M. (2003). Free oxygen radicals regulate plasma membrane Ca²⁺- and K⁺-permeable channels in plant root cells. *J Cell Sci*, 116(Pt 1), 81-88. <https://doi.org/10.1242/jcs.00201>
- Demidchik, V., Straltsova, D., Medvedev, S. S., Pozhvanov, G. A., Sokolik, A., & Yurin, V. (2014). Stress-induced electrolyte leakage: the role of K⁺-permeable channels and involvement in programmed cell death and metabolic adjustment. *J Exp Bot*, 65(5), 1259-1270. <https://doi.org/10.1093/jxb/eru004>
- Deng, F., Yamaji, N., Xia, J., & Ma, J. F. (2013). A member of the heavy metal P-type ATPase OsHMA5 is involved in xylem loading of copper in rice. *Plant Physiol*, 163(3), 1353-1362. <https://doi.org/10.1104/pp.113.226225>
- Diaz-Vivancos, P., de Simone, A., Kiddle, G., & Foyer, C. H. (2015). Glutathione--linking cell proliferation to oxidative stress. *Free Radic Biol Med*, 89, 1154-1164. <https://doi.org/10.1016/j.freeradbiomed.2015.09.023>
- Dolezalova Weissmannova, H., Mihocova, S., Chovanec, P., & Pavlovsky, J. (2019). Potential Ecological Risk and Human Health Risk Assessment of Heavy Metal Pollution in Industrial Affected Soils by Coal Mining and Metallurgy in Ostrava, Czech Republic. *Int J Environ Res Public Health*, 16(22), 4495. <https://doi.org/10.3390/ijerph16224495>
- Doty, S. L., James, C. A., Moore, A. L., Vajzovic, A., Singleton, G. L., Ma, C., Khan, Z., Xin, G., Kang, J. W., Park, J. Y., Meilan, R., Strauss, S. H., Wilkerson, J., Farin, F., & Strand, S. E. (2007). Enhanced phytoremediation of volatile environmental pollutants with transgenic trees. *Proceedings of the National Academy of Sciences*, 104(43), 16816-16821. <https://doi.org/10.1073/pnas.0703276104>
- Drazkiewicz, M., Skorzynska-Polit, E., & Krupa, Z. (2004). Copper-induced oxidative stress and antioxidant defence in Arabidopsis thaliana. *Biometals*, 17(4), 379-387. <https://doi.org/10.1023/b:biom.0000029417.18154.22>
- Duprat, F., Guillemare, E., Romey, G., Fink, M., Lesage, F., Lazdunski, M., & Honore, E. (1995). Susceptibility of cloned K⁺ channels to reactive oxygen species. *Proc Natl Acad Sci U S A*, 92(25), 11796-11800. <https://doi.org/10.1073/pnas.92.25.11796>
- Eprintsev, A., Anokhina, G., & Fedorin, D. (2021). Regulation of Glutamate Dehydrogenase Activity in Maize Leaves (Zea mays L.) with Change in the Light Conditions. *Biology Bulletin*, 48(3), S38-S44. <https://doi.org/https://doi.org/10.1134/S1062359021060066>
- Farago, M., & WA, M. (1979). Plants which accumulate metals. IV. A possible copper-proline complex from the roots of *Armeria maritima*. [https://doi.org/https://doi.org/10.1016/S0020-1693\(00\)91627-X](https://doi.org/https://doi.org/10.1016/S0020-1693(00)91627-X)
- Feng, N.-X., Yu, J., Zhao, H.-M., Cheng, Y.-T., Mo, C.-H., Cai, Q.-Y., Li, Y.-W., Li, H., & Wong, M.-H. (2017). Efficient phytoremediation of organic contaminants in soils using plant-endophyte partnerships. *Science of the Total Environment*, 583, 352-368. <https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.01.075>

- Fernandez, A. P., & Strand, A. (2008). Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. *Curr Opin Plant Biol*, 11(5), 509-513. <https://doi.org/10.1016/j.pbi.2008.06.002>
- Ferraz, P., Fidalgo, F., Almeida, A., & Teixeira, J. (2012). Phytostabilization of nickel by the zinc and cadmium hyperaccumulator *Solanum nigrum* L. Are metallothioneins involved? *Plant Physiol Biochem*, 57, 254-260. <https://doi.org/10.1016/j.plaphy.2012.05.025>
- Fidalgo, F., Azenha, M., Silva, A. F., de Sousa, A., Santiago, A., Ferraz, P., & Teixeira, J. (2013). Copper-induced stress in *Solanum nigrum* L. and antioxidant defense system responses. *Food and Energy Security*, 2(1), 70-80. <https://doi.org/https://doi.org/10.1002/fes3.20>
- Flemotomou, E., Molyviatis, T., & Zabetakis, I. (2011). The effect of trace elements accumulation on the levels of secondary metabolites and antioxidant activity in carrots, onions and potatoes. <https://doi.org/10.4236/fns.2011.210143>
- Fornazari, M., de Paula, J. G., Castilho, R. F., & Kowaltowski, A. J. (2008). Redox properties of the adenosine triphosphate-sensitive K⁺ channel in brain mitochondria. *Journal of Neuroscience Research*, 86(7), 1548-1556. <https://doi.org/https://doi.org/10.1002/jnr.21614>
- Foyer, C. H., & Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology*, 155(1), 2-18. <https://doi.org/https://doi.org/10.1104/pp.110.167569>
- Foyer, C. H., Norton, T. (2009). Redox Regulation in Photosynthetic Organisms: Signaling, Acclimation, and Practical Implications. *Antioxidants & redox signaling*, 11(4), 861-905. <https://doi.org/10.1089/ars.2008.2177>
- Freisinger, E. (2008). Plant MTs—long neglected members of the metallothionein superfamily. *Dalton Transactions*(47), 6663-6675. <https://doi.org/10.1039/b809789e>
- Freisinger, E. (2009). Metallothioneins in plants. *Met Ions Life Sci*, 5, 107-153. <https://doi.org/https://doi.org/10.1039/9781847559531-00107>
- Fry, S. C. (2004). Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. *New Phytologist*, 161(3), 641-675. <https://doi.org/https://doi.org/10.1111/j.1469-8137.2004.00980.x>
- Gajewska, E., & Skłodowska, M. (2009). Nickel-induced changes in nitrogen metabolism in wheat shoots. *Journal of plant physiology*, 166(10), 1034-1044. <https://doi.org/https://doi.org/10.1016/j.jplph.2008.12.004>
- Gao, K., Zhou, T., Hua, Y., Guan, C., & Zhang, Z. (2020). Transcription factor WRKY23 is involved in ammonium-induced repression of Arabidopsis primary root growth under ammonium toxicity. *Plant physiology and biochemistry*, 150, 90-98. <https://doi.org/https://doi.org/10.1016/j.plaphy.2020.02.034>
- Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry*, 48(12), 909-930. <https://doi.org/https://doi.org/10.1016/j.plaphy.2010.08.016>
- Gillespie, K. M., & Ainsworth, E. A. (2007). Measurement of reduced, oxidized and total ascorbate content in plants. *Nature Protocols*, 2(4), 871-874. <https://doi.org/https://doi.org/10.1038/nprot.2007.101>
- Giritch, A., Ganai, M., Stephan, U. W., & Bäumlein, H. (1998). Structure, expression and chromosomal localisation of the metallothionein-like gene family of tomato. *Plant molecular biology*, 37(4), 701-714. <https://doi.org/https://doi.org/10.1023/A:1006001701919>
- Golui, D., Datta, S., Dwivedi, B., Meena, M., Varghese, E., Sanyal, S., Ray, P., Shukla, A., & Trivedi, V. (2019). Assessing soil degradation in relation to metal pollution—A multivariate approach. *Soil and Sediment Contamination: An International Journal*, 28(7), 630-649. <https://doi.org/https://doi.org/10.1080/15320383.2019.1640660>

- Gómez, L. D., Vanacker, H., Buchner, P., Noctor, G., & Foyer, C. H. (2004). Intercellular distribution of glutathione synthesis in maize leaves and its response to short-term chilling. *Plant Physiology*, 134(4), 1662-1671. <https://doi.org/https://doi.org/10.1104/pp.103.033027>
- Gong, P., Zhang, J., Li, H., Yang, C., Zhang, C., Zhang, X., Khurram, Z., Zhang, Y., Wang, T., & Fei, Z. (2010). Transcriptional profiles of drought-responsive genes in modulating transcription signal transduction, and biochemical pathways in tomato. *Journal of Experimental Botany*, 61(13), 3563-3575. <https://doi.org/https://doi.org/10.1093/jxb/erq167>
- Gonzalez-Mendoza, D., Moreno, A. Q., & Zapata-Perez, O. (2007). Coordinated responses of phytochelatin synthase and metallothionein genes in black mangrove, *Avicennia germinans*, exposed to cadmium and copper. *Aquatic Toxicology*, 83(4), 306-314. <https://doi.org/https://doi.org/10.1016/j.aquatox.2007.05.005>
- Greger, M., Brammer, E., Lindberg, S., Larsson, G., & Idestam-Almquist, J. (1991). Uptake and physiological effects of cadmium in sugar beet (*Beta vulgaris*) related to mineral provision. *Journal of Experimental Botany*, 42(6), 729-737. <https://doi.org/https://doi.org/10.1093/jxb/42.6.729>
- Grennan, A. K. (2011). Metallothioneins, a diverse protein family. *Plant Physiology*, 155(4), 1750-1751. <https://doi.org/https://doi.org/10.1104/pp.111.900407>
- Guo, W.-J., Bundithya, W., & Goldsbrough, P. B. (2003). Characterization of the *Arabidopsis* metallothionein gene family: tissue-specific expression and induction during senescence and in response to copper. *New Phytologist*, 369-381. <https://doi.org/10.1046/j.1469-8137.2003.00813.x>
- Hachiya, T., Inaba, J., Wakazaki, M., Sato, M., Toyooka, K., Miyagi, A., Kawai-Yamada, M., Sugiura, D., Nakagawa, T., & Kiba, T. (2021). Excessive ammonium assimilation by plastidic glutamine synthetase causes ammonium toxicity in *Arabidopsis thaliana*. *Nature communications*, 12(1), 1-10. <https://doi.org/https://doi.org/10.1038/s41467-021-25238-7>
- Halliwell, B., and John MC Gutteridge. (2015). *Free radicals in biology and medicine*. Oxford university press. <https://doi.org/https://doi.org/10.1080/09553009014552071>
- Harris, A. T., Naidoo, K., Nokes, J., Walker, T., & Orton, F. (2009). Indicative assessment of the feasibility of Ni and Au phytomining in Australia. *Journal of Cleaner Production*, 17(2), 194-200. <https://doi.org/https://doi.org/10.1016/j.jclepro.2008.04.011>
- Hasan, M. M., Uddin, M. N., Ara-Sharmeen, I., F. Alharby, H., Alzahrani, Y., Hakeem, K. R., & Zhang, L. (2019). Assisting phytoremediation of heavy metals using chemical amendments. *Plants*, 8(9), 295. <https://doi.org/https://doi.org/10.3390/plants8090295>
- Hasanuzzaman, M., Bhuyan, M. B., Parvin, K., Bhuiyan, T. F., Anee, T. I., Nahar, K., Hossen, M. S., Zulfiqar, F., Alam, M. M., & Fujita, M. (2020). Regulation of ROS metabolism in plants under environmental stress: A review of recent experimental evidence. *International journal of molecular sciences*, 21(22), 8695. <https://doi.org/https://doi.org/10.3390/ijms21228695>
- Hasanuzzaman, M., Hossain, M. A., Silva, J. A., & Fujita, M. (2012). Plant response and tolerance to abiotic oxidative stress: antioxidant defense is a key factor. In *Crop stress and its management: perspectives and strategies* (pp. 261-315). Springer. https://doi.org/https://doi.org/10.1007/978-94-007-2220-0_8
- Hassinen, V., Tervahauta, A., Schat, H., & Kärenlampi, S. (2011). Plant metallothioneins—metal chelators with ROS scavenging activity? *Plant Biology*, 13(2), 225-232.
- Hassinen, V., Vallinkoski, V.-M., Issakainen, S., Tervahauta, A., Kärenlampi, S., & Servomaa, K. (2009). Correlation of foliar MT2b expression with Cd and Zn

- concentrations in hybrid aspen (*Populus tremula* × *tremuloides*) grown in contaminated soil. *Environmental Pollution*, 157(3), 922-930. <https://doi.org/https://doi.org/10.1016/j.envpol.2008.10.023>
- Hassinen, V. H., Tervahauta, A. I., Schat, H., & Kärenlampi, S. O. (2011). Plant metallothioneins – metal chelators with ROS scavenging activity? *Plant Biology*, 13(2), 225-232. <https://doi.org/https://doi.org/10.1111/j.1438-8677.2010.00398.x>
- Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., & Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signaling & Behavior*, 7(11), 1456-1466. <https://doi.org/ttps://doi.org/10.4161/psb.21949>
- Heilmeyer, H., & Wiche, O. (2020). The PCA of phytomining: principles, challenges and achievements. *Carpathian journal of earth and environmental sciences*, 15, 37-42. <https://doi.org/10.26471/cjees/2020/015/106>
- Hirel, B., & Lea, P. J. (2001). Ammonia assimilation. In *Plant nitrogen* (pp. 79-99). Springer. https://doi.org/https://doi.org/10.1007/978-3-662-04064-5_4
- Hou, M., Li, M., Yang, X., & Pan, R. (2019). Responses of nonprotein thiols to stress of vanadium and mercury in maize (*Zea mays* L.) seedlings. *Bulletin of Environmental Contamination and Toxicology*, 102(3), 425-431. <https://doi.org/https://doi.org/10.1007/s00128-019-02553-w>
- Huang, G.-Y., & Wang, Y.-S. (2009). Expression analysis of type 2 metallothionein gene in mangrove species (*Bruguiera gymnorrhiza*) under heavy metal stress. *Chemosphere*, 77(7), 1026-1029. <https://doi.org/https://doi.org/10.1016/j.chemosphere.2009.07.073>
- Hunt, A. J., Anderson, C. W. N., Bruce, N., García, A. M., Graedel, T. E., Hodson, M., Meech, J. A., Nassar, N. T., Parker, H. L., Rylott, E. L., Sotiriou, K., Zhang, Q., & Clark, J. H. (2014). Phytoextraction as a tool for green chemistry. *Green Processing and Synthesis*, 3(1), 3-22. <https://doi.org/doi:10.1515/gps-2013-0103>
- Huo, K., Shangguan, X., Xia, Y., Shen, Z., & Chen, C. (2020). Excess copper inhibits the growth of rice seedlings by decreasing uptake of nitrate. *Ecotoxicology and Environmental Safety*, 190, 110105. <https://doi.org/https://doi.org/10.1016/j.ecoenv.2019.110105>
- Ilyas, S., & Rehman, A. (2015). Oxidative stress, glutathione level and antioxidant response to heavy metals in multi-resistant pathogen, *Candida tropicalis*. *Environmental monitoring and assessment*, 187(1), 1-7. <https://doi.org/https://doi.org/10.1007/s10661-014-4115-9>
- INE, I. N. d. E. (2016). *Agricultural Statistics 2015*. INE. https://doi.org/https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_main
- Iwasaki, K. z., Sakurai, K., & Takahashi, E. (1990). Copper binding by the root cell walls of Italian ryegrass and red clover. *Soil Science and Plant Nutrition*, 36(3), 431-439. <https://doi.org/https://doi.org/10.1080/00380768.1990.10416911>
- Jacob, J. M., Karthik, C., Saratale, R. G., Kumar, S. S., Prabakar, D., Kadirvelu, K., & Pugazhendhi, A. (2018). Biological approaches to tackle heavy metal pollution: A survey of literature. *Journal of Environmental Management*, 217, 56-70. <https://doi.org/https://doi.org/10.1016/j.jenvman.2018.03.077>
- Jain, K., Kataria, S., & Guruprasad, K. N. (2004). Oxyradicals under UV-b stress and their quenching by antioxidants. *Indian journal of experimental biology*, 42(9), 884-892. <http://europemc.org/abstract/MED/15462181>
- James, D., Borphukan, B., Fartyal, D., Ram, B., Singh, J., Manna, M., Sheri, V., Panditi, V., Yadav, R., & Achary, V. M. M. (2018). Concurrent overexpression of OsGS1; 1 and OsGS2 genes in transgenic rice (*Oryza sativa* L.): impact on tolerance to abiotic stresses. *Frontiers in plant science*, 9, 786. <https://doi.org/https://doi.org/10.3389/fpls.2018>
- Jha, A. B., & Dubey, R. S. (2004). Arsenic exposure alters activity behaviour of key nitrogen assimilatory enzymes in growing rice plants. *Plant Growth Regulation*,

- 43(3), 259-268.
<https://doi.org/https://doi.org/10.1023/B:GROW.0000045995.49365.df>
- John, R., Ahmad, P., Gadgil, K., & Sharma, S. (2009). Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation by *Brassica juncea* L.
- Karpínski, S., SZECHYŃSKA-HEBDA, M., WITUSZYŃSKA, W., & BURDIĄK, P. (2013). Light acclimation, retrograde signalling, cell death and immune defences in plants. *Plant, Cell & Environment*, 36(4), 736-744.
<https://doi.org/https://doi.org/10.1111/pce.12018>
- Khan, M. S., Zaidi, A., Wani, P. A., & Oves, M. (2009). Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environmental chemistry letters*, 7(1), 1-19. <https://doi.org/https://doi.org/10.1007/s10311-008-0155-0>
- Kim, C., & Apel, K. (2013). 1O₂-Mediated and EXECUTER-Dependent Retrograde Plastid-to-Nucleus Signaling in Norflurazon-Treated Seedlings of *Arabidopsis thaliana*. *Molecular Plant*, 6(5), 1580-1591.
<https://doi.org/https://doi.org/10.1093/mp/sst020>
- Kısa, D., Öztürk, L., & Tekin, Ş. (2016). Gene expression analysis of metallothionein and mineral elements uptake in tomato (*Solanum lycopersicum*) exposed to cadmium. *Journal of plant research*, 129(5), 989-995.
<https://doi.org/https://doi.org/10.1007/s10265-016-0847-7>
- Kohler, A., Blaudez, D., Chalot, M., & Martin, F. (2004). Cloning and expression of multiple metallothioneins from hybrid poplar. *New Phytologist*, 83-93.
<https://doi.org/10.1111/j.1469-8137.2004.01168.x>
- Konishi, N., Ishiyama, K., Beier, M. P., Inoue, E., Kanno, K., Yamaya, T., Takahashi, H., & Kojima, S. (2017). Contributions of two cytosolic glutamine synthetase isozymes to ammonium assimilation in *Arabidopsis* roots. *Journal of Experimental Botany*, 68(3), 613-625. <https://doi.org/10.1093/jxb/erw454>
- Koptsik, G. N. (2014). Problems and prospects concerning the phytoremediation of heavy metal polluted soils: A review. *Eurasian Soil Science*, 47(9), 923-939.
<https://doi.org/10.1134/S1064229314090075>
- Kruk, J., & Trebst, A. (2008). Plastoquinol as a singlet oxygen scavenger in photosystem II. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1777(2), 154-162.
<https://doi.org/https://doi.org/10.1016/j.bbabi.2007.10.008>
- Krupa, Z., Baranowska, M., & Orzol, D. (1996). Can anthocyanins be considered as heavy metal stress indicator in higher plants? *Acta Physiologiae Plantarum*, 18(2). <https://doi.org/bwmeta1.element.agro-article-5fa74918-accb-4841-9357-02f625b7ae85>
- Krzyszowska, M. (2011). The cell wall in plant cell response to trace metals: polysaccharide remodeling and its role in defense strategy. *Acta Physiologiae Plantarum*, 33(1), 35-51. <https://doi.org/https://doi.org/10.1007/s11738-010-0581-z>
- Ku, H.-M., Tan, C.-W., Su, Y.-S., Chiu, C.-Y., Chen, C.-T., & Jan, F.-J. (2012). The effect of water deficit and excess copper on proline metabolism in *Nicotiana benthamiana*. *Biologia Plantarum*, 56(2), 337-343.
<https://doi.org/https://doi.org/10.1007/s10535-012-0095-1>
- Kumar, A., Patel, J. S., Meena, V. S., & Srivastava, R. (2019). Recent advances of PGPR based approaches for stress tolerance in plants for sustainable agriculture. *Biocatalysis and agricultural biotechnology*, 20, 101271.
<https://doi.org/https://doi.org/10.1016/j.bcab.2019.101271>
- Kumar Yadav, K., Gupta, N., Kumar, A., Reece, L. M., Singh, N., Rezaei, S., & Ahmad Khan, S. (2018). Mechanistic understanding and holistic approach of phytoremediation: A review on application and future prospects. *Ecological*

- Engineering, 120, 274-298.
<https://doi.org/https://doi.org/10.1016/j.ecoleng.2018.05.039>
- Kuper, J., Llamas, A., Hecht, H.-J., Mendel, R. R., & Schwarz, G. (2004). Structure of the molybdopter-in-bound Cnx1G domain links molybdenum and copper metabolism. *Nature*, 430(7001), 803-806. <https://doi.org/10.1038/nature02681>
- Kuźniak, E., & Skłodowska, M. (2001). Ascorbate, glutathione and related enzymes in chloroplasts of tomato leaves infected by *Botrytis cinerea*. *Plant Science*, 160(4), 723-731. [https://doi.org/https://doi.org/10.1016/S0168-9452\(00\)00457-X](https://doi.org/https://doi.org/10.1016/S0168-9452(00)00457-X)
- Kwinta, J., & Cal, K. (2005). Effects of Salinity Stress on the Activity of Glutamine Synthetase and Glutamate Dehydrogenase in Triticale Seedlings. *Polish Journal of Environmental Studies*, 14(1). [https://doi.org/Pol. J. Environ. Stud. 2005;14\(1\):125-130](https://doi.org/Pol. J. Environ. Stud. 2005;14(1):125-130)
- Kwinta, J., & Kolik, D. (2006). Glutamine synthetase and glutamate dehydrogenase in cadmium-stressed triticale seedlings. *Acta Physiologiae Plantarum*, 28(4), 339-347. <https://doi.org/https://doi.org/10.1007/s11738-006-0030-1>
- Lange, B. M. (2013). Linking metabolomics with quality traits in crop plants. In *Hot Topics in Metabolomics: Food and Nutrition* (pp. 66-74). <https://doi.org/10.4155/ebo.13.413>
- Lemaire, S. D., Guillon, B., Le Maréchal, P., Keryer, E., Miginiac-Maslow, M., & Decottignies, P. (2004). New thioredoxin targets in the unicellular photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences*, 101(19), 7475-7480. <https://doi.org/https://doi.org/10.1073/pnas.0402221101>
- Lemaire, S. D., Michelet, L., Zaffagnini, M., Massot, V., & Issakidis-Bourguet, E. (2007). Thioredoxins in chloroplasts. *Current genetics*, 51(6), 343-365. <https://doi.org/https://doi.org/10.1007/s00294-007-0128-z>
- Leszczyszyn, O. I., Imam, H. T., & Blindauer, C. A. (2013). Diversity and distribution of plant metallothioneins: a review of structure, properties and functions. *Metallomics*, 5(9), 1146-1169. <https://doi.org/https://doi.org/10.1039/c3mt00072a>
- Li, M., Xiong, Z., Dai, L., & Huang, Y. (2007). Effects of Copper on Nitrogen Assimilation in Copper-tolerant and Non-tolerant Populations of *Elsholtzia haichowensis* S. *Water, Air, and Soil Pollution*, 184(1), 323-333.
- Li, T., Di, Z., Islam, E., Jiang, H., & Yang, X. (2011). Rhizosphere characteristics of zinc hyperaccumulator *Sedum alfredii* involved in zinc accumulation. *Journal of Hazardous Materials*, 185(2-3), 818-823. <https://doi.org/https://doi.org/10.1016/j.jhazmat.2010.09.093>
- Li, Y., Wang, H., Zhang, Y., & Martin, C. (2018). Can the world's favorite fruit, tomato, provide an effective biosynthetic chassis for high-value metabolites? *Plant cell reports*, 37(10), 1443-1450. <https://doi.org/https://doi.org/10.1007/s00299-018-2283-8>
- Lin, T., Zhu, G., Zhang, J., Xu, X., Yu, Q., Zheng, Z., Zhang, Z., Lun, Y., Li, S., & Wang, X. (2014). Genomic analyses provide insights into the history of tomato breeding. *Nature genetics*, 46(11), 1220-1226. <https://doi.org/https://doi.org/10.1038/ng.3117>
- Liu, L., Wang, J., Han, Z., Sun, X., Li, H., Zhang, J., & Lu, Y. (2016). Molecular analyses of tomato GS, GOGAT and GDH gene families and their response to abiotic stresses. *Acta Physiologiae Plantarum*, 38(9), 1-14. <https://doi.org/https://doi.org/10.1007/s11738-016-2251-2>
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *methods*, 25(4), 402-408. <https://doi.org/https://doi.org/10.1006/meth.2001.1262>
- López-Vidal, O., Camejo, D., Rivera-Cabrera, F., Königsberg, M., Villa-Hernández, J. M., Mendoza-Espinoza, J. A., Pérez-Flores, L. J., Sevilla, F., Jiménez, A., & Díaz de León-Sánchez, F. (2016). Mitochondrial ascorbate-glutathione cycle and

- proteomic analysis of carbonylated proteins during tomato (*Solanum lycopersicum*) fruit ripening. *Food Chemistry*, 194, 1064-1072. <https://doi.org/https://doi.org/10.1016/j.foodchem.2015.08.055>
- Lothier, J., Gaufichon, L., Sormani, R., Lemaître, T., Azzopardi, M., Morin, H., Chardon, F., Reisdorf-Cren, M., Avice, J.-C., & Masclaux-Daubresse, C. (2011). The cytosolic glutamine synthetase GLN1; 2 plays a role in the control of plant growth and ammonium homeostasis in *Arabidopsis* rosettes when nitrate supply is not limiting. *Journal of Experimental Botany*, 62(4), 1375-1390. <https://doi.org/https://doi.org/10.1093/jxb/erq299>
- Løvdaal, T., & Lillo, C. (2009). Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. *Analytical biochemistry*, 387(2), 238-242. <https://doi.org/https://doi.org/10.1016/j.ab.2009.01.024>
- Lu, S. C. (2009). Regulation of glutathione synthesis. *Molecular Aspects of Medicine*, 30(1), 42-59. <https://doi.org/https://doi.org/10.1016/j.mam.2008.05.005>
- MacFarlane, G., & Burchett, M. (2001). Photosynthetic pigments and peroxidase activity as indicators of heavy metal stress in the Grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Marine pollution bulletin*, 42(3), 233-240. [https://doi.org/https://doi.org/10.1016/S0025-326X\(00\)00147-8](https://doi.org/https://doi.org/10.1016/S0025-326X(00)00147-8)
- Marques, D., Da Silva, A., Mantovani, J., Magalhães, P., & De Souza, T. (2019). Root morphology and leaf gas exchange in *Peltophorum dubium* (Spreng.) Taub.(Caesalpinioideae) exposed to copper-induced toxicity. *South African Journal of Botany*, 121, 186-192. <https://doi.org/https://doi.org/10.1016/j.sajb.2018.11.007>
- Meyer, A. J., & Hell, R. (2005). Glutathione homeostasis and redox-regulation by sulfhydryl groups. *Photosynthesis research*, 86(3), 435-457. <https://doi.org/https://doi.org/10.1007/s11120-005-8425-1>
- Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, J.-P., & Riondet, C. (2012). Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. *Antioxidants & redox signaling*, 17(8), 1124-1160. <https://doi.org/https://doi.org/10.1089/ars.2011.4327>
- Mhamdi, A., & Van Breusegem, F. (2018). Reactive oxygen species in plant development. *Development*, 145(15). <https://doi.org/10.1242/dev.164376>
- Migocka, M., & Malas, K. (2018). Chapter 4 - Plant Responses to Copper: Molecular and Regulatory Mechanisms of Copper Uptake, Distribution and Accumulation in Plants. In M. A. Hossain, T. Kamiya, D. J. Burritt, L.-S. Phan Tran, & T. Fujiwara (Eds.), *Plant Micronutrient Use Efficiency* (pp. 71-86). Academic Press. <https://doi.org/https://doi.org/10.1016/B978-0-12-812104-7.00005-8>
- Mittova, V., Theodoulou, F. L., Kiddle, G., Gómez, L., Volokita, M., Tal, M., Foyer, C. H., & Guy, M. (2003). Coordinate induction of glutathione biosynthesis and glutathione-metabolizing enzymes is correlated with salt tolerance in tomato. *FEBS Letters*, 554(3), 417-421. [https://doi.org/https://doi.org/10.1016/S0014-5793\(03\)01214-6](https://doi.org/https://doi.org/10.1016/S0014-5793(03)01214-6)
- Miura, K., Shiba, H., Ohta, M., Kang, S. W., Sato, A., Yuasa, T., Iwaya-Inoue, M., Kamada, H., & Ezura, H. (2012). SIICE1 encoding a MYC-type transcription factor controls cold tolerance in tomato, *Solanum lycopersicum*. *Plant Biotechnology*, 12.0303 a. <https://doi.org/https://doi.org/10.5511/plantbiotechnology.12.0303a>
- Møller, I. M., Jensen, P. E., & Hansson, A. (2007). Oxidative Modifications to Cellular Components in Plants. *Annual review of plant biology*, 58(1), 459-481. <https://doi.org/10.1146/annurev.arplant.58.032806.103946>
- Mostofa, M. G., & Fujita, M. (2013). Salicylic acid alleviates copper toxicity in rice (*Oryza sativa* L.) seedlings by up-regulating antioxidative and glyoxalase systems.

- Ecotoxicology*, 22(6), 959-973. <https://doi.org/https://doi.org/10.1007/s10646-013-1073-x>
- Moya, J., Ros, R., & Picazo, I. (1993). Influence of cadmium and nickel on growth, net photosynthesis and carbohydrate distribution in rice plants. *Photosynthesis research*, 36(2), 75-80. <https://doi.org/https://doi.org/10.1007/BF00016271>
- Murphy, A., & Taiz, L. (1995). A New Vertical Mesh Transfer Technique for Metal-Tolerance Studies in Arabidopsis (Ecotypic Variation and Copper-Sensitive Mutants). *Plant Physiology*, 108(1), 29-38. <https://doi.org/10.1104/pp.108.1.29>
- Myouga, F., Hosoda, C., Umezawa, T., Iizumi, H., Kuromori, T., Motohashi, R., Shono, Y., Nagata, N., Ikeuchi, M., & Shinozaki, K. (2008). A Heterocomplex of Iron Superoxide Dismutases Defends Chloroplast Nucleoids against Oxidative Stress and Is Essential for Chloroplast Development in Arabidopsis. *The Plant Cell*, 20(11), 3148-3162. <https://doi.org/10.1105/tpc.108.061341>
- Naila, A., Meerdink, G., Jayasena, V., Sulaiman, A. Z., Ajit, A. B., & Berta, G. (2019). A review on global metal accumulators—mechanism, enhancement, commercial application, and research trend. *Environmental Science and Pollution Research*, 26(26), 26449-26471. <https://doi.org/10.1007/s11356-019-05992-4>
- Navari-Izzo, F., Cestone, B., Cavallini, A., Natali, L., Giordani, T., & Quartacci, M. F. (2006). Copper excess triggers phospholipase D activity in wheat roots. *Phytochemistry*, 67(12), 1232-1242. <https://doi.org/https://doi.org/10.1016/j.phytochem.2006.04.006>
- Nazir, F., Hussain, A., & Fariduddin, Q. (2019). Hydrogen peroxide modulate photosynthesis and antioxidant systems in tomato (*Solanum lycopersicum* L.) plants under copper stress. *Chemosphere*, 230, 544-558. <https://doi.org/https://doi.org/10.1016/j.chemosphere.2019.05.001>
- Ndimele, P. (2010). A review on the phytoremediation of petroleum hydrocarbon. *Pakistan journal of biological sciences: PJBS*, 13(15), 715-722. <https://doi.org/https://doi.org/10.3923/pjbs.2010.715.722>
- Noctor, G., & Foyer, C. H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual review of plant biology*, 49(1), 249-279. <https://doi.org/https://doi.org/10.1146/annurev.arplant.49.1.249>
- Noctor, G., Gomez, L., Vanacker, H., & Foyer, C. H. (2002). Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *Journal of Experimental Botany*, 53(372), 1283-1304. <https://doi.org/https://doi.org/10.1093/jexbot/53.372.1283>
- Nujkić, M. M., Dimitrijević, M. M., Alagić, S. Č., Tošić, S. B., & Petrović, J. V. (2016). Impact of metallurgical activities on the content of trace elements in the spatial soil and plant parts of *Rubus fruticosus* L. *Environmental Science: Processes & Impacts*, 18(3), 350-360. <https://doi.org/10.1039/c5em00646e>
- Olness, A., Nelsen, T., Rinke, J., & Voorhees, W. (2000). Ionic ratios and crop performance. I. Vanadate and phosphate on soybean. *Journal of agronomy and crop science*, 185(3), 145-151. <https://doi.org/https://doi.org/10.1046/j.1439-037x.2000.00429.x>
- Pan, M., & Chu, L. (2016). Phytotoxicity of veterinary antibiotics to seed germination and root elongation of crops. *Ecotoxicology and Environmental Safety*, 126, 228-237. <https://doi.org/https://doi.org/10.1016/j.ecoenv.2015.12.027>
- Pandey, N., Chandra, J., Xalxo, R., & Sahu, K. (2021). Concept and Types of Phytoremediation. In M. Hasanuzzaman (Ed.), *Approaches to the Remediation of Inorganic Pollutants* (pp. 281-302). Springer Singapore. https://doi.org/10.1007/978-981-15-6221-1_14
- Pandhair, V., & Sekhon, B. S. (2006). Reactive Oxygen Species and Antioxidants in Plants: An Overview. *Journal of Plant Biochemistry and Biotechnology*, 15(2), 71-78. <https://doi.org/10.1007/bf03321907>

- Parisy, V., Poinssot, B., Owsianowski, L., Buchala, A., Glazebrook, J., & Mauch, F. (2007). Identification of PAD2 as a γ -glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. *The Plant Journal*, 49(1), 159-172. <https://doi.org/10.1111/j.1365-313X.2006.02938.x>
- Pilon-Smits, E. (2005). PHYTOREMEDIATION. *Annual review of plant biology*, 56(1), 15-39. <https://doi.org/10.1146/annurev.arplant.56.032604.144214>
- Pizarro, I., Gómez-Gómez, M., León, J., Román, D., & Palacios, M. A. (2016). Bioaccessibility and arsenic speciation in carrots, beets and quinoa from a contaminated area of Chile. *Science of the Total Environment*, 565, 557-563. <https://doi.org/10.1016/j.scitotenv.2016.04.199>
- Prasad, M., & Strzałka, K. (1999). Impact of heavy metals on photosynthesis. In *Heavy metal stress in plants* (pp. 117-138). Springer. https://doi.org/10.1007/978-3-662-07745-0_6
- Pryor, W. A., & Squadrito, G. L. (1995). The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 268(5), L699-L722. <https://doi.org/10.1152/ajplung.1995.268.5.L699>
- Quan, X., Shan, L., & Bi, Y. (2007). Cloning of metallothionein genes from *Arachis hypogaea* and characterization of AhMT2a. *Russian Journal of Plant Physiology*, 54(5), 669-675. <https://doi.org/10.1134/S1021443707050159>
- Quinet, M., Angosto, T., Yuste-Lisbona, F. J., Blanchard-Gros, R., Bigot, S., Martinez, J.-P., & Lutts, S. (2019). Tomato Fruit Development and Metabolism [Review]. *Frontiers in plant science*, 10. <https://doi.org/10.3389/fpls.2019.01554>
- Raiola, A., Rigano, M. M., Calafiore, R., Frusciante, L., & Barone, A. (2014). Enhancing the health-promoting effects of tomato fruit for biofortified food. *Mediators of inflammation*, 2014. <https://doi.org/10.1155/2014/139873>
- Ranieri, A., Castagna, A., Pacini, J., Baldan, B., Mensuali Sodi, A., & Soldatini, G. F. (2003). Early production and scavenging of hydrogen peroxide in the apoplast of sunflower plants exposed to ozone. *Journal of Experimental Botany*, 54(392), 2529-2540. <https://doi.org/10.1093/jxb/erg270>
- Rao, M. V., Paliyath, G., & Ormrod, D. P. (1996). Ultraviolet-B- and Ozone-Induced Biochemical Changes in Antioxidant Enzymes of *Arabidopsis thaliana*. *Plant Physiology*, 110(1), 125-136. <https://doi.org/10.1104/pp.110.1.125>
- Rehman, M., Liu, L., Wang, Q., Saleem, M. H., Bashir, S., Ullah, S., & Peng, D. (2019). Copper environmental toxicology, recent advances, and future outlook: a review. *Environmental Science and Pollution Research*, 26(18), 18003-18016. <https://doi.org/10.1007/s11356-019-05073-6>
- Robinson, B., Fernández, J.-E., Madejón, P., Marañón, T., Murillo, J. M., Green, S., & Clothier, B. (2003). Phytoextraction: an assessment of biogeochemical and economic viability. *Plant and soil*, 249(1), 117-125. <https://doi.org/10.1023/A:1022586524971>
- Robinson, B. H., Bañuelos, G., Conesa, H. M., Evangelou, M. W. H., & Schulin, R. (2009). The Phytomanagement of Trace Elements in Soil. *Critical Reviews in Plant Sciences*, 28(4), 240-266. <https://doi.org/10.1080/07352680903035424>
- Robinson, B. H., Brooks, R. R., & Clothier, B. E. (1999). Soil Amendments Affecting Nickel and Cobalt Uptake by *Berkheya coddii*: Potential Use for Phytomining and Phytoremediation. *Annals of Botany*, 84(6), 689-694. <https://doi.org/10.1006/anbo.1999.0970>
- Rucandio, M. I., Petit-Domínguez, M. D., Fidalgo-Hijano, C., & García-Giménez, R. (2011). Biomonitoring of chemical elements in an urban environment using arboreal and bush plant species. *Environmental Science and Pollution Research*, 18(1), 51-63. <https://doi.org/10.1007/s11356-010-0350-y>

- Rucińska-Sobkowiak, R. (2016). Water relations in plants subjected to heavy metal stresses. *Acta Physiologiae Plantarum*, 38(11), 1-13. <https://doi.org/https://doi.org/10.1007/s11738-016-2277-5>
- Sawidis, T., Breuste, J., Mitrovic, M., Pavlovic, P., & Tsigaridas, K. (2011). Trees as bioindicator of heavy metal pollution in three European cities. *Environmental Pollution*, 159(12), 3560-3570. <https://doi.org/https://doi.org/10.1016/j.envpol.2011.08.008>
- Sawyer, D. T., & Gibian, M. J. (1979). The chemistry of superoxide ion. *Tetrahedron*, 35(12), 1471-1481. [https://doi.org/https://doi.org/10.1016/0040-4020\(79\)80032-0](https://doi.org/https://doi.org/10.1016/0040-4020(79)80032-0)
- Saxena, G., Purchase, D., Mulla, S. I., Saratale, G. D., & Bharagava, R. N. (2019). Phytoremediation of Heavy Metal-Contaminated Sites: Eco-environmental Concerns, Field Studies, Sustainability Issues, and Future Prospects. In (pp. 71-131). Springer International Publishing. https://doi.org/10.1007/398_2019_24
- Schweitzer, C., & Schmidt, R. (2003). Physical Mechanisms of Generation and Deactivation of Singlet Oxygen. *Chemical Reviews*, 103(5), 1685-1758. <https://doi.org/10.1021/cr010371d>
- Seneviratne, M., Rajakaruna, N., Rizwan, M., Madawala, H., Ok, Y. S., & Vithanage, M. (2019). Heavy metal-induced oxidative stress on seed germination and seedling development: a critical review. *Environmental Geochemistry and Health*, 41(4), 1813-1831. <https://doi.org/https://doi.org/10.1007/s10653-017-0005-8>
- Shah, F. U. R., Ahmad, N., Masood, K. R., & Peralta-Videa, J. R. (2010). Heavy metal toxicity in plants. In *Plant adaptation and phytoremediation* (pp. 71-97). Springer. https://doi.org/https://doi.org/10.1007/978-90-481-9370-7_4
- Sharma, S. S., Schat, H., & Vooijs, R. (1998). In vitro alleviation of heavy metal-induced enzyme inhibition by proline. *Phytochemistry*, 49(6), 1531-1535. [https://doi.org/https://doi.org/10.1016/S0031-9422\(98\)00282-9](https://doi.org/https://doi.org/10.1016/S0031-9422(98)00282-9)
- Sheoran, V., S. Sheoran, A., & Poonia, P. (2013). Phytomining of gold: A review. *Journal of Geochemical Exploration*, 128, 42-50. <https://doi.org/https://doi.org/10.1016/j.gexplo.2013.01.008>
- Sheoran, V., Sheoran, A. S., & Poonia, P. (2009). Phytomining: A review. *Minerals Engineering*, 22(12), 1007-1019. <https://doi.org/https://doi.org/10.1016/j.mineng.2009.04.001>
- Shi, W., & Chance, M. (2008). Metallomics and metalloproteomics. *Cellular and Molecular Life Sciences*, 65(19), 3040-3048. <https://doi.org/https://doi.org/10.1007/s00018-008-8189-9>
- Sholi, N. J. (2012). Effect of Salt Stress on Seed Germination, Plant Growth, Photosynthesis and Ion Accumulation of four Tomato Cultivars. *American Journal of Plant Physiology*, 7(3), 269-275. <https://doi.org/https://doi.org/10.3923/ajpp.2012.269.275>
- Si-Zhong, Y., Hui-Jun, J., Zhi, W., Rui-Xia, H., Yan-Jun, J., Xiu-Mei, L., & Shao-Peng, Y. (2009). Bioremediation of oil spills in cold environments: a review. *Pedosphere*, 19(3), 371-381. [https://doi.org/https://doi.org/10.1016/S1002-0160\(09\)60128-4](https://doi.org/https://doi.org/10.1016/S1002-0160(09)60128-4)
- Siddique, A., Kandpal, G., & Kumar, P. (2018). Proline accumulation and its defensive role under diverse stress condition in plants: An overview. *Journal of Pure and Applied Microbiology*, 12(3), 1655-1659. <https://doi.org/https://doi.org/10.22207/JPAM.12.3.73>
- Singh, A., Prasad, S. M., Singh, S., & Singh, M. (2016). Phytoremediation potential of weed plants' oxidative biomarker and antioxidant responses. *Chemistry and Ecology*, 32(7), 684-706. <https://doi.org/https://doi.org/10.1080/02757540.2016.1182994>
- Singh, D., Nath, K., & Sharma, Y. K. (2007). Response of wheat seed germination and seedling growth under copper stress. *Journal of Environmental Biology*, 28(2), 409. <https://doi.org/http://www.jeb.co.in/>

- Singh, P., Singh, I., & Shah, K. (2019). Reduced activity of nitrate reductase under heavy metal cadmium stress in rice: An in silico answer. *Frontiers in plant science*, 9, 1948. <https://doi.org/https://doi.org/10.3389/fpls.2018>.
- Singh, S., Zacharias, M., Kalpana, S., & Mishra, S. (2012). Heavy metals accumulation and distribution pattern in different vegetable crops. *Journal of Environmental Chemistry and Ecotoxicology*, 4(10), 170-177. <https://doi.org/https://doi.org/10.5897/JECE11.076>
- Smirnof, N. (1993). The role of active oxygen in the response of plants to water deficit and desiccation. *NewPhy-tologist*, 125, 27-58. <https://doi.org/https://www.jstor.org/stable/2557905>
- Soares, C., Carvalho, M. E., Azevedo, R. A., & Fidalgo, F. (2019). Plants facing oxidative challenges—A little help from the antioxidant networks. *Environmental and Experimental Botany*, 161, 4-25. <https://doi.org/https://doi.org/10.1016/j.envexpbot.2018.12.009>
- Souza, V. L., de Almeida, A.-A. F., de S Souza, J., Mangabeira, P. A., de Jesus, R. M., Pirovani, C. P., Ahnert, D., Baligar, V. C., & Loguercio, L. L. (2014). Altered physiology, cell structure, and gene expression of Theobroma cacao seedlings subjected to Cu toxicity. *Environmental Science and Pollution Research*, 21(2), 1217-1230. <https://doi.org/https://doi.org/10.1007/s11356-013-1983-4>
- Sresty, T., & Rao, K. M. (1999). Ultrastructural alterations in response to zinc and nickel stress in the root cells of pigeonpea. *Environmental and Experimental Botany*, 41(1), 3-13. [https://doi.org/https://doi.org/10.1016/S0098-8472\(98\)00034-3](https://doi.org/https://doi.org/10.1016/S0098-8472(98)00034-3)
- Srivastava, H. (1980). Regulation of nitrate reductase activity in higher plants. *Phytochemistry*, 19(5), 725-733. [https://doi.org/https://doi.org/10.1016/0031-9422\(80\)85100-4](https://doi.org/https://doi.org/10.1016/0031-9422(80)85100-4)
- Swarbreck, S. M., Defoin-Platel, M., Hindle, M., Saqi, M., & Habash, D. Z. (2011). New perspectives on glutamine synthetase in grasses. *Journal of Experimental Botany*, 62(4), 1511-1522. <https://doi.org/https://doi.org/10.1093/jxb/erg356>
- Taiz, L., & Zeiger, E. (2010). Plant physiology 5th Ed. Sunderland, MA: Sinauer Associates, 464. <https://doi.org/http://localhost:8080/xmlui/handle/123456789/1886>
- Talukder, P. (2021). Role of Metallothionein and Phytochelatin in Combating Abiotic Stress Imparted by Copper-Induced Toxicity in Brinjal (Solanum melongena). In (pp. 349-358). Springer Singapore. https://doi.org/10.1007/978-981-15-7409-2_35
- Timofeev, I., Kosheleva, N., & Kasimov, N. (2018). Contamination of soils by potentially toxic elements in the impact zone of tungsten-molybdenum ore mine in the Baikal region: A survey and risk assessment. *Science of the Total Environment*, 642, 63-76. <https://doi.org/https://doi.org/10.1016/j.scitotenv.2018.06.042>
- Tobin, A. K., & Yamaya, T. (2001). Cellular compartmentation of ammonium assimilation in rice and barley. *Journal of Experimental Botany*, 52(356), 591-604. <https://doi.org/https://doi.org/10.1093/jexbot/52.356.591>
- Tosin, R., Pôças, I., Novo, H., Teixeira, J., Fontes, N., Graça, A., & Cunha, M. (2021). Assessing predawn leaf water potential based on hyperspectral data and pigment's concentration of Vitis vinifera L. in the Douro Wine Region. *Scientia Horticulturae*, 278, 109860. <https://doi.org/https://doi.org/10.1016/j.scienta.2020.109860>
- Tózsér, D., Magura, T., & Simon, E. (2017). Heavy metal uptake by plant parts of willow species: a meta-analysis. *Journal of Hazardous Materials*, 336, 101-109. <https://doi.org/https://doi.org/10.1016/j.jhazmat.2017.03.068>
- Turano, F. J., Dashner, R., Upadhyaya, A., & Caldwell, C. R. (1996). Purification of mitochondrial glutamate dehydrogenase from dark-grown soybean seedlings. *Plant Physiology*, 112(3), 1357-1364. <https://doi.org/https://doi.org/10.1104/pp.112.3.1357>

- Van Assche, F., & Clijsters, H. (1990). Effects of metals on enzyme activity in plants. *Plant, Cell & Environment*, 13(3), 195-206. <https://doi.org/https://doi.org/10.1111/j.1365-3040.1990.tb01304.x>
- Veal, E. A., Day, A. M., & Morgan, B. A. (2007). Hydrogen peroxide sensing and signaling. *Molecular cell*, 26(1), 1-14. <https://doi.org/https://doi.org/10.1016/j.molcel.2007.03.016>
- Verbruggen, N., Hermans, C., & Schat, H. (2009). Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, 181(4), 759-776. <https://doi.org/https://doi.org/10.1111/j.1469-8137.2008.02748.x>
- Vernoux, T., Wilson, R. C., Seeley, K. A., Reichheld, J.-P., Muroy, S., Brown, S., Maughan, S. C., Cobbett, C. S., Van Montagu, M., & Inzé, D. (2000). The ROOT MERISTEMLESS1/CADMIUM SENSITIVE2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. *The Plant Cell*, 12(1), 97-109. <https://doi.org/https://doi.org/10.1105/tpc.12.1.97>
- Vinit-Dunand, F., Epron, D., Alaoui-Sossé, B., & Badot, P.-M. (2002). Effects of copper on growth and on photosynthesis of mature and expanding leaves in cucumber plants. *Plant Science*, 163(1), 53-58.
- Viuda-Martos, M., Sanchez-Zapata, E., Sayas-Barberá, E., Sendra, E., Pérez-Álvarez, J., & Fernández-López, J. (2014). Tomato and tomato byproducts. Human health benefits of lycopene and its application to meat products: a review. *Critical reviews in food science and nutrition*, 54(8), 1032-1049. <https://doi.org/https://doi.org/10.1080/10408398.2011.623799>
- Walls, W. (2010). Petroleum refining industry in China. *Energy Policy*, 38(5), 2110-2115. <https://doi.org/https://doi.org/10.1016/j.enpol.2009.06.002>
- Wallsgrave, R. M., Turner, J. C., Hall, N. P., Kendall, A. C., & Bright, S. W. (1987). Barley mutants lacking chloroplast glutamine synthetase—biochemical and genetic analysis. *Plant Physiology*, 83(1), 155-158. <https://doi.org/https://doi.org/10.1104/pp.83.1.155>
- Wang, J., Wang, A., Luo, Q., Hu, Z., Ma, Q., Li, Y., Lin, T., Liang, X., Yu, J., Foyer, C. H., & Shi, K. (2022). Glucose sensing by regulator of G protein signaling 1 (RGS1) plays a crucial role in coordinating defense in response to environmental variation in tomato. *New Phytologist*, 236(2), 561-575. <https://doi.org/https://doi.org/10.1111/nph.18356>
- Wang, R., Shafi, M., Ma, J., Zhong, B., Guo, J., Hu, X., Xu, W., Yang, Y., Ruan, Z., & Wang, Y. (2018). Effect of amendments on contaminated soil of multiple heavy metals and accumulation of heavy metals in plants. *Environmental Science and Pollution Research*, 25(28), 28695-28704. <https://doi.org/https://doi.org/10.1007/s11356-018-2918-x>
- Wang, S., Assmann, S. M., & Fedoroff, N. V. (2008). Characterization of the Arabidopsis Heterotrimeric G Protein*. *Journal of Biological Chemistry*, 283(20), 13913-13922. <https://doi.org/https://doi.org/10.1074/jbc.M801376200>
- Wang, X., Bai, J., Wei, T., Feng, G., Zhao, H., Wei, W., Wang, M., & Zhao, Y. (2018). Oilseed rape cultivation increases the microbial richness and diversity in soils contaminated with cadmium. *Journal of Soils and Sediments*, 18(7), 2451-2462. <https://doi.org/https://doi.org/10.1007/s11368-018-1938-y>
- Waters, D. L., Holton, T. A., Ablett, E. M., Lee, L. S., & Henry, R. J. (2005). cDNA microarray analysis of developing grape (*Vitis vinifera* cv. Shiraz) berry skin. *Functional & integrative genomics*, 5(1), 40-58. <https://doi.org/https://doi.org/10.1007/s10142-004-0124-z>
- Wójcik, M., & Tukiendorf, A. (2003). Response of wild type of *Arabidopsis thaliana* to copper stress. *Biologia Plantarum*, 46(1), 79-84. <https://doi.org/https://doi.org/10.1023/A:1022314201466>

- Xiong, Z.-T., Liu, C., & Geng, B. (2006). Phytotoxic effects of copper on nitrogen metabolism and plant growth in *Brassica pekinensis* Rupr. *Ecotoxicology and Environmental Safety*, 64(3), 273-280. <https://doi.org/https://doi.org/10.1016/j.ecoenv.2006.02.003>
- Yang, J., Chen, Z., Wu, S., Cui, Y., Zhang, L., Dong, H., Yang, C., & Li, C. (2015). Overexpression of the *Tamarix hispida* ThMT3 gene increases copper tolerance and adventitious root induction in *Salix matsudana* Koidz. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 121(2), 469-479. <https://doi.org/https://doi.org/10.1007/s11240-015-0717-3>
- Yoshiyama, K. O., Sakaguchi, K., & Kimura, S. (2013). DNA Damage Response in Plants: Conserved and Variable Response Compared to Animals. *Biology*, 2(4), 1338-1356. <https://www.mdpi.com/2079-7737/2/4/1338>
- Yruela, I., Pueyo, J. J., Alonso, P. J., & Picorel, R. (1996). Photoinhibition of Photosystem II from Higher Plants. *Journal of Biological Chemistry*, 271(44), 27408-27415. <https://doi.org/10.1074/jbc.271.44.27408>
- Yuan, J., Chen, D., Ren, Y., Zhang, X., & Zhao, J. (2008). Characteristic and expression analysis of a metallothionein gene, OsMT2b, down-regulated by cytokinin suggests functions in root development and seed embryo germination of rice. *Plant Physiology*, 146(4), 1637-1650. <https://doi.org/https://doi.org/10.1104/pp.107.110304>
- Zagorchev, L., Seal, C. E., Kranner, I., & Odjakova, M. (2013). A central role for thiols in plant tolerance to abiotic stress. *International journal of molecular sciences*, 14(4), 7405-7432.
- Zhang, H., Lian, C., & Shen, Z. (2009). Proteomic identification of small, copper-responsive proteins in germinating embryos of *Oryza sativa*. *Annals of Botany*, 103(6), 923-930. <https://doi.org/https://doi.org/10.1093/aob/mcp012>
- Zhang, H., Zhang, F., Xia, Y., Wang, G., & Shen, Z. (2010). Excess copper induces production of hydrogen peroxide in the leaf of *Elsholtzia haichowensis* through apoplastic and symplastic CuZn-superoxide dismutase. *Journal of Hazardous Materials*, 178(1-3), 834-843. <https://doi.org/https://doi.org/10.1016/j.jhazmat.2010.02.014>
- Zhang, L.-l., He, X.-j., Chen, M., An, R.-d., An, X., & Li, J. (2014). Responses of nitrogen metabolism to copper stress in *Luffa cylindrica* roots. *Journal of Soil Science and Plant Nutrition*, 14(3), 616-624. <https://doi.org/http://dx.doi.org/10.4067/S0718-95162014005000049>