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Heavy metals’ effect of (copper and zinc) on the parameters: chemical, morphological, biometrical and physiological of tomato (*Lycopersicon esculentum* Mill.)

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Abstract

In agriculture, both heavy metals, copper and zinc, are used as essential elements for plants growth and development. However, the excessive amount of these elements can lead to adverse effects.

In this regard, to assess the heavy metal stress responses which were occurred in leaves and roots of *Lycopersicon esculentum* Mill. the seedlings were subjected to different concentrations of copper and zinc: 0, 100, 200, 300, 400, and 500 ppm. Notably, the phytoxicity and mobility of the heavy metals depend mainly on their chemical forms in the soil. Thus, in the present study, the metal fractionation and chemical speciation were determined by using the Tessier sequential extraction method.

At high level,copper and zinc penetrate into tomato plant tissues leading to major damages on the fundamental and physiological processes (photosynthesis, proteins biosynthesis, transpiration, and cellular water status), therefore; plant reacts rapidly and positively by operating all the mechanisms which can help it to cope against this metal stress, such as the implement of the metabolic processes (antioxidant system and osmotic adjustment). All these internal alterations result in onset of phytotoxicity symptoms, accompanied with great morphological changes such as limiting the plant growth and cell division. Furthermore, our results indicate that the proteins, osmolytes (soluble sugars and proline), antioxidant activity (DPPH), and antioxidant compounds (polyphenols and flavonoids) were highly accumulated in leaves than roots, this might be due to the high level of copper and zinc in the both tissues. The metals content in leaves and roots increased gradually by increasing of their concentration in the soil. Remarkably, zinc was highly accumulated in roots, whereas, a great content of copper was translocated to the leaves. Besides, the distribution of heavy metals in soil was predominantly detected in the organic fraction. However, a little amount was recorded in the exchangeable fraction. A positive correlation was marked between the osmoregulators and antioxidants, as well as between the metal fractions in the soil and those in the plant.

In general, the tomato plant which is growing in the soil contaminated by heavy metals could be stressed depending on the level of these elements in the soil, their bioavailability, and the target plant tissue. This crop might be also suggested for the phytoremediation process due to its accumulative potential, but; this present a risk as tomato is a vegetable crop with edible fruit.

Keywords**:** Copper, chemical speciation, heavy metal stress, *Lycopersicon esculentum* Mill., zinc.

Résumé

En agriculture, les deux métaux lourds, cuivre et zinc, sont utilisés comme éléments essentiels à la croissance et au développement des plantes. Cependant, une quantité excessive de ces éléments peut entraîner des effets indésirables.

A cet effet, pour évaluer les réponses au stress des métaux lourds qui sont produites dans les feuilles et les racines de *Lycopersicon esculentum* Mill., les plantules ont été soumises à des différentes concentrations de cuivre et du zinc : 0, 100, 200, 300, 400 et 500 ppm. Notamment, la phytoxicité et la mobilité des métaux lourds dépendent principalement de leurs formes chimiques dans le sol. Pour celà, dans la présente étude, le fractionnement des métaux et la spéciation chimique ont été déterminés en utilisant la méthode d'extraction séquentielle de Tessier.

A un niveau élevé, le cuivre et le zinc pénètrent dans les tissus de la plante de tomate entraînant des dommages importants sur les processus fondamentaux et physiologiques (photosynthèse, biosynthèse des protéines, transpiration et l’état hydrique cellulaire); la plante réagit rapidement et positivement en actionnant tous les mécanismes qui peuvent l'aider à faire face à ce stress métallique, comme la mise en œuvre des processus métaboliques (système antioxydant et ajustement osmotique). Toutes ces altérations internes entraînent l'apparition des symptômes de phytotoxicité, accompagnés de grands changements morphologiques tels que la limitation de la croissance des plantes et la division cellulaire. De plus, nos résultats indiquent que les protéines, les osmolytes (sucres solubles et proline), l'activité antioxydante (DPPH) et les composés antioxydants (polyphénols et flavonoïdes) ont été fortement accumulés dans les feuilles que dans les racines, cela pourrait être dû au niveau élevé de cuivre et du zinc dans les deux tissus. La teneur en métaux dans les feuilles et les racines a été augmenté progressivement en augmentant leur concentration dans le sol. Remarquablement, le zinc est fortement accumulé dans les racines, alors qu'une grande quantité de cuivre est transférée aux feuilles. En outre, la distribution des métaux lourds dans le sol a été principalement détectée dans la fraction organique. Cependant, une faible quantité a été enregistré dans la fraction échangeable. Une corrélation positive est marquée entre les osmorégulateurs et les antioxydants, ainsi qu’entre les fractions métalliques du sol et celles de la plante.

En général, la plante de tomate cultivée dans un sol contaminé par des métaux lourds peut etre stressée en fonction du niveau de ces éléments dans le sol, leur biodisponibilité, et du tissu végétal cible. Cette culture pourrait également être suggérée pour le processus de phytoremédiation en raison de son potentiel d'accumulation, mais ; cela présente un risque car la tomate est une culture maraichère à fruits comestibles.

Mots clés : Cuivre, spéciation chimique, stress des metaux, *Lycopersicon esculentum* Mill., lourds, zinc.

الملخص

في الزراعة، ستخدم المعدنان الثقيلان ، النحاس والزنك ، كعناصر أساسية لنمو النباتات وتطورها. ومع ذلك، فإن الكمية الزائدة من هذه العناصر يمكن أن تسبب تأثيرات غير مرغوب فيها.

في هذا الصدد، لتقييم استجابات الإجهاد للمعادن الثقيلة التي يتم إنتاجها في أوراق وجذور *Lycopersicon esculentum* Mill. ، تعرضت الشتلات لتركيزات مختلفة من النحاس والزنك : 0 ، 100 ، 200 ، 300 ، 400 و500 جزء في المليون. على وجه الخصوص، تعتمد السمية النباتية وحركة المعادن الثقيلة بشكل أساسي على أشكالها الكيميائية في التربة. لذلك، في هذه الدراسة، تم تحديد تجزئة المعادن والانتواع الكيميائي باستخدام طريقة الاستخراج المتسلسل لـTessier.

على مستوى عالٍ، يخترق النحاس والزنك أنسجة نبات الطماطم مما يتسبب في أضرار جسيمة للعمليات الأساسية والفسيولوجية (التركيب الضوئي ، التصنيع الحيوي للبروتينات ، النتح ، وحالة الماء الخلوي) ، لهذا الغرض ؛ يتفاعل النبات بشكل سريع وإيجابي من خلال تشغيل جميع الآليات التي يمكن أن تساعده على مواجهة هذا الإجهاد المعدني، مثل تنفيذ عمليات التمثيل الغذائي (نظام مضادات الأكسدة والتعديل الاسموزي). تؤدي كل هذه التعديلات الداخلية إلى ظهور أعراض السمية النباتية مصحوبة بتغيرات مورفولوجية كبيرة مثل الحد من نمو النبات وانقسام الخلايا. بالإضافة إلى ذلك، تشير نتائجنا إلى أن البروتينات ، الأسموليات (السكريات القابلة للذوبان و البرولين) ، النشاط المضاد للأكسدة (DPPH) ، والمركبات المضادة للأكسدة (البوليفينول و الفلافونويد) تراكمت بقوة في الأوراق أكثر من الجذور، وهذا يمكن أن يكون بسبب المستوى العالي من النحاس والزنك في كلا النسيجين. زاد المحتوى المعدني في الأوراق والجذور تدريجياً بزيادة تركيزها في التربة. اللافت للنظر أن الزنك تراكم بكثافة في الجذور، بينما تم نقل كمية كبيرة من النحاس إلى الأوراق. بالإضافة إلى ذلك، تم الكشف عن توزيع المعادن الثقيلة في التربة بشكل رئيسي في الجزء العضوي. ومع ذلك، تم تسجيل كمية صغيرة في الجزء القابل للاستبدال. تم تحديد ارتباط إيجابي بين المنظمات الاسموزية ومضادات الأكسدة، وكذلك بين الأجزاء المعدنية في التربة وتلك الموجودة في النبات.

بشكل عام، يمكن اجهاد نبات الطماطم الذي ينمو في التربة الملوثة بالمعادن الثقيلة، اعتمادًا على مستوى هذه العناصر في التربة، توافرها البيولوجي والأنسجة النباتية المستهدفة. يمكن أيضًا اقتراح النبات لعملية المعالجة النباتية نظرًا لقدرته التراكمية، ولكن؛ هذا يمثل خطرا لأن الطماطم محصول نباتي ذو ثمار صالحة للأكل.

الكلمات المفتاحية : النحاس ، الانتواع الكيميائي ، الإجهاد بالمعادن الثقيلة ، *Lycopersicon esculentum* Mill. ، الزنك .

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List of Abbreviations

- **ABA** : Abscisic acid
- **Abs** : Absorbance
- **aq** : Aqueous
- **ATP** : Adenosine triphosphate
- **car** : Carotenoids
- **CAT** : Catalase
- **CEC** : Cation-exchange capacity
- **chl a** : Chlorophyll a
- **chl b** : Chlorophyll b
- **CO_2** : Carbon dioxide
- **DW** : Dry weight
- **EC** : Electrical conductivity
- **Eh** : Redox potential
- **FW** : Fresh weight
- **OM** : Organic matter
- **pH** : Potential of hydrogen
- **ROS** : Reactive Oxygen Species
- **SOD** : Superoxide dismutase
- **t chl** : Total chlorophyll

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- **DPPH** : 2,2-diphényl-1-picrylhydrazyle
- **EDTA** : Ethylenediaminetetraacetic acid (EDTA)
- **H_2O_2** : Hydrogen peroxide
- **HCl** : Hydrochloric acid/ Hydrogen chloride
- **HNO_3** : Nitric acid
- **NaCl** : Sodium chloride
- **NH_4^+** : Ammonium ion
- **NH_4OAc** : Ammonium acetate
- **NO_3^-** : Nitrate ion

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- **Ag** : Silver
- **Al** : Aluminium
- **As** : Arsenic
- **B** : Boron
- **C** : Carbon
- **Ca** : Calcium
- **$CaCO_3$** : Calcium carbonate
- **Cd** : Cadmium
- **Cl^-** : Chloride ion
- **CO** : Carbon monoxide
- **Cr** : Chromium
- **$Cu\ SO_4$** : Copper (II) sulfate
- **Fe** : Iron
- **H** : Hydrogen
- **Hg** : Mercury

- **HMs** : Heavy metals
- **K** : Potassium
- **Mg** : Magnesium
- **Mn** : Manganese
- **N** : Nitrogen
- **Ni** : Nickel
- **NPS** : Nanoparticles
- **P** : Phosphorus
- **Pb** : Lead
- **Si** : Silicon
- **Zn SO_4** : Zinc sulfate
- **ZnO ; CuO** : Zinc oxide ; copper oxide

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- **%** : Percentage
- **C°** : degree Celsius
- **cm^2** : Square centimetre
- **dBSPL ultrasonic** : decibel Sound pressure level
- **$dS.m^{-1}$** : deciSiemens per metre
- **g** : gramme
- **GAE** : Gallic Acid Equivalent
- **h** : hour
- **ha** : Hectare
- **Kg** : Kilogram
- **M** : Molarity
- **meq** : milliequivalent
- **$mg.\ g^{-1}$** : milligram per gram
- **$mg.\ kg^{-1}$** : milligram per kilogram
- **mL** : Milliliter
- **mn** : minute
- **N** : Normality
- **nm** : Nanometer
- **ppm** : Parts per million
- **QE** : Quercetin Equivalent
- **sec** : Second
- **U. mg** : Enzyme activity unit (symbol U)
- **UV** : Ultraviolet
- **V/V** : Volum per Volum
- **µm** : Micrometer

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- **Cys** : Cysteine
- **DNA** : Deoxyribonucleic acid
- **GSH** : Glutathione
- **HSPs** : Heat shock proteins
- **MFA** : Material flows analysis
- **miRNA** : Micro-ARN
- **MT** : Metallothionines;
- **PC** : Phytochelatins
- **PGPR** : Plant growth-promoting rhizobacterial
- **PM health** : Particulate matter (PM) health
- **RNA** : Ribonucleic acid

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" E	**:**	East
" N	**:**	North

FAO: *Food and Agriculture Organization of the United Nations.*

SDGs: *Sustainable Development Goals.*

UNEP: *United Nations Environment Programme*

UNICEF: *United Nations Children's Fund.*

WCA*: World Programme for the Census of Agriculture.*

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Mill: Is the Standard Botanical Abbreviation of Philip Miller.

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GENERAL INTRODUCTION

Plants, as sessile organisms, are often exposed to a broad range of biotic and abiotic stresses during their growth and development, these stresses, either individually or concurrently, have drastic effects on the life cycle of plants **(Ramesh *et al*., 2020 ; Tajima *et al*., 2020 ; Kumar *et al*., 2020)**, moreover, heavy metal stress represents one of the most damaging abiotic stresses **(Jalmi *et al.*, 2018),** so that, large amounts of these elements end up in the environment as an outcome of ever-increasing anthropogenic activities and economic development **(Jiang *et al*., 2019)**.

Anthropogenic activities such as Agriculture, Industry, and Mining, have contributed significantly to the accumulation of heavy metals in the soil **(Correia *et al*., 2018),** this heavy metal pollution of agricultural soils is one of the main concerns which causing different ecological problems, so, the excess accumulation of these metals in soil has deteriorated soil, decreased the growth and yield of the plant, and entered into the food chain **(Etesami, 2018),** as result to that, heavy metals contaminated plants cause losses in crop production and risks for human health **(Gratão *et al*., 2019),** they also cause toxicity by targeting crucial molecules and vital processes in the plant cell **(Jalmi *et al*., 2018).**

Copper (Cu), and zinc (Zn), due to their relatively low levels in the cell compared to abundant metal ions like potassium and magnesium, transition metals are always considered micronutrients and referred to as trace elements **(Li *et al*., 2018a),** these elements have direct roles in plant metabolism **(Pandey, 2018)**, they are also essential for a variety of functions in the living cell **(Lüthje *et al*., 2018)**, that’s why, soils are mainly contaminated by these elements **(Salducci *et al*., 2019)**, because, copper and zinc cause an increase in phytotoxic effect at higher concentrations **(Chrysargyris *et al*., 2019 ; Wolf *et al*., 2017)**, and, the increasing of toxicity of heavy metal ion in soil has been threatening the food security and environments **(Ruan *et al*., 2019).**

Tomato (*Lycopersicon esculentum* Mill.) is a crop of the Solanaceae family, it was selected as the target plant, because, it is the second-largest vegetable crop grown worldwide **(Karniel *et al*., 2020).** However, its productivity is impaired by a wide range of abiotic stresses such as the heavy metals **(Gerszberg and Hnatuszko-Konka, 2017)** and biotic stress **(Bouzroud *et al*., 2018 ; Cumplido-Najera *et al*., 2019 ; Quiterio-Guitierrez *et al*., 2019).**

Tomato is one of the most valuable crops in several countries such as Algeria, specifically in the Mostaganem region, which is grown in fields or greenhouses, and has been bred and genetically improved for centuries. However, Tomato remains vulnerable to diseases, consequently requiring the use of chemical pesticides, mainly in greenhouses **(Ines and Bernacchia, 2018)**, considering that, the crop-growing season is short, so that, the overuse of agrochemicals to protect the plants from insect and pests has greatly increased **(Thwe *et al.*, 2020)**, which contaminate the soil and affect the quality of tomato crop, due to plant's protection products contain copper and zinc as constituents in their composition.

In horticulture, copper sulfate is widely used as fungicide treatment, however, the copper tends to accumulate in soil with a concomitant effect on soil quality and microbial diversity **(Carolina *et al*., 2019).** Therefore, plants must adapt themselves to the prevailing conditions for their survival, resulting in the acquisition of a wide range of metal tolerance mechanisms **(Gratão *et al*., 2019),** because, heavy metals have restricted the plant regular life cycles affecting the plant primer and secondary metabolites by biochemical and physiological pathways **(Kisa *et al*., 2019b).**

Recent decades had witnessed renewed interest to study abiotic factors that influence secondary metabolism during in vitro and in vivo growth of plants **(Isah, 2019),** so that, plants have sophisticated mechanisms to recognize and respond to these stresses **(Kumar *et al*., 2020).** Previous studies had proved that heavy metal stress may affect the plant morphology, water content, and cause an alteration in photosynthetic apparatus, it can also induce alteration in proteins profile, that's why, proteins are involved in heavy metal tolerance and accumulation **(Huang *et al*., 2020b ; Khatiwada *et al*., 2020),** accordingly, several mechanisms are acting together to avoid negative effects of environmental stresses. Among them, accumulation of compatible osmolytes; such as proline and soluble sugar, of antioxidant compounds; such as polyphenols and flavonoids, are an important phenomenon under heavy metal stress **(Gratão *et al*., 2019 ; Huang *et al*., 2020b ; Naikoo *et al*., 2019 ; Zaouali *et al*., 2020).**

Generally, the phytotoxic effect caused by heavy metals can be attributed to their bioavailability and chemical speciation in the soil.

This topic will be treated under three headings:

- Evaluation of toxic effect of copper and zinc on morphological, biometrical, physiological, and biochemical parameters of tomato plant" *Lycopersicon esculentum* Mill."
- Estimation of a total of heavy metal concentrations, which are accumulated in leaves and roots of *Lycopersicon esculentum* Mill.".
- Determination of chemical speciation of copper and zinc in contaminated soil and asssessment of metal mobility.

- From the results, does the plant species selected as model in the present investigation, could be used in phytoremediation strategy.

This research is organized as follows:

- *The first section gives a brief overview of the most recent reports conducted on this topic;*
- *The second section outlines the method, procedure, and implementation of the proposed design;*
- *Experimental results will be analyzed and presented in section relevant to chapter VII;*
- *In section of chapter VIII, results obtained in this study* will *be discussed;*
- *Finally, conclusion, concludes the paper.*

Literature Review Section

CHAPTER I

Challenges Facing Agriculture and the Environment in the Future

CHAPTER I: Challenges facing agriculture and the environment in the future

I.1. Sustainable Development Goals 2030

A healthy environment plays a key role in meeting many of the 17 Sustainable Development Goals. With a little over 10 years left to meet the target date of 2030, the world will need to pick up the pace and put greater efforts into finding the adequated solutions for pollution, climate change, and biodiversity loss to truly transform societies and economies **(UNEP, 2020).**

The 2030 agenda for sustainable development, including the 17 Sustainable Development Goals (SDGs), are new global objectives that succeeded the Millennium Development Goals on 1 January 2016. The SDGs will shape national development plans over the next 15 years. From ending poverty and hunger to responding to climate change and sustaining our natural resources, food, and agriculture lies at the heart of the 2030 Agenda **(FAO, 2020).**

The work of the environment statistics team of the Food and Agriculture Organization of the United Nations (FAO) focuses on providing data to support evidence-based decision making for sustainable agriculture concerning natural resources. The activities support FAO’s strategic objective of making agriculture, forestry, and fisheries more productive and sustainable. Within FAO’s work on the fight against hunger and malnutrition and towards the Sustainable Development Goals (SDGs), the Environment Statistics work focuses in particular on promoting sustainable agriculture and protecting, restoring, and promoting sustainable use of terrestrial ecosystems **(FAO Statistics, 2020a).**

Since the 1940s, the agriculture census team continues to improve and update the decennial World Programme for the Census of Agriculture (WCA) to support countries with their national agricultural census, while applying the latest international standards and methodologies. The “Programme, concepts and definitions” of WCA 2020 is the tenth and latest program, covering national agricultural censuses within the 2016-2025 decade. In 2018, the agriculture census team complemented the WCA 2020 with an “Operational Guidelines” volume **(FAO Statistics, 2020b).**

I.2. Agriculture and abiotic stress 2050

The world population is continuously increasing, with anticipation to reach 9.7 billion by the end of 2050. Otherwise, agricultural productivity facing water shortage, depleting soil fertility, and various abiotic stresses **(Ghoniem *et al*., 2020).**

CHAPTER II

Heavy Metals

II.1. Introduction

Today, synthetic products such as industrial waste, pesticides, and industrial or domestic sludge are widely applied, as well as and manufacturing can adversely result in heavy metal contamination of urban and agricultural soils. Simultaneously, by the growth of industrialization and urbanization, the environmental safety of soil has become of great concern **(Qayyum *et al.,* 2020).** The amount of heavy metal is expected to be more in the future **(Muthusaravanan *et al*., 2020).** As, Ni, Hg, Cd, Cr, Pb, Cu, Al, and Zn are the most common toxic metals in soils and water bodies **(Haq *et al*., 2020 ; Javaid, 2020).** In agricultural areas, the accumulation of heavy metals is of primary concern because of their adverse effects **(Javaid, 2020).** Metals are essential to all organisms; accordingly, cells employ numerous genes to maintain metal homeostasis as high levels can be toxic **(Kang *et al*., 2018).** Due to the property of high solubility in aqueous solutions, heavy metals can quickly be absorbed by all living organisms. Once they enter the food chain, it is challenging to detoxify them. Metals are a part of the biological systems, but up to a certain permissible limit, beyond that limit, it becomes hazardous **(Dhingra *et al*., 2020).** Heavy metals are environmental pollutants that affect the integrity and distribution of living organisms in the ecosystem and also humans across the food chain **(Ghoniem *et al*., 2020).**

II.2. Metals as toxic pollutants in the environment

Heavy metals are naturally present in nature, but if their concentration is higher than the normally accepted threshold levels, they constitute one of the pollutants that are more difficult to remove and also to rehabilitate the contaminated site. Thus heavy-metal pollution is more and more becoming one of the principal issues of global interest because it is common to both industrialized countries and developing countries. These issues are getting hard to be recognized and cannot be followed the simple rules concerning safety and environmental protection, thus fall into the same errors of the already industrialized countries **(Abarca *et al.*, 2020).**

II.3. Ecotoxicity

Environmental contamination due to heavy metals (HMs) is of serious ecotoxicological concern because heavy metal pollution is the worst form of pollution **(Haq *et al*., 2020).** Due to its non-biodegradable and persistent nature, HMs cause serious soil/water pollution and severe health hazards in living beings upon exposure **(Bansod *et al*., 2017 ; Saxena *et al*.,**

2019). Their removal from our natural environment is crucial for the rehabilitation of contaminated sites **(Saxena *et al*., 2019).**

II.4. Heavy metal and health

Heavy metals are causing serious health hazards to human beings and abiotic components of the ecosystem **(Mahmood-ul-Hassan *et al*., 2020).** HMs can be genotoxic, carcinogenic, mutagenic, and teratogenic in nature even at low concentrations. They may also act as endocrine disruptors and induce developmental as well as neurological disorders. Some of the heavy metals including Lead (Pb), Mercury (Hg), Arsenic (As), Chromium (Cr), and Cadmium (Cd) are considered to be highly toxic and hazardous to human health even at trace levels very risky to our environment **(Bansod *et al*., 2017 ; Haq *et al*., 2020).**

II.5. Impacts of metal resources on climate change

The extraction and processing of metals from mined ores have an associated environmental cost. In 2011, metals were responsible for 18 percent of resource-related climate change and 39 percent of PM health impacts. Considering the period 2000-2015, climate change and PM health impacts of metals more or less doubled. The mining and processing of copper and precious metals cause high toxicity impacts compared to their production amounts (Figure 1) **(Oberle *et al*., 2019).**

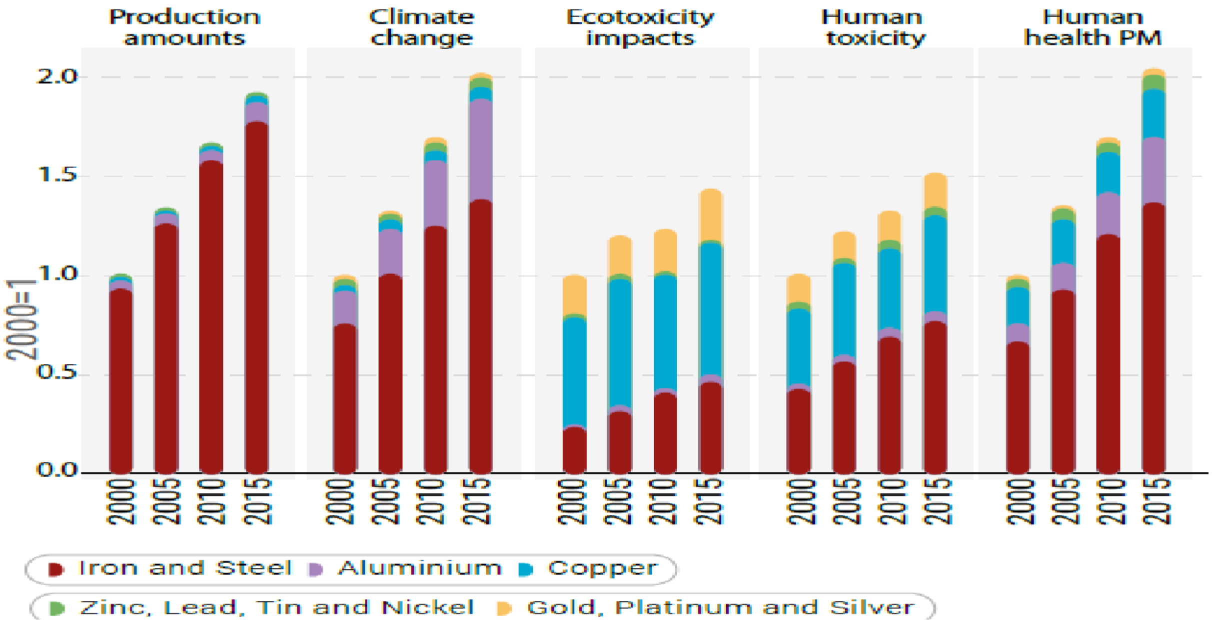


Figure 1: Metal production amounts and environmental impacts of metal mining and processing from 2000 to 2015 (selection of 10 metals covering > 95 percent of global domestic extraction of metal ores in 2015, MFA database) **(Oberle, 2019).**

II.6. How minerals and metals companies can help achieve the 2030 Agenda for Sustainable Development

Minerals and metals underpin national economies; the need for governance reform in the extractive sector is underscored and driven by the adoption in 2015 of the 2030 Agenda for Sustainable Development, including the 17 Sustainable Development Goals (SDGs) and 169 associated targets. Despite enormous efforts to decouple economies from resource use, the extraction of mineral resources has increased markedly in recent decades and, over the last decade, at a faster rate than economic growth. This trend is forecasted to steadily increase in the future, as we struggle to meet the mineral resource needs of a growing global population that is expected to reach 8.5 billion by 2030, 9.8 billion by 2050, and 11.2 billion by the end of the century **(UNEP, 2020).**

II.7. Heavy metal in soil and remediation process

II.7.1 Remediation technologies for heavy metals contaminated soil

Numerous technologies have been used to eliminate heavy metals from our environment, but these techniques are not cost-effective, take a long time, and have mechanical complexity **(Haq *et al.*, 2020).** The remediation of heavy metal-contaminated sites must be viewed seriously as they affect animal and human health **(Muthusaravanan *et al.*, 2020)**. To this aim, physical remediation, chemical remediation, and biological remediation were all paid attention. It is proposed that for effective and economic remediation of soil, a better understanding of remediation procedures and the various options available at the different stages of remediation is highly necessary **(Qayyum *et al.*, 2020)**. At the same time, new environment remediation techniques are developed in the last decade, especially, in these last years. Some of these technologies concern physical or chemical process or effects, such as ion exchanges, flotations, and photocatalysis, while other technologies concern the use of membrane process, especially ultrafiltration or membrane integrated process or hybrid systems, where membranes are generally submerged and used together with another process **(Abarca *et al.*, 2020)**. The techniques for remediation of contaminated soil and groundwater is discussed under ex-situ remediation techniques (dig and dump technique, pump-and-treat technique, incineration technique, oxidation technique, adsorption, ion exchange, pyrolysis remediation technique, physical separation technique, dehalogenation technique, bioremediation technique, solidification remediation technique, constructed wetlands), and in situ remediation techniques (biological treatments, physical or chemical treatments, thermal treatments) **(Hamadani *et al.*,**

2020). When considering the process of HM phytoremediation in soils, people often neglect the sex-specific difference of dioecious plants, especially in soils with multiple HM stresses **(Peng *et al*., 2020),** We can also summarise the following remediation processes:

- Remediation by microbial immobilization with carbon microspheres **(Kazemalilou *et al*., 2020 ; Sun *et al*., 2020b).**
- Remediation by amendments **(Javaid, 2020 ; Tang *et al*., 2019b).**
- Electrokinetic remediation **(Yang, 2020).**
- Heavy metal remediation by natural adsorbents **(Dhingra *et al*., 2020).**
- Phytoremediation **(Cristaldi *et al*., 2020).**

II.7.2 Phytoremediation

Historically, the term phytoremediation was coined in 1991; it describes a technology that uses green plants that can remove, uptake, or render harmless various environmental contaminants such as heavy metals, organic compounds, and radioactive compounds present in soil or water, it was found to be effective, economically viable and safe **(Cristaldi *et al*., 2020 ; Kazemalilou *et al*., 2020 ; Muthusaravanan *et al*., 2020)**. It is the only alternative solution for the remediation of toxic contaminants because its remediation efficiency is high, needs low capital cost, and is clean and environment friendly **(Haq *et al*., 2020 ; Saxena *et al*., 2019)**. The selection of plant species is the most significant aspect for successful phytoremediation **(Ali *et al*., 2020).**

a. Phytoremediation species of heavy metals

Several plants can bioaccumulate and thus are used for the phytoremediation of toxic metals **(Haq *et al*., 2020)**. Plants more efficient in phytoremediation processes are the so-called “hyperaccumulators”. Hyperaccumulator species (e.g. *Thlaspi caerulescens*, *Alyssum bertolonii*, *Arabidopsis halleri*) can accumulate contaminants, but they produce little biomass. It is possible to use species that accumulate fewer contaminants, but which produce more biomass, such as *Brassica* spp., *Arundo donax*, *Typha* spp. and several woody species **(Cristaldi *et al*., 2020).**

The phytoremediation potential of Castor, Sardo, Siciliano, and Spagnolo was evaluated for copper and other heavy metals **(Capozzi *et al*., 2020 ; Palanivel *et al*., 2020).** Coal gangue and *Vetiveria zizanioides* for phytostabilization of copper **(Chu *et al*., 2020 ; Radziemska *et al*., 2020).** It has been proved that castor (*Ricinus communis* L.) is not an hyperaccumulator for Cu, As, Fe, and Zn. However, castor can be used as a phytostabiliser combined with the production of castor oil **(Palanivel *et al*., 2020).** The efficiency of *Saccharum spontaneum* and *Saccharum munja* has been proved for phytoextraction and phytostabilization of metal-contaminated coal. These plants are suitable for extracting Zn, Cu, Cd, Pb **(Banerjee *et al*., 2020).** Other types of hyperaccumulators plants are Atriplex species **(Orrego *et al*., 2020),** *Arabidopsis thaliana* "used as a model plant" **(Ali *et al*., 2020 ; Chen *et al*., 2020b ; Tan *et al*., 2020),** ...etc.

Contaminated plants produced during phytoremediation actions can be further processed for bioharvesting of metals (phytomining) and to produce green energy from organic biomass (e.g., biofuel) **(Capozzi *et al*., 2020).**

b. Mechanism of phytoremediation

Depending on the nature of the contaminants, based on the route of metal uptake and transport and/or the mechanism of phytoremediation, there are various techniques of phytoremediation: phytoextraction (uptake of metals from the environment into the plant biomass), phytostabilization (reducing the mobilization of the metal by plant activities), phytofiltration (adsorb and/or absorb the metal pollutants from the aqueous environment through plant parts) and phytovolatilization (conversion of certain heavy metals into gaseous forms and vaporize into the atmosphere through leaves), phytodegradation, rhizofiltration, rhizodegradation and phytodesalination **(Cristaldi *et al*., 2020 ; Dal Corso *et al*., 2019 ; Rehman *et al*., 2020)**. To enhance the speed and quantity of metal uptake by the plant in the phytoextraction process, application of soil amendments such as metal chelating agents, acidifying agents, fertilizer salts, and plant growth regulators have been proposed **(Kazemalilou *et al*., 2020)**.

Phytoremediation is a green technology wherein plants and associated microbes are used to remediate HM-contaminated sites to safeguard the environment and protect public health **(Saxena *et al*., 2019).** In other studies, the phytoremediation potential of four non-eatable floriculture plants, i.e. antirrhinum, pansy, calendula, and marigold, was explored by growing in heavy metal contaminated soil (collected from a pre-urban area under untreated wastewater irrigation for more than 20 years) amended with bacterial inoculum and EDTA amended soils under greenhouse conditions for 75 days **(Mahmood-ul-Hassan *et al*., 2020).**

c. Factors affecting phytoremediation

The mechanisms and efficiency of the phytoremediation depend on several factors, such as the nature of the contaminant, bioavailability, soil properties and plant species, slow growth rate at higher metal-contaminated sites **(Cristaldi *et al*., 2020 ; Ravi *et al*., 2020).** The circumstance and these factors could be minimized and accelerate the phytoremediation efficiency by incorporating the potential plant growth-promoting rhizobacterial (PGPR) as a combined approach **(Ravi *et al*., 2020).**

II.8. Heavy metal stress and its responses in plants

A recent study shows direct and indirect toxic effects posed by heavy metal stress on diverse plant processes which possibly results in reduced crop production. Figure 2 shows how various plant nutrients including biometals are absorbed from the rhizosphere. After absorption and translocation in various plant tissues, elevated concentrations of biometals impose various direct and indirect effects **(Wani *et al*., 2018).**

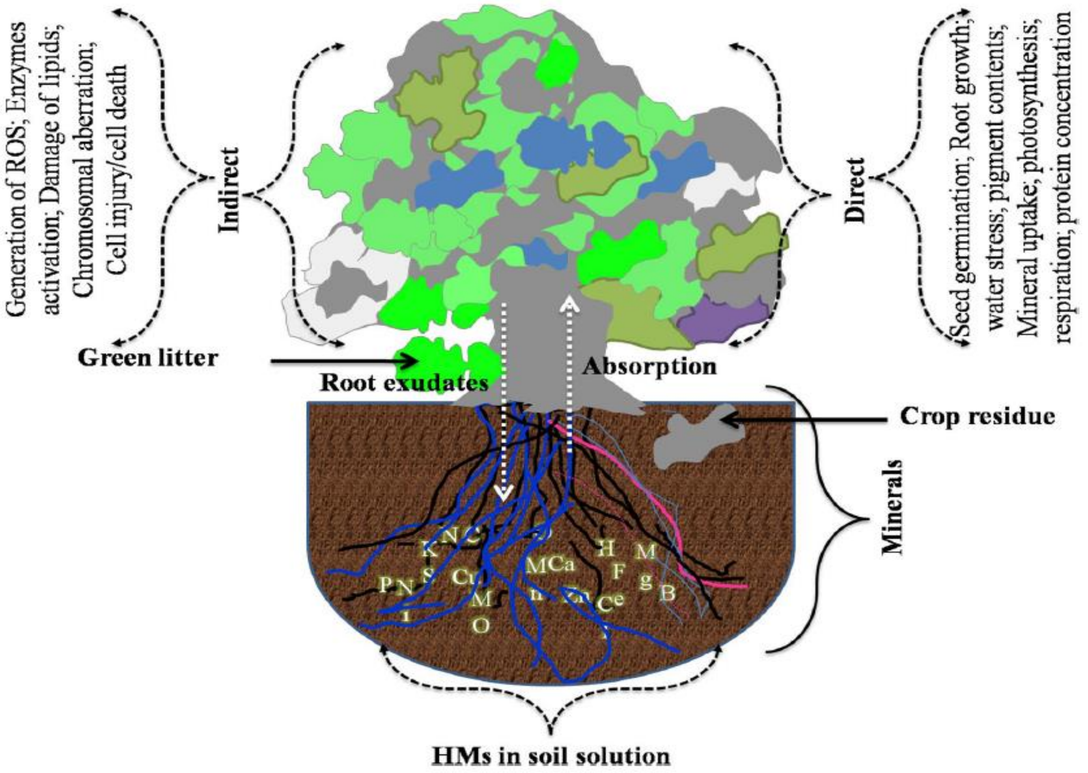


Figure 2: Plant responses to heavy metal stress **(Wani *et al*., 2018)**.

II.8.1 Plant signaling response to heavy metals

Although, some of these metals are essential micronutrients responsible for many regular processes in plants, their excess, however, can have detrimental effects and can directly influence the plant growth, metabolism, physiology, and senescence **(Ghori *et al.*, 2019)**. The inability of plants to escape from environmental stresses such as metal pollution has driven the evolution of multiple mechanisms to efficiently sense, respond, and therefore adapt to such stresses. Sensing of heavy metals by plants generates a response such as modulation of molecular and biochemical mechanisms of the cell **(Jalmi *et al*., 2018).**

Plants have different mechanisms to fight stress, and they are responsible to maintain the homeostasis of essential metals required by plants. These mechanisms also focus on the prevention of plant exposure to heavy metals present in the soil or providing tolerance to the plant by detoxifying the metals. Other mechanisms are specific and are initiated when the respective stress is encountered. The first line of defense provided by a plant is to reduce the uptake of metals when stimulated with the toxicity of heavy metals and includes the help offered by cellular and root exudates that restrict metals from entering the cell. Many plants have exclusive mechanisms for individual metal ions and are involved in sequestering these ions in compartments avoiding their exposure to sensitive components of the cells. As a second line of defense, other mechanisms for detoxification of these metals are introduced that chelates, transports, sequesters, and detoxifies these metal ions in the plant's vacuole. During the time of metal toxicity, oxidative stress is pronounced in the cells, and production of stress-related proteins and hormones, antioxidants, signaling molecules including heat-shock proteins synthesis is initiated **(Ghori *et al*., 2019)**.

II.8.2 Plant regular life cycles under heavy metal stress

Heavy metals have restricted the plant's regular life cycles affecting the plant primer and seconder metabolites by biochemical and physiological pathways. For instance, phenolic compounds considered as products of metabolic alterations have been synthesized in various numbers and typical characteristics of plants. The responses of tomatoes under heavy metals stress resulted in a lower amount of phenolic compounds**.** Proline accumulates in various plants in response to environmental stress, and its accumulation can affect stress tolerance in multiple ways (Figure 3) **(Kısa *et al*., 2019a).**

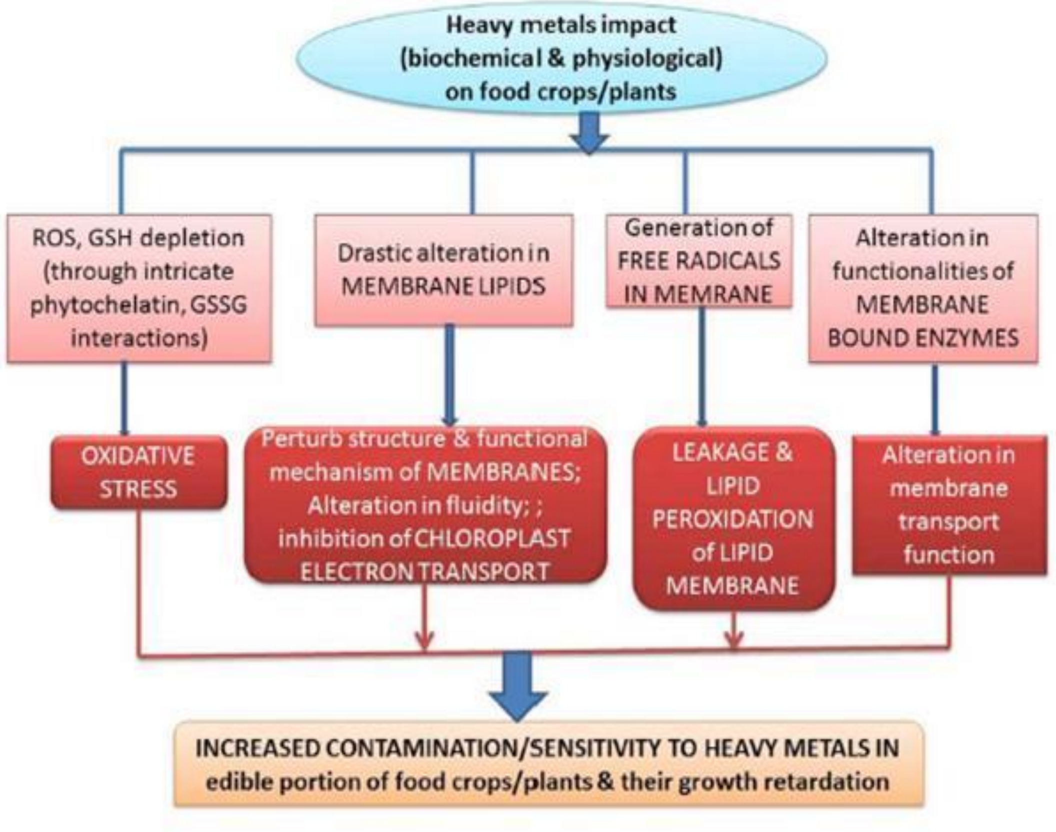


Figure 3: General physiological and biochemical mechanisms of heavy metal contamination in food crops **(Kumar *et al*., 2019).**

II.8.3 Mechanisms involved in plants under heavy metal stress

Physiological, biochemical, and molecular mechanisms of the plants are being studied for a better understanding of metal uptake, translocation, localization, and tolerance capacity. Accumulation of toxic metals/metalloids in plant cells cause the deactivation of cell enzymes, consequently affects plant growth. Detoxification mechanisms of plants to survive and grow in metal-contaminated environments include chelation of metal cations by ligands and sequestered away these toxic metal complexes into less metabolically active sites such as vacuoles and cell wall where the metal cannot readily dissociate. However, physicochemical parameters such as pH, salinity, light, temperature, and the presence of other cations and anions also play important roles in metal/metalloids **(Bora and Sarma, 2020).**

II.8.4 Signal transduction pathways in plants under heavy metal stress

Plants like other organisms have a defense mechanism that is activated during stress conditions to be able to survive. A complicated signal transduction network characterized as synthesis and activation of stress-related proteins and various signaling molecules are activated as soon as heavy metal stress is sensed. The signal transduction pathway usually leads to activation of transcription factors that induce transcription of various metal stress-responsive genes. Different signaling pathways are activated in response to different metals stress and include MAPK cascade, hormonal signaling, ROS signaling pathway, and Ca–Calmodulin pathway (Figure 4) **(Ghori *et al*., 2019).**

This figure displays the involvement of several signaling components working during metal stress. Sensing of a significant level of heavy metals by plants initiates signaling networks causing activation of various metal responsive transcription factors. These transcription factors (TFs) regulate the expression of metal responsive and other stress-related genes ultimately helping the plant to counteract the stressed situation. These stress-related genes are mainly metal transporters, phytochelatins metallothionine, antioxidant genes, and miRNA genes.

The ROS produced in response to metal stress either by respiratory burst oxidase homolog (RBOH) activity or by alteration in electron transport is also known to activate signal transduction. This figure also exhibits the crosstalk between different signaling modules and the feedback regulation of MAPK cascade by miRNA. P = phosphorylated **(Jalmi *et al*., 2018).**

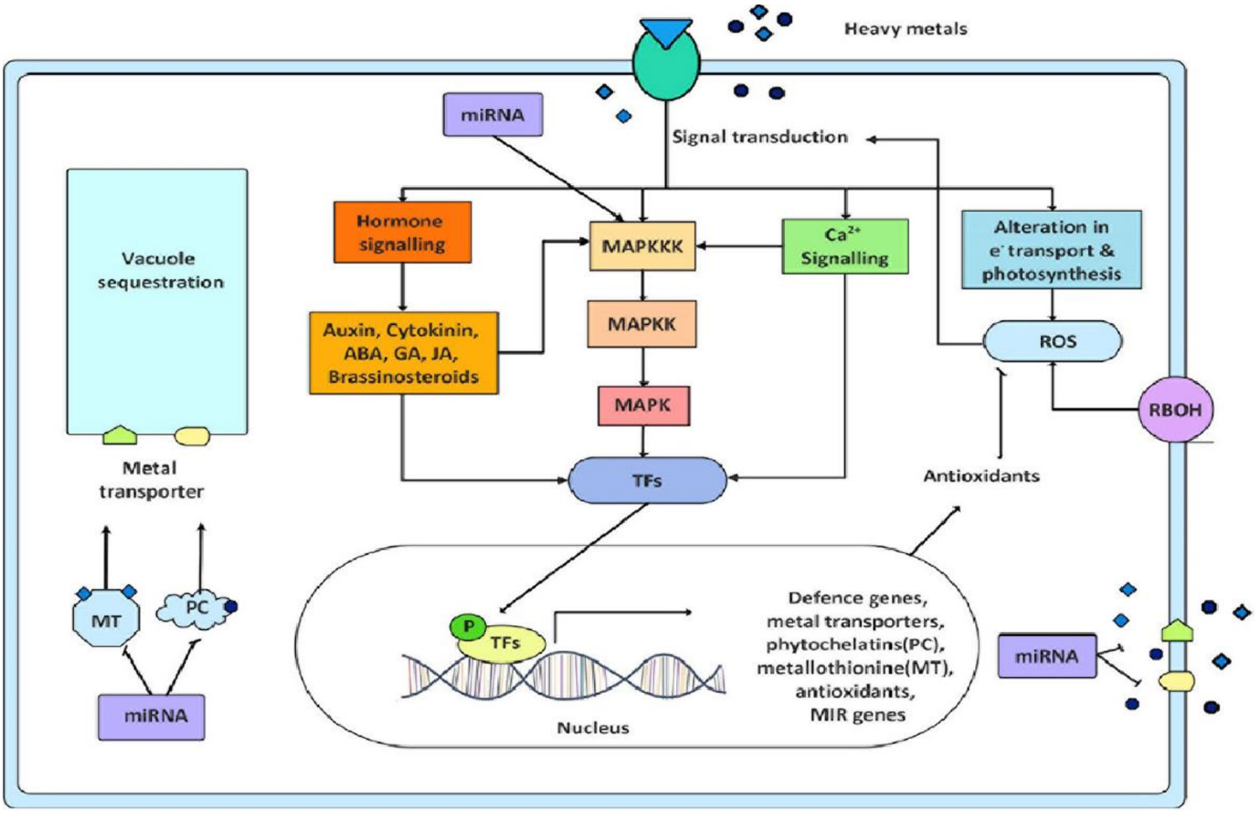


Figure 4: Crosstalk of signaling pathways and its ultimate response in heavy metal stress **(Jalmi *et al*., 2018).**

II.8.5 Genotoxicity due to heavy metal stress

If heavy metals successfully enter the nucleus and bind to it, they may cause promutagenic damage which includes intra- and inter-molecular cross-linkage of DNA and proteins, DNA base modifications, rearrangements, DNA strand breakage, and depurination **(Ghori *et al*., 2019).**

II.8.6 Transport protein and their response during heavy metal stress

Tolerance to heavy metal toxicity can be achieved either by not including the mechanisms pertaining to uptake by roots or else by efflux, compartmentalization, and detoxification of metal after their respective uptake (Figure 5) **(Ghori *et al*., 2019).** This figure displays that different transporters are involved in metal ion uptake. The elevated level of heavy metals triggers different signaling modules that transmit the signals inside the cell, thus triggering the defense response. The toxicity of these metals inside the cell is sequestered by metal chelators like phytochelatins and metallothionines. The chelated metals are then ultimately transported to the vacuoles with the help of metal transporters present on the vacuole membrane. PC, phytochelatins; MT, metallothionines; GSH, Glutathiones **(Jalmi *et al*., 2018).**

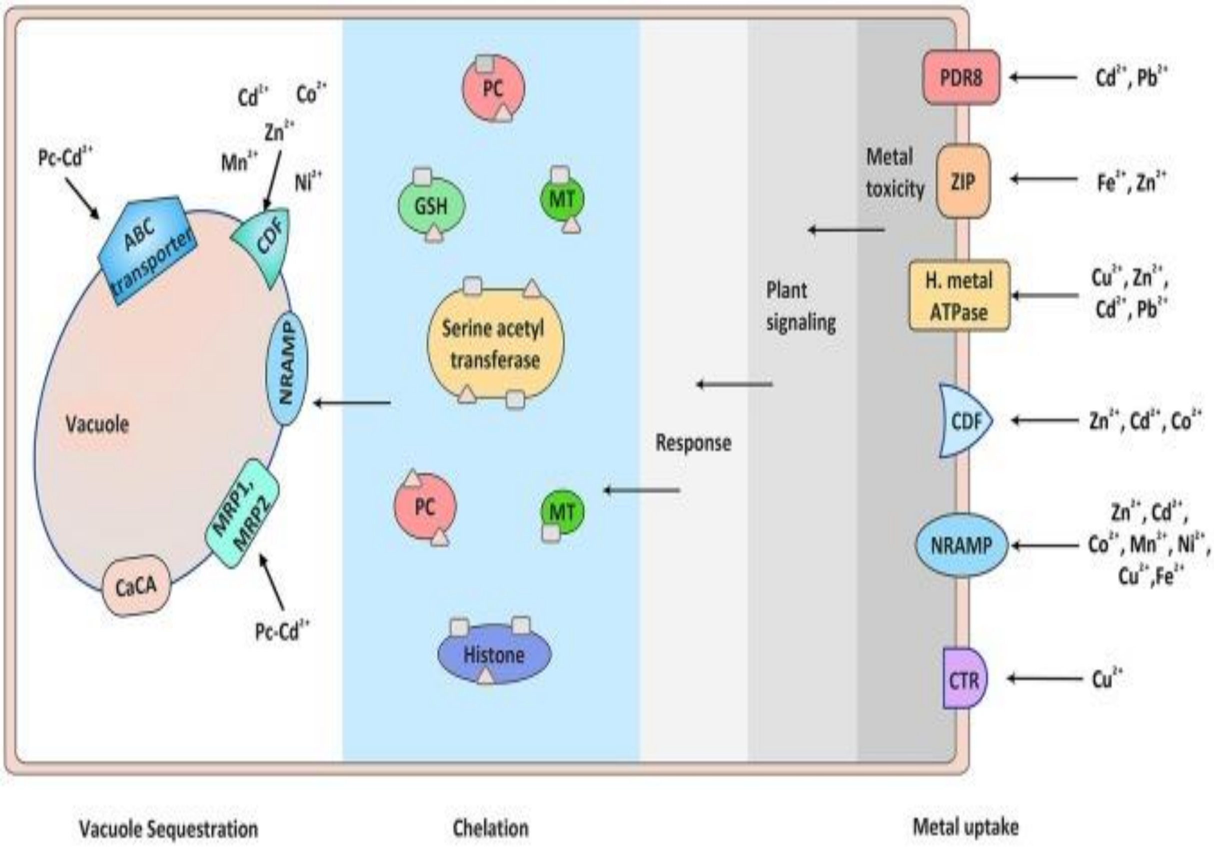


Figure 5: Metal detection, plant signaling, and sequestration **(Jalmi *et al*., 2018).**

II.8.7 Role of HSPs in Plant Tolerance to HM Stress

Most of the members of HSPs are strongly inducible and some are also constitutively expressed under environmental stress conditions. Many newly synthesized proteins are translocated into the endoplasmic reticulum (ER), where proteins are folded into their native three-dimensional structures with the help of chaperones. The correctly folded proteins are then transported to the Golgi complex, followed by delivery to the destination where they eventually function. While the exposure of plants to stress causes oxidative stress by generating an override of ROS and stimulating the misfolding of proteins. The incorrectly folded proteins are then detected by the quality control system, which stimulates another pathway called unfolded protein response (UPR). The terminally misfolded proteins are then eliminated through the endoplasmic reticulum-associated degradation (ERAD) pathway, where they initially ubiquitinated and then degraded in the cytoplasm by proteasome system (UPS) or subjected to autophagy (Figure 6) **(Hasan *et al*., 2017)**.

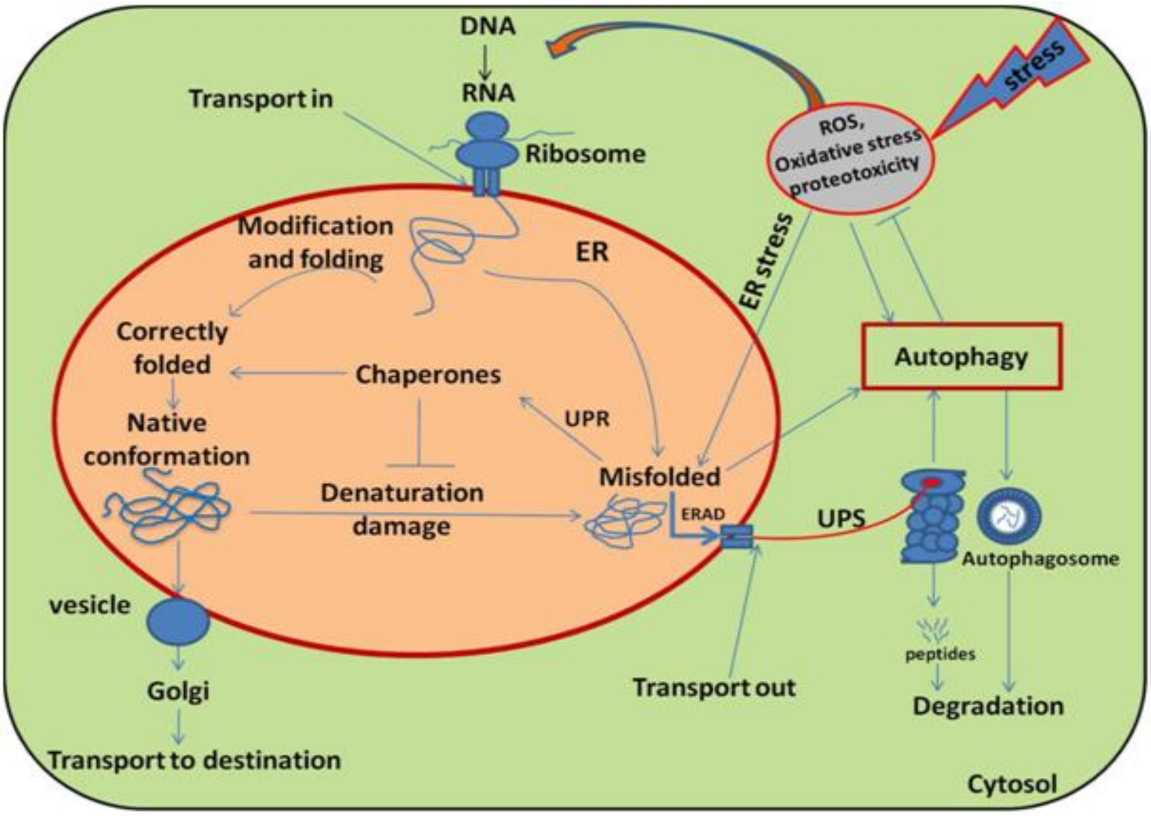


Figure 6: Schematic diagram illustrating the main pathways and regulation of protein folding and modification in the endoplasmic reticulum (ER). Adopted from Dobson (2003) with modification **(Hasan *et al*., 2017).**

II.9. Phytotoxicity

Metal bioavailability and phytotoxicity may be exaggerated when derived from studies based on amending soils with soluble metal salts. It is therefore important to evaluate soil tests for their consistency in estimating plant uptake and phytotoxicity in both field-contaminated and freshly-spiked soils **(Mossa *et al*., 2020).** The evolution of phytotoxicity during composting was analyzed by measuring the germination index.

Physicochemical parameters (pH, electrical conductivity, organic matter, reducing sugars, phenolic compounds, humic substances, soluble organic carbon, $N–NH_4^+$ and $N–NO_3^-$) and heavy metals content were evaluated in final products. Phytotoxicity was attributed to the high electrical conductivity and pH of the vegetal residues compost and heavy metal content of the municipal solid waste compost **(Siles-Castellano *et al*., 2020).**

II.9.1 Heavy metal and oxidative stress

Heavy metal contamination can directly cause strong oxidative stress due to an imbalance between the antioxidant defense system and the overproduction of reactive oxygen species (ROS) in different cellular compartments, leading to membrane disruption (lipid peroxidation) as well as proteins and nucleic acids denaturation, which can negatively affect important metabolic processes such as photosynthesis and carbon fixation (Figure 7) **(Gratão *et al*., 2019).**

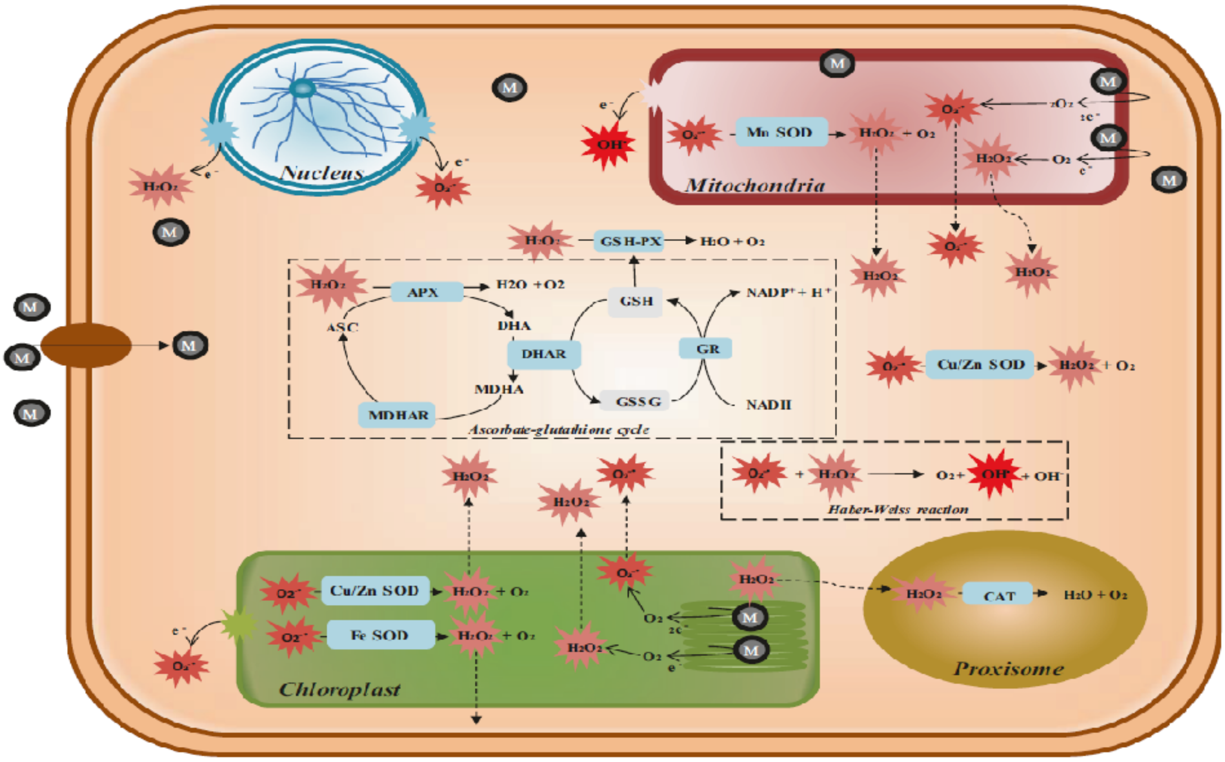


Fig. 1 Induced oxidative stress by heavy metal. Enzymatic reaction of superoxide dismutase (SOD): dismutation of the $O_2^{-\bullet}$, forming O_2 (cell oxygen) and H_2O_2 (hydrogen peroxide) in chloroplast (Cu/Zn and Fe SOD), mitochondria (Mn SOD), and cytosol (Cu/Zn SOD). H_2O_2 degradation catalyzed by the catalase (CAT) enzyme in peroxisomes, resulting in H_2O (water) and O2 (oxygen) formation. Glutathione peroxidase (GSH-Px) activity: H_2O_2 reduction concomitantly with the oxidation of the specific substrate reduced glutathione (GSH). Ascorbate-glutathione cycle enzymes: ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR). Other compounds: hydrogen peroxide (H_2O_2), water (H_2O), ascorbate (ASC), dehydroascorbate (DHA), monodehydroascorbate (MDHA), glutathione (GSH), and oxidized glutathione (GSSG). Haber-Weiss reaction: hydroxyl radical (OH˙) formation from the combination of $O_2^{-\bullet}$ and H_2O_2. The lipid peroxidation is also shown in the image, where the reactive molecules H_2O_2, $O_2^{-\bullet}$ and OH˙ reacts with membrane lipids and disrupts the structure of chloroplast, nucleus, and mitochondria

Figure 7: Induced oxidative stress by heavy metal **(Gratão *et al*., 2019).**

II.9.2 Heavy metal in tomato plant

Results demonstrate that soil contamination with heavy metals harms tomato fruit characteristics, titratable acidity, total soluble solids, lycopene, ascorbic acid, micro-elements, and carbohydrates content. Moreover, It is recommended that fruits cultivated in that area are not to be eaten in large quantities, to avoid excessive accumulation of heavy metals in the human body**.** Tomato fruits harvested from soils contaminated with heavy metals showed elevated amounts of Cd, Co, Ni, and Pb ions indicating that significant amounts of the heavy metals were translocated from contaminated soil to the fruit, thus presenting health concerns **(Hashem *et al*., 2018).**

II.10. Copper in plant

II.10.1 Essential element

Copper is a chemical element with the symbol Cu (from Latin: cuprum) and atomic number 29, extremely ductile metal of group 11 of the periodic table (period 4) **(Sires, 2017)**.

Copper is a vital micronutrient for plants and animals and is highly required for various important physiological and biological functions, such as cellular transportation, mitochondrial respiration, protein trafficking, antioxidative activity, and hormone signaling of plants **(Javed *et al*., 2017 ; Huang *et al*., 2017 ; Vishveshvar *et al*., 2018).** It acts as an essential cofactor of numerous proteins that play key functions in plant cell metabolism, such as the transport of electrons in mitochondria and chloroplast, the regulation of the cellular redox state **(Migocka and Malas, 2018).** However, it works as a beneficial element for plants at a specific concentration (**Andrés-Colás *et al*., 2018 ; Shams *et al*., 2019 ; Zehra *et al*., 2020)**. Cu is an important part of photosynthesis, respiration, glucose, proteins, and cell wall metabolism while excess Cu in the plants can cause alteration in DNA, cell membrane integrity, enzyme activity which ultimately affect crop yield and plant productivity **(Kohli *et al*., 2018 ; Lu *et al*., 2017 ; Rehman *et al*., 2019d ; Rizwan *et al*., 2017).**

II.10.2 Cu deficiency symtoms in plants

Nutrient deficiency occurs when 1 of the 17 essential plant nutrients is not available in sufficient quantity to meet the requirements of a growing plant. Producers, extension agents, and crop consultants should be able to recognize the main symptoms of nutrient deficiencies. It is important to interpret the scenario correctly to determine if timely intervention can minimize current crop yield loss, or whether it is more effective to wait and resolve the nutritional problem for future crops **(Gatiboni, 2020).** Copper deficiency reduces plant growth, male fertility, and seed set. The contribution of copper to female fertility and the underlying molecular aspects of copper deficiency-caused phenotypes are not well known**.** copper deficiency-caused defects in *Arabidopsis thaliana* were also the increased shoot branching, delayed flowering and senescence, and entirely abolished gynoecium fertility. The increased shoot branching of copper-deficient plants was rescued by the exogenous application of auxin or copper. The delayed flowering was associated with the decreased expression of the floral activator, *FT*. Copper deficiency also decreased the expression of senescence-associated genes, *WRKY53* and *SAG13*, but increased the expression of *SAG12*. The latter defect was associated with reduced lignification, the upregulation of copper microRNAs and the downregulation of their targets, laccases, implicated in lignin synthesis. Copper-deficient plants accumulated ROS in pollen and had reduced cytochrome *c* oxidase activity in both leaves and floral buds **(Ishka and Vatamaniuk, 2020)**. By a transcriptomic analysis, it has been observed that *NRAMP4* expression was highly induced in the *copt5* mutant under Cu deficiency, while *COPT5* was overexpressed in the *nramp3nramp4* mutant **(Carrió-Seguí *et al*., 2019).** Sandy soils with high alkalinity are characterized by low copper (Cu) contents that lead to many deficiency symptoms in plants **(Mottaleb *et al*., 2021).** Additionaly**,** copper deficiency in plant growth may appear as wilting with leaf tips turning a bluish green color. In grain-type plants, the tips may become brown and appear to mimic frost damage **(Nikki Phipps, 2011).**

II.10.3 Copper in soil

Copper is considered the most common heavy metal pollutants of soil in many countries, in China **(Huang *et al*., 2020a ; Peng *et al*., 2020)**, in sub-tropical regions **(Lwalaba *et al*., 2020),..**etc. Ecological thresholds and effects of Cu in soil were based on laboratory studies using soils artificially spiked with increasing Cu concentrations. It was necessary to use Cu-spiked soil because generally native soils have poly-metal contamination **(Minkina *et al*., 2019).** Contamination levels of copper (Cu) and other heavy metals are very high in the soils of the abandoned copper mine **(Palanivel *et al*., 2020).** The removal of copper (Cu^{2+}) from aqueous solutions, depending on the biosorption **(Ghoniem *et al*., 2020).** Copper has a fungistatic effect and is used in agriculture as a fungicide **(Ghany *et al*., 2020).** Cu in the soil is enhanced by cuprous and cupric Cu ions, which induce oxidative damage in plant cells/tissues **(Saleem *et al*., 2019a).**

II.10.3.1. Natural and anthropological sources of Cu in soil pollution

After conducting theliterature reviewfor this project, it was found thatthe following sources of Cu in soil pollution were mentioned more than any others:

a. Natural sources

Copper is a reddish metal that occurs naturally in rock, soil, water, sediment, and, at low levels, air. Its average concentration in the earth's crust is about 50 parts copper per million parts soil (ppm) or, stated another way, 50 grams of copper per 1,000,000 grams of soil (1.8 ounces or 0.11 pounds of copper per 2,200 pounds of soil). Copper also occurs naturally in all plants and animals **(Agency for Toxic Substances and Disease Registry).**

Copper is a naturally occurring element that provides benefits to a given ecosystem at the proper concentrations. However, sudden exposure tohigh concentrations, or chronic exposure to slightly elevated concentrations, can be harmful to ecosystems and the species they support **(Sires, 2017)**. As natural sources:

- Natural and environmental variables including flow rates, air and water temperature, water hardness dissolved oxygen, and other water quality parameters related to the development of site-specific copper standards.,
- Natural mineral deposits.

b. Anthropological sources

The source of Cu was a combination of the presence of parent materials as well as human activities **(Wu *et al*., 2018).** As Anthropological sources:

- Vehicle brake pads
- Fertilizers, pesticides, herbicides, and fungicides
- Copper roofing and metal plating
- Mining activity and municipal wastewater discharges
- Boat hull coatings and anti-fouling chemicals
- General urbanization, anthropogenic development, prevalence of impervious surfaces, deforestation, etc **(Sires, 2017).**

II.10.4 Phytotoxic effect of copper

Copper (Cu) is an abundant metal in the environment coming from anthropogenic activities and natural sources that, in excess, easily becomes phytotoxic to most species, being its accumulation in plants considered an environmental threat **(Branco-Neves *et al*., 2017).**

Copper (Cu) is among the main contaminant of agricultural soil **(Napoli *et al*., 2019).** Hence, these properties make Cu ions indispensable for plant growth. However, the presence of Cu in plants can be toxic at even a slightly higher amount than the optimal level **(Juang *et al*., 2019).** Its higher concentration can make disruption in plant growth **(Shams *et al*., 2019)** and may cause adverse effects on plant physiology **(Chrysargyris *et al*., 2019).** The presence of a high level of Cu in the soils reduces biomass, inhibits root growth; bronzing, necrosis, and chlorosis are usually reported symptoms due to increased production of reactive oxygen species at the cellular level **(Lange *et al*., 2017).** Copper (Cu) toxicity has a deleterious effect on plant growth **(Huo *et al*., 2020),** alters the level of chlorophyll content by disturbing the structure of the chloroplast, and acts negatively on photosystem II (Figure 8) **(Peng *et al*., 2020).**

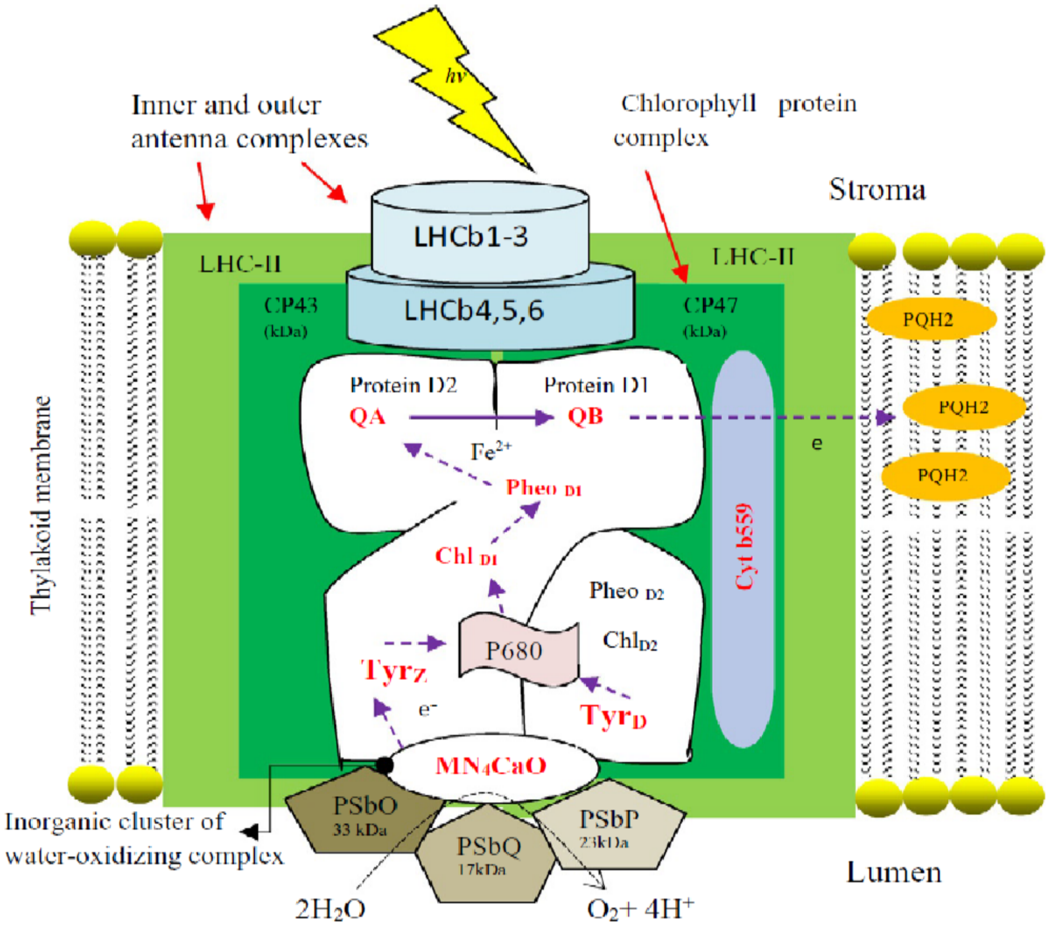


Fig. 4 Schematic scheme of core proteins (highlighted in red) and target sites of Cu action in photosystem II (PSII). Susceptibility of higher plants to photoinhibition (or PSII electron transport impairment) when exposed to toxic levels of Cu is increased (Yruela, 2005); P680 represents special pair chlorophyll molecules (Protein D1 and Protein D2) of PSII; QA and QB are referred to as primary and secondary quinone acceptor of PSII, respectively; Pheo, Pheophytin; Chl, chlorophyll; PQH2, reduced form of plastoquinone molecules. *Courtesy of illustration: Peiman Zandi.*

Figure 8: Target sites of Cu action in photosystem II **(Zandi *et al.*, 2020).**

II.10.5 Bioavailability and toxicity of Cu in plant

The bioavailability and toxicity of Cu in plant and soil/wetland systems depends critically on its chemical speciation**.** Several key plant physiological processes are accomplished on the participation and transformation of Cu which is recognized as a cofactor for many plant metalloproteins. Plants tolerate Cu toxicity by immobilizing excess amounts in harvestable parts including leaves, cell walls, and vacuolar membrane of the root cortex. Concerning environmental Cu bioavailability, plants have evolved different strategies to modulate Cu homeostasis. The immobilized Cu in plants can be remobilized in times of Cu deprivation. Plant Cu acquisition, transportation, and remobilization for growth are managed by several Cu-uptake proteins via Cu transporter COPT/Ctr-like protein family, specific Cu chaperones, and metal chelators (Figure 9). The transfer of Cu from soil/wetland systems to plants is determined by the chemical speciation and bioavailability of Cu, which is critically affected by various factors involving plant species, soil microbial community, and dissolved organic carbon, redox potential as well as other soil/sediment physicochemical factors, particular for S cycling and transformation under flooding conditions. Given the increased consumption of Cu products and enhanced concentration of Cu in the environment in the past several decades, this review recommends continuous and uninterrupted exploring of the biogeochemical behavior and transformation mechanisms of Cu in plants and soil/wetland systems, particular at the molecular levels **(Zandi *et al*., 2020).**

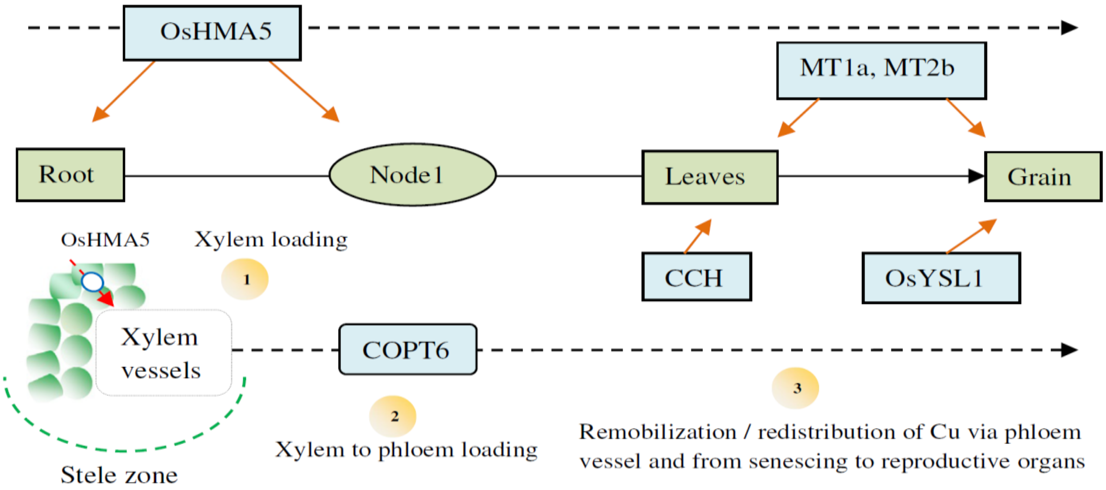


Fig. 2 Word diagram of carrier proteins involved in transportation and remobilization of Cu from green tissues to reproductive organs (root to shoot tissues). *Courtesy of word diagram: Peiman Zandi.*

Figure 9: Transportation of Cu from green tissues to reproductive organs **(Zandi *et al*., 2020)**.

II.10.6 Copper uptake, distribution, and accumulation by plants

Following the entry into the cell, Cu^+ ions are bound to the specific soluble Cu chaperones that serve to deliver Cu to different cellular compartments (Golgi, plastids, mitochondria) and cupro- proteins, or are sequestered in the vacuole or apoplast to eliminate an excessive amount of Cu in the cytosol. Copper is reduced at the root surface to Cu^+ by FRO4/5 and transported across the plasma membrane by COPT1/2 while efflux of Cu^{2+} is mediated by yet unidentified antiporter. In the cytoplasm, Cu^+ is chelated by MTs or specific chaperones: CCS and ATX1 and delivered to different organelles. Import and export to and from vacuoles are carried out by HMA4/5 and COPT5 respectively, while import to GA is mediated by HMA7 and CT1. In mitochondria, the COX17 chaperone in the intermembrane space delivers Cu+ to two other chaperones localized in the mitochondrial inner membrane: HCC1 and COX11. Chloroplastic chaperones PCH1 and CCS deliver Cu^+ to HMA6 and HMA8, respectively, whereas Cu^{2+} ions are imported into the stroma by HMA1. ATX1 delivers copper to HMA5 which loads Cu^+ to the xylem. The complexed Cu^{2+} is further delivered to the leaf by YSL1/2/3, or reduced by FRO4 to Cu^+ and imported to leaf cells by COPT6. YSL16 transfers Cu^{2+} complexes from senescing organs to the phloem.

ATX1, antioxidant protein 1; CCS, Cu chaperone; CHL, chloroplast; COPT, copper transporter; CT1, MFS-type transporter; CYT, cytoplasm; ER, endoplasmic reticulum; FRO, ferric reductase oxidase; G, Golgi apparatus; HMA, heavy metal ATPase; MT, metallothioneins; MT, mitochondria; N, nucleus; PCH1, plastid chaperone 1; V, vacuole; YSL, yellow stripe-like (Figure 10) **(Migocka and Malas, 2018).**

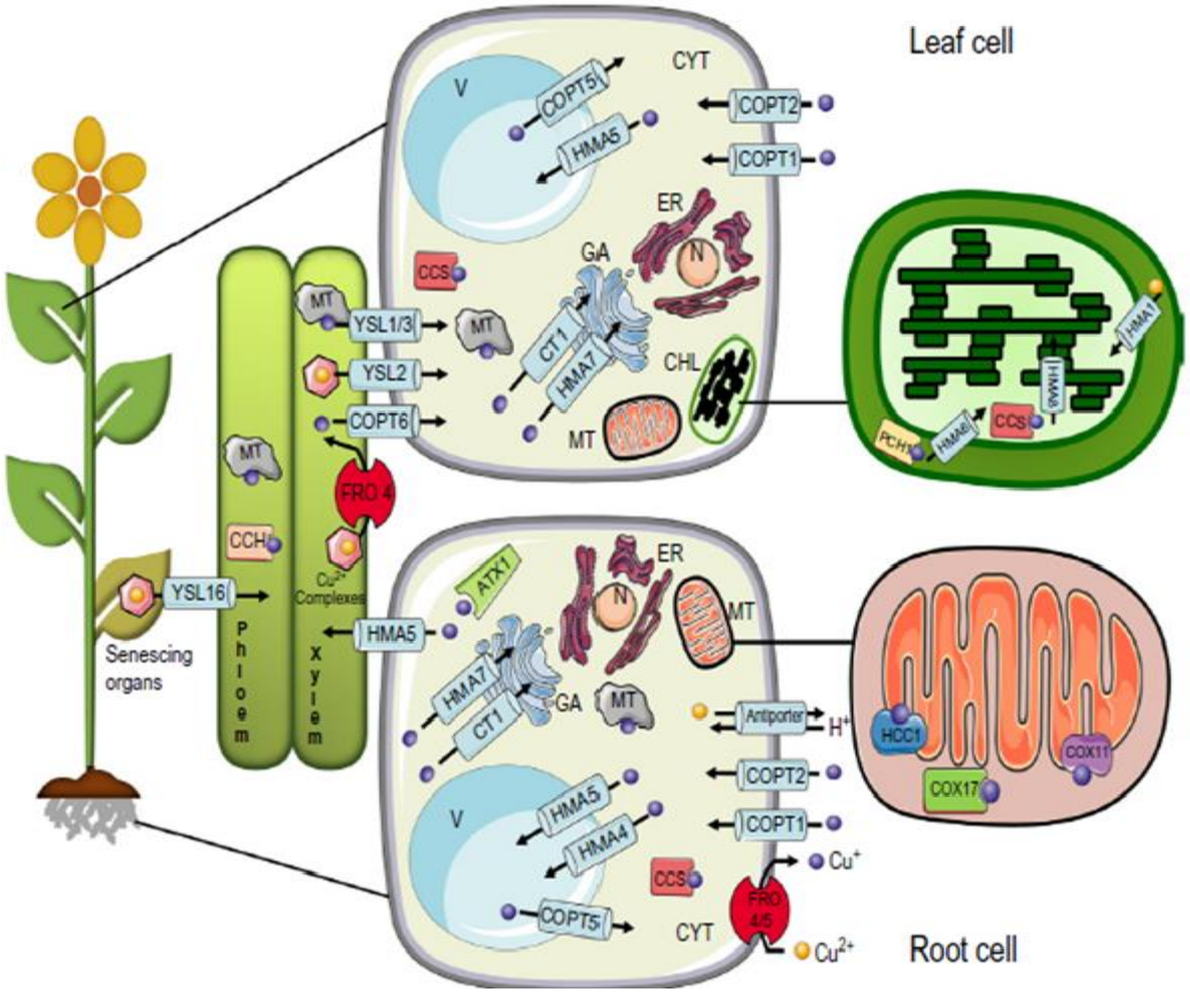


Figure 10: Overview of localization of copper transporters and chaperones in plants **(Migocka and Malas, 2018).**

II.11. Zinc in plant

II.11.1 Essential element

Zinc is a transition metal with the following characteristics: period 4, group 12, atomic number 30, Zinc occurs naturally in soil **(Sires, 2017)**.

Zinc is an essential element, indispensable for all life **(Tilborg, 2020)**. Zinc (Zn) is one of the essential plant micronutrients and is involved in several physiological functions in plants for growth and development **(Moreira *et al*., 2018 ; Rizwan *et al*., 2019).** It is required by plants as it forms important components of zinc finger proteins and also aid in the synthesis of photosynthetic pigments such as chlorophyll. Zinc is required for the catalytic activity of different metabolic enzymes, including dehydrogenases, aldolases, isomerases, transphosphorylases, RNA and DNA polymerases, and it is also involved in the synthesis of tryptophan, cell division, maintenance of membrane structure and potential, and photosynthesis, and acts as a regulatory cofactor in protein synthesis **(Chaudhuri and Malodia, 2017)**. Besides, Zn is required for the synthesis of chlorophyll and pollen viability, the biosynthesis of plant growth regulators, such as indole-3-acetic acid and carbohydrate, and nitrogen metabolism, which leads to increased crop production **(Shukla, 2017).** Zinc, on the other hand, is required for proper growth of plants, and 40 mg Zn. kg^{-1} of grains has been recommended in cereals to avoid Zn deficiency in humans whose main source of food is cereals **(Cakmak *et al*., 2017).** Zn soil application + foliar enhanced grain yield **(Haider *et al*., 2020).** Zinc finger protein is highly expressed in the reproductive organs during *Brassica oleracea* self-pollination, we found that znf1 plays a role in the auxin pathway **(Lian *et al*., 2020).** Its deficiency in plants has been widely reported in many regions of the world **(Moreira *et al*., 2018).**

II.11.2 Zn deficiency symtoms in plants

Zinc (Zn) is an essential micronutrient for plants. Because of its low availability in arable soils worldwide, Zn deficiency is becoming a serious agricultural problem resulting in decreases of crop yield and nutritional quality **(Zeng *et al*., 2021).** Deficiency of zinc in plant causes less oxidation of carbohydrate, improper development of chlorophyll and low level of auxin which in turn reduced the proper growth and development of plant **(Kumar *et al*., 2021).** Zn's activity is to help turn out pigment to the factory. Leaves discoloration if the soil is deficient in Zn and plant growth is scrubbed, deficiency disease induces a kind of leaf discoloration known as iron deficiency, allowing the tissue to appear yellow while the leaf remains inexperienced replanting**.** Zinc deficiency starts on the lower leaves, while iron deficiency starts on the higher leaves due to a lack of carbon, Mn **(Vadlamudi *et al*., 2020).** In tomato plant, zinc defienciency the older plants develop thick leaves with a brownishorange chlorosis, some may show necrotic spots. Stems are normal size but leaves are smaller, commonly called "little leaf **(Akther *et al*., 2020).**

II.11.3 Zinc in soil

Fertilizers, liming materials, pesticides, and fungicides containing Zn have contributed to increasing its concentration in agricultural soils. Zinc in the solution phase of a soil suspension was present mainly as free Zn^{2+} ions. The great Zn availability suppressed Cd uptake by plants. Measurements of soil Zn ‘quantities’ and ‘intensity’ were correlated with Zn uptake and toxicity. The free Zn^{2+} ion activity appears to be a consistent driver for plant uptake and phytotoxic response for both metal-spiked soils and historically contaminated soils. Surprisingly, soil Zn accumulation of up to 100 times the current regulations for normal arable land only produced a mild toxic response suggesting that constituents in biosolids (e.g. organic matter and phosphates) strongly restrict metal bioavailability **(Mossa *et al*., 2020).**

II.11.3.1. Natural and anthropological sources of Zn in soil pollution

After conducting a literature review, the following four sources of zinc were mentioned more than any others:

a. Natural sources

Zinc (Zn) is naturally present in all soils in typical background concentrations 10–100 mg Zn. kg^{-1} in (about 70 mg. kg^{-1} in crustal rocks). Human activities have enriched topsoils with Zn through atmospheric depositions, fertilization and sewage sludge application. Zinc contaminated soils with negative impact on the soil ecosystem are found around Zn smelters, near Zn mining sites and under galvanized structures. The solubility of Zn in soils is almost invariably controlled by sorption reactions. Pure Zn minerals (carbonates, silicates, hydroxides) have been detected at high total soil Zn concentrations (> 1,000 mg Zn. kg^{-1}) but are rarely controlling Zn solubility **(Mertens and Smolders, 2013).**

For instance: natural mineral deposits, mining activity, forest fires, vehicle brake pads **(Sires, 2017).**

b. Anthropic sources

- Galvanized metals and metal surfaces, including metal roofing, construction materials, plumbing and stormwater drainpipes, guardrails, chain-link fencing, etc,
- Vehicles and, more specifically, the wearing of rubber tires,
- Motor oil and hydraulic fluids, industrial processes,
- Lawn and landscaping fertilizers, pesticides, fungicides, and other chemical products, sewage sludge, atmospherique deposition **(Sires, 2017).**

Zn can accumulate in agricultural soils, achieving values considerably higher than its optimum concentration as a nutrient, and it may be toxic to soil organisms **(Mertens and Smolders, 2013).**

II.11.4 Phytotoxic effect of zinc and autophagy

Zinc (Zn) is a micronutrient, but its excessive concentration can impair plant growth and development **(Mossa *et al*., 2020).** However, in excess amount, Zn causes chlorosis of leaf and shoots tissues and generates reactive oxygen species **(Pramanick *et al*., 2017).**

Accordingly, Zn deficiency in agricultural fields is a serious problem, especially in developing regions. However, the relationship between autophagy and deficiencies of other essential elements remains poorly understood, especially in plants. We focused on Zn due to the property that within cells most Zn is tightly bound to proteins, which can be targets of autophagy. Autophagy plays a critical role during Zn deficiency in *Arabidopsis thaliana* L. Autophagy-defective plants failed to grow and developed accelerated chlorosis under Zn starvation. Autophagic degradation plays important functions in maintaining Zn pools to increase Zn bioavailability and maintain ROS homeostasis under Zn starvation in plants **(Shinozaki *et al*., 2020a).**

II.11.5 Zinc uptake, distribution, and accumulation by plants

A model of the mechanisms that occur in hyperaccumulation plants upon exposure to zinc (Zn): Zn ion uptake, chelation, transport, and sequestration. Zn bioavailability can be influenced by several factors, such as microorganisms, branching roots, pH, and exudates. Once absorbed by the roots, Zn can be absorbed by an apoplastic route: A passive diffusion through cells, or by a symplastic route via transporters. Within the latter path, Zn absorption by epidermis cells is mainly promoted by IRT1, ZIP19, and ZIP23. To reach the cortex, Zn can be directly diffused using ZNT2 and ZNT5. Then, Zn can either be stocked in vacuoles (promoted by ZIF2) or transported to the endodermis through the following transporters: ZIP23, ZIP19, ZIP5, and IRT3. Zn following the apoplastic route is stopped by the Casparian strip and then enters the endodermis via ZNT1/ZIP4. At this level, Zn can be chelated by nicotianamine (NA) or directly diffused to pericycle cells where a part can also be associated with histidine (His). The unchelated Zn can reach the xylem through direct diffusion or via the yellow-stripe 1-like (YSL) transporters, ferric reductase defective 3 (FRD3,) and heavy metal pumps P1b-type ATPases (HMA4). Zn then crosses the xylem as a Zn-free form or coupled with His, citrate, or malate. To enter the leaf cells, Zn can passively penetrate in chelated forms or as the Zn-free form via ZIP4 and ZIP6 proteins. It is then sequestrated inside the vacuole through MTP1, MTP8, MTP11, NRAMP3, NRAMP4, HMA3, and HMA4 transporters, or blocked in the cell wall (Figure 11).

IRT, iron-responsive transporter; ZNT, zinc transporter; (Zrt/Irt)-like protein (ZIP), zinc-regulated transporter iron-regulated transporter proteins; ZIF, zinc induced facilitator; HMA, heavy metal pumps P1b-type ATPases; MTP, metal tolerance proteins; MTP, metal tolerance proteins; NRAMP, natural resistance-associated macrophage protein **(Balafrej *et al.*, 2020).**

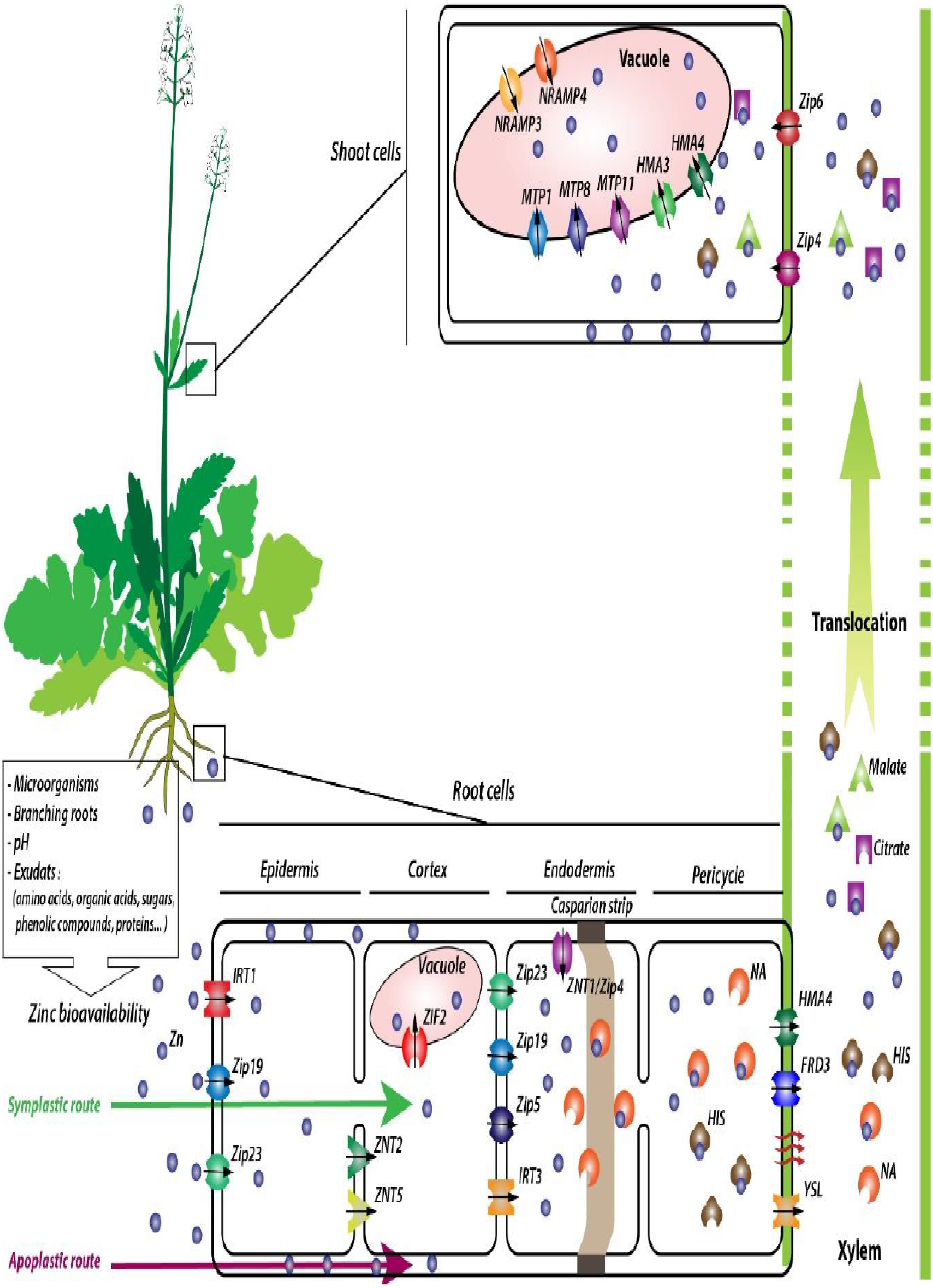


Figure 11: A model of the mechanisms that occur in hyperaccumulation plants upon exposure to zinc (Zn) **(Balafrej *et al*., 2020).**

II.12. Engineering plants for heavy metal stress tolerance

The present collected literature provides new insights in dynamics of metal/metalloid stress response, acclimation, and recovery after stress treatment of various plant processes for developing engineered heavy metal/metalloid transgenic plants with elevated stress tolerance to other abiotic stresses as well as orchestrated crop productivity in decades to come. Up-regulation of the overexpression of genes has resulted in enhanced tolerance of plants to metal stress. Therefore, identifying the genes reported to regulate HM homeostasis, detoxification and exclusion can benefit sustainable agriculture. Further, identifying plant promoters, signal transduction pathways, and transcription factors involved in HM stress tolerance can enlighten the molecular mechanisms behind HM stress resistance in plants. The same knowledge may prove beneficial in translating information to facultative metallophytes as well. Recently, several efforts were made for engineered heavy metal/metalloid stress tolerance through genetic concocting with moderate success. However, owing to the genetically complex nature of heavy metal/metalloid stress tolerance mechanisms and dissemination of this information from lab to field trials make it difficult to achieve (Figure 12) **(Wani *et al.*, 2018).**

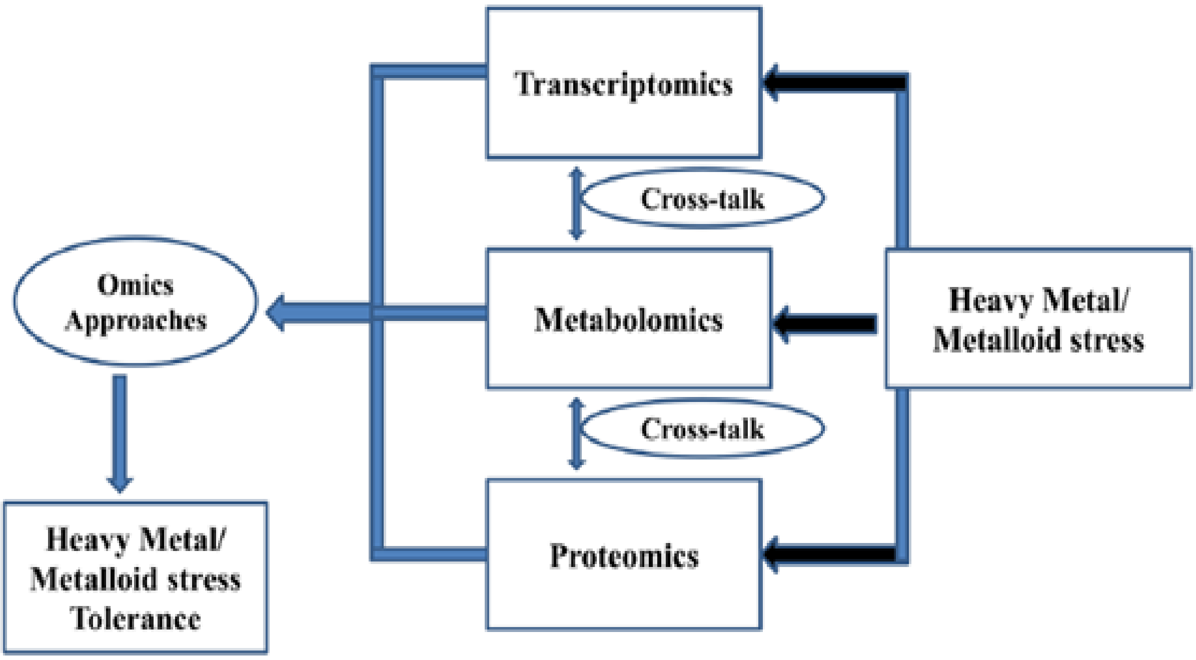


Figure 12: Coordinated approaches in engineering heavy metal stress tolerance in plants **(Wani *et al.*, 2018).**

II.13. Techniques applied to assess the distribution of metals in plant tissues

Different techniques such as electron energy loss spectroscopy (EELS), particle-induced X-ray emission (micro-PIXE), transmission electron microscopy (TEM), nuclear microprobe technique (NMP) are applied to assess the distribution of metals/metalloids in plant tissues at the subcellular level **(Bora and Sarma, 2020).** In tomato plant, a novel combination of transmission electron microscopy and scanning electron microscopy with electron dispersive X-ray analysis was used for understanding the mechanism of zinc toxicity on plant tip morphology, ultrastructure**.** To distinguish between Zn absorbed onto roots surface or accumulated in roots, also to have first views on zinc translocation pathway, a panel of imaging techniques Including scanning electron microscopy (SEM) coupled with EDS (Oxford EDS-Detector), transmission electron microscopy (TEM) was performed **(Min *et al*., 2020).**

CHAPTER III

Tomato

(*Lycopersicon esculentum* Mill.)

III.1. Introduction

Tomato (*Solanum lycopersicon esculentum* Mill.) belongs to the Solanaceae family, it's regarded as one of the most nutritious vegetables **(Thwe *et al*., 2020 ; Waheed *et al*., 2020)**. This vegetable is a part of the major crops in food security **(Alajrami and Abu-naser, 2019)**, it's also known as a protective food, because of its special nutritive value and its widespread production. That's why it's considered the second most highly produced vegetable crop worldwide after potato **(Blanchard-Gros *et al*., 2020 ; Singh *et al*., 2018c)**.

III.2. History and Origin

Following that axis, Tomato plants are originally from South America (Peru), they are grown in temperate and hot regions **(Alajrami and Abu-naser, 2019)**. Although tomato is a temperate crop, it is almost grown in all climatic zones, because of the widespread demand, which makes it a challenge to adapt it in adequated conditions **(Chaudhary *et al*., 2019)**, and extensively cultivated throughout the tropics and sub-tropics around the world **(Singh *et al*., 2018c)**.

III.3. Production, area, and yield

III.3.1 World

Tomato *(Solanum lycopersicum* Mill.) is the major horticultural crop with an estimated global production of 164 million metric tons from 4.73 million hectares of land **(Sa, 2018)**. Recently, Europe surpassed Africa in production, and the combined share of Asia and Europe is 72.83 % of the world's total production **(FAO, 2017)**, meanwhile, The Republic of China is the world leader in tomato production, which is providing more than 50 % of the world's tomato acreage. After China, the United States and India add more than one-third of the world's production; Turkey and Egypt also have a notable contribution.

During the last 20 years, tomato production and the area under its cultivation is continuously increasing. It is very interesting to notice that ~20 years ago, the United States and Europe were the leading tomato producers, but now the scenario has changed (Figure 13) **(Wakil *et al*., 2017).**

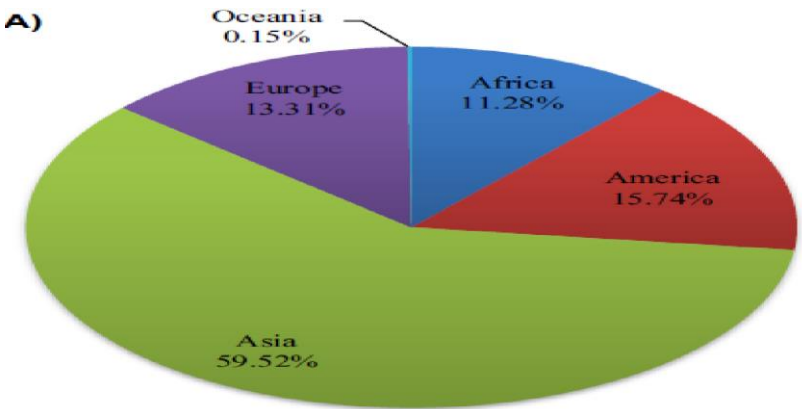


Figure 13: Tomato production in the world **(Wakil *et al*., 2017).**

The area under tomato cultivation has gradually increased globally and reaching 5.02 million ha in 2014 which was 3.27 million ha in 1995. Correspondingly, the total annual production also enhanced from 87.44 to 170.75 million tons from 1995 to 2014, respectively **(Wakil *et al*., 2017)**.

III.3.2 Algeria

Tomato consumption in Algeria continues to indicate a remarkable growth. The most prominent rate of growth was recorded in 2013. Furthermore, tomato consumption peaked in 2018 and is likely to see steady growth soon **(Index Box AI Platform, 2020)**.

By extension, the national production of fresh tomatoes gave 13.72 million quintals (qx) during the 2017-2018 campaign, where the Ministry of Agriculture, Rural Development and Fisheries announced that the yield was 428 qx / hectare for field tomatoes and 1.225 qx / hectare for greenhouse tomatoes. The largest producing regions of fresh tomatoes in Algeria are; Biskra with a production of 2.33 million qx; Mostaganem with a production of 1.33 million qx; Tipaza with 1.04 million qx, and; Ain Defla with 728,250 qx. In addition to fresh tomatoes, industrial tomato production (intended for processing) was 15.4 million qx during the 2017-2018 campaign, with a yield of 651 qx / hectare. The largest producing regions of industrial tomatoes are; Skikda with a production of 4.65 million qx; Tarf with 3.5 million qx; Guelma with 2.06 million qx and; Ain Defla with 1.68 million qx **(MINAGRI, 2018)**. Tomato production in Algeria in 2018 is illustrated in Table 1.

Table 1: Value of agricultural tomato production in Algeria, official data **(FAOSTAT, 2020).**

Tomato in Algeria (2018)	Area harvested (ha)	Yield (hg. ha^{-1})	Production (tonnes)
	22,323.00	586,729.00	1,309,745.00

III.3.3 Mostaganem

The Directorate of Agricultural Services of the Mostaganem region (DSA) enounced the production of 110,000 quintals (qx) of industrial tomatoes for the 2019-2020 agricultural seasons. The Industrial tomato production has experienced stability in Mostaganem with an average harvest of 110,000 qx per season since the 2016-2017 seasons, where the Production did not increase due to the lack of processing units in the region and the inability of producers to transfer the harvest to units in neighboring this region or Wilaya **(DSA Mostaganem, 2020)**. Tomato production in Mostaganem in 2021 is presented in (Table 2).

Table 2: Tomato production in Mostaganem in 2021 **(DSA MOSTAGANEM, 2021).**

Tomato in Mostaganem (2021) **(In greenhouses)**	Area harvested (ha)	Yield (qx. ha^{-1})	Production (qx)
	321	850	272850

III.4. Nutrition value

Tomato is widely consumed in the everyday diet in its raw form for salads or processed in different ways **(Thwe *et al*., 2020).** It contains a lot of vitamin C, vitamin A and B, mineral salts, potassium, iron, and phosphors. Tomato is a source of fiber,but; it doesn't contain sodium salt, fats, or cholesterol **(Alajrami and Abu-naser, 2019 ; Hussein *et al*., 2020)**.This fruit is popular for its outstanding antioxidant content and rich concentration of lycopene, steroidal alkaloids, steroidal saponins, flavonoids, carotenoids, glycosides, and fatty acid derivatives **(Domínguez *et al*., 2020 ; Thwe *et al*., 2020 ; Waheed *et al*., 2020)**. By and large,it is recognized for its anti-inflammatory and anticancer activities **(Salehi *et al*., 2019)**. In general,tomatoes are recommended for people with constipation, diabetes, and patients with heart and body diseases **(Alajrami and Abu-naser, 2019 ; Alam *et al*., 2019)**, that's why, the demand for tomato paste is daily used over the year with the fact that it is abundant in certain seasons and scarce during other seasons **(Hussein *et al*., 2020)**.

III.5. Taxonomy

Indeed, Tomato is classified according to the taxonomy established by **Mill (1763)** and confirmed by **Cronquist (1981) and Gaussen *et al*. (1982)** (Table 3).

Table 3: Taxonomy and nomenclature of the tomato plant (*Lycopersicon esculentum* Mill.)

Kingdom	Plantae.
Subkingdom	Trachenobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Solonales
Family	Solanaceae
Genus	*Solanum* L.
Subgenus	*Lycopersicon*
Species	*Lycopersicon esculentum* Mill.

III.6. Type of tomato classification using deep learning

Modern technologies have given the human community the ability to produce enough food to meet the demand of more than 7.5 billion people. However, with the technological development in botany and the interference in the genetics of plants, a new species of the same plant species has been purified, but in various forms. A tomato classification approach is presented with a data set containing approximately 5,266 images with 7 species belonging to tomatoes. By and large, the Neural Network Algorithms (CNN), a deep learning technique applied widely in image recognition, is used for this task. Also, the Deep Convolutional Neural Network (CNN) does well in classifying tomatoes. Tomatoes are a major crop, and proper automation of the tomato process will help improve crop yields and protect productivity and continuous continuity. The transformation of tomato cultivation using smart agricultural methods can affect economic growth in many countries, therefore, there is a strong relationship between increased productivity and economic abundance **(Alajrami and Abu-naser, 2019)**.

III.7. Culture conditions

Tomato plants are generally cultivated in the field, greenhouse, garden, and pot. This crop is grown under specific conditions of temperature, humidity, light, CO_2. These factors can limit the growth of tomato plants at a high and low level causing abiotic stress.

From another angle, Greenhouse technology is a flexible solution for sustainable year-round cultivation of tomato (*Lycopersicon esculentum* Mill.), particularly in regions with adverse climate conditions or limited land and resources **(Shamshiri *et al*., 2018)**.

III.7.1 Temperature and humidity

Tomato grows well in warm, well-drained fertile lands, and in areas subject to direct sunlight for at least 6 hours a day **(Alajrami and Abu-naser, 2019)**, it grows also under ambient conditions or in greenhouses at a temperature of 20°C - 27°C and 70–95 % relative humidity **(Blanchard-Gros *et al*., 2020 ; Chen *et al*., 2020a ; Hussein *et al*., 2020 ; Pontes and Barros, 2020).** It has been shown that tomato seedlings grown at 30°C have longer hypocotyls than those grown at 25°C, but that does not occur in plants with reduced levels of SlPIF4 (Phytochrome interacting factor (PIF) family of transcription factors). Notably, this study found a SlPIF4-dependent up-regulation of the likely auxin biosynthesis gene SlYUC8 at warm temperatures, suggesting that warm-temperature signal transduction is likely conserved between the two tomato species. Even in the absence of warm-temperature treatments, adult SlPIF4-RNAi lines generally had a compact structure, Eventually, this architectural change may be due to the reduced levels of auxin in these plants, particularly in the leaves **(Hayes, 2019)**.

III.7.2 Light

Tomato plants have been grown under blue or red nets transmitting similar photosynthetic. Plants under the red net were taller with a greater leaf area and a shoot to root ratio. An increased blue to red ratio slightly enhanced the reproductive development of tomato plants. While, an increased red to blue ratio enhanced the tomato glucose (17 %) and fructose (6 %) contents, while the citric (5 %) and malic acids (4 %) contents decreased, triggering a higher sugar/acid ratio. A high blue to red light-limited organ growth and plant light interception was reduced despite a higher photosynthetic yield and a slight enhancement of the organ appearance rate. Besides, a low blue to red light increased fruit weight at harvest and even if it did not significantly improve fruit yield, it favored fruit quality as it enhanced the tomato sugar/ acid ratio **(Thwe *et al*., 2020)**.

III.7.3 CO_2 and light intensity

Undeniably, the Increasing of CO_2, reduce transpiration and water consumption across different levels of light intensity, thus significantly increasing both leaf-level and plant-level water use efficiency, that's why, the combination of increased CO_2 (800 µmol CO_2 mol^{-1}) and the high light intensity (400 µmol. m^{-2} .s^{-1}) works synergistically to promote optimal growth of tomato plant, carbon assimilation, photosynthetic capacity and water use efficiency by upregulation of pigment concentration **(Faizan *et al*., 2020)**.

III.8. Botanical description

III.8.1 Above-ground organs

- **Leaves**

The leaves are alternate and presented without a stipule, and they are composed, pinnate, with 7, 9, or 11 oval segments, incised or roughly serrated and alternating with smaller segments **(Bénard, 2009)** (Figure 14).

Figure 14: Tomato plant (*Lycopersicon esculentum* Mill.) **(Osmane, 2017)**

- **Stem**

The stem is full and angular, it can reach a height of more than 2 m **(Shankara *et al*., 2005)** (Figure 15).

Figure 15: (a) Representation of the stem with the axillary buds of 4th to 6th node at 9 weeks old. (b) Representation of the root growth at 6 weeks old. Scale bars = 1 cm. **(Shinozaki *et al*., 2018a)**.

- **Inflorescence**

A maximum number of inflorescence forty-five was recorded on genotype (1) at the main stem, while a minimum of one inflorescence on the main stem was recorded on genotype 2, from 156 genotypes **(Ali *et al*., 2017)**.

Between two groups that were grown in the greenhouse, a visual screening found a mutant (TOMJPE2753) exhibiting greater plant height than wild-type (WT) and parthenocarpy (Figure 16 a, b). This mutant produced flowers contained an elongated style protruding from the anther (Figure 16 c), and leaflets with smoother margins (Figure 16 d) compared to the WT **(Shinozaki *et al*., 2018a)**.

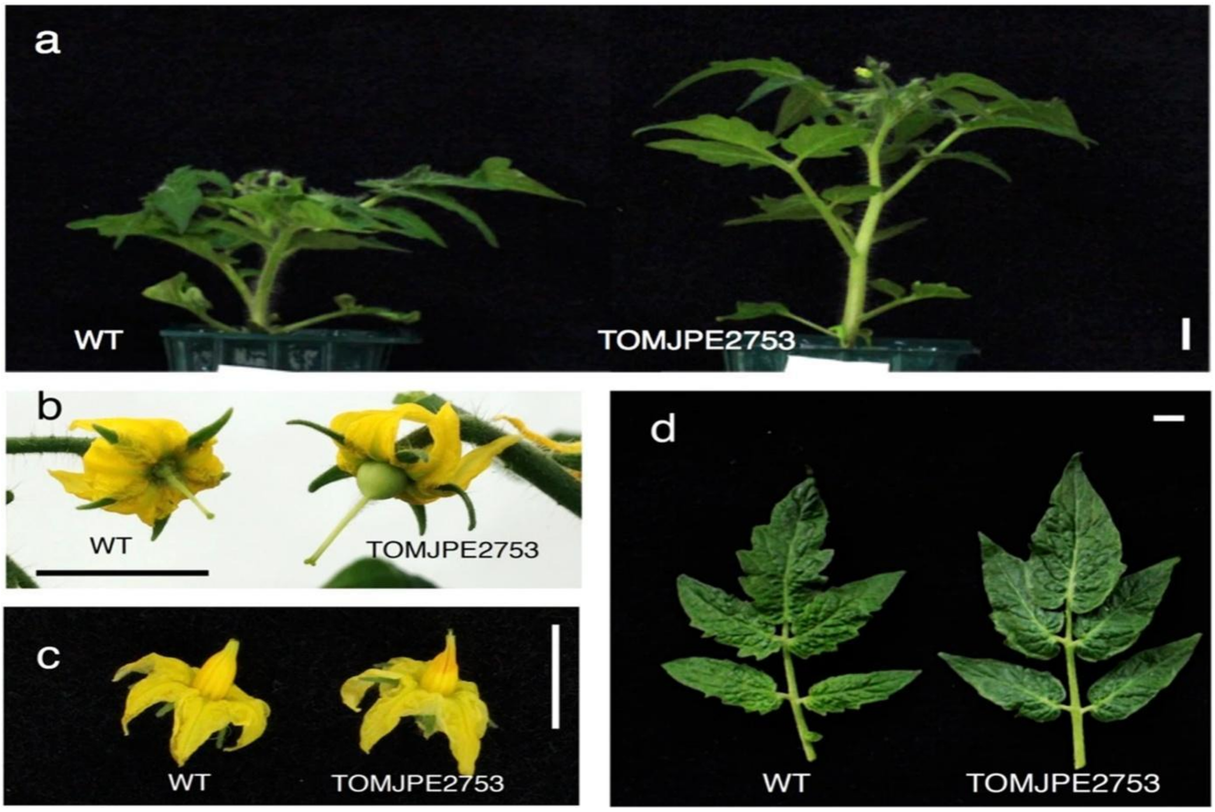


Figure 16: Characteristic mutant phenotypes in the TOMJPE2753 line. (a) Six-week-old plants. (b) Parthenocarpic fruit initiation from an emasculated flower in TOMJPE2753 at 4 days afteranthesis. (c) Flowers with stamen and style. (d) Leaflets of the 6th node. Bars = 1 cm. **(Shinozaki *et al*., 2018a)**.

- **Fruits**

Systematically, tomato is a plant grown to obtain its fruits. When tomatoes are green at first, they turn yellow, orange and red on ripening (Figure 17). Most varieties produce from 4.5 to 7 kg of fruits per plant **(Alajrami and Abu-naser, 2019)**. It has been shown that thirty-three genotypes have very small fruit size, sixty-four genotypes were observed to have small fruit size, forty-three genotypes have intermediate fruit size, and thirteen genotypes were recorded to have large fruit size while three genotypes were having very large fruit size. Fruit length (mm) Maximum fruit length 67.2 mm was observed in genotype LA while minimum fruit length 1.43 mm was observed in genotype N**.** Yield per plant (g) Maximum yield 354.4 g was recorded on genotype 17863 while the minimum yield of 4.8 g was recorded on Tom-2 genotype **(Ali *et al*., 2017)**. Representative pictures of harvested fruit spanning ten developmental stages are illustrated in Figure 17 and the cross-sectional view of tomato in Figure 18.

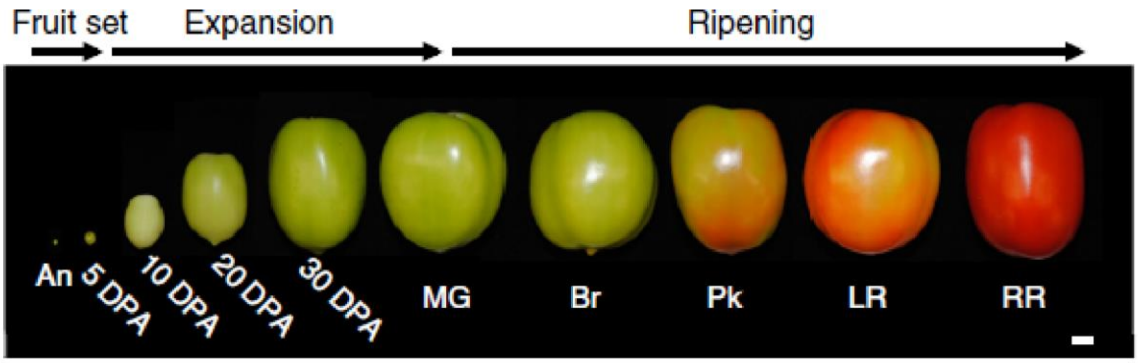


Figure 17: Representative pictures of harvested fruit spanning ten developmental stages **(Shinozaki *et al*., 2018b).**

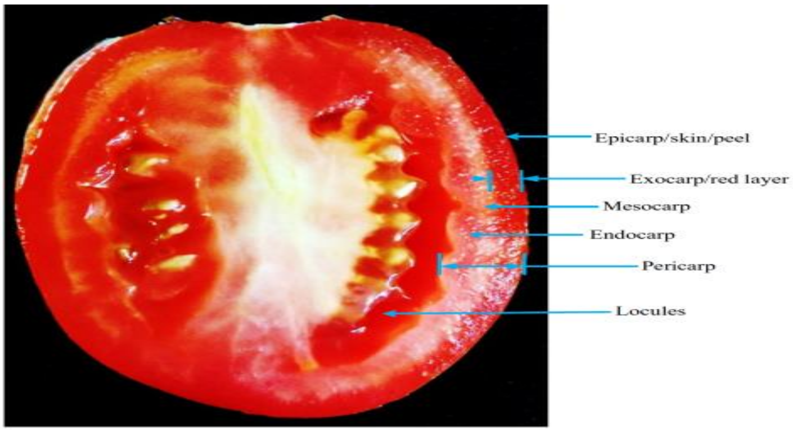


Figure 18: A cross-sectional view of the tomato cultivar **(Vidyarthi, 2017).**

III.8.2 Root system

Plant roots exploit morphological plasticity to adapt and respond to different soil environments **(Alaguero-Cordovilla *et al*., 2018)**. The root system is highly developed and swivel with numerous roots that grow to a depth of 50 cm or more **(Shankara *et al*., 2005)**. **Alaguero-Cordovilla *et al*. (2018)** found extensive differences between the studied lines for several meaningful morphological traits, such as lateral root distribution, lateral root length, or adventitious root development, which might represent adaptations to local soil conditions during speciation and subsequent domestication. This study provides a general quantitative framework for comparing root system architecture in tomato seedlings and other related species (Figure 19). In this investigation, several meaningful traits in tomato root system architecture

(RSA) (such as lateral root (LR) distribution in the soil depth, LR length or adventitious roots (AR) number) displayed significant variation in a reduced number of commercial cultivars and related wild species (Figure 20) **(Alaguero-Cordovilla *et al.*, 2018)**.

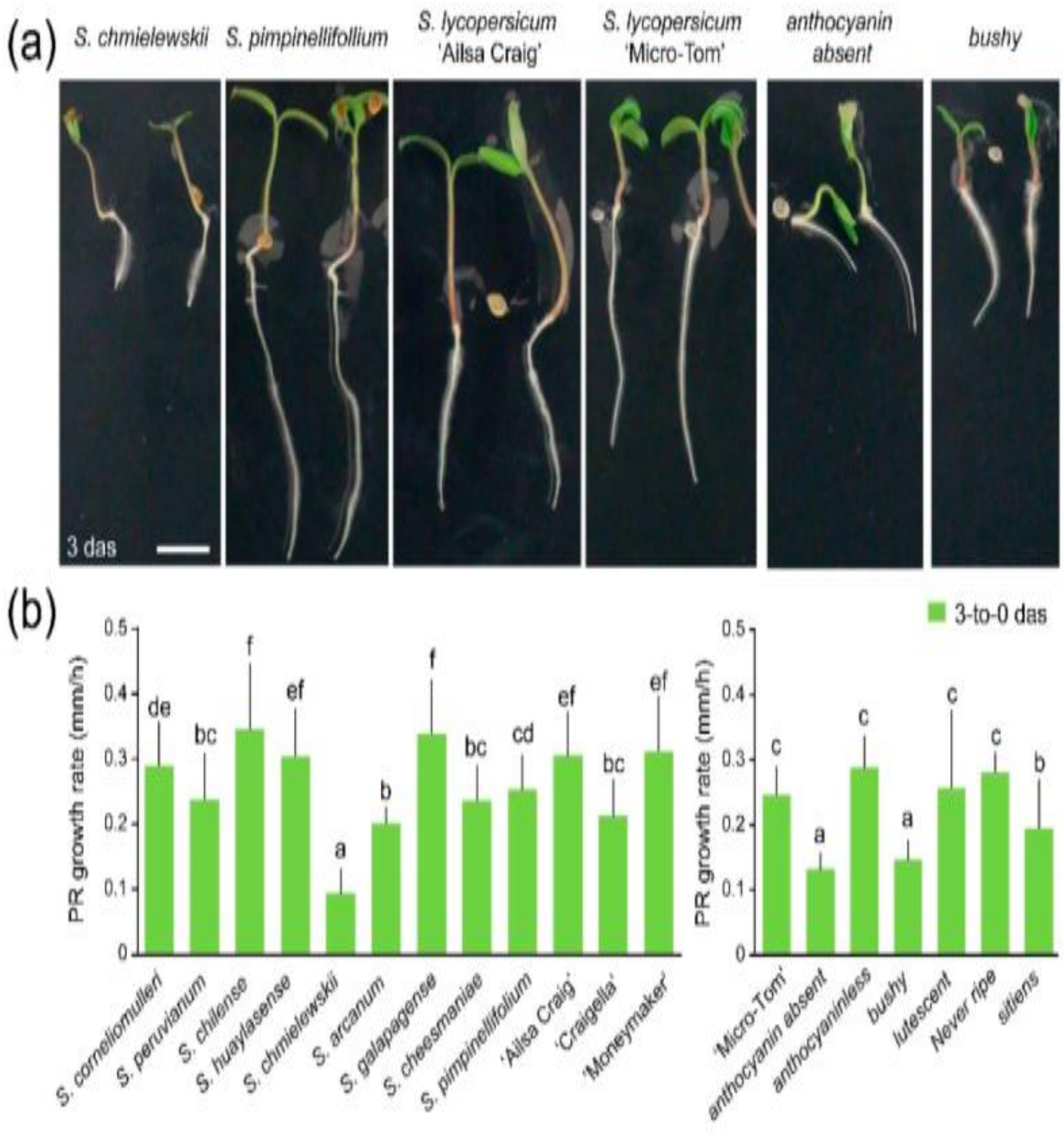


Figure 19: Early growth variation of selected tomato genotypes. (a) Seedlings of tomato genotypes differing in early root growth. Scale bar: 10 mm. (b) Growth rate (mm/h) of primary root (PR) in wild tomato species and commercial tomato cultivars (left), as well as in developmental mutants in the 'Micro-Tom' genetic background (right), das: days after sowing **(Alaguero-Cordovilla *et al.*, 2018)**.

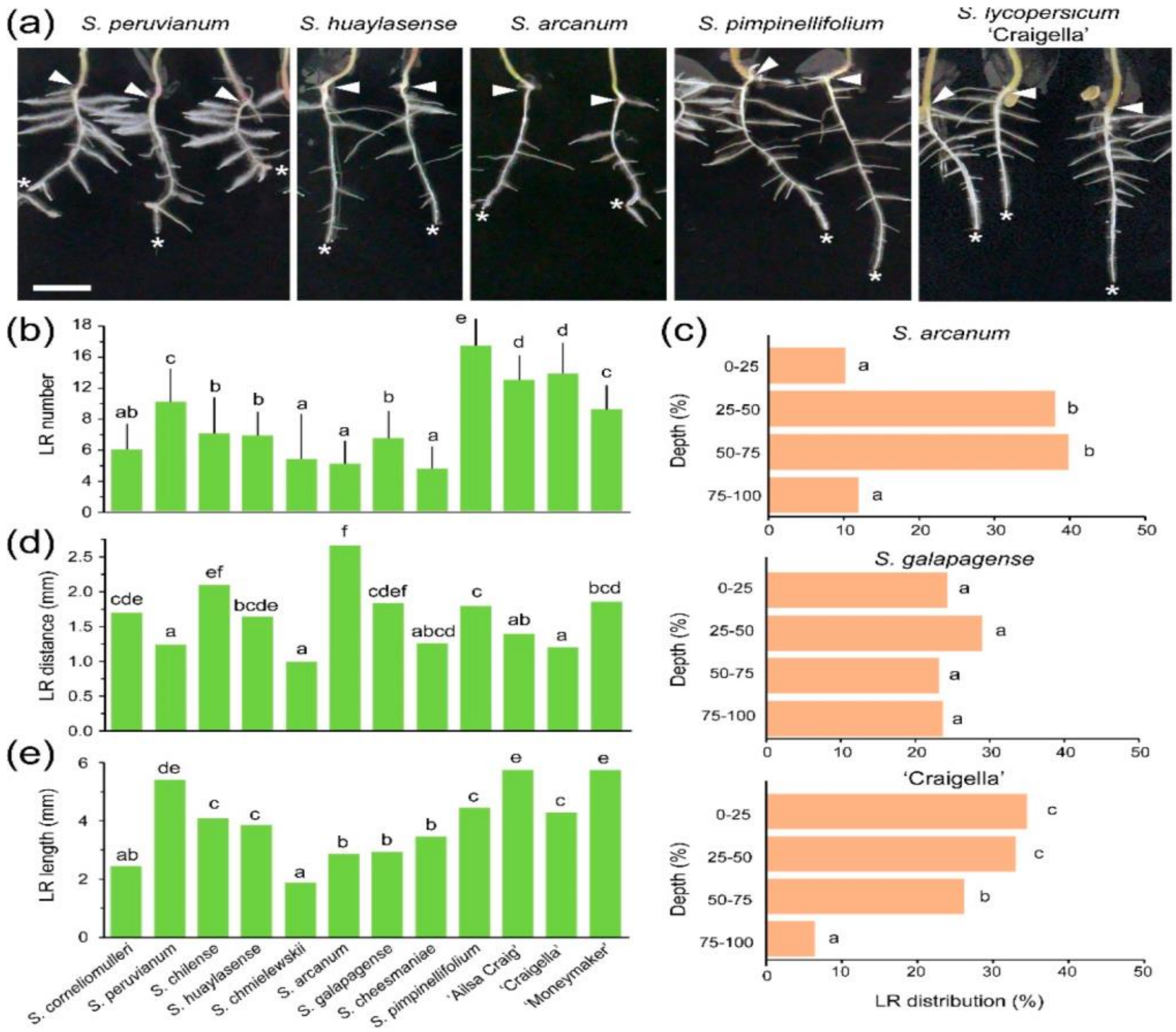


Figure 20: Lateral root (LR) development in wild tomato species and commercial tomato cultivars after root tip excision. (a) Representative images of the entire root system of selected genotypes at 3 days after root tip excision. The arrowhead points to the root-hypocotyl junction, and the asterisk indicates the tip of the primary root (PR). Scale bar: 10 mm. (b) Number of LRs. (c) Average percentages of LR distribution along the length (i.e., depth) of the PR. (d) Distance between consecutive LRs and (e) LR length; median values are shown **(Alaguero-Cordovilla *et al*., 2018)**.

To recapitulate, this section describes characterization of tomato genotypes based on quantitative characteristics. The information generated in this section will be useful for better crop improvement strategies in tomatoes **(Ali *et al*., 2017)**. Therefore**,** these characteristics may be more important in tomato plants under biotic and abiotic stresses.

III.9. Response of tomato to environmental stress

III.9.1 Tomato and biotic stress

The biotic stresses in plants have a significant impact on agricultural productivity **(Amjad *et al*., 2019a)**. Tomato is one of the most crops affected by this type of stress. and Some of its key pests are whiteflies, tomato leafminer, spider mites, dipteran leafminers, thrips, aphids, and noctuid moths. Plant viral diseases transmitted by insects caused severe damages in tomato plants, such as TYLCV, TICV, ToCV and ToTV (by whiteflies), TSWV (by thrips), and PVY (by aphids). Airborne fungal plant pathogens (*Botrytis* spp., *Oidium* spp., *Phytophthora* spp., and *Alternaria* spp.), and soilborne plant pathogens (*Verticillium* spp., *Fusarium* spp., *Rhizoctonia* spp.) can also affect tomato crop **(Castañé *et al*., 2020).**

III.9.1.1. Early biotic stress detection in tomato (*Solanum lycopersicum* Mill.) by BVOC emissions

As a general aim, it has been investigated the impacts of early and mild biotic stress on Biogenic Volatile Organic Compounds (BVOC) emissions from tomato, to test their potential for early (biotic) stress detection. Plants were exposed to methyl jasmonate (MeJA), to identify BVOC emissions related to activation of the jasmonic acid (JA) signaling pathway. These emissions were used as a reference for identifying active JA signaling pathway in plants at the early stages of biotic stress. BVOC emissions during *Myzuspersicae* attack had changed before the occurrence of visual symptoms. Despite low infestation rates, plants emitted methyl salicylate (SA) indicating activation of the SA-mediated defense pathway **(Kasal-slavik *et al*., 2017)**.

III.9.1.2. Effect of stress on tomatoes (Example: Blossom-end rot)

Blossom-end rot is a physiological disorder in tomatoes; is caused by many factors including calcium, abiotic stress, etc. These factors are indicated in (Figure 21) **(Hagassou *et al*., 2019)**.

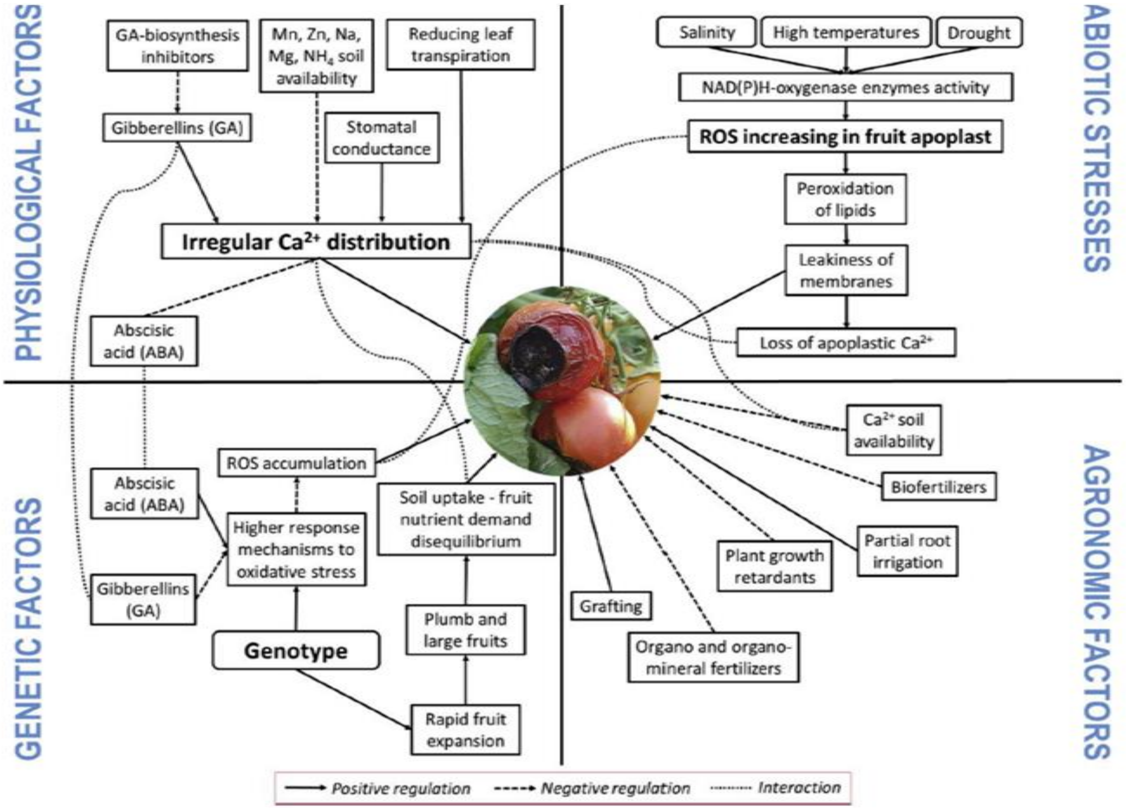


Figure 21: Graphical summary of the factors influencing blossom-end rot. Major components are in bold, while specific interactions are shown with different connectors, as indicated **(Hagassou *et al*., 2019).**

III.9.2 Tomato and abiotic stress

In short, tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops; its production, plant growth, productivity, and quality are adversely affected by abiotic stresses such as drought, high salinity, extreme temperature, and heavy metals, affect almost every stage of the tomato life cycle. Depending upon the plant stage and duration of the stress, abiotic stress causes about 70 % yield loss **(Blanchard-Gros *et al*., 2020 ; Krishna *et al*., 2019)**.

III.9.2.1. Drought stress

The problems of salinity, drought, and soil contamination are increasing rapidly throughout the globe and severely affecting more than 10 % of arable land resulting in a reduction of more than 50 % average yields of major crops including tomato. Therefore, sustainable agriculture is in great demand under the current alarming condition of food security **(Singh *et al*., 2018c)**. Increasing drought, under current climate change scenarios, will reduce the sustainability of tomato cultivation in the Mediterranean region **(Fracasso *et al*., 2020)**. The number of leaves and the plant size of sensitive accessions were reduced by 25 - 40 % underwater stress compared to controls. The resistant accessions did not show any difference in size and number of leaves between treatments (**Blanchard-Gros *et al*., 2020**). A recent study by **Fracasso *et al*. (2020)** revealed the positive effect of *Rhizophagus intraradices* inoculation on tomato plant physiology and yield in response to progressive water deficit conditions, and general response at physiological plane caused by deficit irrigation in tomato fruit illustrated in Figure 22.

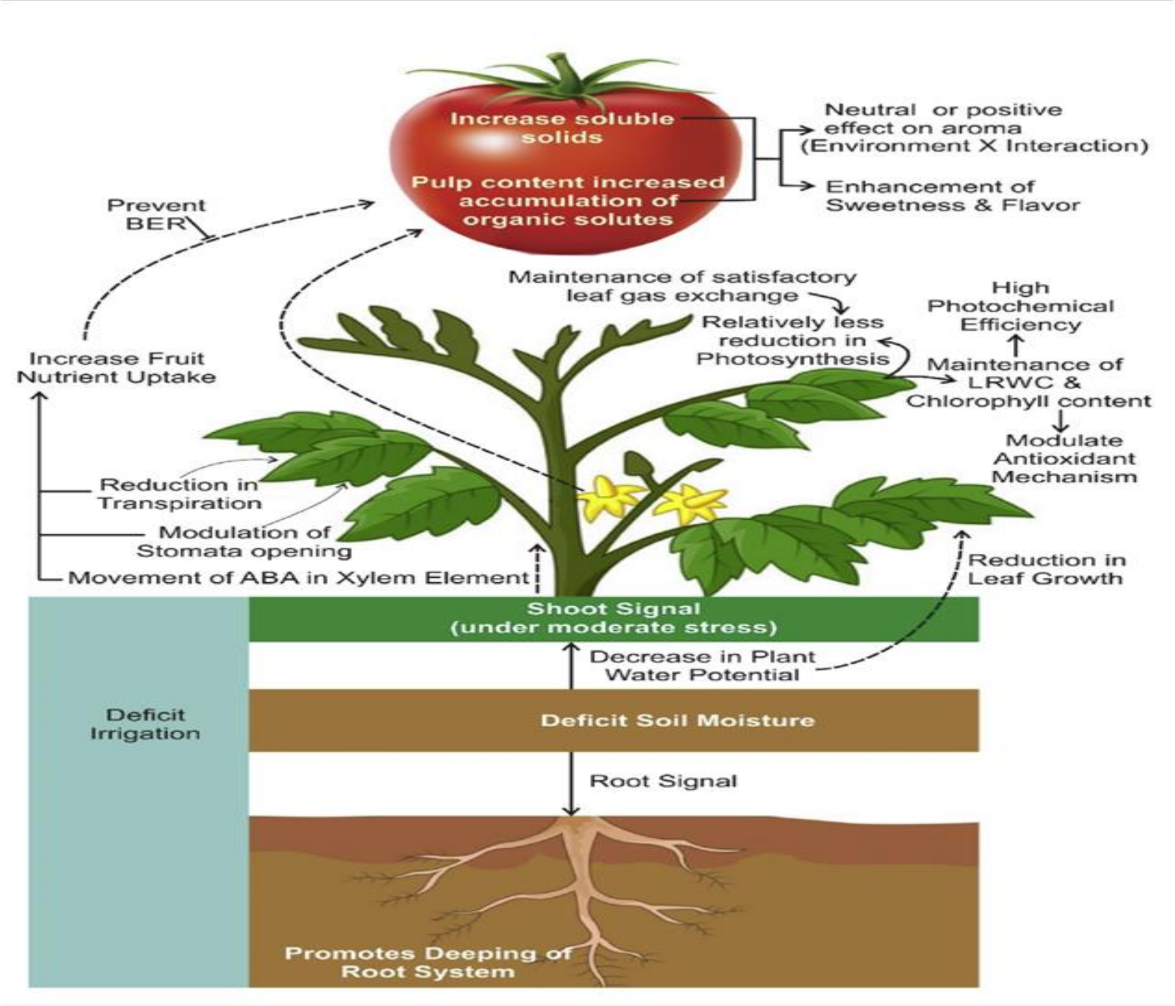


Figure 22: General response at the physiological plant caused by deficit irrigation in tomato fruit **(Khapte *et al*., 2019).**

The response of chlorophyll fluorescence parameters in various grafting tomato seedlings to drought stress are presented in Figure 23. Drought stress decreases all photosynthetic parameters in fully expanded leaves, including net CO_2 assimilation, photosynthetic rate (Pn), and transpiration (Tr). The resulting four groups of seedlings were designated as the following: based on the tomato species: T/T (the combination of tolerant scion and tolerant rootstock), T/S (the combination of tolerant scion and susceptible rootstock), S/T (the combination of susceptible scion and tolerant rootstock), and S/S (the combination of susceptible scion and susceptible rootstock) **(Zhang *et al*., 2019c)**. Therefore, chlorophyll fluorescence analysis of transgenic plants demonstrated an increased photosynthetic activity as well as contents of chlorophyll, proline, and sugar under drought stress **(Muhammad *et al*., 2019)**.

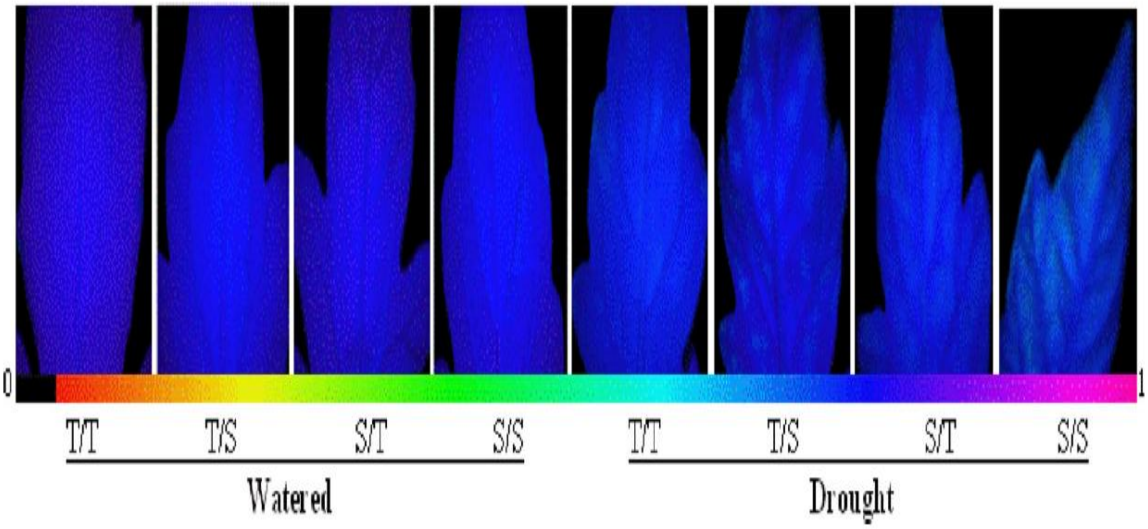


Figure 23: Maximum photochemical efficiency of photosystem II (PSII) (Fv/Fm) was determined 15 days after drought treatment **(Zhang *et al*., 2019b).**

III.9.2.2. Salinity stress

On the whole, Salinity affects glycophyte plants such as tomato (*Solanum Lycopersicum* Mill.) at the whole-plant level. Although soil salinity is a growing problem in the world and tomato is the second most highly produced vegetable crop worldwide, little is known about traits that influence salt resistance at the reproductive stage in this species. The reproductive stage is often more sensitive to abiotic stress than the vegetative stage and represents thus a major issue for the tomato industry**.** The resistance to salinity is a multi-gene mechanism and de novo domestication of wild relative species could be an efficient strategy to improve salt-resistance in cultivated tomato **(Bigot *et al*., 2020).** In synopsis, tomato (*Lycopersicon esculentum* Mill.) is one of the most important fruits and vegetables. The soil salinity is prevalent abiotic stress that adversely affects crop productivity worldwide, that's why it is an environmental stress that limits growth and development in fruits and vegetables due to increased osmotic pressure, imbalance of nutrients, and toxicity of some special ions **(Alavi and Abbaspour, 2020)**.

III.9.2.3. Heat stress

Heat stress is a major limiting factor for crop productivity, besides the tomato is highly sensitive to heat stress, which can result in a total yield loss, and to adapt to current and future heat stress, there is a dire need to develop heat-tolerant cultivars **(Ayenan *et al*., 2019)**. The Heat stress tomato poses a major threat to plant productivity and crop yields. The induction of heat shock proteins (HSPs) by heat shock factors is a principal defense response of plants exposed to heat stress **(Tonhati *et al*., 2020).** The global climate change brings elevated average temperatures and more frequent and extreme weather events, pressure from biotic stresses will become increasingly compounded by harsh abiotic stress conditions **(Havko *et al.,* 2020)**. As such, heat stress affects the physiological and molecular mechanisms of a tomato plant.

III.9.2.4. Heavy metal stress

As essential plant nutrients, mineral sufficiency ranges of Zn and Cu elements in tomato plant at early fruit set stage of growth for optimal plant nutrition was recommended as 25-40 ppm and 5-15 ppm, respectively **(Maynard and Hochmuth, 2007).**

Notably, exposure of tomato plants to heavy metal stress can cause morphological and physiological changes in the stressed plants **(Muhammad *et al*., 2019)**.Treated tomato plants were affected by heavy metal toxicity stress presenting a reduction in the following parameters: dry matter, shoot, root growth, pigment concentration (chlorophyll, carotenoid, and xanthophyll), antioxidative enzymes and soluble proteins, and the high levels of heavy metal in tomato plant cause oxidative stress, as expressed by the increase of lipid peroxidation products, ascorbic acid (ASA), sulphydryl (SH) groups and protein amount compared to control plants **(Heybet and Borlu, 2020 ; Khanna *et al*., 2019a)**. Similar to drought stress, heavy metal stress affects many physiological and metabolic processes including photosynthesis, respiration, and overall plant growth **(Muhammad *et al*., 2019)**.

More details about this stress will be discussed in the present investigation.

a. Tomato tolerance to heavy metal stress by accumulating signaling compounds

Under stress conditions, plant hormone-like ethylene is known to endogenously regulate the homeostasis of plants leading to a significant reduction in root and shoot growth **(Singh *et al*., 2018c)**. Indeed, the plant hormones signaling in tomato plants under heavy metal stress are; auxin, cytokinin, abscisic acid, gibberellic acid, ethylene, jasmonic acid, nitric oxide,.. **(Jahan *et al*., 2019 ; Scalschi *et al*., 2020)**. Osmoregulation and antioxidants compounds such as; proline and thiols, polyphenols, flavonoids, and soluble sugar were the main defense mechanisms activated by tomato plants **(El-Esawi *et al*., 2020 ; Sousa *et al*., 2020)**,also, the heat shock proteins **(Cui *et al*., 2019)**.

b. Tomato tolerance to heavy metal stress by PGPB

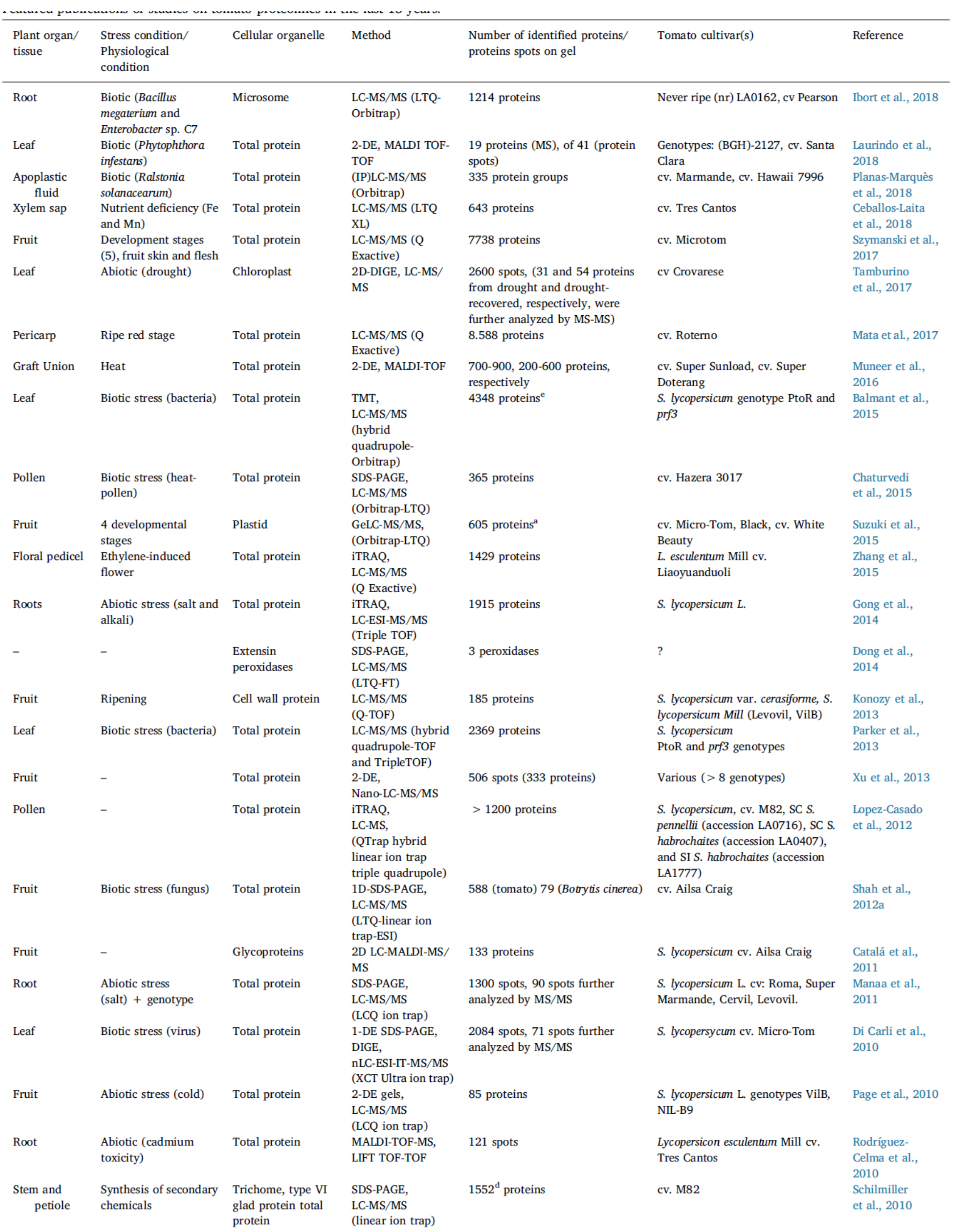
Plant growth-promoting rhizobacteria (PGPR) are very effective in immobilization of heavy metals and reducing their translocation in plants via precipitation, complex formation, and adsorption. Gene expression studies suggested enhanced expression in the metal transporter genes which were further declined in the microbe supplemented tomato seedlings. Therefore, micro-organisms possess growth-promoting traits that enable them to reduce metal toxicity in plants **(Khanna *et al*., 2019a)**. The fungal species belonging to the *Trichoderma* genus are the most promising microorganisms which improve plant growth by enhancing nutrient uptake and preventing them against environmental biotic and abiotic stresses **(Khoshmanzar *et al.*, 2020)**. ACC deaminase (Enzyme 1-aminocyclopropane-1-carboxylate deaminase) production by PGPB reduces ethylene concentration and helps plants to rescue the drought and salt stresses, heavy metals, etc. Certain rhizosphere-associated PGPB produces antioxidants and volatiles that results in significant degradation of ROS **(Singh *et al.,* 2018c)**.

III.10. Proteomics and tomato stress response

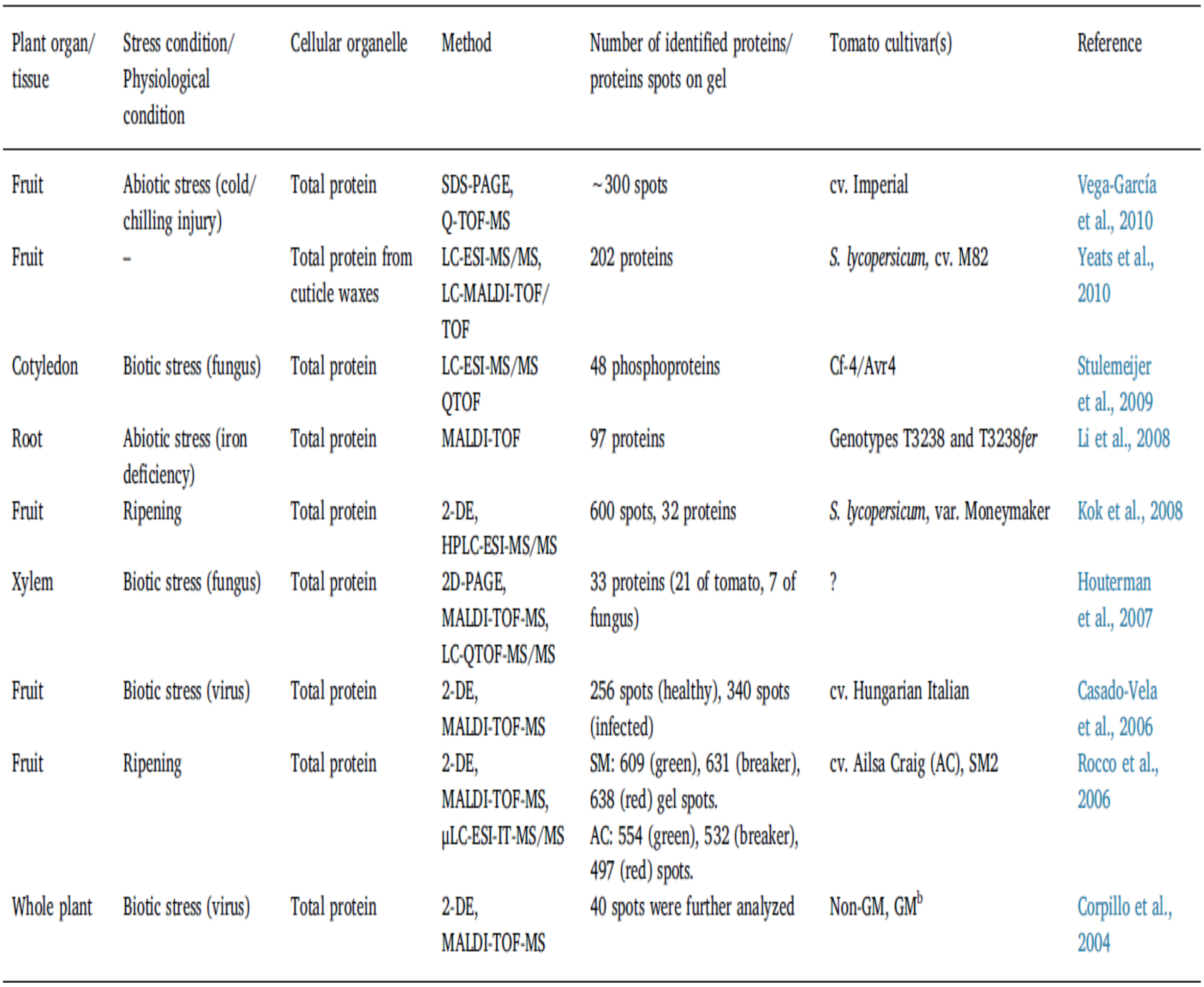
On a larger scale, the understanding of plant stress response is essential to develop crops that are capable of withstanding adverse conditions. The development of proteomics led to the characterization of many of the metabolic pathways involved in plant resistance and adaptation to abiotic stresses. Although these proteomic strategies have been successfully applied to studies with tomato (*Solanum lycopersicum* L.), a major challenge of crop proteomics is the lack of genetic functional information when compared to the model plant, *Arabidopsis thaliana.* Still, the tomato has been the model crop for genetic and molecular research of the Solanaceae family due to its attributes of diploidy, easy genetic transformation, and many genetic resources. Consequently, the necessity of better strategies to increase the genetics and proteomics resources of tomato is in high demand. Many studies have explored the various proteomics methodologies used in studies on tomato plants **(Sant'Ana and Lefsrud, 2018)**.

Featured publications of studies on tomato proteomics in the last 15 years are presented in Table 4.

Table 4: Featured publications of studies on tomato proteomics in the last 15 years **(Sant'Ana and Lefsrud, 2018)**



Plant organ/ tissue	Stress condition/ Physiological condition	Cellular organelle	Method	Number of identified proteins/ proteins spots on gel	Tomato cultivar(s)	Reference
Root	Biotic (*Bacillus megaterium* and *Enterobacter* sp. C7	Microsome	LC-MS/MS (LTQ-Orbitrap)	1214 proteins	Never ripe (nr) LA0162, cv Pearson	Ibort et al., 2018
Leaf	Biotic (*Phytophthora infestans*)	Total protein	2-DE, MALDI TOF-TOF	19 proteins (MS), of 41 (protein spots)	Genotypes: (BGH)-2127, cv. Santa Clara	Laurindo et al., 2018
Apoplastic fluid	Biotic (*Ralstonia solanacearum*)	Total protein	(IP)LC-MS/MS (Orbitrap)	335 protein groups	cv. Marmande, cv. Hawaii 7996	Planas-Marquès et al., 2018
Xylem sap	Nutrient deficiency (Fe and Mn)	Total protein	LC-MS/MS (LTQ XL)	643 proteins	cv. Tres Cantos	Ceballos-Laita et al., 2018
Fruit	Development stages (5), fruit skin and flesh	Total protein	LC-MS/MS (Q Exactive)	7738 proteins	cv. Microtom	Szymanski et al., 2017
Leaf	Abiotic (drought)	Chloroplast	2D-DIGE, LC-MS/MS	2600 spots, (31 and 54 proteins from drought and drought-recovered, respectively, were further analyzed by MS-MS)	cv Crovarese	Tamburino et al., 2017
Pericarp	Ripe red stage	Total protein	LC-MS/MS (Q Exactive)	8.588 proteins	cv. Roterno	Mata et al., 2017
Graft Union	Heat	Total protein	2-DE, MALDI-TOF	700-900, 200-600 proteins, respectively	cv. Super Sunload, cv. Super Doterang	Muneer et al., 2016
Leaf	Biotic stress (bacteria)	Total protein	TMT, LC-MS/MS (hybrid quadrupole-Orbitrap)	4348 proteins[e]	*S. lycopersicum* genotype PtoR and *prf3*	Balmant et al., 2015
Pollen	Biotic stress (heat-pollen)	Total protein	SDS-PAGE, LC-MS/MS (Orbitrap-LTQ)	365 proteins	cv. Hazera 3017	Chaturvedi et al., 2015
Fruit	4 developmental stages	Plastid	GeLC-MS/MS, (Orbitrap-LTQ)	605 proteins[a]	cv. Micro-Tom, Black, cv. White Beauty	Suzuki et al., 2015
Floral pedicel	Ethylene-induced flower	Total protein	iTRAQ, LC-MS/MS (Q Exactive)	1429 proteins	*L. esculentum* Mill cv. Liaoyuanduoli	Zhang et al., 2015
Roots	Abiotic stress (salt and alkali)	Total protein	iTRAQ, LC-ESI-MS/MS (Triple TOF)	1915 proteins	*S. lycopersicum L.*	Gong et al., 2014
–	–	Extensin peroxidases	SDS-PAGE, LC-MS/MS (LTQ-FT)	3 peroxidases	?	Dong et al., 2014
Fruit	Ripening	Cell wall protein	LC-MS/MS (Q-TOF)	185 proteins	*S. lycopersicum* var. *cerasiforme, S. lycopersicum Mill* (Levovil, VilB)	Konozy et al., 2013
Leaf	Biotic stress (bacteria)	Total protein	LC-MS/MS (hybrid quadrupole-TOF and TripleTOF)	2369 proteins	*S. lycopersicum* PtoR and *prf3* genotypes	Parker et al., 2013
Fruit	–	Total protein	2-DE, Nano-LC-MS/MS	506 spots (333 proteins)	Various (> 8 genotypes)	Xu et al., 2013
Pollen	–	Total protein	iTRAQ, LC-MS, (QTrap hybrid linear ion trap triple quadrupole)	> 1200 proteins	*S. lycopersicum*, cv. M82, SC *S. pennellii* (accession LA0716), SC *S. habrochaites* (accession LA0407), and SI *S. habrochaites* (accession LA1777)	Lopez-Casado et al., 2012
Fruit	Biotic stress (fungus)	Total protein	1D-SDS-PAGE, LC-MS/MS (LTQ-linear ion trap-ESI)	588 (tomato) 79 (*Botrytis cinerea*)	cv. Ailsa Craig	Shah et al., 2012a
Fruit	–	Glycoproteins	2D LC-MALDI-MS/MS	133 proteins	*S. lycopersicum* cv. Ailsa Craig	Catalá et al., 2011
Root	Abiotic stress (salt) + genotype	Total protein	SDS-PAGE, LC-MS/MS (LCQ ion trap)	1300 spots, 90 spots further analyzed by MS/MS	*S. lycopersicum* L. cv: Roma, Super Marmande, Cervil, Levovil.	Manaa et al., 2011
Leaf	Biotic stress (virus)	Total protein	1-DE SDS-PAGE, DIGE, nLC-ESI-IT-MS/MS (XCT Ultra ion trap)	2084 spots, 71 spots further analyzed by MS/MS	*S. lycopersycum* cv. Micro-Tom	Di Carli et al., 2010
Fruit	Abiotic stress (cold)	Total protein	2-DE gels, LC-MS/MS (LCQ ion trap)	85 proteins	*S. lycopersicum* L. genotypes VilB, NIL-B9	Page et al., 2010
Root	Abiotic (cadmium toxicity)	Total protein	MALDI-TOF-MS, LIFT TOF-TOF	121 spots	*Lycopersicon esculentum* Mill cv. Tres Cantos	Rodríguez-Celma et al., 2010
Stem and petiole	Synthesis of secondary chemicals	Trichome, type VI glad protein total protein	SDS-PAGE, LC-MS/MS (linear ion trap)	1552[d] proteins	cv. M82	Schilmiller et al., 2010



Plant organ/ tissue	Stress condition/ Physiological condition	Cellular organelle	Method	Number of identified proteins/ proteins spots on gel	Tomato cultivar(s)	Reference
Fruit	Abiotic stress (cold/ chilling injury)	Total protein	SDS-PAGE, Q-TOF-MS	~300 spots	cv. Imperial	Vega-García et al., 2010
Fruit	-	Total protein from cuticle waxes	LC-ESI-MS/MS, LC-MALDI-TOF/ TOF	202 proteins	*S. lycopersicum*, cv. M82	Yeats et al., 2010
Cotyledon	Biotic stress (fungus)	Total protein	LC-ESI-MS/MS QTOF	48 phosphoproteins	Cf-4/Avr4	Stulemeijer et al., 2009
Root	Abiotic stress (iron deficiency)	Total protein	MALDI-TOF	97 proteins	Genotypes T3238 and T3238*fer*	Li et al., 2008
Fruit	Ripening	Total protein	2-DE, HPLC-ESI-MS/MS	600 spots, 32 proteins	*S. lycopersicum*, var. Moneymaker	Kok et al., 2008
Xylem	Biotic stress (fungus)	Total protein	2D-PAGE, MALDI-TOF-MS, LC-QTOF-MS/MS	33 proteins (21 of tomato, 7 of fungus)	?	Houterman et al., 2007
Fruit	Biotic stress (virus)	Total protein	2-DE, MALDI-TOF-MS	256 spots (healthy), 340 spots (infected)	cv. Hungarian Italian	Casado-Vela et al., 2006
Fruit	Ripening	Total protein	2-DE, MALDI-TOF-MS, μLC-ESI-IT-MS/MS	SM: 609 (green), 631 (breaker), 638 (red) gel spots. AC: 554 (green), 532 (breaker), 497 (red) spots.	cv. Ailsa Craig (AC), SM2	Rocco et al., 2006
Whole plant	Biotic stress (virus)	Total protein	2-DE, MALDI-TOF-MS	40 spots were further analyzed	Non-GM, GM^b	Corpillo et al., 2004

III.11. Omics applications: towards sustainable protection of tomato

The transcriptome data and gene expression analysis have a huge potential in the study of multiple relationships involving plants, pathogens, and pests, including the interactions with beneficial microorganisms such as; endophytes or other functional groups. Next-generation sequencing (NGS) and other recent long-read-based sequencing approaches (i.e., nanopore and others) provide unprecedented tools allowing the fast identification of plant information processing systems, in situ and real-time, fundamental for crop management and pest regulation. Other omics approaches such as metagenomics and metatranscriptomics allow high-resolution insights on the rhizosphere ecology. Furthermore, they may highlight key factors affecting belowground biodiversity or processes, modulating the expression of stress-responsive pathways. The application of miRNAs and other small RNAs is a relatively new field of application, with enormous potential for the selective activation of defense pathways. However, limitations concerning the stability of the RNA molecules and their effective delivery must be overcome **(Pentimone *et al*., 2020).**

III.12. Tomato tolerance and gene expression

III.12.1 Transgenic tomatoes for abiotic stress tolerance

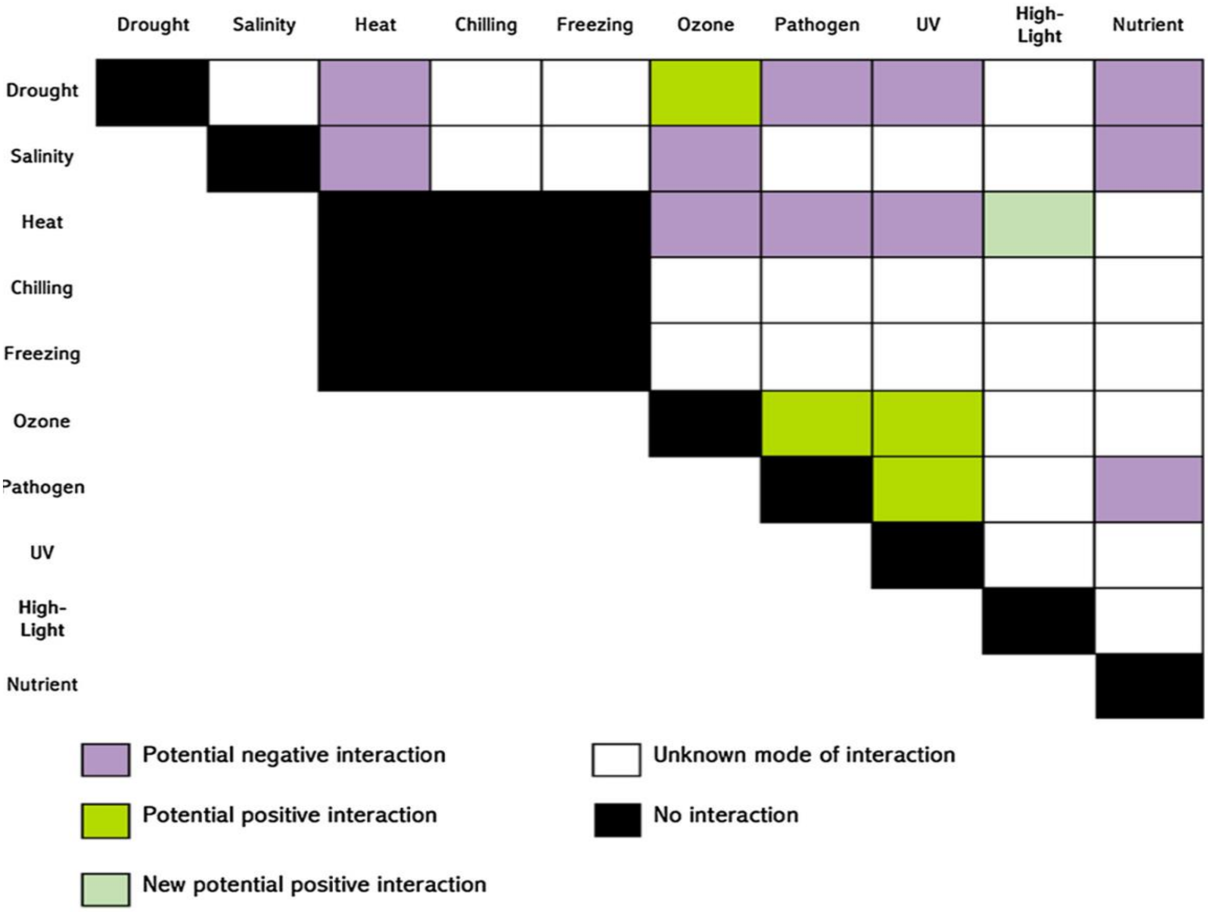
Several wild tomato species have the stress tolerance genes; however, it is very difficult to transfer them into cultivars due to high genetic distance and crossing barriers. Transgenic technology is an alternative potential tool for the improvement of tomato crops to cope with abiotic stress, as it allows gene transfer across species. In recent decades, many transgenic tomatoes have been developed, and many more are under progress against abiotic stress using transgenes such as *DREBs, Osmotin*, *ZAT12*, and *BADH2*. The altered expression of these transgenes under abiotic stresses are involved in every step of stress response, such as; signalling, control of transcription, proteins and membrane protection, compatible solute (betaines, sugars, polyols, and amino acids) synthesis, and free-radical and toxic-compound scavenging. The stress-tolerant transgenic tomato development is based on the introgression of a gene with a known function in stress response and putative tolerance. Despite this, transgenic tomato plants have been developed against drought, heat, and salt, heavy metal stress with the help of various transgenes, the expression of which manages the stress at the cellular level by modulating the expression of downstream genes to ultimately improve growth and yield of tomato plants and help in sustainable agricultural production. The transgenic technology could be a faster way towards tomato improvement against abiotic stress **(Krishna *et al*., 2019)**.

III.12.1.1. Tomato stress tolerance (Example)

Tomato is an important food material and cash crop, as well as a model plant for genetic evolution and molecular biology research. However, as a cold-sensitive crop originating from the tropics, the growth and development of tomato are often affected by low-temperature stress. The overexpressing *LeCOLD1* in tomatoes increases the plants' resistance to low temperatures, and that reducing *LeCOLD1* expression makes the plants more sensitive to low temperatures **(Zhang *et al*., 2020)**.

III.12.2 Tomato and stress combinations (Example)

Today, the matrix represents agricultural important stress combinations (Figure 24). In this version, a piece of new information on the interaction between high-light and temperature is provided. High-light and heat effects were investigated by **Gerganova *et al*. (2016),** reporting a better acclimation of tomato plants under the combined stress conditions. A smaller impact in the photochemical quenching and photosystem II (PSII) quantum yield was seen when both stresses occurred simultaneously, suggesting that high temperature might mitigate the impact of non-photochemical quenching caused by high-light. The Essential elements are including in the frame of "Nutrient".



	Drought	Salinity	Heat	Chilling	Freezing	Ozone	Pathogen	UV	High-Light	Nutrient
Drought	■	□	🟪	□	□	🟩	🟪	🟪	□	🟪
Salinity		■	🟪	□	□	🟪	□	□	□	🟪
Heat			■	■	■	🟪	🟪	🟪	▨	□
Chilling			■	■	■	□	□	□	□	□
Freezing			■	■	■	□	□	□	□	□
Ozone						■	🟩	🟩	□	□
Pathogen							■	🟩	□	🟪
UV								■	□	□
High-Light									■	□
Nutrient										■

🟪 Potential negative interaction

🟩 Potential positive interaction

▨ New potential positive interaction

□ Unknown mode of interaction

■ No interaction

Figure 24: The “Stress Matrix”. Figure modified from reference (Mittler, 2006) **(Sant’Ana and Lefsrud, 2018)**

III.12.3 Tomato tolerance and stress combinations

The genotypes tolerant against one stress can be regarded as tolerant against other stress as well. A previous study revealed that tomato genotypes and hybrids shared the same defense mechanism against salinity and Ni-toxicity tolerance **(Amjad *et al*., 2019b)**. Plant breeding is still needed to develop resistant cultivars and the elucidation of tolerance strategies in wild relatives is a powerful approach to understand local adaptations for potential crop improvement **(Blanchard-Gross et *al*., 2020)**. The development of tomato cultivars with enhanced abiotic stress tolerance is one of the most sustainable approaches for its successful production **(Chaudhary *et al*., 2019)**.

CHAPTER IV

Havy Metals in Soil

IV.1. Introduction

In industrialized countries, there is a growing concern about the possible negative effects on human health induced by high levels of heavy metals in soil. It is recognized that high levels of heavy metals are not necessarily indicative of the adverse effects. These effects are related to bioavailable fractions, which are involved in plant uptake and transfer to the food chain **(Petruzzelli *et al*., 2020).**

Different concentrations of heavy metals (copper and zinc) in soil and plant are presented in Table 5.

Table 5: Soil concentration ranges and regulatory guidelines for some heavy metals **(NJDEP, 1996 ; Salt *et al*., 1995 ;)**, target values are specified to indicate desirable maximum levels of elements in unpolluted soils, the permissible limit recommended by Dutch standard **(Denneman and Robberse, 1994),**WHO permissible limits for heavy metals in plant and soil **(Who, 1996).**

Tolat heavy meatl in soil	Soil concentration range (mg. kg^{-1})	Regulatory limits (mg. kg^{-1})	Target value of soil (mg. kg^{-1})	Permissible value of plants (mg. kg^{-1})
Cu	50- 1550	600	36	10
Zn	150–5 000	1 500	50	0.6

Accordingly, total metal in the soil depending on pH is descirbed in Table 6.

Table 6: Total metal in the soil depending on pH **(Council of the European Communities, 1986).**

Total heavy metal	pH 5- 6 mg. kg^{-1} oven dry soil	pH > 6 mg. kg^{-1} oven dry soil
Cu	50	140
Zn	150	300

IV.2. Bioavailability

Bioavailability is a complex issue that is essential to evaluate to determine if heavy metals present in soil may pose hazards to humans and the environment. In the case of direct ingestion of soil, it is essential to consider also bio accessibility. Bioavailability and bio accessibility are related to several soil processes and may be largely determined by soil characteristics. The concept of bioavailability has long been used in soil science, for example, to define the quantity of an element (N, P, K, etc.) that is available for plant nutrition, which often represents the basis for deciding the amount of fertilizer to be used **(Petruzzelli *et al*., 2020).**

Several studies have attempted to predict the so-called “Phyto available” fraction by correlating plant responses with different soil metal pools **(Lillo-Robles *et al*., 2020).** Determination and recognition of relative distribution of chemical forms of each element and their relationship with physical, chemical, and soil clay minerals can help researchers to manage soil fertility better **(Shakeri and Saffari, 2020).** Besides, it is explained that a scientifically sound risk assessment of soils has to be based on the bioavailable fraction of total essential element present in the soil as well as of the essential element added **(Tilborg, 2020).** This suggests that the environmental risk would mainly be related to the Cu and Zn contamination level rather than to changes in Cu and Zn availability in amended soils **(Laurent *et al*., 2020).** The root-soil interaction affects metal bioavailability in the rhizosphere, thus impacting the uptake and accumulation of metals by plants **(Huang *et al*., 2020a).**

IV.2.1 Bioavailability and metal solubility in soil

The total concentration of metals has been used as an indicator to evaluate soil contamination **(Rong *et al*., 2020).** Important in determining metal solubility, it is the intensity of the exposure, rather than the quantity, of metal in soil that best predicts metal uptake and subsequent phytotoxic response **(Mossa *et al*., 2020).** The bioavailability and eco toxicity of metals in contaminated soils depend largely on their solubility. Assessing the free ion activity of metal in soil solution is tedious and cumbersome **(Golui *et al*., 2020)**. Isotopically exchangeable metals in soil, also termed labile metals, are reversibly bound to soil surface and are a better index of the environmental risk of the metals than are their total concentrations **(Guzman-Rangel *et al*., 2020).**

In soil environments, sorption/desorption reactions as well as chemical complexation with inorganic and organic ligands and redox reactions, both biotic and abiotic, are of great importance in controlling their bioavailability, leaching, and toxicity. These reactions are affected by many factors such as pH, nature of the sorbents, presence, and concentration of organic and inorganic ligands, including humic and fulvic acid, root exudates, microbial metabolites and nutrients **(Violante *et al*., 2010).**

IV.3. Speciation of heavy metals in polluted soil

Heavy metals in agricultural soils exist in diverse dissolved (free cations and complexes species of positive, neutral, or negative charges), particulate (sorbed, structural, and coprecipitation), and colloidal (micro andnanometre-sized particles) species. The fate of different heavy metal species is controlled by the master variables: pH (solubility), ionic strength (activity and charge-shielding), and dissolved organic carbon (complexation). In the rhizosphere, chemical speciation controls toxicokinetics (uptake and transport of metals by plants) while toxicodynamics (interaction between the plant and absorbed species) drives the toxicity outcome. Based on the critical review, the authors recommend omics and data mining techniques to link discrete knowledge bases from the speciation dynamics, soil microbiome, and plant transporter/gene expression relevant to homeostasis conditions of modern agriculture. Such efforts could offer a disruptive application tool to improve and sustain plant tolerance, food safety, and environmental quality **(Uchimiya *et al*., 2020).**

The soil samples were characterized for the distribution and by the chemical speciation method, two extraction techniques (Tessier and Wenzel methods) were applied to fractionate metals **(Ahn *et al*., 2020).**

IV.4. Heavy metal in soil

Heavy metals and metalloids are adsorbed strongly by the organic, and inorganic, colloidal constituents of soil. The overall strength with which metals are retained in soils is a reflection of:

- ‘Residual’ properties attributable to the metal source material (primary minerals within the soil parent material, smelter fallout, sewage sludge, mine spoil, urban anthropogenic artefacts, etc.);
- The intrinsic affinity of individual metal ions for soil adsorption surfaces and soluble soil ligands;
- The suite of adsorption surfaces present in soils (humus, metal oxides, aluminosilicate clays, etc.);
- The more ‘transient’ properties of the soil, including pH, redox potential, water content, temperature, biological activity, salt concentration, etc.

- Soil-metal contact time **(Young, 2013).**

These may exist in a wide range of chemical forms, including soluble ligand complexes (ML; inorganic and organic). Examples will include the following forms in Table 7.

Table 7: Chemical forms of heavy metals in soil **(Young, 2013).**

	Chemicals forms
1	All exchangeable (electrostatically) held hydrated metal ions in clay interlayers and the diffuse double layers of humus acids;
2	Metal ions on the surface of metal compounds which are in solubility equilibrium with the soil solution;
3	Reversibly held metal complexes in solution including most inorganic species and a proportion of the metal-bound to dissolved humic and fulvic acid.
4	Some specifically-adsorbed metal ions on clay edges, Fe/Mn/Al hydrous oxide surfaces and humus

The first three pools are considered as the most important to release available forms of heavy metals for plants, while the remaining pools are characterized by metals with decreasing availability **(Violante *et al*., 2010)**. Factors governing the distribution of inner-sphere surface complexes between 'labile' and 'non-labile' forms are still under investigation **(Young, 2013).**

IV.5. Mobility

The potential toxicity of heavy metals in soil is a function of their mobility and bioavailability. Metal mobility is depending on the phase in which the metal occurs as well as physical and chemical processes that control transformations between phases. Accordingly, only the labile metal species (soluble, exchangeable, and chelated) are available to plants **(Asmoay *et al*., 2019).** Mobilization and immobilization of soil heavy metal mainly depends on heavy metal chemical forms or binding ability, which are governed by soil properties (pH, Eh, CEC, EC, and OM) and environmental processes **(Palansooriya *et al*., 2020)**, besides, the effects of climatic change in heavy metals mobility **(Oyewo *et al*., 2020**).

IV.5.1 Factors affecting heavy metal mobility in soil

IV.5.1.1. Potential of hydrogen (pH)

Soil pH was exponentially related to soil CEC and clay content **(Wei *et al*., 2020).** The pH and organic matter contents of soil were significant parameters affecting speciation of metals in soil samples **(Lu *et al*., 2017).** An acidic pH leads to the dissolution of the metal salts, the dissolution of the retention phases, the cations' desorption, and the anions' adsorption **(Aityoub *et al*., 2020).** At high pH, mobile metal might become immobile **(Zeng *et al*., 2020)**, pH is the most influential factor on the removal of Cu **(Aityoub *et al*., 2020).**

IV.5.1.2. Electrical Conductivity (EC)

Electrical conductivity (EC) is the most important factor affecting metal availability **(Tang *et al*., 2019b).** The soil EC values reflect the soil salinity where, the higher the EC value, the higher the salt concentration in the soil and vice versa **(Othaman *et al*., 2020).** Salinity input likely affected the distributions of Ni, Zn, and Cd, through cation exchange processes **(Yu *et al*., 2020).**

IV.5.1.3. Cation Exchange Capacity and Base Saturation (CEC)

Soil cation exchange capacity (CEC) is a critical property of soil fertility and nutrient retention capacity of the soil **(Mukhopadhyay *et al*., 2019 ; Wan *et al*., 2020).** Soil CEC and clay content may play critical roles in the soil acid-buffering processes from two aspects; it could not only contribute to the soil acid-buffering capacity but also affect the threshold of acidity of acid rain below which abrupt soil acidification may occur **(Wei *et al*., 2020).**

IV.5.1.4. Calcium carbonate ($CaCO_3$)

Calcium carbonate ($CaCO_3$) was used to adjust pH levels into control (pH 4.5), pH 5, 6, and 7, **(Rahman and Othman, 2020).** Soil pH increases of up to 8 after applying $CaCO_3$ assisted heavy metals immobilization through precipitation. Because of their alkaline nature, these materials reduce H^+ concentration, thereby increasing negatively charged sites in the soil. Consequently, positively charged heavy metals can be sorbed onto negative sites **(Palansooriya *et al*., 2020)**

IV.5.1.5. Soil organic matter (SOM)

Soil organic matter (SOM) affects soil biochemical reactions, such as controlling the availability and immobilization of nutrients and heavy metal through its impact on soil pH, and its ability to buffer the effects of contaminants **(Gregory and Blagodatskaya, 2019 ; Palansooriya *et al*., 2020 ; Sun *et al*., 2020b).** The majority of organic matter is bound to clay minerals to form a stable colloidal organo-mineral fraction (COMF) in soil **(Das *et al*., 2019).** Amendments significantly decreased the availability of Cd and Zn, but slightly activated As and Cu **(Tang *et al*., 2019b ; Xu *et al*., 2020).** Soil amendments can immobilize heavy metals through various mechanisms such as ion exchange, precipitation, complexation, and adsorption reactions. However, individual heavy metals had differential effects on the inhibition of soil organic matter decomposition with arsenic and copper **(Enya *et al*., 2020).** Specially, Cu distribution was mainly affected by organic-Cu complexation **(Yu *et al*., 2020).**

IV.5.1.6. Redox potential (Eh)

Heavy metals (HMs) easily react with Redox potential (Eh) sensitive substances by adsorption, complexation, and precipitation, etc **(Mao and Ye, 2018).**

IV.5.1.7. Soil texture

Soil is a medium for plant growth and provides support, minerals, and water to the plant for survival **(Liang and Yang, 2019).** The texture of soil affects the moisture level and EC. Sand, silt, and clay are examples of different types of soil **(Othaman *et al*., 2020).**

CHAPTER V

Abiotic and Biotic Stress

V.1. Introduction

Despite having considerably high productivity, continuous supply as per the market demand is hard to achieve, mostly because of periodic losses occurring due to biotic as well as abiotic stresses **(Chaudhary *et al*., 2019).** In nature, plants are simultaneously exposed to multiple abiotic and biotic stresses **(Llorens *et al*., 2020 ; Ramegowda *et al*., 2020)**, and hence have learned to endure them through a broad range of finely balanced responses. These responses use common signals and/or pathways (crosstalk) leading to either reduced or increased tolerance **(Ramegowda *et al*., 2020).** Biotic factors threaten the production and transportation of products worldwide**.** Thus, it is crucial to improve biotic stress-tolerant plants to overcome a reduction in plant productivity **(Çelik *et al*., 2020).**

V.2. Mechanism plant defence

The ability to perceive the stresses and activate the proper responses is crucial for the survival of the plant **(Llorens*et al*., 2020).** For many years, researchers focused on understanding plant defence mechanisms. Anatomical, physiological, and molecular adaptive mechanisms were investigated for several plant species **(Çelik *et al*., 2020).** Plants possess an intricate network of stress signals including signalling pathways, hormones, and defensive proteins, which activate the most suitable defensive mechanisms against the stresses. This immune system can be stimulated to activate the plant defences and get them ready for subsequent stresses **(Llorens *et al*., 2020).**

Besides the innate immunity mechanisms, including cross-talk, among the phytohormones to manage appropriate defence mechanisms against pathogens, new generation strategies in crop improvements are widely used in plant biotechnology. Although conventional breeding has importance in breeding new varieties, new technologies increase the possibility of success **(Çelik *et al*., 2020).**

V.3. Plant physiological and molecular mechanisms in cross-regulation of biotic-abiotic stress responses

The relationships between different biotic and abiotic stresses in shaping plant responses and performance have diverged in a context-dependent manner**.** The trade-off is often seen in plant adaptation between different biotic and abiotic stresses, however, initial exposure to one stress often leads to an enhanced state of tolerance to different stresses, designated cross-tolerance. Plants often prioritize their response to one stress over that to another**.** This may reflect the allocation of limited resources and the hierarchical interactions between different stress signalling pathways **(Tajima *et al*., 2020).**

V.4. Cross-tolerance

Increased adaptive fitness is called cross-tolerance, wherein exposure of plants to one stress results in tolerance to another. Considerable progress has been made toward understanding the mechanisms of cross-tolerance between abiotic and biotic stresses. Reactive oxygen species and phytohormones are suggested to be the key converging points of abiotic and biotic stress interactions. Recent studies have identified several genes, especially regulatory genes encoding kinases and transcription factors, involved in cross-tolerance **(Ramegowda *et al*., 2020)**. Interestingly, plants that are exposed to single stresses individually are more susceptible than the same plants exposed to simultaneous attacks by multiple stresses. Hence, it is postulated that cross-stress tolerance would have a positive impact on plant health and have profound implications for agricultural systems. Understanding the mechanistic models that underpin how plants adapt to various biotic and abiotic stresses including cross-tolerance is important in developing improved crop phenotypes suitable for climate-smart agriculture **(Ramesh *et al*., 2020).**

V.5. Priming mediated stress and cross-stress tolerance in plants

In this way, cross-tolerance and priming have emerged as potential solutions for enhancing crop resilience. Cross-tolerance is induced by mild primary stress, which activates common defences that reinforce tolerance to different stresses. On the other hand, the application of priming agents prepares the plant to respond faster and more effectively against future stresses. Both mechanisms can improve the resistance of plants against a broad number of stresses simultaneously, providing an effective approach for plant tolerance to environmental perturbations **(Llorens *et al*., 2020).**

During the last decade, the “primed” physiological state has attracted increasing attention since primed-plants can display faster and stronger defence responses. That state can be reached following treatments with various inducers such as chemical compounds or beneficial microbes. Although this phenomenon was discovered a long time ago, new perspectives are still coming up. The possibility to exploit the innate plant silencing pathway using double-stranded RNAs (dsRNAs) and the increasing understanding of defence priming mechanisms are pushing research toward new sustainable frontiers **(Alagna *et al*., 2020)** (Figure 25).

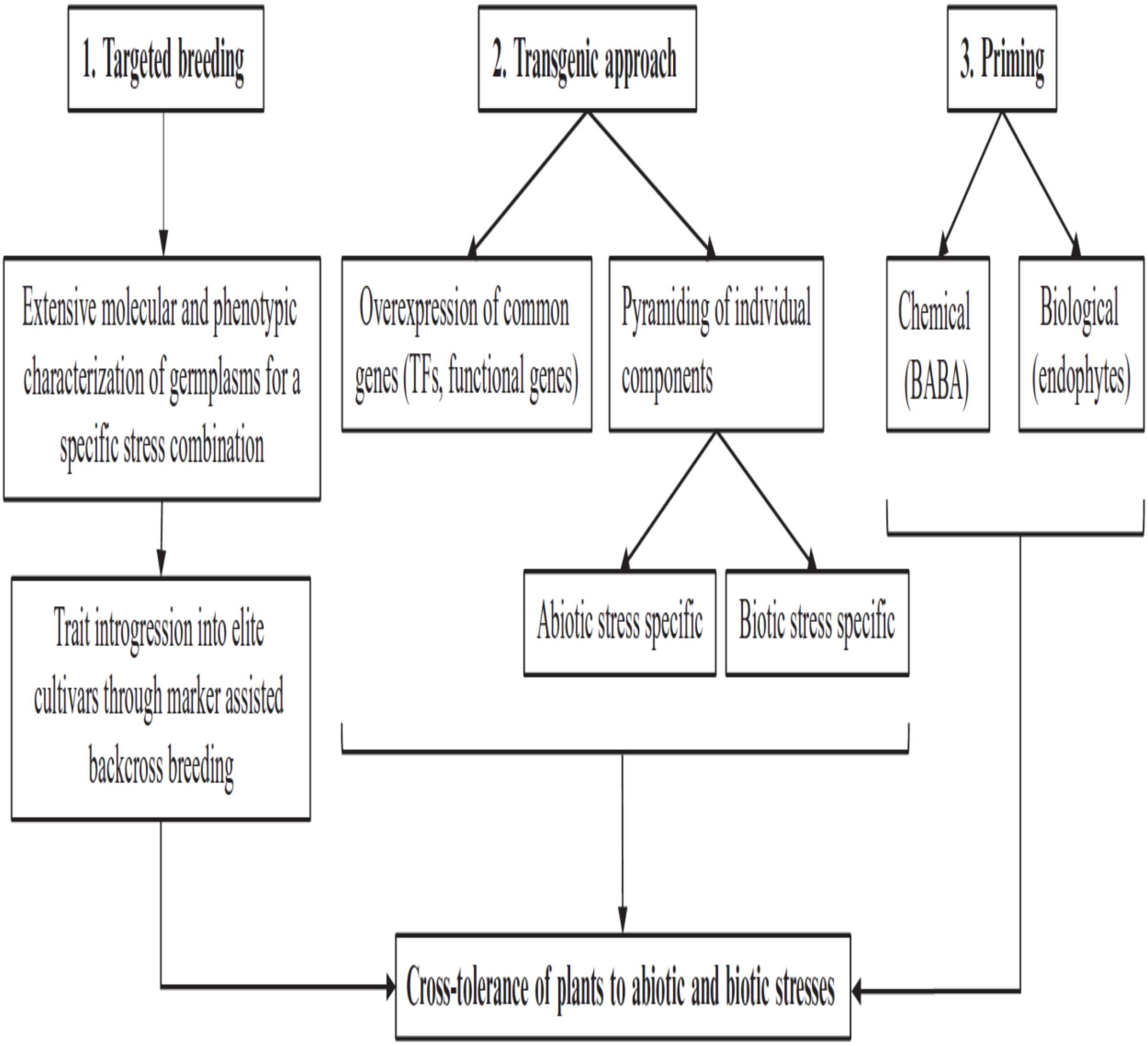


Figure 25: Potential approaches to improve cross-tolerance of plants to abiotic and biotic stresses **(Ramegowda *et al*., 2020).**

V.5.1 Priming technologies triggering plant tolerance traits against biotic and abiotic stresses

Over a long time, the ongoing global changing scenario has led to increasing temperature levels and CO_2 concentration that in turn influenced the severity and frequency of biotic and abiotic stresses threatening agricultural productivity and food security. These stressors, destined to increase in the future, are one of the main challenges that researchers are dealing with by developing sustainable strategies to enhance crop resilience **(Alagna *et al*.,**

2020). Pressure from biotic stresses will become increasingly compounded by harsh abiotic stress conditions **(Havko *et al*., 2020).**

V.6. Signalling molecules

Major components of gene regulatory networks underlying external stress-related adaptation, pest and pathogen recognition, and defence response comprise many known signalling molecules. Predominantly, reactive oxygen species (ROS), modulations in redox status, in-organic ion flux, phytohormones, protein-kinases as transducers, altered miRNAs, and protein modifications act concertedly for adaptive changes in primary and secondary metabolism **(Ramesh *et al*., 2020)** (Figure 26).

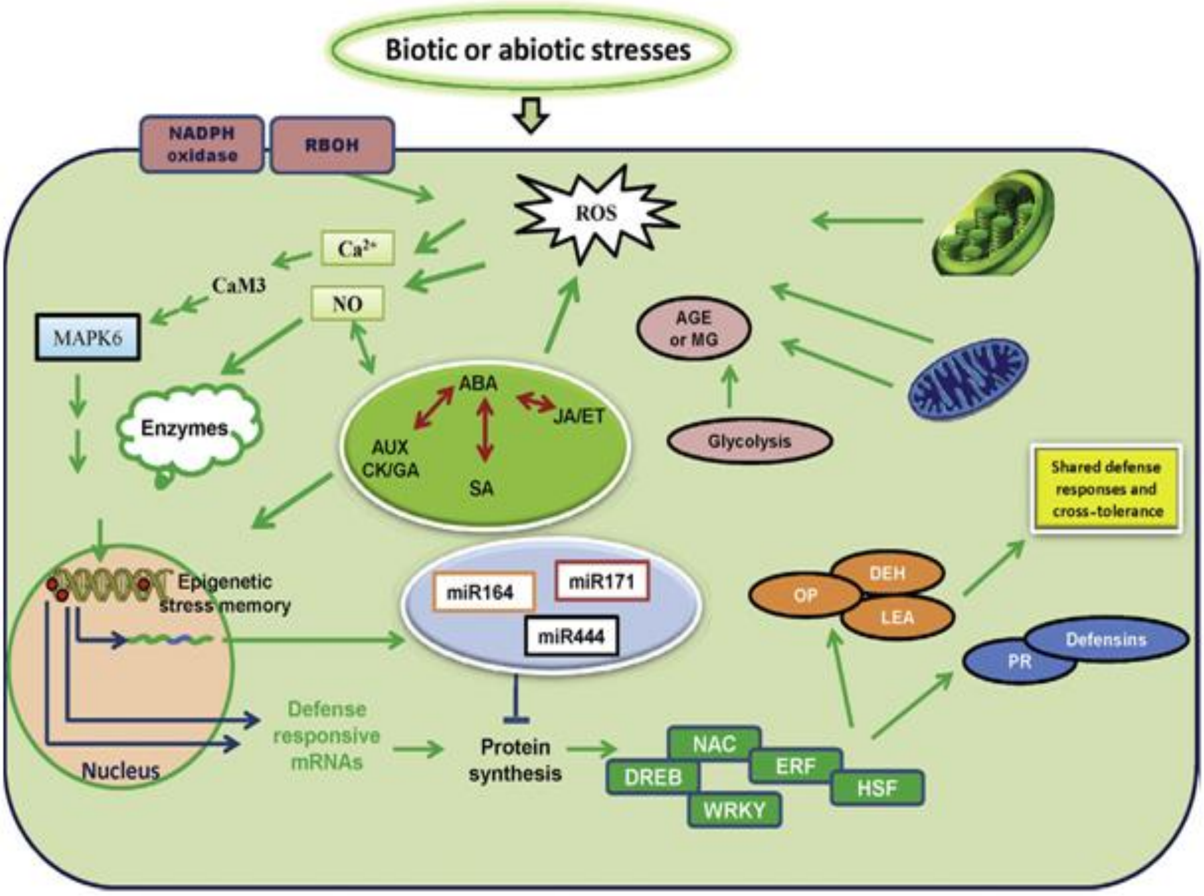


Figure 26: Molecular basis of cross-tolerance in plants: Stress perception, signal transduction, expression of various host factors culminating in cross-tolerance due to shared defence response pathways is depicted. ABA, abscise acid; AGE, advanced glycation end products; DREB, NAC, ERB, HSF, transcription factors; ET, ethylene; JA, jasmonate; MAPK, mitogen-activated protein kinase; MG, methylglyoxal; RBOH, Respiratory Burst Oxidase Homologue; ROS, reactive oxygen species; SA, salicylic acid **(Ramesh *et al*., 2020).**

V.7. Plant transcriptional regulation in modulating cross-tolerance to stress

Plants generally employ gene regulatory signalling networks involving phytohormones, transcriptional factors (TFs), and reactive oxygen species (ROS), and trigger the expression of stress-associated genes (SAGs) to cope with the adverse effects of such stresses. In this signal transduction process, TFs play a pivotal role in signal perception and SAG expression. TFs could be activators/repressors in the transcriptional regulation of SAGs. A large number of TFs have been identified and broadly classified based on the presence of a DNA binding domain into different families. Recent studies have unearthed that a combination of stresses instigates unique molecular responses, which are distinct from those responsive to individual stresses. Also, biotic and abiotic stresses share common signalling pathways, responses, and triggering of defence networks, leading to cross-tolerance. As signalling cascade plays a very important role in cross-tolerance phenomena, microRNAs (miRNAs), which also affect transcriptional regulation of gene expression by epigenetic modifications, requires a special mention. Elucidation of mechanisms underlying transcriptional control of stress-responsiveness in plants is imperative. The invaluable insights into mechanisms underlying the growth and differentiation of plants under adverse conditions will provide ways and means to obtain a better crop phenotype **(Ramesh *et al*., 2020).** The molecular basis of cross-tolerance is the synergistic activation of defense-related nonspecific pathways.

V.8. The signal conflict between biotic and abiotic stress (Example)

The plant defence hormone jasmonate promotes resistance to diverse herbivores, but how this wound signal impacts the plant’s ability to cope with a combination of herbivory and elevated temperature remains unknown. Here, we show that heat shock proteins contribute to enhanced jasmonate responses in tomato plants subjected to simulated heat waves. Herbivore-induced jasmonate signalling at elevated temperature, however, blocked stomatal opening and leaf hyponasty, leading to leaf overheating, reduced photosynthesis, and growth inhibition. The results show how signal conflict between biotic and abiotic stress may exaggerate crop losses under warming conditions that accelerate herbivory, potentially jeopardizing food security **(Havko *et al*., 2020).**

V.9. Plants emit informative airborne sounds under stress

Stressed plants show altered phenotypes, including changes in colour, smell, and shape. The 30 possibilities that plants emit airborne sounds when stressed similarly to many animals has not been investigated. Here we show, to our knowledge for the first time, that stressed plants emit airborne sounds that can be recorded remotely, both in acoustic chambers and in greenhouses. We recorded ~65 dBSPL ultrasonic sounds 10 cm from tomato and tobacco plants, implying that these sounds could be detected by some organisms from up to several meters away. Our results suggest that animals, humans, and possibly even other plants, could use sounds emitted by a plant to gain information about the plant's condition. More investigation on plant bioacoustics in general and on sound emission in plants 40, in particular, may open new avenues for understanding plants and their interactions with the environment, and it may also have a significant impact on agriculture **(Khait *et al*., 2019).**

Experimental Section

CHAPTER VI

Materials and Methods

VI.1. Objective

The present study was carried out to understand the effect of copper and zinc on the *Lycopersicon esculentum* Mill. In this context, major morphological, physiological, and biochemical changes that occurred in response to heavy metal stress were analyzed. Moreover, this effect can be explained by estimating the content of copper and zinc in the leaves and roots of the tomato plant; also, studying the distribution and chemical speciation of both metals in the soil (Figure 27).

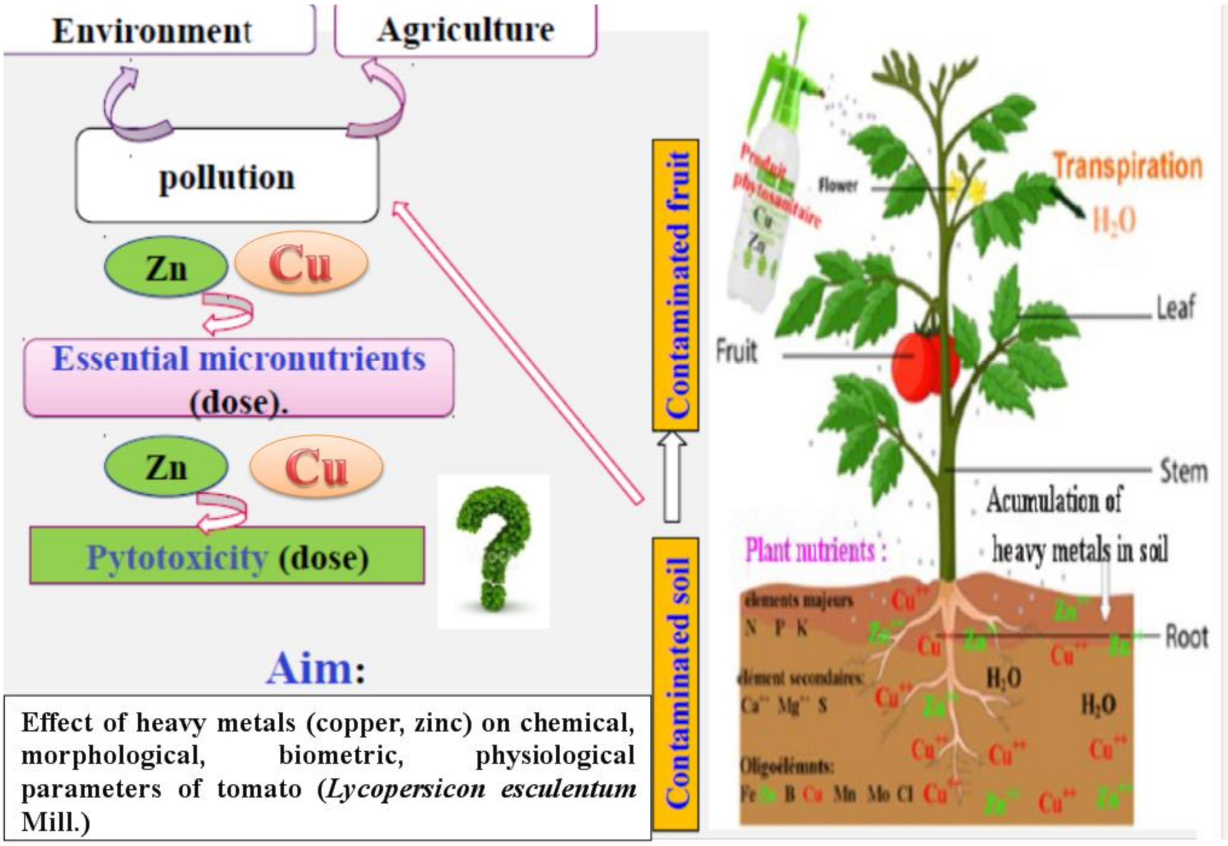


Figure 27: Research project summary

VI.2. Experiment preparation and conditions culture

VI.2.1 Experiment location

The experiment plantation was undertaken in the greenhouse at the National Institute of Plant Protection, located in Saiada, in Mostaganem region, at 35°55'30.1" N and 0°07'30.2" E (Figure 28). Analysis of different parameters was conducted in the laboratory of Biodiversity and Conservation Water and Soil; attached to the department of biology, Abdelhamid Ibn Badis University.

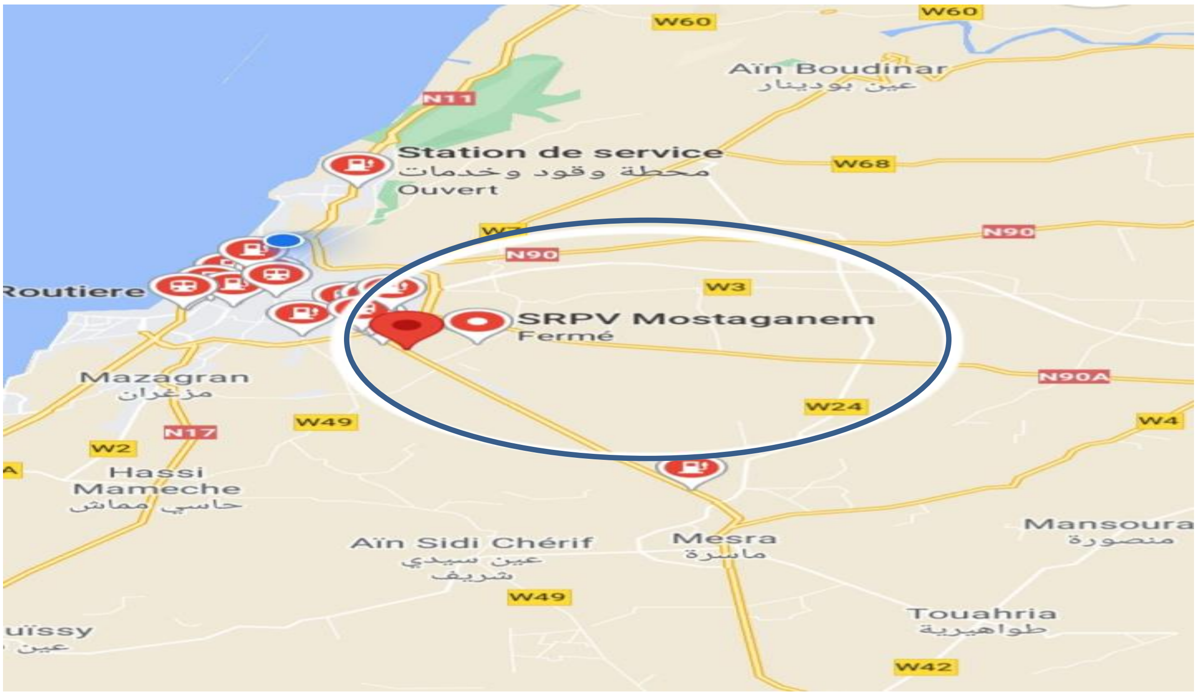


Figure 28: Localization of National Institute of Plant Protection, Mostaganem (greenhouse experiment site) **(Google Maps, 2020).**

VI.2.2 Soil preparation

Growth seedlings were set up in soil (substrate) prepared with some properties. The substrate used in this experiment was formed by the mixture of sand and compost (2V/V) respectively. Sand used was collected from the edge of Wre3a beach situated in the Mostaganem region. It was sieved and treated successively by hydrochloric acid and water to remove debris and living organisms. The final substrate was headed to determine its physicochemical properties including; texture, pH, EC, $CaCO_3$, CEC, before being used in this experiment. All properties and composition related to the compost were presented in detail in (Annex 2).

Growth pots used in this study are types of cylinders 50 cm high and 20 cm in diameter. Each cylinder is linked at the bottom with a grave followed by filling the substrate (soil) prepared above (Figure 29).

Figure 29: Soil preparation

VI.2.3 Material and plant growth conditions

Tomato genotype (*Lycopersicon esculentum* Mill.; Saint Pierre variety) used in this investigation is considered as a major variety planted in the Mostaganem region by tomato farmers (Figure 30). This variety or genotype is characterized by a high resistance capacity against pathogens and insects, it is also recognized by its productive yield in the greenhouse, short season, and growth chamber.

Figure 30: Seeds of *Lycopersicon esculentum* Mill, variety of SainPierre.

VI.2.4 Germination

Tomato seeds are disinfected in sodium hypochlorite solution (5%) for 5 mn and rinsed thoroughly with sterile water. Germination test was carried out in Petri dishes containing a whatman paper as support soaked in distilled water. This test was performed in a growth chamber at 25 °C under dark condition, with a photoperiod of 16h /8h (day/night) and 60–70

% relative humidity (Figure 31). The germination percentage was calculated as represents in the following equation **Ajmal *et al*. (2001)**:

$$Germination\ pourcentage = \frac{Numberof\ total\ germinated\ seeds}{Total\ number\ of\ seeds\ tested} \times 100$$

Tomato seeds were sown in seedling trays containing compost at a rate of 1 plant/cell for 20 days at 25°C until the seedling stage (Figure 31). All the uniformly seedlings were selected for transplantation into the pot.

(a) **(b)**

Figure 31: Germination test (a), tomato seedlings (b)

VI.2.5 Transplanting

Tomato seedlings were transplanted into 60 cylinders at the rate of 1 plant per cylinder. Each seedling was watered with distillate water respecting the AWC. The available water-holding capacity (AWC) of the substrate was given as 1000 ml of water/pot. This one was measured by the difference between the amount of water supplied before watering and that recovered after 24 hours of settling. This capacity takes a value between 1000 ml and 500 ml depending on the cultivation acclimation period (cold or hot), thus, the irrigation can be also performed once or twice a week respectively. A nutrient solution of **Hoagland and Arnon (1950)** was used to promote rapid growth and maintain a high development of tomato crops, to avoid further physiological stresses (Annex 2).

Hoagland's solution has been used recently in several studies, as diluted 1/10 Hoagland's solution **(Ashraf *et al.*, 2020 ; Baruah *et al.*, 2019 ; Huang *et al.*, 2020a ; Khan *et al.*, 2019 ; Liu *et al.*, 2018a ; Montanha *et al.*, 2020 ; Sitko *et al.*, 2019).**

VI.2.6 Stress conditions

Copper and zinc stress applied on tomato seedlings was given in the soil in solution form; as ($CuSO_4$, $5H_2O$) and ($ZnSO_4$, $7H_2O$), respectively. At the seedling stage, heavy metal solutions were applied separately twice during the greenhouse experiment. The stress treatment was performed according to the experimental design which is consisted of a control (plants are not stressed) and five treatments (100, 200, 300, 400, and 500 ppm). Five biological independent replicates were carried out. The plants were kept in the greenhouse until the end of the experiment.

Figure 32 summarizes all the experiment steps conducted in the greenhouse to ensure a successful stress application under ideal conditions.

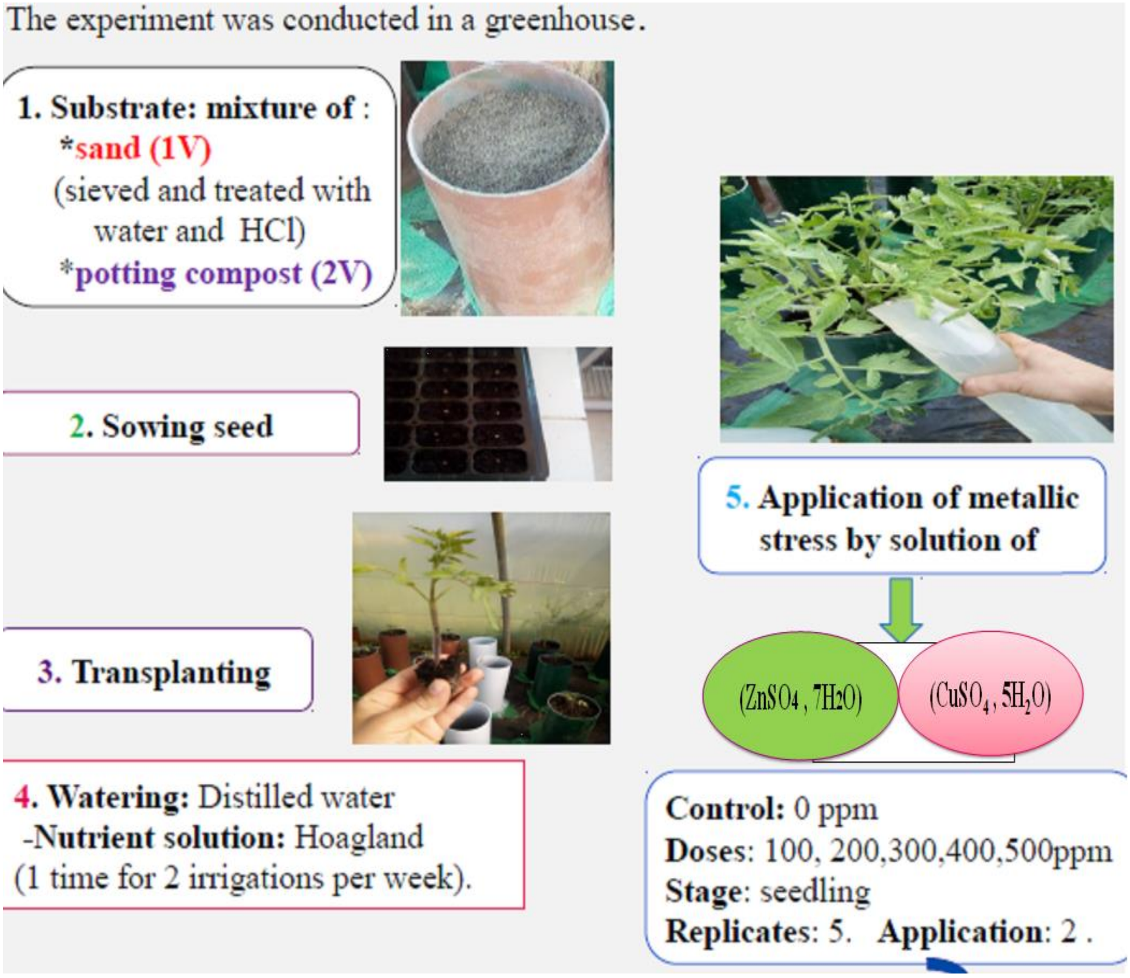
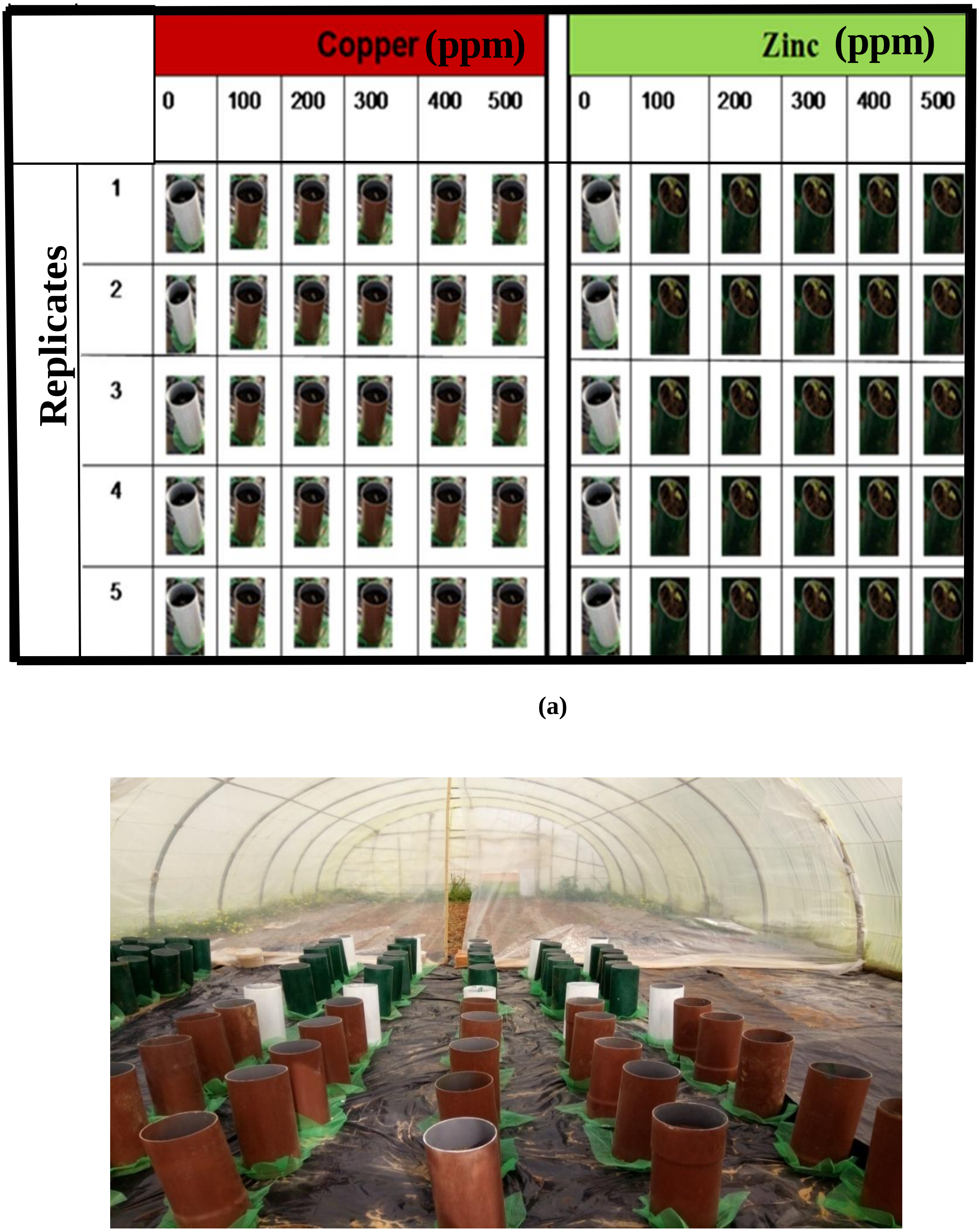


Figure 32: Overview of the experimental steps conducted in the greenhouse.

VI.2.7 Experimental design

The experimental design adopted in this approach is outlined in (Figure 33).



(a)

(b)

Figure 33: Greenhouse experimental design

VI.3. Parameters analysis

VI.3.1 Morphological parameters

Differences in measurements between the control and the contaminated plants were used to evaluate metal toxicity **(Pérez Harguindeguy *et al*., 2013)**. Overall, to observe the effects of heavy metals on tomato seedlings is necessary to measure different growth indicators such as shoot length, root length, fresh and dry weight of leaves, and roots **(Shah *et al*., 2020)**.

At the end of the greenhouse experiment, tomato seedlings were harvested and separated into the below and above-ground parts. Each sample was washed carefully with tap water. After that, all growth traits measurements (plant height, root length, number of leaves, dry and fresh weight, and leaf area) were recorded for further laboratory analysis.

VI.3.1.1. Plant height and root length

Stem and root length were measured by using a centimeter-scale and expressed in cm.

VI.3.1.2. Number of leaves

The total number of leaves per plant was obtained using a standard count.

VI.3.1.3. Measurement of biomass

Fresh and dry weights of aerial and root parts were measured using analytical weight balance. Each weight was expressed in gram (g).

a. Fresh weight Measurement

Fresh weight (FW) of below and above ground parts was measured after harvesting the tomato seedlings.

b. Dry weight Measurement

For dry weights (DW), below and above ground parts harvested were dried at 65-70 °C to constant weights.

VI.3.1.4. Leaf area

Leaf area was measured according to the standard method of **Bazzela (2005)**, leaves were collected randomly from each plant. Leaf area was calculated using the following formula:

$$LA = \frac{(\pi \times a \times b)}{4}$$

Where,

- LA: Leaf area (cm^2)
- a : Leaf blade length (cm).
- b : Leaf blade width (cm).

VI.3.2 Biometrical parameters

VI.3.2.1. Relative water content (RWC)

Relative water content (RWC) was determined in fresh leaves according to the method of **Barrs and Weatherly (1962)** and **Scippa *et al*. (2004)**. It was calculated by the following formula:

$$RWC(\%) = \frac{FW-DW}{TW-DW} \times 100$$

Where,

- FW: Fresh weight;
- TW: Turgid weight;
- DW: Dry weight.

VI.3.2.2. Rate water loss (RWL)

Rate water loss (RWL) from excised leaves was estimated by the method described by **Clarke *et al*. (1989)** with some modifications. The final measurements were performed at 27C° after 60 minutes. Rate water loss was presented as g. cm^{-2}. mn^{-1}. It can be calculated by the following equation:

$$RWL = \frac{FW - W60mn}{LA \ X \ t}$$

Where,

- FW: Fresh weight (PW)
- W_{60mn}: Weight measured after 60 minutes
- LA: Leaf area
- RWL: Rate water loss.
- t : 60 minutes

VI.3.3 Physiological parameters

VI.3.3.1. Determination of photosynthetic pigments content

Photosynthetic pigments were extracted in acetone 80% and estimated according to the method described by **Lichtenthaler and Wellburn (1983).**

The absorbance was measured at 663, 645, 470 nm using a spectrophotometer (UV-2550, SHIMADZU, Japan). chl a, chl b, t chl, and carotenoids content were calculated by the following equation:

$$\textbf{Chl a} = 12.21 \times A663 - 2.81 \times A645;$$

$$\textbf{Chl b} = 20.13 \times A645 - 5.03 \times A663;$$

$$\textbf{t Chl} = \textbf{Chl (a+b)} = 7.15 \times A663 + 18.71 \times A645;$$

$$\textbf{Car} = (1000 \times A470 - 3.27 \times \text{chl a} -104 \times \text{chl b}) / 229.$$

Chlorophyll and carotenoids contents were expressed in milligrams per gram leaf fresh weight (mg. g^{-1} FW).

VI.3.4 Biochemical parameters

VI.3.4.1. Estimation of soluble proteins content

Protein content extracted from leaves and roots was determined by the method of **Bradford (1976)**. Absorbance at 595 nm was measured by a spectrophotometer (UV-2550, SHIMADZU, Japan). Protein concentration was estimated by using BSA (bovine serum albumin) as standard to plot a standard curve. Protein content was expressed in milligrams per gram leaf fresh weight (mg. g^{-1} FW).

VI.3.4.2. Estimation of soluble sugars content

The level of the soluble sugars content of leaves and roots was measured by the method of **Dubois *et al*. (1956).** The absorbance was determined at 485 nm by a spectrophotometer (UV-2550, SHIMADZU, Japan). Soluble sugars content was estimated by using glucose as standard. The amount was expressed in milligrams per gram leaf dry weight (mg. g^{-1} DW).

VI.3.4.3. Estimation of proline content

Proline content in roots and leaves of the tomato plant was achieved according to the method of **Bates *et al.* (1973).** The absorbance was measured at 528 nm. The proline content calculated using a calibration curve was plotted and prepared with proline, this content was expressed as milligrams per gram of dry weight (mg. g^{-1} DW).

VI.3.4.4. Estimation of polyphenols content

Polyphenol content in roots and leaves of the tomato plant was estimated according to the method of Folin-Ciocalteau phenol reagent described by **Singleton *et al.* (1999).** The concentration of polyphenol was calculated using a calibration curve prepared with standard Gallic acid. The data were expressed as milligrams of Gallic acid equivalents per gram of dry weight (mg GAE. g^{-1} DW).

VI.3.4.5. Estimation of flavonoids content

The flavonoids content in the roots and leaves of the tomato plant was determined through the method indicated by **Zhishen *et al.* (1999).** The concentration of flavonoids was calculated using a calibration curve prepared and plotted with standard quercetin. The data were expressed as milligrams of quercetin equivalents per gram of dry weight (mg QE. g^{-1} DW).

VI.3.4.6. DPPH Radical-scavenging activity

Antiradical activities of various antioxidants were determined using the free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH°). In its radical form, DPPH° has an absorption band at 515 nm which disappears upon reduction by an antiradical compound. Twenty compounds were reacted with the DPPH° and shown to follow one of three possible reaction kinetic types. Ascorbic acid is ascorbic acid and isoeugenol reacted quickly with the DPPH° reaching a steady-state immediately **(Brand *et al*., 1995).**

a. DPPH assay

To evaluate the antioxidative activity of specific compounds or extracts, the latter is allowed to react with a stable radical, 2, 2-Diphenyl-picrylhydrazyl (DPPH °) in a methanol solution. The reduction of DPPH ° as indicated below is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH ° absorbs at 515 nm, but upon reduction by an antioxidant (AH) or a radical species (Re), the absorption disappears **(Brand *et al*., 1995).**

$$DPPH^{\circ} + AH \rightarrow DPPH - H + A^{\circ}$$

$$DPPH^{\circ} + R^{\circ} \rightarrow DPPH - R$$

Free radical scavenging activity was assessed spectrophotometrically at 517 nm by turning the deep violet color solution of DPPH into colorless or pale yellow color **(Brand *et al.*, 1995).**

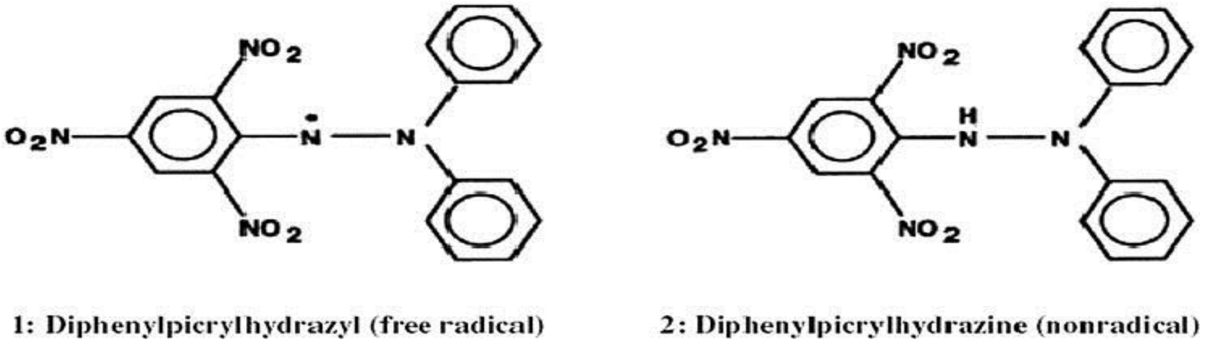


Figure 8: DPPH radical and its stable form **(Kedare and Singh, 2011).**

DPPH radical scavenging activity was determined according to the protocol proposed by **Brand *et al*. (1995)** with some modifications, using DPPH as free radical. The decrease in absorbance was measured at 517 nm using a UV / visible spectrophotometer. The antioxidant activity was expressed as percentage inhibition of the DPPH radical, and calculated by the following equation:

$$(\%) Radical\ scavenging\ activity = \frac{Abs\ of control - Abs\ of sample}{A\ 517\ of control} \times 100$$

Where,

- Abs: Absorbance
- 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is stable nitrogen radical available commercially as a solid.

VI.3.5 Chemical parameters

VI.3.5.1. Soil physical and chemical properties

Routine and detailed analysis (sample preparation, extractions, metal fractionations, wet ashing of plant and soil samples, determination of physicochemical properties) were carried out in the Environmental and Organic agriculture laboratory of Akdeniz University, Vocational School of Technical Sciences, Antalya Turkey.

Soil samples were collected, air-dried for a period of 2 to 3 weeks, and sieved to be analyzed. The physicochemical properties of soil (pH, EC, CEC, $CaCO_3$) were determined according to the following methods:

a. Potential of hydrogen (pH)

The potential of hydrogen was measured from a soil-water suspension (1: 2.5) using a pH meter (HANNA instruments HI8314), as described by **Greweling and Peech (1960).**

b. Texture

Soil texture was determined by the method suggested by **Bouyoucos (1951)**, using a hydrometer. The hydrometer method is a sedimentation procedure used extensively in soil science and engineering for particle size analysis. **Bouyoucos (1962)** developed a hydrometer test which he used to estimate sand, silt, and clay percentages by taking hydrometer readings at 40 sec, 4 mn, and 2 hours, respectively, in a suspended soil solution. Empirical corrections were made for temperature and solution viscosity**.** The Bouyoucos method is still used in several science laboratories **(Jacob *et al.*, 1971; Moodie and Koehler, 1975)** as a rapid way to approximate texture. Sand, silt, and clay percentages were calculated as defined by the following formulas;

$$Clay + Slit\ (\%) = \frac{A}{Soil\ weight(g)} \times 100$$

$$Clay\ (\%) = \frac{B}{Soil\ weight\ (g)} \times\ 100$$

$$Slit\ (\%) = \ (Clay + Slit) - Clay$$

$$Sand\ (\%) = \ 100 - (Clay + Slit)$$

$$*\boldsymbol{Corrected\ hydrometer\ reading\ for\ A\ or\ B}:$$

$$(Temperature\ at\ that\ time - \ 20) \times 0.36 + hydrometer\ reading$$

Where; temperature: 18C°.

Hydrometer reading is given in Table 8.

Table 8: Hydrometer reading

	A	B
Time	40 sec	120 mn
Hydrometer reading	10.30	10.05

Soil texture was determined by using the texture triangle presented by USDA soil taxonomy (ST) developed by the United States Department of Agriculture (Annex1).

c. Lime ($CaCO_3$)

One of the most important parameters in determining soil chemistry is $CaCO_3$ content **(Şenlikci *et al*., 2015)**. Lime content was analyzed by a Scheibler altimeter according to the method of **Woodward (1961)**. $CaCO_3$ percentage is decided by calculating the gas pressure of CO_2 resulting from the reaction of HCl with soil lime ($CaCO_3$) **(Şenlikci *et al*., 2015).**

$$Lime(\%) = \frac{V0 \times 0.4464}{sample\ amount(g)}$$

Where;

$$Vo\colon \frac{Vt(b-e) \times \ 273}{760 \ \times (273 + t)}$$

b: Corrected barometric pressure according to temperature (764.31 mm Hg).

e: Vapor pressure of water in measured temperature (16.27 mm Hg).

t: Measured temperature (18.8 C°).

Vt: CO_2 volume of the soil sample in altimeter.

d. Cation exchange capacity of the soil (CEC)

Cation exchange capacity (CEC) of a soil is a measure of the quantity of negatively charged sites on soil surfaces that can retain positively charged ions (cations) such as calcium (Ca^{2+}), magnesium (Mg^{2+}), and potassium (K^{+}), by electrostatic forces. Cations retained electrostatically are easily exchangeable with cations in the soil solution so a soil with a higher CEC has a greater capacity to maintain adequate quantities of $Ca^{2+,}$ $Mg^{2+,}$ and K^{+} than a soil with a low CEC. Soil CEC is normally expressed in one of two numerically equivalent sets of units: meq.100 g^{-1} (milliequivalents of charge per 100 g of dry soil) or cmol. kg^{-1} (centimoles of charge per kilogram of dry soil). Because of the different methods to estimate CEC, it is important to know the intended use of the data. For soil classification purposes, a soil’s CEC is often measured at a standard pH value **(Ross and Ketterings, 1995).**

In the present experiment, the cation exchange capacity (CEC) of soil was determined according to the protocol suggested by **Chapman (1995).** The principle of this method is to saturate the absorbent complex with a normal solution of sodium acetate (1N) buffered to pH 8.2 (Annex1).

e. Electrical conductivity (EC)

Soil electrical conductivity (EC) is an approximate measure of the concentration of soluble salts in soil (salinity of soil). Soil solution EC was regulated by pH, as well as the sum of cation and anion concentrations, and the C contents determined in the soil liquid phase. The electrical conductivity of the soil water suspension (1:2.5 soil: water) was estimated using an electrical conductivity meter (HANNA instruments HI8633) as indicated by **Rhoades (1993).** Electrical conductivity was measured at ambient temperature (25°C) after settling of the soil particles. Electrical conductivity was expressed in (dS. cm^{-1}). Soil EC does not directly affect plant growth but has been used as an indirect indicator of the number of nutrients available for plant uptake and salinity levels. EC has been used as a surrogate measure of salt concentration; organic salinity class was given according to the classification adopted by NRCS soil survey Handbook (Annex1).

VI.3.5.2. Heavy metals analysis in plant and soil

a. Sequential extraction procedure for the speciation of particulate trace metals

An analytical procedure involving sequential chemical extractions has been developed for the partitioning of particulate trace metals (Cd, Co, Cu, Ni, Pb, Zn, Fe, and Mn) into five fractions: exchangeable, bound to carbonates, bound to Fe-Mn oxides, bound to organic matter, and residual**.** The use of sequential extractions, although more time consuming, furnishes detailed information about the origin, mode of occurrence, biological and physicochemical availability, mobilization, and transport of trace metals. It has been proved the merits of a method of sequential “selective” extractions for partitioning particulate trace metals into chemical forms likely to be released in solution under various environmental conditions **(Tessier *et al*., 1979).** In the present investigation, we analyzed the heavy metal (copper and zinc) in two fractions: exchangeable and organic fraction.

- **Fraction 1. Exchangeable**

Numerous studies performed on sediments or their major constituents (clays, hydrated oxides of iron and manganese, humic acids) have demonstrated the adsorption of trace metals; changes in water ionic composition (e.g., in estuarine waters) are likely to affect sorption-desorption processes. Water-soluble and exchangeable forms are considered readily mobile and available to plants. The exchangeable fraction is removed by changing the ionic composition of water allowing metals sorbed to the exposed surfaces of sediment to be removed easily. A salt solution is commonly used to remove the exchangeable fraction **(Tessier *et al*., 1979).**

- **Fraction 4. Bound to organic matter**

The complexation and peptization properties of natural organic matter (notably humic and fulvic acids) are well recognized, as is the phenomenon of bioaccumulation in certain living organisms. Trace metals may be bound to various forms of organic matter: living organisms, detritus, coatings on mineral particles, etc. To remove metals bound in the organic phase, the organic material must be oxidized **(Tessier *et al*., 1979).**

b. Sequential extraction of trace metals (Protocol)

In the present study, Tessier’s sequential extraction procedure was adopted. It consists of the following steps:

The soil samples were dried at 105°C in a forced-air oven. They were subsequently ground in an agate mortar, homogenized. The quantities indicated below refer to 1 g soil samples and 0.1g for a dry matter of plant analysis **(Tessier *et al*., 1979).**

- **Metals in the exchangeable fraction**

The soil samples were extracted at room temperature for 1 h with 40 mL of either magnesium chloride solution (1 M $MgCl_2$, pH 7.0) with continuous agitation **(Tessier *et al*., 1979).**

- **Metals bound to the organic fraction**

A method described by Gupta and Chen was adopted. To the residue from (F3) were added 10 mL of 0.02 M HNO_3 and 15 mL of 30 % H_2O_2 adjusted to pH 2 with HNO_3, and the mixture was heated to 85 °C for 2 h with occasional agitation. This step was repeated.

After cooling, 5 mL of 3.2 M NH_4OAc in 20 % (V/V) HNO_3 was added and the sample was diluted to 50 mL and agitated continuously for 30 min. The addition of NH_4 OAc is designed to prevent the adsorption of extracted metals onto the oxidized sediment **(Tessier *et al*., 1979).**

Between each successive extraction, the separation was obtained by centrifuging at 3000 rpm for 10 min. The supernatant was removed with a pipette and analyzed for trace metal, whereas the residue was washed with 10 mL of deionized or distilled water; after centrifugation for 30 min, this second supernatant was discarded. The volume of rinse water used was kept to a minimum to avoid excessive solubilizing of solid material, particularly organic matter **(Tessier *et al*., 1979).**

VI.3.5.3. Heavy metal analysis

Heavy metals content (copper and zinc) accumulated in the plant (leaves and roots) and soil were analyzed by the inductively coupled plasma mass spectrometry (ICP-MS) **(According to international standard ISO 11466, 1995).**

The metal determination was carried out in Akdeniz University Centre of Agricultural Researches and Food Security laboratories, Antalya Turkey.

VI.4. Factors and indices

VI.4.1 Contamination factor (CF)

The level of contamination of soil by metals expressed in terms of a contamination factor (CF) calculated as following **(Memet, 2011)**:

$$CF\colon \frac{Metal\ concentration\ in\ sample}{Metal\ concentration\ in\ soil}$$

where the contamination factor CF < 1 refers to low contamination; 1 ≤ CF < 3 means moderate contamination; 3 ≤ CF ≤ 6 indicates considerable contamination and CF > 6 indicates very high contamination **(Hakanson, 1980).**

VI.4.2 Evaluation of phytoremediation efficiency

Both tools (BCF and TF) are important for assessing the feasibility of a plant species for phytoremediation purposes. Plants having both a phytostabilization and metal-tolerance capacity could potentially be useful for phytoremediation purposes **(Mishra and Pandey, 2018).**

VI.4.2.1. Translocation factor (TF)

Translocation factor (TF) is the ratio of the ability of plants to translocate heavy metals from roots through shoots **(Zhang and Wong, 2007).** It was claculated by estimating the concentration of metal in one part of plant as follow:

$$Translocation\ factor\colon\ \frac{Metal\ concentration\ in\ leaves}{Metal\ concentration\ in\ roots}$$

TF > 1: Signifies that the plant effectively translocates heavy metals from roots to the shoots or leaves.

TF ≤ 1: Indicates that heavy metals are accumulated in the roots. The plant is ideal for phytostabilization **(Zhang and Wong, 2007).**

VI.4.2.2. Bioconcentration factor (BCF)

Bioconcentration factor (BCF) is described as the ability of plants for elemental accumulation from the substrate **(Qihang *et al*., 2011).** It can be measured for each plant part, such as roots, stems, and leaves. In this study BCF was calculated for root using the equation:

$$BCF\colon\ \frac{Metal\ concentration\ root}{Metal\ concentration\ in\ soil}$$

* Metal concentration in soil: the exchangeable fraction was used in the present experiment to calculate the bioconcentration factor.

BCF (root) > 1**:** it indicates the phytostabilisation.

Indeed, plants having BCF values > 1 are referred to as hyper-accumulators for a particular metal. While a lower BCF shoot value compared to BCF root in amended Cu soil indicates lower accumulation of heavy metals affinity in shoots **(Munir *et al*., 2020).**

VI.4.3 Phytotoxicity

Phytotoxicity for each organ was calculated as given below **(Bouziani *et al.*, 2019)**:

$$Stem\ toxicity\ (\%): \frac{Stem\ length\ of\ control - Stem\ length\ of\ treatment}{stem\ length\ of\ control} X100$$

$$Root\ toxicity\ (\%): \frac{Root\ length\ of\ control - Root\ length\ of\ treatment}{Root\ length\ of control} X100$$

VI.4.4 Tolerance index (TI)

The tolerance index (TI) was calculated according to **Zhang *et al*. (2020)**

$$Tolerance\ index\ (\%): \frac{Mean\ root\ length\ in\ stress}{Mean\ root\ length\ in\ control} X\ 100$$

VI.5. Statistical analysis

All analyses were performed through the use of STAT BOX software. The data were calculated by variance analysis (ANOVA). The significance of differences between control and treatment was determined at the 0.05 level of probability. Data presented in this study were expressed as mean values ± standard deviation (SD). The averages are compared according to the Newman- Keuls test. Each treatment was carried out with five replicates. The correlation between parameters was performed by Pearson's correlation.

CHAPTER VII

Results

VII.1. Germination rate

In non-stress conditions, the germination rate obtained by tomato seeds, Saint Pierre variety, exhibited a high percentage of 90 % (Figure 34).

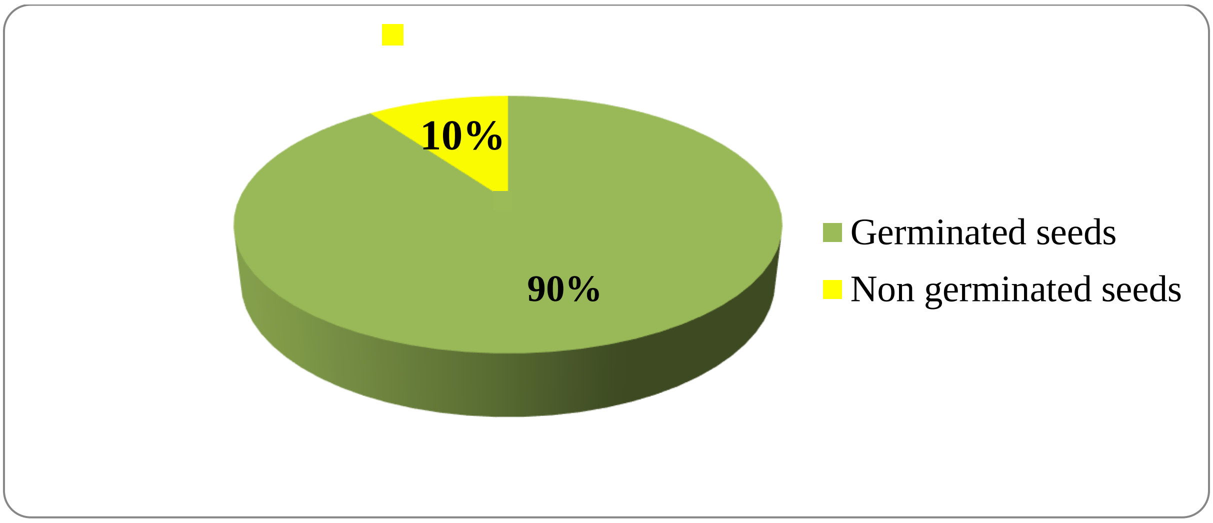


Figure 34: Germination rate of *Lycopersicon esculentum* Mill.

VII.2. Effect of copper on the morphological, biometrical, physiological, and biochemical parameters of *Lycopersicon esculentum* Mill.

VII.2.1 Morphological parameters

VII.2.1.1. Effect of copper on stem length

Plants exposed to different concentrations of copper showed a significant decrease in stem length of *Lycopersicon esculentum* Mill. (Figure 35). This effect was observed at 500 ppm compared to the control treatment (0 ppm) with 42.5 and 60 cm, respectively. However, the stem length was enhanced by the application of 300 ppm Cu to give a value of 46.66 cm.

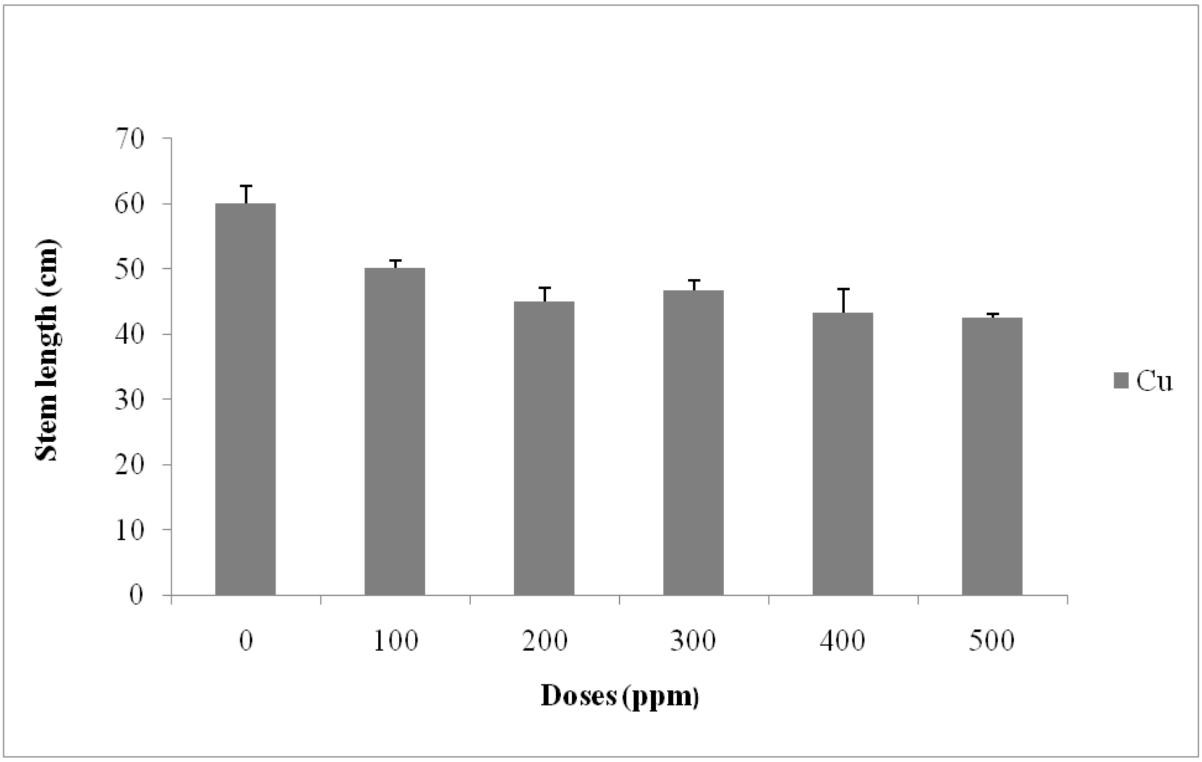


Figure 35: Effect of copper on stem length of *Lycopersicon esculentum* Mill.

Table 9: Shows values of stem length of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Stem length (cm) **P= 0.00**	60 a ± 2.64	50.16 b ± 1.04	45 c ± 2	46.66 bc ± 1.52	43.33c ± 3.51	42.5c ± 0.5

Statistical analysis revealed a highly significant effect of copper on the stem elongation of *Lycopersicon esculentum* Mill., compared to the control plants (Annex 4).

As shown in Table 9, a presence of four homogenous groups, group a (0 ppm), group b (100 ppm), group bc (300 ppm), and group c (200, 400, and 500 ppm).

VII.2.1.2. Effect of copper on root length

Figure 36 shows that copper treatments inhibited gradually the root growth of *Lycopersicon esculentum* Mill.

Control seedlings exhibited the highest value of root length by 62.33 cm; whereas, the minimum lengths of 41.33 and 40.66 cm were observed at 400 and 500 ppm Cu doses, respectively. However, a noticeable increase was recorded at 300 ppm, to reach a similar level as that obtained at 100 ppm, with a value of 49.33 cm.

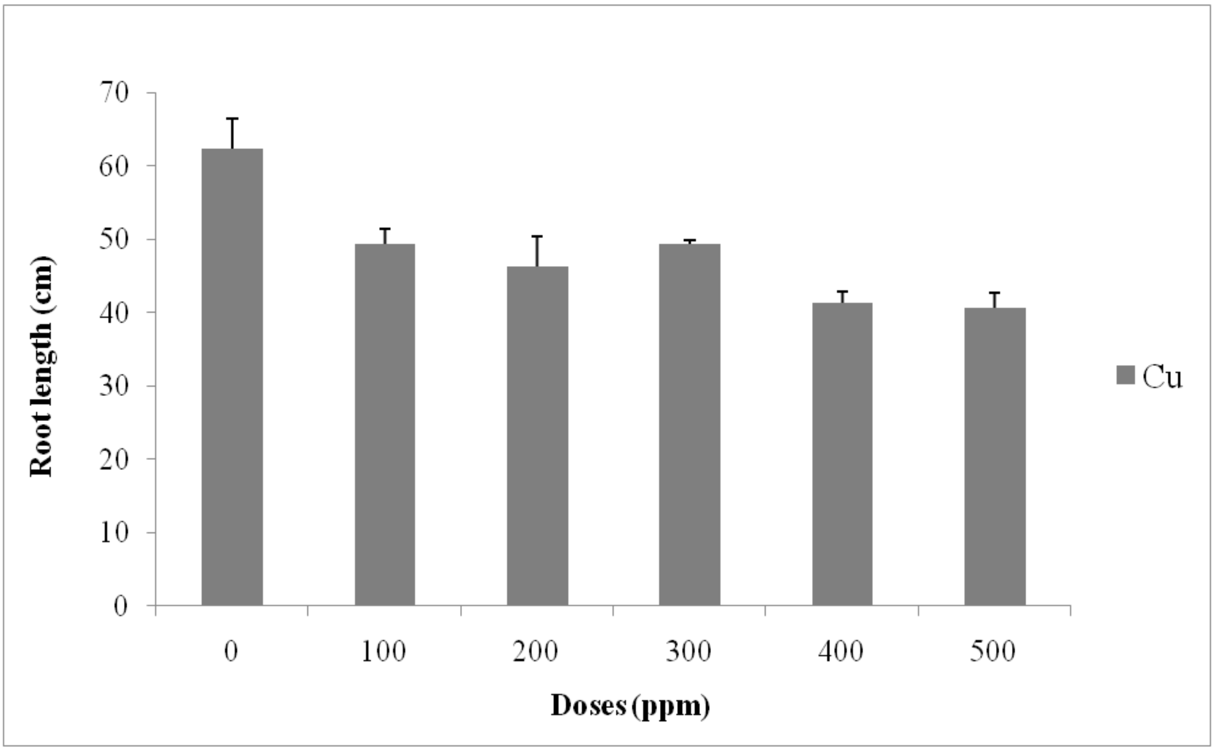


Figure 36: Effect of copper on root length of *Lycopersicon esculentum* Mill.

Table 10: Shows values of root length of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, and c) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Root length (cm) **P= 0.00**	62.33 a ± 4.16	49.33 b ± 2.082	46.33bc ± 4.04	49.33 b ± 0.577	41.33 c ± 1.52	40.66 c ± 2.08

Statistical analysis showed a highly significant effect of copper on root growth of *Lycopersicon esculentum* Mill., compared to the control (Annex 4).

As displayed in Table 10, four homogeneous groups were recorded with root growth, group a (0 ppm), group b (100 and 300 ppm), group bc (200 ppm), and group c (400 and 500 ppm).

VII.2.1.3. Effect of copper on the number of leaves

Figure 37 indicates that the total number of leaves noted in *Lycopersicon esculentum* Mill., decreased consistently as copper doses in the soil increased.

In control seedlings (0 ppm), the highest number of leaves was marked by 350 leaves per plant; whereas, copper with a high level (500 ppm) led to the lowest number of 157 leaves per plant. Moreover, almost a similar effect was obtained with 200 and 400 ppm by 194 and 192 leaves, respectively.

Copper treatment at 300 ppm acted differently by a slight increase of 200 leaves per plant, which was lower than that recorded at 100 ppm (224 leaves).

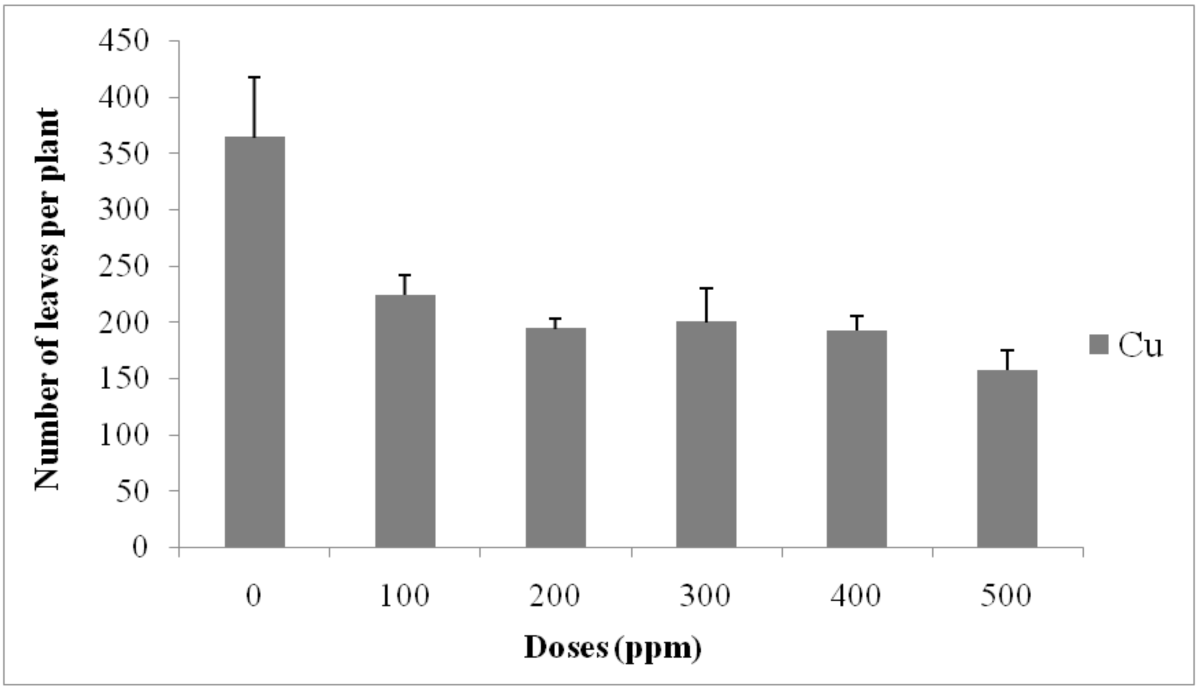


Figure 37: Effect of copper on the number of leaves of *Lycopersicon esculentum* Mill.

Table 11: Shows values of the number of leaves of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a and b) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Number of leaves per plant P=0.00	364 a ± 53	224 b ± 17.77	194.33 b ± 9.01	200 b ± 30	192.33 b ± 13.57	157 b ± 17.57

Compared to control seedlings of *Lycopersicon esculentum* Mill., statistical analysis demonstrated a highly significant effect of copper on the total number of leaves (Annex 4).

Two homogenous groups were presented in Table 11, group a (0 ppm) and group b (100, 200, 300, 400, and 500 ppm).

VII.2.1.4. Effect of copper on aerial and root fresh biomass

Aerial and root fresh biomass of *Lycopersicon esculentum* Mill. decreased gradually with the increase of copper doses in the soil; exceptionally, a slight increase was detected at 300 ppm Cu, as shown in Figure 38. Compared to the control conditions, treatment with 100 ppm Cu induced a moderate decline in aerial fresh biomass from 281.8 to 221.527 g, respectively. A sharp decrease was recorded at 500 ppm with an incredible value of 122 g. Moreover, copper at 200, 300, and 400 ppm presented the following biomass values of 161.967, 173, and 149.333 g, respectively.

A similar trend was also observed in root fresh biomass by a reduction of 65.9 g at 0 ppm to 62.567 g at 100 ppm, and to18.9 at 500 ppm, respectively.

It is worth mentioning that, aerial fresh biomass was more important than that of roots, in normal as in stress conditions.

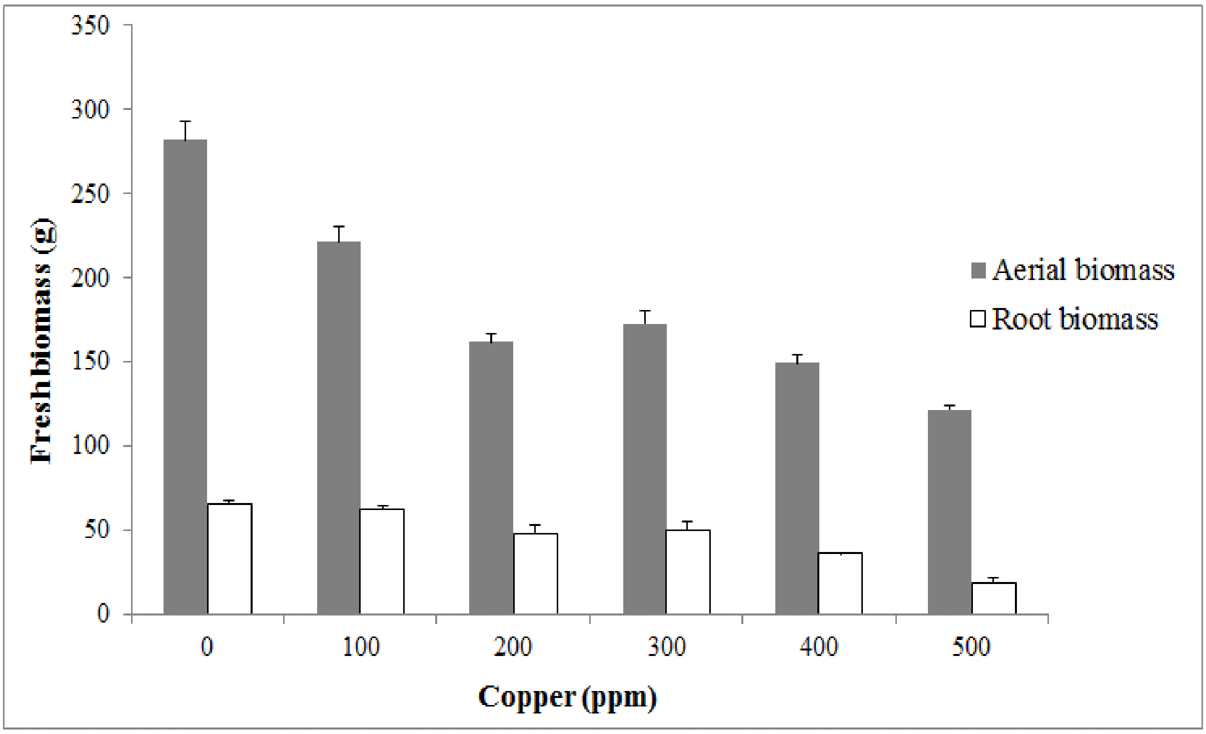


Figure 38: Effect of copper on aerial and root fresh biomass of *Lycopersicon esculentum* Mill.

Table 12: Shows values of aerial and root fresh biomass of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Aerial fresh biomass (g) P=0	281.8 a ± 11.557	221.527b ± 8.525	161.967 c ± 5.4	173 c ± 7.55	149.333 d ± 5.132	122 e ± 1.943
Root fresh biomass (g) P=0	65.9 a ± 1.825	62.567 a ± 2.159	48.2 b ± 5.632	49.7 b ± 5.188	36.633 c ± 0.462	18.9 d ± 2.914

Compared to control plants, statistical analysis revealed that copper-induced a highly significant effect on aerial and root fresh biomass of *Lycopersicon esculentum* Mill. (Annex 4).

In aerial fresh biomass, five homogenous groups were given in Table 12, group a (0 ppm), group b (100 ppm), group c (200 and 300 ppm), group d (400 ppm), and group e (500 ppm). However, four homogenous groups were found with root fresh biomass, group a (0 and 100 ppm), group b (200 and 300 ppm), group c (400 ppm), and group d (500 ppm).

VII.2.1.5. Effect of copper on aerial and root dry biomass

As shown in Figure 39, dry weight of *Lycopersicon esculentum* Mill. was considerably reduced by increasing copper doses in the soil, compared to the control seedlings.

In absence of Cu, aerial and root dry biomass was 90.12 and 19.31 g, respectively. A moderate decrease in the aerial weight (63.5 g) was observed at 100 ppm Cu; while no significant reduction in root weight (18.126 g) was obtained by this concentration. Remarkably, copper at a high level (500 ppm) caused a sharp decrease in the aerial and root dry biomass by 37.12 and 5.4 g, respectively.

Although, the reduction of aerial and root dry biomass recorded at 200 ppm (45.531 and 14.335 g) and 400 ppm (44.57 and 10.11g), treatment by 300 ppm Cu slightly improved the aerial and root dry biomass to 50.262 and 16.528 g.

Overall, aerial dry weight was higher than root dry weight.

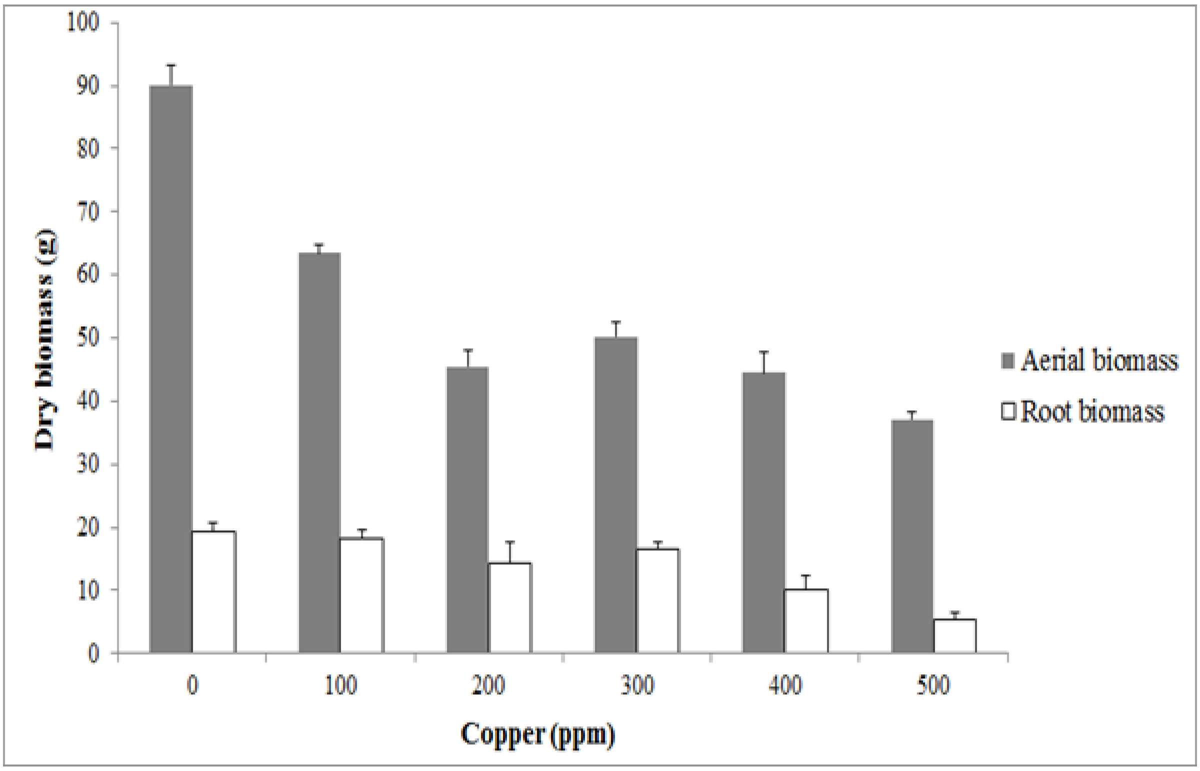


Figure 39: Effect of copper on aerial and root dry biomass of *Lycopersicon esculentum* Mill.

Table 13: Shows values of aerial and root dry biomass of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Aerial dry biomass (g) **P= 0**	90.12 a ± 3.1	63.5 b ± 1.23	45.531 c ± 2.541	50.262 d ± 2.21	44.57 c ± 3.315	37.12 e ± 1.215
Root dry biomass (g) **P= 0**	19.31 a ± 1.32	18.126 a ± 1.35	14.335 b ± 3.212	16.528b ± 1.22	10.11 c ± 2.123	5.4 d ± 1.139

Statistical data revealed that copper treatments induced a highly significant effect on aerial and root dry biomass of *Lycopersicon esculentum* Mill. (Annex 4).

In aerial dry biomass, five homogeneous groups were shown in Table 13, group a (0 ppm), group b (100 ppm), group c (200 and 400 ppm), group d (300 ppm), and group e (500 ppm). However, four homogenous groups were given with root dry biomass, group a (0 and 100 ppm), group b (200 and 300 ppm), group c (400 ppm), and group d (500 ppm).

VII.2.1.6. Effect of copper on leaf area

Excess amount of copper caused a significant reduction in the leaf area of *Lycopersicon esculentum* Mill. (Figure 40). This reduction was proportional to the copper concentrations applied in the soil. In comparison to control seedlings (17.79 cm²), a sharp decrease was recorded from 100 and 200 ppm by 9.93 and 9.35cm²; whereas, treatment with 300 ppm slightly increased the leaf area by 9.39 cm². However, the most important reduction was observed at 400 and 500 ppm by 7.32 and 6.8 cm^2 respectively, compared to the control.

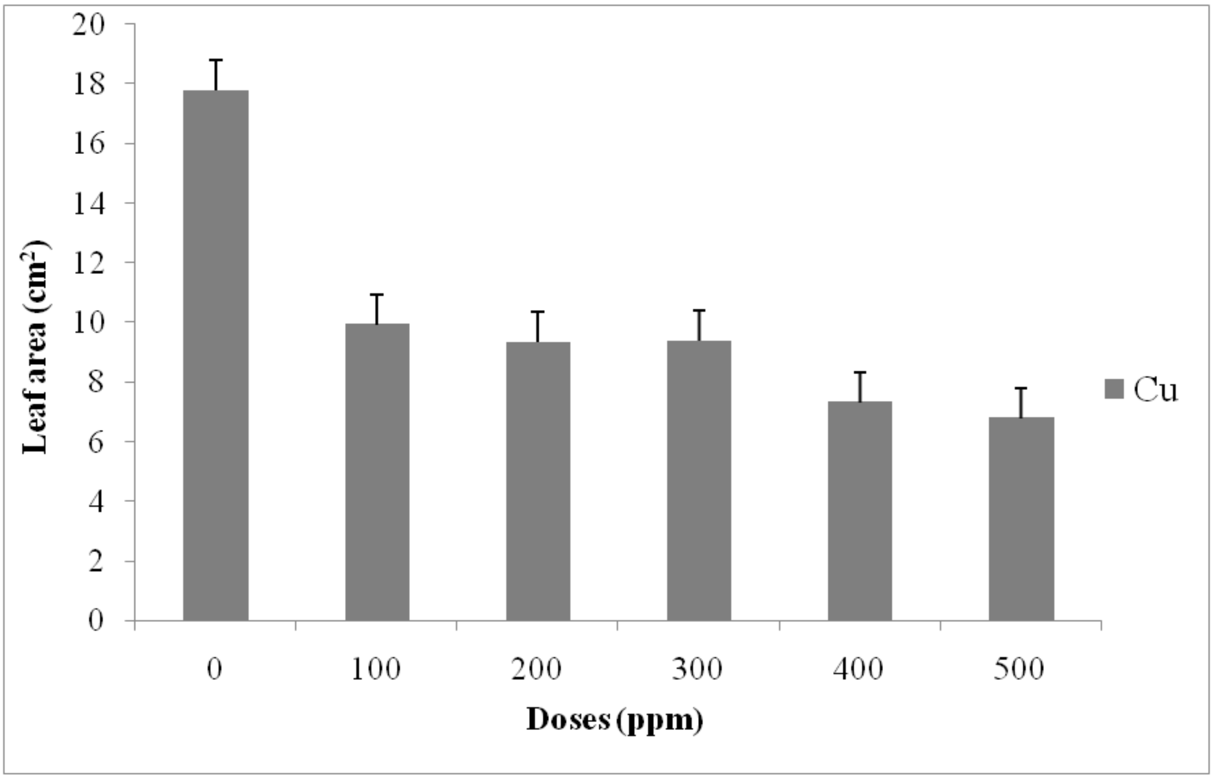


Figure 40: Effect of copper on leaf area of *Lycopersiconesculentum* Mill.

Table 14: Shows values of leaf area of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a and b) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf area (cm^2) **P=0.00**	17.79 a ± 3.26	9.93 b ± 0.44	9.35 b ± 1.24	9.39 b ± 0.32	7.32 b ± 0.90	6.8 b ± 0.45

Variance analysis revealed a highly significant effect of copper on leaf area of *Lycopersicon esculentum* Mill., compared to control seedlings (Annex 4).

As shown in Table 14, a presence of two homogeneous groups, group a (0 ppm) and group b (100, 200, 300, 400, and 500 ppm).

VII.2.2 Biometrical parameters

VII.2.2.1. Effect of copper on leaf relative water content (RWC)

As shown in Figure 41, leaf water content decreased substantially as the different copper doses in the soil increased.

In comparison to leaves control, in which RWC maintained the highest level of 74.90 %, the lowest water content (36.33 %) was observed at 500 ppm. Moreover, a remarkable reduction of 63.37 % was recorded at 100 ppm. However, plants exposed to 200 and 300ppm Cu exhibited a slight difference in RWC between them, as given by the following percentages: 55.48 and 54.14 %, respectively.

Indeed, water status remained superior to 50 % until the application of 400 ppm Cu that provided 46 % of RWC.

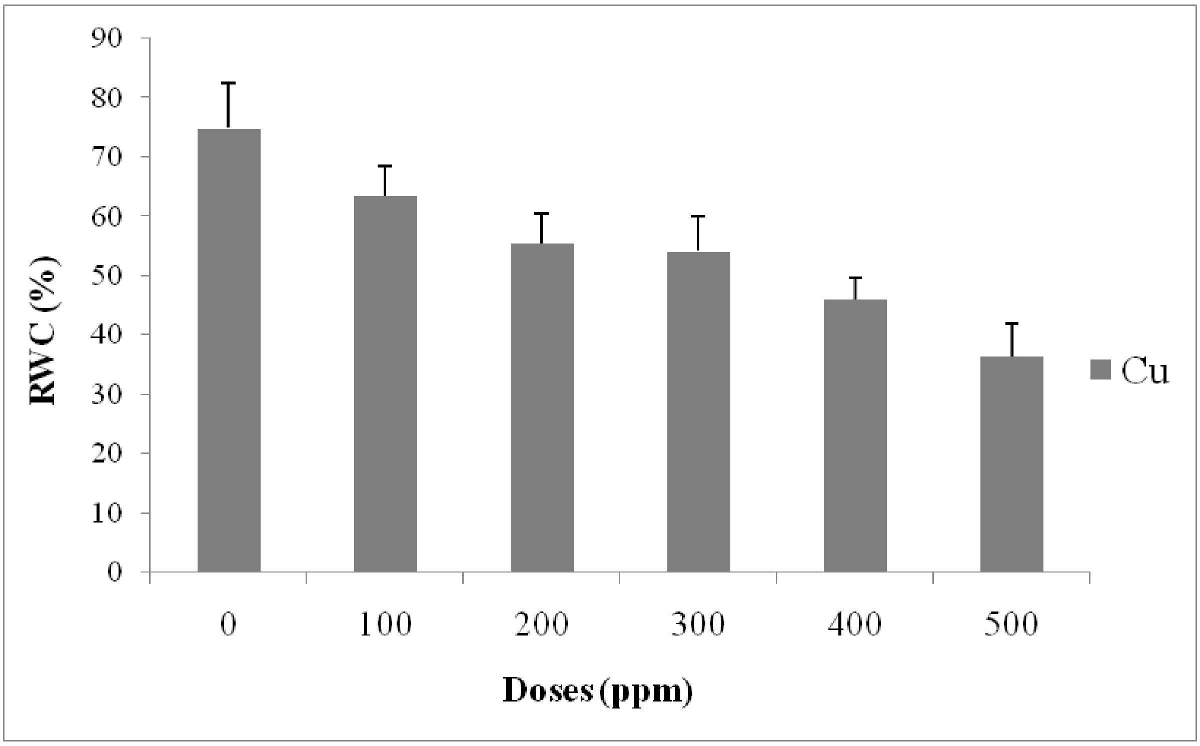


Figure 41: Effect of copper on leaf relative water content (RWC) of *Lycopersicon esculentum* Mill.

Table 15: Shows values of leaf relative water content of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, and d) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Relative water content (%) **P= 0.00**	74.90 a ± 7.64	63.37 b ± 5.19	55 .48 bc ± 5.07	54.14 bc ± 5.791	46 cd ± 3.06	36.33 d ± 5.68

Statistical data showed that the effect of copper treatment was highly significant on relative water content, compared to control plants of *Lycopersicon esculentum* Mill. (Annex 4).

As described in Table 15, five homogenous groups were found with RWC, group a (0 ppm), group b (100 ppm), group bc (200 and 300 ppm), group cd (400 ppm), and group d (500 ppm).

VII.2.2.2. Effect of copper on relative water loss (RWL)

Results depicted in Figure 42 demonstrate the loss of water content in the eaves of *Lycopersicon esculentum* Mill. was influenced by copper treatments at high concentrations.

Moreover, this negative effect was moderately increased with increasing copper doses in the soil, compared to RWL in the control leaves (0.361 g. cm^{-2}.min^{-1}). A maximum decrease in relative water loss was observed at 500 ppm by 0.232 g. cm^{-2}. min^{-1}; while, copper treatments with 200 ppm did not show a significant difference compared to100 ppm, as given by the following values: 0.289 and 0.282 g. cm^{-2}.min^{-1} respectively. Likewise, 300 ppm Cu was revealed to have a similar effect as that of 400 ppm Cu with 0.253 g. cm^{-2}. min^{-1}.

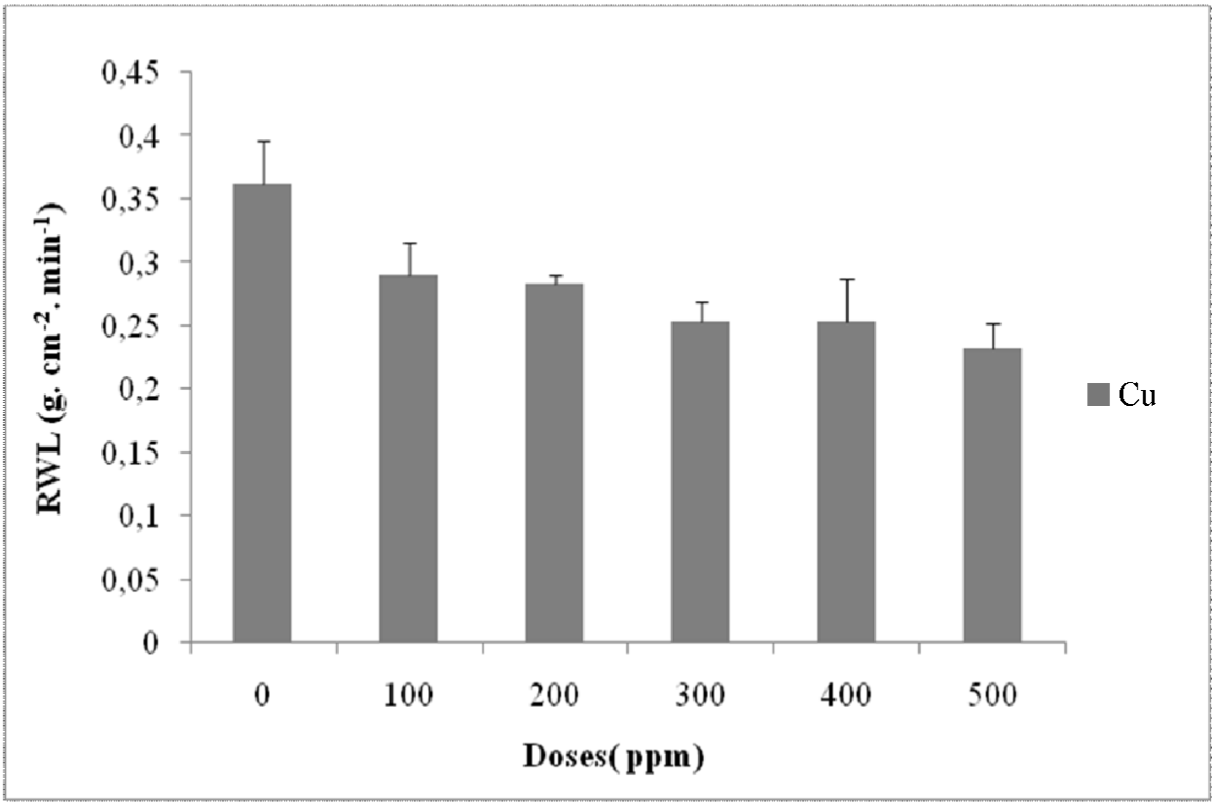


Figure 42: Effect of copper on relative water loss of *Lycopersicon esculentum* Mill.

Table 16: Shows values of leaf relative water loss of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a and d) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
RWL ($g.cm^{-2}.min^{-1}$) P= 0.00	0.361 a ± 0.034	0.289 b ± 0.026	0.282 b ± 0.008	0.253 b ± 0.015	0.253 b ± 0.033	0.232 b ± 0.02

Variance analysis pointed out a highly significant effect of copper on relative water loss, compared to the control plants of *Lycopersicon esculentum* Mill. (Annex 4).

As shown in Table 16, two homogenous groups were determined for RWL, group a (0 ppm), group b (100 ppm, 200, 300 ppm, 400 ppm, and 500 ppm).

VII.2.3 Physiological parameters

VII.2.3.1. Effect of copper on chlorophyll and carotenoids pigments content

As a result of heavy metal stress, pigments biosynthesis in leaves of *Lycopersicon esculentum* Mill. was affected by different concentrations of copper (Figure 43).

All treatments showed that: Total chlorophyll > chlorophyll a > cholophyll b > carotenoids. Despite this, all chlorophyll pigments exhibited a similar trend by the gradual decline in their content, as copper concentration increased.

Compared to the control pigments, copper at 500 ppm lowered the content of chlorophyll a and b from 5.914 to 3.295 and 2.832 to 1.311 mg. g^{-1} FW, respectively. Furthermore, total chlorophyll content at 0 ppm was 8.745 mg. g^{-1} FW; while, at 500 ppm it was 4.606 mg. g^{-1} FW.

Notably, no significant decrease was obtained by 100 to 200 ppm for chlorophyll a (5.046 to 5.041mg. g^{-1} FW), b (2.769 to 2.395 mg. g^{-1} FW), and total chlorophyll (7.815 to 7.435 mg. g^{-1} FW).

Compared to the control (1.176 mg. g^{-1} FW), carotenoids content declined slightly at 100 ppm Cu by 0.789 mg. g^{-1} FW. After that, a remarkable increase was observed with 200, 300, and 400 ppm to reach a value of 1.12 mg. g^{-1} FW; whereas, a decrease of carotenoid content to 0.911mg. g^{-1} FW was found at 500 ppm Cu.

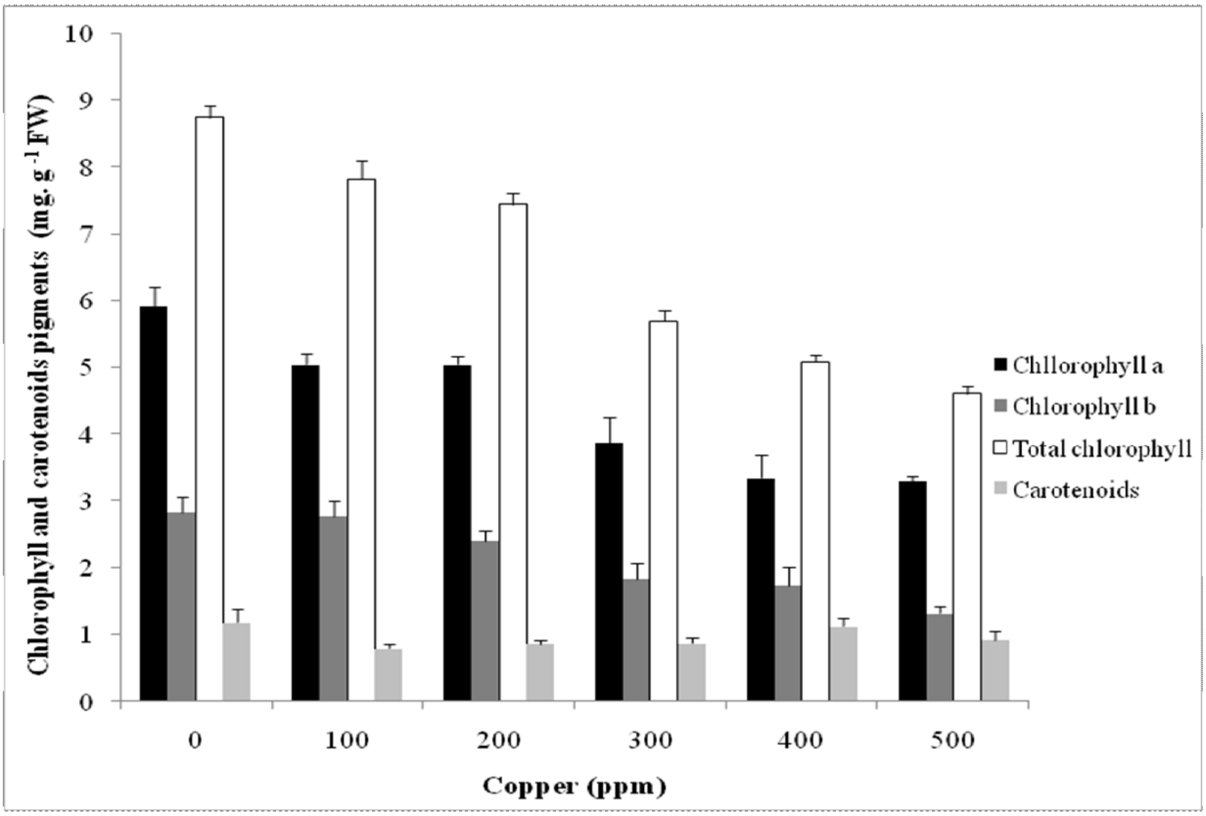


Figure 43: Effect of copper on chlorophyll and carotenoids pigments content of *Lycopersicone sculentum* Mill.

Table 17: Shows values of chlorophyll and carotenoids pigment content of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, and d) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Chlorophyll a content (mg. g^{-1} FW) P=0	5.914 a ± 0.293	5.046 b ± 0.162	5.041 b ± 0.124	3.861 c ± 0.383	3.346 d ± 0.331	3.295 d ± 0.066

Chlorophyll b content (mg. g^{-1} FW) P= 0.00	2.832 a ± 0.228	2.769 a ± 0.222	2.395 b ± 0.158	1.831 c ± 0.235	1.734 c ± 0.275	1.311 d ± 0.113
Total chlorophyll content (mg. g^{-1} FW) P=0	8.745 a ± 0.164	7.815 b ± 0.281	7.435 b ± 0.164	5.692 c ± 0.164	5.08 c ± 0.113	4.606 d ± 0.107
Carotenoids content (mg. g^{-1} FW) P=0	1.176 a ± 0.212	0.789 b ± 0.063	0.853 b ± 0.056	0.865 b ± 0.076	1.12 a ± 0.124	0.911 b ± 0.142

Statistical data indicated a highly significant effect of copper on the level of photosynthetic pigments, compared to non-stressed leaves of *Lycopersicone sculentum* Mill. (Annex 4).

As shown in Table 17, four homogenous groups (a, b, c, and d) were found with chlorophyll a, b**,** and total chl; while two groups (a and b) were recorded with carotenoids pigments.

VII.2.4 Biochemical parameters

VII.2.4.1. Effect of copper on proteins content

Plant subjected to different concentrations of copper exhibited a fluctuating trend in proteins content, either in leaves or roots of *Lycopersicon esculentum* Mill. (Figure 44).

Copper at 100 ppm induced a significant elevation of proteins content in the leaves by 12.7 mg. g^{-1}FW, compared to the control by 10.38 mg. g^{-1} FW. However, a slight decrease was noted at 200 and 300 ppm Cu by 10.42 and 10.55 mg. g^{-1} FW respectively; to achieve a similar level as that in control seedlings. Likewise, a maximum decline in proteins content was obtained at 400 and 500 ppm Cu, respecting the following values: 6.58 and 5.63 mg. g^{-1}FW.

Besides, copper stress substantially induced the accumulation of proteins content in the roots. It reached the maximum level at 300 ppm by 7.077 mg. g^{-1} FW, compared to 0, 100 and 200 ppm by 1.584, 4.54, and 5.953 mg. g^{-1} FW, respectively. However, proteins content recorded at 400 and 500 ppm Cu decreased steadily by 6.285 and 6.256 mg. g^{-1} FW.

All copper treatments indicated that proteins content in leaves was higher than roots, conversely to the results obtained with 500 ppm Cu.

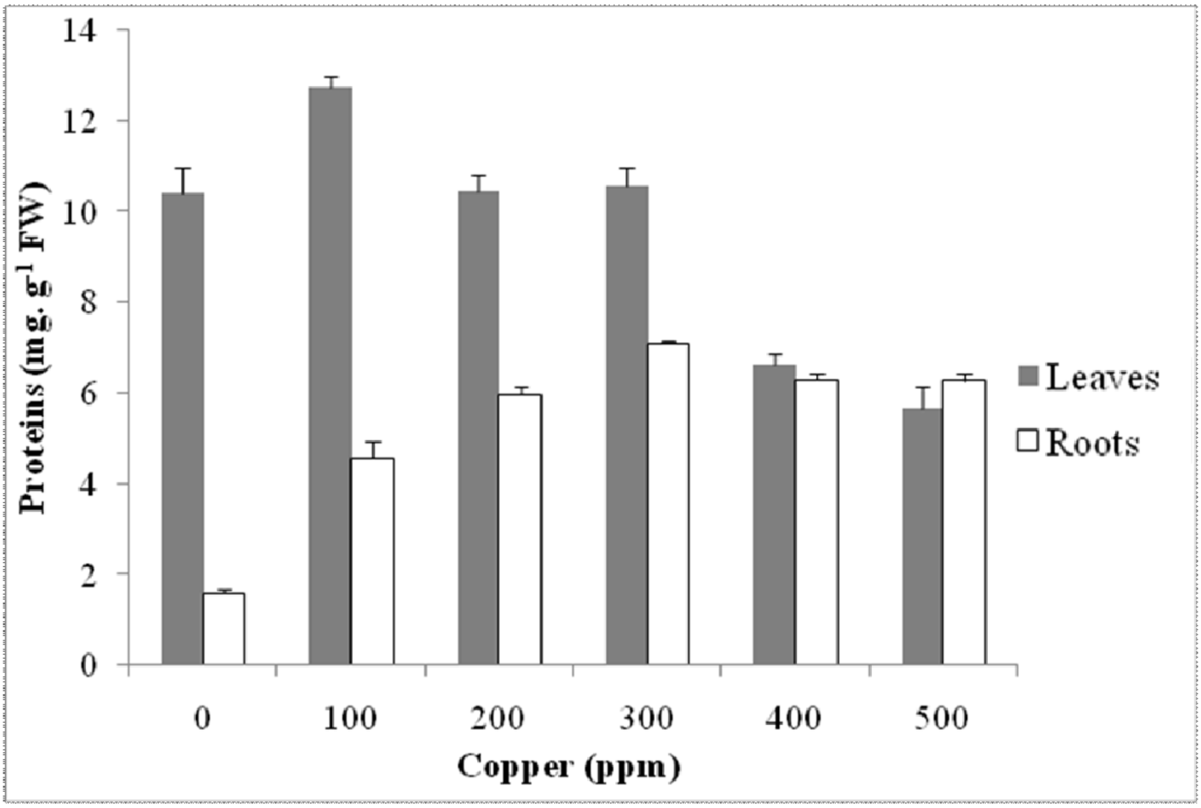


Figure 44: Effect of copper on proteins content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 18: Shows values of proteins content of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, and d) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf proteins content (mg. g^{-1}FW) P=0	10.381 b ± 0.566	12.708 a ± 0.237	10.42 b ± 0.358	10.557 b ± 0.406	6.588 c ± 0.271	5.63 d ± 0.511
Root proteins content (mg. g^{-1}FW) P= 0	1.584 d ± 0.078	4.54 c ± 0.395	5.953 b ± 0.163	7.077 a ± 0.045	6.285 b ± 0.139	6.256 b ± 0.15

A highly significant effect was noted in proteins content due to the presence of different concentrations of copper in the soil, compared to the control of *Lycopersicon esculentum* Mill. (Annex 4).

As shown in Table 18, four homogeneous groups were stated for leaf proteins content, group a (100 ppm), group b (0, 200, 300 ppm), group c (400 ppm), and group d (500 ppm). Also, for root proteins content four groups were given, group a (300 ppm), group b (200, 400, and 500 ppm), group c (100 ppm), and group d (0 ppm).

VII.2.4.2. Effect of copper on soluble sugars content

Copper stress induced the accumulation of soluble sugars content in leaves and roots of *Lycopersicon esculentum* Mill. This accumulation was depending on the increasing copper doses in the soil (Figure 45).

Soluble sugars were highly accumulated in the leaves compared to roots, inversely to the level recorded at 0 and 300 ppm Cu.

Besides, the highest amount of soluble sugars was observed in leaves at 500 ppm by 9.124 mg. g^{-1}DW and roots at 300 and 500 ppm by 8.393 and 8.331 mg. g^{-1}DW, respectively. However, the lowest content in both organs was noted at 0 ppm by 4.5 and 4.854 mg. g^{-1}DW, respectively, this content increased slightly by 100 ppm Cu to give values of 5.59 and 4.933 mg. g^{-1}DW.

Remarkably, in the leaves, no significant increase in soluble sugars content was marked from 200 ppm to 300 ppm Cu treatments, as given by the following amounts: 6.292 and 6.483 mg. g^{-1}DW, respectively.

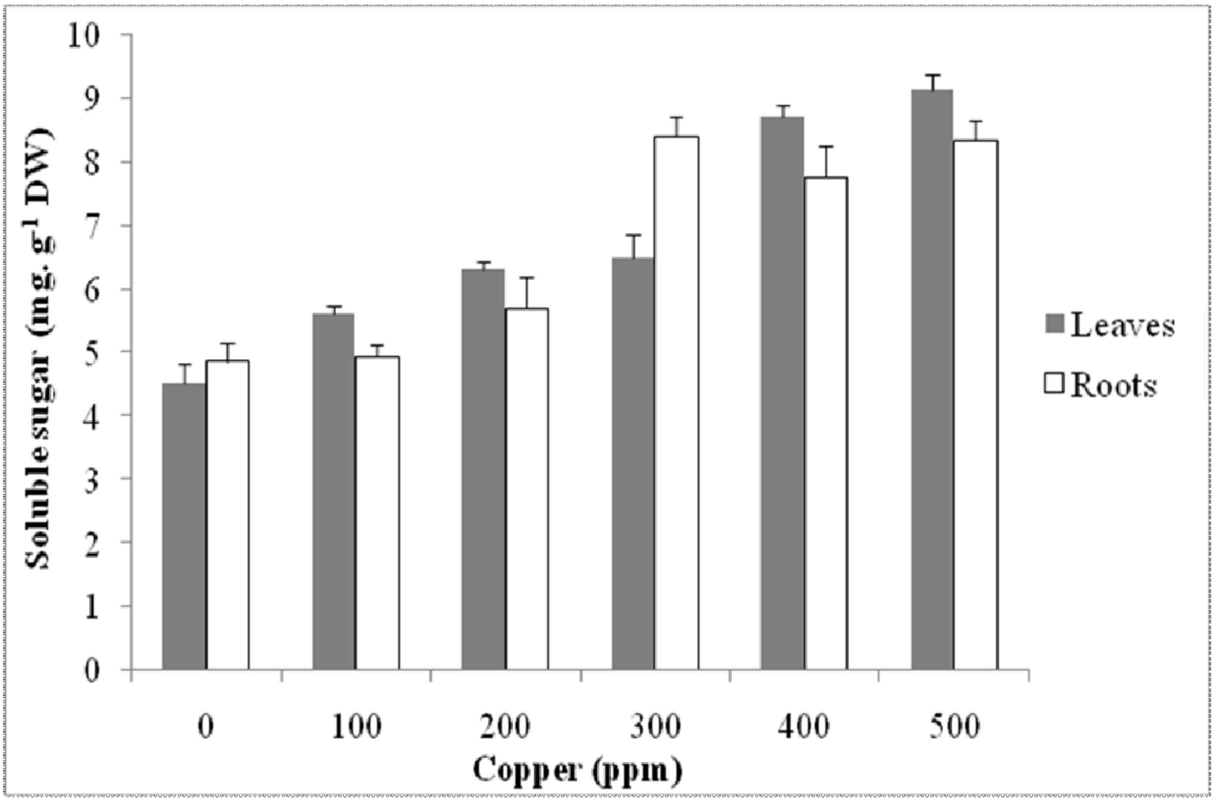


Figure 45: Effect of copper on soluble sugars content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 19: Shows values of soluble sugars content of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, and c) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf soluble sugars content (mg. g^{-1}DW) P=0	4.5 c ± 0.321	5.59 b ± 0.122	6.292 b ± 0.136	6.483 b ± 0.354	8.691 a ± 0.189	9.124 a ± 0.256
Root soluble sugars content (mg. g^{-1}DW) P=0	4.854 b ± 0.307	4.933 b ± 0.173	5.68 b ± 0.488	8.393 a ± 0.294	7.764 ab ± 0.484	8.331a ± 0.318

Analysis data state that the copper effect was highly significant on the content of soluble sugars in leaves and roots, compared to control seedlings of *Lycopersicon esculentum* Mill. (Annex 4).

As displayed in Table 19, three homogenous groups were determined with leaf soluble sugars content, group a (400 and 500 ppm), group b (100, 200, and 300 ppm), and group c (0 ppm). However, root soluble sugar content exhibited three groups, group a (300 and 500 ppm), group b (0, 100, and 200 ppm), and group ab (400 ppm).

VII.2.4.3. Effect of copper on proline content

Proline content increased gradually by copper treatment, in leaves and roots of *Lycopersicon esculentum* Mill. This trend was directly proportional to the different doses of copper (Figure 46).

As is observed, no significant increase in proline content was observed between the following copper concentrations: 0, 100 and 200 ppm; in leaves by 9.214, 9.543, and 9.675 mg. g^{-1}DW, in roots by 3.225, 3.291 and 3.949 mg. g^{-1}DW, respectively. However, the highest level of proline content was obtained by copper at 500 ppm, in leaves with 13.624 mg. g^{-1}DW and roots with 9.478 mg. g^{-1}DW.

Besides, proline was lowlily accumulated in the roots than leaves, for all treatments.

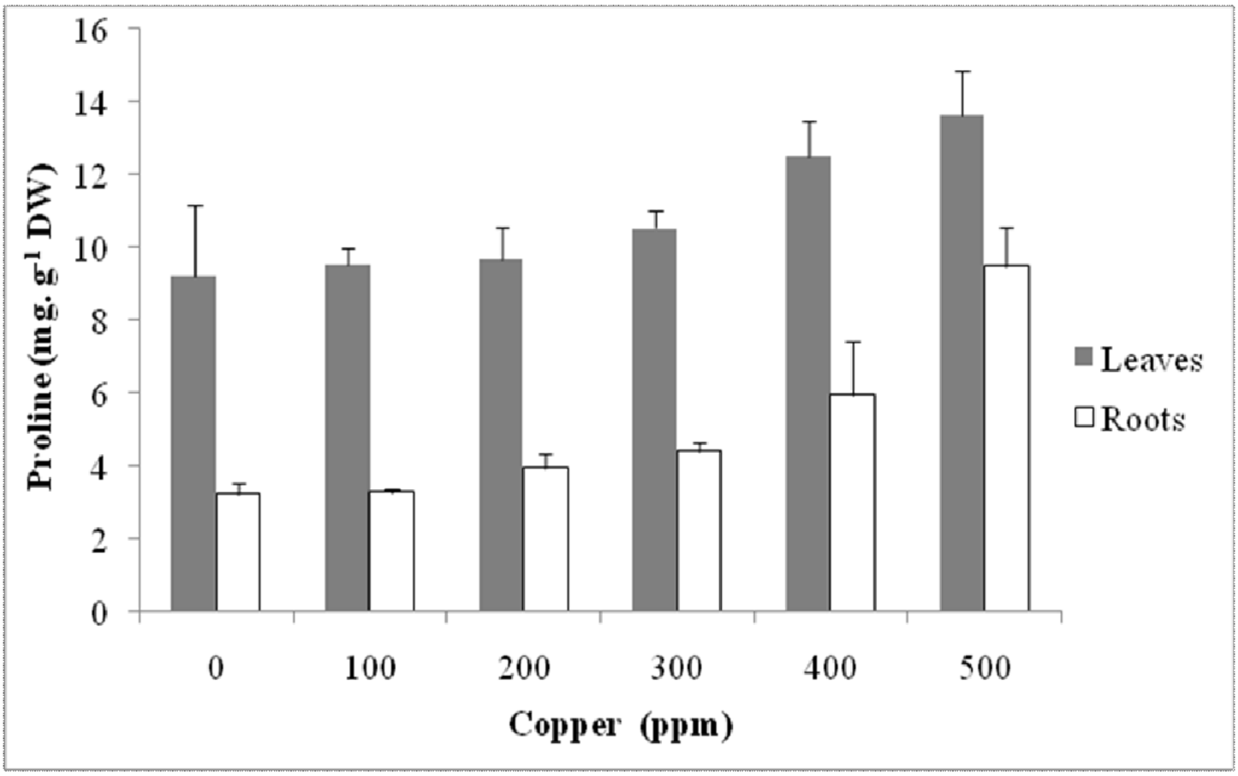


Figure 46: Effect of copper on proline content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 20: Shows values of proline content of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, and c) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf proline content (mg. g^{-1}DW) P=0	9.214 c ± 1.978	9.543 c ± 0.456	9.675 c ± 0.861	10.531bc ± 0.497	12.505 ab ± 0.994	13.624 a ± 1.233
Root proline content (mg. g^{-1}DW) P=0	3.225 c ± 0.302	3.291c ± 0.114	3.949 c ± 0.395	4.41 c ± 0.228	5.924 b ± 1.491	9.478 a ± 1.099

A highly significant effect of copper treatment on proline content was observed in leaves and roots of *Lycopersicon esculentum* Mill., compared to the control (Annex 4).

Four homogenous groups were found with leaf proline content, group a (500 ppm), group ab (400ppm), group bc (300 ppm), and group c (0, 100, and 200 ppm). However, root proline content presents three homogeneous groups, group a (500 ppm), group b (400 ppm), and group c (0,100, 200 and 300 ppm) (Table 20).

VII.2.4.4. Effect of copper on polyphenols content

As a consequence of copper stress exposure, an increase in polyphenols content was recorded in leaves and roots of *Lycopersicon esculentum* Mill. (Figure 47). Moreover, this accumulation increased positively with the increase of copper doses in the soil.

At treatments of 0 and 100 ppm Cu, polyphenols content was 12.37 and 13.7 mg GAE.g^{-1}DWin the leaves, and 6.543 and 9.922 mg GAE.g^{-1}DW in roots, respectively. However, the maximum level of polyphenols was marked at 400 and 500 ppm by 22.6 and 23.407 mg GAE.g^{-1}DW in the leaves, and 17.448 and 18.861 mg GAE.g^{-1}DW in the roots.

Overall, the leaves have a tendency to accumulate a high level of polyphenols compared to the roots.

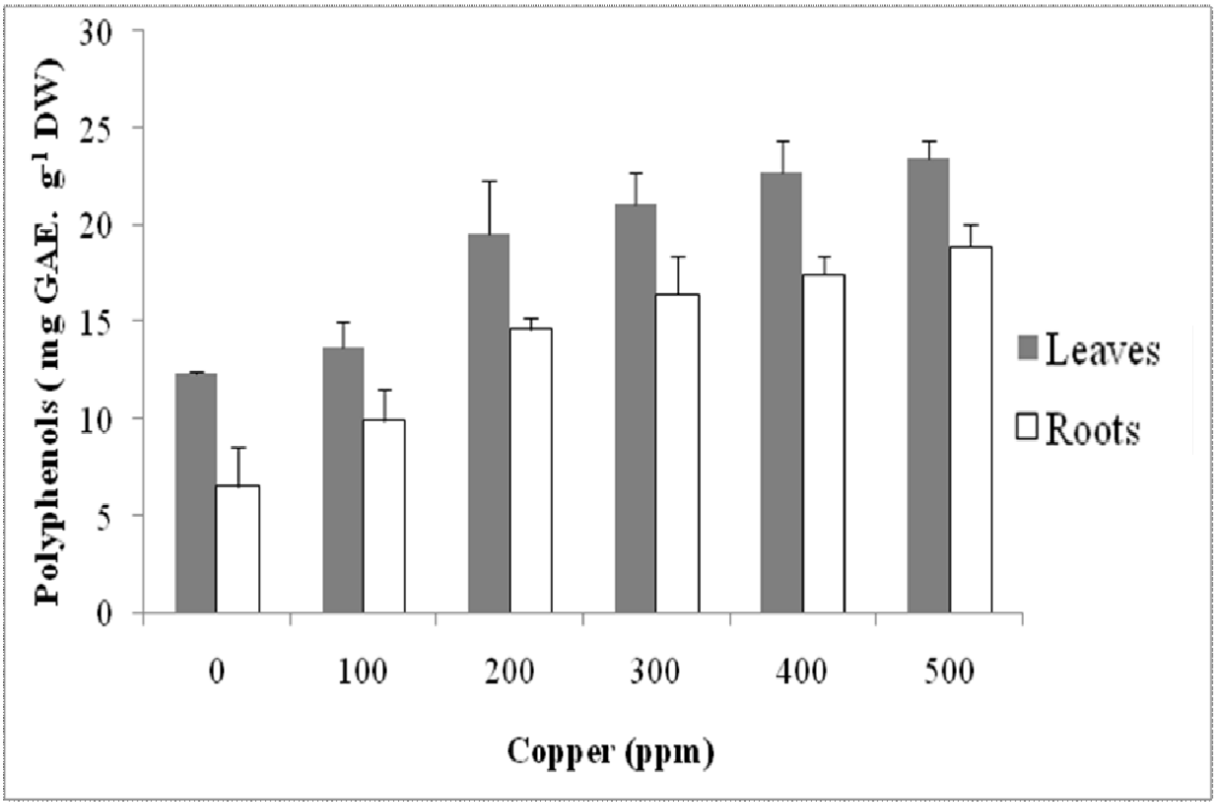


Figure 47: Effect of copper on polyphenols content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 21: Shows values of polyphenols content of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a and b) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf polyphenols content (mg GAE. g^{-1} DW) P=0	12.379 b ± 0.106	13.7 b ± 1.324	19.506 a ± 2.767	21.103 a ± 1.575	22.67 a ± 1.661	23.407 a ± 0.975
Root polyphenols content (mg GAE. g^{-1} DW) P=0	6.543 c ± 2.438	9.922 b ± 1.918	14.622 a ± 0.698	16.403 a ± 2.488	17.448 a ± 1.185	18.861 a ± 1.446

Effect of copper treatment on polyphenols content in roots and leaves of *Lycopersicon esculentum* Mill. was highly significant, compared to the control plants.

As shown in Table 21, two homogenous groups were determined for leaf polyphenols content, group a (200, 300, 400, and 500 ppm) and group b (0 and 100ppm). However, three groups were found with root polyphenols content, group a (200, 300, 400, and 500 ppm), group b (100 ppm), and group c (0 ppm) (Annex 4).

VII.2.4.5. Effect of copper on flavonoids content

Under copper stress, an increasing trend in flavonoids content was detected in leaves and roots of *Lycopersicon esculentum* Mill. (Figure 48).

In comparison to the control, a slight increase was recorded at 100 ppm in the leaves by 0.211 and 0.288 mg QE. g^{-1}DW, and roots by 0.147 and 0.177 mg QE. g^{-1}DW, respectively. However, the flavonoids content at 200 and 300 ppm remained steady, after that, it continued to increase up 400 ppm; while, the highest level has occurred at 500 ppm.

Flavonoids were accumulated with a large amount in leaves compared to roots.

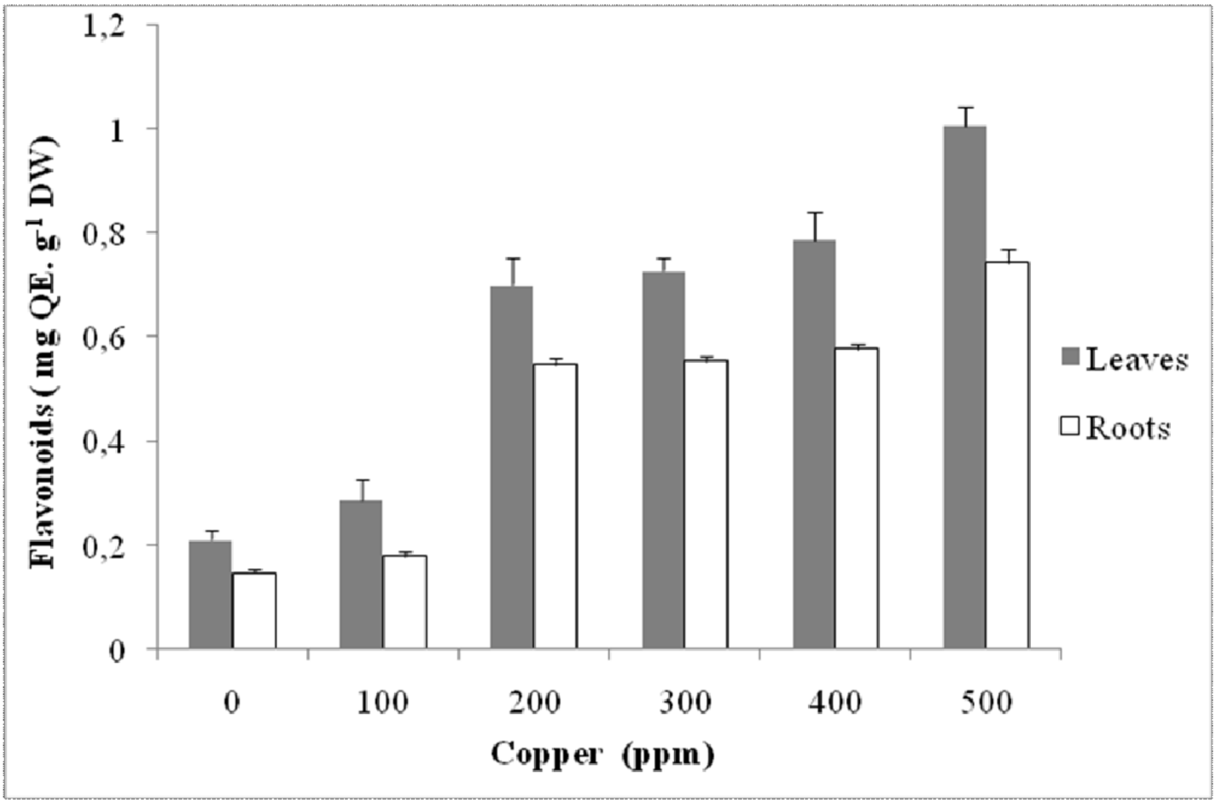


Figure 48: Effect of copper on flavonoid content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 22: Shows values of flavonoids content of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf flavonoids content (mg QE. g^{-1}DW) P=0	0.211 e ± 0.018	0.288 d ± 0.038	0.701 c ± 0.051	0.728 bc ± 0.023	0.787 b ± 0.054	1.007 a ± 0.035
Root flavonoids content (mg QE. g^{-1}DW) P=0	0.147 e ± 0.009	0.177 d ± 0.011	0.548 c ± 0.014	0.554 bc ± 0.011	0.578 b ± 0.009	0.744 a ± 0.024

Copper stress caused a highly significant increase in flavonoids content of leaves and roots of *Lycopersicon esculentum* Mill., compared to the control (Annex 4).

As shown in Table 22, six homogenous groups were observed with leaf and root flavonoids content, group a (500 ppm), group b (400 ppm), group bc (300 ppm), group c (200 ppm), group d (100 ppm) and group e (0 ppm).

VII.2.4.6. Effect of copper on the antioxidant activity (DPPH test)

Copper stress highly increased the antioxidant activity in leaves and roots of *Lycopersicon esculentum* Mill. (Figure 49). This capacity was elevated as copper concentrations in the soil increased, compared to the control seedlings.

The most important activity was recorded in the leaves; following by roots with little differences, as indicated by the inhibition percentages. Remarkably, a low concentration of copper (100 ppm) was able to trigger 50% of the antioxidant activity in the leaves, while this percentage in the roots was obtained by Cu treatment at 300 ppm

In general, this scavenging activity was induced from 0 to 500 ppm by 45.15 to 67.95% in the leaves and 40.25 to 59.62%, in the roots, respectively.

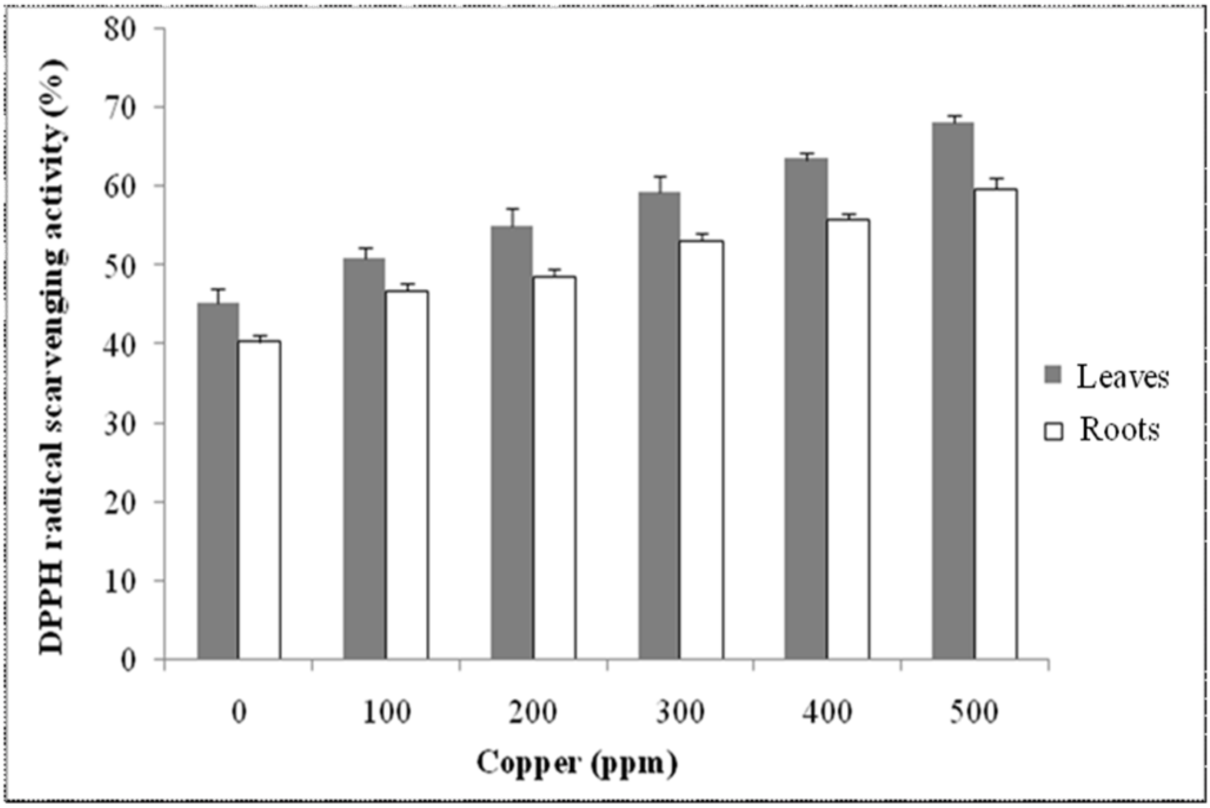


Figure 49: Effect of copper on the scavenging activity DPPH in leaves and roots of *Lycopersicon esculentum* Mill.

Table 23 : Shows values of scavenging activity in *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, d, e, and f) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Scavenging activity in leaves (%) P=0	45.159 f ± 1.841	50.735 e ± 1.388	54.902 d ± 2.208	59.252 c ± 1.913	63.358 b ± 0.696	67.953 a ± 0.925
Scavenging activity in roots (%) P=0	40.257 e ± 0.919	46.752 d ± 0.925	48.468 d ± 1.012	53.125 c ± 0.842	55.699 b ± 0.663	59.62 a ± 1.392

Copper stress exhibited a highly significant effect on the antioxidant activity in roots and leaves of *Lycopersicon esculentum* Mill., compared to the control seedlings (Annex 4).

As described in Table 23, six homogenous groups were determined for scavenging activity in leaves, group a (500 ppm), group b (400 ppm), group c (300 ppm), group d (200 ppm), group e (100 ppm) and group f (0 ppm). However, scavenging activity in roots presented five homogenous groups, group a (500 ppm), group b (400 ppm), group c (300 ppm), group d (100 and 200 ppm), and group e (0 ppm).

VII.3. Effect of zinc on the morphological, biometrical, physiological, and biochemical parameters of *Lycopersicon esculentum* Mill.

VII.3.1 Morphological parameters

VII.3.1.1. Effect of zinc on stem length

Zinc at different concentrations negatively affected the stem growth of *Lycopersicon esculentum* Mill. (Figure 50). Compared to control seedlings, length stem decreased steadily by increasing zinc doses in the soil.

Stem elongation exhibited a maximum-length growth in normal conditions by 57 cm; whereas, the minimum length was recorded at 500 ppm by 37 cm. Remarkably, the important inhibitory effect of zinc on the stem elongation has mainly occurred at 100 ppm by 48 cm; followed by the effect of 200, 300, and 400 ppm Zn, where no significant difference between them was observed (43.33, 42.66, and 40.33 cm), respectively.

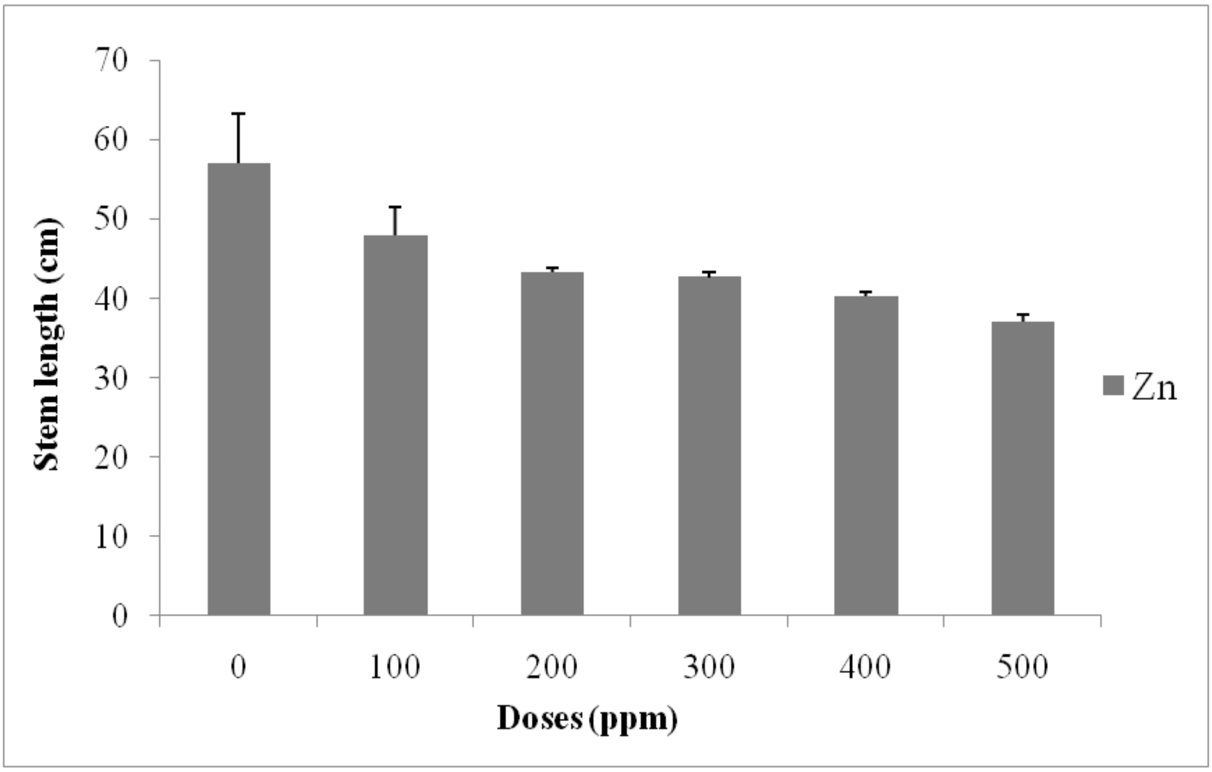


Figure 50: Effect of zinc on stem length of *Lycopersicon esculentum* Mill.

Table 24: Shows values of stem length of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, and c) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Stem length (cm) **P=0.00**	57 a ± 6.24	48 b ± 3.46	43.33 bc ± 0.57	42.66 bc ± 0.57	40.33 c ± 0.57	37 c ± 1

A highly significant effect was obtained by zinc treatment on stem length of *Lycopersicon esculentum* Mill., compared to the control seedlings (Annex 4).

As shown in Table 24, four homogenous groups were recorded for stem length, group a (0 ppm), group b (100 ppm), group bc (200 and 300 ppm), and group c (400 and 500 ppm).

VII.3.1.2. Effect of zinc on root length

As depicted in Figure 51, zinc treatments resulted in a moderate reduction of root growth of *Lycopersicon esculentum* Mill. This reduction was inversely proportional to the zinc doses in the soil.

In the absence of zinc, roots length measurements displayed a high value of 56.33 cm; while a slight decrease was noted at 100 ppm by 52.33 cm, compared to the control seedlings. Moreover, the effects caused by 200, 300, and 400 ppm were 45.66, 45, and 43.33 cm, respectively. Zinc at high concentration (500 ppm), did not show a substantial inhibitory effect on the root’s growth (42.66 cm); but among all treatments, it remained to have the minimum length.

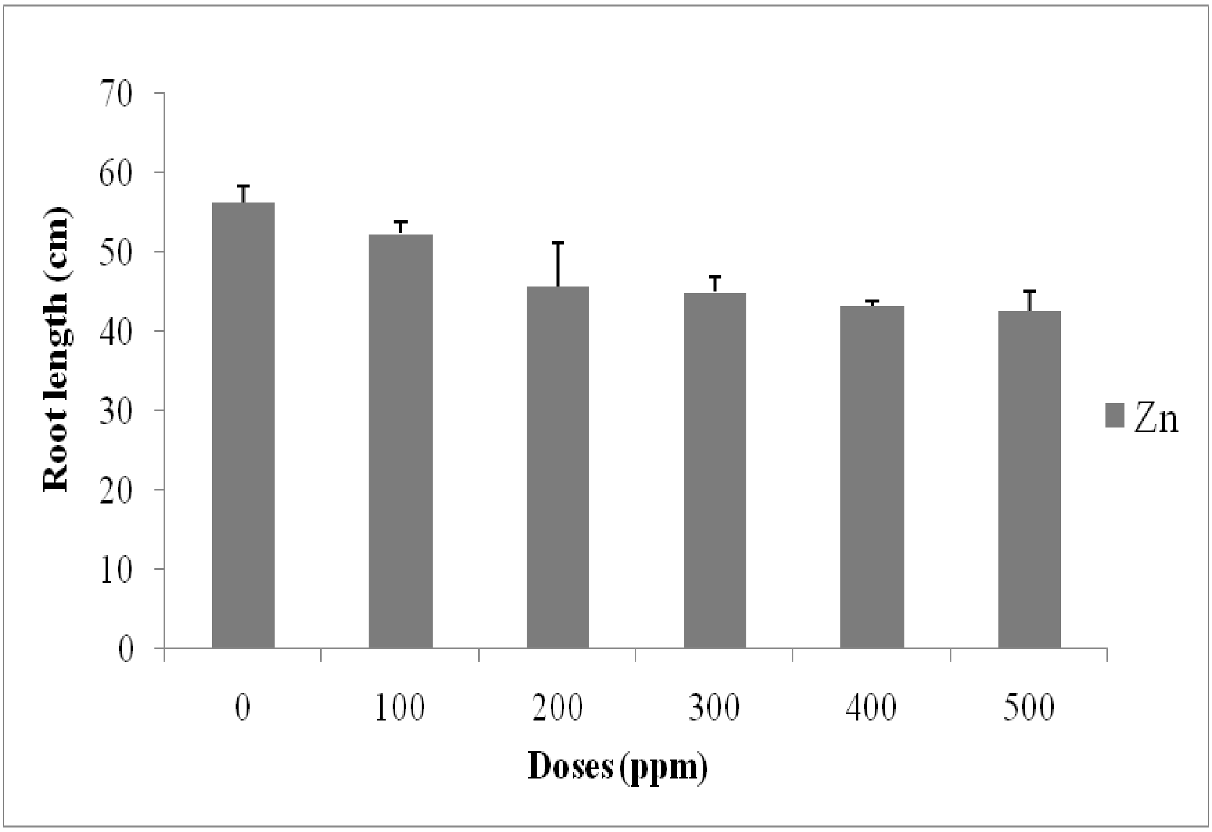


Figure 51: Effect of zinc on root length of *Lycopersiconesculentum* Mill.

Table 25: Shows values of root length of *Lycopersiconesculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a and b) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Root length (cm) **P=0**	56.33a ± 2.08	52.33 a ± 1.52	45.66 b ± 5.50	45 b ± 2	43.33 b ± 0.57	42.66 b ± 2.51

The zinc effect was highly significant on roots growth of *Lycopersicon esculentum* Mill., compared to the control plants.

As shown in Table 25, two homogenous groups were provided for root length, group a (0 and 100 ppm) and group b (200, 300, 400, and 500 ppm) (Annex 4).

VII.3.1.3. Effect of zinc on the number of leaves

As shown in Figure 52, control seedlings of *Lycopersicon esculentum* Mill. produced a greater number of leaves per plant (327 leaves), compared to those treated by zinc solution. This number of leaves diminished slightly when the plants were exposed to a high level of zinc.

A sharp decrease was observed at 100 ppm Zn by 201 leaves per plant. Furthermore, the application of 200, 300, and 400 ppm Zn reduced the number of leaves to 189, 185, and 168 leaves per plant, respectively. However, a severe loss in leaves (125 leaves) was obtained at a high zinc concentration of 500 ppm, it exceeded the 50 % of total leaves recorded at 0 ppm.

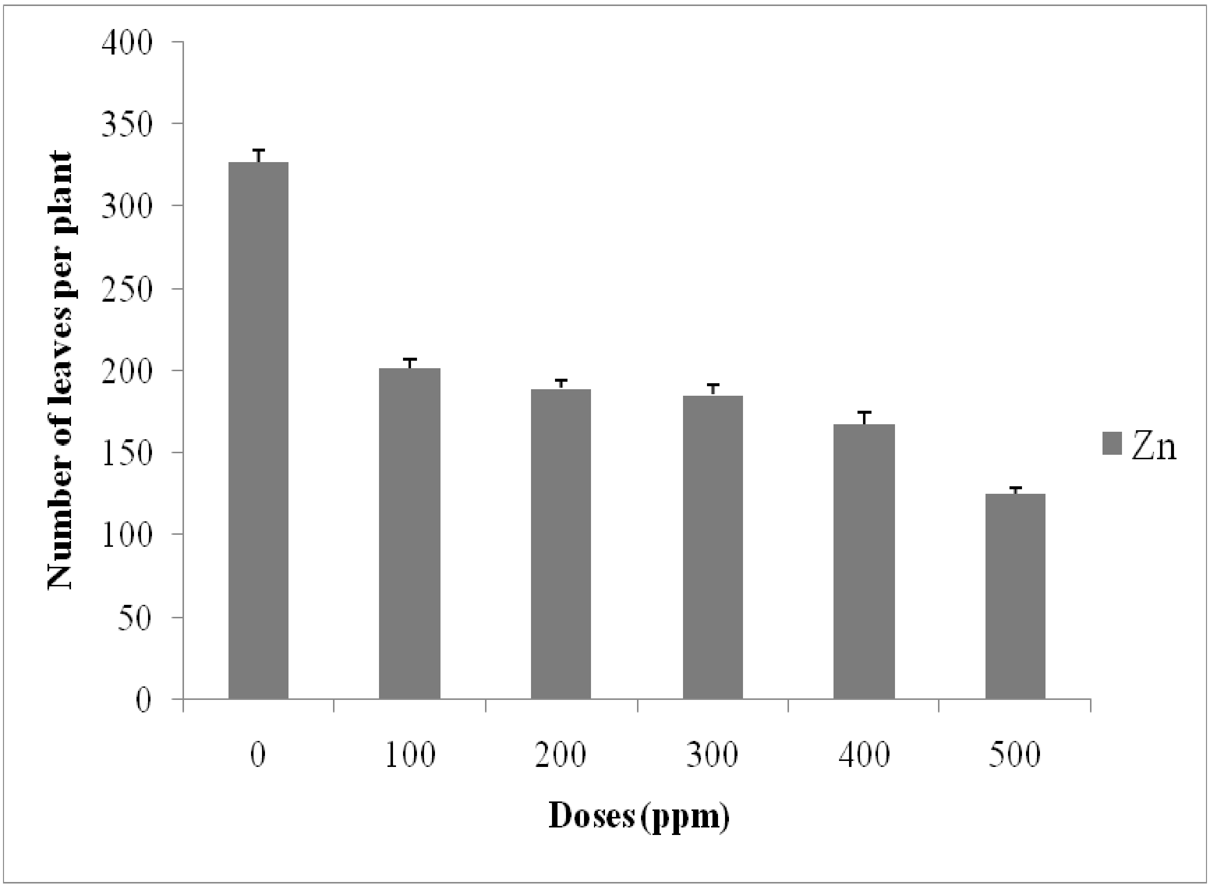


Figure 52: Effect of zinc on the number of leaves of *Lycopersicon esculentum* Mill.

Table 26: Shows values of several leaves of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Number of leaves per plant P=0	327.33 a ± 7.024	201.66 b ± 5.85	189.33 c ± 5.13	185.66 c ± 6.028	168 d ± 7	125.33 e ± 3.78

A highly significant effect was caused by zinc treatments on the number of leaves of *Lycopersicon esculentum* Mill., compared to control seedlings (Annex 4).

Five homogenous groups were recorded with several leaves per plant, group a (0 ppm), group b (100 ppm), group c (200 and 300 ppm), group d (400ppm), and group e (500 ppm) (Table 26).

VII.3.1.4. Effect of zinc on aerial and root fresh biomass

Seedlings of *Lycopersicon esculentum* Mill., exposed to different concentrations of zinc, showed a drastic decrease in areal and root fresh biomass, compared to the control (Figure 53).

Seedlings exposed to 0 and 100 ppm Zn exhibited a decline in the production of aerial fresh biomass by 251.233 and 211.33 g; while, roots fresh biomass was 89.333 and 47.433 g, respectively. No remarkable difference was observed between the effect of the followings zinc doses: 200, 300 and 400 ppm, in aerial biomass (146.28, 139.033 and 137.26 g) as well as the roots biomass (33.8, 33 and 27.453 g). However, zinc at 500 ppm lowered the aerial leaf fresh weight to 125.933 g; while, the root weight was highly affected to hit the value of 21g, as compared to the control.

Overall, aerial fresh biomass values were higher than roots fresh biomass, for all zinc treatments.

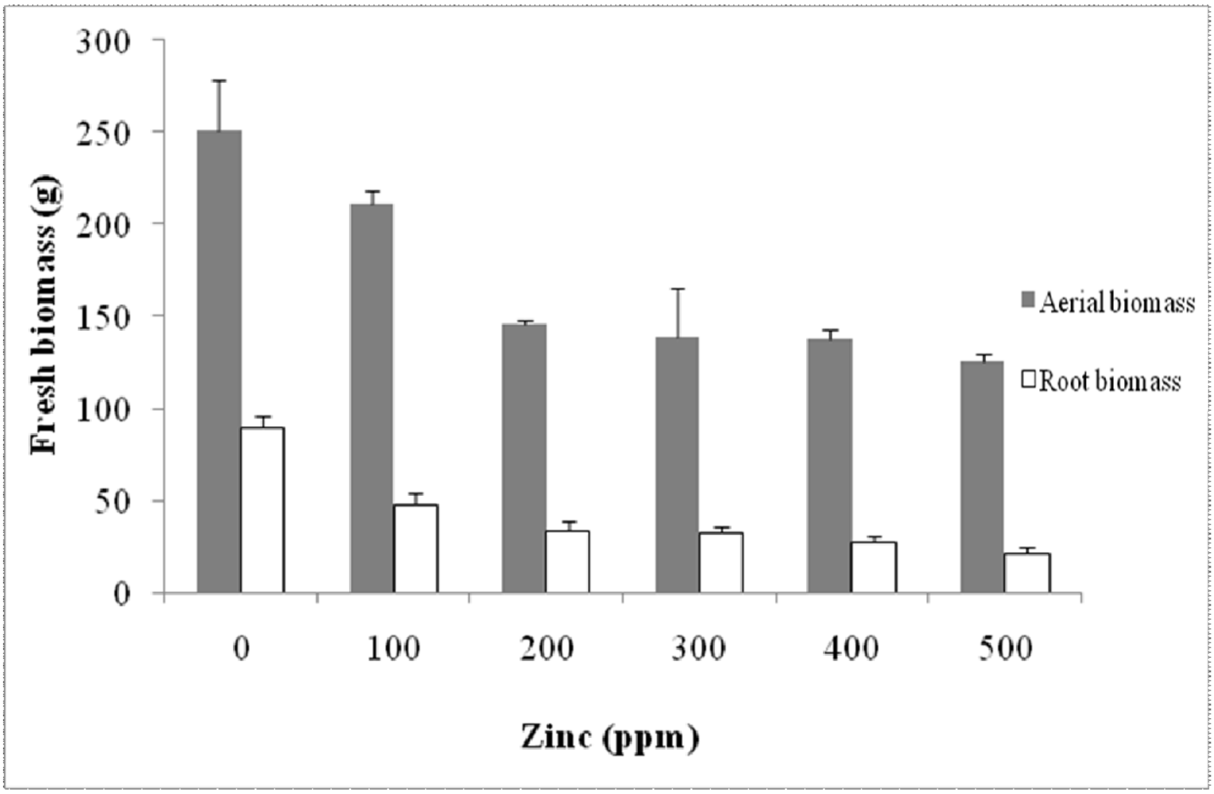


Figure 53: Effect of zinc on aerial and root fresh biomass *Lycopersicon esculentum* Mill.

Table 27: Shows values of aerial and root fresh biomass of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Doses du zinc (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Areal fresh biomass (g) P=0	251.233 a ± 27.547	211.333 b ± 6.506	146.28 c ± 1.583	139.033 d ± 25.962	137.26 d ± 5.741	125.933 e ± 3.573
Root fresh biomass (g) P=0	89.333 a ± 6.11	47.433 b ± 6.734	33.8 c ± 4.484	33 c ± 2.905	27.453 cd ± 3.083	21 d ± 3.606

Statistical data indicated that zinc treatments were highly significant on aerial and root biomass of *Lycopersicon esculentum* Mill., compared to control seedlings (Annex 4).

As shown in Table 27, five homogenous groups were determined for aerial fresh biomass, group a (0 ppm), group b (100 ppm), group c (200 ppm), group d (300 and 400 ppm), and group e (500 ppm). Likewise, five homogenous groups were recorded for root fresh biomass, group a (0 ppm), group b (100 ppm), group c (200 and 300 ppm), group cd (400 ppm), and group d (500 ppm).

VII.3.1.5. Effect of zinc on leaf and root dry biomass

As shown in Figure 54, zinc negatively affected the aerial and root dry weight of *Lycopersicon esculentum* Mill.

Compared to the control (81.312 and 22.35g), a significant reduction in biomass was observed at 100 ppm by 65.202 and 12.125 g; while, 500 ppm induced a sharp decrease by 26.1 and 3.213 g, of aerial and root parts respectively. However, dry biomass recorded at 200, 300 and 400 ppm were 40.257, 36.352 and 31.02 g for aerial parts and 8.153, 7.226 and 5.231 for roots parts.

Notably, control seedlings as well as those treated by zinc have an aerial dry weight higher than that of roots.

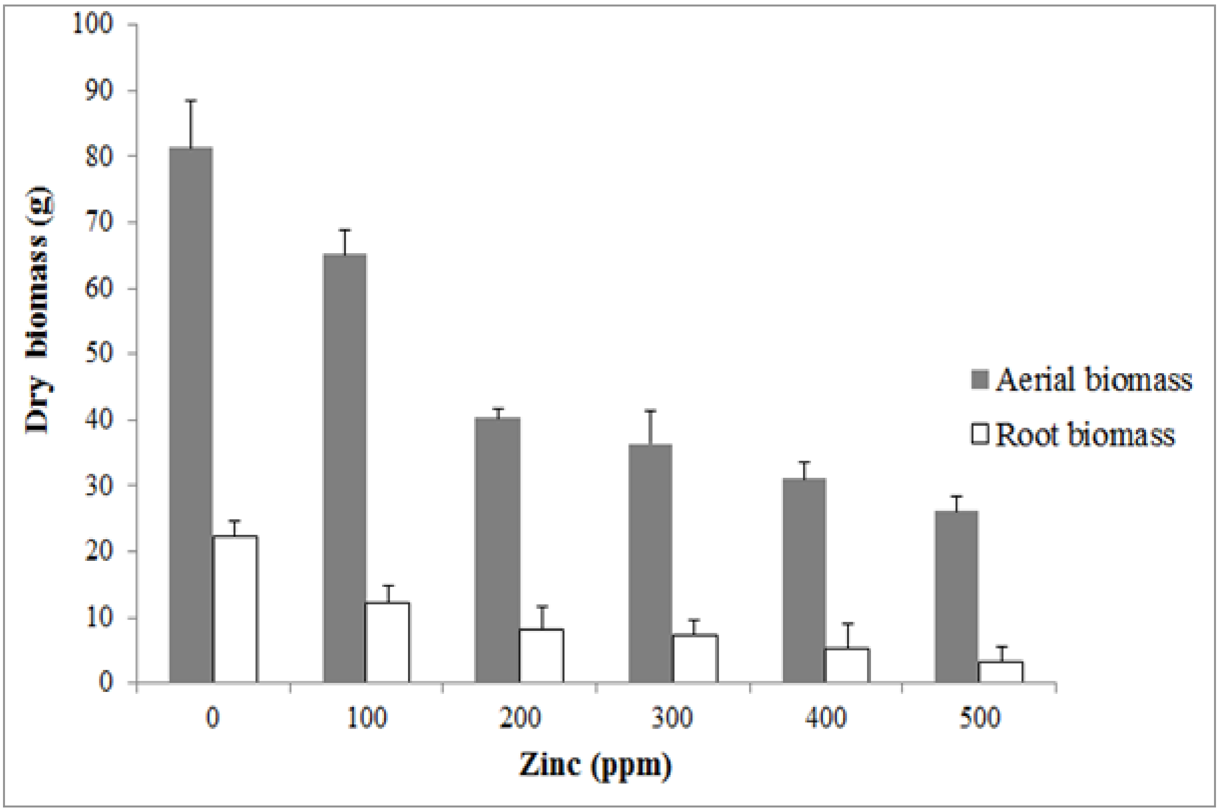


Figure 54: Effect of zinc on aerial and root dry biomass of *Lycopersicon esculentum* Mill.

Table 28: Shows values of aerial and root dry biomass of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Doses du zinc (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Areal dry biomass (g) P=0	81.312 a ± 7.123	65.202 b ± 3.61	40.257 c ± 1.3	36.352 d ± 5.125	31.02 d ± 2.54	26.1 e ± 2.256
Root dry biomass (g) P=0	22.35 a ± 2.123	12.125 b ± 2.564	8.153 c ± 3.36	7.226 c ± 2.214	5.231 cd ± 3.65	3.213 d ± 2.223

Statistical data indicated that zinc treatments were highly significant on dry and root biomass of *Lycopersicon esculentum* Mill., compared to control seedlings (Annex 4).

As shown in Table 28, five homogenous groups were determined for aerial dry biomass, group a (0 ppm), group b (100 ppm), group c (200 ppm), group d (300 and 400 ppm), and group e (500 ppm). likewise, five homogenous groups were recorded for root dry biomass, group a (0 ppm), group b (100 ppm), group c (200 and 300 ppm), group cd (400 ppm), and group d (500 ppm).

VII.3.1.6. Effect of zinc on leaf area

Exposure to different concentrations of zinc considerably reduced the leaf area of *Lycopersicon esculentum* Mill. seedlings. This reduction was directly proportional to zinc doses in the soil.

Moreover, less negative effect on leaf area was recorded at 0 and 100 ppm Zn, with close values of 10.99 and 10.46 cm^2, respectively. Nevertheless, the most pronounced effect hit the lowest leaf area by 4.92 cm^2 at 500 ppm Zn, compared to the control seedlings (Figure 55).

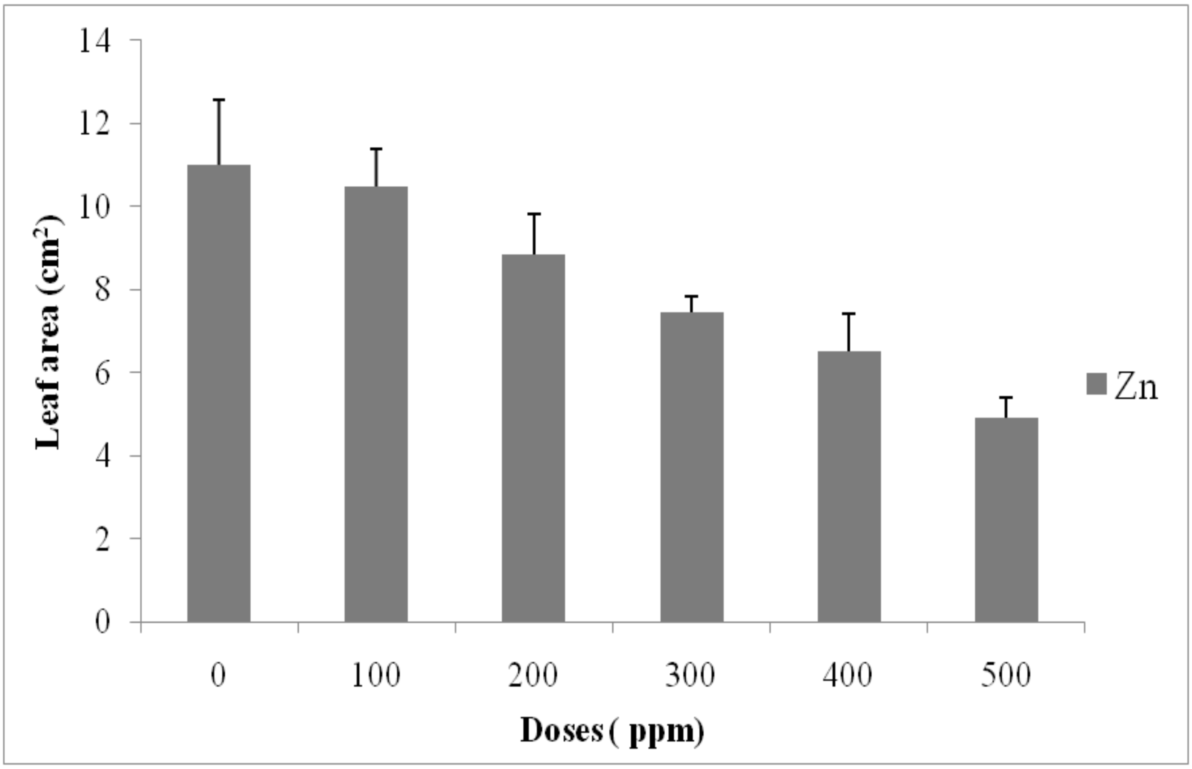


Figure 55: Effect of zinc on leaf area of *Lycopersicon esculentum* Mill.

Table 29: Shows values of leaf area of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf area P= 0.00	10.99 a ± 1.57	10.46 a ± 0.90	8.83 b ± 0.98	7.45 c ± 0.39	6.51d ± 0.89	4.92 e ± 0.49

Variance analysis revealed a highly significant effect of zinc on leaf area of *Lycopersicon esculentum* Mill., compared to the control (Annex 4).

Five homogenous groups were indicated for leaf area, group a (0 and 100 ppm), group b (200 ppm), group c (300 ppm), group d (400 ppm), and group e (500 ppm) (Table 29).

VII.3.2 Biometrical parameters

VII.3.2.1. Effect of zinc on leaf relative water content (RWC)

Relative water content in leaves of *Lycopersicon esculentum* Mill. declined steadily with an elevated level of zinc in the soil, as compared to the control leaves (Figure 56).

Zn at 0 ppm maintained the highest level of water content in the leaves by 73.2%; while, this level decreased to 63.11% by 100 ppm Zn. Besides, a reduction of leaf water content to 50 % was only achieved by the application of 400 ppm Zn. However, the negative effect caused by zinc was highly pronounced at 500 ppm by 34.33% of RWC, among all treatments applied.

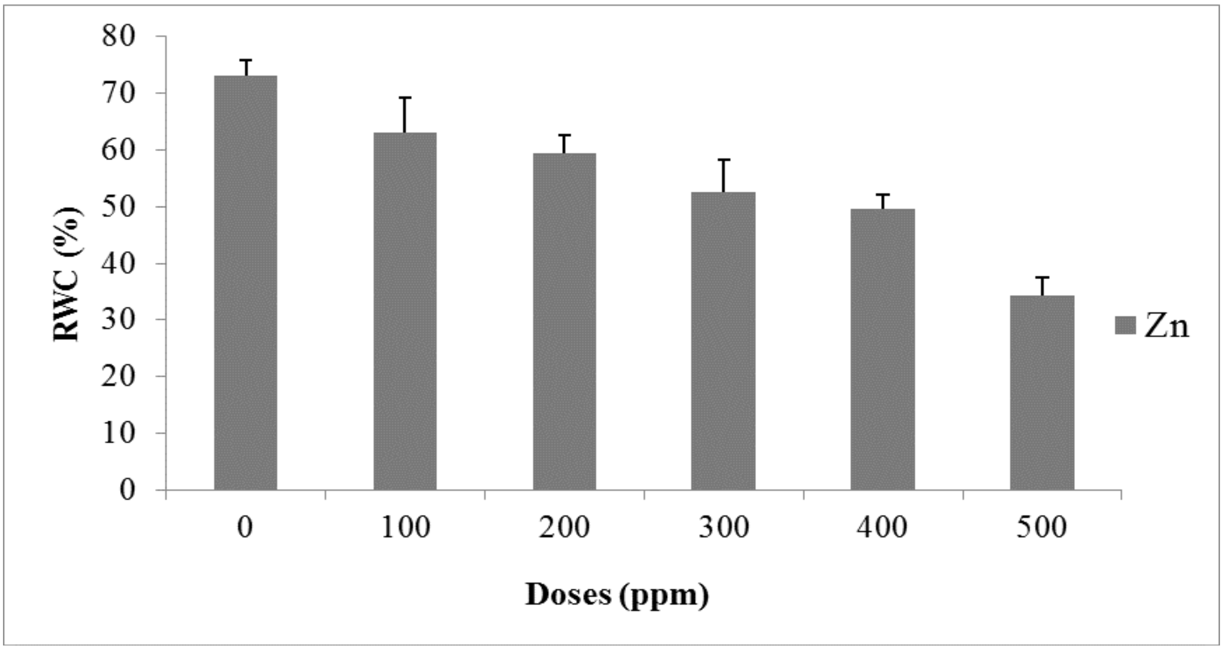


Figure 56: Effect of zinc on leaf relative water content (RWC) *Lycopersicon esculentum* Mill.

Table 30: Shows values of leaf relative water content of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Relative water content (%) P=0	73.2 a ± 2.71	63.11 b ± 6.04	59.31bc ± 3.27	52.66 c ± 5.508	49.66 d ± 2.51	34.33 e ± 3.21

Effect of zinc treatment on leaf relative water content of *Lycopersicon esculentum* Mill. was highly significant, compared to the control seedlings (Annex 4).

As shown in Table 30, six homogeneous groups were determined for RWC, group a (0 ppm), group b (100 ppm), group bc (200 ppm), group c (300 ppm) and group d (400 ppm), and group e (500 ppm).

VII.3.2.2. Effect of zinc on relative water loss (RWL)

Transpiration rate in leaves of *Lycopersicon esculentum* Mill. was negatively affected by zinc treatments, as illustrated in Figure 57. Moreover, relative water loss in the leaves decreased gradually as zinc concentrations in the soil increased.

In the absence of zinc, tomato leaves lost an ideal volume of water by approximately 0.548 g. cm^{-2}. min^{-1}. However, zinc at low concentrations (100 and 200 ppm) caused a moderate decline in RWL by 0.32, and 0.29 g. cm^{-2}. min^{-1}; while, high doses as 300 and 500 ppm Zn significantly limited the loss of water by 0.164 and 0.137 g^{-1}. cm^{-2}. min^{-1}, respectively. Remarkably, an appreciable dose of 400 ppm exhibited an apparent increase of RWL by 0.256 g. cm^{-2}.min^{-1}, this measure was less to that noted at 200 ppm.

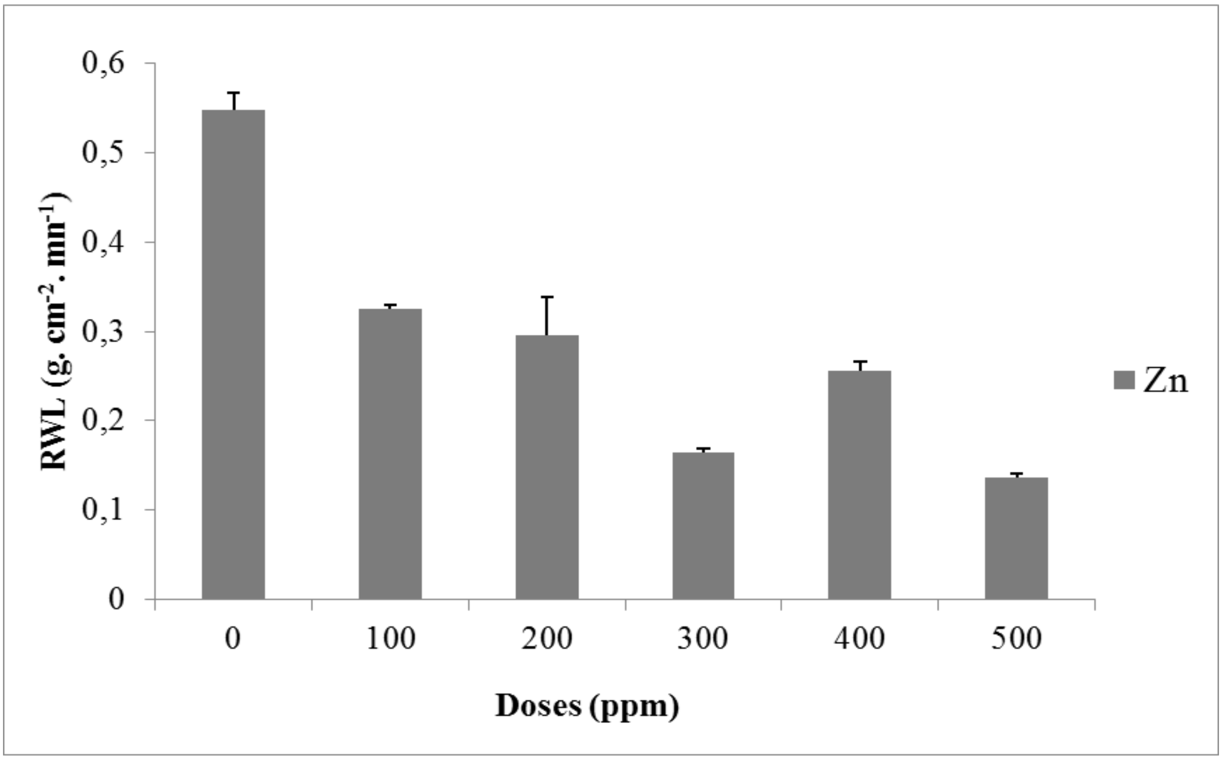


Figure 57: Effect of zinc on relative water loss of *Lycopersicon esculentum* Mill.

Table 31: Shows values of leaf relative water loss of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, and d) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
RWL (g. cm^{-2}. min^{-1}) P=0	0.548 a ± 0.018	0.32 b ± 0.005	0.29 b ± 0.044	0.167 d ± 0.005	0.256 c ± 0.01	0.137 d ± 0.004

Statistical analysis showed that the effect of zinc treatment on leaf relative water loss was highly significant, compared to the control plants.

As displayed in Table 31, four homogenous groups were recorded for RWL, group a (0 ppm), group b (100 and 200 ppm), group c (400 ppm), and group d (300 and 500 ppm) (Annex 4).

VII.3.3 Physiological parameters

VII.3.3.1. Effect of zinc on chlorophyll and carotenoids pigments content

Content of chlorophyll pigments (chla, chlb, chlt, and car) in leaves of *Lycopersicon esculentum* Mill. was considerably reduced by zinc treatments. This reduction was proportional to the different zinc doses in the soil, compared to the control (Figure 58).

At 0 ppm chlorophyll a was 5.096 mg. g^{-1} FW; while, it declined to 2.585 mg. g^{-1} FW at 500 ppm. Likewise, chlorophyll b decreased from 2.795 mg. g^{-1} FW at 0 ppm to 0.531mg. g^{-1} FW at 500 ppm. Besides, no noticeable changes in pigment content were observed between100 and 200 ppm by 4.289 and 4.185 mg. g^{-1} FW for chlorophyll a, and between 0, 100, and 200 ppm by 2.795, 2.71 and 2.69 mg. g^{-1} FW for chlorophyll b, respectively.

Total chlorophyll was higher (7.89 mg. g^{-1} FW) at 0 ppm; whereas, it declined to 3.116 mg. g^{-1} FW at 500 ppm.

In the case of carotenoids pigments, no significant decrease was recorded from 100, 200 to 300 ppm Zn, as noted by 0.588, 0.524, and 0.538 mg. g^{-1} FW respectively. However, in comparison to the control content (0.832 mg. g^{-1}FW), a moderate decrease was observed at 400 and 500 ppm Zn by 0.358 and 0.222 mg. g^{-1} FW.

Along with treatments, pigments content was found to follow the order of total chlorophyll a > chlorophyll b > total chlorophyll > carotenoids.

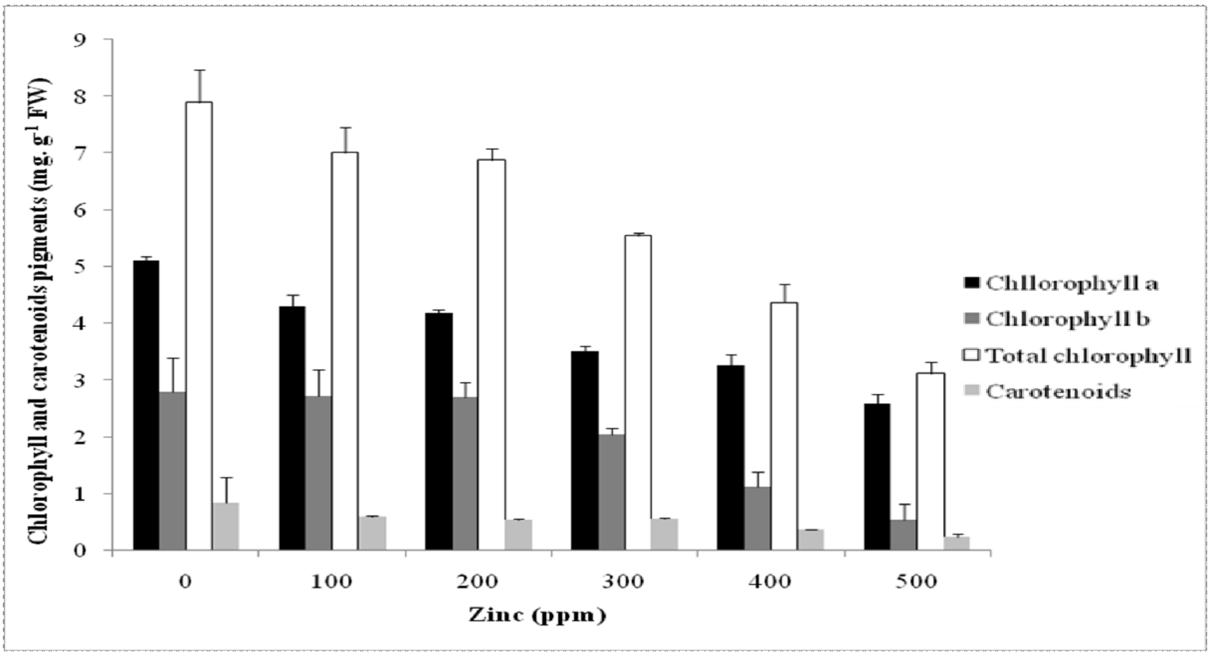


Figure 58: Effect of zinc on chlorophyll and carotenoids pigments content of *Lycopersicon esculentum* Mill.

Table 32: Shows values of chlorophyll and carotenoids pigments content of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, and c) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Chlorophyll a content P=0	5.096 a ± 0.074	4.289 b ± 0.205	4.185 b ± 0.06	3.497 b ± 0.105	3.263 bc ± 0.177	2.585 c ± 0.156
Chlorophyll b content (mg. g^{-1} FW) P=0,00003	2.795 a ± 0.602	2.71 a ± 0.469	2.69 b ± 0.27	2.039 b ± 0.104	1.103 bc ± 0.278	0.531 c ± 0.277
Total Chlorophyll content (mg. g^{-1} FW) P=0	7.89 a ± 0.579	6.999 b ± 0.464	6.875 b ± 0.211	5.536 bc ± 0.05	4.366 c ± 0.319	3.116 c ± 0.19
Carotenoids content (mg. g^{-1} FW) P= 0,02506	0.832 a ± 0.44	0.588 b ± 0.015	0.524 b ± 0.029	0.538 b ± 0.033	0.358 c ± 0.006	0.222 c ± 0.068

Variance analysis of zinc effect on the content of chlorophyll pigments produces highly significant results, compared to the control plants (Annex 4).

Four homogenous groups (a, b, bc and c) were given for chlorophyll a, b, and total chl, while three groups (a, b and c) were recorded for carotenoids pigments (Table 32).

VII.3.4 Biochemical parameters

VII.3.4.1. Effect of zinc on proteins content

Figure 59 illustrates the accumulation of proteins content in leaves and roots of *Lycopersicon esculentum* Mill., treated by different concentrations of zinc.

A substantial accumulation of proteins content in leaves and roots was marked at 100 ppm Zn by 19.472 and 9.658 mg. g^{-1} FW, compared to the control by 7.038 and 3.069 mg. g^{-1}FW, respectively. This appreciable concentration induced the most important accumulative effect of proteins content, in both tissues. However, a significant decrease was mainly provoked with 200 ppm Zn, following by 300, 400, and 500 ppm Zn. Despite this, total proteins accumulated in leaves and roots at 500 ppm Zn were greater than the level recorded with zinc at 0 ppm.

Remarkably, proteins content was highly accumulated in leaves than roots.

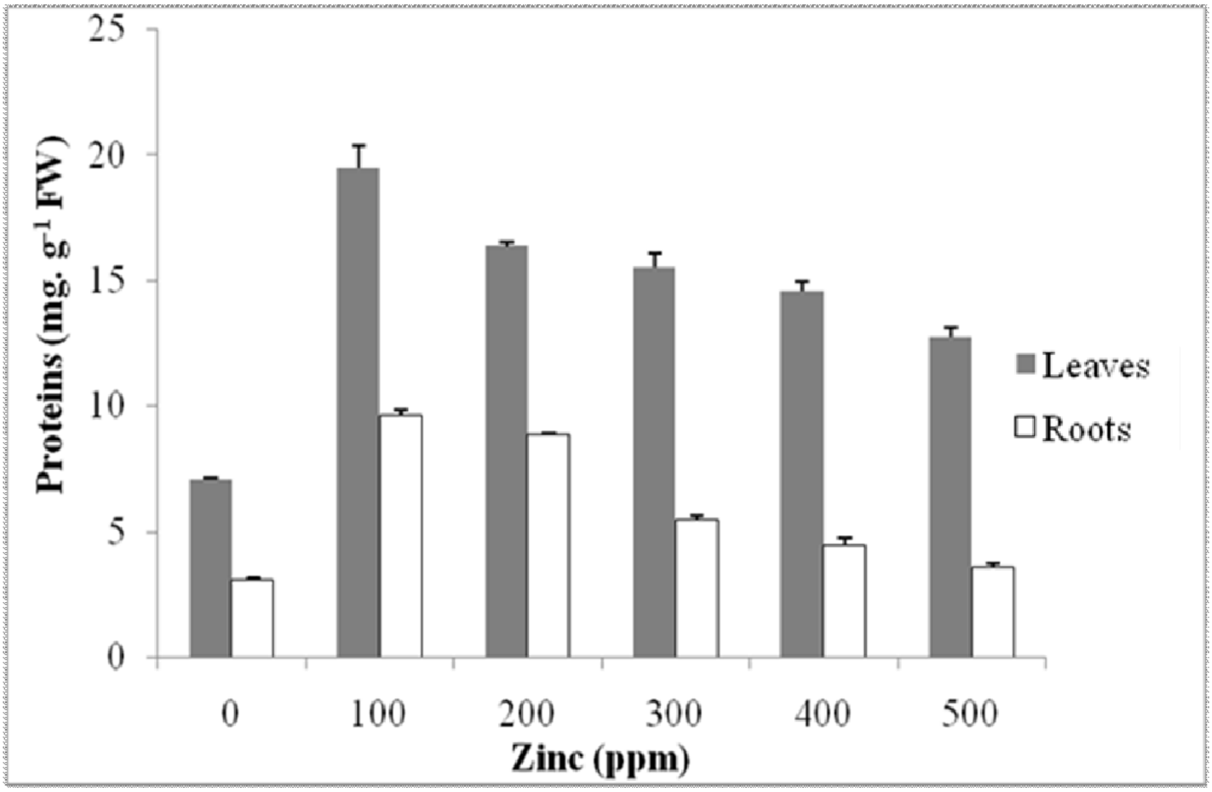


Figure 59: Effect of zinc on proteins content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 33: Shows values of proteins content of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, and d) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf proteins content (mg. g^{-1} FW) P=0	7.038 d ± 0.102	19.472 a ± 0.914	16.344 b ± 0.148	15.503 b ± 0.564	14.545 bc ± 0.385	12.708 c ± 0.391
Root proteins content (mg. g^{-1} FW) P=0	3.069 c ± 0.09	9.658 a ± 0.189	8.856 a ± 0.059	5.494 b ± 0.09	4.497 b ± 0.237	3.578 c ± 0.155

One-way ANOVA revealed a highly significant effect of zinc treatments on the level of proteins content in *Lycopersicon esculentum* Mill., compared to control seedlings.

As shown in Table 33, five homogenous groups were recorded in leaf proteins content, group a (100 ppm), group b (200 and 300 ppm), group bc (400 ppm) group c (500 ppm), and group d (0 ppm). However, in root proteins content three groups were marked, group a (100 and 200 ppm), group b (300 and 400 ppm), and group c (0 and 500 ppm) (Annex 4).

VII.3.4.2. Effect of zinc on soluble sugars content

Zinc stress triggered the biosynthesis and accumulation of soluble sugars content in leaves and roots of *Lycopersicon esculentum* Mill. This accumulation showed a positive trend with the increasing of zinc doses in the soil, compared to the control (Figure 60).

Soluble sugars content in the leaves was 6.124 mg. g^{-1} DW at 0 ppm Zn; while, no significant accumulation (6.938 mg. g^{-1} DW) was recorded with 100 ppm Zn. Besides, zinc treatment with 500 ppm, led to the highest level of soluble sugars in the leaves by 10.702 mg. g^{-1} DW.

In the roots, no noticeable increase in soluble sugars content was found between the following zinc doses: 0, 100, 200, and 300 ppm for 5.382, 5.584, 5.955, and 5.994 mg. g^{-1} DW, respectively. Nevertheless, a remarkable increase of soluble sugars was recorded at 400 and 500 ppm zinc for 6.489 and 8.556 mg. g^{-1} DW, respectively.

Overall, leaves tend to accumulate a high level of soluble sugars than roots.

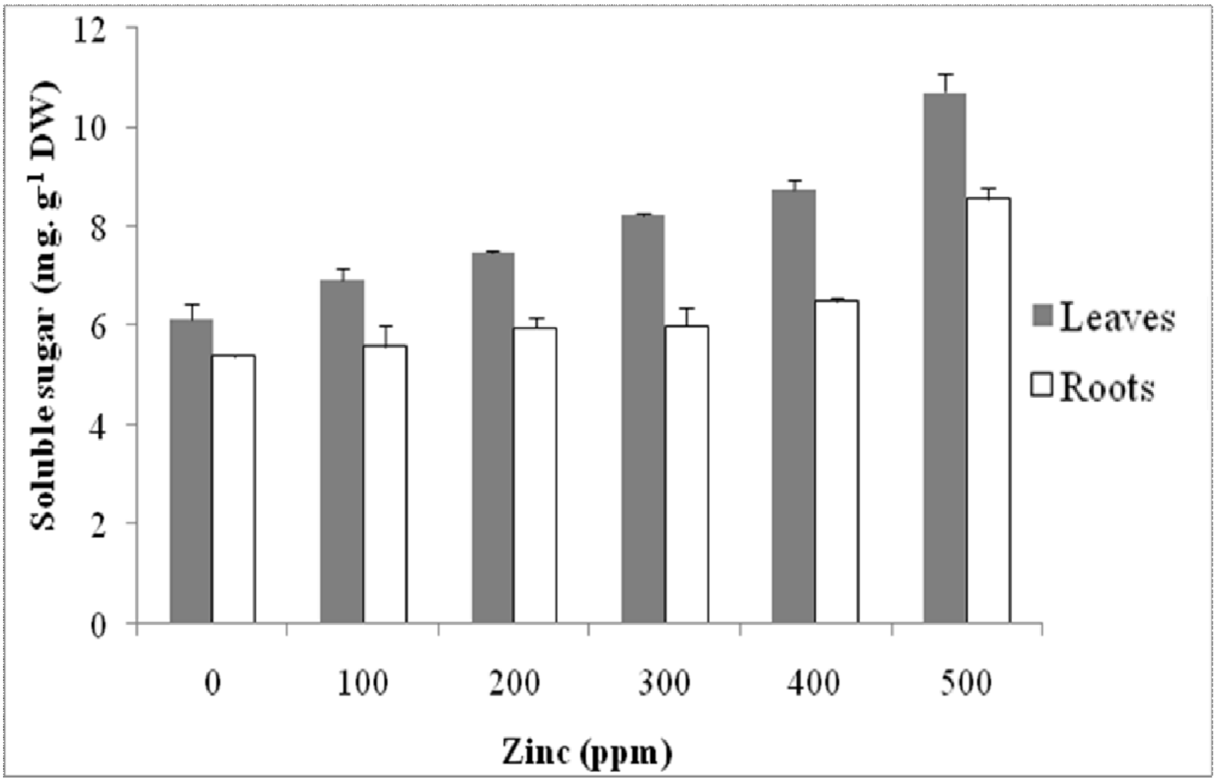


Figure 60: Effect of zinc on soluble sugars content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 34: Shows values of the soluble sugars content of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, and d) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf soluble sugars content (mg. g^{-1} DW) P=0	6.124 cd ± 0.329	6.938 c ± 0.212	7.489 c ± 0.035	8.225 b ± 0.045	8.753 b ± 0.18	10.702 a ± 0.376
Root soluble sugars content (mg. g^{-1} DW) P=0	5.382 b ± 0.035	5.584 b ± 0.428	5.955 b ± 0.195	5.994 b ± 0.358	6.489 b ± 0.077	8.556 a ± 0.211

Statistical analysis revealed the effect of zinc treatments on soluble sugars content in leaves and roots of *Lycopersicon esculentum* Mill. was highly significant, compared to the control plants (Annex 4).

As shown in Table 34, four homogenous groups were recorded for leaf soluble sugars content, group a (500 ppm), group b (300 and 400 ppm), group c (100 and 200 ppm), and group cd (0 ppm). However, for root soluble sugars content two groups were presented, group a (500 ppm) and group b (0, 100, 200, 300, and 400 ppm).

VII.3.4.3. Effect of zinc on proline content

Exposure of *Lycopersicon esculentum* Mill. to different concentrations of zinc induced the accumulation of proline content in the leaves and roots. This increase has a positive correlation with the increase of zinc doses in the soil (Figure 61).

Proline content at 500 ppm was highly accumulated in leaves and roots by 16.454 and 9.214 mg. g^{-1} DW respectively, compared to that at 0 ppm by 8.161 and 2.238 mg. g^{-1} DW. However, zinc treatment with 100 ppm slightly increased the proline content by 9.017 mg. g^{-1} DW in the leaves and 3.422 mg. g^{-1} DW in the roots.

No elevation of proline content in leaves was observed from 200 to 300 ppm Zn, as given by the similar value of 9.609 mg. g^{-1} DW.

Besides, leaves accumulated a high level of proline compared to the roots.

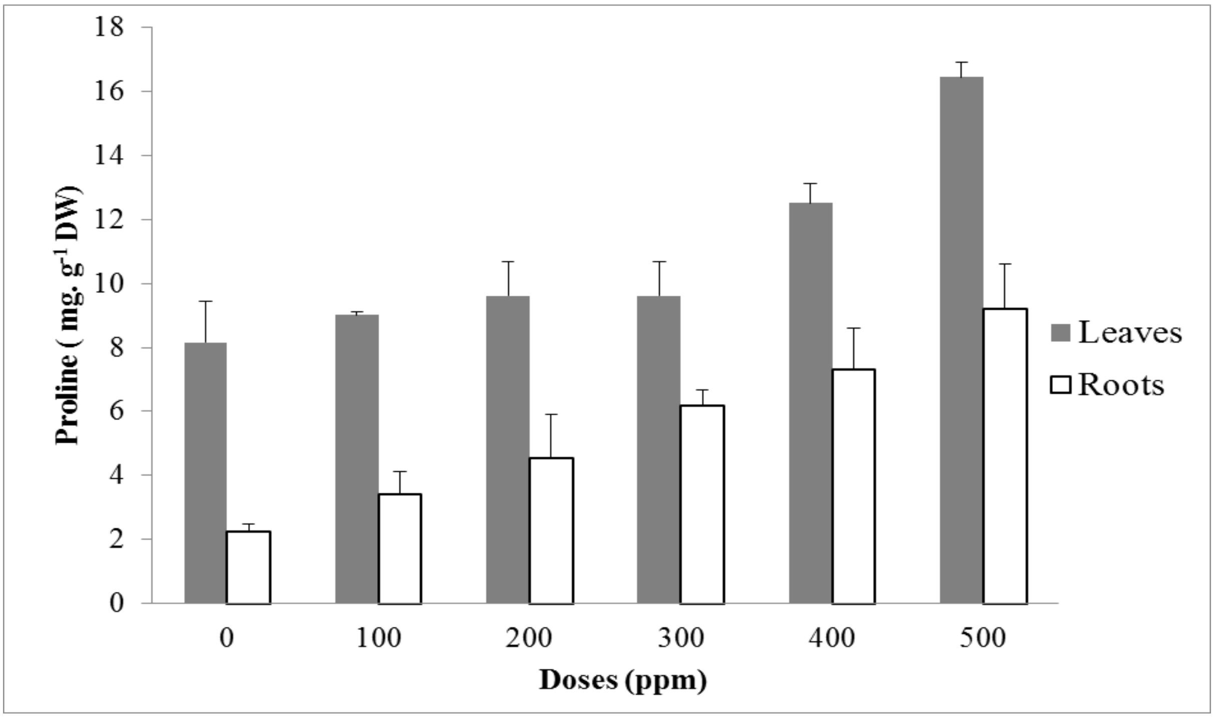


Figure 61: Effect of zinc on proline content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 35: Shows values of proline content of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d and e) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf proline content (mg. g^{-1}DW) P= 0	8.161c ± 1.269	9.017 c ± 0.114	9.609 c ± 1.087	9.609 c ± 1.087	12.505 b ± 0.603	16.454 a ± 0.456
Root proline content (mg. g^{-1}DW) P= 0	2.238 e ± 0.228	3.422 de ± 0.693	4.541 cd ± 1.382	6.187bc ± 0.497	7.306 b ± 1.295	9.214 a ± 1.387

The zinc effect was highly significant on proline content in leaves and roots of *Lycopersicon esculentum* Mill, compared to control seedlings (Annex 4).

Three homogenous groups were observed for leaf proline content, group a (500 ppm), group b (400 ppm), and group c (0, 100, 200, and 300 ppm). However, six homogenous groups were found with root proline content, group a (500 ppm), group b (400 ppm), group bc (300 ppm) and group cd (200 ppm), group de (100 ppm), and group e (0 ppm) (Table 35).

VII.3.4.4. Effect of zinc on polyphenols content

Polyphenols content in leaves and roots of *Lycopersicon esculentum* Mill. increased gradually as zinc doses in the soil increased (Figure 62). Exceptionally, a decrease in polyphenols content was observed at 400 ppm Zn, where; these antioxidant compounds were highly accumulated in roots than leaves by 21.687 and 18.338 mg GAE. g^{-1} DW respectively.

Moreover, no significant increase in polyphenols content was obtained by zinc concentration from 0 to 100 ppm, for 17.693 and 17.601mg GAE. g^{-1}DW content in leaves, and 7.464 and 8.632 mg GAE. g^{-1} DWcontent in roots, respectively.

However, the response pattern of polyphenols content reached the maximum level at 500 ppm by 28.567 and 22.823 mg GAE. g^{-1} DW, in both organs respectively.

Overall, leaves accumulated a high level of polyphenols compared to the roots, for different treatments.

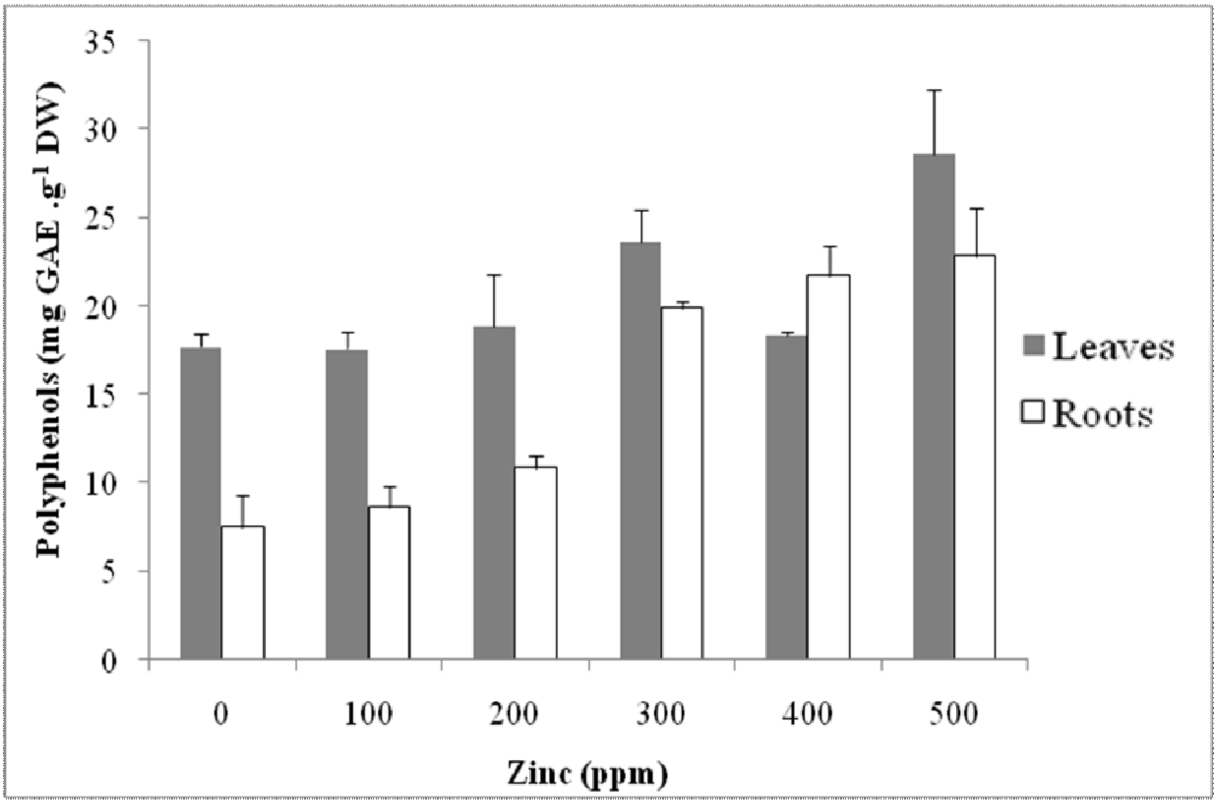


Figure 62: Effect of zinc on polyphenols content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 36: Shows values of polyphenols content of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b and c) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf polyphenols content (mg GAE. g^{-1} DW) P= 0	17.693 c ± 0.787	17.601 c ± 0.922	18.891c ± 2.904	23.653 b ± 1.778	18.338 c ± 0.184	28.567 a ± 3.641
Root polyphenols content (mg GAE. g^{-1} DW) P= 0	7.464 b ± 1.843	8.632 b ± 1.199	10.874 b ± 0.638	19.874 a ± 0.416	21.687 b ± 1.764	22.823 a ± 2.675

Compared to control seedlings, the statistical test showed a highly significant effect of zinc on the polyphenols profile in the leaves and roots of *Lycopersicon esculentum* Mill. (Annex 4).

Three homogenous groups were observed for leaf polyphenols content, group a (500 ppm), group b (300 ppm), and group c (0, 100, 200, and 400 ppm). However, two homogenous groups were recorded with root polyphenols content, group a (300 and 500 ppm), group b (0, 100, 200, and 400 ppm) (Table 36).

VII.3.4.5. Effect of zinc on flavonoids content

As can be seen from Figure 63, a clear trend in increasing flavonoids content in leaves and roots of *Lycopersicon esculentum* Mill. was obtained by zinc treatments. A positive correlation was noted between the flavonoids content and the elevation of zinc doses in the soil.

The highest level of flavonoids was observed at 500 ppm by 1.446 and 0.933 mg QE. g^{-1} DW, compared to the control by 0.187 and 0.132 mg QE. g^{-1} DW, in leaves and roots respectively. Besides, no significant increase in flavonoids content was noted at 300 to 400 ppm, as given by 1.203 to 1.298 mg QE. g^{-1} DW in the leaves, and by 0.811 to 0.814 mg QE. g^{-1} DW in the roots, respectively.

Furthermore, leaves accumulated a high level of flavonoids compared to the roots, along with all treatments.

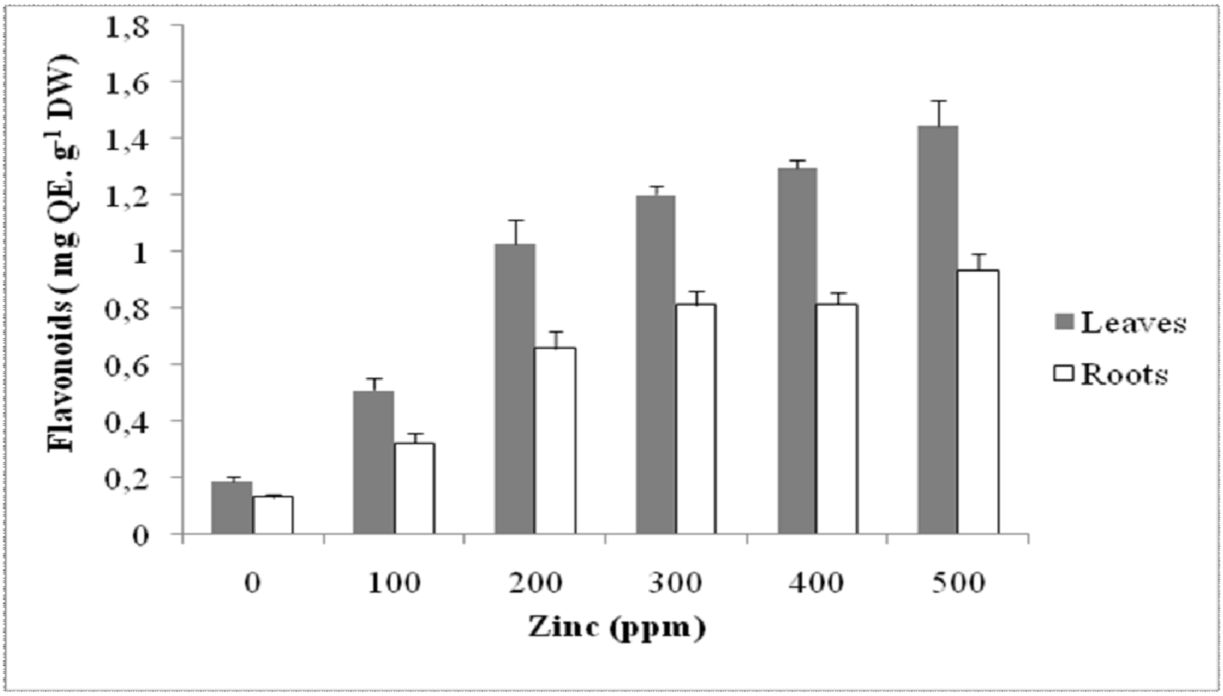


Figure 63: Effect of zinc on flavonoids content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 37: Shows values of flavonoids content of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, and e) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Leaf flavonoids content (mg QE. g^{-1}DW) P=0	0.187 e ± 0.019	0.511d ± 0.043	1.03 c ± 0.082	1.203 b ± 0.033	1.298 b ± 0.03	1.446 a ± 0.09
Root flavonoids content (mg QE. g^{-1}DW) P=0	0.132 e ± 0.011	0.321 d ± 0.037	0.658 c ± 0.06	0.811 b ± 0.052	0.814 b ± 0.043	0.933 a ± 0.06

A highly significant increase in flavonoids content with zinc stress was noted in leaves and roots as compared to control plants (Annex 4).

Five homogenous groups were provided for leaf and root flavonoids content, group a (500 ppm), group b (300 and 400 ppm), group c (200 ppm), group d (100 ppm), and group e (0 ppm) (Table 37).

VII.3.4.6. Effect of zinc on the antioxidant activity (DPPH test)

The exposure to different concentrations of zinc strongly stimulated the antioxidant activity in leaves and roots of *Lycopersicon esculentum* Mill. This positive effect was dramatically increased by increasing zinc doses in the soil, in both organs (Figure 64).

The minimum level of scavenging activity was recorded in non-stressed seedlings by 46.569 and 33.088%, in leaves and roots respectively. Nevertheless, the maximum inhibition was detected at 500 ppm by approximately 71.875% and 62.745%, in both organs. Differently, 50% of scavenging activity in leaves and roots was obtained at 100 and 200 ppm Zn, respectively.

Overall, scavenging activity in the leaves was greater than that observed in the roots.

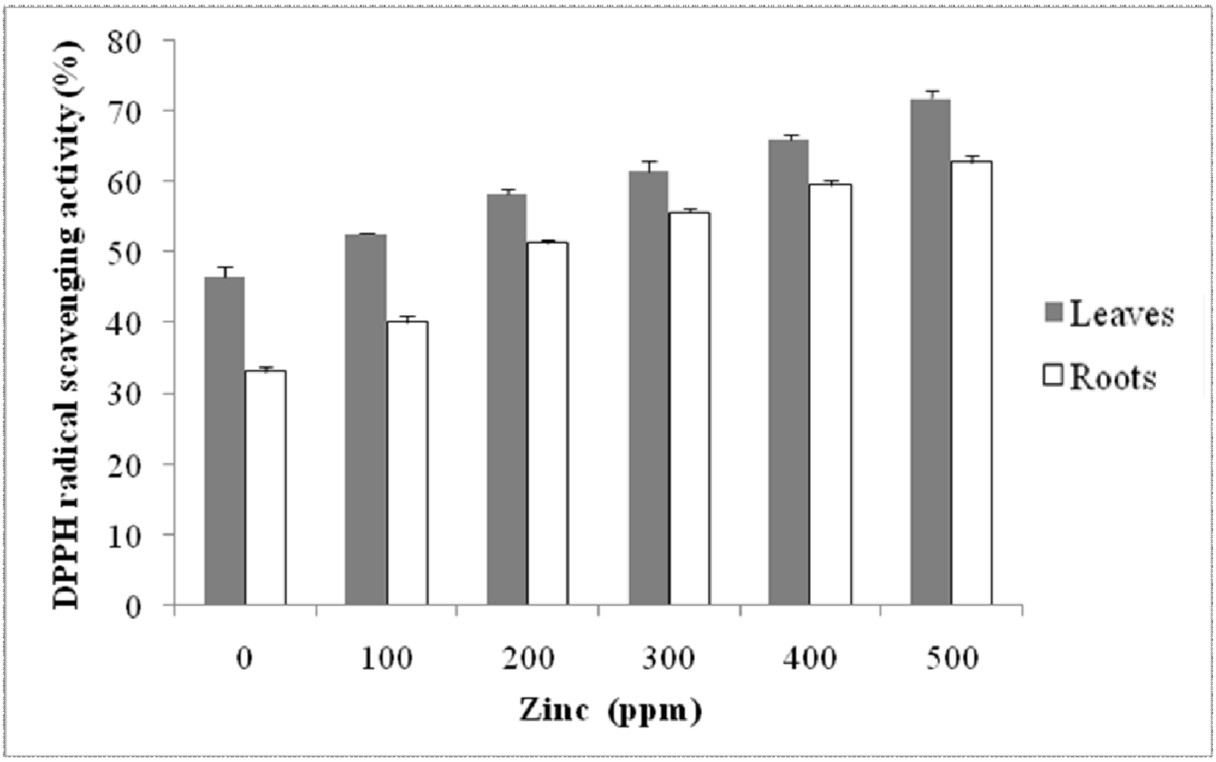


Figure 64: Effect of zinc on the scavenging activity DPPH in leaves and roots of *Lycopersicon esculentum* Mill.

Table 38: Shows values of scavenging activity in *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, e, and f) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Scavenging activity in leaves (%) P=0	46.569 f ± 1.428	52.696 e ± 0.212	58.272 d ± 0.663	61.52 c ± 1.531	66.054 b ± 0.743	71.875 a ± 1.024
Scavenging activity in roots (%) P=0	33.088 f ± 0.735	40.012 e ± 1.012	51.287 d ± 0.552	55.699 c ± 0.663	59.498 b ± 0.829	62.745 a ± 1.012

Zinc treatment induced a highly significant effect on the antioxidant activity in leaves and roots of *Lycopersicon esculentum* Mill. (Annex 4).

Six homogenous groups were found with scavenging activity in the leaves and roots, group a (500 ppm), group b (400 ppm), group c (300 ppm), group d (200 ppm), group e (100 ppm), and group f (0 ppm) (Table 38).

VII.4. Chemical parameters

VII.4.1 Soil physicochemical characterization

Soil physicochemical characterization is given in Table 39.

Table 39: Soil physicochemical properties.

Soil parameters	Values
pH	6.57
EC (dS. m^{-1})	23.6
Lime ($CaCO_3$) %	28.29
CEC meq.100g^{-1} soil	40.76
Soil fractions	Clay: 14.47% Silt: 0.36% Sand: 85.16%
Texture class	Loamy fine sand
Copper (ppm)	2.368
Zinc (ppm)	15.023

Results are shown in Table 39 indicate that soil has a slightly acidic reaction, which may be caused by the high rate of organic matter in the soil mixture. The slightly acidic reaction of soil mixture causes mobility of heavy metals added in experimental design. Besides, this soil has a high salinity degree; this may cause growth retardation, plant stress, and toxicity. This is very difficult for many cultural plant growths, but only salinity-resistant plants can be survived. As we can see, this mixture soil has high lime content, and this may cause fixation and immobilization of some minerals in the soil system. As can be expected this soil has high CEC content. Although it has high sand content, the high CEC of this soil may cause by the high organic content of the soil mixture (organic matter added to the soil for pot experiment). Texture percentages demonstrated that the mixture soil has high sand content and this may cause leaching of minerals.

Assessment of metal contamination soil (contamination factor) of control indicated that the contamination factor obtained was **1,** for both soil controls of copper and zinc. Hence, soil control is moderately contaminated by copper and zinc. This is maybe due to the nutrients solution and compost used or sand contaminated.

VII.4.2 Heavy metals content in plant

VII.4.2.1. Copper content in leaves and roots of *Lycopersicon esculentum* Mill.

As provided in Figure 65, leaves and roots of *Lycopersicon esculentum* Mill. tend to accumulate high content of copper, compared to the control. This level increased substantially by increasing the copper doses in the soil.

Application of 100 ppm Cu caused a moderate increase of copper content by 42.598 ppm in the leaves and 9.892 ppm in the roots, compared to the control content by 11.837 and 5.861 ppm in both organs, respectively.

However, a high amount of copper was detected at 400 and 500 ppm Cu by 302.273 and 331.758 ppm in the leaves, and 50.108 and 83.299 ppm in the roots, respectively. Copper at both doses was highly translocated to the leaves, compared to all treatments.

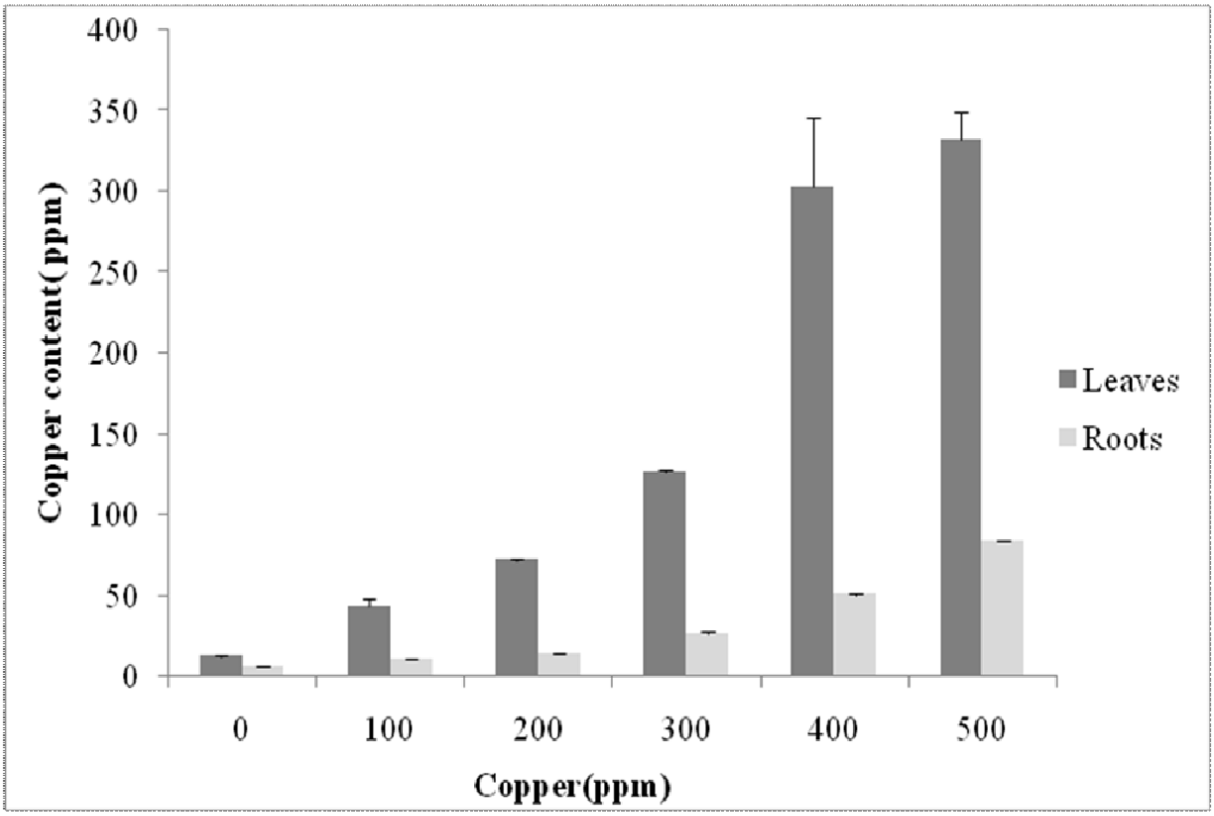


Figure 65: Copper content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 40: Shows values of the copper content in leaves and roots of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, d, e and f) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Copper content in leaves (ppm) P= 0	11.837 d ± 0.186	42.598 cd ± 4.563	71.455 c ± 0.632	125.69 b ± 1.425	302.273 a ± 42.820	331.758 a ± 17.491
Copper content in roots (ppm) P= 0	5.861 f ± 0.019	9.892 e ± 0.303	13.575 d ± 0.059	25.939 c ± 0.605	50.108 b ± 0.259	83.299 a ± 0.304

Copper at different concentrations induced a highly significant effect on the copper accumulation of leaves and roots of *Lycopersicon esculentum* Mill., compared to the control seedlings (Annex 4).

Table 40, five homogenous groups were recorded with leaf copper content, group a (400 and 500 ppm), group b (300 ppm), group c (200 ppm), group cd (100 ppm), and group d (0 ppm). Likewise, six homogenous groups were recorded with root copper content, group a (500 ppm), group b (400 ppm), group c (300 ppm), group d (200 ppm), group e (100 ppm), and group f (0 ppm).

VII.4.2.2. Zinc content in leaves and roots of *Lycopersicon esculentum* Mill.

Zinc content accumulated in leaves and roots of *Lycopersicon esculentum* Mill. increased proportionally as zinc doses in the soil increased (Figure 66). Zinc was highly accumulated in the roots than leaves.

The minimum content of zinc was obtained at 0 ppm in the roots by 17. 91 ppm; whereas, this level was negligible in the leaves by 1.502 ppm. Although, zinc at 100 and 200 ppm accumulated in the roots with a high level of 104.2 and 142.12 ppm, it was lowly translocated to the leaves by 5.7 and 7.864 ppm, respectively.

Besides, zinc at 400 and 500 ppm was highly accumulated in the roots by maximum values of 443.38 and 435.76 ppm, and at 300 and 500 ppm in the leaves by 11.927 and 21.647 ppm. Differently, roots of *Lycopersicon esculentum* Mill. showed a sharp decrease in zinc content at 400 ppm by 66.293 ppm.

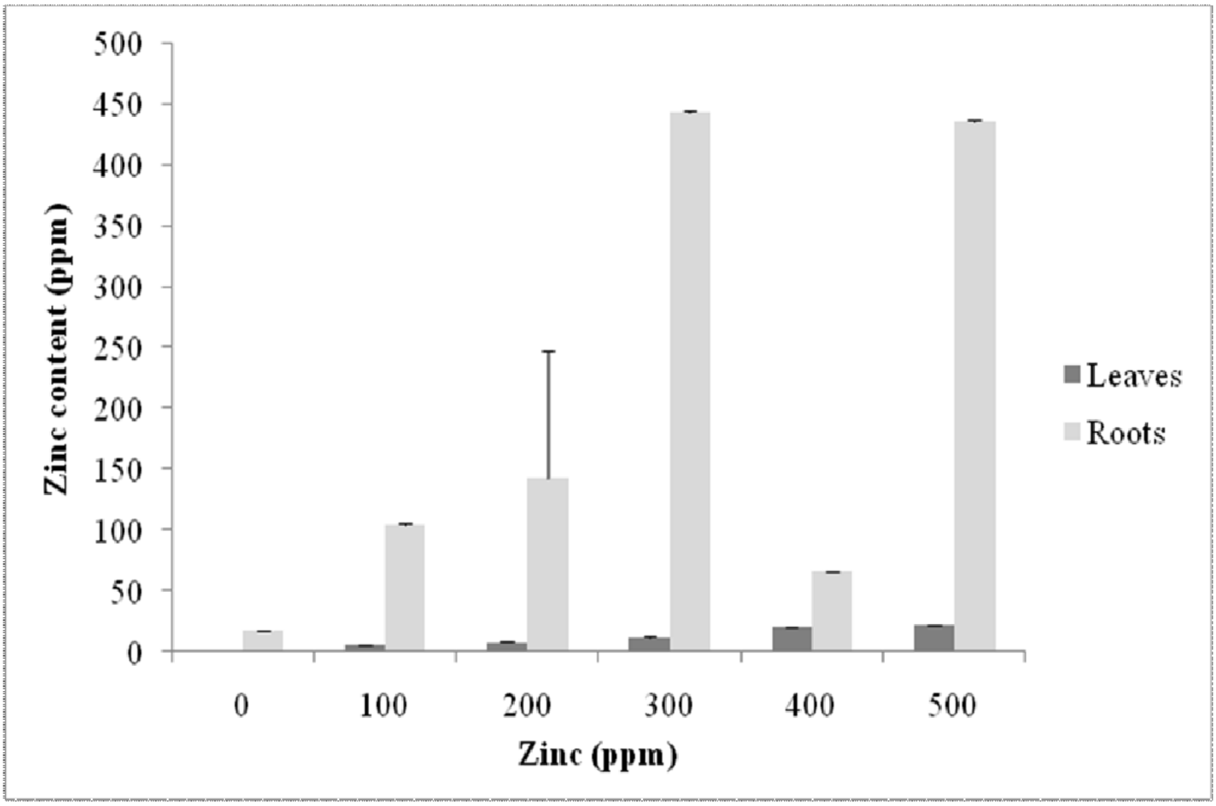


Figure 66: Zinc content in leaves and roots of *Lycopersicon esculentum* Mill.

Table 41: Shows values of the zinc content in leaves and roots of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, e, and f) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Zinc content in leaves (ppm) P = 0	1.502 f ± 0.144	5.700 e ± 0.398	7.864 d ± 0.421	11.927 c ± 0.561	20.553 b ± 0.387	21.647 a ± 0.428
Zinc content in roots (ppm) P = 0	17.91c ± 0.151	104.42 bc ± 2.01	142.12 b ± 105.62	443.38 a ± 0.78	66.293 bc ± 0.162	435.76 a ± 1.36

Zinc concentration in the soil caused a highly significant accumulation of zinc in the leaves and roots of *Lycopersicon esculentum* Mill., compared to the control (Annex 4).

As shown in Table 41, six homogenous groups were found for leaf zinc content, group a (500 ppm), group b (400 ppm), group c (300 ppm), group d (200 ppm), group e (100 ppm) and group f (0 ppm). However, four homogenous groups were observed with root zinc content, group a (300 and 500 ppm), group b (200 ppm), group bc (100 and 400 ppm), and group c (0 ppm).

VII.4.3 Chemical speciation, mobility and distribution of heavy metals in soil

VII.4.3.1. Copper content in the organic and exchangeable fraction

The copper content in the soil profile was distributed in the organic and exchangeable fractions. This content increased gradually as the copper concentration in the soil increased. Moreover, copper was highly bounded to the organic fraction, compared to the small content recorded in the available fraction (Figure 67).

At 0 ppm, a little portion of copper was found in the organic and exchangeable fractions by 1.401 and 0.238 ppm. However, this level increased by the addition of 100 ppm Cu in the soil, with 118.958 and 0.357 ppm in both fractions, respectively. Although the greatest content of copper linked to the organic matter was recorded at 400 ppm Cu by 432.417 ppm, the less content was given with 500 ppm Cu by 104.271 ppm. Inversely, the highest level of copper in the soluble fraction was observed at 500 ppm by 5.692 ppm.

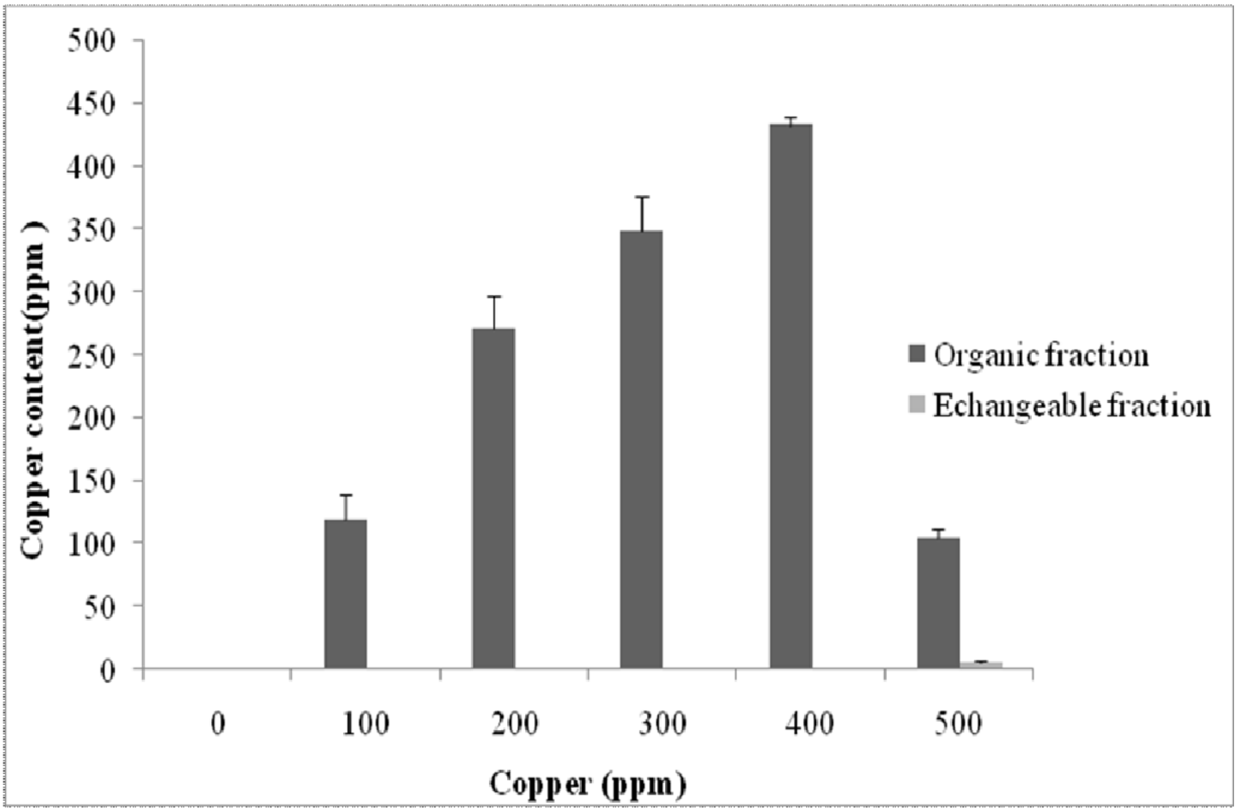


Figure 67: Copper content in the organic and exchangeable fraction

Table 42: Shows values of the copper content in an organic and exchangeable fraction of *Lycopersicon esculentum* Mill. treated by different concentrations of copper ± standard deviation. Letters (a, b, c, d and e) indicate the homogeneous groups as determined by ANOVA.

Copper doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Copper bound to organic fraction (ppm) P= 0	1.401 e ± 0.146	118.958 d ± 19.553	271.083 c ± 26.064	348.083 b ± 28.009	432.417 a ± 6.006	104.271 d ± 8.062
Copper in the exchangeable fraction (ppm) P= 0	0.238 d ± 0.006	0.357 c ± 0.012	0.549 b ± 0.006	0.563 b ± 0.015	0.581 b ± 0.002	5.692 a ± 0.13

Statistical data showed that copper level in the soil caused a highly significant elevation of the copper content in the organic and exchangeable fraction, compared to the control (Annex 4).

Five homogenous groups were recorded for copper in the organic fraction, group a (400 ppm), group b (300 ppm), group c (200 ppm), and group d (100 and 500 ppm), and group e (0 ppm). However, four homogenous groups were obtained for copper content in the exchangeable fraction, group a (500 ppm), group b (200, 300, and 400 ppm), group c (100 ppm), and group d (0 ppm) (Table 42).

VII.4.3.2. Zinc content in the organic and exchangeable fraction

As shown in Figure 68, zinc with different concentrations was distributed in the organic and exchangeable fractions. This level increased substantially with the increasing of zinc doses in the soil of *Lycopersicon esculentum* Mill. Moreover, zinc was highly associated with organic matter, but it was lowly dissolved in the soluble fraction.

Control samples provided a low amount of zinc in the exchangeable and organic fraction by 0.637 and 17.375 ppm. Remarkably, zinc at 100 ppm was strongly bound to the organic matter by 120.63 ppm, compared to its low available content of 3.416 ppm.

Only for the organic fraction, zinc treatment at 300 ppm acted negatively by reducing the zinc content to 177.75 ppm, this was compared to the level obtained at 200 and 300 ppm Zn by 197.708 and 213.348 ppm.

However, the affinity of zinc to the organic matter was promoting by 500 ppm, resulting in the highest content of 246.375 ppm, compared to the control samples. This level was highly superior to that recorded in the exchangeable fraction by 20.413 ppm.

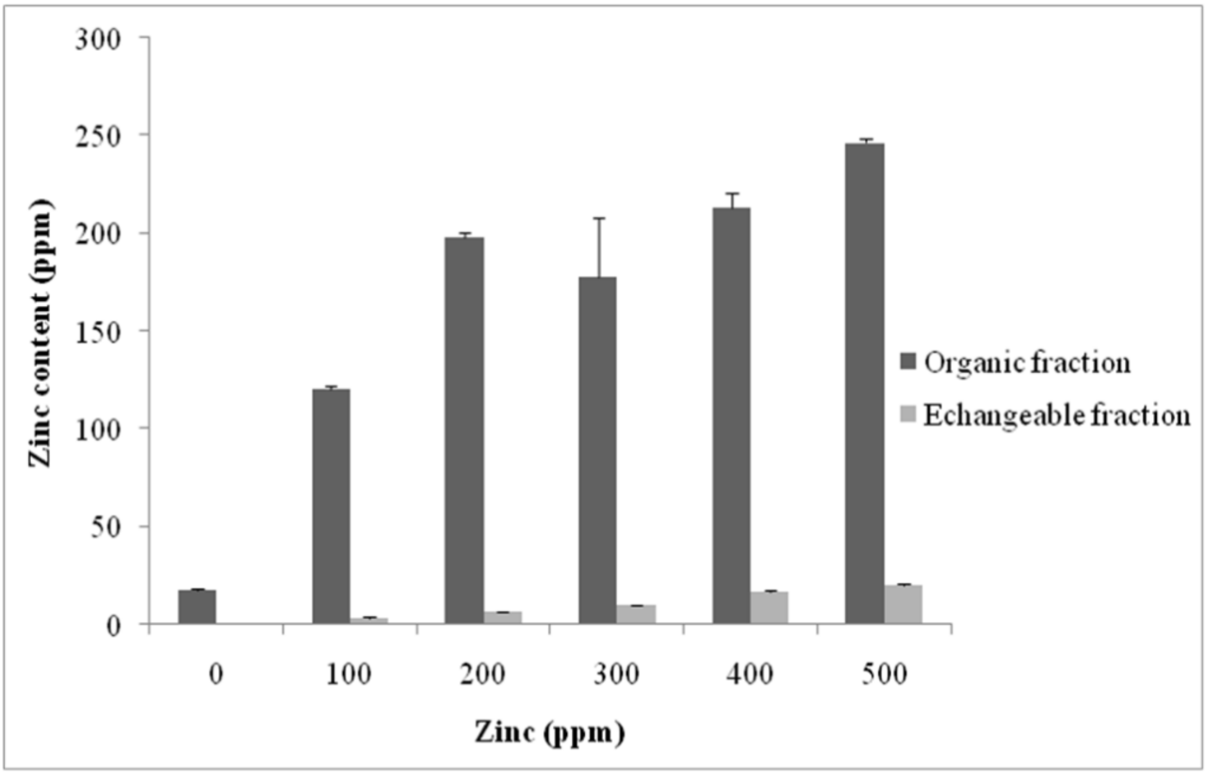


Figure 68: Zinc content in the organic and exchangeable fraction

Table 43: Shows values of the zinc content in an organic and exchangeable fraction of *Lycopersicon esculentum* Mill. treated by different concentrations of zinc ± standard deviation. Letters (a, b, c, d, e, and f) indicate the homogeneous groups as determined by ANOVA.

Zinc doses (ppm)	0 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
Zinc bound to organic matter (ppm) **P= 0**	17.375 e ± 0.672	120.63 d ± 1.259	197.708 bc ± 2.32	177.75 c ± 30.225	213.348 b ± 7.508	246.375 a ± 1.984

Zinc in the exchangeable fraction (ppm) P= 0	0.637 f ± 0.005	3.416 e ± 0.245	6.278 d ± 0.085	9.685 c ± 0.23	16.995 b ± 0.824	20.413 a ± 0.279

Zinc level in the soil induced a highly significant increase of zinc content in the organic and exchangeable fraction, compared to the control (Annex 4).

Six homogenous groups were obtained for zinc content in the organic fraction, group a (500 ppm), group b (400 ppm), group bc (200 ppm), group c (300 ppm), group d (100 ppm), and group e (0 ppm). However, six homogenous groups were obtained with zinc content in the exchangeable fraction, group a (500 ppm), group b (400 ppm), group c (300 ppm), group d (200 ppm), group e (100 ppm), and group f (0 ppm) (Table 43).

VII.5. Correlation analysis between the biochemical parameters in *Lycopersicon esculentum* Mill.

VII.5.1 Correlation obtained by copper treatment

Lycopersicon esculentum Mill. subjected to different concentrations of copper displayed a high correlation between the following parameters, antioxidant compounds, osmolytes, and proteins content, in the leaves and roots (Table 44).

In terms of organ, the level of polyphenol, flavonoids, antioxidant activity, proline, and soluble sugars in the leaves was positively correlated to its content in the roots (r = 0.989, 0.996, 0.994, 0.953, and 0.804), respectively. However, a negative correlation was recorded between the proteins content in the leaves and that in the roots (r= -0.364).

Additionally, the level of antioxidants compounds and the osmolytes in leaves as well as roots of *Lycopersicon esculentum* Mill. was negatively correlated with proteins content in the leaves; whereas, this level was highly positively correlated with proteins content in the roots. Besides, a low positive correlation was recorded between the proteins content in roots and proline content in leaves (r= 0.556) as well as in the roots (r= 0.477).

A negative correlation was noted between the antioxidant activity in leaves and roots and the content of proteins in the leaves, while a positive correlation was recorded with the proteins content in the roots.

Furthermore, a significant positive correlation was noted between osmolytes content (proline and soluble sugars) in the leaves and roots and the antioxidants content (polyphenols, flavonoids, and antioxidant activity) in these organs.

Besides, a highly positive correlation, varied from 0.904 to 0.968 depending on the organ and the compounds, was rcorded between the antioxidant activity in leaves and roots and the content of the antioxidants coumpounds in these organs under copper treatment.

Table 44: Pearson correlation matrix between osmolytes, antioxidants compounds, and proteins content in leaves and roots of *Lycopersicon esculentum* Mill. stressed by copper (L: leaves, R: roots)., (DPPH test).

Copper stress	L-poly	R-poly	L-flav	R-flav	L-DPPH	R-DPPH	L-prol	R-prol	L-sugar	R-sugar	L-prote	R-prote
L-poly	1											
R-poly	0.989**	1										
L-flav	0.981**	0.976**	1									
R-flav	0.977**	0.967**	0.996**	1								
L-DPPH	0.956**	0.968**	0.956**	0.931**	1							
R-DPPH	0.936**	0.961**	0.933**	0.904**	0.994**	1						
L-prol	0.824*	0.817*	0.836*	0.797	0.93**	0.912**	1					
R-prol	0.757	0.757	0.826*	0.792	0.878*	0.856*	0.953**	1				
L-sugar	0.903**	0.906**	0.899*	0.867*	0.968**	0.954**	0.964**	0.89*	1			
R-sugar	0.905**	0.894*	0.867*	0.855*	0.9**	0.898*	0.806**	0.729	0.804*	1		
L-prote	-0.761	-0.705	-0.775	-0.754	-0.807	-0.753	-0.928	-0.889	-0.877	-0.698	1	
R-prote	0.869*	0.915**	0.831*	0.83*	0.817*	0.8415*	0.556	0.477	0.707	0.782	-0.364	1

*Correlation is significant at the 0.05 level (2-tailed);

**Correlation is significant at the 0.01 level (2-tailed);

Underline values indicate a negative correlation.

VII.5.2 Correlation obtained by zinc treatment

A significant positive correlation was observed between the content of antioxidants compounds (polyphenols, flavonoids), antioxidant activity (DPPH), osmolytes, and proteins in leaves and their content in the roots of *Lycopersicon esculentum* Mill. (r= 0.710, 0.998, 0.978, 0.919, 0.952, 0.842), respectively.

Pearson analysis proved that the accumulation of antioxidants, antioxidant activity and osmolytes compounds in leaves and roots was negatively correlated with proteins contained in the roots; inversely to the results obtained by copper.

No correlation was revealed between proteins level in leaves and the content of polyphenol (r= -0.08 and 0.06), soluble sugar (r= 0.096 and-0.068), and proline (r=-0.038 and 0.117), in the leaves and roots respectively. A low correlation was recorded between protein content in leaves and the accumulation of flavonoids (r= 0.294 and 0.279) and the antioxidant activity (r= 0.212 and 0.258), in leaves and roots of *Lycopersicon esculentum* Mill, respectively.

Osmolytes compounds (proline and soluble sugar), accumulated in roots and leaves, were positively correlated with the antioxidant content (polyphenols and flavonoids) and the antioxidant activity in both organs.

Besides, a positive correlation was noted between the antioxidant activity in leaves and roots and the content of the antioxidants coumpounds in these organs under zinc treatment (Table 45).

Table 45: Pearson correlation matrix between osmolytes, antioxidants compounds, and protein content in leaves and roots of *Lycopersicon esculentum* Mill. stressed by zinc (L: leaves, R: roots), (DPPH test).

Zinc stress	L-poly	R-poly	L-flav	R-flav	L-DPPH	R-DPPH	L-prol	R-prol	L-sugar	R-sugar	L-prote	R-prote
L-poly	1											
R-poly	0.710	1										
L-flav	0.673	0.911**	1									
R-flav	0.699	0.915**	0.998**	1								
L-DPPH	0.743	0.931**	0.966**	0.959**	1							
R-DPPH	0.677	0.929**	0.997**	0.994**	0.978**	1						
L-prol	0.766	0.795	0.761	0.746	0.897*	0.793	1					
R-prol	0.785	0.956**	0.937**	0.934**	0.99**	0.955**	0.919**	1				
L-sugar	0.845**	0.898*	0.892*	0.888*	0.972**	0.91**	0.958**	0.984**	1			
R-sugar	0.858**	0.75	0.735	0.729	0.867*	0.76	0.98**	0.891*	0.952**	1		
L-prote	-0.08	0.06	0.294	0.279	0.212	0.258	-0.038	0.117	0.096	-0.068	1	
R-prote	-0.401	-0.457	-0.146	-0.161	-0.248	-0.192	-0.414	-0.358	-0.342	-0.404	0.842*	1

Correlation is significant at the 0.05 level (2-tailed);

**Correlation is significant at the 0.01 level (2-tailed);

Underline values indicate a negative correlation.

VII.5.3 Correlation between the level of copper in soil fractions and its content in leaves and roots of *Lycopersicon esculentum* Mill.

Pearson correlation indicated that the level of Cu in the exchangeable fractions was negatively correlated with Cu content in the organic fraction (r=-0.264). Nevertheless, a significantly positive correlation was observed between Cu in the exchangeable fraction and the content of Cu in leaves and roots of *Lycopersicon esculentum* Mill. (r= 0.649 and 0.868), respectively. No correlation was detected between the Cu content in the organic fraction and Cu level in the roots; but, a low positive correlation was noted with Cu content in the leaves (r= 0.432) (Table 46).

In term of organ-organ, it was recorded a highly positive correlation between the copper content in leaves and that roots.

Table 46: Pearson correlation matrix between organic fraction, exchangeable fraction, and leaf and root copper content in *Lycopersicon esculentum* Mill. stressd by copper.

Copper	Organic fraction	Exchangeable fraction	Leaf copper content	Root copper content
Organic fraction	1			
Exchangeable fraction	-0.264	1		
Leaf copper content	0.432	0.649	1	
Root copper content	0.167	0.868*	0.938**	1

VII.5.4 Correlation between the level of zinc in soil fractions and its content in leaves and roots of *Lycopersicon esculentum* Mill.

In soil profile, zinc associated with organic fraction was significantly correlated with its level in the exchangeable fraction (r= 0.853). Furthermore, these both fractions were highly positively correlated to zinc content in the leaves of *Lycopersicon esculentum* Mill. (r=0.859 and r= 0.993), and lowly correlated to zinc content in the roots (r= 0.579 and r= 0.545).

Similarly, zinc content in the leaves was positively correlated to the zinc content in the roots by a coefficient of 0.503 (Table 47).

Table 47: Pearson correlation matrix between organic fraction, exchangeable fraction, and leaf and root zinc content in *Lycopersicon esculentum* Mill. stressed by zinc

Zinc	Organic fraction	Exchangeable fraction	Leaf zinc content	Root zinc content
Organic fraction	1			
Exchangeable fraction	0.853*	1		
Leaf zinc content	0.859*	0.993**	1	
Root zinc content	0.579	0.545	0.503	1

VII.6. Evaluation of phytoremediation efficiency

VII.6.1 Translocation factor (TF)

The translocation factor (TF) of copper and zinc in *Lycopersicon esculentum* Mill. is depicted in Table 48. TF of copper was >1, whereas; it was < 1 for zinc. These observations were detected in control seedlings (TF: 2.019 and 0.083) as well as in the different concentrations of both metals. Inversely to the zinc, as copper content increased in the soil as it more translocated to the leaves.

Copper and zinc at 300 ppm were lowly translocated to the leaves as showing by the following TF values: 4.850 and 0.026. However, 400 ppm induced the maximum translocation of copper and zinc (TF: 6.034 and 0.310), whereas, this potential was reduced by 500 ppm to give values of 3.982 and 0.05 for Cu and Zn, respectively.

Table 48: Translocation factor (TF) of copper and zinc in *Lycopersicon esculentum* Mill.

Metals doses (ppm)	**Translocation factor (TF) of Copper**	**Translocation factor (TF) of Zinc**
0	2.019 ± 0.04 e	0.083 ± 0.007 b
100	4.306 ± 0.325 cd	0.054 ± 0.003 b
200	5.263 ± 0.064 b	0.055 ± 0.201 ab
300	4.850 ± 0.122 bc	0.026 ± 0.001 b
400	6.034 ± 0.874 a	0.310 ± 0.006 a
500	3.982 ± 0.215 d	0.05 ± 0.001 b

VII.6.2 Accumulation efficiency (Bioconcentration factor (BCF))

Results illustrated in Table 49 revealed that copper and zinc content was lowly accumulated in roots of *Lycopersicon esculentum* Mill. As an indicator of phytostabilization capacity, the bioconcentration factor (BCF) was higher than the critical value. For instance, control seedlings exhibit a BCF of 24.637 for copper and 28.124 for zinc.

A positive correlation was noted between the elevation concentration of copper and zinc in the soil and the bioconcentration factor. Unexpectedly, a slight decline in BCF was pointed at 200 ppm for both metals.

Moreover, BCF reached the maximum value at 400 ppm for copper with 86.245 and 300 ppm for zinc with 45.799. However, the lowest value was observed at 400 ppm for zinc by 3.906 and 500 ppm for copper by 14.639.

Table 49: Bioconcentration factor of copper and zinc in *Lycopersicon esculentum* Mill.

Copper (ppm)	**Bioconcentration Factor (BCF), root**	
	Copper	**Zinc**
0	24.637 ± 0.641d	28.124 ± 0.441 b
100	27.748 ± 1.745 c	30.705 ± 2.87 b
200	24.743 ± 0.238 d	22.59 ± 16.77 b
300	46.103 ± 1.978 b	45.799 ± 1.185 a
400	86.245 ± 0.357 a	3.906 ± 0.177 c
500	14.639 ± 0.381e	21.349 ± 0.224 b

VII.7. Phytotoxicity

In absence of copper and zinc treatments, no toxicity was recorded in the stem and root of *Lycopersicon esculentum* Mill. (Table 50).

In the case of the stem, a similar toxic effect of copper and zinc was induced at 100 ppm by approximately 16 %. Contrarily to the roots, a moderate toxic effect (20.856 %) was given by copper at 100 ppm; whereas, the lowest toxicity was marked for zinc by 7.101% at this concentration, among all treatments. Remarkably, low toxicity in roots and stems was occurred with 300 ppm, compared to 200 and 400 ppm.

Overall, total mean values showed that zinc content in the stem was more toxic than copper; while, this content in roots was less toxic.

In roots as well as stem, the toxic effect does not exceed the value of 35% either with zinc or copper.

Table 50: Phytoxicity and tolerance index of copper and zinc in *Lycopersicon esculentum* Mill.

Doses (ppm)	Stem toxicity (%)		Root toxicity (%)		Tolerance index (TI) Root (%)	
	Copper	**Zinc**	**Copper**	**Zinc**	**Copper**	**Zinc**
0	0	0	0	0	100	100
100	16.4	15.789	20.856	7.101	79.143	92.898
200	25	23.982	25.669	18.941	74.330	81.058
300	22.233	25.157	20.856	20.113	79.143	79.886
400	27.783	29.245	33.691	23.078	66.308	76.921
500	29.166	35.087	34.766	24.267	65.233	75.732
Total mean values	24.11	25.853	22.640	15.583	77.359	84.416

VII.8. Tolerance index (TI)

The tolerance index, calculated for roots of *Lycopersicon esculentum* Mill., was reduced by increasing copper and zinc doses in the soil (Table 50).

The highest tolerance potential (100%) was recorded in the absence of Cu and Zn (0 ppm) treatment. However, this tolerance decreased slightly with the concentration of 100 ppm to give values of 79.143 and 92.898%; while, the lowest potential was recorded at 500 ppm by 65.233 and 75.732%, for copper and zinc respectively. Remarkably, roots treated by Cu and Zn at 300 ppm exhibited a similar tolerance index of 79%.

Besides, roots subjected to zinc doses were relatively tolerant compared to those exposed to copper. Overall, the tolerance index of roots had a prominent value exceeding 75%, for both metals.

CHAPTER VIII

Discussion

VIII.1. Effect of copper and zinc on the morphological parameters

VIII.1.1 Stem length

Plants' tolerance to substantial metal toxicity was evaluated by their roots or shoots development limited by the toxic metal in the soil **(Amin *et al*., 2019)**.

- **Effect of copper**

Many studies have reported the reduction of stem length under copper stress in different plants species; in *Melissa officinalis* **(Adamczyk-Szabela *et al*., 2019)**, in *Brassica juncea* **(Yadav *et al*., 2018)**, in *Verbascum* populations **(Morina *et al*., 2016)**, in *Cassia Angustifolia Vahl* **(Nanda and Agrawal, 2018)**, in *Lycopersicum esculentum* **(Pérez-Labrada *et al*., 2019)**.

In the same context, the current study revealed a reduction in stem length of *Lycopersicon esculentum* Mill. under copper stress. This decrease was proportional to the increase of copper doses in the soil; however, an unexpected improvement in stem length was marked at 300 ppm Cu.

Our results were consistent with the findings of **Shukla (2017)** who suggested that the foliar application of $CuSO_4$ at 150, 250, and 350 ppm increased the height of *Solanum lycopersicum* by 1.54%, 1.33%, and 1.67% respectively compared to the control treatment. By contrast, the findings observed by **Nazir *et al*. (2019)** in *Solanum lycopersicum* plants treated with a low concentration of Cu (10 mg of Cu. kg^{-1}) showed that this level improved all the growth traits; however, higher concentration (100 mg of Cu. kg^{-1}) triggered a reduction in all the growth parameters. Besides, Nazir's calculation only referred to the limited case that 10 ppm improve stem length of the tomato plant, our conclusion that the beneficial dose was 300 ppm would thus seem to be defensible. Similarly, according to **Zehra *et al*. (2020)**, the maximum shoot length of *Artemisia annua* was recorded at the application of 10 mg. kg^{-1} Cu in all the genotypes while maximum inhibition of shoot length was found when 60 mg. kg^{-1} Cu was applied compared with control.

Remarkably, some plant species required a minimum level of copper for their growth. In this case, copper was found to be beneficial to plant growth at 3 ppm and 5 ppm for all the selected species given below showing an increase in growth; whereas, copper was toxic at 100 ppm and onwards, showing inhibition of root and shoot elongation. Based on the observations, severe inhibition was noted at 200 ppm to 500 ppm **(Menon *et al*., 2020)**. These findings are in good agreement with our data noted at 500 ppm. Consequently, **Saleem *et al*. (2020b)** demonstrated that increasing Cu levels in the nutrient solution causes a significant reduction in plant height and plant diameter compared to the control. Similar results were also obtained by many researchers **(Huo *et al*., 2020 ; Zehra *et al*., 2020)**. By contrast, in tomato, rice, maize, and wheat, as well, the use of copper significantly increased growth parameters **(Jyothi and Hebsur, 2017 ; Singh *et al.*, 2017).**

Compared to the findings obtained by previous research, stem elongation was affected by many factors including the concentration as given above, the species sensibility to heavy metals, duration of application, the development stage, combination metal, type of the metal, and the tissue and metal toxicity.

Depending on the plant species sensibility, it has been demonstrated that in the morphological responses of selected plants to copper, the highest growth was recorded for *Vigna radiata*. to the other selected species. The germination and growth of *Trigonella foenum graecum* and *Oryza sativa* seedlings were found to be most sensitive. The resistance of the plants can be represented as *Vigna radiata* > *Vigna aconitifolia* > *Pennisetum glaucum* > *Sorghum vulgare* > *Trigonella foenum* **(Menon *et al*., 2020).**

Moreover, the stem elongation affected by copper in terms of application duration was reported by many researchers. Obvious growth inhibition in seedlings of *Brassica rapa* was observed under 200 mg. L^{-1} Cu^{2+} treatment after nine and 12 days compared with other treatments with lower Cu^{2+} concentration **(Ruan *et al*., 2019)**. Similarly, **Mostofa *et al*. (2015)** found that the height of the Cu-stressed seedlings decreased by 13 and 18% at days 4 and 7 respectively, compared with control.

Furthermore, a crucial study depending on the plant tissue was conducted by **Huo *et al*. (2020),** who revealed that copper at 10 µmol. L^{-1} nearly inhibited root growth, whereas 20 µmol. L^{-1} Cu entirely inhibited the shoot growth of plants. These data probably justify the copper effects observed in roots and shoots length of *Lycopersicon escculentum* Mill., in our study.

Depending on the stage development, percentage germination, root length, and shoot length were decreased in different concentrations of Cu in a dose-dependent manner and the highest decrease of 34% in seed germination, 82.80% in root length, and 45.92% in shoot length was observed in 10 mM Cu **(Sharma *et al*., 2017).** A similar decrease in seed germination, root length as well as shoot length was seen in *Lycopersicon esculentum* under Cu stress **(Ashagre *et al*., 2013)**. These observations support the hypothesis which indicates that reduction in all growth parameters follows the same trend under heavy metal stress either by increasing or decreasing. This is much well with our observations, where copper doses reduced the growth plant (stem and roots length) with an exceptional increase at 300 ppm for both organs.

Depending on the metal toxicity, it has been demonstrated that copper revealed maximum effect (51.2%) on germination followed by lead (47.5%) and cadmium (35.3%). The growth parameters wee depending on the metal, species, development stage, *Solanum lycopersicum* seeds were most sensitive in the germination stage (lengths of plumules and radicals) followed by *Pisum sativum* and *Triticum aestivum*. In the seedling stage (length root, height stem, fresh biomass), tomatoes also showed the highest sensitivity to both Cd and Cu. However, *Pisum sativum* seedlings showed higher tolerance to Pb and *Triticum aestivum* seedlings had the highest tolerance to both Cu and Cd. Toxicity and tolerance of metals were found to vary with crops and growth stages **(Baruah *et al*., 2019)**. These findings may argue the different effects obtained in our study by copper and zinc. More details are given in the discussion section of tolerance and toxicity.

Many studies proved the effect of combination metal as a limiting factor of heavy metal stress. The treatment of Co or Cu alone significantly reduced the growth (tissue height and dry weight) of the two barley genotypes. Meanwhile, the combined treatment caused more severe lipid in comparison with the treatment of Co or Cu alone **(Lwalaba *et al*., 2020)**.

Conversely, some species seem to be more tolerant to metals than others, for instance, **Pietrini *et al*. (2019)** tented to evaluate the morpho-physiological response of *Arundo donax*, a well-known energy crop, when treated with increasing concentrations of Cu (0, 150, and 300 ppm) in a semi-hydroponic growing system (mesocosm) for one month. The plant morphology (height and base diameter of the stem, number of stems) was not affected by the treatments. The presence of Cu led to the disequilibrium of Fe and Zn foliar concentration and caused an impairment of photosynthetic parameters: at 150 and 300 ppm the chlorophyll content and the electron transport rate (ETR) were significantly lower than the control. The study demonstrated that, although the presence of Cu may initially affect the plant physiology, the *Arundo* plants can tolerate up to 300 ppm of Cu without any adverse effect on biomass production, even when grown in semi-hydroponic conditions. The findings of this study are consistent with that of the **Pietrini *et al*. (2019).**

According to previous research, morphological alterations in stem length could be attributed to the physiological, biometrical, biochemical, and chemical mechanisms; pointed to mineral interruption, metal content in the tissue, ultrastructural damages, biochemical, oxidative stress, phytohormone pathway, gene regulation, and transcription factor;

The probable reason for the decrease in stem length could be mainly due to changes in ion toxicity and mineral interruptions in plants which led to inhibition of cell division and cell elongation **(Feigl *et al*., 2015 ; Kopittke and Menzies, 2006).** Generally, reduced growth plant occurs in the presence of excess heavy metals is correlated with the amount of metal present in their organs **(Kang *et al*., 2017)**. Furthermore, it has been found that additions of Cu could inhibit the growth and the activity of antioxidant enzymes, increase the degree of membrane lipid peroxidation, and decrease the podophyllotoxin content of *Dysosma vermicelli* compared with the control **(Luo *et al*., 2020)**. Similar effects were also obtained at higher concentrations of Cu **(Zehra *et al*., 2020)**.

Cu is also known for impairing water and nutrients uptake, and the negative effect on plant growth is doubled by the diminished chlorophyll pigments, which has an immediate effect on photosynthesis and respiration **(Nanda and Agrawal, 2016)**. Previous studies suggested that, in higher plants, chloroplasts are the major site of Cu accumulation and this may affect the metabolic processes of photosynthesis and respiration at a genetic level **(Habiba *et al*., 2014 ; Rehman *et al*., 2019d)**. Moreover, Cu stress significantly increased the electrolyte leakage **(Nazir *et al*., 2019)**.

As given by **Lwalaba *et al*. (2020)**, the treatment of Cu alone significantly induced the cell oxidative stress, reflected by the accumulation of ROS molecules in leaves of both genotypes of barley.

The surprising increase of stem length recorded at 300 ppm Cu in our study might be due to the role of this element in the formation of regulatory proteins as well as in mitochondrial respiration and hormone signaling **(Singh *et al*., 2017)**. Also, this might be because of the accumulation of photosynthates and which in turn increased dry matter in plants and resulted in increased height **(Shukla, 2017).**

Different effects occurred on stem elongation as results of copper stress were regulated by plant hormones, this is supported by many scientific reports. For instance, in plants, cytokinins are known to promote shoot bud proliferation in dormant shoot meristems. Therefore, an exogenous supply of cytokinin is usually essential for inducing bud break or shoot differentiation in the explants. On increasing or decreasing the concentration of the hormone, a gradual decline in mean shoots number and length was noted **(Alam *et al*., 2020)**. It has been also indicated that Brassinosteroids play an important role in the growth and development of tomato plants **(Soto *et al*., 2020)**.

Importantly, ultrastructural damages in the stem of *Lycopersicon esculentum* Mill. under copper, stress was not analyzed, but we can refer to other studies for more explanations;

Based on the electron microscopic examination of the *Hordeum sativum* plant's stem, **Minkina *et al*. (2019)** demonstrated that plants exposed to supplementary Cu, cytoplasm showed deep invaginations into the central vacuole in individual cells, and the majority of cells exhibited as narrow dense (dark) bands. In some parts of the cytoplasm, accumulated clusters of lipid droplets showed considerable thickening. Cu toxicity caused a significant increase in electron density of plastids and expanded the space of thylakoids. There was also an increase in the plastoglobule sizes (up to 150 nm in diameter) and a decrease in electron density in the central parts of the matrix.

Effect of zinc

The current study showed the reduction in length stem of *Lycopersicon esculentum* Mill. under zinc treatments. This decrease was proportional to the increasing of zinc doses in the soil.

Our results concur with the findings of **Salimi *et al*. (2019)** who suggested that tomato seedlings treated with different concentrations of zinc (0, 50, 100 mg. L^{-1}) presented a reduction in the height plant of the *Lycopersicon esculentum* (28.67, 37.33, and 37 cm). The concentration of 100 ppm was appreciable and confirmed our findings, while 50 ppm was essential for the growth of the tomato plant. Furthermore, to evaluate the toxicity and tolerance capacity of the plant, another study has been undertaken by using high level of zinc, it indicated that *Plantago ovata* seedlings grown in presence of 500, 800, and 1000 µM of $ZnSO_4$ showed a significant reduction in the root and shoot length in comparison to the untreated seedlings **(Pramanick *et al*., 2017)**. These data confirmed the sharp reduction in stem length at 500 ppm in our results. It supports also the hypothesis that all the growth parameters exhibited the same trend under heavy metal stress. Therefore, our results indicated that the concentration plays a crucial role in the effect of zinc on plant growth parameters.

Our findings have several similarities with **Subba *et al*.'s (2014)** outcomes, which emphasized the effect of zinc on stem elongation in terms of high and low concentration. In this study, the height of the plants (*Citrus reticulata* Blanco) increased with an increase in Zn concentration from 3 to 5 mM but afterward the plant height was reduced with additional Zn concentration. Plants supplied with 4 and 5 mM Zn were superior in growth than the plants that received higher doses (10, 15, and 20 mM) of Zn. Growth retardation, defoliation, and sluggish root growth were the prime features in the plants supplied with 10, 15, and 20 mM Zn. However, among all the Zn supplemented treatments, 5 mM Zn induced prolific growth and sprouted abundantly.

According to the previous research, stem length affected by zinc treatment highlighted the impact of many factors as; metal concentration, duration of application, type of metal, the tissue or organ, species sensibility to heavy metals. etc;

According to the duration of application, it has been observed that the parameters like plant height, root length, shoot length, leaf length, and the number of leaves per plant decreased by different degree of percent (84.06, 69.16, 92.36, 46.80, and 75.00%, respectively) in 200 µM of Zn when compared to control plants of 7-day treatment. On the other hand, trends like 74.03, 66.83, 77.76, 62.50, and 62.50% of decrement against respective parameters (plant height, root length, shoot length, leaf length, and the number of leaves per plant) were also observed in 200 µM of Zn after 14 days treated plants **(Rout *et al*., 2019)**.

Depending on the metal, **Tiecher *et al.* (2017)** demonstrated that the highest doses of Cu and Zn caused a decrease in plant growth and photochemical efficiency associated with the decrease in photosynthetic pigment content. Also, it has been observed the effect of heavy metals, $CuSO_4$ and $ZnSO_4$ on the morphogenic response of cotyledonary node explants of *Mucuna pruriens* **(Alam *et al*., 2020)**. However, depending on the tissue, the impact of both HMs was highly pronounced in roots when compared with shoots **(Garg and Singh, 2018)**, this is in contradiction with our findings.

Zinc has a negative and a positive effect on stem growth depending on the zinc concentration in the soil, plant genotype, and the zinc formula. Notably, the positive effect was not observed in our investigation, but we propose a dose inferior to 100 ppm to improve the stem elongation of tomato, as was discussed above. **Haider *et al*. (2020)** indicated that the plant height of both genotypes was improved with the application of Zn through either method. This increase in plant height was due to an improvement in growth cascades. **Alam *et al.* (2020)** confirmed that the optimization of $ZnSO_4$ can help in enhancing morphogenic response from the explants. Similarly, the results obtained by **Eneida *et al*. (2020)** showed that ZnO-NP-treated tomato plants significantly increased plant height, stem diameter, and plant organs (leaves, stem, and root) dry weight compared to plants without NP treatment.

Many research had compared the effect of copper and zinc on stem elongation, in a study conducted by **Alam *et al*. (2020),** copper is supplied in the medium in the form of $CuSO_4$ in trace amount (0.1 µM) for normal growth and development of the plant in natural habitat as it is an essential micronutrient. However, the optimal requirement of copper varies with the species. Of the tested concentrations of $CuSO_4$, a medium containing 5.0 µM $CuSO_4$ proved to be optimum in inducing the number of shoots (11.20) per explant with an average shoot length (2.34 cm) after 56 days. On further increasing the concentration of $CuSO_4$, a sharp decline in various parameters was noticed, indicating the toxic effect of a higher concentration of $CuSO_4$. The basal medium contains 30 µM $ZnSO_4$, but the optimal concentration varies with the species and explant type. Therefore, optimization of $ZnSO_4$ can help in enhancing morphogenic response from the explant. The cotyledonary node explants cultured on media augmented with 80.0 µM $ZnSO_4$ gave better response in terms of the number of shoots (13.40) per explant and average shoot length (2.70 cm) after 56 days. These findings indicated that copper was more toxic than zinc, which is in contradiction with our results.

In terms of the heavy metal sensibility of tomato, it has been indicated that after 39 days of exposure to the metal, the tolerant cultivar exhibited a lower height than control plants, but this effect disappeared in advanced stages of development **(Carvalho *et al*., 2018).**

All the examples above were provided to elucidate the different effects of heavy metals obtained in our results compared to those reported recently.

The morphological outcomes of stem length might likely be justified by the participants of physiological, biometrical, biochemical, and chemical mechanisms involving in response to heavy metal stress such as mineral interruption, metal content in the tissue, oxidative stress, phytohormone pathway, gene regulation, and transcription factor, ultrastructural damages, etc.

Essential heavy metals, e.g., Zn or Fe, are vital for plants to achieve intact growth and development **(Nosek *et al*., 2020)**. Moreover, zinc is an important essential metal and micronutrient directly promoting plant growth **(Adamczyk-Szabela *et al*., 2020)**. Both metal Zn and Cu at higher concentrations had a pronounced effect on tissue Fe concentration in both shoots and roots of the *Verbascum* (NMET) populations. At the highest Zn concentration tested (60 µM Zn), Fe concentration was significantly reduced in the shoots of both NMET1 and NMET2 plants but was not significantly affected in MET1 plants. Correspondingly, 60 µM Zn treatment caused a large increase in Fe accumulation in the roots of both NMET populations **(Morina e*t al*., 2016)**. Besides, the toxicity is related to the zinc content in shoots **(Samardjieva *et al*., 2015).**

Furthermore, Zn treatments significantly promoted the activity of the superoxide dismutase (SOD) enzyme (about two-fold) **(Salimi *et al*., 2019)**. The previous results demonstrated that glutathione-S-transferase (GSTs) was involved in fighting heavy metal stress, and might also have a role in vitro regeneration **(Alam *et al*., 2020)**. It has been observed that single treatments of Zn significantly induced phenylalanine ammonia-lyase activity by 30%, respectively **(Salimi *et al*., 2019)**. Besides, it has been suggested that the antioxidant defense system did not sufficiently protect the plants under rigorous Zn stress which was also corroborated by the alteration in cell ultrastructure as revealed by transmission electron microscopy. Remarkably, among the ultrastructural damages, the mitochondrial membranes of excess Zn treated plants were disorganized and the cristae were randomly distributed. The excess level of Zn treatment caused a collapse of the cristae and localized condensations in the mitochondrial matrix **(Subba *et al*., 2014).** However, supplementation of both Zn and Si promoted plant biomass, photosynthetic parameters, ionic balance, and improved chloroplast ultrastructure with minimized Cd uptake and malondialdehyde (MDA) content due to the activation of antioxidant enzymes in Cd stressed plants. Relative to control, root length, shoot height, plant tillers, and dry weight of root, stem, and leaf of all Cd treated plants were lowered in both genotypes regardless of Zn or Si supplementation. The addition of Zn managed to significantly alleviate the reduction in root length and shoot height of both genotypes **(Mapodzeke *et al*., 2021).**

The stem elongation could be also influenced by the metal combination and the type of this element as a toxic or essential metal for growth. A study conducted by **Ribeiro *et al*. (2020b)**, indicated that cadmium (Cd) and zinc (Zn) toxicity causes physiological disorders and harms plants, interfering with the rehabilitation of areas affected by mining activities. This study evaluated how the exposure to Zn and/or Cd affects the growth of native andropogon grass (*Andropogon gayanus* Kunth) plants originally found in areas contaminated with Cd and/or Zn due to zinc mining activities. Plants were cultivated for 7 weeks in a nutrient solution treated with Zn (142.3–854.0 μM) or Cd (0.9–13.3 μM) separately or combined. Andropogon grass tolerated both metals better separately than when applied together. Zinc doses significantly increased andropogon grass height in comparison to the control (up to 22%) except for the dose of 854.0 μM, for which a non-significant effect was observed. For plant height, the treatments with combined doses of Zn (284.7) and Cd (4.4) or higher showed significant negative effects on plant height. These results indicate that in the presence of Cd, the beneficial effects obtained by increasing the Zn contents were canceled, and the harmful effects of Cd increased. The individual doses of 0.9 μM of Cd and 569.3 μM of Zn promoted an increase (up to 40%) in stem diameter. For the other treatments, no differences were observed concerning the control treatment.

Unfortunately, in our study, ultrastructure analysis on stem length exposed to different doses of zinc was not achieved. Therefore, the interpretations were given according to the previous research. Ultrastructure analysis was identified that zinc (Zn) and silicon (Si) markedly improved chloroplast formation, whereas the formation of plastoglobuli was higher with Si than Zn in both rice genotypes. Starch granule formation also increased significantly by Zn and Si addition. In Xiushui-110 (rice genotype), Zn alleviation caused fragmentation of stromal thylakoids **(Mapodzeke *et al*., 2021).** For instance, transmission electron microscopy analyses showed modifications such as vesiculation and vacuolation in the ultrastructure of andropogon tissues by Cd and/or Zn. The andropogon grass was tolerant to the doses tested, evidencing that it has potential for recovering areas contaminated with Zn and/or Cd **(Ribeiro *et al.*, 2020b).**

A satisfactory explanation for the zinc effect has been pointed that Zinc-treated plant stems also presented a generalized AMG staining in all the tissues contrary to the controls. At low magnification, autometallography (AMG) staining was observed to be associated with the cell walls of stem medullary parenchyma, vascular tissues, cortical parenchyma, and epidermis. The stem structure of *Solanum nigrum* L. has collateral vascular bundles, characterized by internal and external phloem. At higher magnification, it was noticeable that the Zn deposits of the internal phloem and xylem parenchyma were more conspicuous than at the xylem tracheary elements. The interfascicular parenchyma cells presented black precipitates in the vacuoles indicative of Zn accumulations in these compartments. Large Zn deposits were observed in the vacuoles of starch sheath cells, and in the vascular cylinder, an intense AMG staining appeared to be associated with the cell walls of both the cambium and external phloem. Smaller deposits were also present in the vacuoles of outer cortical parenchyma cells. Our results suggest that Zn flux through the plant occurs via the xylem and phloem and associated parenchyma until it is conducted to permanent storage sites, namely, the apoplast and vacuoles of cortical parenchyma cells of the root, stem, and the leaf mesophyll**.** This Zn concentration (0.025 g Zn. L^{-1} during 35 days) used in this study resulted in stunted plant growth but no other symptoms of Zn toxicity **(Samardjieva *et al*., 2015).**

Early reports have demonstrated that the reduction in plant growth was closely related to the decreases in gibberellins (GA), Indole acetic acid (IAA) contents **(Li *et al*., 2018c)**. Heavy metal affects phytohormones (abcisic acid (ABA), salycilic acid (SA), Indole acetic acid (IAA), gibberellins (Gas)). Apart from these, plant hormone-like cytokinins (CKs), contributes to the regulation of plant development by stimulating cell division and elongation **(Sharma *et al*., 2020)**. In the apical plant stem cell system, local accumulation of the small, highly mobile phytohormone auxin triggers differentiation while at the same time, pluripotent stem cells are maintained throughout the entire life-cycle **(Ma *et al*., 2019)**.

Moreover, auxin controls multiple aspects of plant growth and development. Auxin acts on the transcriptional regulation of target genes, mainly through Auxin Response Factors (ARF). This study focused on the involvement of SlARF4 in tomato tolerance to salinity and osmotic stress. ARFs play a crucial role in auxin signaling **(Bouzroud *et al*., 2020)**. It has been established that stem cells require auxin signaling for their activity **(Ma *et al*., 2019)**. Also, it has been demonstrated that ZnF1 may play a negative regulatory role and influence reproduction through the auxin pathway in *Brassica*. ZnF1 delayed plant growth and development **(Lian *et al*., 2020)**.

Furthermore, the plant growth hormone auxin stimulates target of rapamycin (TOR) activity through a physical interaction between TOR and auxin-activated Rho-like GTPase 2 (ROP 2) to promote the activation of shoot meristem **(Li *et al*., 2017a ; Schepetilnikov *et al*., 2017)**. Indeed, **Ma *et al*. (2019)** found that stem cells are resistant to auxin-mediated differentiation but require low levels of signaling for their maintenance. They demonstrated that the WUSCHEL transcription factor confers this behavior by rheostatically controlling the auxin signaling and response pathway. Finally, it showed that WUSCHEL acts via regulation of histone acetylation at target loci, including those with functions in the auxin pathway.

It has been examined that RNAs associated with epidermis cell differentiation, Wax biosynthesis, and cell wall organization were all significantly enriched in the epidermis, confirming known physiological functions. As another example, genes encoding extensions and expansins were also enriched in the epidermis. Particularly, gene regulatory networks control development via domain-specific gene expression. A large number of unusual floral organs UFO' domain-specific transcripts may correspond to active cell proliferation in this domain **(Tian *et al*., 2019)**. According to **Ma *et al*. (2019)**, the shoot apical meristem (SAM) is a highly dynamic and continuously active stem cell system responsible for the generation of all above-ground tissues of plants.

Overall, heavy metal stress induced by copper and zinc was depending on the concentration and the metal as well as other factors. Consequently, these elements could improve or inhibit the stem elongation of plants.

VIII.1.2 Root length

- **Effect of copper**

Results illustrated in this study indicate that copper stress reduced the root length of *Lycopersicon esculentum* Mill. Although that this reduction was proportional to the increase of copper doses in the soil, an unexpected slight increase was observed at 300 ppm Cu. These results are in good agreement with the findings of **Hu *et al.* (2020)**. Also, in similar experimental conditions **Hemeir (2015)** found that copper decreased the length root of tomato at 100 ppm with a slight increase at 200 ppm; while, the most reduced length was obtained at a high level of copper.

The negative effect caused by the lowest concentration (100 ppm Cu), in our study, corroborates the findings of **Işeri *et al.* (2018)**, who proved that 30 ppm Cu^{2+} application reduced the root growth of tomato seedlings. A similar effect was also obtained by **Nanda and Agrawal (2018)** in *Cassia Angustifolia* seedlings for all concentrations, where she found that root length was slightly decreased to 6.14 cm at 10 mg. L^{-1}; whereas, a sharp reduction in root length was marked at high concentrations to 2.35, 1.35, and 0.34 cm at 50, 100, and 200 mg. L^{-1}. According to **Zehra *et al.* (2020),** the maximum increase in growth parameters (including roots) was reported when 10 mg. kg^{-1} Cu was applied to *Artemisia annua*, whereas root length decreased significantly at 60 mg. kg^{-1} Cu. As noticed earlier, increasing the Cu rate to 2 times significantly reduced the root lifespan. It has been shown that both root types declined to about 1/3 of the controls for the highest Cu treatment as was indicated by the main effect means **(Hamido *et al*., 2019)**.

The evidence we found points to a high concentration of copper induce a toxic effect on the root growth; while, low concentration reduces the length, this because of deficiency in copper as essential elements. Besides, differences observed between the present findings and the reports mentioned above may be due to dose-effect and the growth stage.

As confirmed in this investigation, the concentration of copper is considered a limited parameter of plant growth; however, the root elongation could be also influenced by other factors including, duration of exposure to the metal, plant species sensibility, formula product, metal content in the tissue, root types, and nutrient elements;

Depending on the duration of exposure to metal and plant species sensibility, it has been demonstrated that the presence of Cu toxicity (50 and 100 mM) for 10 days significantly retarded the growth attributes in tomato plants **(Kang *et al*., 2017)**. On the other hand, depending on the plant species, **Branco-Neves *et al.* (2017)** showed that after 28 days in hydroponic culture, the presence of 250 μmol. L^{-1} Cu promoted a decrease in root and shoot length in both *Solanum cheesmaniae* and *Solanum lycopersicum* plants and inhibited biomass production, but these effects were more pronounced in *S. lycopersicum* individuals, clearly showing that *S. cheesmaniae* is more tolerant to the toxic effects of the heavy metal. In a study conducted by **Wang *et al*. (2017)** noted that quantitative analysis of Cu treated seedlings showed that root length, root tip number, root surface area, root volume, and leaf surface area were lowlily decreased by 5.9%, 58.3%, 76.2%, 39.1%, and 4.4%, respectively, compared to the control results. Furthermore, depending on the formula and the content of copper in the roots, it has been found that sugarcane roots from plants grown in soil treated with Kocide 3000, nCuO, Cu NP, and $CuCl_2$ at 60 mg. kg^{-1} exhibited Cu concentrations of 146%, 46%, 191%, and 56% higher than controls **(Tamez *et al*., 2020)**. As also confirmed by many researchers, the most pronounced decrease of biomass and organ length observed in *Solanum lycopersicum* Mill. in response to Cu is likely correlated with the higher levels of Cu found in *S. lycopersicum* organs **(Branco-Neves *et al*., 2017)**.

All the suggestions provided above, as well as the results obtained in our study on the root growth of *Lycopersicon esculentum* Mill. could be explained by the recent researches established at different stages, including; cell wall alterations, membrane permeability, ultrastructural damages, oxidative stress, genotoxicity, etc;

Root development is closely related to cell wall formation **(Hu *et al*., 2020)**. The plants grown in the presence of Cu (100 mg. kg^{-1}) have distorted the morphology of roots; whereas, the root morphology of the control plants was not affected **(Nazir *et al*., 2019)**. This effect because Cu tends to be strongly absorbed by plant roots and is not readily translocated to above ground parts of the plant **(Rehman *et al*., 2019b)**, this result was also consistent with **Zaouali *et al*. (2020).**

The study conducted by **Wang *et al*. (2017)** demonstrated that *Oenothera glazioviana* seedlings exhibited visible damage when exposed to 50 μM $CuSO_4$ for 3 days. The roots became slightly brown, and their growth was markedly inhibited, these observations were also noted in our results. Similarly, **Hu *et al*. (2020)** showed that untreated control seedlings had long primary roots and many lateral roots. The inhibition of transposition of phytotoxic amounts related to the heavy metals from the roots to the leaves can be one of the mechanisms of heavy metals resistance that plants may use **(Tiwari and Lata, 2018)**. Such behavior was also observed by **Ambrosini *et al*. (2015)**, who found alterations in the root system of young grapevines after 70 days of exposure in soil with an excess of Cu. Additionally, root thickening was observed, which may be a defense strategy of plant in response to abiotic stress, increasing the area of the apoplast and, consequently, the retention of metals in the roots **(Tiecher *et al.*, 2017)**.

The copper effects caused an increase in non-specific membrane permeability, which may be responsible for the imbalance in nutrient concentration in plants grown in environments with high levels of heavy metals **(Cambrollé *et al.*, 2013)**. It has been indicated that decreased elongation of the primary root, impaired secondary growth, increased root dieback, or reduced root hair caused by toxic ions all exert a deleterious effect on the root-absorbing area and water uptake **(Rucińska-Sobkowiak, 2016)**. Elongation of plant roots and shoots demonstrated a notable sensitivity toward excess heavy metals in soil. The decrease in root length could be primarily due to the interference of heavy metals with the uptake of water and mineral nutrient. Moreover, the decreased rate of water and mineral absorption was induced due to mineral deficiency in plants and reduced root cell division, cell elongation along the cell cycle that in turn reduced root length of plants **(Adrees *et al.*, 2015)**. Besides, it has been demonstrated that Fe and Mo, Mn, B and Zn accumulation in the roots decreased when this organ was treated with nCuO, Cu NP, and Cu; whereas, S content increased in the roots **(Tamez *et al.*, 2020)**. The Cu-induced deficiency in other essential elements due to competition has been described by many studies, while the accumulation of Ca, Fe, and Zn in the roots are unexpected (due to competition in the case of Ca, and the interconnection between the acquisition pathways for Fe and Zn) **(Zaouali *et al.*, 2020)**. Nevertheless, the increase in root Ca could be regarded as an adaptive strategy to counteract the Cu-induced oxidative burst which activates the Ca-influx channels leading thereby to the activation of a caspase-like driven programmed cell death and ultimately to the inhibition of root elongation **(Printz *et al*. 2016)**. Hence, low-Ca plants exhibited higher Cu concentration in the roots compared to control plants both with the highest dose of Cu in the nutrient solution, enhancing damages caused by the metal toxicity, as confirmed by the decrease in internal CO_2 concentration (C_i), electron transport rate (ETR) and nitrate reductase (NRase) activity **(Hippler *et al.*, 2018)**. In this way, excess Cu can alter mainly the ability of plants to assimilate N in the form of nitrate **(Ademar *et al.*, 2018)**. The reduced content of Mg in roots could be responsible for the inhibition of root growth and represents a further piece of evidence to the susceptibility of root growth to Cu stress **(Guo *et al*. 2016).** Roots are also major metabolic sinks for carbon acquired in terrestrial net primary productivity **(Ogden *et al.*, 2020)**.

It has been proved that excess Cu caused ultrastructural damage to the cell membranes of the cytoplasm, endodermis, mitochondria, and vacuoles in the roots. Indeed, this was one of the causes of disruption and disabling of radial transport and translocation of mineral nutrients from roots **(Minkina *et al*., 2019)**. A distorted or broken cell nucleus, damaged organelles, rough cell wall, and large size vacuoles were observed under metal stress conditions **(Li *et al*., 2018b)**. Especially, it has been demonstrated that copper-induced DNA damage in root nuclei of tomato seedlings **(Işeri et *al*., 2018)**. Furthermore, the malondialdehyde (MDA) content of the CK2 (300 µmol L^{-1} $CuSO_4$) seedlings increased by 40% compared with that of the control seedlings (CK1). The root vigor of the CK2 seedlings was reduced by 69% relative to that of the CK1 seedlings **(Hu *et al*., 2020)**. Besides, Cu deficiency altered root architecture (RA) with more lateral root (LR) emergence from the main cord roots compared with that of control **(Patel *et al*., 2019)**.

Our results could be also more explained by discussing the role of phytohormones as controller factors of root growth and development; for instance, jasmonic acid (JA) can inhibit plant roots by inhibiting root cell elongation and cell division **(Liu *et al*., 2018c)**. Indole-3-acetic acid (IAA) was significantly increased concerning the concentration gradient of Cu toxicity **(Kang *et al*., 2017)**. However, in the tomato plant, it has been indicated that the contents of bioactive gibberellins (GA_4) were significantly decreased relative to the concentration gradient of Cu toxicity. Thus, the slight increase observed at 300 ppm Cu, in the current investigation might be also attributed to the regulation of these phytohormones.

Particularly, soluble sugar content could also participate in root development. It has been observed that trehalose biosynthesis genes, 6 trehalose phosphate synthases (TPSs), were differentially regulated during root growth **(Ogden *et al*., 2020)**. As important regulators of heavy metal stress, plant micro RNAs (miRNAs) control growth, development, and stress tolerance **(Patel *et al*., 2019)**.

The trend of increasing and the reducing in root length of *Lycopersicon esculentum* Mill., observed in this study, could be argued by the regulation of transcription factors (TF) families, as important factors involved in specific phases of rooting, and possibly in the regulation of transition to plant reproductive stages. The trend of WRKY TFs upregulation from leaf development stage (T1) to the early-tillering stage (T4) indicated that this family of transcription factors (TFs) could play a positive role in accelerating root growth, and further suggested their possible involvements in lateral root formation **(Ogden *et al*., 2020)**.

Effect of Zinc

The current study showed a reduction in root length of *Lycopersicon esculentum* Mill. under zinc stress. This decrease was depending on the elevated zinc doses in the soil.

Our results consistent well with previous findings, which reported that the root and shoot length of the tomato plant gets reduced, and also the total area of leaves and their morphology gets altered by zinc toxicity **(Emamverdian *et al*., 2015 ; Vijayarengan and Mahalakshmi, 2013).**

All the morphological observations could be relevant to many factors including the zinc concentration, duration of exposure to the metal, species sensibility, stage development, formula product, metal depending;

A kind of reduction in primary and lateral root length has been observed in 16 days old seedlings of *Brassica napus* treated with 50, 150, and 300 l M $ZnSO_4$ **(Feigl *et al*., 2015)**. These results, corroborate our findings which indicated that root length was reduced at a low concentration of zinc (100 ppm). In contrast, **Guo *et al*. (2020)** showed that the root length, root surface area, root volume, and numbers of root tips of both populations of *Hylotelephium spectabile* were severely suppressed under high levels of Cd or Zn, while no significant inhibition was found with low levels of Cd or Zn. On the other hand, He also revealed that root growth was inhibited in both populations by the addition of 20 mg. L^{-1} Zn under Cd stress, while 10 mg. L^{-1} Zn showed little impact on the root growth inhibition of HB2 (population).

Notably, it has been proved that all the studies described in the previous research were based on the negative plant growth with increasing concentration of Zn and suggested the high levels of Zn develop the first visible symptoms like retardation of root growth which is due to abnormality cell division **(Rout *et al*., 2019)**. **Ahmed *et al*. (2017)** distinctly demonstrated that the dose-dependent inhibition of *Allium cepa* root length and root cell viability upon exposure to ZnO-NPs under in vitro conditions, which suggests induced phytotoxicity and oxidative stress at the cellular level. These reports confirmed the toxic effect of zinc on the *Lycopersicon esculentum* Mill. at high concentration in our study. Conversely, **Shivaji and Dronamaraju (2019)** showed that Zn treated cultures of *Scenedesmus rotundus* tolerated the heavy metal up to the highest concentration of 1.21 mM studied, however a dose-dependent reduction in growth was observed.

The zinc formula can also have a positive effect on root growth. This was proved by a study conducted by **Haider *et al*. (2020)**, who declared that Zn application methods enhanced root length as compared with no Zn application. The genotype NM-92 attained the highest root length with combined Zn application as osmopriming + foliar application + soil application. The genotype NM-2006 sown without Zn application showed lesser root length.

Compared to the control, in our study, the positive effect on root growth might be induced by zinc at a concentration inferior to 100 ppm, so, further research needs to be done to estimate this dose.

Results obtained in the current study could be justified by many reports published recently, which defined the main mechanism triggered by zinc stress, including the ultrastructural damages, genotoxicity, cytotoxicity, and nutrient elements, oxidative stress, phytohormone pathway, etc;

$ZnSO_4$ is beneficial for plants and is required up to a certain threshold level but causing oxidative stress in a higher dosage which is evident from the phenotypic and biochemical alterations **(Pramanick *et al*., 2017)**.

The diminish of growth parameters during nutritional stress is due to low water potential, irregularity in symplastic, or apoplastic uptake of nutrients, and secondary stress such as oxidative stress **(Rout *et al*., 2019)**, these observations were also confirmed by **Rucińska-Sobkowiak (2016)**. Additionally, it has been demonstrated that 60 µM Zn treatment caused a large increase in Fe accumulation in the roots of both populations of *Verbascum thapsus* (from a zinc-contaminated site MET1) and *Verbascum lychnitis* population (from an open-cast copper mine MET2) **(Morina *et al*., 2016)**.

Phytotoxicity resulted in our study was depending on the zinc doses, as well as the duration of exposure to the metal. These observations were examined by the changes in cell count recorded at regular intervals up to a period of 24 days that revealed a concentration-dependent inhibition in growth under zinc **(Shivaji and Dronamaraju, 2019)**. By contrast, better root growth due to Zn application was due to Zn involvement in the stimulation of several enzymes in the roots and plant body **(Rehman *et al*., 2019a ; Singh *et al*., 2019 ; Shojaei and Makarian, 2015 ; Ullah *et al*., 2019 a)**. Similarly, it has been proved that the increase in Zn supply improved the root growth **(Rehman *et al*., 2018)**. According to **Guo *et al*. (2020)**, the root growth was more severely affected by metal stress than aerial parts, which is disagrees with our findings. Besides, the resupplied Zn via root autophagy seems to be locally used in the root to alleviate the inhibition of root growth by Zn starvation. The phenotype of NahG atg5/NahG plant also suggested that resupplied Zn via autophagy in root is unlikely to be remobilized to leaves and cannot suppress Fe uptake/translocation under Zn **(Shinozaki *et al*., 2020b)**.

After harvesting, the symptoms and the various observations on the primary, lateral roots, root tip, and root cap of *Lycopersicon esculentum* Mill. were marked as follows: a diminish in primary and lateral roots, root thickening with brown color, compared to the control seedlings. In fact, according to **Shivaji and Dronamaraju (2019)** marked changes in the architecture of the cell wall were observed in both treatments of zinc or cadmium.

It has been demonstrated that bulbs of *Allium cepa* exposed to ZnO-NPs for 12 h exhibited a significant decrease in percentage mitotic index and increase in chromosomal aberrations (18 ± 7.6%), in a dose-dependent manner. Moreover, cells at each stage were scored for chromosomal aberrations (CA) such as irregular prophase, vacuolated nucleus at prophase, stickiness, and disorientation at metaphase, polar deviation at anaphase, chromosome bridges with lag, multipolar anaphase, and vagrant chromosomes. The cells exhibiting major chromosomal damage were quantified and genotoxicity was represented in terms of mitotic index (MI) and % CAs, as a function of the concentration of ZnO-NPs, ZnO-Bulk, and Zn^{2+} ions **(Ahmed *et al*., 2017)**. In the same context, another study conducted by **Reis *et al*. (2018)** showed that the number of counted cells ranged from 4387 in control root-tips to 6183 in priming with 2 mg. L^{-1}Zn. Therefore, it was found statistically significant differences for the average number of cells in prophase and telophase cells among the seed priming treatments. Globally, it was verified a trend of increase of abnormal prophase and metaphase cells with the augment of Fe or Zn concentrations in the single micronutrient treatments.

It has been indicated that analysis of root tissues demonstrated symmetric and asymmetric P=O stretching of $>PO_2^-$ at 1240 cm^{-1} and stretching of C-O ribose at 1060 cm^{-1}, suggestive of nuclear damage. Overall, the results elucidated *Allium cepa*, as a good model for assessment of cytotoxicity and oxidative DNA damage with ZnO-NPs and Zn^{2+} in plants **(Ahmed *et al*., 2017)**. Furthermore, an excess of the GLV6/RGF8 peptide disrupts these initial asymmetric cell divisions, resulting in more symmetric divisions and the failure to achieve lateral root organogenesis **(Fernandez *et al*., 2020)**.

Zinc at different levels induce also oxidative stress. Notably, it has been indicated that Zn treatment led to an increase in the activity of catalase and superoxide dismutase and replete glutathione (GSH) pools **(Shivaji and Dronamaraju, 2019)**.

Phytotoxicity was also based on the metal-dependent. The findings described above by **Guo *et al*. (2020)**, confirm that zinc (essential element) and cadmium (a toxic metal) caused a toxic effect; but, cadmium appeared more toxic than zinc. In the current study, the reason why copper and zinc act differently on root elongation might be argued by **Morina *et al*. (2016)**, who demonstrated that root tips were more susceptible to Cu than Zn; Zn induced higher ROS accumulation compared to control plants (about 3- and 5-fold at 60 and 100 µM Zn, respectively), while Cu-induced increase in ROS accumulation was about 3-fold compared to controls.

As growth regulators, phytohormones play a crucial role in root growth and development. That's why, as team research lab directed by professors **Reguieg Yassad and Bülent Topcuoglu**, we hypothesis that zinc and its effect elucidated in our study act on the root's gene regulation of tomato (*Lycopersicon esculentum* Mill.) to reduce its elongation. In hence, all the explanations given below might help to understand which phytohormone could be involved in this regulation to confirm our hypothesis.

It has been shown that auxin and cytokinin converge at the regulation of *SYNERGISTIC ON AUXIN ANDCYTOKININ 1* (*SYAC1*), encoding for a protein of unknown function. SYAC1 is a component of the secretory pathway and when overexpressed can impact the cell wall composition. Modulation of SYAC1 activity affects the growth of plant organs such as roots, hypocotyls, and interferes with apical hook development. Intriguingly, in the zone of differentiation and rapid elongation, *SYAC1* expression is dependent on the simultaneous action of both hormonal pathways. In the roots of germinating seedlings, *SYAC1* expression remains low but can increase when the levels of auxin and cytokinin rise. This indicates that under optimal conditions, the expression of *SYAC1* is suppressed and thus does not limit root growth. However, in heterogeneous soil environments roots might be challenged by various abiotic stresses such as excess of heavy metals which have been shown to affect the auxin–cytokinin balance and thereby modulate root growth and development. SYAC1 might be a downstream effector by which these stresses lead to decreased root growth **(Hurný *et al.*, 2020)**.

In brief, copper, and zinc improve the root growth of the tomato plant; but, at the excess level, they reduced significantly the growth. This effect was depending on many factors including the dose and the type of heavy metal supplied.

VIII.1.3 Number of leaves

It was found a reduce in the number of leaves in tomato seedlings under copper and zinc treatments. Exceptionally, a slight increase at 300 ppm Cu was observed.

These results agree with the previous findings. It has been indicted that the presence of $ZnSO_4$ and $CuSO_4$ in Murashige and Skoog medium affected severely many growth parameters (shoot length, number of roots and leaves, and fresh weight) of both *Solanum nigrum* and *Solanum lycopersicum* at high levels. On the other hand, $CdCl_2$ significantly reduced most of the studied growth parameters for both species. *S. nigrum* exhibited higher tolerance than *S. lycopersicum* for all types of stress **(Khateeb and Qwasemeh, 2014).**

Similarly, **Baroni-nezhad *et al*. (2021),** showed that the morphological related traits such as plant height, lateral branch number, leaf number, leaf area index were reduced in *Stevia rebaudiana* by the treatment of copper and zinc. The limited number of leaves under heavy metals might be attributed to either a reduction in the number of cells.

Inversely, it has been show that the application of nano-zinc and nano-copper levels (0,1, 2 g. L^{-1}), (0,2,3 g. L^{-1}) and (0, 0.5,1 g. L^{-1}) sprayed twice increased the number of leaves increased per transplant, on some vegetative growth **(Al-Janabi *et al*., 2021).**

VIII.1.4 Biomass

The high plant biomass has the first prerequisite for high plant yield. The biomass was mainly based on the growth performance of a particular plant **(Adrees *et al.*, 2015)**. If plant biomass is increased, their net capacity to extract metals from soil is also improved; hence, better growth of plants greatly improves phytoremediation efficiency **(Jian *et al.*, 2019)**. The majority of reports emphasize the negative effects leading to biomass decline **(Adamczyk-Szabela *et al.*, 2019)**.

- **Effect of copper**

a. Fresh weight

As shown in the present study, different concentrations of copper significantly reduced the fresh weight of leaves and roots of *Lycopersicon eculentum* Mill. Remarkably, as copper doses in the soil increased consequently both weights increased.

Our results corroborate the findings of **Saleem *et al.* (2020b),** who confirmed that plant fresh and dry biomass was affected due to the high concentration of Cu in the nutrient solution. The fresh and dry biomass reduced by 14% and 17% respectively at 60 µmol. L^{-1}, 33%, and 40% at 120 µmol. L^{-1} and 78% and 75% at 180 µmol. L^{-1} compared to the control. This reduction in biomass under copper was also observed by **Carvalho *et al.* (2018)** in tomato, **Zaouali *et al.* (2020)** in *Coriandrum sativum* L., **Shen *et al.* (2019)** with combination treatment of Pb and Cu in *Kandelia obovata.*

Our results indicated that the biomass was significantly decreased when the Cu concentration increased, these findings are similar to those of **Hak *et al.* (2020)**. Besides, it was also found that using copper at 300 ppm induced a little increase in fresh leave and root biomass of tomato seedlings, after that, it decreased; these results much well with the findings of **Huang *et al.*, (2020b)**, who proved that in *Citrus sinensis* the biomass remained little changed as Cu concentration in the nutrient solution elevated from 0.5 to 300 (200) µM, then declined at 400 (300–400) µM Cu.

Under heavy metal stress, plant biomass could be influenced by many factors as, metal concentration, organ, plant status (fresh or dry), copper formulation, the application method (by root and foliar), development stage, genotype, plant species sensibility, **duration of application, and the type of metal, etc;**

Depending on the organ, it was found that leaf biomass was highly affected by copper compared to the roots, for both statuses (fresh or dry). This is in good agreement with **Peng *et al.* (2020)**, who demonstrated that the total biomass, root biomass, shoot, and leaf biomass, also the root/shoot ratio decreased significantly in response to Cu in *Populus yunnanensis*. Nevertheless, our results showed that the root biomass was lower than leaves, in control as in treated seedlings. Similarly, **Du *et al*. (2018)** revealed that μCu at 50 mg. kg^{-1} significantly decreased root biomass and length by 48.6 % and 20.5 %, respectively. Moreover, Cu treatments decreased shoot biomass by 21.6–58.5%, when compared to the control.

For Cd, and Cu the highest cumulative percentage (CPC) was for the root fresh weight (23, and 19% respectively), indicating that root fresh weight was significantly reduced by the uptake of these metals **(Khan *et al*., 2019)**. These findings support ours and the cumulative content justifies the negative effect of copper on the biomass. Additionally, the decline in our biomass disagrees with that observed by **Adisa (2019)**; Despite this, his findings can assist to get an insight into the enhancement of tomato biomass and support at the same time the beneficial effect of 300 ppm Cu obtained in our study. Adisa showed that tomato seedlings were exposed to copper oxide nanoparticles (nCuO at 250 or 500 mg. L^{-1}, by root and foliar treatments), $CuSO_4$ (25 or 50 mg. L^{-1}, by foliar treatment). Biomass percentage was found to be increased in Cu stress treatment as compared to the control, and the highest increase was seen in 5 mM Cu stress, while root exposure to nCuO at 500 mg kg^{-1} increased shoot fresh weight by 18%. Root fresh weight increased in plants exposed to foliar treatment with nCuO at 250 mg. L^{-1} (36%), and root exposure at 250 and 500 mg. kg^{-1} by 33%, compared with untreated infected control. Overall, nCuO improved the chlorophyll content, increased plant biomass, and improve the defense mechanism against the pathogen. So, the copper formulation given as a nanoparticle or as a copper sulfate have an impact on the plant biomass; as well as the application method (foliar or root application) where the plant can absorb easily the copper from leaves compared to that uptake it by roots, root application was used in our study.

According to the plant species sensibility to heavy metal, the tolerant tomato genotype showed less reduction in growth **(Amjad *et al*., 2019b)**. This observation emphasizes the interest in using the Saint Pierre variety in our experiment. Despite this, this variety still sensible compared to the other genotypes used previously, which justifies all the differences in the results.

b. Dry weight

The current study showed the reduction of leaves and roots dry weight of *Lycopersicon esculentum* Mill. under copper stress. Our results presented that the dry biomass was significantly decreased with the highest Cu concentration.

Our results are in line with the findings of **Walter *et al*. (2020)**, who noted that root dry weight decreased with increased Cu doses applied to the soil. However, dry weights of leaves and twigs of the new part were not affected by the Cu application to the soil. Our results also confirm the previous findings suggested by **Shams *et al*. (2019).** Besides, various studies showed that the severe negative effect on tomato dry biomass(for leaves and roots) was induced by a high content of copper, this result was also proved by **Hippler *et al*. (2018)** who indicated that the highest level of copper sulfate (400 µM) decreased root dry matter by 79% as compared to the control plants. Additionally, plants exposed to 200 and 400 µM of $CuSO_4$ showed a reduction of 34.94 and 32.23 g of shoot dry matter weight compared to the non-treated plants, respectively. The highest reduction in shoot dry matter (13%) was observed in the highest copper concentration (400 µM of $CuSO_4$) in comparison to the control plants. Similarly, our findings are also in agreement with **Li *et al*. (2018b)** who reported significant reductions in the biomass of Cu-treated plants of *Brassica napus* L.

Remarkably, it was found that copper acts highly differently on leaves compared to the roots either for fresh or dry weight of *Lycopersicon esculentum* Mill. **Wang *et al*. (2017)** found that the shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight of Cu-treated seedlings were significantly reduced by 9.2%, 16.9%, 47.2%, and 14.8%, respectively, compared with those of the control. Furthermore, the fresh weight of the Cu-stressed seedlings decreased by 33 and 65%, whereas dry weight decreased by 17 and 39% at days 4 and 7, respectively, compared with control **(Mostofa *et al*., 2015)**. These results are similar to those observed by **Saleem *et al*. (2019b)**. Examining all these data, we can confirm that the duration of application, organ, and plant status (dry or fresh weight) play a crucial role in evaluating the metal effects on plants.

Similarly, it has been demonstrated that the biomass was reduced depending on the duration of application, compared with the control, 10 μmol. L^{-1} Cu treatments for 15 days inhibited seedling's growth and significantly decreased the height and dry weight of shoots **(Huo *et al*., 2020)**. In contrast to our results, and depending on the type of metal, the highest decline in dry matter yield was recorded in tomato under the exposure of 220 ppm of Cd (74.9 %) followed by copper (62.4 %) at the same concentration **(Baruah *et al*., 2019)**. These findings point out that copper as an essential element was less toxic than cadmium as toxic metal.

Inversely to the decline obtained at 100 ppm Cu, the surprising increase at 300 ppm Cu, in dry or fresh biomass of leaves and roots recorded in our study, was confirmed by the earlier study of **Nazir *et al*. (2019),** who demonstrated that the plants exposed to lower Cu dose (10mg Cu. kg^{-1} soil) showed subtle increase in the growth biomarkers, whereas the plants exposed to

Cu (100 mg. kg^{-1} soil) had significant reduction of 27.52 % (shoot length), 31.88 % (root length), 28.91 % (shoot fresh mass), 29.48 % (root fresh mass), 28.63 % (shoot dry mass), 29.32% (root dry mass) and 30.92 % (leaf area) respectively, compared to control. Remarkably, this study also states the hypothesis of trend growth parameters citing along of the present discussion; Where, copper at different concentrations affect negatively the biomass as well as the length root, length shoot, leaf area, and the number of leaves.

In contrast to our outcomes, some plant species did not present any severe effect even at a high level of copper, this may be depending on the plant responses against copper stress. According to the results of **Saleem *et al*. (2020a)**, *Corchorus capsularis* L. showed a considerable tolerance of up to 300 mg. kg^{-1} Cu without significant decreases in plant growth or biomass. A similar increase at 400 mg. kg^{-1} was noted by **Amin *et al*. (2019)**.

Effect of zinc

a. Fresh weight

In the present research, the application of zinc reduced significantly the fresh weight of leaves and roots of *Lycopersicon esculentum* Mill. This decrease was proportional to the elevation of zinc concentration in the soil.

Our results are in good agreement with the findings of **Chen *et al*. (2019b)**, who found that no difference in biomass between 100 mg. kg^{-1} Zn only treatment (22.497 g. $plant^{-1}$) and the control, but the biomass in 500 mg. kg^{-1} Zn only treatment was 21.314 g. $plant^{-1}$, which was lower than the control. Similar results were also obtained by **Hak *et al*. (2020)** under copper and zinc in *Ceratophyllum demersum*. By contrast, **Adamczyk-szabela *et al*. (2020)** found that the highest good quality plants were observed in samples subjected to 300 µg. g^{-1} Zn supplementation; likewise, they also reported that the biomass increased as compared to the control sample.

Depending on the plant species, significant differences were recorded in the shoot and root biomasses between the two populations **(Guo *et al*., 2020).**

b. Dry weight

It was found that increasing zinc doses in the soil decreased consequently the dry weight of leaves and roots of *Lycopersicon esculentum* Mill.

Similar to our results, **Min *et al*. (2020)** showed that compared with healthy plants, the dry weights of tomato seedlings significantly decreased (81 % for shoot and 66.7 % for root). By, contrast, at 400 mg. kg^{-1} Zn, the dry weights of tomato seedlings shoots and roots 5.7 and 1.5 times more than those of the control. Furthermore, **Hatam *et al*. (2020)** found that the highest dry weight was observed at 100 kg. Zn ha^{-1} where this upsurge was equal to 32 % compared to Zn control, whereas 50 kg. Zn ha^{-1} significantly decreased the Root/Shoot ratio by 23 % and higher concentration did not affect this ratio compared to the control. **Aldoobie *et al.* (2013)** found a reduction in root fresh weights, shoot and root dry weights, number of leaves, and leaf fresh weight.

Depending on the duration of application, it has been demonstrated that the decreasing trends of growth parameters were noticed under Zn excess both in day 7 and day 14 treated plants **(Rout *et al.*, 2019)**.

Interestingly, the effects of copper and zinc observed in our study distinguished from those provided in the literature. These elements remain stable and are incorporated into the formulation of agriculture products such as pesticides, nutritional products, etc. In this case, zinc and copper are used separately or in combination; so, it is important to highlight the interaction risks. Therefore, to confirm our hypothesis I suggest referring to some recently supports; where, **Tiecher *et al.* (2017)** revealed a reduction in dry matter production of the shoots and roots upon addition of Zn doses applied to the soil for the three Cu doses. Treatments without Cu addition showed the highest dry matter production of the shoots and roots. There was no interaction between Cu and Zn in dry matter production of the roots and shoots of young grapevine plants.

Additionally, as team research, we are also interested in the formulations using nanoparticles as a novel technology widely used, this to reinforce the future investigations and improve the biomass quality. So, in this context, depending on the metal formulation, **Ashraf *et al.* (2020)** disagree with our results and showed that the greatest fresh (32.57 g) and dry (10.85) weight of basil aerial parts were observed in 4000 ppm Zn NPs + 2000 ppm Cu NPS treatment.

All the results obtained in this study have been explained by many scientific reports. The heavy metal effects observed in tomato biomass were attributed to the alterations marked at different levels; including, the physiological, biometrical, biochemical, and chemical. Thus, we summarized all the effects upon the following parameters, growth, genotoxicity, photosynthesis, oxidative stress, membrane, ultrastructural damages, essential elements;

Growth inhibition and reduction of biomass production are general responses of plants to metal toxicity, which results due to an inhibition in elongation and division of cells by metal addition **(Azmat and Riaz, 2012)**. Disruption in some vital processes (transpiration and photosynthesis, respiration, and enzyme activity,…) could contribute to the biomass reduction under metal. This suggestion was confirmed by **Hippler *et al*. (2018)** who demonstrated that copper absorption was greater with increased Cu concentration in the nutrient solution, which reduced plant biomass accumulation, gas exchange measurements, the activity of nitrate reductase, and affected Cu partitioning between roots and shoots.

Also, prolonged Cu stress resulted in a decrease in the overall growth of rice seedlings, perhaps by disturbing the functions of Cu-containing proteins and the cell metabolism of plants, which was attributed to Cu-induced phytotoxicity **(Mostofa *et al*., 2015**). Particularly, it has appeared that the biomass reduction was not associated with Cu ion release or uptake/translocation because plants exposed to 50 mg. kg^{-1} showed more severe adverse effects on a shoot or root growth **(Du *et al*., 2018)**. Similarly, lower dry matter yield recorded in all the treatments compared to control revealed the toxic effect of applied concentrations of metals on the tested crops **(Baruah *et al*., 2019)**.

By contrast, it has been indicated that the higher leaves, shoots, stems, and roots dry matter production of plants cultivated in soil with low Zn rates is probably justified because soil Zn content was only sufficient to supply plant demand since excess of such elements leads to toxicity **(Tiecher *et al*., 2017)**. Moreover, the observed decrease in dry weight caused by the inhibitors may result from decreased leaf area **(Higashide *et al*., 2014)**. So, such fact indicates that high heavy metal concentration, such as Cu and Zn, potentiate oxidative stress damage, phytotoxicity, and DNA damage, resulting in lower photosynthetic rates and total plant biomass reduction **(Hammerschmitt *et al*., 2020 ; Liu *et al*., 2018a ; Nanda and Agrawal, 2018 ; İşeri *et al*., 2018)**.

These decrease in biomass at low and high concentrations may be due to the cell water status, either Cu-starvation or Cu-toxicity significantly reduced the net photosynthetic rate, stomatal conductance, and transpiration rate resulting in reduced water use efficiency **(Zaouali *et al*., 2020)**. Biomass reduction was also explained by the damages at the molecular level; where excess Cu in the soil causes alterations in DNA and cell membrane integrity, ultimately reducing plant growth and biomass **(Ballabio *et al*., 2018 ; Celis-Plá *et al*., 2018 ; Li *et al*., 2018b ; Rehman *et al*., 2019 b)**. Similar effects of copper and zinc were also noted by previous reports **(Ashraf *et al*., 2020 ; Mossa *et al*., 2020)**.

By contrast, exposure to zinc during micropropagation enhanced the plant biomass **(Kumar *et al*., 2019).** Remarkably, plant metal uptake at harvest showed a significant negative correlation with dry matter yield and tolerance index **(Baruah *et al*., 2019)**. This indice will be discussed in the section tolerance and phytotoxicity.

On the other hand, the reduction in biomass was also attributed to the damage in the membrane, as demonstrated by **Peng *et al*. (2020)** who evaluated the membrane lipid peroxidation by esteeming the level of MDA. He found that the content of malondialdehyde (MDA) significantly increased under Cu stress. Additionally, copper-exposed plants showed an accumulation of free radicals and lipid peroxidation **(Yadav *et al*., 2018)**. Similar results were noted in tomato plants by **Zhou *et al*. (2019b)**. In particular, depending on the tissue, excess Cu in the nutrient solution ameliorates contents of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and electrolyte leakage (EL) which showed that Cu induced oxidative damage in the roots and leaves of *Hibiscus cannabinus* L. **(Saleem *et al*., 2020b)**. Furthermore, in the roots of copper mine plants, the membrane integrity was preserved up to 4 h, and the degree of electrolyte leakage was about 20 % in the highest Zn treatment, and about 25 % in the highest Cu treatment **(Morina *et al*., 2016)**. Phytotoxicity of Cu causes membrane damage through the binding of Cu to the sulfhydryl groups of membrane proteins **(Saleem *et al*., 2020a)**.

According to previous studies, structural analysis was argued upon the decline in tomato biomass under copper stress. It has been indicated that the cell ultrastructure of spinach seedlings substantially changed with the increases of $CuSO_4$ treated concentrations. The chloroplast in leaf cells became rounder, the chloroplast membrane became thinner, the stroma and basal granule layer became less, and the layer accumulation height decreased. The nucleus was broken up and small black spots were found in vacuoles and cell walls, which might be attributed to the enhancement of intracellular swelling pressure caused by high accumulation of Cu^{2+} **(Gong *et al*., 2019b)**. Consequently, all these damages resulting in the reduction of biomass as well as other growth parameters. According to **Huang *et al*. (2017)**, the castor bean exhibited a strong tolerance to Cu, which accumulated mainly in the cell wall. This way we can observe great biomass at a high level of copper.

It has been indicated that the reduction in biomass may be due to the imbalance in mineral elements. **Chrysargyris *et al*. (2019)** showed that the increased Cu content in nutrient solution affected mineral accumulation in the upper part (shoots and leaves) and roots of spearmint plants. Exposure to Cu increased Ca (up to 119.4 %), Mg (up to 19.0 %), and Cu (up to 30.5 %) content but decreased K, N, P, and Zn content. In addition, the copper treatment at either 20 $\mu g.\ g^{-1}$ or 100 $\mu g.\ g^{-1}$ had a significant impact on the Mn, Pb, Cd, and Zn content in roots only **(Adamczyk-Szabela *et al*., 2019).**

Ademar *et al*. (2018) proved that the high concentrations of Cu can also cause effects on nitrogen (N) metabolism. Similarly, it has been shown that when the $CuSO_4$ concentrations exceeded 600 mg. L^{-1}, foliar N concentration increased while that of P, K, Ca Mg, and Fe decreased. The concentrations of N, P, K, Ca, Mg and Fe in roots declined **(Gong *et al*., 2019)**. This explains the greatest biomass of leaves compared to that of roots illustrated in our study.

Conversely, iron plays an important role in enhancing plant biomass as evidenced by the significant increase of biomass in roots, stems, and leaves **(Wei *et al*., 2018)**. So, the elevated in root and leaf biomass at 300 ppm Cu observed in our study might be because copper increased highly the iron level in both tissues; Evidently, these results were also confirmed by those mentioned above by **Gong *et al's*. (2019)** on the decline of iron by copper. Noteworthy, higher levels of Cu taken up either by roots or leaves increased Fe concentration in the root tissue **(Walter *et al*., 2018)**. These data also justify the negative effect recorded in the root's biomass.

Differently, the highest Zn and Cu concentrations in the nutrient solution induced Ca and Fe accumulation in the roots, at the same time causing Ca, Fe, and Mn deficiency in the leaves which may result in chlorosis. This could be explained by Fe competition with Zn and Cu for loading into the xylem **(Morina *et al*., 2016)**. This way we found that leaves were more affected than leaves. Nevertheless, the remarkable increase at 300 ppm Cu was also argued by **Mallhi *et al*. (2019)**, who suggested that improvement in plant growth and biomass under heavy metal stress condition might be due to the chelating role of citric acid (CA), which helps to increase nutrient uptake by the plant **(Parveen *et al*., 2020)**.

On the other hand, **Zaouali *et al*. (2020)** demonstrated that the contents of C and H were virtually unaltered in control and stressed plants, whereas the N content increased remarkably with increasing Cu concentrations in both roots and shoots. Considering the drastic decrease in root and shoot biomasses and the insignificant changes of C and H contents in both plant parts, the hypothesis that a part of C and H was lost through root exudation could make sense.

Overall, *Lycopersicon esculentum* Mill. exposed to heavy metals stress (copper and zinc) exhibited dry and fresh biomass substantially reduced. This depending on many factors, intern or extern, the metal (copper or zinc) and its concentration applied (0, 100, 200, 300, 400, 500 ppm).

VIII.1.5 Leaf area

Leaves are an active border of exchanging energy, carbon, and water in between plants, canopies, and the atmosphere. These verdurous organs help to feed plants concerning photosynthesis and evapotranspiration **(Ghadami Firouzabadi *et al*., 2015)**. Plants have evolved several different strategies to counter stress conditions including root exploration, water conservation, osmotic adjustment, root-soil isolation, leaf orientation, root to shoot ratio, stress recovery **(Gilbert and Medina, 2016).**

- **Effect of copper**

Copper remarkably inhibited the leaf growth of *Lycopersicon esculentum* Mill. depending on the copper increasing concentration. Differently, at 300 ppm, this element stimulated the leaf growth.

Our results are in agreement with the findings of **Walter *et al*. (2018)** who suggested that leaf area (from the second and third vegetative flushes) decreased in the plants that received the highest dose of the metal 8.0 g per plant. Moreover, the supply of the highest dose of Cu as hydroxide also reduced the leaf area of the second and third vegetative flushes compared to other Cu-foliar sprayed treatments.

Referring to previous studies, leaf area was depending on the development stage, the dose, copper formulation, and the organ. As dose depending, our results corroborate the findings of **Shams *et al*. (2019)** who indicated that the leaf area, shoot, and root dry matter significantly decreased with increasing the copper concentration in the growing medium of lettuce, however, 400 µM of copper sulfate had the highest negative impact. Copper sulfate treatment (400 µM) decreased leaf area by 63% as compared to the untreated one. Similarly, **Hamido *et al*. (2019)** showed that a higher Cu rate significantly reduced the leaf area growth by 52%. All these findings much well with our results obtained at a high concentration of copper (500 ppm).

Conversely, it was found that copper at 100 ppm could not promote the satisfying leaf growth of tomato, compared to the control; this may be due to copper deficiency. My suggestion is argued by **Timofeeva *et al*'s. (2016)** findings, where they indicated that the differences with the control were significant even though all tested concentrations of copper inhibited leaf growth of *Alisma gramineum* only at concentrations of 10, 50, and 100 mg. L^{-1}. That is to say, this research can support that copper at 300 ppm acts beneficially on the leaf growth of tomatoes.

Remarkably, it has been observed that significant changes in the structure leaves of *Hordeum sativum* plants due to exposure to Cu were not found. These plants exhibited stunted stems, reduced leaf sizes, and decreased primary and lateral root lengths **(Minkina *et al*., 2019)**. By contrast, the ratio of the transpiration surface area of the canopy to the uptake surface area of the root system (Tsa/EUsa) was not affected by Cu treatment **(Hamido *et al.*, 2019).** Unfortunately, limited research was conducted on leaf areas.

The negative effects recorded at 100 ppm as well as 500 ppm, comparatively to the positive effect at 300 ppm, might be explained by the fact that elongation of tomato leaves was depending on the nature and the role of copper within the cell, as an essential or a toxic element.

Effect of zinc

In the current study, the application of zinc at different concentrations induces a reduction in the leaf area of *Lycopersicon esculentum* Mill. Additionally, it was noted that leaf area was depending on the zinc concentration with an inversely proportional correlation.

Our results were consistent with the findings of **Vijayarengan and Mahalakshmi (2013)** who suggested that zinc treatment at all levels (except 50 and 100 mg. kg^{-1}) decreased the various growth and yield parameters such as the area of leaves of tomato plants. These results also support the finding of **Haleema *et al*. (2017);** but, it contrasts with the decrease observed at 100 ppm in our study which evokes the first toxic symptoms. In fact, not only the heavy metal concentration can affect the elongation of leaves but also the application methods, plant development stage, the duration of application, and the type of heavy metal supplied. In this way, our results have not confirmed the previous research conducted by **Haider *et al*. (2020),** who indicated that mungbean genotypes and Zn application methods had a highly significant effect on leaf area index, crop growth rate (CGR), and net assimilation rate (NAR) at both sites, etc... In that leaf area per plant and plant growth rate was increased with Zn application methods as compared with no Zn treatment. Besides, it has been demonstrated the efficacy of the prolonged foliar Zn supply could be enhanced if the ZnHN suspension is sprayed over a large leaf surface area at the peak vegetative or early flowering stage **(Heuvelink *et al*., 2005)**. Similar observations in tomatoes have been reported by various studies; leaf area was generally decreased in Cd **(Carvalho *et al*., 2018 ; Godinho *et al*., 2018 ; Rahmatizadeh *et al*., 2019).**

All the results observed in this study may be related to many factors, and the leaf area could be also regulated by various mechanisms pointed on the physiological, biometrical, biochemical, and chemical interactions such as ultrastructural damages,

phytohormones, proteins, and gene expression, signaling pathway, leaf development stage, **etc. Therefore, the reduction in leaf area has been justified by several reports;**

Leaf elongation rate (LER) is an important factor controlling plant growth and productivity. The relative importance of each cell process accounting for the variations in leaf elongation rate is also variable, depending on plant species and environmental factors. The cellular and molecular factors accounting for the genetic variation in leaf elongation are still not well understood **(Xu *et al*., 2016b)**. Morphology changes were observed for plants grown in the soil spiked with Cu **(Minkina *et al*., 2019)**. One of the reasons for the reduction in leaf area under copper stress conditions can be due to lignin accumulation in xylem cells of leaves **(Shams *et al*., 2019)**. This causes the cell wall to get thicker and harder, thus diminishing cell growth and leaf expansion by reducing its elasticity **(Garcia *et al*., 2018)**. Moreover, a decrease in leaf area rate can lead to a significant decrease of the shoot, root dry matter, and leaf dry of lettuce **(Shams *et al*., 2019)**. This result implied that the leaf enlargement was inhibited before the decrease in dry matter production and that the leaf enlargement inhibition could cause the inhibition of dry matter production **(Higashide *et al*., 2014)**. These results are in good agreement with our finding, where it was found a decrease in leaf area and the growth parameters (root length, stem length, biomass, number of leaves) of tomato seedlings. Remarkably, these findings also contribute to confirm the hypothesis suggested on the trend of growth parameters under metal stress.

It has been indicated that low-calcium plants exhibited lower leaf area even at the lowest Cu concentration compared to control ones (**Hippler *et al*., 2018)**. It is known that copper and zinc can affect calcium content.

On the other hand, further investigations proved that Cu contamination caused ultrastructural changes in the cells of the mesophyll. Plastid electron density increased significantly, as did disorganization of the thylakoid membranes. Concentrations of nutrients, such as calcium, potassium, magnesium, and manganese decreased with the increase in the copper bioaccumulation, resulting in a smaller leaf area and biomass **(Garcia *et al*., 2018)**.

Besides, most transpiration of citrus canopies occurs through stomates on leaves and since they are on the abaxial surface, the total plant leaf area can serve as transpiration surface area. Unexpectedly, it has indicated that Cu treatments did not impact the xylem water potential of the stem. That could be a result of the balanced leaf area with tree roots development reduction (**Hamido *et al*., 2019)**. This result confirms our findings.

Phytohormones are the main regulators of plant growth parameters, particularly the leaf area. According to **Xu *et al*. (2016)**, exogenous application of gibberellic acid

(GA_3) significantly enhanced leaf elongation rate (LER) while tranexamic-ethyl (TE) treatment inhibited leaf elongation due to GA_3-stimulation or TE-inhibition of cell elongation and production rate in leaves for both cultivars. Although the mechanism of the inhibition by auxin biosynthesis inhibitors and the active site of inhibitors also remain unclear, it appears that the inhibitors inhibit the leaf enlargement. Also, it has been found that L-a-aminooxy-b-phenyl propionic acid (AOPP) decreased the endogenous indole acetic acid (IAA) levels in tomato and rice seedlings and acts as an inhibitor that directly blocks auxin biosynthesis **(Higashide *et al.*, 2014)**. Thus, in the current study, reduction in leaf area occurred at 100 to 500 ppm might be attributed to the elevation of the auxin level in the cell, however, its inhibition by increasing L-a-aminooxy-b-phenyl propionic acid or the synthesis of gibberellic acid interpret the stimulation of leaf area at 300 ppm.

As team research, we can also appreciate our results by additional explanations referring to the protein's function, involved in reducing or enhancing the leaf area. Expansins were discovered two decades ago as cell wall proteins that mediate acid-induced growth by catalyzing the loosening of plant cell walls without lysis of wall polymers. Transgenic modulation of expansin expression modifies the growth and stress physiology of plants, but not always in predictable and even understandable ways **(Cosgrove, 2015)**. Moreover, expansins (EXPs) facilitate non-enzymatic cell wall loosening during several phases of plant growth and development including fruit growth, internode expansion, pollen tube growth, leaf, and root development, and abiotic stress responses **(Mayorga-Gómez and Nambeesan, 2020)**. It has been indicated that three α-expansins, one β-expansin, and three xyloglucan endotransglycosylase (XET) genes were associated with GA-stimulation of leaf elongation, of which, the differential expression of EXPA4 and EXPA7 was related to the genotypic variation in LER of two cultivars. Those differentially-expressed expansin and XET genes could play major roles in genetic variation and GA-regulated leaf elongation in tall fescue **(Xu *et al.*, 2016b)**. Remarkably, reduced the expression of several expansin genes in *Arabidopsis thaliana* using an inducible micro RNA construct and found that the decreased expansin gene expression led to a repression of leaf growth **(Goh *et al.*, 2012)**. This explanation highlighted at the molecular level justifies the reduction of leaf area observed in our study. XET is another important protein located in the cell wall that has been associated with cell elongation in various plant species **(Eklöf and Brumer, 2010).** Moreover, there are various members of expansins and XET genes, but the specific genes related to genetic variation in leaf elongation are not well documented **(Xu *et al.*, 2016b)**.

knowledge about how the plant's nutritional status can interfere with ABA biosynthesis and signaling mechanisms is necessary to optimize stress tolerance in horticultural crops **(Carrió-Seguí *et al*., 2016)**. It has been highlighted that abscisic acid (ABA) is an essential phytohormone in plant responses to heavy metal stress. That is a reason why we suggest the involvement of abscisic acid (ABA) in the elongation of leaf area under copper stress. However, few explanations have been reported on the role of ABA in the regulation of leaf area by acting on the copper transporters. **Carrió-Seguí *et al*. (2016)** pointed out that ABA plays role in the inhibition of plasma membrane copper transporters and regulates the master regulator Squamosa Promoter-Binding Protein-Like 7 (AtSPL7) and its targets.

In tomato, post-mitotic cell expansion results in an overall increase in cell volume by over 30,000-fold **(Chevalier *et al*., 2014)**. Also, in an earlier study, Expansins (EXPs) were identified from bell pepper and spatial expression patterns of CaEXPA transcripts in seedlings, root, stem, leaves, and fruit were determined **(Mayorga-Gómez and Nambeesan, 2020)**. Importantly, the genes of α-expansins of woody plants are of great interest for genetic engineering (**Kuluev *et al*., 2017).**

The leaf development stage (young or old leaves) acts as a limiting factor in determining the leaf area, which can help to justify the difference between our results and those published recently. Accordingly, the response to heavy metal stress in both types of leaves might be attributed to the gene expression. It has been indicated that the highest *PtrEXPA3* mRNA level was detected in young intensely growing aspen leaves, furthermore, expression of the gene was induced by exogenous cytokinins and auxins. In response to NaCl and constitutive expression of the *PnARGOS-LIKE* gene, the *PtrEXPA3* mRNA level decreased. Transgenic aspen plants with constitutive *PnEXPA3* expression were characterized by the decreased size of leaves, petioles, and internodes, as well as the increased size of leaf epidermal cells, while the stem size remained unchanged. Thus, the data obtained enable the suggestion that the *PtrEXPA3* and *PnEXPA3* genes encode cytokinin and auxin-regulated, leaf-specific expansins that are involved in the cell expansion **(Kuluev *et al*., 2017).** *The BLADE-ON-PETIOLE1 (BOP1)* gene was first identified as a suppressor of lamina differentiation on the petiole in Arabidopsis **(Chan *et al*., 2004 ; Ha *et al*., 2003)**. Also, **Xu *et al*. (2016a)** proposed that *OBP4* is a novel regulator of cell cycle progression and cell expansion. Here, he reported that the DOF transcription factor *OBP4* negatively regulates cell proliferation and expansion. OBP4 is a nuclear protein. Constitutive and inducible overexpression of *OBP4* reduced the cell size and number, resulting in dwarf plants. Inducible overexpression of *OBP4* in *Arabidopsis* also promoted early endocycle onset and inhibited cell expansion, while inducible overexpression of *OBP4* fused to the VP16 activation domain in *Arabidopsis* delayed endocycle onset and promoted plant growth. Furthermore, gene expression analysis showed that cell cycle regulators and cell wall expansion factors were largely down-regulated in the *OBP4* overexpression lines. After auxin (IAA) and salicylic acid (SA) treatment, *OBP4* expression in the leaves increased substantially at 3 h and remained high at 24 h, while after 8 h of GA treatment, *OBP4* expression increased only slightly. Furthermore, it has been stated that synergistic on auxin and cytokinin 1(SYAC1) is a regulator of the secretory pathway, whose enhanced activity interferes with deposition of cell wall components and can fine-tune organ growth and sensitivity to soil pathogens **(Hurný *et al.*, 2020)**. For this reason, we suggest that the elongation of leaf area could be regulated by SYAC1.

Overall, because of the limited studies on the leaf area which is affected by copper and zinc, we could not provide more details on how these elements interfere precisely in the leaf growth of *Lycopersicon esculentum* Mill.

VIII.2. Effect of copper and zinc on the biometrical parameters

VIII.2.1 Leaf relative water content (LRWC)

Water is an essential element for the plant development **(Vezza *et al*., 2017)**. Besides, relative to the water content (RWC), as the metabolically available water, could reflect the metabolic activity in plant tissues **(Yan *et al*., 2016)**. So, the water content decrease could act as a signal to activate mechanisms that allows a reduction in the loss of the water and the restoration of the water status. supporting the hypothesis that the metalloid participates in the induction of responses tending to maintain water equilibrium **(Vezza *et al*., 2017).**

Many studies have reported that the effect of the abiotic stress on the relative water content, in *Triticum aestivum* under drought stress **(Abid *et al*., 2018),** in *Lycopersicon esculentum* under drought stress **(Zhang *et al*., 2019b)**, in *Solanum tuberosum* under salinity stress **(Mahmoud *et al*., 2019)**, in *Glycine max* under arsenic **(Vezza *et al*., 2017)**, in *Triticum aestivum* under colloidal solution of copper and zinc nanoparticles **(Taran *et al*., 2017)**, in *Nicotiana benthamiana* under copper **(Ku *et al*., 2012)**,

- **Effect of copper**

The present study showed that the copper treatment contributes to the reduction in relative water content of *Lycopersicon esculentum* Mill.'s leaves. This reduction was depending on the copper concentration increase.

Compared to the previous studies, the relative water content varied according to many factors of: metal concentration, plant species, heavy metal and combination, metal formulation, duration of application, temperature, and organ;

Our results consist with the findings of **Gong *et al*. (2017)** who suggested that RWC decreased as Cu concentration increased, but for A5 (fungus) treated plants, RWC declined by around 1.7 and 3.3-fold in 100 and 200 mg. kg^{-1} Cu treated plant. With the addition of 25, 100 and 200 mg. kg^{-1} Cu, water saturation deficit (WSD) was enhanced by around 6.5 and 4.8 and 3.1-fold, respectively, in *Kandelia candel*. This reduction in RWC under copper stress was also marked by **Negazz (2019)** in *Raphanus sativus* and **khedim (2019)** in *Atriplex canescens.* Especially, in the tomato plant, the reduction in the leaf water potential has been observed in the presence of the different heavy metals stresses such as, boron **(Kaya *et al*., 2020)**, copper **(Martins and Mourato, 2006)**, and antimony Sb **(Espinosa-Vellarino *et al*., 2020)**, also under the osmotic stress **(Parvin *et al*., 2019)**. In contrast to our findings, the combined effect of the colloidal solution of nanoparticles and drought induced the increase of RWC in leaves **(Taran *et al*., 2017)**, this is confirmed by **Nguyen *et al*. (2020)** who showed that copper nanoparticle priming plants displayed enhanced drought tolerance indicated by their higher leaf water content. However, leaf RWC did not change under copper in *Zea mays* **(Cetinkaya *et al.*, 2014)**, under cadmium **(Godinho *et al.*, 2018)** and AgNPs **(Özlem Çekiç *et al.*, 2017)** in *Lycopersicum esculentum* . Similar to our results, **Shariat *et al*. (2017)** and **Gong *et al*. (2017)** found that RWC decreased when copper supply increased.

According to **Vezza *et al*. (2017),** these responses show that the reduced water absorption rate in the treated plants causes a decrease in water content at the beginning of the treatment. Similarly, at day 4 and 7, the decreases were notably drastic (32% RWC) after 7 days of copper treatment **(Mostofa *et al*., 2015)**. Indeed, the water status was affected by the presence of heavy metals in growing substrata, and also by the high temperatures during the months of May and June in glasshouse **(Bahri *et al*., 2015)**.

Differently, depending on the organ and the copper formulation, it has been demonstrated that the shoot water content was 6.9 –12.5% higher in all Cu treatments, compared with the control. However, the root water content decreased when exposed to µCu at 100 and 200 mg. kg^{-1} (by 1.8 % and 3.9%, compared to control; 3.8 % and 4.7%, compared to nCu, respectively). In addition, µCu decreased water content in shoot by 2.8 % at 100 mg kg^{-1} but increased it by 4.7 % at 200 mg. kg^{-1}, compared with nCu **(Du *et al*., 2018)**. Similar decrease of RWC in shoot has been also obtained by **Shiyab (2018).** From these reports we can conclude that the reduction in water content of leaves was also related to the decline of water level in the other plant parts (roots and shoots), which justify mainly the inhibition of the growth stem and root of tomato seedlings observed in our study.

Effect of zinc

The current study showed that different concentration of zinc substantially decreased the relative water content (RWC) in leaves of *Lycopersicon esculentum* Mill. This water level reduced as zinc doses in the soil increased.

Our results are in line with earlier findings of **Garg and Singh (2018)**, who showed that cadmium and zinc stress significantly decreased leaf relative the water content (LRWC) depending upon the concentration of heavy metals and genotype. Plants treated with Cd reduced these parameters to a greater extent than Zn. Besides, depending on the duration of application, **El and Deef (2008)** demonstrated that RWC was significantly lower in the tomato seedlings treated with Cd and Zn from the fourth day as compared with the control group. This decrease persisted up to the 12 day. The decrease was more in Cd (42.1%) than in Zn (35.2%) compared with the C group at 12 day for 10 µmol. L^{-1} treatments

Our results showed that RWC was reduced at 100 ppm Zn. By contradiction, **Alamer *et al.* (2020)** indicated that zinc-fertilizer at 100 mg. L^{-1} enhanced RWC in marigold leaves. This is depending on the plant species sensibility or growth conditions, etc.

Heavy metal stress induces a decline in the relative water content of leaves, which could be regulated by physiological and biochemical mechanisms of: water absorption, nutrient uptake, ultrastructural damages, gene regulation, proteins, and phytohormones. All these alterations and mechanisms were reported by many reviews;

An excess of metal ions in the tissues may affect water absorption from the soil, and in the turn decrease water content in the root **(Rucińska-Sobkowiak, 2016)**. Remarkably, metal-induced water deficiency, on the other hand, is a less explored process **(Vezza *et al*., 2017)**.

It has been demonstrated that nutrient status (N, P, K, Mg, Fe and Ca) decreased significantly with application of Cd and Zn stress in a concentration and genotype-dependent manner **(Garg and Singh, 2018)**. Restriction in the nutrient acquisition is one of the primary causes for reduced growth and yield in water deficient soils **(Usmani *et al*., 2020)**. Moreover, the application of Cu stress decreased Zn, N and K (leaves), K, Ca, P and Mg (roots) content, whereas copper application increased Ca and Mg in leaves **(Chrysargyris *et al*., 2019)**. For these reasons, the decline in nutrient uptake resulted in loss of leaf water status (LRWC) under Cd and Zn stresses **(Garg and Singh, 2018)**.

A visual loss of turgor correlated with the general decline in the water content (WC) and significant differences were observed between the response of the different organs **(Castañeda *et al*., 2018)**. It has been reported that water content decreased in the various organs of plants grown in solutions containing toxic levels of metal ions. Additionally, the reduction in water absorption rate induced by metals such as Cd, Ni, Pb, Cu, Co, Cr and Zn causes root and leaf dehydration in several plant species **(Rucińska-Sobkowiak, 2016)**. Consequently, a decrease in water content in the leaves and roots of As-treated plants was identified as an early stress response **(Vezza *et al*., 2017)**. It has been proved that water content of leaves and taproot (tapR) decreased around 15% under severe water deficit (SD), while fibrous roots (fibR) water content decreased 30 and 65% under invertase (MD) and severe water deficit (SD), respectively **(Castañeda *et al*., 2018)**. Furthermore, Water transport through the roots is important because hydraulic conductivity (Lp) influences the water supply for the whole plant **(Rucińska-Sobkowiak, 2016)**. Reduced plant growth and productivity under drought are caused by altered plant water relations **(Abid *et al*., 2018)**.

The Previous study conducted by **Bahri *et al*. (2015)** noted the effect of some limiting factors on the water content in the plants. He demonstrated that the degree of membrane disruption in *Paulownia plantlets*, which is represented by the electrolyte leakage, and was affected by the heavy metal (Zn, Pb and Cd) concentrations in the three substrates and by the time exposure to stress. According to **Przedpelska-Wasowicz and Wierzbicka (2011)**, Zn, Pb, Cd and Hg can change aquaporin (AQP) conductivity, leading to a decrease in membrane water permeability; aquaporin are water channels. Thus, plants respond to this situation by the storing water and limiting the rate of transpiration, resulting in higher water content in leaves **(Du *et al*., 2018)**. **Rucińska-Sobkowiak *et al*. (2013)** observed a relation between increased cell vacuolization and high RWC values in *Lupinus luteus* treated with Pb, suggesting that water can be stored in vacuoles in response to the metal in order to maintain the water status. These structural changes reduce the capacity of plants to explore the soil and increase the resistance to symplastic and apoplastic water transport **(Vezza *et al*., 2017).**

Similar to our study, other factors can have also an impact on the relative water status in the plant, and leaves, which is the concentration and osmotic stress. This is clearly confirmed by **Rucińska-Sobkowiak (2016)** who suggested that the concentrations of heavy metals in soil seldom reach a level sufficient to cause osmotic disturbances in plants. This explain the negative effect recorded at 500 ppm for zinc and copper. Recently, another study suggest that the osmotic stress-induced water shortage can lead to loss of turgidity and dehydration of cells, and ultimately, death of plants. Osmotic adjustment is a fundamental mechanism plants adopt to continue their growth under saline conditions **(Farooq *et al*., 2015)**. Moreover, it has been indicated that drought stress at both stages altered leaf water relations by decreasing leaf water potential, osmotic potential, turgor osmotic potential , and RWC but increasing osmotic adjustment (OA) as compared to WW plants **(Abid *et al*., 2018)**. Accordingly, leaf water potential and magnitude of OA was lower in the sensitive cultivar than the tolerant cultivar.

On the other hand, it has been indicated that physiological analysis revealed that miR1916 affected the osmoregulation and reactive oxygen species (ROS) accumulation. Moreover, the reduction in water potential observed in leaves of As treated plants could increase the ability to retain water within the plant and could also be a consequence of osmotic potential reduction through the accumulation of compatible solutes such as proline and soluble sugars **(Vezza *et al*., 2017)**. Thus, the accumulation of proline and malondialdehyde (MDA) were at least partially resulting from the deterioration of water status in plant leaves. It may also inhibit leaf transpiration, and thus retain more water in soil **(Tang *et al*., 2019a)**. These biochemical molecules were accumulated simultaneously as leaf water content decreased; this observation is in good agreement with our findings.

GmSYP24 was identified as a dehydration-responsive gene. GmSYP24ox alters the expression of some aquaporins under osmotic or drought, salt, or ABA treatment. Moreover, syntaxin proteins are not limited to vesicle trafficking, and they may be involved in abiotic stresses **(Chen *et al*., 2019a).**

Importantly, endogenous hormones and polyamines (PAs) could interact to regulate the growth and tolerance to the water stress in white clover. In response to water deficit, indole-3-acetic acid (IAA), gibberellic acid (GA), and cytokinins (CK) content significantly declined, but ABA content significantly increased in all treatments in spite of the application of indole-3-acetic acid (IAA) or L-2-aminooxy-3-phenylpropionic acid (L-AOPP) **(Li *et al*., 2018c)**. These data support our findings of the reduction in growth parameters and the decrease in water content. Hence, disturbance in leaf water content disrupt the plant growth.

Overall, copper and zinc play an important role in decreasing the water content in leaves of *Lycopersicon esculentum* Mill.; but, this can be ensure depending on their concentration and the different factors that may be acted positively.

VIII.2.2 Leaf relative water loss (RWL)

- **Effect of copper**

In the presence of high level of copper, we found that leaves of *Lycopersicon esculentum* Mill. Were exhibited a decrease in relative water loss RWL (or transpiration). This decline was depending on the increase of copper doses in the soil.

Relative water loss was not well documented, Accordingly, recent reports prefer studying precisely the transpiration parameters (stomatal conductance, gaz exchangen, ..) rather than the relative water loss RWL. Obviously, leaf transpiration is a vital process defined as the loss of water escaped through stomata in the form of vapor. Thus, all the explanations given below discuss the transpiration.

Our result consistent with the findings of **Parveen *et al*. (2020)** who found that Cu stress (50 and 100 µM) significantly reduced transpiration rate (T*r*), stomatal conductance and intercellular CO_2. However, these results showing that the application of calcium (Ca) to copper treated plants significant increased gaseous exchange attributes compared with the plants grown under Cu-only treatment. Similarly, **Hippler *et al*. (2018)** noted that Cu toxicity reduced the transpiration parameters up to 50 %, for plants in 0.50 µM Cu. Furthermore, low-Ca plants under Cu excess also exhibited a reduction of internal CO_2 concentration (C i) compared to the controls. In the Ca-experiment, plants that were grown with 0.01 µM Cu exhibited intermediate photosynthetic rate (P_N), stomatal conductance (g_S) and values relative to those grown at the higher Cu concentrations. This is also in agreement with the findings of **Fu *et al*. (2015)**, for copper stress. Besides, all these observations confirm that the damages occurred on the transpiration process minimise the photosynthetic rate, the reason why the chlorophyll content and RWL in our study were concurrently decreased, and more details were provided in section "discussion- chlorophyll"

In contradiction with our results, **Huang *et al*. (2020b)** demonstrated that leaf CO_2 concentration and stomatal conductance (gs) kept unchanged or increased as Cu concentration in the nutrient solution rose from 0.5 to 200 µM, then declined with further rise in Cu concentration. According to **Hamzah *et al*. (2019)**, the effects of different levels of Cu (0, 100, 200, 300, and 400 mg. kg^{-1}) on the gas exchange attributes of the plants were presented. He showed that photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (gs) decreased steadily with increasing Cu concentration in all treatments. In contrast, internal CO_2 concentration (Ci) showed non-significant results up to a concentration of 300 mg. kg^{-1} Cu, while further increases in Cu, to 400 mg. kg^{-1}, significantly reduced it. Pn was highest in the treatment without Cu and was reduced by 44.3% at 400 mg. kg^{-1} Cu. The maximum reductions in Tr and Gs were found at 400 mg. kg^{-1} Cu (23.5 % and 70.5 %, respectively) when compared to the values in the groups without Cu. However, Ci exhibited non-significant values up to 300 mg. kg^{-1} Cu but was reduced by 7.9 % compared to the control at 400 mg. kg^{-1} Cu. It has been suggested that leaf gas exchange decreased progressively to zero during drought and embolism induced loss of hydraulic conductance in petioles peaked to approximately 50 % in correspondence with strong daily limitation of stomatal conductance **(Tombesi *et al*., 2015)**.

Effect of zinc

we found that the presence of zinc in the soil significantly limited the loss of water in the leaves of *Lycopersicon esculentum* Mill. This decline was proportional to the zinc increases concentration.

Our results corroborate with the findings of **Cruz *et al*. (2019)** who demonstrated that neither the transpiration rate nor the photosynthesis and conductance to H_2O were affected during the first hour of treatment exposure. On the other hand, the measurement performed 24 hours later showed that the transpiration rate decreased whereas the photosynthetic rate was only reduced in the plants treated with $ZnSO_4$. In addition, past 48 hours of exposure, the photosynthesis of the plants treated with 40 nm ZnO also decreased. At the end of the experiment, the leaves of plants treated with 1000 mg. L^{-1} $ZnSO_4$ (aq) became wilted. Similarly, **Hatam *et al*. (2020)** indicated that the application of 50 and 100 kg Zn. ha^{-1} significantly decreased vapour pressure deficit from 7.54 in the control to 7.12 and 6.84, respectively. While the application of 50 and 100 kg Zn. ha^{-1} decreased water use efficiency by 57 and 68%, respectively. However, at 15 dSm^{-1}, only 50 kg Zn. ha^{-1} resulted in 70% increase in water use efficiency. However, when Zn was raised to 100 kg. ha^{-1}, it antagonized 50 and 100 kg K. ha^{-1}, which resulted in a decrease of water use efficiency by 72 and 58%, respectively, water use efficiency is a ratio between the photosynthesis rate and the transpiration rate. In concordance with our results, **Shen *et al*. (2019)** found that minimum value of transpiration was observed at the highest Zn concentration (100 kg. ha^{-1}) where this decline was 35% compared to the control. However, stomatal conductance (Gs) and transpiration rate (Tr), decreased with increasing concentration of treatments (except for Pb + Cu and Pb + Zn + Cu). Moreover, previous studies conducted by **Ashraf *et al*. (2020) and Chandra and Kang (2016)** confirmed that transpiration rates were significantly affected by increased heavy metals (Cd, Cr, Cu, and Zn). **Cruz *et al*. (2019)** suggested that the Zn content in the upper part of stem affects the transpiration rate more than the Zn the lower stem. The data in suggest negative correlation between the Zn content in the petiole and the transpiration rate on leaves.

Under heavy metal stress, zinc and copper, leaves of *Lycopersicon esculentum* Mill. loss a volume of water through the transpiration process. This effect was insured by involving a cascade of molecules (such as, proteins, proline, plant hormones, metabolites,…), and the biochemical and molecular mechanisms (such photosynthesis, genes expression, etc). Consequently, other negative effects could be also induced, such as deprivation of nutrient elements; So, in this context and according to previous investigations, further explanations were discussed below.

A decrease of the transpiration rate or the loss water in leaves might be due to the effect of heavy metals on the nutrient elements, this suggestion was clearly explained by **Sitko *et al*. (2019),** who demonstrated that the most significant inhibition of the transpiration rate by 15% was observed in the plants with a Ca and Mg deprivation. In addition, the decrease in stomatal conductance in the Mg- and P-deprived plants was similar and reached 79 and 76% of the control, respectively.

Importantly, stomata and guard cells, are essential elements participating in loss water and the transpiration process, should be obviously discussed in this section. It has been indicated that stomata, which are surrounded by a pair of guard cells, are the main gateways plants use to efficiently take up CO_2 for photosynthesis while simultaneously regulating water transpiration **(Qi *et al*., 2018)**. Precisely, stomatal aperture regulation is a prime example of such switches between growth and stress responses in plants **(Rosenberger and Chen, 2018)**. Abiotic stress conditions like drought, heat, or intense light as well as biotic stresses close stomata **(Devireddy *et al*., 2018 ; Tardieu, 2016)**. Especially, the toxicity caused by heavy metals can also promote stomatal closure and consequently the reduction of stomatal conductance; besides, it leads to the reduction of transpiration and decreases the loss of water from the plant to the atmosphere **(Sousa Paula *et al.*, 2015)**, these stomatal movements justify our results. On the other hand, all these responses consequently cause closure of stomata and inhibition of cell growth and photosynthesis, diminish osmotic potential of plant tissues, lower the transpiration rate, and enhance respiration rate **(Qazi *et al*., 2019)**. These observations concur well with our findings where, a simultaneous reduce was revealed in the biometrical parameters (the transpiration or water loss) and those of physiological and biochemical.

On the other hand, zinc act differently on RWL to copper, this might be depending on their toxicity. In this context, some reports demonstrate that the number of stomata might decline on account of metal toxicity. It appears evident that specific mechanisms are employed for specific metals in particular species. Stomata closure is induced by direct interaction of toxic metals with guard cells and/or as a consequence of the early effects of metal toxicity on roots and stems **(Rucińska-Sobkowiak, 2016)**. Indeed, water uptake from the soil is mainly mediated by roots **(Batool *et al*., 2018)**.

In Cu stressed plants, closed stomata with deformed guard cells were found partially open in presence of H_2O_2 **(Nazir *et al*., 2019)**. Moreover, stomatal closure resulted in reduced transpiration rate which in turn limited the translocation of Cu to the shoots via the flow of transpiration contributing thereby to its accumulation in the roots. Also, the lower Cu-translocation from roots to shoots might be attributed to the reduced transpiration rate **(Zaouali *et al*., 2020)**. It has been indicated that, the transpiration cycle in trees is powered by a negative water potential generated within the leaves, which pumps water up a dense array of xylem conduits **(Shi *et al*., 2020)**.

It was highlighted the crucial role of plant regulators for all the discussed parameters; likewise, phytohomone and the signalling pathway regulate the water loss by mediating the stomata and guard cells movement, which explain the mechanism implied by plant to close its stomata and thereby lead to reduce the water loss in leaves of tomato.

The opening and closing of stomata are controlled by the integration of environmental and endogenous signals **(Habermann *et al*., 2019)**; such as abscisic acid and apoplastic reactive oxygen species (ROS) **(Qi *et al*., 2018)**. Notably, abscisic acid (ABA) acts as a signaling hormone in plants against abiotic and abiotic stress **(Li *et al*., 2020 ; Parmar *et al*., 2019)**. The accumulation of ABA in roots is one of the fastest responses to water or drought stress. It is loaded to xylem vessels and transported to the leaf cells via the transpiration stream **(Dikilitas *et al*., 2019)**. Importantly, guard cells also harbor the machinery to produce ABA autonomously and it has been shown that this biosynthesis is sufficient to close stomata in response to decreased relative humidity **(Bauer *et al*., 2013).** It has been indicated that leaf ABA content was initially low, but then gradually increased during both root and leaf stress treatments. So in response to root stress, ABA is initially synthesized in root where it accumulates before being transported to other tissues including leaf, where it induces stomatal closure **(Hu *et al*., 2016)**. This experiment confirms the presence of a cross talk between leaves and roots of *Lycopersicon esculentum* Mill. stressed by copper and zinc, which consequently support the reason behind the diminished of root length and the root biomass as well as the leaf area, leaves biomass, and number of leaves, noted previously. Referring to **Barickman *et al*. (2019)**, who proved that the most dramatic change in nutrient concentrations was for Mg in the leaf tissue when ABA was applied to both leaf and root tissue of tomato plant. The imbalance of other nutrient elements caused also water loss, **Waidyarathne and Samarasinghe (2018)** suggested that Ca^{2+} accelerates stomatal closure by enhancing plasma membrane slowly activating anion channel SLAC1 and actin rearrangement.

According to many studies, decline in the transpiration the water loss shows also an impact on various vital processes in plant. It has been suggested that stomatal closure is expected to produce a decrease in photosynthesis due to lower CO_2 levels in mesophyll cells **(Müller *et al*., 2018)**. Furthermore, **Avinash (2017)** state that reduction in photosynthetic rate could be attributed to the Cu inhibition of stomatal conductance and photosynthetic pigment content as also observed in *Sorghum bicolour* under Cu stress. For example, leaf stomata or leaf surfaces are closed under drought stress and cell osmotic potential decreases. As a result, photosynthesis is reduced. Plants, therefore, must constantly adjust stomatal conductance to allow enough CO_2 uptake and avoid unnecessary water loss during water stress **(Dikilitas *et al*., 2019)**.

Remarkably, it has been indicated that the disturbances in water relations trigger differential regulation of aquaporin gene expression, which may contribute to further reductions in water loss **(Rucińska-Sobkowiak, 2016)**.

Conversely, an expected elevation in water loss was obtained at 400 ppm in our study; it may be explained by the involved of ethylene signal. **Zhang *et al*. (2016)** confirmed that ethylene responsive factors (ERFs) play important roles in the abiotic stress. *BpERF11* inhibits the expression of an *AtMYB61* homologous gene, resulting in increased stomatal aperture, which elevated the transpiration rate, which results in reduced proline levels and increased reactive oxygen species (ROS) accumulation. *BpERF11* also significantly inhibits the expression of *LEA* and dehydrin genes that involve in abiotic stress tolerance. Therefore, *BpERF11* serves as a transcription factor that negatively regulates salt and severe osmotic tolerance by modulating various physiological processes**.**

Interestingly, proteins are physiological parameter analysed in our study, they play a crucial role in water loss. It has been indicated that the core ABA signalling components are snf1-related protein kinase2s (SnRK2s), which are activated by ABA-triggered inhibition of type-2C protein-phosphatases (PP2Cs). SnRK2 kinases are also activated by a rapid, largely unknown, ABA-independent osmotic-stress signalling pathway **(Takahashi *et al*., 2020)**.

Thus, to sustain future generations, a great challenge is to improve crop yield and water use efficiency, which is the carbon gained per water lost **(Müller *et al*., 2018).**

Overall, in excess copper and zinc, leaves of *Lycopersicon esculentum* Mill. reduce the loss of water content by limiting the transpiration rate. Each heavy metal and its concentration have an impact on RWL. Notably, water loss regulation is related to the physiological, biometrical, morphological, and biochemical mechanisms.

VIII.3. Effect of copper and zinc on the physiological parameters

VIII.3.1 Chlorophyll and carotenoids pigments content

Photosynthetic pigments of a plant are important biological parameters in elevating of abiotic stress **(Rehman *et al.*, 2019d)**. Chlorophyll (Chl) is essential for photosynthetic reactions and chloroplast development **(Zhang *et al.*, 2020)**. On the other hand, chlorophyll is one of the primary photosynthetic pigments; any alterations to its concentration can affect the photosynthetic process, thereby reducing the plants ability to produce and store sugar, affecting crop yield **(Tamez *et al.*, 2020)**. Moreover, chlorophyll in leaves are vital to the synthesis of photosynthetic related pigments and enzymes in the Calvin cycle **(Dai *et al.*, 2017)**. Furthermore, determination of chlorophyll contents from the leaves are important biological parameter for the evaluation of plant stress **(Saleem *et al.*, 2019b)**. Notably, malfunction in gas exchange and photosynthesis are the first responses of plants to environmental stresses **(Hatam *et al.*, 2020)**. Plant-based pigments have been used as substances to improve crop yield and quality, but the mechanisms of their action on plant growth and stress tolerance are not well understood **(Zhang *et al.*, 2019b)**.

In general, alterations in the pigment content are generally considered as a measure of physiological competence **(Shivaji and Dronamaraju, 2019)**. For instance, tomato plants subjected to drought stress had higher levels of chlorophyll and carotenoids in leaves **(Zhang *et al.*, 2019b).** Thus, efficient photosynthesis, plant fitness, and grain yield are critically dependent on the dynamic regulation of chlorophyll level in response to various developmental and environmental cues **(Wang *et al.*, 2020).** Because of the crucial role of photosynthesis in the plant growth, this discussion part is well extended. Copper and zinc are essential part in the function of this process; so, depending on the specific mechanism, each metal is explained separately.

- **Effect of copper**

Several research report a decline in chl a, chl b, tchl, and carotenoids content when plant species were exposed to heavy metals stress; For instance, under copper stress **(Walter *et al.*, 2018)** in *Citrus trees*, and in *Mentha spicata* **(Chrysargyris *et al.*, 2019)**, under copper, zinc, cadmium and lead **(Kutrowska *et al.*, 2017)** in *Brassica junce*, under lead and copper **(Peng *et al.*, 2020)** in *Populus yunnanensis*, under cadmium and copper **(Adamczyk-Szabela *et al.*, 2019)** in *Melissa officinalis*, under cadmium **(Khanna *et al.*, 2019b)** in *Solanum lycopersicum*

.

As the current study showed, chlorophyll content in leaves of *Lycopersicon esculentum* Mill. exposed to copper stress was declined respectively by increasing the copper concentration in the soil. Our results are in agreement with the findings of **Saleem *et al*. (2020a)** who suggested that the most common impact of Cu stress is a decrease in photosynthetic pigments in the leaves. Additionally, Saleem *et al*'s results also revealed that high concentrations of Cu in the soil significantly affected the chlorophyll content of *Corchorus capsularis*. The maximum reduction in chlorophyll content was observed at 400 mg. kg^{-1} Cu (48 % lower than the control). The maximum chlorophyll content (2.5 mg. g^{-1} FW) was observed at 0 mg. kg^{-1} Cu while the minimum chlorophyll content (1.3 mg. g^{-1} FW) was observed at 400 mg. kg^{-1} Cu. Moreover, **Nazir *et al*. (2019)** demonstrated that the plants raised in the presence of Cu (100 mg. kg^{-1}) had significant reduction in chlorophyll content by 27.11% compared with the control.

Different variations in the set of photosynthesis pigments is relevant to many factors of, vegetative stage, copper formulation, the supply mode of metal, plant species, metal concentration, chlorophyll type (a, b, total), chlorophyll or carotenoid, plant sex;

Depending on the vegetative stage, recently, **Walter *et al*. (2018**) reported that the amount of chlorophyll in leaves of the first and second vegetative flushes reduced with the increase of Cu doses applied to the soil, but not in those grown with the third one.

In contradiction with our results, depending on copper formulation and the supply mode, chlorophyll content was elevated in tomato plants treated foliarly with nCuO at 250 (10 %), 500 (14%), and $CuSO_4$ (15%), and via root to nCuO at 500 mg. kg^{-1} (14%), compared with plant treated with Kocide 3000 **(Adisa, 2019)**. Depending on the concentration, **Zehra *et al*. (2020)** suggested that copper at higher concentrations decreased photosynthesis significantly while increased at lower concentration. Similarly, **Shams *et al*. (2019)** showed that sulfate treatment (200 µM) increased the chlorophyll content, but 400 µM of $CuSO_4$ did not have a positive effect on the chlorophyll content in leaves when compared to the plants treated with 200 µM of copper sulphate. This result is similar to the finding proposed by **Cuchiara *et al*. (2015) and Sharma *et al*. (2017)** in sweet potato and **Zhou *et al*. (2019)** in tomato. However, no changes in the final content of both chlorophylls and carotenoids were observed in both species *"Solanum cheesmaniae, Solanum lycopersicum"*. in response to 250 µmol. L^{-1} Cu **(Branco-Neves *et al*., 2017)**". These findings are similar to the results of **Du *et al*. (2018)** in *Origanum vulgare*.

Depending on the chlorophyll type, it was found an increase content in chlorophyl a, b, and tchl; while, chlorophyll a was higher than chlorophyll b. Many reports support the findings obtained in the present study; for instance, **Al-Shemmary *et al*. (2020)** showed that Cu nanoparticle at concentration of 5 M caused decreasing in chl.a content of tomato leaves, Cu nanoparticles of all it concentration caused decreasing in chl.b content of tomato leaves significantly comparing with control. These results were also confirmed by **Amin *et al*. (2019)** in all tested plant species, who found that the maximum chlorophyll contents (a, b, and total mg. g^{-1} FW) were observed at low Cu concentration (25 mg Cu. kg^{-1}) as compared to control, while the lowest value for chlorophyll contents was found at 300 mg Cu. kg^{-1}. The heavy metal stress negatively affected the photosynthesis process and decreased the chlorophyll-a, chlorophyll-b, and total chlorophyll contents in all plant species at higher concentration. In contrast to our results, it has been indicated that the addition of 0 and 100 mg. kg^{-1} Cu had negligible impact on chlorophyll a and b, while 25 mg. kg^{-1} slightly increased chlorophyll **(Gong *et al*., 2017)**. Similar to our results, more than 50 % decline in chl a content was observed at 100 and 200 mg L^{-1} Cu. However, chl b content declined by 22.68 % and 32.86 % at 1 and 10 mg. L^{-1} Cu. Chlorophyll contents (a, b, and total mg. g^{-1} FW) were decreased significantly with steady raise of Cu concentration from 25 to 300 mg Cu. kg^{-1}. Chlorophyll a and b showed dose dependent decrease in Cu stressed seedlings at all concentrations **(Nanda and Agrawal, 2018)**. A recent study described by **Hak *et al*. (2020)** indicate the impact of the time on the chlorophyll content, he showed that the highest chlorophyll a content was found in Cu 0.05 mg. L^{-1} (694.37 ± 57.27 µg. g^{-1}) of fresh weight after 15 days. The highest chlorophyll b content was found in Cu 0.15 mg. L^{-1} (154.76 ± 16.91 µg. g^{-1}) after 10 days.

The present study demonstrated that chlorophyll content was higher than carotenoid; otherwise, both of them were reduced by copper treatments. In concordance, **Saleem *et al*. (2020b)** suggested that the contents of total chlorophyll and carotenoids were significantly affected by high concentrations of Cu in the nutrient solution. Similarly, in tomato leaves, carotenoid and total chlorophyll content diminished with two doses (50 and 100 µM Cd) significantly **(Heybet and Borlu, 2020)**. Moreover, Cu stress (100 mg. kg^{-1}) decreased lycopene and b-carotene content by 32.47 %, and 29.43 % in comparison with the control **(Nazir *et al*., 2019)**. According to **Tiecher *et al*. (2017)**, carotenoid levels were lower in treatments with the addition of 30 mg Cu. kg^{-1}, compared to the other Cu doses by (0.76, 0.57, 0.72 mg. kg^{-1}) for 0, 30, and 60 mg Cu. kg^{-1}, respectively. This reduction in carotenoid contents was also obtained by **Parveen *et al*. (2020).** These Findings much well with ours. However, **Nguyen *et al*. (2020)** revealed an increase in this parameter. It has been noticed that maximum contents of total chlorophyll (2.9 mg. g^{-1} FW) and carotenoid (0.84 mg. g^{-1} FW) were observed in the treatment where the Cu concentration in the nutrient solution is 0 µmol. L^{-1} while increasing Cu levels to the nutrient solution significantly reduced total chlorophyll and carotenoid contents in the leaves of *Hibiscus cannabinus*. Total contents of chlorophyll and carotenoid were reduced by 81 % and 133 % respectively were observed in the treatment of 180 µmol. L^{-1} compared to the control **(Saleem *et al*., 2020b)**.

Under copper stress, all the damages noted in photosynthetic pigments are related to many reactions regulated by several mechanisms (photosystems, ultrastructral damages, transpiration, etc); so, referring to the scientific reports, more explained were provided below;

Copper is an essential part of the electron transfer reactions interceded by proteins, such as cytochrome-c oxidase, and plastocyanin involved in the photosynthetic activity **(Migocka and Malas, 2018)**. Additionally, in plants, Cu plays key roles in chlorophyll formation and photosynthesis **(Rehman *et al*., 2019c)**. However, photosynthesis is one of the main plant metabolic processes affected by copper deficiency and toxicity **(Cuchiara *et al*., 2015)**. Accordingly, the increase in Cu accumulation and corresponding increase in oxidative stress resulted in reduction of photosynthetic and growth characteristics **(Nazir *et al*., 2019)**. A study conducted by **Saleem *et al*. (2020b)** indicated that elevating concentration of copper reduced net photosynthetic rate Pn. This decrease in net photosynthetic rate may be the consequence of the weakening activity of related enzymes under heavy-metal stresses, such as Cu **(Zhou *et al*., 2019a)**. Moreover, reduction in Pn could be attributed to the Cu inhibition of stomatal conductance and photosynthetic pigment content as also observed in *Sorghum bicolour* under Cu stress **(Avinash, 2017)**. Importantly, other vital plant processes might be simultaneously altered with photosynthesis. **Zaouali *et al*. (2020)** proved that under Cu toxicity, the variations in gas exchange parameters were associated with a remarkable decline in the photosynthetic pigments including chlorophyll a, chlorophyllb, chlorophyll (a+b) and carotenoids. These findings justify the concordance noted in our research regards the trend of the transpiration and photosynthesis. It has been demonstrated that the decreased activities of photosynthetic apparatus might be due to excess Cu in the soil, which affects PS II machinery, resulting in lower photosynthetic electron transport activities **(Habiba *et al*., 2014 ; Rehman *et al*., 2019d)**. Similar results were showed by **Rehman *et al*. (2019b)** who found that toxic amount of metal reduced photochemistry of *Boehmeria nivea*. Although Cu phytotoxicity affects both photosystems in plants, photosystem II (PS II) is more sensitive than photosystem

I (PS I) to Cu stress **(Sağlam *et al.*, 2015)**. This photosynthesis could be affected by many factors as plant sex. It has been demonstrated that the greater reduction in pigment contents in the female plants indicates that their photosynthesis machinery is more sensitive overall to Cu and Pb than that of males **(Peng *et al.*, 2020)**.

Furthermore, Cu and Cr induced reduction in chlorophyll content and chlorosis might be due to the reduction of Fe availability to the leaves and negative effects of Cr on chlorophyll metabolism **(Li *et al.*, 2018b).** In addition to the reduction of the content of Mg, Fe and Zn which are directly involved in chlorophyll synthesis, the loss in chlorophyll contents could be due to the activation of chlorophyllase, peroxidation of chloroplast membranes or replacing of magnesium in chlorophyll molecule by copper **(Girotto *et al.*, 2016)**.

Despite of relatively higher Cu concentration in root tissues, efficient translocation of Cu was noticed to leaves which damaged chlorophyll pigment and consequently caused biomass reduction **(Zehra *et al.*, 2020)**. This is confirmed also by **Li *et al*. (2018b)**. Heavy metal at toxic levels interfered with normal enzyme functions in plants and drastically affected the process of photosynthesis **(Ali *et al.*, 2015)**. Furthermore, heavy metals cause oxidative destruction of membrane lipids by free radical formation, in this way chlorophyll degradation is increased and synthesis is inhibited **(Heybet and Borlu, 2020)**.

Although, chloroplast is the main site of Cu accumulation as mentioned in the details in the review of literature by **Adrees *et al*. (2015)**. However, similar results have been noticed in another pot experiment that Cu toxicity disrupts the ultrastructure of chloroplast **(Saleem *et al.*, 2020a)**. Moreover, a decrease in photosynthesis activity and alteration in the ultrastructure of the chloroplast is directly linked with photosynthetic pigments in the leaves **(Li *et al.*, 2018b ; Rehman *et al.*, 2019d)**. It has been observed that low concentration of Cu causes a little effect on the structure of the chloroplast, nucleus, mitochondria and ribosomes, etc. In T1 plants, the organelles of the leaves were damaged heavily by exposure to excess Cu in the soil. It was also observed that a large number of chloroplast particles was accumulated inside the cell wall and outside the chloroplast in T1 plants **(Saleem *et al.*, 2020a)**. Similar result was obtained by **Shams *et al*. (2019)**. These studies reported that Cu toxicity reduced the number and volume of chloroplasts, decreased chlorophyll content, and subsequently reduced photosynthesis. These results indicated an increased electron density of chloroplasts, disorganized thylakoid membranes, expanded inter-thylakoid spaces, a growing number of grana, large-sized plastoglobules, disturbances in the peroxisome matrix and endoplasmic network, swollen vacuoles and mitochondrial cristae, rare starch grains, and lipid droplet clusters in leaf and stems cells **(Minkina *et al.*, 2019)**. The decline in the levels of photosynthetic pigments is mainly due to loss of cell wall and membrane integrity of thylakoid membrane **(Khanna *et al*., 2019b)**.The damage to the integrity of the thylakoid membrane due to the accumulation of Cu can change the composition of fatty acid molecules and also stop various enzymes binding with chlorophyll **(Adrees *et al*., 2015 ; Rehman *et al*., 2019d ; Zaheer *et al*., 2015).**

Inversely, the increase in chlorophyll content may be a compensatory growth caused by temperature, heavy metal stresses, or interaction of multiple factors to maintain the structure and functions of the chloroplast **(Zhou *et al*., 2019a).** However, increases in chlorophyll content could be due to the role of copper in giving rise to the PSI efficiency in plants as well as less sensitive action of chlorophyll-a and chlorophyll-b to metals than the growth rate under copper treatment **(Cuchiara *et al*., 2015)**.

Besides, it was found that carotenoids content was relatively reduced by copper treatments, this observation was confirmed by many researches. Tomato fruit synthesizes carotenoids **(Karniel *et al*., 2020)**, it may be proposed that the exposure of plants to copper fungicide induced the accumulation of β-carotene in tomato fruits **(Kristl *et al*., 2018)**. Moreover, carotenoids protect the plant's photosynthetic machinery from photo-oxidative disruptions through ROS scavenging. Reduction in carotenoids lead to PSII damage by retrogression of D1 protein that inhibits chlorophyll synthesis **(Huang and Wang, 2010)**. Oxalis corniculata leaves showed high radical scavenging activity attributed to the high levels of carotenoids **(Zeb and Imran, 2019)**. In contrast, the ratio Chl (a + b)/Car remained unchanged indicating that carotenoid metabolism was more resistant to the Cu excess **(Zaouali *et al*., 2020)**.

The possible explanation for the different chlorophyll responses may be related to various tolerant mechanisms **(Chai *et al*., 2014)**.This reduction in chlorophyll could be due to different responses of plant species to heavy metals **(Shams *et al*., 2019)**. This suggests that the various concentrations of heavy metals affect the pigments contents **(Chandra and Kang, 2016)**.

Effect of zinc

Many studies report a decline in chlorophyll a, b, tchl, and carotenoids content, under heavy metal stress, in *Solanum lycopersicum* L. exposed to cadmium **(Heybet and Borlu, 2020 ; Rodrigues *et al*., 2020 ; Wei *et al*., 2018 ;)**, to cadmium, lead and nickel **(Zeeshan *et al*., 2020)**, to silver (AgNPs) **(Özlem Çekiç *et al*., 2017)**, to boron **(Kaya *et al*., 2020)**, to zinc **(Akanbi-Gada *et al*., 2019)**. In other plant species, exposed to cadmium **(Chen *et al*., 2019b)** in *Kandelia Obovata*, to cadmium and zinc **(Shivaji and Dronamaraju, 2019)** in *Scenedesnus* *rotundus*, to cadmium **(Khan *et al*., 2019)** in *Petunia hybrida* L., to nickel **(Turan, 2019)** in *Lactuca sativa*. On the other hand, in photosynthetic pigments could be also impaired by different stresses; salinity **(Siddiqui *et al*., 2019)** in *Solanum lycopersicum* L., drought stress **(Abid *et al*., 2018)** in *Triticum* aestivum L., salinity **(Sarker and Oba, 2019)** in *Amaranthus spinosus*, heat stress **(Han *et al*., 2019)** in *Brassica chinensis* L.

It was demonstrated that zinc stress caused a decline in the chlorophyll content (chla, b, and tchl) of *Lycopersicon esculentum* Mill. Relatively, this level was depending on the increase of zinc doses in the soil. Our results corroborate with the findings of **Akanbi-Gada *et al*. (2019)** who proved that ZnO-treatments caused reduction of chl-a, b and total-chl in tomato leaves by at least 54.3%, 99.6% and 105.4%, respectively at 30-day exposure. These results were depending on zinc formulation and the duration of treatment. In addition, depending on plant species and the type of chlorophyll, **Pramanick *et al*. (2017)** indicated a reduction in chlorophyll a, chlorophyll b and total chlorophyll content on further increasing the concentration of $ZnSO_4$ in the medium. However, Zn only treatments exhibited significant decreases in chlorophyll a content in *Kandelia Obovata* leaves, but no distinct difference was found in chlorophyll b content **(Chen *et al*., 2019b)**.

In term of dose depending, our results concurred with those of **Hammerschmitt *et al*. (2020)** who found that chl a, chlb, tchl, decrease with increasing of zinc concentration in peach leaves. It was found that carotenoid content in tomato leaves reduced at 100 ppm and increased at 200 and 300 ppm of zinc. Similarly, **Espinosa-Vellarino *et al*. (2020)** found that the contents in chlorophyll a and b decrease, as does the photosynthetic efficiency. On the similar the carotenoids increase, indicating a possible action as antioxidants and protectors against Sb in tomato. It has been indicated that relative to the control soils, the greatest Zn additions resulted in a 10 –28% reduction in the chlorophyll content **(Mossa *et al*., 2020)**.

In contradiction to our findings, **Hatam *et al*. (2020)** showed that various Zn concentrations (50, 50, 100 kg Zn. ha^{-1}) had no effects on chlorophyll content index. The application of 100 kg Zn. ha^{-1} increased the photosynthesis rate by 38 and 44%, respectively. This increase in total and chlorophyll a under zinc stress was also indicated by **Zaman *et al*. (2020)**. Moreover, depending on the application mode, foliar application of Zn significantly improved the chl a content but did not influence the chlb contents in mungbean. Zn alone significantly improved the chlt contents while it maintained non-significantly higher carotenoids contents **(Ashraf *et al*., 2020)**. Moreover, **Alamer *et al*. (2020)** found that chlorophyll and carotenoids content were enhanced due to supplying Zn. A comparison of the usage treatments showed that the higher dose of Zn was better than the lower one. Also, this increase in chlorophyll content has been observed in tomato under salinity stress **(Cao *et al*., 2018)**. Depending on the duration of application, a recent study described by **Hak *et al*. (2020)** demonstrated that in the separate Zn treatment, there was significant difference of chl a, chl b and chl b/a ratio only on day 5 of the experiment. The highest chl a was found in Zn 10.00 mg. l^{-1} on day 10. While, the highest chl b content was found in day 15 at Zn 10.00 mg. L^{-1}. As compared with control, combined stress induced a decline in the chlorophyll a content of cultivar CV_2 on stress day 4 until recovery day 2 **(Zhou *et al*., 2019b)**.

Besides, **Chandra and Kang (2016)** confirmed the trend fluctuation of carotenoids content observed in our study, where he proved that the photosynthetic rate decreased with increasing heavy metal (Cd, Cr, Cu, and Zn) concentrations; however, an increase was observed at the highest concentration of 500 ppm at 14.54 μmol. m^{-2}. s^{-1}. However, the carotenoid contents decreased with increasing heavy metal concentrations and at 500 ppm increased dramatically to 3.17 mg. g^{-1} **Cruz *et al*. (2019)** also reported that the photosynthetic rate in the plants treated with ZnO was diminished by an increase of 50% of the Zn concentration. Chl a and b and carotenoid contents showed similar trends with the addition of Cu and Zn. This is in good agreement with our results.

Importantly**, Tiecher *et al*. (2017)** found that in the treatments without Cu addition, there was an increase in concentration of these pigments up to Zn dose of 30 mg. kg^{-1} and a decrease in treatments with 60 and 120 mg Zn. kg^{-1}, with subsequent increase in treatment with 180 mg Zn kg^{-1}. In treatments with the addition of 30 mg Cu. kg^{-1}, the highest amounts of photosynthetic pigments were observed in the treatment without Zn, and there was a decrease in these levels due to the addition of Zn. In treatments with the addition of 60 mg Cu. kg^{-1}, there was a decrease in the pigment levels upon addition of Zn, levels exceeding 60 mg Zn. kg^{-1} dose, and the lowest levels were observed in the treatment with the addition of the highest Zn dose (180 mg Zn. kg^{-1}). However, metal combination is also considered as a limited factor in stress plant, **Rizwan *et al*. (2019)** noted an increase in leaf chlorophyll content under combined stress Zn and Cd. Furthermore, the combination of Cu and Zn caused more toxic effect on chlorophyll content than either Cu or Zn separately. Importantly, responses to Cu were slower than for Zn even though in the longer run Cu was considerably more toxic than Zn **(Hak *et al*., 2020)**. This research confirms our findings which indicated that copper effect on the photosynthetic pigments was lower than zinc.

According to the amount of zinc uptake from the contaminated soil, plant metabolic react immediately by different responses of, oxidative stress, respiration rate,

gene expression, and signalling phytohormone, etc. These reactions lead to several variations in the photosynthesis process. In this case, many reports conducted recently have explained the reason behind the decline in photosynthetic pigments;

The photosynthesis was impaired by the increasing Zn content in the leaflet. A surprising piece of information regards the amount of Zn necessary to trigger the deleterious effects on photosynthesis **(Cruz *et al*., 2019)**. The reduction in chlorophyll which ultimately would have reduced photo assimilation and photosynthesis as required for the crop growth and development **(Turan, 2019)**.In addition, Zn application significantly decreased photosynthesis rate probably due to inhibitory effect on electron transfer within photosystem II **(Hatam *et al*., 2020)**. Similarly, it has been confirmed that there was a reduction in the values of maximum quantum yield of PSII , basal quantum yield of PSII and electron transport rate upon addition of Zn **(Tiecher *et al*., 2017)**.

Compared to the biomass and chlorophyll content, the photosynthetic rate was less sensitive to Zn toxicity **(Mossa *et al*., 2020)**. Some reports showed that decrease in leaf chlorophyll contents in plants is the result of reduction in synthesis of 5-aminolinolic acid a necessary precursor of protochlorophyllide that converts into chlorophyll on light exposure **(Radi *et al*., 2013 ; Santos, 2004)**. Moreover, reduced chlorophyll contents may be observed due to the increased activity of enzymes like chlorophyllase and deficiency of nutrients **(Bilal Shakoor *et al*., 2014 ; Khan *et al*., 2016)**. Another factor can be also including in this problematic is that, the decline in nutrient uptake resulted in loss of chlorophyll pigments (chl a, chl b, total chl) under Zn stresses **(Garg and Singh, 2018)**. On the other hand, Zn treatments raised the contents of N, P, K and Zn in leaves relative to unfertilized ones **(Alamer *et al.*, 2020)**. Variations in P contents was also observed by **Ashraf *et al.* (2020)** under zinc stress. It has been demonstrated that increased photosynthetic pigment accumulation in peach leaves cultivated in low Zn dose soils can be explained by a better plant nutritional state as well as by lower Zn absorption and translocation, which contributes to reduce its phytotoxic effects **(Tiecher *et al*., 2017)**.

Importantly, oxidative stress in leaves was also induced under zinc stress. Malondialdehyde (MDA) content, electrolyte leakage (EL), and H_2O_2 contents were found strongly positively correlated, while were found negatively correlated for chl a, chl b, t chl and car with metal uptake **(Khan *et al*., 2019)**. The carotenoids might have played an important role in restraining ROS accumulation in the chloroplasts via photoprotection of the photosystem and preventing membrane lipid peroxidation **(Abid *et al*., 2018** ; **Tang *et al*., 2019a)**. Carotenoid plays a key photoprotective role in leaf senescence **(Tang *et al*., 2019a)**. The results indicated by **Singh *et al*. (2015)** implied that carrotenoids extract-assisted elevation of sugar and free amino acid levels might have contributed to osmotolerance of salt-exposed maize plants by improving water status, stabilizing membranes and protein complexes, and protecting enzymes from denaturation.

The negative effects observed on photosynthetic pigments, in the present study, should be explained at molecular level. It has been indicated that *TDDF1* is involved in abiotic stress resistance in tomato through up-regulating abiotic stress responsive genes. In the same manner, *TDDF1* overexpression enhanced the chlorophyll (A and B) synthesis by up-regulating the expression levels of biosynthetic genes *HEMA*, *HEML1*, *HEMB1*, *HEMC*, *HEME1*, *HEMG1*, *CHLD*, *CHLM*, *CRD* and *CAO* in tomato **(Ewas *et al*., 2017)**. Additionally, the overexpression of *SlARF6A* (Auxin response factors) increased chlorophyll contents in the fruits and leaves of tomato plants, whereas downregulation of *SlARF6A* decreased chlorophyll contents compared with those of wild-type (WT) plants **(Yuan *et al*., 2019)**. Thus, downregulation of *TDDF1* and *SlARF6A* justify the decline of chlorophyll content in our study.

Furthermore, Zn is a constituent of RUBISCO enzyme involved in CO_2 fixation during photosynthesis process, and it is also essential for structural integrity of membranes and proteins in plants **(Sadeghi and Nazemosadat, 2011)**. In this context, it has been demonstrated that *SlARF6A* has important roles in photosynthesis via the direct regulation of the *RbcS* gene in tomato **(Yuan *et al*., 2019)**. Morover, **Wang *et al*. (2020)** showed that two *Arabidopsis* paralogs of BALANCE of CHLOROPHYLL METABOLISM (BCM) act as functionally conserved scaffold proteins to regulate the trade-off between chlorophyll synthesis and breakdown. Meanwhile, BCM1's interaction with Mg-dechelatase promotes degradation of the latter, thereby preventing chlorophyll degradation. In addition, it has been indicated that reduced chl levels in *bcm1* was correlated with decreased levels of LHCa1 and LHCb1 (representative LHC proteins of PSI and PSII), in agreement with the highly synchronized synthesis of chls and LHC proteins **(Dall'Osto *et al*., 2015 ; Wang and Grimm, 2015).** BCMs delay chl breakdown by destabilization of SGR1. Phytohormones exhibit also a main role in regulation of photosynthetic pigments. ABA signalling can efficiently induce chl breakdown **(Wang *et al*., 2020).** Additionally, the results presented by **Cao *et al*. (2018)** showed that phytochrome B1 mediated tomato seedlings tolerate high salinity conditions under different Red: far-red (FR) light treatments.

Overall, chlotophyll and carotenoids pigments of *Lycopersicon esculentum* Mill. leaves. declined in the presence of high-level copper and zinc. This effect due to the different damages occurred in the photosynthesis process.

VIII.4. Biochemical parameters

The adaptation of plants to their environment requires tight regulation of metabolism and growth processes through central and highly connected signalling pathways **(Ryabova *et al.*, 2019).**

VIII.4.1 Proteins content

Proteins are the essential macromolecules accountable for the fundamental biological processes in the plant cell. In this context, it has been indicated that leaf quantitative proteomic profiling revealed drastic differences in proteins related to cell cycle, flowering, hormone signalling and carbon metabolism between transgenic lines and wild-type **(Müller *et al.*, 2018)**. In addition, protein is an important constituent of the plant cell that easily damage environmental stress condition. Hence, quantification and qualitative analysis of protein indicate oxidative stress in plants. The protein contents of the organism or cell get altered during stress to adopt the extremity of the surrounding environment created by the stress **(Rout *et al.*, 2019)**.

- **Effect of copper**

Under copper stress, leaves and roots of *Lycopersicon esculentum* Mill. accumulated a considerable amount of proteins compared to the control. This content decreased at a high level of copper. Notably, proteins content in leaves was greater than roots.

Our results corroborate the findings of **Nazir *et al.* (2019)**, who suggested that tomato plants subjected to Cu (100 mg. kg^{-1}) showed decreased protein content. The plants treated with H_2O_2 as root dipping or Cu (10 mg. kg^{-1}) supplementation or their combined application possessed higher total protein content than their respective controls. Similarly, protein content decreased by 9.43% in *Brassica juncea* plants grown in Cu-treated soil compared to protein content in control plants **(Yadav *et al.*, 2018)**.

Depending on the metal sensibility, **Li *et al.* (2018b)** found that the total soluble protein content decreased under Cu or Cr stress, the sensitive cultivar, a more prominent decline was recorded compared with the less sensitive one. On the other hand, depending on the metal, this study indicates that the effect of Cr on total soluble protein degradation was stronger than Cu. Many authors report decreased protein content in the tomato plant under cadmium stress **(Manara *et al.*, 2020 ; Rodrigues *et al.*, 2020).**

It was found that protein content in leaves increases up to 100 ppm of copper. However, the minimum content was recorded at 500 ppm. In contrast to our results, the protein content of copper treated *Sesuvium portulacastrum* shoot increased appreciably with increasing concentration of applied copper in the soil up to 200 mg. kg^{-1} of copper level. The minimum protein content was observed at 600 mg. kg^{-1} of copper level in all the sampling days **(Kalaikandhan *et al*., 2018)**.

Depending on the duration of application, it has been indicated that after induction with heavy metals (Hg, Zn, Ni), the mRNA of HSPA1A (heat shock proteins) showed peak activation at similar concentrations as seen in the luciferase assay but with higher sensitivity. The results for the different heavy metals show that even with a short treatment of 1 h, the cells continuously increase their heat shock response (HSR) activation up to 24 h **(Steurer *et al*., 2018)**. Depending on the metal and plant species, a minor decrease was noted from copper and cadmium exposure of 175 ppm in pea and 220 ppm in tomato **(Baruah *et al*., 2019)**.

According to the current study, the accumulation of soluble proteins under copper and stress was higher in leaves than roots. It was found in our study that proteins accumulated in leaves were higher than in roots. These results are following the earlier study conducted by **Walter *et al*. (2018)**, who proved that the total protein content in leaves (8.6 ± 0.7 mg. g^{-1} FW) and roots (5.3 ± 0.6 mg. g^{-1} FW) did not vary with the different levels of Cu.

Protein content under heavy metal stress could be attributed to the level of cell enzymes. Many studies report increased antioxidants enzymes in roots and leaves under copper stress **(Hippler *et al*., 2018 ; Nanda and Agrawal, 2018 ; Parveen *et al*., 2020 ; Ruan *et al*., 2019 ; Zehra *et al*., 2020).** It has been indicated that the level of these enzymes increased with increasing Cu concentration **(Hamzah *et al*., 2019)**. However, the decrease of proteins level recorded in our study may be explained by various studies that revealed a decrease in these enzymes **(Chrysargyris *et al*., 2019 ; Hu *et al*., 2020)**. In this regard, 200 µM of copper sulfate increased the peroxidase (POD) content. However, the highest concentration of copper sulfate (400 µM) significantly decreased its activity compared to the plants treated with 200 µM of copper sulphate. However, copper sulfate treatment (400 µM) did not represent a decreasing effect on the superoxide dismutase (SOD) and Ascorbate peroxidase (APX) content **(Shams *et al*., 2019)**. Low concentration Cu stresses can stimulate SOD activity more than high-concentration Cu stress **(Zhou *et al*., 2019).** This increase of SOD and decrease of catalase (CAT) in the root of *Solanum lycopersicum has* been demonstrated by **Branco-Neves *et al*. (2017).**

The variation in proteins content obtained in the present study was reported by many investigations. They found that protein content in both organs was independent of the metal concentration and was different from leaves to roots.

Nitrogen is an essential element required for protein synthesis; thereby, the protein trend in both tissues may also be explained by the cell nitrogen (N) level. **Saleem *et al*. (2019a)** showed that the exposure to 60 µM of Cu decreased (up to 20.6 %) N content in the upper part but not in roots. From a biochemical standpoint, it has been reported that the increase in N content under stressful conditions could be ascribed to increased activity of the enzymes involved in N metabolism, including nitrate reductase (NR), nitrite reductase, glutamine synthetase, and glutamine synthase **(Meng *et al*., 2016)**. According to **Hippler *et al*. (2018)**, high-N citrus plants exhibited lower phytotoxic effect by Cu toxicity because of the greater activities of SOD in roots and CAT in leaves and roots. However, the hydrogen peroxide (H_2O_2) concentrations in the leaves and roots and the MDA levels in the leaves were lower in high-N than in control plants, both under Cu toxicity.

It was found that proteins level in leaves was higher than roots; this might be due to the different proteins expressed in each organ and the tolerance metal-organ. Protein, as an important macromolecule, needs deep explanations. Importantly, in the previous studies, the proteomic analysis indicated that proteins. N^{o}. 49, 50, 51, and 53 were down-regulated in the roots of Cu treated seedlings, revealing that these proteins might be involved in growth inhibition. However, phosphomannomutase (No. 54), GDP-mannose 3,5-epimerase (No. 56), and peroxidase 73 (No. 55) that play crucial roles in ROS scavenging were accumulated in the roots of *Oenothera glazioviana* seedlings exposed to Cu, suggesting that they might be associated with oxidative stress response **(Wang *et al*., 2017)**. These results also justify the elevation and the decrease in proteins content under different concentrations of copper.

Also, proteins varied depending on the plant species, **Huang *et al*. (2020b)** detected that forty-one and 37 differentially abundant protein (DAP) spots were identified in Cu-treated *Citrus grandis* and *Citrus sinensis* leaves, respectively, including some novel DAPs that were not reported in leaves and/or roots. Most of these DAPs were identified only in *C. grandis*, or *C. Sinensis* leaves. Over 50 % of DAPs were associated with photosynthesis, carbohydrate, and energy metabolism. Forty-one and 37 DAP spots were identified in 200, 300 and/or 400 µM Cu-treated. Cu-effects on photosynthetic electron transport chain (PETC) were more pronounced in *C. Sinensis* leaves than in *C. grandis* leaves. DAPs related to antioxidation and detoxification, protein folding and assembly (chaperones and folding catalysts), and signal transduction might involve Citrus Cu-toxicity and Cu-tolerance. The abundance of chl a-b binding protein eight was increased and decreased in 200 and 400 µM Cu-treated *C. Sinensis* leaves, respectively. Thus, the decreased abundance of PSI protein LHCA3 in 400 µM Cu-treated *C. Sinensis* leaves might contribute to the Cu-induced photoinhibition. This is in good agreement with our results which present an increase at 300 ppm Cu following by a decrease at 400 ppm; also, similar to 100 and 200 ppm Cu. Furthermore, the abundance of PsbP domain-containing protein 3 (PPD3, S13) involved in PSII light reaction was decreased in 300 and 400 µM Cu-treated *C. Sinensis* leaves. Similarly, the abundance of PSII stability/assembly factor HCF136, an essential protein for the stability/assembly of PSII, was increased in 300 (G31) and 400 (G30) µM Cu-treated *C. grandis* leaves, but not in Cu-treated *C. Sinensis* leaves. In agreement with our outcomes, these studies give insight into the fluctuation trend in proteins content obtained between 200, 300, 400 ppm Cu and confirm that the accumulation of proteins in leaves is because of their main role in the photosynthesis process.

Notably, the label-free proteomic analysis indicated 58 differentially abundant proteins (DAPs) of the total 3149 proteins in the roots of *O. glazioviana* seedlings, of which 58 proteins (1.8 % of the total proteins) were classified as DAPs; 36 were upregulated and 22 were downregulated under Cu stress conditions. Gene Ontology (GO) analysis was performed. It showed that DAPs were annotated to protein metabolism (18 DAPs), carbohydrate and energy metabolism (15 DAPs), signal transduction (eight DAPs), detoxification and stress defence (seven DAPs), development (five DAPs), oxidoreduction (three DAPs), and other unknown functions (two DAPs). This study confirms our results, indicating that proteins play an important role in the accumulation of soluble sugar.

Also, this study on the proteins related to carbohydrate and energy metabolism analysis indicated that six pathways (involved 13 DAPs), including the citric acid cycle (CA), carbon metabolism, pyruvate metabolism, fructose and mannose metabolism, glycolysis/gluconeogenesis, and amino sugar and nucleotide sugar metabolism, were significant. The CA cycle was the most significantly enriched, and the citrate synthase was the most upregulated among these 13 DAPs. Under Cu stress conditions, we observed the up-regulation of No. 1, 9, and 10 that suggested the accumulation of damaged or misfolded proteins under Cu stress. Whereas the down-regulation of No. 5, 7, and 8 indicated the synthesis of inappropriate proteins that led to the abnormal growth of *O. glazioviana* seedlings **(Wang *et al.*, 2017).** These findings could explain our results regards the reduction of growth parameters.

Remarkably, it has been identified some new abundant proteins (DAPs) (viz., LFNR2, SBPase, probable PGL4, ferritin, AdoHcy hydrolase and abscisic stress-ripening protein 1-like) were not reported in leaves and/or roots. The abundances of the other 16 DAPs matched well with the expression levels of the corresponding genes regardless of whether PRPF31 **(Huang *et al.*, 2020b).**

Many studies have reported the reasons behind the accumulation of proteins in leaves and roots stressed by heavy metals. The level of cell proteins might explain the

negative effect of copper observed on the morphological, biometrical, physiological parameter;

Proteins that have a high affinity toward different metal ions contain cysteine (Cys), methionine (Met), and histidine (His) residues play a role in plant cell homeostasis and tolerance **(Qazi *et al*., 2019)**. Soluble proteins are important osmotic adjustment substances and nutrients. Their increase and accumulation can improve the water retention capacity of plants and play an important role in protecting the vital substances and biofilms of cells **(Liang and Yang, 2019)**. It has been proved that several proteins involved in antioxidant defence, superoxide dismutases, and glutathione-s-transferases were upregulated. In contrast, protein synthesis and degradation proteins were modulated in rice seedlings under Cu stress, indicating oxidative stress **(Chen *et al*., 2015).** The presence of Cu is crucial for successful protein synthesis. The most abundant Cu proteins in leaves are plastocyanin, essential for photosynthesis in higher plants, and Cu/Zn SOD, involved in ROS detoxification **(Rehman *et al*., 2019c)**.

Moreover, the total free amino acid content decreased by 8.98% in Cu-stressed **(Yadav *et al*., 2018)**. Some plants activate the production of small proteins such as glutathione S-transferase (GST) and small heat shock protein (HSP) **(Sun *et al*., 2020c)**. Plants also produce specific metallothionines and phytosiderophores **(Zúñiga *et al*., 2019)** to chelate heavy metals or to activate heavy metals transporters **(Shafiq *et al*., 2019)**. It has been indicated that GSH and PC2-PC5 are accumulated with significant differences with the control in copper treated plants **(Contreras *et al*., 2018)**. Importantly, excessive Cu can react with membrane protein–SH or phospholipids in the molecular layer of phospholipids and result in changes in the structure of membrane protein phospholipids. These changes then reduce the cell membrane structure changes, damage the membrane system, increase permeability, extravasation of intracellular soluble substances, and increase relative membrane permeability (MRP). The podophyllotoxin content in the roots and shoots of *D. versipellis* decreased significantly with the increase of Cu concentration **(Luo *et al*., 2020)**. It turns out that miR528 predominantly regulates relevant genes encoding Cu-containing proteins **(Zhu *et al*., 2020)**. It has been indicated that the levels of transcripts encoding proteins belonging to Light-Harvesting Complex II (LHCII), photosystem II (PSII), cytochrome b6f, PSI, LHCI, ATP synthase and proteins involved in the repair of PSII and protection of PSI were increased in the alga cultivated with copper. Also, the level of transcripts encoding proteins of the mitochondrial electron transport chain. The higher percentages of increase in the level of transcripts were mainly observed on days 3 and 5.

In contrast, transcripts involved protein synthesis and degradation, signal transduction and replication, and decreased DNA repair. Furthermore, the activities of enzymes involved in C, N and S assimilation, rubisco, glutamine synthase and cysteine synthase, respectively, were also increased, mainly at days 1 and 3. At time point 0 vs 1, the number of differentially expressed transcripts was 28,510, those upregulated were 13,855, and those down-regulated were 14,655, representing 48.6 and 51.4%, respectively. At time point 0 vs 3, the number of differentially expressed transcripts was 8174, those upregulated were 6047, and those down-regulated were 2127, representing 74 and 26%, respectively. At time point 0 vs 5, the number of differentially expressed transcripts was 30.589, those upregulated were 17,218, and those down-regulated were 13,371, representing 56.3 and 43.7%, respectively. Thus, the higher percentages of upregulated transcripts were observed at days 3 and 5 in the alga exposed to copper excess. On the other hand, the levels of transcripts encoding the chaperone methyltransferase gene (MET1), the ATP-dependent metalloproteases were increased **(Laporte *et al*., 2020)**. Another type of protein, translationally controlled tumour protein, is considered a major regulator of cell growth in plants. Additionally, prohibitins play an important role in root hair elongation, cell division, and development **(Wang *et al*., 2010)**. The reduction in grain yield (GY) by $CuSO_4$ application might be due to the toxic effects of Cu^{2+} on the photosynthetic electron transport chain resulting in protein denaturation and deactivation of antioxidant enzymes in the plant cell **(Barbosa *et al*., 2013)**.

Proteins are considered a part of the mechanisms regulating the biometrical parameters such as the relative water content and water loss. These types of proteins are aquaporins, the main transmembrane proteins or the water channel, **Wang *et al*. (2017)** found that Cu stress-induced the up-regulation of aquaporin that might influence the intracellular transport of Cu, as well as the up-regulation and activation of V-type proton ATPase that led to the excessive accumulation of Cu in the vacuole. Thus, the mechanism by which nutritional stress affects protein synthesis is still unknown and needs further analytical study **(Rout *et al*., 2019).**

Effect of Zinc

In *Lycopersicon esculentum* Mill. exposed to different concentrations of zinc, leaves and roots accumulate a high level of proteins, compared to the control. Proteins content in leaves was higher than roots. This content was greater at low concentrations; however, it was reduced significantly under a high zinc level in both tissues.

Our results are in good agreement with the findings of **Kalaikandhan *et al*. (2018)**, who showed that amino acids and protein content of *Sesuvium portulacastrum* were higher at a low level of copper (100-200 mg. kg^{-1}) and zinc (100-300 mg. kg^{-1}) in the soil than in the control plants. Further, the values decreased with a gradual increase in copper (300- 600 mg. kg^{-1}) and zinc (400- 600 mg. kg^{-1}) levels in all the sampling days.

Our investigation showed that the proteins content accumulated in leaves was higher than in roots. Similarly, **Rout *et al*. (2019)** demonstrated that leaf content of soluble proteins was higher than root after seven days and the roots higher than leaves after 14 days; under different zinc concentrations (0, 25, 50, 100, 200 µM). Additionally, the protein content was maximum in leaves of 25 µM Zn, which was 1.37 times higher than leaf samples of control after seven days of treatment. When the concentration of Zn was increased, the amount of protein gradually decreased to the minimum, and the highest minimum was noticed in 200 µM of treatment. A similar trend is also noticed in leaves of day 14 treated plants, but in exceptional that the maximum protein concentration was observed in 50 µM of Zn. In the case of roots, the maximum amount of proteins was exhibited at 25 µM concentration in both 7 and 14 days of treatment than the controls. But the inhibitory effect was noticed at 50 µM and onwards. The maximum negative effect was 200 µM of treatment in both 7 and 14 days of exposure. These results corroborate our findings and indicate the impact of other factors, as the duration of application, in plant response to zinc.

Some reports indicate increased protein content in algae under metal stress **(Shivaji and Dronamaraju, 2019)**. It has been observed that proteins enzyme increase in roots under zinc and copper **(Morina *et al*., 2016).** In addition, **Baruah *et al*. (2019) ; Heybet and Borlu (2020)** showed that proteins content in tomatoes was increased under cadmium. Furthermore, the activity of antioxidant enzymes increased in intermediate doses of Zn **(Tiecher *et al*., 2017)**. This result is consistent with many studies **(Alam *et al*., 2020 ; Baruah *et al*., 2019; Hammerschmitt *et al*., 2020 ; Liang and Yang, 2019 ; Shivaji *et al*., 2019)**. Moreover, under Zn stress, the maximum activity of superoxide dismutase (SOD) was observed in 100 µM of leaf tissues at the end of 7 days treatment which was 1.65 times higher than the control. Whereas in leaves, the maximum (82.90 ± 8.74 U. mg^{-1} protein) activity of SOD was obtained with 50 µM of treatment and then suddenly declined in higher concentrations **(Rout *et al*., 2019)**.

Numerous proteins accumulated in leaves and roots of *Lycopersicon esculentum* Mill. Varied depending on the concentration, which explains the fluctuate trend observed in our study. According to the proteomic analysis performed by **Rout *et al*. (2019)**, a total of 15 numbers protein bands were found at 25, 50, and 100 µM Zn treatment in leaf samples of 7 days plants. Polypeptides with the molecular weight of 23.15 and 18.65 kDa have newly appeared in 25, 50, and 100 µM, and the peptide-like 18.65 kDa vanished in 200 µM of treatment. But the long-term exposure (after 14 days) did not enhance the number of polypeptides except 25 µM of treatment. Similarly, in the control root samples of 7 days plants, six peptides were observed, whereas, in 50, 100, and 200 µM of treatment, 9, 8, and 6 numbers of peptides were visualized, respectively. Polypeptide, like 87.05 kDa was disappeared upon 25, 100, and 200 µM of treatment; however, 17.57 kDa newly appeared in 25, 50, and 100 µM of treatment. After seven days, the 50 µM of treatment synthesized three new proteins (87.05, 54.21, and 40.23 kDa), which were disappeared in 200 µM of treatment. But a reverse pattern of changes was detected in root tissues that the number of polypeptides increased after 14 days of exposure. Ten peptides were noticed in 50 µM treatment of Zn compared to six numbers of peptides in control tissues. Polypeptides like 81.52, 27.62, and 22.88 kDa were newly noticed in 50 µM, where 27.62 kDa was missing in higher concentrations (100 and 200 µM) treatment. Notably, this study also gives a reason for the content of the different proteins in leaves compared to roots of *Lycopersicon esculentum* Mill., observed in our results.

Zinc stress induces a balance in the proteins content of leaves and roots, where degradation and synthesis of these macromolecules were observed in the present study. Several reports explained these effects;

Synthesis of various stress-responsive proteins (dehydrins, osmosis, cold shock domain proteins, salt shock proteins, phytochelatins, metallothioneins, PR proteins, defensins) plays an important role regulating plant response to environmental conditions **(Bleackley *et al*., 2020 ; Qazi *et al*., 2019)**. Additionally, heavy metal-associated proteins (HMPs) participate in heavy metal detoxification **(Fu *et al*., 2020 ; Khatiwada *et al*., 2020 ; Li *et al*., 2020)**. Also, there was a substantial increase in the abundance of thiol-rich proteins that are paramount in metal chelation and sequestration, as well as proteins involved in cellular stress response **(Khatiwada *et al*., 2020)**. It has been demonstrated that water deficit stress provoked a reduction of the soluble protein content in both root tissues, which is more pronounced in the fibre **(Castañeda *et al*., 2018)**. The decline in soluble protein results may be due to the loss of genetic material by fragmentation of the chromosome, micronuclei, and particularly on lagging chromosomes, with repercussions on the synthesis of proteins encoded by the genes **(Olteanu *et al*., 2013)**. The disappearance and de novo synthesis of proteins or polypeptides with response to Zn stress signify the development of strategies by causing a major reshuffle of protein profiles of *Withania somnifera*. Zn toxicity influences the decrease in growth parameters, alteration of proteins, and sudden rise in activities of enzymatic antioxidants **(Rout *et al*., 2019)**; this study supports our results where the proteins content declined simultaneously with the growth parameters.

Furthermore, protein oxidation was significant in Zn treated cells compared to the control **(Shivaji and Dronamaraju, 2019)**. The soluble protein content of the oily seedlings of Zn and Cu combined with heavy metals was significantly increased, which also indicated that under this stress, the plants could withstand stress and always protected the normal physiology and differentiation of the plants **(Liang and Yang, 2019)**. Nevertheless, excess Cu and Zn may cause inhibition of enzyme activity by the structural alteration of enzymes, the binding of these elements to specific active sites, or the induced deficiency of other elements necessary for enzymatic activation **(Ademar *et al*., 2018)**. Recent studies conducted by **Kumar *et al*. (2020)** and **Sable *et al*. (2018)** suggested that heat shock protein (OsHsp18.0) may be required for preventing misfolding or aggregation and thus maintaining the proper conformations of the proteins involved in nucleo-cytoplasmic trafficking under stress conditions. Furthermore, in response to mental stress, the CeHSP17 protein expression was significantly induced by cadmium and zinc at high concentrations of clearly cytotoxic range in wild-type *Caenorhabditis Elegans* **(Ezemaduka and Li, 2017)**. A recent study proved that metallocarboxypeptidase inhibitor (TCMP) interacts with the metal-ion binding protein SlHIPP26 **(Manara *et al*., 2020)**. Indeed, cadmium and zinc ions induce AtHIPP26 expression, while Cu and Pb do not enhance AtHIPP26 expression **(Gao *et al*., 2009).**

Overall, a class of proteins was synthesis in the presence of stress; otherwise, other proteins were altered and denatured in the same conditions, depending on the metal and its concentration (copper or zinc). Proteins profile in leaves was different to roots in *Lycopersicon esculentum* Mill. All these ascertainments explain the proteins trend recorded in our study.

VIII.4.2 Soluble sugars content

In plants, the best-understood examples of nutrient signalling metabolites are carbohydrates **(O'Leary *et al*., 2020).** Sugars are significant products of carbon and energy during photosynthesis. Sugars play essential roles in plant growth and development, crop yield and quality, and responses to abiotic stresses **(Ma *et al*., 2017)**. **.**The soluble sugar content increases with worsening heavy-metal pollution **(Zhou *et al*., 2019).**

Many studies have indicated that the accumulation of soluble sugar was noted under heavy metals, copper and cadmium **(Zhou *et al*., 2019),** copper **(Gong *et al*., 2019b)**, copper and lead **(Jiang *et al*., 2019**), copper, zinc and arsenic **(Salducci *et al*., 2019)**, nickel **(Ribeiro *et al*., 2020a)**, copper and zinc **(Hammerschmitt *et al*., 2020)**, also, under salt **(Abdel *et al*., 2019)**, salt stress in *Lycopersicum esculentum* **(Alavi and Abbaspour, 2020)**, drought stress in *Lycopersicon esculentum* **(Ewas *et al*., 2017)**, drought stress **(Abid *et al*., 2018)**, under light **(Bender *et al*., 2017)**, a decreased under drought stress **(Dien *et al*., 2019)**. However, many genetic and environmental factors influence sucrose metabolism and transport **(Julius *et al*., 2017)**.

- **Effect of copper**

The current study indicated that the leaves and roots of *Lycopersicon esculentum* Mill, exposed to different concentrations of copper, accumulated a high content of soluble sugar compared to the control seedlings. Notably, this sugar level in the leaves was higher than roots.

Our results corroborate the findings of **Yadav *et al*. (2018)**, who demonstrated that total sugar content was observed to increase by 22.76% under Cu toxicity conditions relative to control plants. Except for the entire content of soluble sugar in Cu 50 treatment, a significant increase was observed when compared with the control; in *Spartina alterniflora* **(Chai *et al*., 2014)**. This significant increase in soluble sugar has been noticed in Cu-treated plants by many studies; in *Coriandrum sativum* **(Zaouali *et al*., 2020)**, *Cinnamomum camphora* leaves **(Zhou *et al*., 2019a)**, and in *Colobanthus quitensis* **(Contreras *et al*., 2018)**. Similar to our data, **Shariat *et al*. (2017)** found that the concentration of soluble sugars in the leaves of *Eucalyptus camaldulensis* was more than two times for the 20 mM copper treatment than the control. Morover, **Shariat *et al*. (2017) and Zaouali *et al*. (2020)** showed that the soluble sugars increased progressively by increasing copper supply. These findings are in good agreement with our results.

As provided by several studies, the trend soluble sugar was depending on the copper concentration, plant species, toxic or essential metal, copper formulation, duration of application, sugar class, and the organ;

In contradiction with our findings, a reverse relation of metal concentration on carbohydrate content was noted by **Baruah *et al.* (2019)**. It has been demonstrated that an increase in total carbohydrate content was seen in different concentrations of Cu, and the highest increase of 63.0 % was observed in 5 mM. Reducing sugars were decreased in a dose-dependent manner, and the highest decrease of 208.8% was seen in 10 mM Cu. However, non-reducing sugars were highest in 51.71% in 5 mM Cu **(Sharma *et al.*, 2017)**. Along with decreased total sugar and reducing sugar, non reducing sugar also decreased in leaves **(Du *et al.*, 2018)**. In addition, depending on the metal toxicity, **Baruah *et al.* (2019)** showed that the highest reduction in carbohydrate content was noted at 220 ppm of cadmium exposure compared to lead and copper. According to **Kalaikandhan *et al.* (2018)**, the total sugar content of *the Sesuvium portulacastrum* shoot was found to be highest at 200 mg. kg^{-1} of copper level in all the sampling days. There was a gradual decrease in the total sugar content with a further increase in copper level, which occurred in all the sampling days. There was a gradual decrease in the starch content with increasing copper levels. The maximum decrease of starch content was recorded at 600 mg. kg^{-1} of copper level in all the sampling days. However, the starch content increased up to 200 mg kg^{-1} of copper level in all the sampling days.

Moreover, depending on the copper formulation, **Du *et al.* (2018)** showed the different trends in total/reducing sugar contents between nCu and µCu**.** For nCu, the highest inhibition was shown at 50 mg. kg^{-1}, lower inhibition at higher concentrations but insignificant between 100 and 200 mg. kg^{-1}. For µCu, higher inhibition was shown at higher applied concentrations, consistent with soluble Cu in soil. It indicates that excess Cu induced significant changes in plant nutrient quality, resulting in either the release of the ions into the soil (µCu) or combined with the presence of particles (nCu).

Differently, depending on the duration of application, it has been demonstrated that under copper stress, the endogenous level of trehalose (Tre) increased in the stressed seedlings by 45 and 50 % at days 4 and 7, respectively unstressed control. A further increase in the level of Tre was noted in the Cu-stressed seedlings due to Tre pretreatment, making the endogenous Tre level in the Tre-treated Cu-stressed seedlings significantly higher (41 and 39 % at days 4 and 7, respectively) than that in the Cu-stressed only seedlings **(Mostofa *et al.*, 2015)**.

Depending on the organ, our results concur well with previous findings, which proved that the accumulation of soluble sugar in leaves was higher than in roots **(Khedim *et al*., 2019 ; Negazz *et al*., 2019)**.

- **Effect of zinc**

It was found that zinc stress induces an accumulation of soluble sugar in leaves and roots of *Lycopersicon esculentum* Mill. This level depended on the zinc concentration. Remarkably, the sugar content in the leaves was higher than roots.

Our results corroborate the findings of **Kalaikandhan *et al*. (2018)**, who suggested that zinc treatment at low levels (100, 200 and 300 mg. kg^{-1} of soil) increased the total sugars content of shoot of *Sesuvium portulacastrum* in all the sampling days. With further increase of zinc levels (400, 500 and 600 mg. kg^{-1}), the total sugar content of *S. portulacastrum* was reduced in all the sampling days. Zinc treatment beyond this level decreased the starch content of *S. portulacastrum*. The lowest starch content was recorded at 600 mg. kg^{-1} of zinc in all the sampling days. The total sugar content of *S. portulacastrum* showed a progressive trend up to the growth stage (30th, 60th and 90th day) and gradually declined on the 120th day due to the senescence of leaves.

These findings prove that the accumulation of soluble sugar depended on the zinc doses and the growth stage. It also appears that other factors could be involved, such as the class of sugar, the metal (toxic or essential element), depending on the organ, plant species, tolerant or sensible species, and the combination treatment;

In contrast to our data, **Godinho *et al*. (2018)** found that the amount of both sucrose and glucose plus fructose decreased in tomato plants exposed to low concentrations of Cd, having the lower values at 0.5 mM. In plants exposed to 0.75 mM of Cd, the levels of sugars peaked to values higher than in control plants but then decreased again for higher concentrations to values lower than on control plants. A similar reduction has also obtained by **El-Esawi *et al*. (2020)**.

According to the current study, soluble sugar accumulation under copper and zinc stress was higher in leaves than roots. This result is in line with the findings obtained by **Negazz *et al*. (2019)** in *Raphanus sativus* under copper and zinc. **Khedim *et al*. (2019)** also indicated a similar result in *Atriplex canescens* as tolerant plant under higher doses.

Additionally, depending on the organ, **Zaouali *et al*. (2020)** found that the accumulation of these metabolites was higher in shoots than in roots. Interestingly, it has been shown that metabolite modifications in *Solanum lycopersicum* roots and leaves under cadmium stress. In the roots of control plants, sucrose content was about three-fold higher than that of glucose and fructose. In plants treated for ten days with 30 µM of Cd, glucose and fructose contents in roots were 2.6 and 4.8-fold higher, respectively, than in control, but sucrose content was not significantly modified. However, in the roots of plants treated for ten days with 300 µM $CdCl_2$, glucose and fructose contents did not change significantly compared to the control **(Zoghlami *et al*., 2011)**.

In contradiction with our study, depending on the metal toxicity and metal combination, soluble sugar content increased in the leaves of As-treated soybean plants, whereas a decrease in soluble sugars was recorded in roots **(Vezza *et al*., 2017)**. It is also confirmed by **Shen *et al*. (2019)** that soluble sugar content in the roots was lower under binary than trinary treatments, indicating that the combination of Zn and Cu exhibited improved effects of alleviating toxicity than each of them alone in Pb-containing combined treatments. In the leaves of *Kandelia obovata*, Zn-containing combined treatments significantly decreased soluble sugar in low concentration, while Pb + Cu treatments significantly increased these parameters.

The accumulation of soluble sugar in leaves and roots might be attributed to various factors relevant to the physiological, biochemical, and molecular mechanisms. Many scientific reports have clearly explained the findings;

Accumulation of osmoprotectants such as soluble sugars and amino acids help in maintaining the equilibrium across the plasma membranes, reduces cell osmotic potential and increases the turgor pressure in the cell **(Dikilitas *et al*., 2019)**. A similar hypothesis was also noted by **Bouazzi *et al*. (2019)**. Nevertheless, **Thalmann *et al*. (2016)** proposed that the regulation of leaf starch degradation was necessary for osmotic stress tolerance in plants. Significantly, these outcomes correlate favourably with our findings that highlighted a disruption in the level of relative water content (RWC), which was therefore regulated by the accumulation of soluble sugars in leaves of *Lycopersicon esculentum* Mill. This role is also a reason why the sugar compounds were defined as osmolytes.

These findings were similarly confirmed by **Sharma *et al*. (2019)**, who indicate that these soluble sugar compounds stabilize the osmotic differences between the cell's surroundings and the cytosol. Additionally, they also protect the plant cells from oxidative stress by inhibiting the production of harmful ROS like hydroxyl ions, superoxide ions, hydrogen peroxide, and other free radicals. Moreover, it has been suggested that soluble sugar might act as an antioxidant rather than an osmoprotectant since there is a significant decrease in water use efficiency (WUE) in response to increased Cu concentration **(Zaouali *et al*., 2020)**. Accordingly, as anticipated, under copper and zinc stress conditions, there is a positive correlation between the accumulation of soluble sugar and antioxidant compounds in the leaves and roots of *Lycopersicon esculentum* Mill.; so, all the hypotheses mentioned above match well with our study.

On the other hand, despite the over-accumulation of carbon compounds, the decline in respiration suggests a modulation at sucrose cleavage level by sucrose synthase and invertase **(Castañeda *et al*., 2018)**. This fits well with **O'Leary *et al*. (2020)** and confirms our results where soluble sugar accumulation participates in modulating the reduced transpiration (RWL) in leaves of *Lycopersicon esculentm* Mill.

It has been identified that the glucose-6-phosphate isomerase, cytosolic, suggested that the glycolytic pathway might be involved in plant response to Cu stress. Here, most of the identified glycolysis-related proteins were up-regulated, indicating that *Oenothera glazioviana* seedlings could maintain their essential respiration and provide more glycolytically generated ATP by reinforcing the citric acid cycle (CA) and glycolytic pathway under Cu stress conditions **(Wang *et al*., 2017)**. This reasonably well with our data, where the level of soluble sugar increase in concordance with elevation in the content of a particular group of proteins.

Hence, soluble sugar contents increase accordingly to protect biological macromolecular structure and stability and improve plant resistance to stresses to maintain normal metabolic activities **(Zhou *et al*., 2019a)**.

Many factors could affect the level of soluble sugar and justify the negative effect of heavy metals, such as duration of application, sugar class, phytohormone, gene expression, metal formulation, reduction, organ-organ, in the same tissue;

Depending on the duration of application, prolonged exposure to abiotic stress interferes with carbon metabolism and alters the cellular levels of certain sugars and polyols (alcohol derivatives of sugars) **(Abid *et al*., 2018)**. However, the increments in soluble sugar may also be due to short-term stress, which stimulates the plant leaves to release abundant carbohydrates to maintain normal metabolism **(Zhou *et al*., 2019a).** In this context, an approach was conducted to prove that plant tissues accumulate soluble sugar content under copper treatments to enhance their tolerance against heavy metal stress and consequently limited the phytotoxic damages. For instance, they were selected from the recent investigations the trehalose (Tre), a primary soluble sugar, as a model or marker of abiotic stress. It has been demonstrated that pretreatment of rice seedlings with trehalose (Tre) enhanced the endogenous Tre level and significantly mitigated the toxic effects of excessive Cu on photosynthesis and plant growth-related parameters. The improved tolerance induced by Tre could be attributed to its ability to reduce Cu uptake and decrease Cu-induced oxidative damage by lowering the accumulation of reactive oxygen species (ROS) and malondialdehyde in Cu-stressed plants. (Mostofa et al., 2015)(Mostofa et al., 2015)More critical, Tre-induced restoration of the levels of RWC and Chl in the Cu-stressed seedlings indicated an osmoprotective and membrane-protecting role of Tre for plants exposed to Cu stress as well as reduced lipoxygenase (LOX) activity and MDA (malondialdehyde) content **(Mostofa *et al*., 2015)**. These findings confirm why the soluble sugar was accumulated simultaneously in leaves and roots of *Lycopersicon esculentum* Mill., treated by copper and zinc. As expected, these compounds levels have the objective to improve the plant growth, low level of RWC and the content of chlorophyll and carotenoids recorded in our research.

Similarly, it has been demonstrated that the same positive effect was also obtained by other types of soluble sugars such as glucose, fructose and sucrose **(Zaouali *et al*., 2020)** and sorbitol, mannitol, fructans, and trehalose **(Qazi *et al*., 2019)**. Notably, **Contreras *et al*. (2018)** discarded the possible role of galactose to maybe act as a precursor of ascorbate because the enzyme analysis reveals that the ascorbate-glutathione (Asc-GSH) cycle did not participate actively in the tolerance to copper and aluminium, and ascorbate did not show changes along with the treatments.

As discussed in the previous sections related to the morphological and physiological parameters, plant hormones are major elements in cell plant communication; it has also interfered with biochemical and molecular mechanisms for regulating the heavy metal stress. As highlighted by **Sharma *et al*. (2019)**, the accumulation of osmolytes is further modulated by phytohormones. As depicted from the scientific reports, many researchers showed substantial interest in a specific plant hormone, which is the ABA. Abscisic acid (ABA) was pointed as the main regulator in plant stress and could also be accountable for plant growth, RWL, RWC, ... The results indicated by **Ma *et al*. (2017)** demonstrated that the abscisic acid ABA-responsive transcription factor MdAREB2 directly activates the expression of amylase and sugar transporter genes to promote soluble sugar accumulation, suggesting a mechanism by which ABA regulates sugar accumulation in plants. Significantly, ABA can be transported long-distance within the plant via its inactive form of ABA-glucose (ABA-GE) and is released by β-D-glucosidase under stress conditions **(Hartung *et al*., 2002)**. The physiological action of ABA justifies the decline of RWL and the increase of soluble sugar in tomato seedlings stressed by copper and zinc in the present study. Hence, these suggestions confirm the biological network between the signalling molecules and the metabolites. Moreover, ABA supports the implication of sugar compounds as osmoprotectants...

For more explanations, another class of phytohormones should also be pointed, is the BRs, brassinosteroids (BRs) are known for regulating cellulose synthase (CESA) gene expression required for the synthesis of cellulose, and they are considered as significant carbohydrate regulators and sugar accumulation under stress **(Kaur *et al*., 2014)**. This is was confirmed by many reports **(Zhang and He, 2015 ; Zhang *et al*., 2015).**

The genes involved in synthesising osmoprotectants such as proline, glycine betaine, sugars showed increased tolerance to abiotic stresses **(Dikilitas *et al*., 2019)**. At the molecular level, it has been demonstrated that overexpression of *TDDF1* activates some stress-responsive genes such as *DREB2*, *MAPK2* and *PR1*, which led to the accumulation of proline and soluble sugars to alleviate oxidative stress by eliminating ROS production **(Ewas *et al*., 2017)**. These demonstrations confirm our observations and justify the interest of the tomato plant to accumulate the osmolytes compounds of proline and soluble sugar in their parts (roots and leaves) concurrently, under copper and zinc stress. Moreover, even though for their osmoprotectant role, this hypothesis emphasis also the vigour function of these compounds as antioxidants. Their positive correlation might confirm this with antioxidants compounds (polyphenols, flavonoids, DPPH test).

Besides, auxin response factors (ARFs) are involved in auxin-mediated transcriptional regulation in plants. The SlARF6A gene is located in the nucleus and exhibits transcriptional activator activity. SlARF6A plays a vital role in the regulation of fruit quality and development. Overexpression of SlARF6A increased chlorophyll contents in the fruits and leaves of tomato plants, whereas downregulation of SlARF6A decreased chlorophyll contents compared with those of wild-type (WT) plants. It has been observed that the increased chlorophyll accumulation and photosynthesis rate in OE-SlARF6A tomato plants resulted in the increased contents of starch and soluble sugars in fruits. In this study, SlARF6A was positively correlated with the expression of AGPase genes, suggesting the important role of AGPase genes in starch biosynthesis in tomatoes. Auxin-responsive motifs were detected in the promoters of AGPase S1 and AGPase S2 genes. Evidence suggests that sucrose induces the expression of AGPase genes in leaves and fruits in tomato 472013. Overexpression of SlARF6A led to increased sucrose content in tomato fruits, while the RNAi-SlARF6A fruits displayed decreased sucrose accumulation. The altered accumulation of starch in OE-SlARF6A and RNAi-SlARF6A lines may be explained by the altered expression of AGPase genes influenced by sucrose in tomatoes. Overexpression of SlARF6A also resulted in increased glucose and fructose content, which was likely due to the increased starch content degraded into increased contents of soluble sugars in tomato fruits **(Yuan *et al*., 2019)**. Apart from this slight discordance with our data, the finding confirms the correlation between the elevation in chlorophyll content and soluble sugar accumulation. These results refer only to the particular class of sugar, which act differently depending on their function within a cell in stress conditions.

Inversely, in tomatoes, **Bouzroud *et al*. (2018)** found that the antisense down-regulation of SlARF4 promotes root development and density, increases soluble sugars content and maintains chlorophyll content at high levels under stress conditions. This justifies the simultaneous accumulation of soluble sugar in the roots and leaves of tomato seedlings to modulate the harmful stress effects in both tissues (for root elongation and chlorophyll content in leaves), corresponding to our study.

Besides, it has been indicated that the MdAREB2 transcription factor was found to bind to the promoters of the sugar transporter and amylase genes and activate their expression. Furthermore, MdAREB2 promoted the accumulation of sucrose and soluble sugars in a MdSUT2-dependent manner. These results demonstrate that the ABA-responsive transcription factor MdAREB2 directly activates the expression of amylase and sugar transporter genes to promote soluble sugar accumulation, suggesting a mechanism by which ABA regulates sugar accumulation in plants **(Ma *et al*., 2017)**.

It was found that soluble sugar content increase under copper and zinc stress, whereas **Du *et al*. (2018)** found that the downregulation of sugar by Cu was a possible indicator of stress. Significantly, this disagreement depended on the class of sugar, which was accumulated or decreased in the presence of stress. Additionally, different factors such as the plant tissues, metal concentrations and plant species exhibit a major position in determining the stress effects. In the tomato plant, **Chen *et al*. (2018)** speculated that the reduction or induction of soluble sugars contents in transgenic plants after drought treatment might be due to miR1916 affecting the expression of UDP-glycosyltransferases (UGTs) genes. Moreover, as proved in this discussion section, the copper formulation is also considered a limiting factor.

Further experiments with various particle sizes are needed to confirm the size-dependent effects of the toxicity induced by copper **(Du *et al*., 2018)**. It has been suggested that the concentration-dependent damage of polysaccharides by ZnO-NPs, ZnO-bulk and Zn^{2+} ions. Notably, a decrease in the absorbance and shift of the peaks supports the damage of phosphate backbone and C-O bond between ribose/deoxyribose sugars of nucleic acids (DNA and RNA) compared to untreated control **(Ahmed *et al*., 2017)**.

Subsequent reduction of soluble sugar may be due to blockage of decomposition and transportation of insoluble sugar caused by heavy metal stress **(Zhou *et al*., 2019a)**. Additionally, Cu may affect the biosynthesis of starch and sugar by disturbing the accumulation and translocation of nutrient elements **(Du *et al*., 2018)**. The reduced carbohydrate content of the emerging seedlings under metal exposure might be due to inhibition of chlorophyll biosynthesis as observed from the lower chlorophyll content **(Baruah *et al*., 2019)**. Furthermore, reduced sugars are related to altering the photosynthesis parameters **(Bahri *et al*., 2015).** In contrast with what was previously thought, we found that a decline in chlorophyll content induces an increment in soluble sugar. These findings confirm the hypothesis suggesting the emergence of different classes of soluble sugar under stress conditions that were consequently altered by the level of heavy metals. These effects might depend on each class's cell function as osmoprotectants and antioxidants or as photosynthesis products. Therefore, the fluctuation trend observed in some parameters could be attributed to the expression of different sugar classes.

Thus, all the present data need to be treated with caution; moreover, it is necessary to monitor the growth condition before and after stress treatment, affecting the trend level of soluble sugar in roots and leaves. Besides, extreme sensibility and highly controlled conditions must be taken for plant stress performance. Notably, an explicative experiment was performed by **Abid *et al*. (2018)** pointed out that after re-watering, a rapid reduction in sugar levels might be an indication of a quick breakdown of sugars upon relief from stress providing the plants with sufficient energy to repair damaged tissues**.**

Depending on the organ, it was found that leaves accumulate a high content of soluble sugar compared to the roots. This level might be due to the migration of heavy metals towards the leaves, where the more severe damages are the emphasis. Additionally, the leaf is considered a factory of sugar production using the photosynthesis process through a manipulative experiment conducted by **Camisón *et al*. (2020),** induced growth-limiting conditions in *Castanea sativa* seedlings to identify differences in the dynamics of soluble sugars, starch and total non-structural carbohydrates (NSC) in leaves, stems and roots. However, it has been indicated that root respiration rates corresponded with root carbohydrate and starch concentrations irrespective of treatments **(Tang *et al*., 2017a)**.

Notably, twice as many metabolites were modulated in leaves than in roots. Under cadmium stress, plants significantly reprogram the metabolic profiles of leaves rather than roots, which might subsequently impact harvest and crop quality. The leaves, pathway analysis revealed that six metabolic pathways were significantly disrupted. The larger number of disturbed metabolic pathways in the leaves than in the roots was unexpected, given that the leaves were not directly exposed to cadmium stress **(Tian *et al*., 2020)**. The accumulation in leaves regulates transpiration and stomatal aperture; alternatively, the lower stomatal aperture in the transgenic lines may also be due to the higher sugar content in the phloem **(Müller *et al*., 2018)**. These findings justify the role of soluble sugar in modulating the loss of water content in leaves of *Lycopersicon esculentum* Mill., recorded in our study. Furthermore, the concentrations of sucrose and glucose in the root followed the pattern of photosynthesis, indicating direct transduction of shoot sugar levels to the root **(Brauner *et al*., 2018)**.

Crucially, the accumulation of soluble sugar depended on the organ, either leaves or roots, but could also vary within the same organ. Subsequently, further analysis should be performed to precise the different patterns in the tissue of interest. For instance, it has been observed that upon water deficit stress, sucrose content increased 3- and 8- fold in the primary/taproot (tapR) and the lateral/fibrous roots (fibR), respectively, while the hexoses, glucose and fructose, accumulated exclusively in the fibre. An early blockage of sucrose metabolism occurs at the level of sucrose synthase activity leading to a marked accumulation of sucrose in the root **(Castañeda *et al*., 2018)**. These data confirm that soluble sugar analyses were based on the root section.

Differently, many studies proved that soluble sugar content depended on the cross-talk between leaves and roots, which was regulated by the stress signal of abscisic acid (ABA). This suggestion gives another reason for the high level in leaves than roots. **Yang *et al*. (2020)** indicate that the roots had a stronger water retention capacity than the leaves. This result was attributed to ABA accumulation, which promoted an increased accumulation of trehalose to maintain cell osmotic pressure. The results provided by **Fan *et al*. (2019)** showed that the soluble sugar content in the root, stem and shoot increased with the ABA concentration increasing at first and then decreased. The soluble sugar content in the leaf decreased with the ABA concentration increasing. The soluble sugar content in the root and stem peaked when the ABA concentration was five µmol. L^{-1}. Furthermore, the soluble sugar content in the leaf peaked when the ABA concentration was one µmol. L^{-1}.

VIII.4.3 Proline content

Several studies proofed that the abiotic stress induce the accumulation of proline content in the different part of plants. This effect was clearly marked under heavy metals, in *Cinnamomum camphora* in response to Cu and Cd **(Zhou *et al*., 2019a),** in spinach under copper **(Gong *et al*., 2019b),** in wheat under copper and pb **(Jiang *et al*., 2019)**, in *Astragalus tragacantha* under copper, zinc and arsenic **(Salducci *et al*., 2019)**. Precisely, the accumulation of proline was given in the tomato species, under heavy metals as Cd **(Lima *et al*., 2019),** Salt stress **(Poór *et al*., 2019 ; Siddiqui *et al*., 2019)**, drought stress **(Chen *et al*., 2018 ; Olivier and Nunes-nesi, 2018)**, Chilling tolerance **(Aghdam *et al*., 2018 ; Ghanbari and Sayyari, 2018)**, heat and drought **(Zhou *et al*., 2019b).**

- **Effect of copper**

Under copper stress, leaves and roots of *Lycopersicon esculentum* Mill. exhibited a high level of proline content compared to the control seedlings. As observed in the present study, leaves accumulate a high amount than leaves.

Our results are consistent with the findings of **Nazir et *al*. (2019)**, who suggested that Cu stress increased the level of proline in leaves of tomato in comparison to the control. Similar results were obtained in tomato leaves under copper stress by **Kisa (2019a).**

As provided by several studies, the accumulation of proline was depending on the concentration of copper or zinc, plant species, toxic or essential metal, duration of application, and the organ;

In term of depending dose, our results concurred with those of **Hanafy *et al*. (2017),** who indicated that proline content of tomato plants significantly increased gradually by increasing the Cu levels, as compared with control plants. Depending on the plant species and metal toxicity, similar results were also confirmed by **Rizvi and Khan (2018)** in *Zea mays* under Cu and Pb, by **Kalaikandhan *et al*. (2018)** in *Sesuvium portulacastrum* under Cu and zinc, by **Nannda and Agrawal (2018)** in *Cassia angustifolia* under copper. Similar to our findings, it has been reported that the content of proline in *Raphanus sativus* was elevated in plants treated with an excess of copper (100 to 250 mg. kg^{-1}), while the lower level of this osmolyte was obtained only by low doses of copper (50 mg. kg^{-1}) **(Chrithuuthayam *et al*., 2018)**.

In contrast to our results, **Maleva *et al.* (2018)** proved that Cu treatment caused a significant decrease in the proline content in *Egeria densa* and did not change its amount in *Ceratophyllum demersum***.**

- **Effect of zinc**

It was found that the application of different concentration of zinc caused the accumulation of proline in leaves and roots of *Lycopersicon esculentum* Mill.

Our results are in a good agreement with the findings of **Salimi *et al.* (2019)**, who showed that the increases in proline contents were provoked by applying the treatments of zinc 0, 50 and 100 mg. L^{-1} and obtained the content of 0.45, 0.54, 0.6, mg. g^{-1} FW, respectively.

Depending on the concentration, proline content in shoot of *Sesuvium portulacastrum* increased with increasing the concentration of zinc **(Kalaikandhan *et al.*, 2018)**, these results corroborate our findings. Moreover, depending on the metal toxicity, Menon ***et al.* (2018)** proved that the exposure to either metal (Co/Zn/Pb) in concentration resulted in a sharp rise in the proline content in the test plants.

On the other part, it was found that low and high content of proline was noted at 100 and 500 ppm, respectively, for copper and zinc. According to **Menon *et al.* (2018)**, depending on the duration of application, there was a progressive increase in the proline content up to the 28th day in the order 50 ppm < 100 ppm < 300 ppm < 500 ppm, these data are in line with our results. This result was also confirmed by who proposed that proline accumulation was of greater magnitude in the test plants especially at 500 ppm concentration in all salt. Another study conducted by **Kalaikandhan *et al.* (2018)** demonstrate that the minimum and maximum proline accumulation was recorded at 100 mg. kg^{-1} and 600 mg. kg^{-1} respectively, either for zinc or copper in *S. portulacastrum*.

Depending on the organ, it was found that content of proline accumulated in leaves was higher than that in roots, for copper and zinc. These results are in accordance with earlier studies conducted by **Girilal *et al.* (2018)**, who proved that proline content was accumulated in all the vegetative organs as well as the fruit when plants were subjected to stress; but, the highest concentration was found in growing leaves. Similar results were also indicated by **Alves *et al.* (2018)** in tomato under salinity. It was reported by **Saif and Khan (2018)** that proline accumulation followed the order: leaves > roots > shoots. In contradiction, these results differ from some published studies which suggested that proline content is higher in roots than leaves of tomato in control and salt stress **(Horchani *et al.*, 2010)**, similar results were noted by **Ullah *et al.* (2019a)**, and higher in roots than shoots in tomato plants **(Natarajan *et al.*, 2018)**.

According to **Kebert *et al*. (2017)**, root proline showed a strong linear relationship with endogenous Cu accumulated after exposure to the metal. Importantly, it has been reported that application of proline significantly increased the number of roots and root fresh weight **(The *et al*., 2016).**

Accumulation of proline in many plant species under stress has been correlated with plant tolerance, and its concentration has been shown to be generally higher in stress tolerant plants such as *Atriplex lentiformis* than in stress-sensitive plants **(Eissa and Abeed, 2019 ; Goni *et al*., 2018)**.

Under abiotic stress including heavy metal, many studies have justified the reasons behind the accumulation of proline in different part of plants.

Various investigations have been conducted to identify the role of a particular amino acid(s) during Cu heavy metal stress **(Kang *et al*., 2017).**

Chandrakar *et al*. (2018) argued that proline has a role as protective agent, where it enhanced the growth plant. Moreover, it is well documented in the literature that the compatible osmolytes such as proline regulate the osmotic potential of cells exposed to abiotic stresses **(Alyemeni *et al*., 2018 ; Chandrakar *et al*., 2017 ; Wiesenthal *et al*., 2019 ; Yadu *et al*., 2016).**

On the other hand, it has been observed that the net photosynthesis and transpiration were decreased by the application of proline in both control and salt stressed plants **(Orsini *et al*., 2018)**. Furthermore, proline mediates the elimination of ROS **(Alves *et al*., 2018 ; Alyemeni *et al*., 2018)** and directly scavenges OH radicals **(Chandrakar *et al*., 2018 ; Per *et al*., 2017)**. It also plays important roles during stress as a metal chelator **(Aslam *et al*., 2017)**. Additionally, proline protects folded protein structures against denaturation, stabilizes cell membranes by interacting with phospholipids, or serves as an energy and nitrogen source **(Arroussi *et al*., 2018 ; Per *et al*., 2017),** This osmolyte, proline, protects the plant cells from the lipid peroxidation damage **(Alves *et al*., 2018).**

From the previous researches, is difficult to elucidate the exact mechanism by it the proline can helps plant to cope up with heavy metal stress.

The available evidences suggest that proline acts by protecting the key enzymes from being inactivated by toxic metal ions **(Menon *et al*., 2018)**. This could be another reason why proline was considered as osmoprotector. In addition, there was a direct correlation between enhanced proline content and activities of anabolic enzymes namely P5CS Pyrroline-5-carboxylate synthetase and glutamate dehydrogenase (GDH) involved in its biosynthesis

(Aswani *et al*., 2018 ; Garg and Singh, 2018). However, it has been observed that proline dehydrogenase (ProDH) activity was induced by water deficit in both root types, exhibiting a higher activity in the primary or taproot tapR than in the fibR lateral or fibrous roots (fibR) **(Castaneda *et al*., 2018)**. **Aswani *et al*. (2018)** suggested that proline metabolism can help to mediate inter-organelle interactions. Furthermore, **The *et al*. (2019)** indicated that proline-treated rice roots showed up-regulation and down-regulation of nine and eight proteins, respectively, when compared to those in the control.

From all the explanations given above; in agriculture, improving the plant tolerance against the sensibility of heavy metals is among the challenges of the Sustainable Agenda 2030. Hence, recent approaches have been used to regulate and enhance the accumulation of proline content;

For example, inoculation plants by different types of bacterial species such as plant growth promoting rhizobacteria (PGPR) **(Bindu *et al*., 2018)**, and the inoculation with fungal species such as *Piriformospora indica* significantly enhanced proline content as compared to Cu alone **(Nanda and Agrawal, 2018)**. Other applications could have a similar effect; such as, exogenous glutathione **(Hasanuzzaman *et al*., 2018)**, exogenous melatonin **(Siddiqui *et al*., 2019)**, application of exopolysaccharide **(Arroussi *et al*., 2018)**. Also, by phytohormones; such as, jasmonic acid and nitric oxide **(Ahmad *et al*., 2018b),** with bacterial genus; such as, *Pseudomonas aeruginosa* on tomato plant under heavy metal stress **(Khanna *et al*., 2019b).**

Overall, the accumulation of proline in *Lycopersicon esculentum* Mill. was depending on the heavy metal (copper or zinc), its concentration in the soil, and the target organ (leaves and roots). So, proline as osmoprotector compound could be used to limit the phytoxicity by enhancing the tomato plant tolerance.

VIII.4.4 Polyphenols and flavonoids content

The accumulation of total phenols and flavonoids content in different organs of tomato plant under heavy metal stress has been reported by many studies, in seedlings under Cd **(Khanna *et al*., 2019),** in fruits under selinium **(Andrejiová *et al*., 2019)**, in roots and leaves under Cr and Pb **(Ullah *et al*., 2019b).**

The accumulation of the antioxidant compounds under copper under zinc stress was depending on the plan species, formulation of heavy metal as nanoparticles or other, organ, and the exogenous application;

- **Effect of copper**

Different concentrations of copper induced the accumulation of polyphenols and flavonoids content in leaves and roots of *Lycopersicon esculentum* Mill. These results corroborate with the findings of **Hanafy *et al*. (2017)**, who demonstrated that Cu stress caused a significant increase in the phenols contents of tomato leaves under different concentration of Cu. Similar results were also obtained by **Chrysargyris *et al*. (2019)** in *Mentha spicata* under copper. According to **Chung *et al*. (2018)** and **Singh *et al*. (2018a)**, the application of CuO nanoparticles significantly enhanced the polyphenols and flavonoids content, these findings were also confirmed by **Pérez-Labrada *et al*. (2019)** who discovered an increase of the phenols in the leaves by 16 % and the fruits by 7.8 %, compared with the control of tomato plant. Particularly relevant was the observation that flavonoids increased substantially in roots of *Solanum cheesmaniae* in response to excess Cu and decreased in shoots **(Branco-Neves *et al*., 2017)**. Besides to that , our findings contrast with those of **Kisa *et al*. (2019b)**, who found that the applications of Cu, Cd, and Pb significantly reduced the total phenolic content in tomato leaves.

- **Effect of zinc**

Under zinc stress, leaves and roots of *Lycopersicon esculentum* Mill. accumulate a high level of polyphenols and flavonoids. Notably, this content increased gradually by increasing the level of zinc in the soil.

Our results are in good agreement with the findings of **Ibiang *et al*. (2018),** who reported that the total polyphenols in fruits and shoot of tomato were significantly increased due to excess Zn. Similarly, **Chen *et al*., (2019b)** indicated that total phenolic compounds in roots and leaves was improved by increasing the gradient of Cd or Zn concentrations; total phenolic compounds significantly increased by 3.6 –44.6 % in the roots, and by 0.4–126.6% in the leaves.

In contrast to our results, the synthesis of antioxidants such as phenolic compounds decline as the concentration of Cd and Zn in leaves increase. These phenomena might be related to stress or the manifestation of a mechanism for tolerance to Cd and Zn accumulation **(Sakurai *et al*., 2019)**. Moreover, it has been proved that the contents of total phenol and flavonoids in the mature green fruits of tomato were reduced compared to control, following the n-ZnO nanoparticles treatment **(Akanbi-Gada *et al*., 2019)**.

It was found that polyphenols and flavonoids compounds were highly accumulated in leaves than roots. This result was obviously confirmed by **Ullah *et al*. (2019b)**. Also, by **Pérez-Labrada *et al*. (2019)** in tomato, where the level of these compounds was higher in leaves than fruit, in roots than shoot **(Natarajan *et al*., 2018)** in tomato, in fruits than shoots **(Ibiang *et al*., 2018)** in tomato treated by zinc.

The explanations given below justify the raison behind the accumulation of polyphenol and flavonoids compounds in the plants stressed by heavy metals.

It has been found that phenolic compounds involved in one of the defensive systems that the plants used against Cd and Zn stress **(Chen *et al*., 2019b)**. Furthermore, these compounds provide a defence against oxidative stress by acting as metal chelators and quenching of ROS **(Ullah *et al*., 2019b)**. Flavonoid and other phenolic compounds of plant origin have been reported as free radical scavengers **(Singh *et al*., 2018a).**

On the other hand, it has been indicated that Zn is a part of the antioxidant enzyme SOD (ZnSOD), thus it enhances the activity of quenching the ROS **(Ahmad *et al*., 2018c)**. However, Zn concentrations had a moderate negative correlation with both polyphenol levels and radical scavenging activity **(Sakurai *et al*., 2019)**. According to **Girilal *et al*. (2018)** phenolics possess hydroxyl and carboxyl groups and can bind to the metals. This may be the reason for the elevated level of secretion total phenolics in plants treated with silver nitrate. In this way, another reason was also given by **Chen *et al*. (2019b),** who indicated that an increase in soluble phenolic compounds such as intermediates in lignin biosynthesis increase cell wall endurance by the creation of physical barriers that protect cells against the harmful action of heavy metals, as well as influence the transition of metal ions within plant tissues since the lignification probably retains a substantial portion of metals into the cell wall fraction.

Many studies have explained the mechanism by which the phenolic compounds were synthesis;

It has been reported that plant phenolics are biosynthesized in plants from a biosynthetic intermediate, phenylalanine and shikimic acid through the shikimic acid pathway

(Naikoo *et al*., 2019). Under stressful conditions, **Zaho *et al*. (2018)** indicated that Shikimate phenylpropanoid biosynthesis was perturbed by excess copper. Therefore, Cd and Zn treatments affected phenolic compounds metabolism in *Kandelia. obovata* **(Chen *et al*., 2019b)**. According to **Soleimani *et al*. (2019)**, increasing phenols and flavonoids in tomatoes may arise from increasing PAL (phenylalanine ammonia-lyase) enzyme activity. This confirmed by **Aghdam *et al*. (2018).** However, the decreases in the phenolics should be results of the decline in the activity of crucial enzymes involved in the biosynthesis of phenolic compounds under the heavy metal stress **(Kisa *et al*., 2019b)**.

Earlier strategies have been used to enhance the plant tolerance against the heavy metal stress, this by elevating level of the cellular phenolic compounds within plant.

For instance, exogenous applications are used to improve antioxidant metabolism; such as, nitric oxide NO that increased the flavonoids and total phenol content in Cd- stressed tomato plants **(Ahmad *et al*., 2018b)**. The same effect was observed under the application of jasmonic acid **(Ahmad *et al*., 2018a),** and exopolysaccharide treatment in tomato **(Arroussi *et al*., 2018)**.

Overall, soil contaminated by different concentration of copper and zinc, affects the biochemical mechanism of *Lycopersicon esculentum* Mill. seedlings which contribute to the accumulation of the antioxidant compounds including polyphenols and flavonoids. However, these compounds were preferably found in leaves than roots. Thus, this accumulation of was depending on heavy metal (copper or zinc) and their concentration, and the organ (leaves and roots).

VIII.4.5 Antioxidant activity (DPPH radical scavenging activity)

DPPH is considered as a stable free radical which is used as a substrate to evaluate antioxidant activity **(Sanjari *et al.*, 2019)**. DPPH technique measures antioxidant activity by combining the antioxidants and analyzing the elimination of DPPH radicals **(Alam *et al.*, 2013)**.

- **Effect of copper**

The current study showed the elevation percentage of radical scavenging activity inhibition (DPPH) in leaves and roots of *Lycopersicon esculentum* Mill. under copper stress. The increase was proportional to the concentration of copper.

In addition, DPPH scavenging activity inhibition was depending on many factors such as; the stress (alone or in combination), plant species, the metal and its concentration, organ, metal formulation, metal toxicity;

Depending on the stress, our results are consistent with the findings of **Chrysargyris *et al*. (2019),** which demonstrated that polyphenols and antioxidants activity (DPPH) increased in single stress treatments (Salt or Cu), but decreased in the combined stress (Salt+Cu). Additionally to that , similar results have been reported on the positive effect of using copper in increasing the antioxidant activity of rice **(Hossain *et al.*, 2015),** of cauliflower and tomato plants **(Singh *et al.*, 2017)**. These results were also confirmed by **Ibrahim *et al*. (2017),** depending on the metal and its concentration**,** who found that DPPH antioxidant activity of two-heavy metals (cadmium and copper) exposure ranged from 29.2 % to 57.5 %, respectively. Cd 2 mg. L^{-1} treatments showed the highest DPPH activity compared to Cu 70 mg. L^{-1} treatments. With a two-fold increase in Cd and Cu levels the DPPH activity of the plant extracts decreased significantly in *Gynura procumbens*. Depending on the concentration, **Nazir *et al*. (2019)** indicated that Cu grown tomato plants showed greater ascorbic acid content, but the marked increase of 27.85 % was observed under Cu stress (100 mg. kg^{-1}) compared with control.

Depending on the organ, it was found that the antioxidant activity in leaves was higher than roots. According to **Pérez-Labrada *et al*. (2019)**, in tomato plant, the foliar tissue had a higher content of glutathione (GSH), total phenols, and flavonoids, as well as greater antioxidant activity by DPPH, compared with the fruit. However, in this regard, it was possible to appreciate that the antioxidant activity in the leaves was similar between the NaCl-treated and control plants.

According to the metal formulation and the type of stress, in this same study of **Pérez-Labrada *et al*. (2019),** the lowest activity was found in plants under salt stress with the application of Cu NPs, and the second lowest activity was observed in plants treated with Cu

NPs. Antioxidant activity by DPPH increased in the fruit of plants treated with Cu NPs and decreased in those exposed to salinity (− 1.2%). Similarly, **Pinedo-Guerrero *et al.* (2017)** reported an increase in the antioxidant content of DPPH in *Jalapeno pepper* fruits by applying Cu NPs to chitosan–polyvinyl alcohol in the growth substrate. Their observation of increased activity disagrees with the reduction observed by **Pérez-Labrada *et al.* (2019)** after applying Cu NPs, and the discrepancy is likely caused by interactions between ROS and antioxidants and a decrease in total antioxidant capacity. According to the metal toxicity and its concentration, a dramatic enhance in free radical scavenging activity DPPH was recorded under 250 and 500 µM cadmium levels by 101 % and 149 % respectively when compared with the plants control **(Sanjari *et al.*, 2019)**. In contrast to our results, **Bhagyawant *et al.* (2019)** proved that particularly free radical scavenging activity too show almost similar suppression either and/or assayed by DPPH, each metal treatment induces further steady reduction in antioxidant activity with increasing dose and accumulation of metal.

- **Effect of zinc**

It was found an elevation of scavenging activity inhibition (DPPH) in leaves and roots of *Lycopersicon esculentum* Mill. under zinc stress. The increase was proportional to the concentration of zinc. Notably, the percentage was higher in leaves than roots.

DPPH scavenging activity inhibition was depending on many factors such as; metal concentration, plant species, metal formulation;

These results are in agreement with the findings of **Pramanick *et al.* (2017)** which demonstrated an increase in the total antioxidant activity by increasing the dose of $ZnSO_4$. Total antioxidant activity of control plant (untreated) is found to be 5.49 mg AAE per g of FW tissue; whereas that of 1000 µM $ZnSO_4$ treated seedlings is found to be 9.66 mg AAE per g of FW tissue. There has been about 1.3, 1.5 and 1.8 fold increases in the total antioxidant activity with the addition of 500, 800 and 1000 µM $ZnSO_4$, respectively. Though there is not much variation observed in the free radical scavenging activity on increasing the dose of $ZnSO_4$.

Increases in DPPH radical scavenging activity with zinc fertilization have been reported in different plants, including cabbage, cauliflower, tomato **(Singh *et al.*, 2013)** black mustard **(Zafar *et al.*, 2016)** and pepper **(García-Gómez *et al.*, 2017),** these outcomes are in line with the results observed by **Abbasifar *et al.* (2020).** Furthermore, depending on the metal formulation and its concentration, and the metal combination, Abbasifar et al's study indicated that the minimum of antioxidant activity (30.69 mg. g^{-1} DW) was found in 2000 ppm Zn NPs + 2000 ppm Cu NPs treatment, whereas plants treated with 4000 ppm Zn NPs + 0 ppm Cu NPs showed the highest antioxidant activity (191.58 mg. g^{-1} DW) followed by 4000 ppm Zn NPs + 2000 ppm Cu NPs (190.14 mg. g^{-1} DW) and 2000 ppm Zn NPs + 0 ppm Cu NPs (187.73)**.**

May reasons act in the elevation of DPPH scavenging activity; phenolic compounds, flavonoids, brassinoids, oxidative stress, metal formulation, lipid peroxidation, vitamin B, pH, metal concentration, the presence or absence of metal;

The non-enzymatic antioxidant system measured as free radical-scavenge DPPH assay, and as reducing power, or as complementary assays, shows an induction of non-enzymatic antioxidant machinery, as a concentration-dependent in copper, concluding that *Colobanthus quitensis* responds to treatments **(Contreras *et al*., 2018).** This hypothesis is in good agreement with our results. On the other hand, the experiments described by **Costa *et al*. (2018)** indicated that the metal ions (Cu and Zn) in solution contribute to the reduction of the antioxidant activity as the antioxidant effect over the DPPH radicals occurs due to their capacity of donating hydrogen. The characterization of the synthesized compounds proves them as prooxidant agents. Moreover, **Mosa *et al*. (2018)** suggested that the increase of antioxidant content DPPH in tomato fruit after applying Cu NPs may be due to the capacity of NPs to induce oxidative stress. According to **Pramanick *et al*. (2017),** the increase of DPPH may be due to the increase in the rate of lipid peroxidation on treatment with $ZnSO_4$.

Pekal and Pyrzynska (2015) showed that the DPPH scavenging activity of the effluents were higher than those of the primary extracts and this may be related to the antioxidant capacity of these samples. This result argued that DPPH activity was depending on the extract or more specifically on the metal, this could justify the different level of DPPH scavenging activity obtained in our study by copper and zinc.

It has been demonstrated that the strong inhibition of DPPH radical, may be linked to the content of phenolic compounds which are capable of donating electrons or transferring hydrogen atom to neutralize free radicals **(Sharma and Ramawat, 2014)**. Furthermore, spearmint plants subjected to salinity and/or Cu stress presented increased levels of antioxidative mechanisms to overpass the induced stress conditions. In details, salinity or exposure to Cu did not affect total phenolic compounds content or antioxidant capacity as assayed by DPPH methods. In contrast, the combined effect of salinity and Cu decreased total phenolic compounds content and antioxidant activity (DPPH) indicating an effective amelioration of stress conditions **(Chrysargyris *et al*., 2019)**. According to **Sanjari *et al*. (2019)**, phenolic compounds were reported to be highly related with DPPH assay, suggesting that all the phenolic compounds contributed to the antioxidant potential of canola plant. These findings consistent with the results of **Xi *et al.* (2013)** in grape seedling treated by 24-epibrassinolide.

It has been indicated that the possible reason for the strong inhibition of DPPH radical is that these brassinolides (BRs) could increase DPPH radical scavenging capacity under Cu stress in *Raphanus sativus* plants with increasing production of secondary metabolites related to phenylpropanoid path enzymes **(Choudhary *et al*., 2011)**. Moreover, enhancing the DPPH radical scavenging potential along with increasing phenylalanine ammonia lyase (PAL) activity indicates the major influence of 24-epibrassinolide in the reduction of Cd-induced oxidative stress in canola plants **(Sanjari *et al*., 2019)**. In addition to higher ROS scavenging enzymes activity, higher phenylpropanoid pathway activity as shown by higher PAL enzyme activity resulting higher phenols and flavonoids accumulation, giving rise to higher DPPH scavenging capacity may be crucial for conferring chilling tolerance in tomatoes in response to exogenous phenylalanine application. Additionally, the higher proline and lycopene accumulation in tomatoes **(Soleimani *et al*., 2019)**.

All these reactions given above confirm the positive correlation between the DPPH and the phenolic compounds observed in our study.

Another relevant result, in tomato plant, application of H_2O_2 decreased the ascorbic acid content in stressed as well as in stress free plants in comparison to control **(Nazir *et al*., 2019b)**. Moreover, the relation between increasing of DPPH and vitamin B in many seeds were reported by **Chaichana (2016)**. Furthermore, DPPH radical scavenging assay performed, accounts for the ROS scavenging activity of the natural antioxidants present in the ethanolic extracts of *Plantago ovata* seedlings. Many studies indicated that metallothionein proteins has ROS scavenging activity **(Pramanick *et al*., 2017)**.

The decrease in antioxidant potential in the leaves under combined heavy metal treatment could be related to the phytochemicals, such as total phenolics, total flavonoids and saponin under these conditions. There was a positive correlation between total phenolics, flavonoids and saponin content with DPPH activity **(Ibrahim *et al*., 2017)**. However, **Sakurai *et al*. (2019)** showed a negative correlation between Zn leaf concentrations and polyphenol/2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. This finding might be related to stress or the manifestation of a mechanism for tolerance to Cd and Zn accumulation.

In some cases, flavonoids were less efficient in the reaction with DPPH radical in the presence of Al(III) ions in comparison with the systems without added metal **(Pekal and Pyrzynska, 2015)**. This study proves the effect of metal on the radical scavenging activity.

Moreover, it has been indicated that the moderate concentrations of $ZnSO_4$ were supported for the overproduction of CAT activity **(Rout *et al*., 2019)**. When the plants were exposed for 7 days, an enhancement of antioxidant activity was observed in both leaves and roots. This indicates to what extent pH has the influence on the results obtained in DPPH assay for natural samples, leading to differences in the estimation of their antioxidant activity **(Pekal and Pyrzynska, 2015)**. Generally, DPPH activity was influenced by the heavy metal treatments **(Ibrahim *et al*., 2017)**.

Thus, the high scavenging DPPH activity recorded in leaves than root could be explained by the fact of the great accumulation of the hydrogen's donating compounds in leaves compared to roots, including the antioxidants such as, polyphenols, flavonoids, proline, ..., which proved our results of the correlation between them.

Briefly, DPPH scavenging activity noted in the leaves and roots of *Lycopersicon esculentum* Mill. was depending on the metal (copper or zinc), its concentration, the organ. The Inhibition percentage was higher in leaves than roots. According to the previous study this level could be elevated by many factors and interactions.

VIII.5. Chemical parameters

Metals content are defined as a product of concentration and biomass of particular plant follow the metal concentration distribution **(Adamczyk-szabela *et al*., 2020)**. That's why , it is an urgently needful to do the metal profiling after treating plants under metal **(Rout *et al*., 2019)**.

The metal bioaccumulation mechanism modifies structural, biochemical, and physiological properties in vegetables. These modifications depend on the metal chemical species, concentration, and exposure time **(Wolf *et al*., 2017)**. The "Accumulation" means Here the concentration of a metal in the plant is higher than that in the soil **(Rong *et al*., 2020).** Metal uptake by plants strongly depends on their health status and should not be discussed without connection to the plant growth **(Adamczyk-Szabela *et al*., 2017).**

The efficiency of living plants at removing heavy metals mainly depends on plant species, type of metal, and plant metabolism **(Tang *et al*., 2017b).**

It is well known that plant species suitable for phytoremediation tend to accumulate heavy metals at the root level, avoiding the translocation to aerial parts to prevent and/or avoid their deleterious effects on the photosynthetic machinery **(Bonanno *et al*., 2017).** The Accumulation potential of plants depends on two main factors, i.e., metal concentration in soil and plants biomass for accurate metal quantity measurements **(Vymazal, 2016)**.

The accumulation of copper and zinc in the different tissue of tomato plant (leaves and root) will be discussed in the following section.

VIII.5.1 Heavy metals in plant

- **Copper content in leaves and roots of *Lycopersicon esculentum* Mill.**

Sevreal studies have reported the accumulation of heavy metals in the different plant species under, copper **(Khan *et al.*, 2020 ; Nazir *et al.*, 2019)**, copper and chromium **(Karimi *et al.*, 2020 ; Li *et al.*, 2018b ; Tamez *et al.*, 2020)**, copper and lead **(Peng *et al.*, 2020)**.

The present study demonstrated that copper was accumulated in leaves and roots of *Lycopersicon esculentum* Mill., this level was increase by increasing the copper doses in the soil. Our results are in agreement with the findings of **Saleem *et al.* (2020b)**, who indicated that this accumulation was also identified in the different parts of plant, Cu concentration in the nutrient solution enhances Cu concentration in roots, leaves and stems of *Hibiscus cannabinus*. Similarly, it has been found that *Cinnamomum camphora* can absorb Cu and Cd from contaminated soil and transfer them from root to stem and then to leaf **(Zhou *et al.*, 2019a).**

Copper accumulation was depending on the organ, plant species, metal formulation, the application mode of the metal, plant's sex, cultivated period, growth stage, and fertilizer application,

It was found that the copper content accumulated in leaves was higher than that in the roots. This corroborate the findings of **Amin *et al.* (2019)**, depending on the organ, which proved that for all plant species, the highest Cu accumulation was found in shoots than roots per plant because of high shoot biomass. Depending on the plant species, the results showed that *Avena sativa* accumulated the greatest amount of Cu in both root and shoot from 25 to 300 mg Cu. kg^{-1} treatment; whereas, a minimum content was accumulated in *Guizotia abyssinica*. Differently, it has been recorded that Cu concentration was lowered in roots and leaves, increased in the stem, and in total lowered by a factor of around 2. Zn content changed in a more diverse way, it was induced by a factor of 1.5-2 in whole plants, with stems having up to 4 or 5 times more of Zn than in control. Plants treated with Cd, Pb and Cd +Pb were generally distributing more essential metals toward their stems **(Kutrowska *et al.*, 2017)**. By contrast, the data obtained related to the copper accumulation in leaves and roots of lettuce suggested that most of copper accumulation was accrued in roots and the little portion of this metal was translocated in the leaves **(Shams *et al.*, 2019)**. Compared to the control, the maximum increased in Cu concentration was observed in the roots (103 mg. kg^{-1}) followed by leaves (88 mg. kg^{-1}) and stems (75 mg. kg^{-1}) **(Saleem *et al.*, 2020b)**, this is similar to the results of **Lwalaba *et al.* (2020)**. Additionally, it has been demonstrated that copper concentrations in the studied plants were up to 32.9 and 13 times higher in roots, stems and leaves, respectively, than in plants from non-spiked soils **(Minkina *et al*., 2019).** This result proved also the impact of the contaminated soil by copper on the plant, that why the minimum content of copper in the leaves and roots of *Lycopersicon esculentum* Mill. was observed in the control. Another study conducted by **Rehman *et al*. (2019c),** confirm that Cu has a propensity for the accumulation in the root tissues with little upward movement towards shoots.

Depending on the metal formulation, even though nCu treatments accumulated more copper in roots, no difference in shoot Cu was found, compared with μCu treatments at 50 and 100 mg. kg^{-1} **(Du *et al*., 2018)**. Accordingly, to our findings, and depending on the application methods, it has been found that Cu concentrations were higher in leaves that received foliar sprays, mainly as $Cu(OH)_2$. With the highest level of Cu applied via foliar as sulfate, Cu was eliminated by plant roots, increasing the rhizospheric soil metal levels **(Walter *et al*., 2018)**. Furthermore, depending on the sex of plant, it has been observed that males had a significantly lower stem Cu concentration, but higher leaf and root Cu concentrations than females. At the same time, under Cu stress, the stem Cu content in males was significantly lower than in females, which indicates that males have a higher ability to inhibit metal translocation from roots to shoots **(Peng *et al*., 2020).**

Depending on the cultivated period, the highest mean concentration of copper in the root was 0.208 mg. kg^{-1} occurred in *Brassica campestris* grown in winter, and the lowest mean concentration was 0.037 mg. kg^{-1} observed in *Sorghum vulgare* grown in summer **(Khan *et al*., 2020)**.

In *Lycopersicon esculentum* Mill., it was observed that the copper content in leaves and roots increase as the copper concentration in the soil increase. This result is in good line with **Huang *et al*. (2020b),** who found that leaf Cu concentration increased with Cu supply, a similar results was also obtained by **Lwalaba *et al*. (2020)** in root and shoot. Meanwhile, 20, 40, and 60 mg. kg^{-1} treatments of both Cu NP and $CuCl_2$ showed a significant increase, from 2.4 mg. Cu kg^{-1} leaf tissue (dry mass) to a range of 3.9 to 4.6 mg. Cu kg^{-1} leaf tissue (dry mass) **(Tamez *et al*., 2020)**. Differently to this positive trend in roots, the surprising decrease at a specific doses in leaves might be argued by the findings of **Tiecher *et al*. (2017)**, which indicated that there was no difference of Cu content in shoots with the addition of 30 and 60 mg Cu. kg^{-1}. Cu content in roots was higher with increasing Cu doses applied to the soil. The highest shoot/root ratio of Cu content was observed in treatments without Cu addition (1.0), while this ratio reduced significantly in treatments with the addition of 30 and 60 mg Cu. kg^{-1} (0.2 and 0.1, respectively). A similar trend was observed in relation to Cu distribution between the shoots and roots. Similarly, Cu accumulation results revealed that increasing Cu concentration in the soil significantly enhanced Cu contents in the roots and shoots of *Corchorus capsularis* compared to that in the plants grown in Cu-free soil **(Saleem *et al*., 2020a) .**

According to the outcomes of this study, *Lycopersicon esculentum* Mill. is an accumulator plant. Indeed, plant species vary in their capacity for Cu accumulation depending on growth stage and fertilizer application **(Rehman *et al*., 2019c)**. On the other hand, a hyperaccumulator plants had the ability to absorb and accumulate heavy metals in high concentrations in their above ground tissues without severe damage to vital physiological processes and plant growth **(Muszyńska and Hanus-Fajerska, 2015)**.

Many studies have explained the reasons of why plants accumulate the heavy metals, especially copper, in their tissues (leaves and roots) with different levels.

Besides, it has been indicated that the reason for the high contents of Zn and Cu in the leaves is that these elements are essential nutrients for tobacco plants **(Rong *et al*., 2020).** The trend of increased leaf Cu with increasing applied treatment supports the idea of the internalized Cu in all tissues being in the same form. However, the inconsistencies in root Cu throughout treatments suggests the source of Cu and concentration play a role in its transport to aerial tissues **(Tamez *et al*., 2020).** Importantly, the concentration in the above ground parts may be a poor indicator of plant status **(Rehman *et al*., 2019c).** The increasing concentration of Cu in the aboveground parts of *Corchorus capsularis* with aging seems to indicate that there must be some physiological mechanism working in *C. capsularis* that is associated with the bioaccumulation of Cu in different parts of plant. The uptake of heavy metals from the soil and its transport to different parts of the plant mainly depends on the Cu supply and growth conditions **(Saleem *et al*., 2020b)**. Moreover, the HMs compartmentalization with in *Petunia hybrida* L. was found to vary for each of HMs. With Cr, Ni, and Pb, significantly higher compartmentalization occurred in *Petunia hybrida* L. leaf. This represented that translocate HMs to above ground parts *P. hybrida* L. can lead to reduced plant vigour and aesthetics, as observed in this work that enhanced HM uptake occurred with facilitation of EDTA but with negative impacts on plant growth **(Khan *et al*., 2019)**.

The previous study showed that the uptake of metals, partition, and translocation to different plant parts as well as the degree of tolerance depends on the metal concentration and availability, the plant species, and metabolism **(Amin *et al*., 2019)**. According to the distribution of metals in the tobacco leaves, Zn absorbed more easily in the middle leaves and could be transferred to the lower and upper leaves **(Liu, 2010).** In contrast, Cu uptake in tobacco is more likely to occur in immature leaves **(Rong *et al*., 2020)**. Notably, according to **Rout *et al*. (2019),** the rate of absorption became lower with a higher concentration of stress which maybe due to the injuries caused by the applied metal.

However, depending on the growth stage, it has been noticed that at earlier stage of the growth Cu was highly accumulated in the roots while at the lateral stages of the growth Cu was highly transported to the shoots (aboveground parts of the plants) due to the formation of iron-plague in the roots **(Parveen *et al*., 2020).** The higher concentration of Cu in the root tissues in the earlier stages of the growth was associated with the low transportation of Cu in the Cu-tolerant species. Accumulation of Cu in plant roots may lead to the inhibition of fine root development and reduce the uptake of other trace element, e.g., iron **(Rehman *et al*., 2019c)**. On the other hand, the acquisition of Cu was found markedly higher in root followed by shoot. It is considered that the restriction of HM translocation from root to shoot could be a possible mechanism that reinforced the plants to riposte damage caused by Cu toxicity **(Rizwan *et al*., 2016 ; Adrees *et al*., 2015)**. During the initial stage, the Cu^{2+} adsorption rates of the root cell walls in both plants were fast. As time increased, the changes in the adsorbed quantities became increasingly smaller. These results further demonstrate that *Oenothera glazioviana* maintains its high resistance to Cu by limiting Cu accumulation and Cu adsorption in cell walls **(Fu *et al*., 2015).** It has been proved that at 50 µM Cu, the Cu distribution percent in organelles in the root and leaf cells decreased, but that in the cell wall and soluble fraction increased, as compared to that at 0 µM Cu. The decrease in Cu distribution percent observed at exposure concentrations that affect the structure of many organelles may just be due to effects on transport systems. For the same Cu exposure level and exposure time, Cu is mainly deposited in the cell wall, then chloroplast and the soluble fraction in the plant leaf cells. After increasing Cu to 100 µM, the distribution percent of Cu in the cell wall and chloroplast was increased distribution percentage in the cell wall and decreased distribution percentage in the chloroplast were noted in the plant leaf cells **(Parveen *et al*., 2020).** This result might jdtify the decrease of zinc content at 400 ppm noted in tomato roots. Hence, it has been inferred that the cell walls of *Oenothera glazioviana* roots prevent additional Cu accumulation in the cells and that fewer negatively charged cationic binding sites are present in the cell walls, thus resulting in higher copper exclusion when treated with high Cu concentrations. Remarkably, long-distance transportation in plants is an important topic of research in plant physiological ecology **(Fu *et al*., 2015).** Moreover, depending on the concentration, the normal concentration of Cu in the plants is 20–30 mg. kg^{-1} of dry mass. The Cu level accumulated by the plants must be transported and distributed to various parts of the plants for healthy growth and development **(Saleem *et al*., 2019b).**

In term of metal distribution, the connection between the different part of plant (leaves and roots) could be justified by the different mechanism of metal transport;

It has been indicated differences in copper (Cu) absorption and transport, physiological responses and structural characteristics between two types of Cu-resistant plants **(Fu *et al*., 2015).** Metal ions enter via highly active membrane transporters, they are compartmentalized into organelles such as the vacuole **(Sun *et al*., 2019).** Additionally, it has been indicated that plants absorb Cu^{2+} from the soil through the root, which further reach the aboveground part of plants through the xylem vessels. These ions are sequestered in the cell walls, vacuoles and the Golgi apparatuses through membrane transporter carriers **(Luo *et al*., 2016).** According to **Rehman *et al*. (2019d)**, there are two types of Cu transporters identified in plants. The first class is HMA family (P-type ATPases) and The COPT proteins belong to the Cu transport family (CTR). Among these, COPT1 is considered to be responsible for Cu acquisition by roots, while, COPT2 also localizes to the plasma membrane in the root tissues. Since reduction and complexation mechanisms of iron have been suggested to affect Cu speciation **(Ryan *et al*., 2013)**. However, the vacuolar targeting and degradation of the exporters AtHMA5 and SvHMA5II in response to high Cu concentrations seems a conundrum, as under these conditions the exporters are most needed for Cu extrusion **(Li *et al*., 2017b)**. According to **Huo *et al*. (2020),** not only Cu uptake in roots but also the change in cell wall constitution may play an important role in higher Cu concentration and accumulation in roots of rice seedlings supplied with NO_3^- rather than with NH_4^+ .

Indeed, plants tolerate Cu toxicity by immobilizing excess amounts in harvestable parts including leaves, cell walls and vacuolar membrane of root cortex **(Zandi *et al*., 2020).**

Regardless to our results, more explanations are needed to highlight the detoxification mechanism of copper in the plant;

The interaction of Cu^{2+} with mitogen activated protein kinase (MAPK) seems to be an important parameter to explore the mechanism of a possible detoxification effect of Cu^{2+} **(Liu *et al*., 2018a).** Detoxification of heavy metals occurs through a complex formation of phytochelatins (PCs) with metals through thiol coordination of the cysteine (Cys) present in the PC structure **(Piotrowska-Niczyporuk *et al*., 2020)**. Furthermore, detoxification of Cu through complexation is an important defence **(Chrysargyris *et al*., 2019)**. Thus, it could be hypothesized that other metabolic and cellular strategies may be used to detoxify *Spartina alterniflora* of Cu ions besides, complexation with organic acids, which deserves further investigation. In fine roots, citric acid might have no function in detoxification of Cu due to the unchanged level of citric acid in control and Cu 50-800 treatments and was significantly reduced when stressed by 1,000 mg kg^{-1} Cu **(Chai *et al*., 2014).** It has been also revealed that metal chelating compounds such as total thiols, protein bound thiols and non-protein bound thiols were enhanced in seedlings under Cd stress. These metal chelators usually consist of sulfhydrl (-SH) groups that effectively bind to metals in order to immobilise them **(Rizwan *et al*., 2016).**

Phytotoxicity of copper and zinc observed in *Lycopersicon esculentum* Mill. was due to their bioavailability. In this context, and according to **Zandi *et al*. (2020)**, the bioavailability and toxicity of Cu in plant and soil/ wetland systems depends critically on its chemical speciation. This characteristic will be discussed in the second part of soil speciation.

Zinc content in leaves and roots of *Lycopersicon esculentum* Mill.

Many studies indicated the accumulation of heavy metals in the different parts of plant such as, cadmium by **Liu *et al*. (2019)**, chromium by **Ponce *et al*. (2019).** Additionally, there are large genotypic variations in Zn accumulation by plants **(Cakmak *et al*., 2017 ; Caldelas and Weiss, 2017 ; Hussain *et al*., 2019).**

It was observed that zinc content accumulated in leaves and roots of *Lycopersicon esculentum* Mill. increased proportionally with zinc doses in the soil, while unexpected decrease was noted at 400 ppm. Similarly, depending on the concentration, it has been recorded that the results of increment was also obtained by **Hammerschmitt *et al*. (2020)** under zinc after 15 mg. kg^{-1}, who revealed that the zinc content in the shoots, roots, and soil changed with increased zinc concentrations in the soil. As the zinc concentration increased from 0 to 400 mg. Kg $^{-1}$, the zinc uptake by shoots in the control increased from 0.8 to 35.8 mg. kg^{-1} **(Min *et al*., 2020).** Additionally, **Guo *et al*. (2020)** demonstrated that zinc concentration in root, stem, and leaves increased by increasing of the zinc concentration, in comparison to the control. This is ties well with previous studies **(Ahmed *et al*., 2017 ; Rout *et al*., 2019 ; Xue *et al*., 2020).**

By contrast, **Cruz *et al*. (2019)** observed that the Zn concentration decreased from P1 (point1) of stem to the petiole, i.e. from root to shoot. The exception was the $ZnSO_{4(aq)}$ at 1000 mg Zn. L^{-1} that presented a different behavior, for example by the end of the experiment the concentration of Zn in the P3 point was higher than in P1. Moreover, the concentration of Zn in P2 was the same as in the petiole.

Zinc accumulation was depending on multiples factors such as, the metal concentration and the application period, organ,

Depending on the metal concentration and the application period, the results presented in our study could be more explained by **Rout *et al*. (2019),** who showed that the intake of supplemented Zn was increased in treated tissue samples and the maximum intake was found in 14 days treated plants. In day 7 treated plants, the maximum absorption (86.7 ± 1.6 ppm) was observed with 200 µM, which was two folds higher than the control (41.5 ± 1.0 ppm). About 3.9-fold increment. It was also noticed at the end of 14 days with 200 µM (166.7± 1.7 ppm) than control (41.9 ± 1.4 ppm).

Depending on the organ, it was found that zinc content in roots was higher than leaves, our results concur well with the data of **Montanha *et al*. (2020),** who found that the concentration of Zn in the roots was higher to the leaves of soybean (*Glycine max*), in control (50 mg. kg $^{-1}$, 37 mg. kg $^{-1}$) and $ZnSO_4$ (1580 mg. kg $^{-1}$, 320 mg. kg $^{-1}$) treatments respectively. On the other hand, it was demonstrated unexpected decrease at 400 ppm Cu. according to **Min *et al*., (2020),** who observed that compared with healthy tips, the most obvious change of tomato exposed to 400 mg. kg^{-1} Zn was the accumulation of metal in stems and root tips, especially in stem cells, many metal crystals were precipitated in the parenchymal cells, which might hinder nutrient transport.

Comparatively, it was found that copper was highly accumulated in leaves, whereas zinc was stored with high amount in roots. These results are in contradiction with previous findings which indicated that most heavy metals such as Pb, Cr, and Cu are absorbed and stored in crop root, while Cd, Ni, and Zn, etc. are easily transported to the upper part of crops **(Ahmad and Ashraf, 2011 ; Gaiss *et al*., 2019 ; Yan *et al*., 2019).** Furthermore, the non-edible part of tomato accumulated 84 and 80 % of Zn and Cu, 16 and 20 % was moved to the fruit. Similarly, tomato roots stored 33 – 47, 30 – 48 and 76 – 91 % of the total Zn, Cu and Pb absorbed by tomato, while the shoots stored 39–50, 37–51 and 9–18 %. Zn, Cu and Pb concentration in the root was higher than that stored in the shoot of tomato plants **(Almaroai and Eissa, 2020)**. Moreover, it has been proved that metal content in plant tissues varied among different combinations and followed the order Pb > Cu > Zn > Cd in roots, Zn > Cu > Pb > Cd in stems and Zn > Cu > Cd > Pb in leaves **(Kutrowska *et al*., 2017).** According to **Almaroai and Eissa (2020),** metal concentrations in tomato root were found to increase in the order: Ni > Cd > Cu > Zn > Pb but these orders in tomato shoot were increase in the order Ni > Cd > Pb > Cu >Zn.

The results obtained in the current study need to be more explained by the physiological and biochemical mechanisms. According to the previous studies, it has been showed that the distribution and accumulation of zinc in the leaves and roots was affected by many factors such as; bioavailability, mobility, transport of metal from root to leaves, metal dissolution, complexation, chemical speciation;

As positive factor, **Wolf *et al*., (2017)** demonstrated that bioaccumulated metal concentrations increased with the increase in the growth time, and zinc concentrations were always higher than copper concentrations. This clearly explain our results. On the other hand, it was also confirmed by **Cruz *et al*. (2019)**, who proved that the Zn content followed a linear function of time. By contrast, another factor could acts negatively, according to **Wolf *et al*. (2017)** the lower mobility of copper and its strong interaction with organic and inorganic colloids in soil lead to lower bioaccumulation in lettuce leaves.

According to **Hammerschmitt *et al*. (2020),** the increased Cu and Zn concentrations was observed in roots, but mainly increased Zn concentration in stems, shoots and leaves could be explained by increased availability of both elements in soil. Highest Cu and Zn tissue concentrations were observed in roots, where, mainly Cu, was more retained.

Remarkably, **Tiecher *et al*. (2017)** suggested that Cu content in shoots and roots were not affected by the addition of Zn, and there was not interaction between Cu and Zn treatments for these variables. Another study conducted by **Hammerschmitt *et al*. (2020),** indicated that the accumulation of zinc in the roots, these might be plants root system mechanisms to decrease translocation of Cu and Zn excess, but mainly Cu, to shoot organs. Due to this tendency of accumulating metals in the root system, the concentration of Cu and Zn in the roots can be a sensitive indicator to the presence and levels of these elements in the soil **(Ademar *et al*., 2018**). Importantly, the amount of dissolved Zn increased in the presence of roots. This effect is likely caused by the action of organic acids exuded by roots **(Cruz *et al*., 2019)**.

Similar to our findings, it has been also identified, that the concentrations of Zn in peach leaves cultivated in the soil with the addition of Zn rates higher than 30 mg. kg^{-1} exceeded these reference values. Such result may be attributed to the high Zn amounts found in the soil solution, along with similarity between the ionic radii of bivalent cations such as Cu, Manganese (Mn) and Iron (Fe) **(Hammerschmitt *et al*., 2020)**. This result might justify the surprising high content of zinc obtained in the roots at 100 and 300 ppm Zn compared to that supplied in the soil.

It was found that at 400 ppm Zn the content in root was lower than other doses. These findings might be argued by **Cruz *et al*. (2019),** who showed that the content of Zn in the stem also held a linear relationship with the concentration of soluble Zn in contact with roots. In addition to the decreasing Zn content from root to shoot, the uptake velocity diminished. It also shows that the plant trended to store Zn in the lower tissues, as there is a gradient from P1 towards the petiole. available again, a divergent behaviour was observed for $ZnSO_{4(aq)}$ at 1000 mg. L^{-1}, for this treatment the uptake velocity increased in the upper tissues. This study could also help to understand why Zinc was highly accumulated in root than leaves in *Lycopersicon esculentum* Mill. Another reason, is that the mechanism that trend to accumulate Zn in the lower tissues may be associated with an enhanced of Zn compartmentalization in vacuoles.

The accumulation observed in the lower tissue is also discussed by the effect of zinc on the stem as is a connector organ between leaves and roots; depending on the level of the metal, its solubility, and the formulation;

Special distribution of zinc in the stem regardless of the treatments, Zn is mostly concentrated at primary and secondary xylem vessels of Soybean seedlings **(Montanha *et al*., 2020)**. It has been observed that the high amount of Zn quickly saturated the lower tissues of the stem which gradually stopped accumulating, whereas the upper tissues were still able to keep storing Zn. Also, depending on the metal formulation, **Cruz *et al*. (2019)** show that the key factor controlling the uptake of Zn is rather the concentration of dissolved Zn than the concentration of the nanoparticle itself. The uptake of Zn from the 300 nm ZnO was smaller due to its lowest solubility. The amount of dissolved Zn, in turn, varied only slightly with the concentration of the dispersed ZnO and depended more on the nanoparticle size**.** This may be justifying the limited zinc absorption at 400 ppm Zn compared to 300 and 500 ppm Zn.

Differently, according to **Montanha *et al*. (2020),** the amount of zinc absorbed by the plant as defined in the root- solution interface, factors such as charge and diffusion coefficient might play significant roles. On the other hand, the zinc source did not affect transport velocity in the stem. The stem seems to act as buffer storing the Zn excess protecting the photosynthetic apparatus in soyban leaves, no symptoms of intoxication such as stain, spots, or leaf wilting were observed past 48h exposure.

Studying the accumulation and distribution of zinc in the plant is considered as a key to define the toxicity of the metal. It has been suggested that the toxicity of ZnO-NPs may not alone be attributed to their dissolution at the root surface, but also inside the tissue **(Lee *et***

***al*., 2013)**. This why the present study was based on the accumulation of metals in the organs. Hence, it is important to determine the different mechanisms involved in the accumulation of copper and zinc in *Lycopersicon esculentum* Mill.

The accumulation of copper and zinc in tomato plant may be due to the high absorption. This is explained by the fact that Zn and Cu in solution in the soil occur mainly in the Zn^{2+} and Cu^{2+} forms, being these forms preferentially absorbed by plants. The absorption of metal ions from the soil solution is mediated by specialized transporters present in the plasma membrane of the root cells **(Ademar *et al*., 2018)**. Moreover, **Zúñiga *et al*. (2019)**, proved the presence of three Metallothioneins that allowed copper and zinc accumulation in vivo.

On the other hand, it has been indicated that the limiting factor controlling the content of Zn in the stem is the entrance of Zn in the root appoplast and symplast rather than the loading of zinc from roots into the primary and secondary xylem or the transport along the xylem itself **(Montanha *et al*., 2020)**.

Notably, increased Zn concentration in soil could stimulate apoplastic transport of ions or/and activate non-specific ion channels **(Kutrowska *et al*., 2017)**. Depending on the plant species, in the case of Coontail, it has no roots and so it does not have the benefit of the discriminatory selective transport of metals from roots to shoots found in rooted plants. The rooted plants described above took up Zn by the roots but did not transfer it to the rest of the plant because of selective loading of metals into the xylem stream at the root cortex **(Hak *et al*., 2020).** This might justify why the Zinc was highly accumulated in root than leaves.

All these findings support the reason why the root was selected as a main organ in the current study, and justify the supply methods of copper and zinc by root and not by foliar application.

Inside the plant, metal accumulation was influenced by other factors such as, the complexation and chemical speciation. These factors could be the main reason for the metal toxicity and the pattern distribution;

Montanha *et al*., (2020) showed that zinc complexed to phytate / phosphate is frequently reported in plant tissues. Additionally, the complexation also ensures that the metal ions reach the target tissue either than precipitating in the way up. Regardless the source, the zinc distribution and its transport in the stem were specially correlated to the bundles and cortex nearby the epidermal cells. The chemical speciation showed that Zn is neither transported as $ZnSO_4$ (aq) nor as Zn-EDTA (aq), indicating that these compounds are retained in the roots or biotransformed on in the root- solution interface. Zn^{2+} was long distance transported complexed by organic molecules such as histidine, malate, and citrate, and the proportion of ligands was affected by the concentration of Zn^{2+} in the stem rather than by the type of Zn source. Additionally, it has been proved that the coexistence of two ring pattern and the accumulation of zinc in the epidermal region, were previously reported for *Phaseolus vulgaris* **(Cruz *et al.*, 2017)**, *Zea mays* **(Lv *et al.*, 2015).** Since there is no concentration gradient from the xylem to the epidermis, it is still not clear how zinc is loaded in the epidermal layer and how that image pattern is formed. In any case, the epidermal layer is supposed to act as storage tissue under high Zn concentration **(Cruz *et al.*, 2017)**. According to **Ponce *et al*. (2019),** it is noteworthy that the accumulation of metal in the former was significantly lower in buffered plants, whereas in the latter it was lower in unbuffered plants.

Clearly, it has been demonstrated that the accumulation of copper and zinc could act positively on the plant. In this context, it has been demonstrated that heavy metal stress affects nutrient uptake in plants by interacting with other essential minerals **(Sharma *et al.*, 2020)**, increasing in the concentration in the soil has main role in enhancing the plant nutrients. On the other hand, it is necessary for plant to accumulate the zinc in the upper part as well as the roots parts of *Lycopersicon esculentum* Mill.

These two hypothesis were confirmed by **Rout *et al*. (2019)** which indicated that the accumulation of Zn facilitated the uptaking capacity of macronutrients like K and Ca which were more absorbed in high concentrations of Zn. However, unequivocally indicates that combined cadmium-zinc interactions significantly alter manganese, lead and copper uptake by *Melissa officinalis* **(Adamczyk-szabela *et al*., 2020).**

Overall, the different concentration of copper and zinc in the soil were absorbed by roots system, translocated into the upper organ (leaves), distributed and accumulated depending on the organ, the metal and its concentration.

VIII.5.2 Chemical speciation, mobility and distibution of heavy metals in soil

Generally, this section (link between tow compartments of: soil and plant), it is considered as thesis’s axe for the reason that could explain the metal transfer from soil to the different part of the plant, depending on their concentration, chemical forms, and bioavailability.

The total concentration of metals has been used as an indicator to evaluate soil contamination **(Rong *et al*., 2020)**. Notably, soil is a medium for plant growth and provides support, minerals and water to the plant for survival **(Liang and Yang, 2019)**. Several studies have attempted to predict the so-called “phytoavailable” fraction by correlating plant responses with different soil metal pools **(Lillo-Robles *et al*., 2020)**. Determination and recognition of relative distribution of chemical forms of each element and their relationship with physical, chemical and soil clay minerals can help researchers to manage soil fertility better **(Shakeri and Saffari, 2020)**. Additionally, it is explained that a scientifically sound risk assessment of soils has to be based on the bioavailable fraction of total essential element present in the soil as well as of the essential element added **(Tilborg, 2020)**. This suggests that the environmental risk would mainly be related to the Cu and Zn contamination level rather than to the changes in Cu and Zn availability in amended soils **(Laurent *et al*., 2020)**. According to **Huang *et al*. (2020a)**, the root soil interaction affects metal bioavailability in the rhizosphere, thus impacting the uptake and accumulation of metals by plants.

Chemical speciation of copper in soil

Toxic concentration of Cu in soil can cause nutrient imbalance by binding with organic matter, clay minerals, and hydrated oxides of iron (Fe), aluminum (Al), and manganese (Mn), which affects the plant productivity and crop yields **(Rizwan *et al*., 2016).** Additionally, it has been indicated that shoot biomass was well explained by exchangeable soil Cu free Cu^{2+}. However, the coefficient of determination was higher for exchangeable soil Cu **(Lillo-Robles *et al*., 2020)**. All these reasons make our research focus on the both fractions (organic and exchangeable fractions).

According to the sequential extraction results obtained in this study, the copper content in the organic and exchangeable fraction increased proportionally with its dose applied in the soil, exceptionally a sharp decrease at 500 ppm Cu was noted for the organic fraction. It was also indicated that the content of copper bound to organic fraction was higher than that presented in the exchangeable fraction.

Metal distribution was related to many factors of: metal fraction, rhizosphere, study area, season, amendments, the period application, and the metal formulation, ...

1- Exchangeable fraction

Regarding to the rhizophere, our experiment corroborates with earlier results obtained by **Huang *et al*. (2020a),** who suggested that the percentage of Cu fractions in the non-rhizosphere soil of control treatment was ranked as: residual > reducible > oxidizable > exchangeable. The percentages of exchangeable Cu, reducible Cu, and oxidizable Cu to the total Cu increased significantly with the addition of Cu. By contrast, in the non-rhizosphere of Cu 400 treatment, the proportions of Cu fractions were ranked as: reducible (31.77%) > exchangeable (29.74%) > residual (19.41%) > oxidizable (19.08%). This hypothesis may confirm our results.

In contradiction with our findings **Almendros *et al*. (2020)** showed that the recoveries for heavy metals Cd, Cr, Cu, Ni, Pb and Zn in the standard reference material varied between 88% and 94% for the exchangeable fraction (F1). The ratios were determined to vary between 90 % and 103 % for the reducible fraction (F2) and between 90% and 92% for the oxidizable fraction (F3). **Huang *et al*. (2020a)** demonstrated that after cultivating castor bean plants, the concentration of acid exchangeable Cu in rhizosphere soil was higher than that in the bulk soil for the same Cu addition, whereas the concentrations of reducible Cu, oxidizable Cu, and residual Cu in the rhizosphere soil 2 were all lower than those in the bulk soil, respectively.

Importantly, the differences in the copper values obtained in the various studies could depend on the study areas **(Khan *et al*., 2020).** Similarly, it has been proved that the Cu content in oxidizable (F3) fraction and sum of fractions can be ordered as mineralization area soil > agricultural soil > control soil **(Sungur *et al*., 2020).**

Depending on the season, **Ideriah *et al*. (2013)** revealed that at the spill site, the highest concentrations of Cu 22.8% (0-15 cm) and 24.9% (0-15cm) were obtained in the exchangeable fraction in the dry and rainy seasons respectively. At the control sites the highest concentrations of Cu, 34.9% (15-30 cm) and 35.2% (15-30 cm) obtained in the Fe-Mn oxide fraction in the dry and rainy seasons respectively occurred at control 1.The lowest concentrations of Cu 11.7 % and 12.6 % (0-15 cm) were obtained in the residual fraction in the dry and rainy seasons respectively. According to **Taghipour and Jalali (2019)**, in treated soils, the highest percentage of Cr, Cu, Ni, Pb, and Zn were associated with residual (RES) fraction and the order of fractions was the same as in control soils. In most treatments, the majority of Cd was in the exchangeable

(EXC) fraction indicating potential mobility of Cd in these treatments. These findings confirm the order of fractions in the control soils recorded in our study.

Returning to the amendment, **Hamid *et al*. (2019)** found that the organic amendment converted the soluble forms of metals (EXC fraction) to organically bound fraction and thus decreased their availability to plants, this explain our data. Similarly, it has been also shown that the contents of all heavy metals were decreased in exchangeable fraction (EXC), while they increased in organic matter (OM) and residual (RES) fractions after the addition of both organic wastes **(Taghipour and Jalali, 2019).** All these data are in good agreement with our findings where the exchangeable fraction decrease as the organic fraction of the metal in the soil increased. Moreover, sequential extractions results obtained by **Munir *et al*. (2020)**, showed a reduction in the exchangeable fraction of Cu, Cd, Pb, and Zn and elevation in the residual fraction following the addition of biochar (BC-2%) and BC-HTCG (hydrothermally treated coal gangue HTCG). According to the metal, in unamended copper mine tailing (Cu-MT), Cu, Pb, and Zn were distributed mostly in the residual (RS) fraction ranging from (28.87 % – 41.76 %), carbonate (CB) fraction (20% – 29%) and exchangeable (EX) fraction (17.34% –30.54%), whereas Cr and Ni were dominantly distributed in the organic (OM) fraction ranging from 30.23% to 54.14%. The most primarily Cd occurred in CB, RS, and EX forms, occupying 42.39%, 24.32%, and 12.56%, respectively of the total Cd in the copper mine tailing (Cu-MT).

Basing on the period application and the metal formulation, it has been demonstrated that copper fractions in soil after 60 days, only 1.1–2.4% and 0.5 – 0.8% of total Cu applied was found as soluble Cu for nCu and µCu, respectively. In addition, compared to soil amended with µCu, the acid-extractable Cu in soil amend with nCu was 25.5% and 150.0% higher at 50 and 200 mg. kg^{-1}, respectively, but without significant difference at 100 mg. kg^{-1} **(Du *et al*., 2018)**. These results confirm the impact of the amendment and the metal formulation on the solubility and the distribution of copper in the organic and especially on the exchangeable fraction, this directly justify the results of our experiment which based on the use of mixed substrate (sand and the organic matter as compost).

2- Oxidizable fraction

The high level of Cu in oxidizable fraction obtained in the present study was also reported by **Du *et al*. (2018),** who showed that among the different fractions, oxidizable fraction contributes to 80.7%, 70.0%, and 81.2% in nCu treatments of 50, 100, and 200 mg. kg^{-1}, respectively, and 58.1% and 62.5% for µCu at 50 and 100 mg. kg^{-1}, respectively; the residual fraction represented 56.5% of µCu applied at 200 mg. kg^{-1}. **Shakeri and Saffari (2020),** found that the distribution of chemical fractions of both Zn and Cu followed the order: residual > carbonate > organic fractions. Previous studies have shown that nCu is complexed with soil organic matter in potting soil **(Zuverza-Mena *et al.*, 2015)**, while the not complexed μCu explain for the vast majority of residual fraction **(Du *et al.*, 2018)**. The behaviour of free metals was expectedly the exact opposite of what was observed with fulvic acid bound metals (FA-Metal) species. In general, the acid- sandy- forest soils had the highest free metal species as single and mixtures. For metal mixtures, the pattern of percentage metal bound to fulvic acid was Co < Ni < Zn < Pb < Cu which was the opposite pattern for free metals. Metal speciation did not have particular patterns both for metal bound to Fulvic acid and free metal ion concentration **(Jegede *et al.*, 2020)**.

According to **Du *et al*. (2018)**, the mechanism underlying the morphological distribution of nCu in soil associated with the added concentrations still needs further studies.

The bioavailability of heavy metals in soil is closely related to the distribution of their different fractions and mineral components **(Zhang and Zhou, 2020)**. It has been suggested that exchangeable soil Cu was a better indicator of phytotoxicity than either total soil Cu or free Cu^{2+} in the soil solution, in soil series **(Lillo-Robles *et al*., 2020)**. Copper in soil can be present in several soluble (hydroxy and carbonate) and insoluble (oxide and sulfide) forms, while the availability of soluble Cu depend on soil pH, clay content, and concentration of organic matter **(Rehman *et al*., 2019c).** Furthermore, clay, silt, and CEC showed the greatest effect on the chemical forms of Cu, respectively. The results suggested by **Shakeri and Saffari (2020)**, showed that different forms of Zn and Cu are directly related with 2:1 silicate mineral. All these explanations give the reason of why the soil physical-chemical properties were determined in the present study.

The decrease of copper in the exchangeable fraction could be explained either by the chelation and precipitation of copper by organic matter present in these soils or by the use of copper by the plant for the maturity of fruits **(López-Vargas *et al*., 2018)**. Soils with very low bioavailable Cu can result in poor growth and losses in crop yield, especially in intensive farming systems, even when all other nutrients are present in required amounts **(Rehman *et al*., 2019c)**, Additionally, a study conducted by Rehman *et al* reported also prominent reduction of 96% in Cu availability after addition of 10% rice straw biochar in a Cu polluted soil.

It has been explained that polyvalent metal ions may serve as cross-linking agents within the organic phase by binding to multiple functional groups from different strands of humic macromolecules. At the same time, the soil organic matter (SOM) plays a major role in the interception of exogenous inputs **(Zhang and Zhou, 2020)**. As a consequence, SOM-bound heavy metals could also be removed from the soil along with the released SOM. Generally, higher concentrations could supply more active groups to bind and remove more heavy metal ions **(Zhang *et al*., 2019a)**. So, according to our results, copper and zinc as bivalent ions have high affinity for the organic matter.

Compared with other heavy metals (e.g. Pb), Cu showed a stronger affinity for organic matter, which suggested that the dissolution of SOM from soils is another probable mechanism for Cu extraction by EDTA. Soil copper and lead were mainly in the combination of fulvic humic acid, organic binding state, and reducible oxide. In the case of Cu, the content of exchangeable copper with high bioavailability was the same in each sampling point **(Zhang and Zhou, 2020)**.

Furthermore, the soil texture, as a crucial parameter in this study, could be one of the reasons that induced copper and zinc ions to be highly attracted for the organic fraction sites compared to the exchangeable (As found, the soil is loamy fine sand). In this context, **Jegede *et al*. (2020)** proposed that the loamy soil had the highest lead bound to Fulvic acid (FA-lead), and together with Loam-Sandy had the highest FA- copper. However, Loamy-Alluvial had the highest FA-cobalt, nickel and zinc. Only zinc maintained the same speciation pattern from single to mixtures across all the five soils. This proposal could explain why the level of copper was higher in organic fraction.

Although Cu and Zn show some mobility in sandy soils, there is generally a gradient of concentration that is higher in the top soil and decreases with depth **(Brunetto *et al*., 2014).**

In the present research, the lower content of Cu in the organic fraction obtained at 500 ppm might be explain by the hypothesis given by **García-Gómez *et al*. (2020)** who pointed out that at higher soil concentrations, possible agglomeration processes, together with higher pH levels and greater adsorption to the soil solid components, led to a lower percentage of available Zn in relation to the total. Additionally, it was noted that the sharp decrease of copper content in the organic fraction at 500 ppm Cu was simultaneously accompanied by an increase in the exchangeable fraction. This result gives an overview on the exchange occurred between the both fractions, to ensure an equilibrium reaction in the soil and release the metal by removing or desorpting the ions from solid phase to the soluble one. All these chemical mechanisms are highly activated in the presence of tomato rhizosphere, and the desorption should be in term of metal-selective. Another reason of the detected decease at 500 ppm Cu might be of the manipulations error.

Chemical speciation of zinc in soil

Cu and Zn content available in soil increased according both element dose increases **(Hammerschmitt *et al*., 2020)**. Similar results were also obtained by **García-Gómez *et al*. (2020)** with the zinc oxide (ZnO) application. According to the results indicated by **Shakeri and Saffari (2020**), the amount of DTPA-extracted Zn was obtained as available form of Zn for the plants in the range of 0-3.8 mg. kg $^{-1}$ (the average of 0.73 mg. kg $^{-1}$) in the studied soils. The behaviours of Zn in soil and its availability to crops depend on the concentration and percentage of each Zn fraction to soil total Zn **(Liu *et al*., 2020)**. It has been demonstrated that about 10–100 ppm of zinc is present in soil which is generally immobile **(Bhatt and Maheshwari, 2019)**.

It was found that zinc level increase in the both fractions as its concentration in the soil increased. An expect dcrease in the organic fraction was detected at 300 ppm Zn compared to 200 ppm.

Zinc content in the soluble fraction was lower than that bounded to the organic fraction.

According to many reports, chemical speciation of metal in the soil was depending on the concentration, the concentration and the cultivated crop, the season, soil depth, growth period, metal, plant species, pH soil, ...

1- Exchangeable fraction

Similar to our findings, **Liu *et al*. (2020)** indicated that repeated Zn fertilization over the multiple years increased concentrations of all soil Zn fractions. However, the percentage of each Zn fraction to total Zn varied with Zn inputs. Increasing Zn input increased the percentages of Ex-, Carb-, MnO- and FeO-Zn to soil total Zn, whereas reduced that of OM- and Res-Zn. Our results are also consistent with the findings of **Min *et al*. (2020),** who demonstrated that as the Zn level increased in the soil, the water-extractable Zn content remaining in the soil increased from 6.5 to 255.5 mg. kg^{-1}. In contrast to our findings, Zn applied to soil increased the Zn concentration in labile fractions in decreasing order as follows: ZnExc > ZnOM > ZnCarb **(De Carvalho Leite *et al*., 2020)**. The majority (67 – 90 %) of the added Zn remained isotopically exchangeable after 60 days. Zinc in the solution phase of a soil suspension was present mainly as free Zn^{2+} ions **(Mossa *et al*., 2020).**

Relatively to the concentration and the cultivated crop, it has been shown that the average concentration of extracted ZnExc ranged from 0.40 (control) to 2.72 mg. kg $^{-1}$ (8 mg. kg $^{-1}$ rate) in the soil cultivated with rice, which demonstrates how the applied rates increases Zn in the exchangeable fraction **(De Carvalho Leite *et al*., 2020)**. Depending on the season, ZnExc, which is weakly bound in soil particles, but became available easily in the soil solution through exchanges of chemical bonds. These chemical forms can be related to many factors described by **Ideriah *et al*. (2013),** who found that the highest concentration of Zn at the spill site, 23.4% (15-30 cm) was obtained in the carbon fraction in the dry season and 22.8% (0-15 cm) in the exchangeable fraction in the rainy season. Whereas, at the control sites the highest value of Zn, 23.7% (0-15 cm) was in the exchangeable fraction at control 1 in the dry season and 26.5% (15-30 cm) in the exchangeable fraction at control 2 in the rainy season. On the other hand, ZnE was determined throughout the growth period (4 times) and revealed no change in Zn lability, indicating that any variation in Zn uptake or toxicity cannot be attributable to a change in Zn lability during the growth period **(Mossa *et al*., 2020).**

Focusing on the metal, our results share a number of similarities with **Rong *et al*. (2020)** findings who suggested that the exchangeable fractions of Cd, Pb, Cu, and Zn and the reducible fractions of Pb and Cu are the main bioavailable fractions. Additionally, the reducible fractions of Cd and Zn, the oxidizable fractions of Pb, Cu, and Zn, and all residual fractions of metals were non bioavailable fractions in the soil. In another test, **Leite *et al*. (2020)** verified a total Zn content close to 15 mg. kg^{-1} in the same soil, despite the low concentration of Zn available 0.81 mg. kg^{-1}. Importantly, Zn fractionation without $ZnCl_2$ fertilization revealed the predominance of Zn associated with residual fraction, followed by the fraction associated with oxides and lower participation of Zn fractions bound to soil organic matter and carbonates. This explain the low level of zinc in both fractions of the control soil.

2- Oxidizable fraction

In addition to occurring as free Zn or chelate complex in the soil solution, Zn in soil may be adsorbed on surfaces of solid particles such as carbonate, metal oxides of iron (Fe), alumimium (Al), manganese (Mn), and organic matter **(Liu *et al*., 2020).** Thus, use of zinc solubilizer (Zn SB) could be beneficial for increasing solubilisation of Zn in soil and its consequent availability to plants **(Patra *et al*., 2020)**. The concentration of soluble Zn ($ZnSol_n$) increased linearly as a function of Zn added for individual amended soils, suggesting that a constant proportion of the Zn added was potentially available for plant uptake **(Mossa *et al*., 2020).** This was in good agreement with our results.

In contradiction with our data, **Rong *et al*. (2020)** revealed that with increasing amounts of humic acid (HA), the concentrations of exchangeable fractions of Cd, Pb, Cu, and Zn, and the reduction fraction of Pb and Cu decreased with a corresponding increase of the other fractions. Overall, the soils treated with HA showed shifts of the soil metals away from exchangeable fractions to forms that are stable phases and less bioavailable. It is worth emphasizing that the binding order of metals to HA in a typical multimetal contaminated soils is Pb > Zn ≈ Cu > Cd **(Kulikowska *et al*., 2015)**, these findings confirm the same trend recorded in the present study.

It has been reported that three forms including organic Zn, crystalline iron oxide-bound Zn, and iron and aluminium oxides-bound Zn were the dominant forms of Zn after Car and Res forms. In general, the average of Zn chemical forms in the studied soils, irrespective of exchangeable and sorbed forms, have the following order: Om < Car <<Res. Additionally, it is possible that Zn would be adsorbed more intensity on minerals, and enter minerals network structure; therefore, it would be less leached and consequently its contents would be higher on the soil surface **(Shakeri and Saffari, 2020).**

Similar to our data, depending on the plant species, it has been indicated that Zn res was the most abundant phase for the highest rate, i.e. 8 mg. kg^{-1}, followed by ZnOxi, ZnExc, ZnOM and ZnCarb in soil cultivated with rice. Additionally, Znres was also the most expressive phase for soybean cultivation, followed by ZnExc, ZnOxi = ZnOM and ZnCarb, with no difference between the ZnOxi and ZnOM proportions. Soil samples treated with 4 mg. kg^{-1} had the following designs: Znres > ZnOxi > ZnExc > ZnOM = ZnCarb for soil cultivated with rice and Znres > ZnOxi > ZnExc = ZnOM > ZnCarb for soil cultivated with soybean. However, the control samples, without any Zn addition, were described by Znres > ZnOxi > ZnOM > ZnExc > ZnCarb for soil cultivated with rice and Znres > ZnOxi > ZnOM > ZnExc= ZnCarb for soybean **(De Carvalho Leite *et al*., 2020).**

In the same context, a survey study has been conducted recently by **Topcuoğlu (2018)** in groundwater and soils of intensive agricultural areas of Kaş, one of the major greenhouse production regions of Antalya, Turkey to assess the heavy metal pollution and their relationships with metal parameters. This author suggested that the distribution of metals in greenhouse soil samples generally followed the order; Zn: metals associated with Fe and Mn oxides (F3) < exchangeable forms (F1) < metals associated with organic matter (F4) < carbonate fraction (F2) < residual fraction (F5). While this order changed with Cu: metals associated with Fe and Mn oxides (F3) < exchangeable forms (F1) < metals associated with organic matter (F4) < carbonate fraction (F2) < residual fraction (F5). These results are in complete agreement with our findings where the fraction of organic matter was higher than the exchangeable. Additionally, the distribution of metals showed that the greatest percentage of all metals was present in the residual fraction. However, F1, F2 and F3 fractions of Zn, Cu, Cd and As metals were higher than other metals. This property possibly gives these metals a high mobility. These results indicate the higher percentages of metals in control soil were bound in organic matter and residual fractions.

De Carvalho Leite *et al*. (2020) identified that the concentrations of the ZnOM fraction ranged from 5 to 10% for soil cultivated with rice and from 5 to 11% for soybean. The lower abundance of the ZnOM fraction is due to the clayey-textured. Thus, soybean plants have higher access to available Zn bound to organic matter than rice plants, especially at the 4 and 8 mg kg^{-1} rates. This result can be attributed to the protonation and deprotonation in functional groups of organic matter, which may favor binds with Zn^{2+} initially available in the soil solution. Moreover, Zn bioavailability is related to soluble inorganic compounds and the weak adsorption of organic and inorganic soil particles which characterize the cation exchange pool.

It has been proved that after harvest, the immobile fractions (strongly bound to organic matter and left in residue mineral) considerably increased, whereas the proportions of weakly bound to organic matter, bound to carbonate and bound to manganese oxide decreased by 28.9% – 88.1% in the single Zn treatment compared with the corresponding treatments before planting **(Xue *et al*., 2020).**

Generally, in low pH soils, soil-applied fertilizers are fixed rapidly within aluminosilicate clays and/or bind to negatively charged oxides or fixed rapidly to Ca carbonates in high-pH soils **(Gregory *et al*., 2017).** This justify the high affinity of copper and zinc for the organic fraction, in our study with a slightly acid soil.

Finally, the comparison of Zn and Cu chemical forms indicates an increase in the organic matter form of Cu, which can be attributed to the high potential of Cu to be bound with organic matters. Higher value of mobility factor in Cu than Zn in the studied soils can help to justify why the lack of available Zn in the studied soils is more tangible than available Zn **(Shakeri and Saffari, 2020)**. The differences in soil phases can be associated with distinct rhizospheres behavior between the crops with the addition of a soluble Zn source in the soil, which changes the bioavailability condition and results in labile phases in plants. the plants' rhizospheres are different in terms of the root exudates concentrations "as organic acids" that change the rhizosphere pH and thus the Zn availability **(De Carvalho Leite *et al*., 2020).** All these findings explain the noteworthy decline of zinc content at 300 ppm Zn in the organic fraction. This level justifies also the low bioconcentration and translocation of zinc at this specific dose. Additionally, the affinity of zinc to organic matter sites is reduced because of the soil pH, or might be this dose is a point of releasing the metal from the solide to soluble fraction. Other suggestions are that: the reacting surfaces with organic fraction are not uniform, the metal affinity is more important to the fulvic acide, or it precisely reacts with metal oxides (iron and manganese). In some cases, the low zinc concentrations cause a sorption of zinc dependings on the ligands; but, the feedback reaction still as the most explaing effect causing the decrease of zinc content in the organic fraction.

In overall, I suggest that added the organic matter to soil is not only considerd as a feritiliser but have also a crucial role in limiting and solving the zinc and copper deficiency problems in the soils through.

VIII.6. Correlation between the biochemical parameters in *Lycopersicon esculentum* Mill.

Basically, the correlation of the biochemical parameters of *Lycopersicon esculentum* Mill. in the different organs (leaves and roots) was analyzed and discussed below. As given by many studies the Pearson's correlation between the biochemical parameters was depending on the stress concentration, the metabolomic and the transcriptome. This is in good agreements with the analysis of **Li *et al*. (2021)** on *Nitraria sibirica* Pall. exposed to salt stress, who revealed that 18 differentially expressed genes (DEGs) and 8 differential metabolites between the 100 mM NaCl and 0 mM NaCl treatments were negatively correlated, and the remaining 113 DEGs and differential metabolites were positively correlated. A total of 25 DEGs and 11 differential metabolites between the T400L and T0L treatments were negatively correlated, and the synthesis of 8 differential metabolites, including ribitol, proline, methionine, and oxaloacetate, was only positively correlated with regulation of the DEGs. A comprehensive analysis showed that in *N. sibirica*, metabolites such as 2-aminophenol, chlorophyll, and L-cysteine, and DEGs such as *AMY2*, *BAM1*, and *GPAT3*, occupied important positions in the correlation network under T100L treatment, while under T400L treatment, metabolites such as 4-aminobutyric acid, butyraldehyde, proline, oxaloacetate, and uracil, and DEGs such as *ASP1*, *CML38*, *RPL4*, and *YDA*, played important roles in the correlation network.

a) In term of organ

In term of organ, it was found a positive correlation between the level of antioxidants, osmolytes compounds, and the scavenging activity in leaves and their content in the roots of *Lycopersicon esculentum* Mill., this positive correlation was marked by both treatments of copper and zinc. Similarly, **Singh *et al*. (2018b)** noted the correlation between these accumulations in different tissues; in which demonstrated that the total phenol content in shoots showed a significant positive correlation with total phenol content in roots. It also showed a positive correlation with flavonoid content in shoots. Furthermore, the total phenol content in roots showed a positive correlation with flavonoid content in shoots. On the other hand, flavonoid content in roots and shoots also showed a positive correlation.

Moreover, it has been found that, Cu contents in the roots of the plant were positively linked with Cu contents in the harvestable parts of the plants, but was negatively correlated with plant growth, biomass, fibre yield and quality and gaseous exchange. Similarly, antioxidant enzyme activities and oxidative stress were positively correlated with each other as well as Cu accumulation in the belowground parts and shoots, but were negatively linked with plant growth, fibre quality and gaseous exchange. Plant height was positively correlated with plant biomass, fibre quality and gaseous exchange, but was negatively correlated with Cu accumulation, antioxidant enzyme activities and oxidative stress. This correlation analysis revealed a close relationship between different parameters of *Corchorus capsularis* plants **(Saleem *et al*., 2020a).**

b) In term of antioxidants - antioxidant activity

It was found a positive correlation between the antioxidants (polyphenols and flavonoids) and the scavenging activity, in leaves and roots of *Lycopersicon esculentum* Mill. this positive correlation was obtained by both treatments of copper and zinc.

Similarly, **Tungmunnithum *et al*. (2018)** showed the positive correlation between phenolic content and the activity of free-radical scavenging**.** Inversely, total phenolic compounds were correlated negatively with DPPH. A negative correlation was found between total flavonoides compounds and DPPH scavenging rate. These results show that phenolic and flavonoids compounds are positively associated with antioxidant activity. The damages were explicated by the correlation between yield loss was positively and significantly correlated with electrolyte leakage and DPPH; while negatively correlated with protein content, proline content, total phenolic compounds, and total flavonoids compounds. Similarly, it has been demonstrated a strong correlation between antioxidant activity with total phenolic and total flavonoid content **(Sushant *et al*., 2019).** Additionally, **Kholodova *et al*. (2018)** established the positive correlation between the level of oxidative stress and proline content in the presence of $CuSO_4$. Moreover, a significant negative relationship between Zn leaf concentrations and polyphenol concentration/radical scavenging activity has been found by **Yilmaz *et al*. (2016).**

c) In term of osmolyte- osmolyte (proline – soluble sugars)

We noticed a positive correlation between the osmolytes (proline and soluble sugars) in leaves and roots of *Lycopersicon esculentum* Mill. exposed to copper and zinc stress. These results corroborate the findings of **Zhou *et al*. (2019a)**, who indicated that the proline and soluble sugar contents of leaves are significantly positively correlated with each other, thereby indicating that these contents protect plants effectively as major osmotic adjustment substances under adversity stresses. Indeed, in exogenous proline medium condition, it has been assisted to the presence of sugar in the wild lame and their absence on the mutant one **(Bouazzi *et al*., 2019).** In order to explain the connection root-leaves, proline and amino acid production were correlated with a decrease in leaf water potential **(Abid *et al*., 2018)**. Moreover, it has been proved that leaf membrane lipid peroxidation (MDA); which indicate the damage cell membrane and proline content were negatively correlated with Zn concentration. The positive correlations between MDA content and the osmotic parameters showed that osmotic stress and lipid membranes oxidation exist simultaneously under multiple heavy metal stresses **(Shen *et al*., 2019).** Another justification was given by **Zhou *et al*. (2019a)**, in *Cinnamomum camphora* under copper and cadmium, who demonstrated that the net photosynthetic rate is significantly positively correlated with the total chlorophyll, MDA, soluble sugar, and proline contents but is significantly negatively correlated with the SOD activity. A significantly negative correlation was observed among MDA content, proline content, and SOD activity, whereas a significantly positive correlation is observed between soluble sugar and proline contents.

Another research described by **Martins *et al*. (2018)**, makes our results clear by compared them with other physiological parameters. Positive correlations were presented between chlorophylls and some acidic metabolites such as: isocitrate, glucuronate and galactonate; chlorophylls and sugars and sugar-derivatives such as trehalose; sucrose, ribose, gentibiose, myo-inositol and squalene. Additionally, most sugars were negatively correlated with chlorophylls, proteins, and some acids, more specifically those with positive correlation with chlorophyll and within their own group. It is noteworthy that there were also compounds which had little or no correlation within their own group including the amino acids GABA (γ-aminobutyric acid), cysteine, β-alanine, alanine, asparagine, aspartate, pyroglutamate, valine and tyrosine; the sugars.

d) In term of osmolytes – antioxidants

It was marked a positive correlation between the osmolyes and the antioxidants under copper and zinc stress of *Lycopersicon esculentum* Mill.

- **Proline - antioxidants**

Under salt stress, a positive correlation between the antioxidant enzymes, high-level of α-tocopherol as well, and a negative correlation with an enzyme activity incorporates in proline catabolism. It has been demonstrated that flavonoid was positively correlated with proline. For phenylpropanoid and favonoid biosynthesis, both 113 DEGs and 22 DEMs related to phenylpropanoid and favonoids biosynthesis were carried out Pearson correlation analysis. The result showed that 43 DEGs (differentially expressed genes) had strong positive and negative correlation coefficient values with 12 metabolites. For example, there was signifcantly positive correlation between the gene expression (CCR, DFR, CHI3, PER42) and metabolite abundances (gallocatechin, apigenin O-malonylhexoside, delphinidin 3-O-glucoside), but there was signifcantly negative correlation between the gene expression (CAD) and metabolite abundances (apigenin O-malonylhexoside, gallocatechin, dihydromyricetin and mirtillin) **(Sushant *et al*., 2019)**.

- **Soluble sugars- antioxidants**

It was found a positive correlation between the level of the antioxidants and the soluble sugars content in leaves and roots of *Lycopersicon esculentum* Mill. stressed by copper and zinc.

Sugars play diverse roles during plant growth and development, therefore, their abundance, direct participation in stress tolerance as osmo-protectants/antioxidants, signaling function, a significant correlation with photosynthesis or source-sink **(Gangola and Ramadoss, 2018).**

It has been indicated that phenylalanine ammonia-lyase (PAL) activity had a significant positive correlation with total phenolics total flavonoids and saponin content (heterosides comple), suggesting when plants are exposed to Cd and Cu, PAL activity would be enhanced and subsequently increase the production of plant secondary metabolites. In this study, there was a positive correlation between total phenolics, flavonoids and saponin content with DPPH. A positive correlation was noted between osmolytes content in leaves and roots and the antioxidant content in these organ **(Borges *et al*., 2019)**. A similar result was also obtained by **Guo *et al*. (2017),** a significantly positive correlation was found between soluble sugar and proline, SOD, CAT, and ABA under the experimental treatments. Being a compatible osmolyte and a scavenger of reactive oxygen species (ROS).

e) In term of proteins-organ

According to the present results, it was recorded a negative correlation between the proteins content in leaves and that in roots of *Lycopersicon esculentum* Mill. stressed by copper. However, the application of zinc caused a positive correlation between the proteins content in leaves and that in roots. The different level of protins under copper and zinc is relevant to the distribution of each metal in the both organs (Zinc in roots and copper in leaves).

The negative correlation my be explained by the biosynthesis of some proteins in roots (as sHSPs), and simultaneously a denaturations of proteins involved in the vital processes in leaves such as photosynthesis, respiration, water regulation, homeostasis, the unfolded protein response (UPR),…..

f) In term of antioxidants, scarvenging activity and osmolytes - proteins

Under copper stress, it was noted that the level of antioxidants, osmolytes, and the antioxidant activity in leaves and roots of *Lycopersicon esculentum* Mill. was negatively correlated with the content of proteins in the leaves, and was positively correlated with the content recorded in the roots. This positive correlation with proteins content in the roots may be explained by the accumulation of the antioxidant and osmolytes compounds and the high synthesis of a specific class of proteins as a defence system against heavy metals stress, such as heat shock proteins. Another hypothesis is that the roots exposed to copper stress are less tolerant than zinc. However, the negative correlation with proteins content in leaves may be due to the high degradation of proteins in leaves under copper stress, which is accompanied with the synthesis of the antioxidants componds and an increase in the antioxidant activity. Another hypothesis is that copper is translocated to the leaves and was highly accumulated compared to the roots, which contribute to a high stress.

Inversely, with zinc treatments the accumulation of antioxidants, osmolytes compounds and antioxidants activity in leaves and roots was negatively correlated with the proteins in the roots. A low correlation was recorded between proteins content in leaves and the antioxidant activity and the content of flavonoids in leaves and roots. In overall, no correlation was notd between proteins content in leaves and the polyphenols, soluble sugars, and proline content in leaves and roots of *Lycopersicon esculentum* Mill. The negative correlation with the proteins content in roots may due to the degradation of different cell proteins or to a limit of the biosynthesis of stress preoteins in roots exposed to zinc. Another hypothesis is that zinc is highly accumulated in the roots than leaves, wich induce a degradation of proteins and the increase of antioxidants, osmolytes and antioxidant activity. Additionally, the roots of *Lycopersicon esculentum* Mill. tolerate zinc and the phytotoxicity in roots was lower than copper.

The absence and low correlation observd with proteins content in leaves may be due to the fact that zinc content was highly lower in leaves compard to roots, this why a limite connection and crosstalk-organ-organ was noted between the differents coumpounds. In addition, it is clearly demonstrated that the zinc content in the leaves exhibit a low positive correlation with its content in the roots; this correlation was also lower than copper.

Moreover, in metal stress cell induce the biosynthesis of some proteins which are protected by accumulation of proline in leaves and roots as stabilizer of proteins structure.

VIII.7. Correlation between the level of metals in soil fractions and their contents in leaves and roots of *Lycoprsicon esculentum* Mill.

The metal correlation was depending on: the organ (leaves or roots) and soil fraction (valaible or unvailable fraction), that make the following distribution as: organ-organ, organ-fraction, fraction-fraction.

a) In term of organ-organ

It was recorded a highly positive correlation between the copper content in leaves and that in roots of *Lycopersicon sculentum* Mill. Besides, the zinc content in the leaves was also positively correlated to that content in the roots. Remarkably, the correlation in the case of zinc was lower than copper. This result is in good agreements with the findings of **Saleem *et al*. (2020a)**, who indicated that the Cu concentration in the roots was positively correlated with the Cu concentration in the fibers, stem core, and leaves while it was negatively correlated with all other growth parameters (i.e., plant height, plant fresh weight, stem fresh weight, bark fresh weight, and plant dry weight) and gases exchange parameters, except plant and bark diameter. This correlation demonstrates a close relationship between Cu uptake, growth, and gases exchange parameters in *Corchorus capsularis*. Similarly, it has been demonstrated that Cu concentration in the roots is positively correlated with Cu concentration in the leaves while negatively correlated with plant height, plant diameter, plant fresh weight, plant dry weight, total chlorophyll, carotenoid contents, net photosynthesis, transpiration rate, stomatal conductance and intercellular CO_2. This correlation reflected the close connection between Cu uptake and growth in *Hibiscus cannabinus* seedlings **(Saleem *et al*., 2020b)**. This similar to *Linum usitatissimum* **(Saleem *et al*., 2019b).** Inversely, according to **Yilmaz *et al*. (2016)**, the total amount of Zn in shoot during the early growth stage did not correlate with the initial seed Zn concentrations. In addition, Cd soil concentrations significantly correlated with Cd leaf concentrations. Importantly, there is a statistically significant highly negative correlation between the content plants of chlorophyll a and the concentration of zinc in the leaf of *Trapa natans* L. This study supports the analysis conducted by **Zhou *et al*. (2019a)**, who demonstrated that the physiological and biochemical characteristics of *Cinnamomum camphora* are correlated with the Cu and Cd contents in leaf, stem, and root of *C. camphora*.

b) In term of organ- unvailable fraction

As it was indicated in the present study, no correlation was detected between the Cu content in the organic fraction and Cu level in the roots, but a low positive correlation was noted with Cu content in the leaves *Lycopersicon esclentum* Mill. However, a positive correlation was obtaind between zinc content in the organic fraction and its level in the leaves and roots. As suggestion, this results of organ- fractions need to be explained by calculating the transfert factor, also the mobility factor of zinc and copper.

In accordance with our data, **De Carvalho Leite *et al*. (2020)** showed that there was no difference between unavailable fractions, ZnOxi and Znres, when there was no correlation between the rates either with total accumulated Zn in plants (Zn total), or the contents extracted by DTPA.

In overall, a low affinity was concluded for copper and zinc content in leaves and roots to the organic fraction.

c) In term of organ-vailable fraction

The results of the current study show a positive correlation between the exchangeable fraction and the content of copper and zinc in leaves and roots of *Lycopersicon esclentum* Mill. These finding consistent with the results of **Tran *et al*. (2018)** who demonstrated a significant positive correlation between Ex-Zn in soil and the Zn concentration in the shoots of pak choi. Similarly, it has been indicated that total cumulative Zn content in rice and soybean affected by the $ZnCl_2$ rates applied were positively correlated with Zn content extracted by both solutions. Both extractant solutions used in this study presented positive correlation between available contents of Zn with Zn bound to labile fractions. Zn available to rice plants determined by a DTPA extractant solution showed positive correlation for both subsamples (ZnDTPAt1 and ZnDTPAt2) in the soil fractions **(De Carvalho Leite *et al.*, 2020).** Additionally, **Xue *et al*. (2020)**, showed that soil Zn fractions were related to Zn concentration in shoots and roots of pak choi after Se–Zn co-amended soil. Zinc concentration in shoots and roots of pak choi had the best correlation with soil available Zn (DTPA-Zn), Zn weakly bound to organic matter (Wbo-Zn), Zn bound to carbonate fraction (Carb-Zn). Moreover, this research found that exchangeable Zn (Ex-Zn) was only positively correlated with Zn concentration in shoots of pak choi and Wbo-Zn. A significant positive correlation was observed between the Zn concentration in pak choi tissues and the soil DTPA-Zn and Wbo-Zn. Consequently, the increase in Wbo-Zn is the main reason for the elevated Zn available in soil, a similar result was observed by **Chen *et al*. (2017).** Importantly, the strong positive correlation between Zn in plant roots and Zn in the soil indicates that the amounts of Zn in roots strongly depend on its concentration in the soil **(Andrejić *et al*., 2018).**

In overall, a high affinity was concluded for copper and zinc content in leaves and roots to the exchangeble fraction.

d) In term of fraction-fraction

It was demonstrated that Cu content in the exchangeable fraction was negatively correlated with Cu content in the organic fraction. Inversely, a high positive correlation was noted between the zinc content in the exchangeable and the organic fraction, which explain the high mobility of zinc in soil and justify its toxicity in the plant compared to copper. This distribution of both metals is by the high cation exchange capacity of the soil used in this experiment, the mixture of organic matter with sand.

According to **Xue *et al*. (2020)**, the indirect path coefficients indicated that merely Zn in Wbo and Carb fractions positively indirectly affected soil DTPA-Zn. This finding reveals that the bioavailability of soil Zn depends on the potential available Zn sources in soil (Wbo-Zn and Carb- Zn) due to the low Ex-Zn fraction in soil. The other Zn fractions could also affect DTPA-Zn through Zn in Wbo and Carb fractions. The result of the correlation analysis suggests that in addition to DTPA-Zn, the semi-mobile Zn fractions (Wbo-Zn and Carb-Zn), the mobility factor (MF) and distribution index (DI) of Zn in soil values can also predict the Zn bioavailability in soil and the Zn uptake capability by plants.

VIII.8. Relationship between physicochemical properties and chemical forms of copper and zinc in the soil

The diversity in distribution and correlation between the different fractions noted above may be explained by the relationship between these fractions and the physicochemical properties of soil indicated an influence of soil properties, primarily soil pH and organic matter content. Lower pH values increased Zn solubility; correlation coefficients between soil pH and ZnSoln or the free ion activity (Zn^{2+}) were 0.77 and 0.79 respectively. Soils with greater organic matter contents had lower values of ZnSoln **(Mossa *et al*., 2020).**

As has been demonstrated, the Om form of Zn has shown a significant positive correlation with organic carbon and a significant negative correlation with pH. Also, the results show a significant positive correlation between DTPA-extracted Zn, CEC and organic matter and a significant negative correlation with soil pH. The Om form of each element includes the ions, chelated or complexed with organic matter, justifying the positive correlation of this form with organic carbon. Explaining the relation with other fractions, various forms of Zn and Cu are directly related to 2: 1 silicate minerals. The clay minerals of 2:1-layer types have a high permanent negative charge in high-pH and thus are able to sorb various cations. In evaluating the effect of clay on elements sorption, the amount and type of clay are considered together. According to the studies, low concentrations of Cu and Zn are adsorbed by iron and aluminum oxides through formation of inner sphere complexes (chemical sorption), but the hydroxide deposit of these metals are formed at high concentrations of these elements. Cu and Zn sorption occurs through formation of outer sphere complexes (physical sorption) on surfaces with a negative charge of 2: 1 layered silicate minerals and may occur by the formation of the inner sphere complexes (chemical absorption) on kaolinite surfaces **(Shakeri and Saffari, 2020).**

VIII.9. Evaluation of phytoremediation efficiency

VIII.9.1 Translocation and bioconcentration factors

According to **Saleem *et al*. (2019a),** the bioaccumulation and translocation factors could be used to assess the level of phytoextraction by a plant. The BCF and TF values help to identify the suitability of plants for phytoextraction and phytostabilization by explaining the accumulation characteristics and translocation behaviors of metals in plants. Since plants exhibiting TLF values > 1 are considered promising phytoextractors **(Chaturvedi *et al*., 2018).**

It was found that the translocation factor **(TF)** was superior to 1 for copper and inferior for zinc. Additionally, as the copper content increased in the soil as it more translocated to the leaves. Remarkably, at 300 ppm, zinc and copper were lowly translocated to the leaves. However, 400 ppm induce the maximum translocation for both metals following by a decline at 500 ppm. This may due to the saturation of transfer sites at this high dose.

Besides, as an indicator of pytostabilization, the bioconcentration **(BCF)** of copper and zinc indicate their lowly accumulation in the roots of *Lycopersicon esculentum* Mill., it was recorded a decline at 200 and 400 ppm for zinc and 200 and 500 ppm for copper. Therefore, according to the translocation and the bioconcentration factors, the results indicated that zinc was retained in the roots, inversely to copper which was highly translocated to the leaves.

Justifying our results, the poor translocation of toxic metals to the aerial parts especially the zinc might be due to sequestration of metals inside root cell vacuoles to render it harmless, which is considered the natural toxicity response of the plant **(Shanker *et al*., 2005)**. Depending on the concentration, at low zinc doses, this element was highly concentrated in the roots with maximum absorption at 300 ppm, but the higher doses of 400 and 500 ppm limit the zinc absorption which may be due to a saturation of the transport sites in the roots. This is clearly confirmed by the highest zinc content in the root at 300 ppm and the highest value of BCF at the same dose. Similarly, the same relations trend was given at 400 ppm give by zinc content in roots and the lowest value of BCF.

BCF might be also limited by another factor, of the zinc content in the soil or precisely in the exchangeable fraction. Inversely, copper content in the root at 200 and 500 ppm clearly explained the impact of the exchangeable fraction on the level of copper in this organ, where the highest level in the root was at 500 ppm Cu, whereas this concentration gives the lowest BCF value. The high level of copper in root may be due to its mobility and biodisponibility in the exchangeable fraction. As demonstrated before, the zinc was higher in the exchangeable fraction than copper, which is in accordance with the BCF_{zinc}, this finding indicates that the transfer of zinc from soil to plant was higher compared to copper. Inversely, the low BCF values might be due to the chemical interactions, exudates, transfer sites in the plant, the organ absorption.

As hypothesis, plant absorb zinc from the exchangeable fraction and concentrate its in the roots better than copper to limit its translocation, this mecanism in roots because of many factors of; short contact time(metal-organ), root geometry, uptake at the root solution interface, the rhizosphere, its strongly influence dynamics in soil, interinstely, there is competitive adsorption of these two cations on soil, especially mineral exchange sites,

Previous studies have proved the effect of copper and zinc on the translocation and bioconcentration;

Many factors might affect the bioconcentration and the translocation of metals in the plant depending on the, organ, soil, the absorbed element, hyperaccumylator potential;

Similar to our results on the reduction of BCF at higher doses, it has been indicated that the bioconcentration factor (BCF) (0.70−1.11) and bioaccumulation factor (BAF) (0.23−0.61) for Zn were lower than 1 in all tested plants, except those of the treatments T250, where BCF slightly exceeded 1. There were no statistically significant differences of BCF values between control and Zn-treated plants, whereas plants in T1,000 had significantly lower BCF than in T250 and T500. However, the progressive decrease of BCF with increasing Zn concentration in the soil indicated that *Miscanthus* × *giganteus* plants limited absorption of Zn to roots at higher Zn concentrations in the soil **(Andrejić *et al*., 2018).**

Besides, this research also explained another factor, depending on the concentration, it was found that zinc was highly translocated to leaves at 400 ppm (0.31) but it still inferior to 1 for zinc. Similarly, the translocation factor (TF) for Zn was lower than 1 (0.27−0.55) in all *M.* × *giganteus* plants. In this study, T 250 and T 1,000 plants had higher TF values than that in the control plants. At the same time, there were no significant differences of TF values between Zn-treated plants (between T250 and T500, between T250 and T1,000, and between T500 and T1,000). This is in good agreement with our results, between 100 and 200 ppm for zinc and 100 and 300 ppm for copper). Furthermore, according to relatively low BAF and TF values and the mostly uniform concentration of Zn in leaves between treatments, the Zn translocation from roots to leaves was restricted. Concentrations of Zn in the rhizome were 2‑3 times lower than that in the roots, indicating that most of the Zn was retained within the roots or was translocated to the stem. On the basis of the concentrations of Zn in its organs, it can be asserted that *M.* ×

giganteus represents an excluder plant species suitable for Zn phytostabilization. These observations are in good agreement with our results to investigate *Lycopersicon esculentum* Mill. plant for phytostabilization of zinc and phytoextraction of copper. **Pietrini *et al*. (2019)** corroborate our findings, who indicated that the copper concentrations in the tank were greatly lower than those at the leaf level. This suggests that the plants adsorbed the element from the complex root system perlite and transferred it to the leaf.

Relatively to the concentration, metal combination, and the plant species, a study conducted by **Chaturvedi *et al*. (2018)**, showed the Cd and Zn transfer factors in different parts of *Hylotelephium spectabile*. The transfer factor from stem to leaf (TF1) for Cd in HB2 was significantly higher than that in HB1 under Cd stress conditions, but a sharp decrease was observed in HB2 with the addition of Zn. In contrast to HB2, the TF1 for Cd in HB1significantly increased under Cd5 + Zn10 treatment. Additionally, the transfer factor from root to stem (TF2) for Cd in HB1 was significantly higher than that in HB2 under 2-mg. L^{-1} Cd but was greatly decreased with increased Cd stress or the addition of Zn. For HB2, the TF2 for Cd significantly increased by adding 10 mg. L^{-1} Zn under 2 or 5 mg. L^{-1} Cd stress but significantly decreased with addition of 20 mg. L^{-1} Zn. There was no significant effect of different Zn or Cd + Zn treatments on the TF1 for Zn in either population of *H. spectabile*. The TF2 for Zn in HB1 was significantly decreased with increased Zn stress but was not affected by adding Cd under Zn stress. Inversely to our findings, where the low value of TF_{copper} was marked in the control plant, it has been indicated that the minimum TF values of Cu for leaf (0.53) and stem (0.39) were recorded at 400 mg. kg^{-1} Cu while the maximum TF values for leaf (0.89) and stem (0.54) were observed at 50 mg Cu. kg^{-1}. Additionally, the highest bioaccumulation factor was recorded at 50 mg. kg^{-1} which were 0.30, 0.26, and 0.16 for root, leaf, and stem, respectively. Cu uptake was highest in roots followed by leaves and stems, respectively, at all treatments of *Boehmeria nivea*. Uptake and translocation of Cu in *B. nivea* mainly depend on levels of Cu supply and growth conditions, this is in contradiction with our data where the highest BCF was at the high dose of 400 ppm **(Rehman *et al*., 2019d).**

According to **Muszyńska and Hanus-Fajerska (2015),** around 500 plant species have been known to accumulate extraordinarily high content of metals in their tissues without any noxious effect. In this study, inversely to our results, most of Cu was accumulated in roots and values for both BAF and TF < 1. The highest BAF values in the leaf and stem were 0.26 and 0.16, respectively, while the highest TF values for leaf and stem were 0.89 and 0.54, respectively. Explaining our results, **Xiong *et al*. (2017)** indicated that the low values for both BAF and TF might also be due to middle type roots variety. In another study on ramie growing in a mining area, **Jian *et al*. (2016)** reported that the highest bioaccumulation coefficient and the transfer factor of Cu were 0.08 and 0.37, respectively. Similarly, it has been showed that BAF and TF for Cu in *B. nivea* grown in a mining site were 0.06 and 0.98, respectively **(She *et al*. 2011)**. The both studies are in contradiction with our findings where the highest TF_{copper} was 6.03.

Remarkably, depending on the organ, **Yilmaz *et al*. (2016)** indicated that durum wheat genotypes expressed highly significant differences in root uptake and root-to-shoot translocation of Zn and translocation of Zn from flag leaves into grains. However, none of these parameters showed a significant correlation either with the initial seed Zn concentrations at sowing or the grain Zn concentrations at harvest. Differences in grain Zn concentration of wild emmer and cultivated wheat could not be explained by root Zn uptake and Zn translocation from flag leaf into grains during seedling and reproductive growth stages, respectively. It seems that there are additional key factors affecting the expression of genetic variation for grain Zn accumulation. This give another justification at molecular level about the translocation and distribution of zinc in plant.

Depending on the soil, the element absorbed, and the hyperaccumylator potential, compared to tomato plant, a study conducted on a hyperaccumulatrice plant of castor showed a low BCF for Cu in the Cu-mine soils and slag. The values ranged between 0.11 and 0.36. Castor displayed low BCF values for As, Fe, and Zn in all the examined soils and slag; however, castor showed a BCF value of > 1 for boron (B) in D and E soils. The highest BCF value (1.03) for Mn was found when castor was grown in A soil. The obtained results suggested that castor is a hyperaccumulator only for B and Mn **(Palanivel *et al*., 2020).**

Depending on the concentration and the plant species, it has been indicated recently that metal uptake was noted to rise in plant parts in lower concentration of metal in culture media, while at higher concentration of metal, it was noted to decrease metal uptake. This result is in good agreement with our data, especially those recorded at high concentration 500 ppm Cu or Zn for low value of TF and BCF. This recent research demonstrated also that BCF value was highest (15.1) in tomato at 20 ppm of cadmium exposure followed by pea (14.9) under the same treatment. Exposure to copper (Cu_{175}) also showed higher BCF value (14.2) in tomato roots. The calculated greater BCF values (> 1) under the exposure of lower to moderate concentrations of both the metals suggested higher accumulation. This confirm obviously our result were the BCF was 27.7 for Cu at 100 ppm. Irrespective of the tested metals in this recent research, highest TF noted in wheat compared to other crops reveals higher metal translocation from root to shoot. Similarly, higher TF and TI value of wheat under Cd and Cu treatments indicated the existence of internal metal detoxification mechanism for both the metals. Higher accumulation of cadmium in wheat and tomato shoots shows its greater mobility and hence the risk of food chain contamination even at lower concentration **(Baruah *et al*., 2019),** this also justify the high translocation factor and tolerance index of Cu in *Lycopersicon esculentum* Mill. Inversely, Castor as hyperaccumulator plant demonstrated a low TF value (< 1.0) for Cu when it was grown in the Cu-mine soils and ranging from 0.02 to 0.03, whereas castor exhibited a TF value > 1 for boron (B) in all the examined soils and slag; for instance, the TF values of B in native soils and slag were 9.15 and 7.30, respectively. Similarly, castor showed the highest TF value for Mn (2.87) in E soil, whereas castor displayed very low TF values for Fe, ranging from 0.03 to 0.14 in Cu-mine soils and slag **(Palanivel *et al*., 2020).** This study supports that TF in the same plant species such as *Lycopersicon esculentum* Mill. depend on the metal, TF value > 1 for copper and < 1.0 for zinc.

Depending on the metal combination and the root system, it has been proved that the total concentrations of Cd and Zn were higher in roots compared to shoots for all treatments. Andropogon grass showed a TF smaller than 1 for both metals. The translocation of Cd and Zn to plant shoot reduced when the metals were combined in the doses (4.4 + 284.7 µM) and (8.9 + 569.3 µM). The TF was twice higher for Cd and from 6.5 to ten-fold higher for Zn, in the treatments 4.4 + 284.7 µM and 8.9 + 569.3 µM, respectively. A high BAC_{Shoot} values for this element observed in the combined treatments, except for one dose. No differences were observed for BAC_{Root} between single and combined Cd treatments. Andropogon grass showed characteristics typical of metalexcluder plants, as it accumulated high amounts of metals but presented a TF < 1. This behavior combined with high biomass production as well as a great tolerance to Cd and Zn make this grass species a promising candidate for phytostabilization programs of Zn- and Cd-polluted sites **(Ribeiro *et al*., 2020b).**This result much well with our findings where TF < 1 for zinc, beside, the accumulation in root and the tolerance of this metal by *Lycopersicon esculentum* Mill. were higher, Therefore, the tomato plant can be suggested for the phytostabilisation approach.

A crucial hypothesis was signalled by **Lago-Vila *et al*. (2014)**, suggested that even if the specific plant could not be considered a hyperaccumulator, enrichment in the tissue could still be indicative for bioavailability of the respective elements since they crossed the cellular membrane. This might be supporting our suggestion on tomato *Lycopersicon esculentum* Mill. as an accumulator plant.

It has been demonstrated that many factors could affect positively the translocation and the bioconcentration of metals in the plant;

In accordance with our results, **Chaturvedi *et al*. (2018)** proved that tomato plant can be stated as an efficient phytoextractor for Cd, whose efficiency can further be improved by synchronizing with arbuscular mycorrhizal fungus (AMF) amendment. Also, AMF inoculation led to an increase in TF plant.

Inversely, other factors could negatively or positively affect the translocation and the bioconcentration including the: exogenous application (Citric acid (CA), Zinc), organ,..

As found in the present study, the soil was characteristic by a high level of the carbonated calcium, so this could affect negatively the mobility and the transfer of metals from soil to plant. However, the application of calcium to plant interact positively as demonstrated by many studies. It has been noticed that the application of CA to Cu stressed plants showed higher values of BAF and TF as compare to the plants without CA. The minimum value of TF was observed in 100 µM with the application of CA (0.64) while maximum TF value was observed in 50 µM with the application of CA (0.83). The highest BAF value was recorded at 50 µM with the application of CA (0.84) in the roots while (0.70) in the shoots. Cu uptake was highest in the roots than shoots while application of CA increases the value of BAF and TF. Thus, application of CA is helpful in increasing phytoremediation potential of the plant by increasing Cu concentration in the roots and shoots of the plants which is manifested by increasing the values of bioaccumulation (BAF) and translocation factors (TF) also. These observations depicted that application of CA could be a useful approach to assist Cu phytoextraction and stress tolerance against Cu in *Corchorus capsularis* seedlings grown in Cu contaminated site **(Parveen *et al*., 2020)**. Moreover, the application of exogenous Zn significantly increased the Zn concentration in the shoots of pak choi by promoting the translocation of Zn to shoots in plants **(Tran *et al*., 2018).**

VIII.10. Phytotoxicity

The present study shows that the toxicity obtained by zinc on the root and stem of *Lycopersicon esculentum* Mill. was higher than copper. In addition to that, this toxicity increases as the metal doses in the soil increase. It was noticed that in the stem, 100 ppm of copper and zinc exhibited a similar toxic effect. Inversely, in the root, zinc was less toxic than copper despite its high bioconcentration compared to the second metal. This might be justifying by the high tolerance ability (TI) of roots for the zinc compared to copper.

Remarkably, for stem and root, the low toxicity recorded at 300 ppm Cu compared to 200 and 400 ppm Cu, might be explained by the fact that plant used copper as essential element at this concentration. Inversely, it could be suggested also that Cu < 300 ppm induce a sort of toxicity that tend to be in term of deficiency, however, in execs, Cu > 300 ppm, the effect is considered as a phytotoxicity. Hence, this morpho-physiological status depends on the metal, its concentration, organ, tolerance degree of the plant species.

Inversely to the toxic effect in the roots, it was also recorded that zinc content in the stem was highly toxic than copper, this might be due that zinc change its chemical form, which defined as the chemical speciation of the metal in the plant, by linking to other molecules in sense to be transported within stem. Importantly, all these forms were modulated by plant as a defence system to reduce the toxic effect by limiting the zinc translocation.

Generally, all the results obtained in this study, including the metal effect on the morphological, physiological and biochemical parameters obviously confirm that the zinc was more toxic than the copper, this finding was also supported by the toxicity level calculated in the stem and roots of *Lycopersicon esculentum* Mill.

Indeed, according to the obtained results, the level of toxicity does not exceed 35%, which mean that the phytotoxicity caused by the copper and the zinc was not highly important to expose plant for hazardous damages, compared to the that resulted by toxic metals such as; cadmium, lead, and mercury.

Hence, *Lycopersicon esculenum* Mill. plant tolerate the different doses of copper and zinc; moreover, at 500 ppm, this plant species accumulates the both elements in their organs with low level of toxicity. Consequently, this species could be suggested for the process of the phytoremediation. Besides, *Lycopersicon esculentum* Mill. showed a high affinity for zinc to be absorbed and stored in the roots, while copper was highly translocated to the leaves.

The phytotoxicity still depending on the metal concentration, the tolerance of plant species, the soil, the low mobility and transfer of these element from the soil to the plant, as consequence of using the organic matter.

Our data are in accordance with the results obtained by **Ashagre *et al*. (2013)**, on the effect of copper and zinc on seed germination, phytotoxicity, tolerance and seedling vigor of tomato *Lycopersicon esculentum* L. cultivar Roma VF. As a result, the tolerance and vigor were expressed at 600 ppm zinc (our results: at 500 ppm); however, copper ≥ 300 ppm induced total failure on the tomato seeds germination. The highest toxicity to shoot (92.3%) and root (93.4%) appeared at 600 ppm zinc, (our results: at 500 ppm Zn for stem (35%) and roots (24.26 %)), whereas ≥ 300 ppm copper caused 100% toxicity on shoot and root (our results at 400 ppm Cu: for stem (27.78%) and roots (33.69%), no concentration was able to give a toxicity of 100 %). Additionally, at 100 ppm, copper was toxic to shoot (61%) and roots (85%) (our results: for stem (16.4 %), roots (20.85 %), while zinc showed toxicity of 68% of shoot and 66% of root toxicity (our results: for stem (15.78%) and roots (7.1%)). Hence, other than 100 ppm, copper is more toxic than zinc for germination and seedling growth of Roma VF. This comparative result confirms the best select of Saint Pierre variety in our experiment as a resistant species to abiotic stress, which indicate also that the tolerance and the phytotoxicity depends on the concentration, development stage; but mainly on the sensibility of the plant species.

Even that the copper and the zinc are toxic for plants at higher concentration, a good in vitro propagation response was recorded at lower concentration. The role of the copper as cofactors in some of the key enzymes of electron transport chains, carbohydrate and protein biosynthesis, and polyphenol metabolism accentuate its role in regeneration **(Alam *et al*., 2020).** Additionaly, it has been indicated that copper (Cu) is an essential heavy metal for plants, but high Cu concentration in the soil causes phytotoxicity **(Saleem *et al*., 2020a).** The phytotoxicity of Cu in reducing the growth and biomass of the plants has been reported in many previous studies **(Celis-Plá *et al*., 2018 ; Liu *et al*., 2018a ; Rehman *et al*., 2019d ; Saleem *et al*., 2020b).** For instance, it has been demonstrated that in tomato Cd exhibited leaf chlorosis and necrosis **(Carvalho *et al*., 2018).** According to **Timofeeva *et al*. (2016),** a reduced color intensity of various organs of seedlings and, eventually, their browning showed different stages of necrosis of plant tissues (marginal, spot, or full), which was associated with inhibition of the photosynthetic apparatus by HMs. These observations were also confirmed by a reduction of chlorophyll and carotenoids recorded in *Lycopersicon esculentum* Mill.

According to **Peng *et al*. (2020),** the impact on growth rate was more severe in females than in males. It has been demonstrated that the maximum growth retardation was found in 200 µM, but the symptoms like chlorosis, necrosis, and leaf discoloration were noticed even in low concentration (25 µM) of $ZnSO_4$ or at the doses of 0.25, 50, 100, 200µM zinc **(Rout *et al*., 2019).** The appearance of visual toxicity symptoms correlated closely with the effect of Zn on the biomass production: at 60 µM Zn, shoots of NMET plants were slightly wilted and chlorotic, with necrotic spots, and the root browning and the necrotic spots on the root tips were evident, whereas for MET1 and MET2 only the oldest leaves were slightly yellowish, with the roots being slightly darker than control **(Morina, 2016).**

Typically, the symptoms of toxicity induced by high levels of Cu and Zn may be related to the high levels of both elements in the leaves, causing oxidative stress, or in the roots, interfering with the nutrition of the plant **(Ademar *et al*., 2018)**. Besides, **Singh and Dwivedi (2019)** reported that micronutrients, zinc and boron greatly affect plant growth and development**.** It has been demonstrated that foliar chlorosis is a clear symptom of the heavy metal, especially Cd, stress in which the cellular photosynthetic apparatus is severely affected. The energy from light photons is the driving force to oxidize water and different pigments, within photosystem I and II, and protein molecules in order to generate an electron flow in the thylakoid membrane to produce NADPH and ATP. Different membrane proteins are involved in the electron transport chain in chloroplasts, and different metals such iron (Fe) and cupper (Cu) are responsible for the oxido-reduction reactions in the system **(Gratão *et al*., 2019).**

The satisfied results, leads to announcement of, **Wolf *et al*. (2017)** who proved that phytotoxicity was greater in the zinc-contaminated soils than copper-contaminated soils. Moreover, the toxic effects were much more significant with 2400 mg of zinc per kilogram of soil. Lettuce leaves from metal-contaminated soil exhibited discoloration and poor development, indicating more significant phytotoxicity. Similar results were previously observed for lettuce grown in soils containing copper and zinc separately. It has been demonstrated that the concentration of 250 µmol. L^{-1} Cu was adopted as it enabled the growth of both plant species (*Solanum lycopersicum* and *Solanum cheesmaniae*) plants, while inducing several toxic symptoms and antioxidant responses **(Branco-Neves *et al*., 2017).**

Differently, as it was suggested above, *Lycopersicon esculentum* Mill. plant might be investigated in the phytoremediation process. This suggestion is in the good agreement with the research conducted by **Mustapha and Tukur (2020),** who demonstrated the potential of tomato (*Lycopersicon esculentum* Mill.) plants in phytoremediation of multi-metal (Cu, Fe, Mg, Mn, and Zn) in contaminated soils and may serve as uneconomical and innocuous candidates for phytoremediation strategy in the future for controlling metal contamination level in soil. The bioaccumulation and translocation factors were found to be greater than one for *Lycopersicon esculentum* Mill.), which indicate that this plant is good hyper-accumulators and have the potentials for remediation processes. This suggestion might be also confirmed by the tolerance level of *Lycopersicon esculentum* Mill.

VIII.11. Tolerance index

It was found that tolerance index for root reduced by increasing the copper doses in the soil. Roots subjected to zinc doses were tolerant compared to those exposed to copper. The highest tolerance was recorded at in the absence of copper and zinc. The high tolerance obtained in this study, may be due to the high level of the antioxidants and osmolytes recorded.

Many studies have reported the effect of metals on the tolerance index depending on the plant species as tolerant or sensitive, the soil, organ, concentration, exposure duration,

It has been demonstrated that *Boehmeria nivea* can tolerate Cu ≤ 100 mg. kg^{-1} in Cu polluted soils with higher biomass production **(Rehman *et al*., 2019d)**. On the other hand, according to **Palanivel *et al*. (2020),** showed that castor displayed a high tolerance index TI when it was cultivated in Cu- mine soils. Castor grown in E soil demonstrated the maximum values of TI for castor height (133%), fresh mass (406%), and dry mass (328%) of shoots, whereas castor grown in the D soil exhibited highest TI in the fresh mass (358%) and dry mass (360%) of the roots.

Similar to what we have obtained in our study, it has been indicated by **Baruah *et al*. (2019),** that with increased concentration of the applied metals, tolerance index (TI) decreased gradually in all the. Depending on the plant species and the metal, among the tested crops (Wheat, Pea, Tomato), pea showed higher tolerance to lead with maximum TI (0.97) at 110 ppm of exposure (Pb_{110}). While, wheat documented higher tolerance to both copper (Cu) and cadmium (Cd) with maximum TI value of 0.97 and 0.96 respectively at an exposure of 65 ppm. Despite its lowest CAT activity, the higher tolerance of wheat crop to copper and cadmium indicates the existence of avoidance and homeostatic mechanism leading to lower metal build-up at sensitive sites. Least tolerance observed in tomato seedlings for both cadmium and copper (at 200 ppm) with lowest TI value of 0.25 and 0.38 respectively. The lower TI value of cadmium compared to copper indicates higher toxicity of cadmium than copper to tomato seedlings. Highly significant difference of interactions between crops, metals, and metal levels on tolerance index was observed.

Considerably, **Piotto *et al*. (2018)** indicated that tolerance index (TI) as an estimator of tomato tolerance degree to Cd toxicity in general. TI was able to rank tomato cultivars according to their ability for biomass accumulation in Cd-containing solution when compared to the control condition, allowing the identification of trends associated to plant tolerance/ sensitivity to Cd toxicity. However, depending on the metal and the exposure duration, certain tomato cultivars such as Pusa Ruby and Olena Ukrainien presented considerable variations in their TI values, especially when $CdCl_2$ concentrations were lower than 25 µM. The intersection between $CdCl_2$ concentrations containing higher TI stability (single asterisk) with the confidence interval that was associated to reductions by 50 % in plant growth revealed 25 to 50 µM $CdCl_2$ as the best concentrations for segregation of tomato cultivars according to their tolerance degree after Cd exposure for 7 days. Accordingly, when plants under exposure to 50 µM $CdCl_2$ were evaluated, TI was able to segregate tomato cultivars with similar performance, when based on their total dry weight. Among the range of $CdCl_2$ concentrations that were considered the most suitable for determination of plant tolerance, 35 µM $CdCl_2$ was estimated as the concentration that can amplify, at maximum, the separation of tomato cultivars according their TI. Since this concentration was not included in the primary experimental settings, and the initial set of tomato cultivars was relatively small (5 cultivars), an extra trial with 35 distinct tomato accessions (*S. lycopersicum and S. pimpinellifolium*) was run in order to check TI efficiency as an estimator of tomato tolerance degree to Cd toxicity. After 7 days of plant exposure to 35 µM $CdCl_2$, accession dependent variations in the total plant biomass were observed, and TI ranged from negative to positive values.

It has been indicated that plants with both bio-concentration factor and translocation factor greater than one (BCF and TF > 1) have the potential to be used in phytoextraction. Besides, plants with bioconcentration factor greater than one and translocation factor less than one (BCF > 1 and TF<1) have the potential for phytostabilization **(Lasat, 2002).**

In overall, *Lycopersicon esculentum* Mill., especially Saint Pierre variety, cultivated in soil contaminated by copper and zinc, present:

- For zinc; a low toxicity, a high tolerance, BCF > 1 and TF <1, thereby, it could be used in the phytostabilisation,
- For copper; a low toxicity, a high tolerance, BCF and TF > 1, thereby, it could be used in the phytoextraction.
- So, all the results confirm our hypothesis that tomato plant species used in the present study could be investigated in the phytoremediation of contaminated soil by copper and zinc.

CONCLUSION

In the present research, we noticed that the contaminated agricultural soils by heavy metals negatively affect the physiological performance of *Lycopersicon esculentum* Mill. This problematic open a new insight in agriculture field regarded to abiotic stress and the phytoremediation soil. In this context, the present project was undertaken to test the effect of copper and zinc on leaves and roots of *Lycopersicon esculentum* Mill., and evaluate the accumulation potential of these elements in the both organs. Therefore, it is possible to conclude that:

- The heavy metal stress caused by copper and zinc at high concentrations restricts stem and root growth of *Lycopersicon esculentum* Mill. Remarkable morphological changes were recorded in leaf and root biomass, accompanied by a reduction in leaf area and the total in the number of leaves compared to the control plants.
- A disruption in leaf water status was marked in the presence of different concentrations of copper and zinc, in *Lycopersicon esculentum* Mill., thereby limiting transpiration rate by reducing leaf relative water loss was necessary.
- The Photosynthesis process was impaired under copper and zinc treatment, the resulted effect was mainly elucidated by a reduction in chlorophyll pigment content in leaves of *Lycopersicon esculentum*, inducing the chlorosis symptoms and retardation in plant growth. The less content of carotenoid was also accumulated in leaves under heavy metal stress, with a fluctuation trend depending on the metal concentration.

As functional macromolecules, metal stress contributes to proteolytic breakdown set of proteins, and at the same trend, metal can also promote a synthesis of proteins involved in stress defense, in leaves and roots.

- The findings obtained in this study indicate that both tissues (Leaves and roots) of *Lycopersicon esculentum* exposed to different level of copper and zinc, regulate their cellular osmotic adjustments by accumulating a high level of osmolytes compounds as; proline and soluble sugar. The endogenous level of these osmoprotectors in leaves was higher than roots.
- In order to alleviate the toxic effect and considerable damages caused by copper and zinc, tomato plant involves an antioxidant mechanism by triggering the synthesis of flavonoids and phenolic compounds, thereby increasing the antioxidant activity to protect intracellular reactions in leaves and roots. It was appeared that leaves accumulate a high content of flavonoids and phenolic compound than roots.

Generally, our research shows that copper play an important role as an essential element at a dose ranged between 200 and 300 ppm. Inversely to zinc, the dose needed varied between 100 and 200 ppm. Thus, toxic effect may be occurred by exceeding these norms.

- Pysicochemical analysis revealed that the soil used in this study (a mixture of sand and compost) elucidate slightly acidic reaction and characterised by loamy fine sand texture with high salinity degree, high lime content and high cation exchange capacity. Toxicity and mobility of heavy metals depends mainly on their chemical forms in the soil. These forms were extracted using Tessier sequential extraction method. Two fractions were easily determined: organic and exchangeable fraction, referring to the immobile and mobile forms respectively. It is important to note that toxic effect induced by heavy metals is attributed to their chemical speciation in the soil, and no toxic risks can be estimated using the total concentration of these metal elements.
- This study demonstrates that copper and zinc ions were accumulated in leaves and roots of *Lycopersicon esculentum* Mill. The metals content in both tissues increases gradually with increasing metal doses applied. Depending on the metal and its solubility in the soil, copper ions were absorbed by roots and substantially translocated to the aboveground parts, whereas zinc ions still stored in the roots and only a low content was found in the leaves.
- Metal fractionation showed that copper and zinc exhibit a high affinity to organic matter compared to their low content in the exchangeable fraction. Moreover, metal amount in both fractions increase according to the increases concentrations of copper and zinc supplemented to the soil. The high metal content associated with organic matter explain its low mobility in the soil. Overall, morphological, physiological, biochemical and chemical parameters relevant to plant and soil showed a positive correlation between them. Leaves were more susceptible than roots in both treatments for all parameters highlighted in this study. All the various mechanisms mentioned above help *Lycopersicon esculentum* Mill. plant to detoxify and tolerate the negative effects induced by copper and zinc, which obviously reduces the phytotoxic symptoms.
- Both factors of BCF and TF indicated that, *Lycopersicon esculentum* Mill. absorb both copper and zinc from the soil; however, zinc was more retained by roots than copper which was highly translocated to the leaves.
- Depending on the organ, the concentration and the effects recorded at the physiological and biochemical level, zinc was toxic compared to copper.
- Roots subjected to zinc doses were relatively tolerant compared to those exposed to copper.

Significance of the findings with a qualification

Physico-biochemical disturbances occurred in different parts (leaves and roots) of *Lycopersicon esculentum* Mill. under copper and zinc treatment may consequently act negatively on the quality of tomato fruit. Although it is a very effective plant in phytoremediation, as reported in previous studies, *Lycopersion esculentum* Mill ability can follow another trend by accumulating the metals in the edible organ and consequently risks being toxic to human and animals.

Research contribution

- One of the most significant findings to emerge from this study is that the genotype of tomato crop “Saint Pierre” is characterized by an immediate response and tolerance trait to heavy metals. It is able to adapt to high concentration of copper and zinc applied without being create severe cell injuries or contribute to plant cell death.

Limitations of the current study

The major limitation of this study is pointed on:

- The incorporation of organic matter in soil limits the absorption and translocation of metals to leaves of *Lycopersicon esculentum* Mill., which lower their bioavailability. Thus, it will be better to exploit this protocol using compost in the context of immobilization of soil polluted by heavy metal to avoid the phytotoxicity, this with respecting the level of physico-chemiclal properties.
- Organic matter used in this study impeded the extraction of residual and oxides fractions, whereas it allows ultimately obtaining only the organic and exchangeable fractions. In considering that the mobility factor requires the total proportion of each fraction to be measured.
- Zinc was found highly emphasis in roots with a low mobility in the soil.

General conclusion

Successful tomato crop is based on specific conditions corresponding to plant physiology and the soil stability. It is very important to monitor the environment conditions, in where nutrient elements must be applied with appropriate doses. Soil physicochemical properties including CEC, pH, EC, $CaCO_{3,}$ and soil texture, must be analyzed and equilibrated before planting *Lycopersicon esculentum* Mill. In contaminated soil by heavy metals, the balance between these parameters is established by addition of organic matter; which is recognized for its immobilization efficiency. Edaphics factors also greatly influence the growth, development, and associated biota of *Lycopersicon esculentum* Mill. plant such as; temperature, humidity, season, species, plant growth stage, biotic and abiotic stress etc.). These parameters maintain the plant performance to avoid the phytotoxicity impact and prevent tomato plant against various environmental stresses. It is crucial to respect the application period for copper and zinc, as well as the growing type of *Lycopersicon esculentum* Mill. in field or greenhouse. Finally, the present study which focus on using tomato crop attempt to be one of projects of agriculture and sustainable development to achieve the goal of 2025 by increasing the production yield on global scale, limiting stress plant, preserving a healthy and safety fruit, removal pollutant from agricultural contaminated soil.

Table 51 provides an overview of the main findings and comparisons relevant to the consequences resulting from the application of copper and zinc on different systems of *Lycopersicon esculentum* Mill., including morphological, physiological, biochemical, and chemical.

Table 51: Main results of the effect of copper and zinc on morphological, biometrical, physiological, biochemical, and chemical parameters of *Lycopersicon esculentum* Mill.

Parameters		**Copper**	**Zinc**
Morphological	Stem length	↘	↘
	Root length	↘	↘
	Number of leaves	↘	↘
	Fresh biomass (aerial and roots)	↘	↘
	Dry biomass (aerial and roots)	↘	↘
	Leaf area	↘	↘
Biometrical	RWC	↘	↘
	RWL	↘	↘
Physiological	Chlorophyll content	↘	↘
	Carotenoids content	↘ ↗ ↘	↘ ↗ ↘
Biochemical	Proteins content (in leaves and roots)	↗ ↘	↗ ↘
	Soluble sugars content (in leaves and roots)	↗	↗
	Proline content (in leaves and roots)	↗	↗
	Flavonoids content (in leaves and roots)	↗	↗
	Polyphenols content (in leaves and roots)	↗	↗
	Antioxydant activity “DPPH test” (in leaves and roots)	↗	↗
Chemical	Heavy metals in plant (in leaves and roots)	↗	↗
	Heavy metal in soil (in the organic and excheangeable fractions)	↗	↗
BCF	Roots	Zn > Cu	
TF	From root to leaves	Cu > Zn	
Phytotoicity		Zn > Cu	
Tolerance (roots)		Zn > Cu	

↗ An increase.
↘ A decrease.

RECOMMENDATIONS

Recommendations for further research work

In our future research we intend to concentrate on

- **Morphological**
    - Ultrastructural analyses in roots and leaves are necessary to determine the localisation and distribution of metals in the targets cell. These analyses can help to identify the alterations resulted in cell organelles (chloroplasts, mitochondria, endoplasmic reticulum, membrane integrity, etc.), and in different plant tissues (epidermis and number of parenchyma cells, etc.).
    - Determine the toxic effect of copper and zinc at the molecular level; by detecting the DNA damage and cell division cycle alterations (prophase, metaphase, anaphase, telophase), to give more details and explain the findings recorded in tomato plant growth.
    - Identify the gene expression profiling of plant hormones participated in regulation of plant growth and development (Axin, gibberellin, cytokinin, etc.).
- **Biometrical**
    - Disruption of leaf water status might be explained by analysing the implication of aquaporines expression and its impact on stomata aperture and stomata density. Study gene expression of abscisic acid that acts as a regulator hormone of several mechanisms including; leaf water potentiel, transpiration and stomatal movement.
- **Physiological**
    - Proteomic, metabolomic, ionomic, and transcriptomic approaches have to be implemented to identify proteins, metabolites, mineral elements, and genes involved in tomato responses to copper and zinc stress. Determine also the gene expression of proteins involved in Cu and Zn remobilization and elements transport. It is also necessary to study the fundamental mechanisms engendered for heavy metal detoxification. All these approaches help detecting the differences between the response occurred in leaves and roots.

- **Biochemical**
    - Identifying the genes responsible for accumulation and synthesis of proline and different profiles of solubles sugar in roots and leaves.
    - Study the oxidative stress caused by copper and zinc in both organs.

- Recent biotechnological advances and transgenic approaches are effective strategies to enhance heavy metal stress tolerance of tomato crops, create tomato species resistant to high level of copper and zinc, and help to reduce the accumulation of heavy metals in the contaminated soil. In this context, a future research will be conducted on transgenic tomato plant by inducing the gene expression of proline and trehalose; as major osmolytes involved in tolerance to heavy metal stress. Besides, the exogenous application of these metabolites can be also promoting the plant resistance to environments conditions.

- **Plant**
  - Test the effect of copper and zinc on germination of *Lycopersicon esculentum* Mill.
  - Determine the critical toxic dose able to suppress the growth of tomato crop (at different stages).
  - Pot conditions do not present significant and real findings, it is important to undertake the experiment in field condition where agriculture soils highly contaminated by heavy metals. Determine the rate accumulation in edible organ (tomatoes).
  - Test the sensibility and the tolerance of other tomato varieties growing in Mostaganem and Antalya regions.
- **Soil**
  - Determine the level of copper and zinc in the contaminated soils of both regions.

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ANNEXES

Annex 1: Soil analysis

Table 1: Soil parameters and properties

Soil parameters	**Classes**	**References**
EC (dS/m) ≥ 16	Strongly saline	Classes of salinity and EC (1dS. m^{-1}= 1 mmhos.cm^{-1}; adapted from NRCS Soil Survey Handbook)
25 < $CaCO_3 \leq$ 50%	Highly calcareous	United States Department of Agriculture
CEC > 25	CEC is higher	United States Department of Agriculture

Table 2: Relationship between soil texture and CEC **(OHIO Agricultural Researcher and Development Center)**

Soil Texture	Typical CEC (meq.100 g^{-1} soil)
Sands	3 - 5
Loams	10 - 15
Silt loams	15- 25
Clay and clay loams	20 - 50
Organic soils	50 - 100

Triangle: Texture triangle presented by USDA soil taxonomy (ST) developed by United States Department of Agriculture

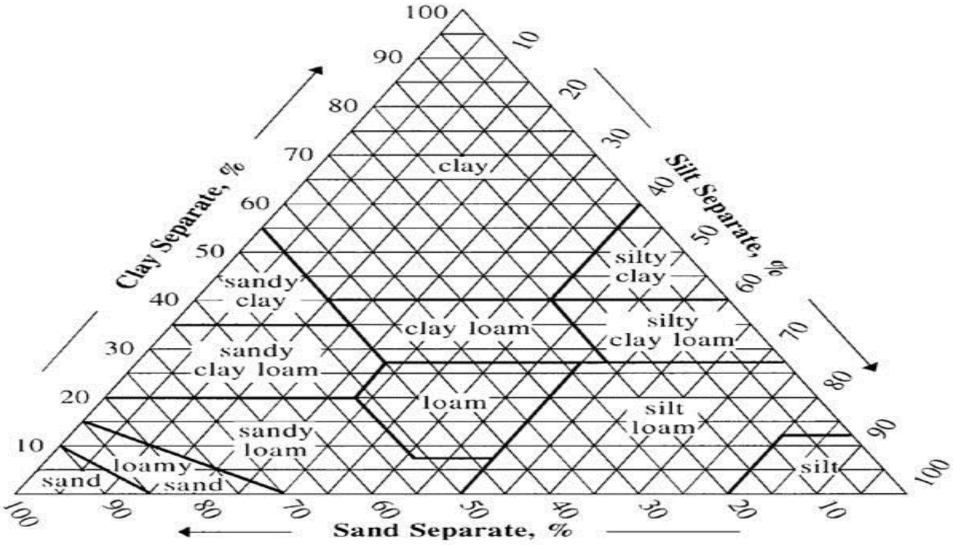

Figure 1: Texture triangle

Annex 2: Compost and nutrient solution

Table 3: Composition of the nutrient solution described by **Hoagland and Arnon, (1950)**

Macronutrients (mM)		**Micronutrients (µM)**	
KNO_3	5	$MnSO_4$	11
$Ca(NO_3)_2$	5	$ZnSO_4$	1.53
KH_2PO_4	2	$CuSO_4$	0.235
$MgSO_4$	1.5	$H3BO_3$	24.05
-	-	Na_2MoO_4	0.1
-	-	Fe-EDTA	268.6

Table 4: Compost composition

Type of product	Peat moss product, growing Media, blocking substrate.
Composition	The mixture of more strongly decomposed peat (frozen black peat), slightly to medium decomposed peat(white peat) and NPK-fertilizer (18-10-20) including trace elements
pH ($CaCl_2$)	5.2 - 6.0
Moisture	50 - 60%
Salt content (H_2O)	< 1.5g. l^{-1}
Nutrients (available for plants)	Nitrogen (N) 110-250 mg. l^{-1} Phosphate (P_2O_5) 60-140 mg. l^{-1} Potassium (K_2O)140-280 mg. l^{-1}

Structure	Fine
Application	For plants with low and medium nutrient requirements. For the production of blocks.
volume	70 liters (EN 12580)
Country of origin	Germany
Manufacturer	-----
Storing	Cool and dry

Annex 3: Calibration curves

1- Calibration curves of biochemical analysis

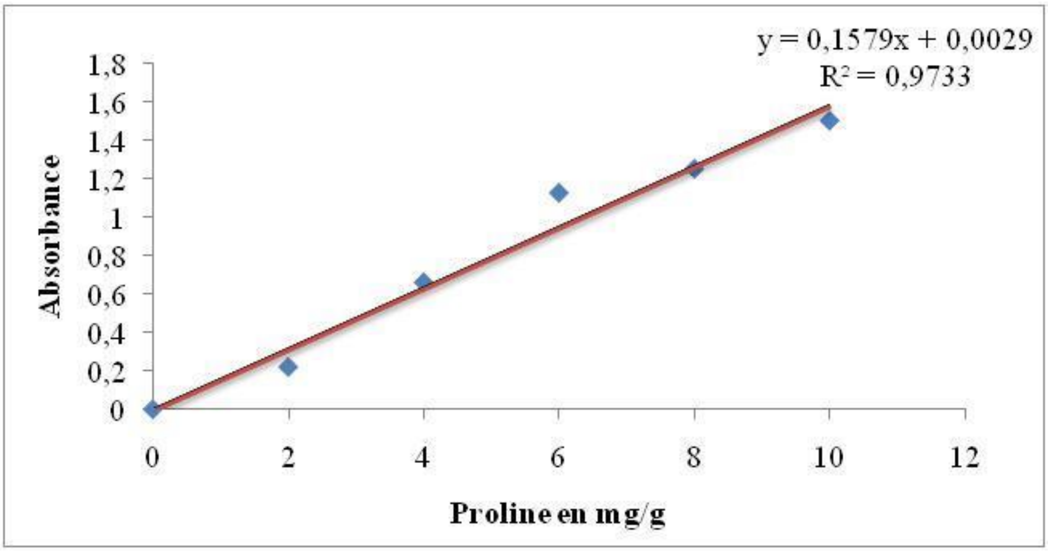

Figure 1: Proline calibration curve

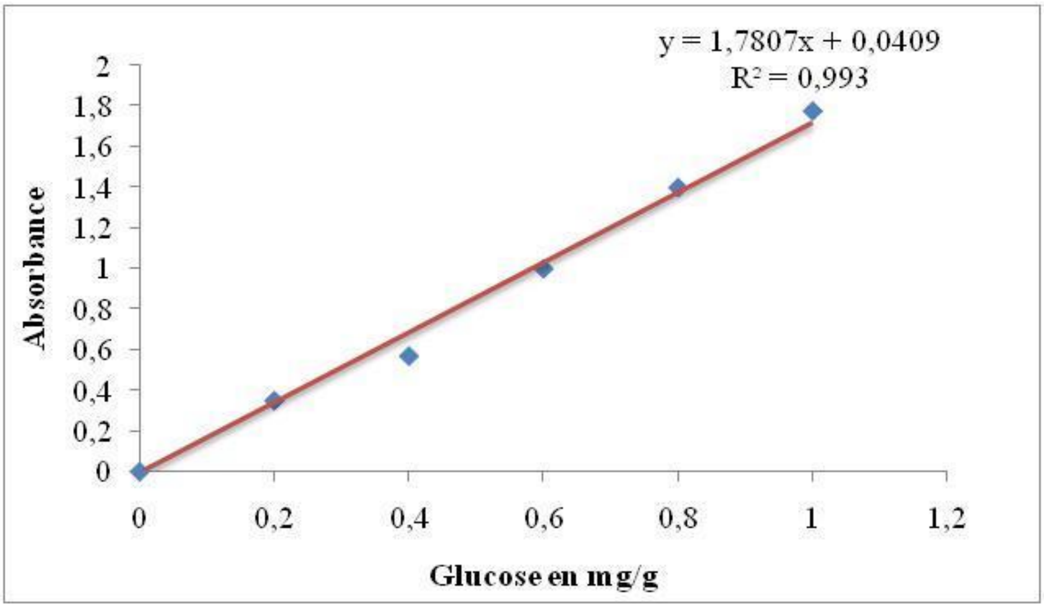

Figure 2: Soluble sugar calibration curve

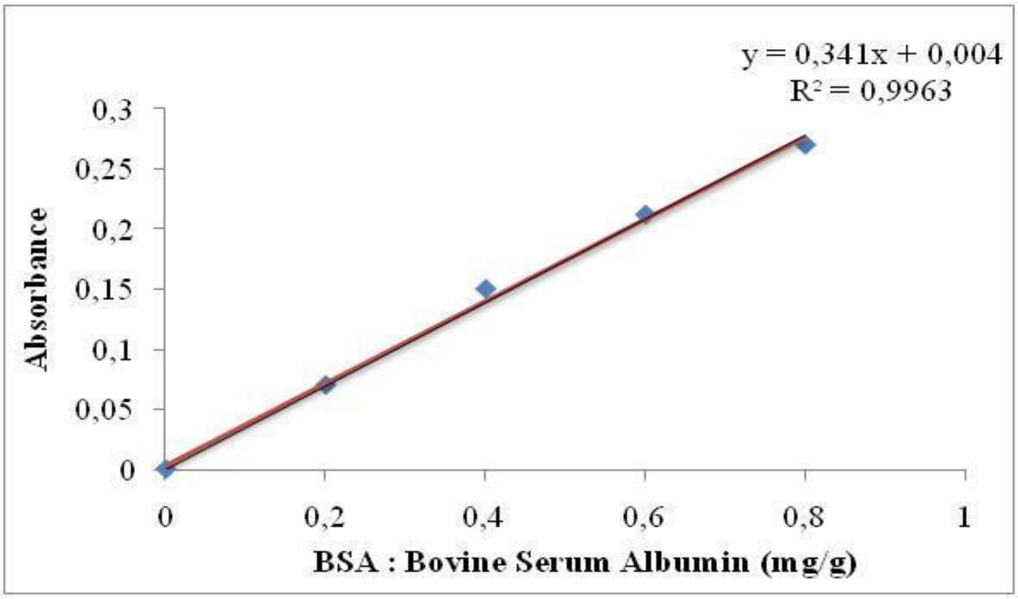

Figure 3: Proteins calibration curve

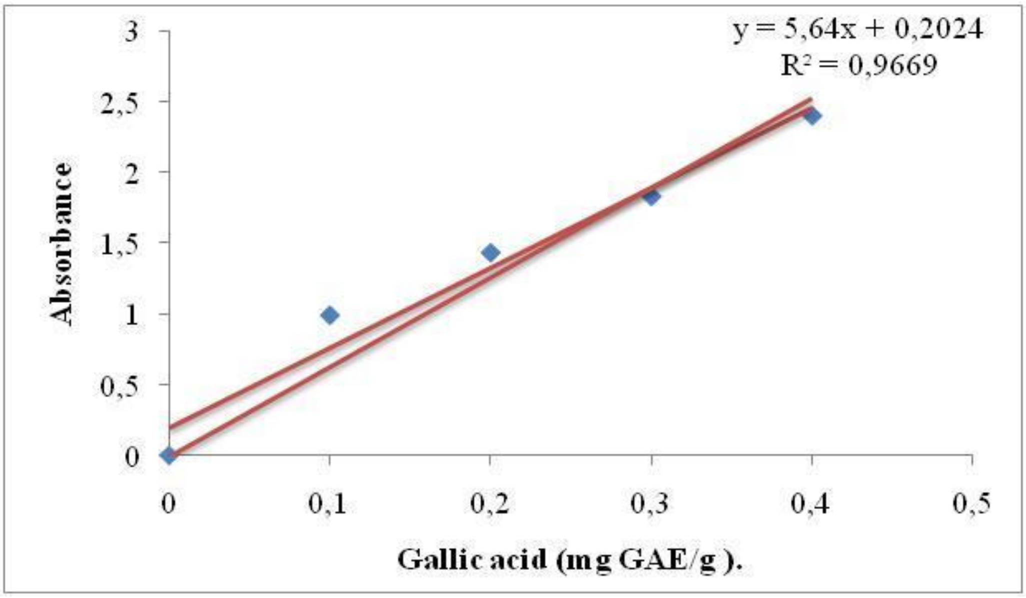

Figure 4: Polyphenols calibration curve

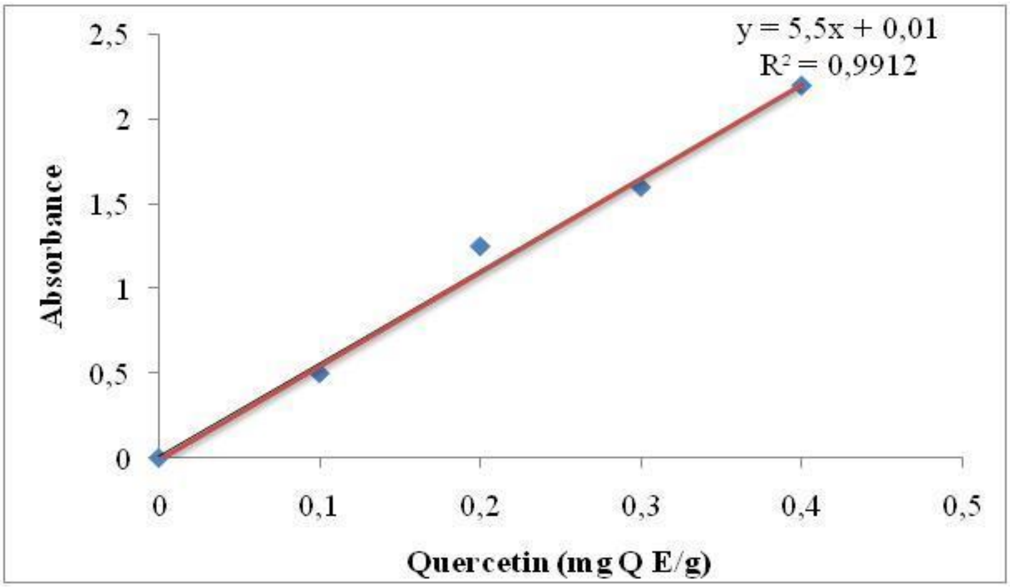

Figure 5: Flavonoids calibration curve

2- Calibration curves of Heavy metal analysis

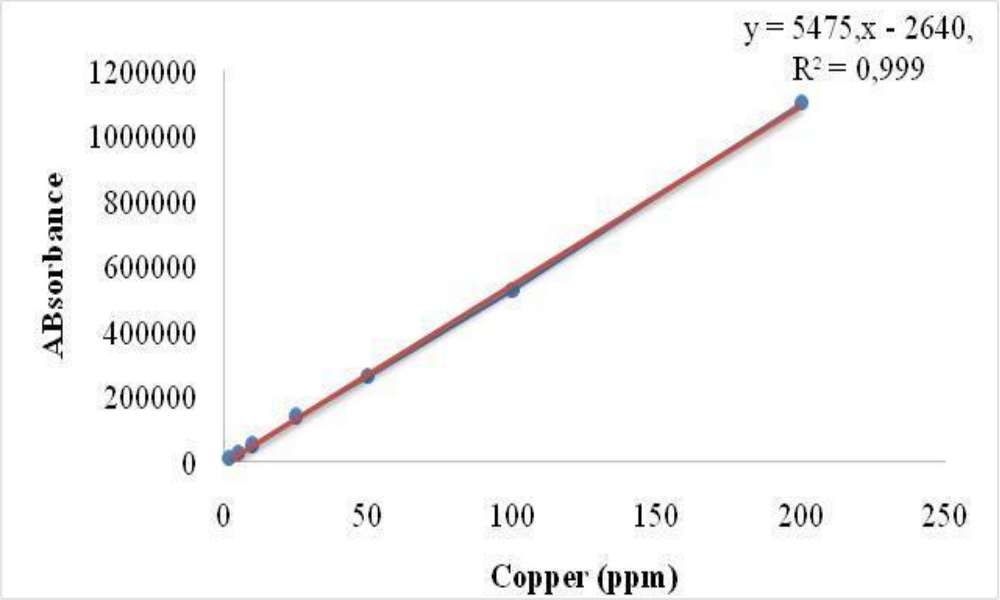

Figure 6: Copper calibration curve

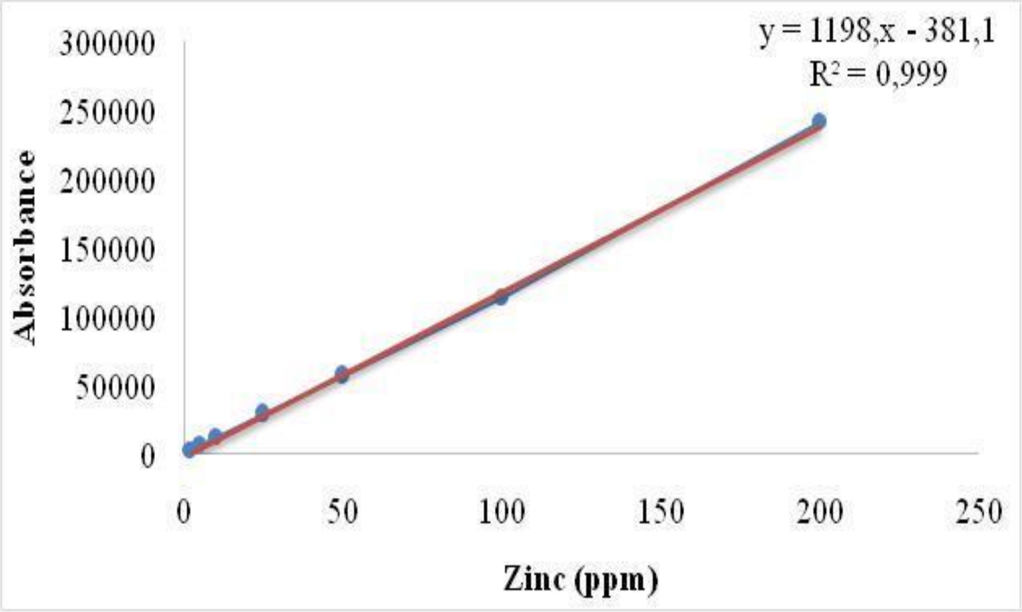

Figure 7: Zinc calibration curve

Annex 4: Statistical analysis

Table 1: Variance Analysis of copper effect on the different parameters of *Lycopersicon esculentum* Mill.

Variance factor	S.C.E	DDL	C.M.	TEST F	Probability
Stem lengh	634,444	5	126,889	28,198	0,00001
Number of leaves	79567,61	5	15913,52	20,758	0,00002
Leaf area	236,558	5	47,312	20,924	0,00002
Root lengh	929,111	5	185,822	24,776	0,00001
Biomass L	21356,128	5	122,126	23,126	0
Biomass R	82.125	5	17.152	10.26	0
chla	20.12	5	9.124	8.012	0
chl b	15.123	5	8.123	6.025	0
ar	10.004	5	6.124	5.682	0
RWC	2690,389	5	538,078	16,983	0,00006
RWL	0,031	5	0,006	10,344	0,00057
Proteins L	60.302	5	10.241	13.201	0
Proteins R	92.13	5	23.125	32.125	0
Proline L	48,901	5	9,78	7,705	0,002
Proline R	85,244	5	17,049	27,33	0,00001
Soluble sugar L	68,102	5	14.23	23.128	0
soluble sugar R	70,531	5	16.244	25,520	0
Polyphenol L	327,709	5	65,542	25,195	0,00001
Polyphenol R	341,725	5	68,345	20,713	0,00003
Flavonoids L	1,42	5	0,284	189,46	0

Flavonoids R	0,867	5	0,173	882,158	0
DPPH L	1047,764	5	209,553	82,746	0
DPPH R	720,105	5	144,021	148,679	0

Table 2: Variance Analysis of zinc effect on the different parameters of *Lycopersicon esculentum* Mill.

Variance factor	S.C.E	DDL	C.M.	TEST F	Probability
Stem lengh	739,611	5	147,922	16,746	0,00007
Number of leaves	69401,11	5	13880,22	397,206	0
Leaf area	82,274	5	16,455	18,16	0,00005
Root lenght	455,111	5	91,022	11,457	0,00037
Biomass L	1246.123	5	841.146	10.250	0
Biomass R	62.3215	5	21.18	12.410	0
RWC	2627,15	5	525,43	31,013	0
RWL	0,325	5	0,065	164,16	0
Chlorphyll a	30.162	5	12.563	5.215	0
Chlorphyll b	15.534	5	9.528	5.849	0
Carotenoids	6.782	5	5.265	5.000	0
Proteins L	100.0125	5	18.352	15.620	0
Proteins R	121.123	5	12.562	15.253	
Proline L	143,414	5	28,683	37,728	0
proline r	100,215	5	20,043	19,119	0,00004
Soluble sugar	95.162	5	14.132	13.128	0
Soluble sugar	103.102	5	25.325	25.246	0
Polyphenol L	294,167	5	58,833	13,395	0,00018

Polyphenol R	731,2	5	146,24	55,962	0
Flavonoids L	3,637	5	0,727	230,824	0
Flavonoids R	1,504	5	0,301	136,675	0
DPPH L	1245,582	5	249,116	231,191	0
DPPH R	2014,887	5	402,977	601,283	0

Table 3: Variance Analysis of copper and zinc content in leaves and roots of *Lycopersicon esculentum* Mill.

Variance factor	**S.C.E**	**DDL**	**C.M.**	**TEST F**	**Probability**
Cu in R	13517,54	5	2703,508	26116,91	0
Cu in L	280819	5	56163,79	155,804	0
Zn in R	1337416	5	267483,1	57,504	0
Zn in L	248942,7	5	497885,3	1188,034	0

Table 4: Variance analysis of chemical specition of copper and zinc in soil (organic and the exchangeable fraction).

Variance factor	**S.C.E**	**DDL**	**C.M.**	**TEST F**	**Probability**
Cu in O.F	405613,3	5	81122,66	249,959	0
Cu in E.F	68,787	5	13,757	4759,92	0
Zn in O.F	101719,4	5	20343,89	124,392	0
Zn in E.F	903,703	5	180,741	1236,431	0

Table 5: Variance analysis of translocation and bioconcentration factors of copper and zinc.

Variance factor	S.C.E	DDL	C.M.	TEST F	Probability
TCF Cu	28,423	5	5.685	36.422	0
TCF Zn	0.17	5	0,.034	4.657	0.01373
BCF Cu	10187.61	5	2037.523	1588.547	0
BCF Zn	2813.851	5	562.77	11.586	0.00035

Annex 5: Laboratory experiment photos

Photo 1: Tomato seedlings before treatment by copper

Photo 2: Tomato seedlings after treatment by copper

Photo 3 : Tomato seedlings before treatment by zinc

Photo 4: Tomato seedlings after treatment by zinc

Photo 5 : Root length: at 0 ppm (a) ; at 500 ppm: Cu (b) and Zn (c)

Photo 6: Tomato leaves stressed by zinc

Photo 7: Dispositif for RWC

Photo 8: Leaf area, stressed by copper (a) and zinc (b), for RWL.

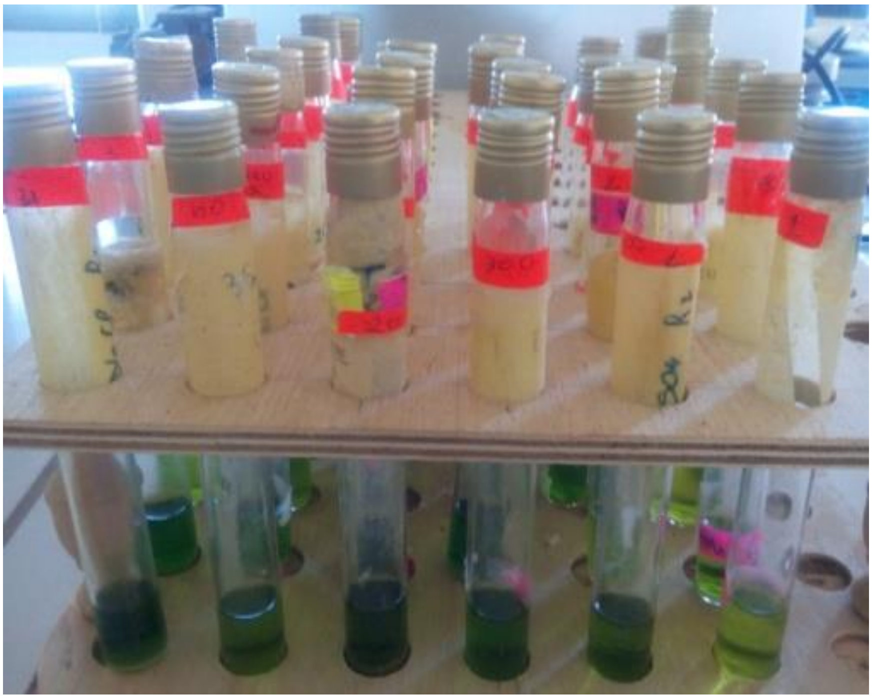

Photo 9: Chlorophyll pigments obtained by different concentrations of copper

Photo 10: Extraction of heavy metals (the beginning)

Photo 11: Extraction of heavy metals (the end)

Photo 12: Scheibler altimeter

Photo 13: Hydrometer

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EFFECT OF HEAVY METALS (COPPER AND ZINC) ON PROLINE, POLYPHENOLS AND FLAVONOIDS CONTENT OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

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Abstract

Current research on heavy metal toxicity in the agricultural field is focused on the toxic effects of copper and zinc on the most important crop which is tomato (*Lycopersicon esculentum* Mill.). To test the responses of tomato crop to heavy metal stress, leaves and roots were exposed to different concentrations of copper and zinc (0, 200, 300, 400 and 500ppm). All comparisons to the control indicate that the stress application induces the accumulation of proline content and promotes the synthesis of antioxidants such as polyphenols and flavonoids. This effect was directly proportional to the concentrations. Furthermore, these results suggest that the highest accumulation was occurred in leaves compared to the roots with all parameters studied. Additionally, the highest effect was marked with 500 ppm in both parts of the plant. The statistical analysis shows a highly significant increase in all biochemical parameters. A positive correlation was obtained between the osmolytes and the antioxidants. This study revealed that copper and zinc are essential elements for the plant. However, these metals at high concentrations become toxic for tomato by affecting and triggering the tolerance system in response to this stress.

***Key words**: Lycopersicon esculentum* Mill., copper, zinc, proline, polyphenols, flavonoids, phytotoxicity.

Introduction

Plants confront multifarious environmental stresses widely divided into abiotic and biotic stresses, of which heavy metal stress represents one of the most damaging abiotic stresses (Jalmi *et al*., 2018). Large amounts of heavy metals end up in the environment as a result of ever-increasing anthropogenic activities and economic development (Jiang *et al*., 2019). Anthropogenic activities such as agriculture, industry and mining have contributed significantly to the accumulation of heavy metals in the soil (Correia *et al*., 2018). Heavy metal pollution of agricultural soils is one of main concerns causing some of the different ecological and environmental problems. Excess accumulation of these metals in soil has deteriorated soil, decreased the growth and yield of plant and entered into the food chain. (Etesami, 2018). Heavy metals contaminated plants cause losses in crop production and risks for human health (Gratão *et al*., 2019). Heavy metals cause toxicity by targeting crucial molecules and vital processes in the plant cell (Jalmi *et al*., 2018).

Manganese (Mn), iron (Fe), copper (Cu) and zinc (Zn), due to their relatively low levels in the cell compared to abundant metal ions such as potassium and magnesium, transition metals are often considered micronutrients and referred to as trace elements (Li *et al*., 2018). Essential micronutrients, such as zinc (Zn) and copper (Cu) have direct roles in plant metabolism (Pandey, 2018), they are essential for a variety of functions in the living cell (Lüthje *et al*., 2018). Soils are mainly contaminated by copper and zinc (Salducci *et al*., 2019).

Zinc (Zn) is one of the essential plant micronutrients and is involved in several physiological functions in plants (Rizwan *et al*., 2019) for plant growth and development (Moreira *et al*., 2018). Zinc (Zn), an essential metal, is required by plants as they form important components of

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zinc finger proteins and also aid in synthesis of photosynthetic pigments such as chlorophyll. However, in excess amount Zn causes chlorosis of leaf and shoots tissues and generates reactive oxygen species (Pramanick *et al*., 2017). Its deficiency in plants has been widely reported in many regions of the world (Moreira *et al*., 2018).

Copper is an essential element in plants (Andrés-Colás *et al*., 2018 ; Shams *et al*., 2019), It acts as an essential cofactor of numerous proteins that play key functions in plant cell metabolism, such as the transport of electrons in mitochondria and chloroplast, the regulation of the cellular redox state (Migocka and Malas, 2018). Copper (Cu) contamination has been increasing in land ecosystems due to economic development activities. Excessive amount of Cu in soils is toxic to both plants and microorganisms (Meier *et al*., 2017). Its higher concentration can make disruption in plant growth (Shams *et al*., 2019) and may cause adverse effects on plant physiology (Chrysargyris *et al*., 2019). Copper (Cu) is among the main contaminant of agricultural soil (Napoli *et al*., 2019).

Deprivation of these elements (copper and zinc) is causing symptoms of deficiency, whereas excess can be toxic due to the production of reactive oxygen species and an imbalance of the cellular redox state. Both deficiency and toxicity are the cause of reduced growth and crop yields (Lüthje *et al*., 2018). These elements cause an increase in phytotoxicity effects in higher concentration (Wolf *et al*., 2017). The toxicity of increasing heavy metal ion in soil has been threatening the food security and environments (Ruan *et al*., 2019).

Tomato (*Lycopersicon esculentum* Mill.), this crop is of the Solanaceae family. The tomato is one of the most important crops worldwide (Marti *et al*., 2018), However, its productivity is impaired by a wide range of abiotic stresses such as the heavy metals (Gerszberg and Hnatuszko-Konka, 2017) and biotic stress (Bouzroud *et al*., 2018; Quiterio-Guitierrez *et al*., 2019; Cumplido-Najera *et al*., 2019). Tomato is an agronomically valuable crop in many countries as in Algeria and specifically in Mostaganem region, either grown in fields or greenhouses and therefore has been bred and genetically improved for centuries. Nonetheless, it remains vulnerable to diseases, consequently requiring the use of chemical pesticides, mainly in greenhouses (Ines and Bernacchia, 2018), but the overuse of these products contributes to soil contamination and has harmful effects on the microfauna, microflora of cultivated soils and on the quality of the tomato crop. In other side there is a wide range of plant protection products applied to tomatoes containing some heavy metals such as zinc and copper. In horticulture copper sulphate is utilized for the inhibition of phytopathogenic fungi. However, copper tends to accumulate in soil with a concomitant effect on soil quality and microbial diversity. The effect of the metal highlights the importance of the analysis of the consequence of copper utilization as fungicide on microbial activities (Carolina *et al*., 2019).

Plants must adapt themselves to the prevailing conditions for their survival, resulting in the acquisition of a wide range of metal tolerance mechanisms (Gratão *et al*., 2019). Heavy metals have restricted the plant regular life cycles affecting the plant primer and secondary metabolites by biochemical and physiological pathways (Kisa *et al*., 2019). The past recent decades had witnessed renewed interest to study abiotic factors that influence secondary metabolism during in vitro and in vivo growth of plants (Isah, 2019).

For instance, the antioxydants compounds such as polyphenol and flavonoids (Giordano *et al*., 2019; Sarker *et al*., 2019; Aryal *et al*., 2019). Flavonoids, a class of polyphenol secondary metabolites, are presented broadly in plants and diets (Wang *et al*., 2018).

Plant phenolics or polyphenols, the aromatic compounds with one or more hydroxyl groups, are produced by plants mainly for protection against stresses. Plants accumulate phenolic compounds in their tissues as an adaptive response to adverse environmental conditions and have a key role in the regulation of various environmental stresses (Naikoo *et al*., 2019). Furthermore, multivariate analyses, in which information about the antioxidant machinery was also included, were performed in order to identify the set of parameters related to plant tolerance (Borges *et al*., 2019).

The increased occurrence of phenols was recorded as a response to abiotic stress (Piccolella *et al*., 2018**)** and biotic stress (Eitle *et al*., 2019). According to Marti *et al.*, (2018) stress deficit irrigation can increase the amount of polyphenols and flavonoids in tomato.

Proline is an important amino acid that accumulates in plants in response to different environmental constraints (Ashraf *et al*., 2018; Jogawat, 2019). According to Li *et al*., (2018) under stress proline was accumulated in tomato**.** Plants' tolerance to heavy metal stress needs to be improved in order to allow the growth of crops with minimum or no accumulation of heavy metals in edible parts of the plant that satisfy safe food demands for the world's rapidly increasing population (Etesami, 2018). This is why this study was undertaken to evaluate the toxic effects of copper and zinc on biochemical parameters (proline, total polyphenols and flavonoids) in roots and leaves of tomato.

Material and Methods

Material and plant growth conditions

The seeds of tomato (*Lycopersicon esculentum* Mill.; Sain pierre variety) were chosen in this study as they are the one of the major variety planted in Mostaganem region by tomato farmers. The tomato seeds are disinfected in sodium hypochlorite solution (5%) for 5 min and rinsed thoroughly with sterile water and then, the seeds were sown in seedling trays containing compost for a period of 20 days at 25°C.

Sowing

The experiment was carried out in a greenhouse. The tomato seedlings were transplanted into cylinders (h=50 cm, d=20 cm) at the rate of 1 plant per cylinder. Each cylinder is lined at the bottom with a grave followed by filling a substrate consisting of a mixture of sand and potting compost (2V/V) respectively. The sand was sieved and treated successively by hydrochloric acid and water. A nutrient solution of Hoagland and Arnon, (1950) was used to maintain the development of the tomato plant.

Stress conditions

The copper stress was given as ($CuSO_4$, $5H_2O$) and zinc stress as ($ZnSO_4$, $7H_2O$). At the seedling stage, the solutions were prepared and applied separately twice during the experiment. The stress treatment was performed according to the experiment design which is consisted of a control (plants are not stressed) and five treatments (100, 200, 300, 400 and 500 ppm). Five biological independent replicates were carried out. The plants were kept in the greenhouse until the end of the experiment.

Parameters studied

- Estimation of proline content:

Proline content in roots and leaves of the tomato plant was achieved according to the method of Batles *et al.*, (1973). The absorbance was measured at 528 nm. The proline content calculated using a calibration curve was plotted and prepared with proline, this content was expressed as milligrams per gram of dry weight (mg g^{-1} DW).

- Estimation of polyphenols content:

Polyphenol content in roots and leaves of the tomato plant was estimated according to the method of Folin-Ciocalteau phenol reagent described by Singleton *et al.*, (1999). The concentration of polyphenol was calculated using a calibration curve prepared with standard gallic acid. The data were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

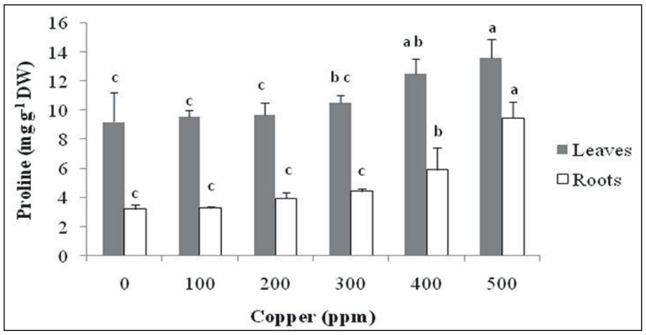

Fig. 1: Proline content in roots and leaves of tomato plants exposed to different concentrations of copper.

- Estimation of flavonoids content

The amount of flavonoids in roots and leaves of the tomato plant was determined through the method indicated by Zhishen *et al.*, (1999). The concentration of flavonoids was calculated using a calibration curve prepared and plotted with standard quercetin. The data were expressed as milligrams of quercetin equivalents per gram of dry weight (mg QE /g DW).

Statistical analysis

All analyses were performed through the use of STAT BOX software. The data were calculated by variance analysis (ANOVA). The significance of differences between control and treatment was determined at the 0.05 level of probability. Data presented in this study were expressed as mean values ± standard deviation (SD). The averages are compared according to the Newman- Keuls test. Each treatment was carried out with five replicates. The correlation between proline, flavonoids and polyphenols was performed by Pearson's correlation.

Results

Proline content

- Proline content in leaves and roots under copper stress: The results outlined in fig. 1, revealed that the proline content was increased by copper treatment in leaves and roots. This increase was directly proportional

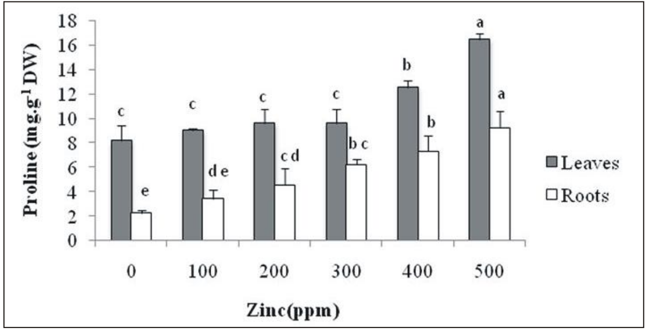

Fig. 2: Proline content in roots and leaves of tomato plants exposed to different concentrations of zinc.

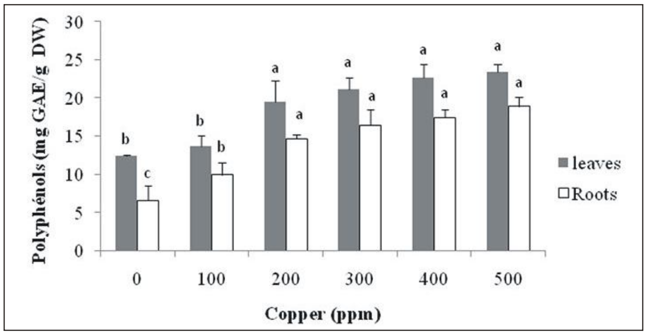

Fig. 3: Polyphenols content in roots and leaves of tomato plants exposed to different concentrations of copper.

to the different concentrations of copper stress. As is clearly observed in figure below there are no differences between the control and doses of 100, 200 ppm (9.21, 9.5, 9.6 mg g^{-1} DW) for leaves and (3.22, 3.29, 3.9 mg g^{-1} D.W) for roots respectively. This effect was more pronounced by 500 ppm with 13.62 in leaves and 9.47 mg g^{-1} DW in roots. The statistical analysis showed that the effect of copper treatment was highly significant in both leaves and roots compared to the control plants.

- Proline content in leaves and roots under zinc stress: fig. 2, illustrates the accumulation level of proline in roots and leaves exposed to different concentrations of zinc (0, 200, 300, 400 and 500 ppm). As it is given below the higher effect was reported with 500 ppm in leaves and roots (16.45, 9.21 mg.g^{-1} DW respectively) compared to the control that present values of (8.16, 2.23 mg.g^{-1} DW) in the same parts respectively. These results suggest that the highest accumulation occurred in leaves compared to roots. Compared to the unstressed plants, the variance analysis indicated that zinc stress increased the proline content in leaves and roots; this effect was highly significant in both parts of the tomato plant.

Polyphenols content

- Polyphenols content in leaves and roots under copper stress: In this test, an increase in polyphenols content was recorded in leaves and roots as a consequence of copper stress exposure. The results depicted in fig. 3, show that the best effect was obtained at 500 ppm. The differences between 200, 300, 400 and 500 ppm were not dramatic (19.5, 21.1, 22.6 and 23.4 mg GAE/g DW). However, these values remain superior to the control and those treated by 100 ppm (12.37, 13.7 mg GAE/g DW). This test highlighted that there is a positive correlation between concentrations and the accumulation level of polyphenols. The statistical analysis plays a crucial role to explain all the data presented in figure below. As it was found, the effect of copper treatment on polyphenols content was highly significant in roots and leaves compared to the unstressed plants. Overall, these results indicate that the high content in the leaves was not much greater than that contained in the roots.

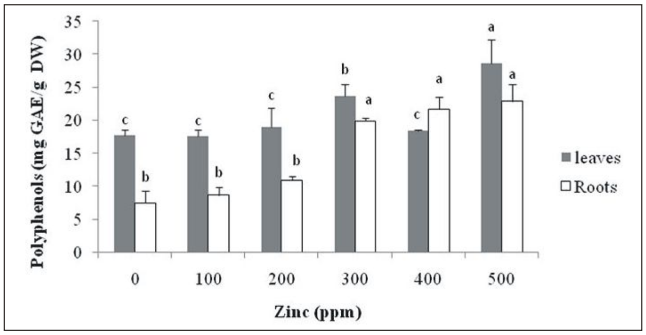

Fig. 4: Polyphenols content in roots and leaves of tomato plants exposed to different concentrations of zinc.

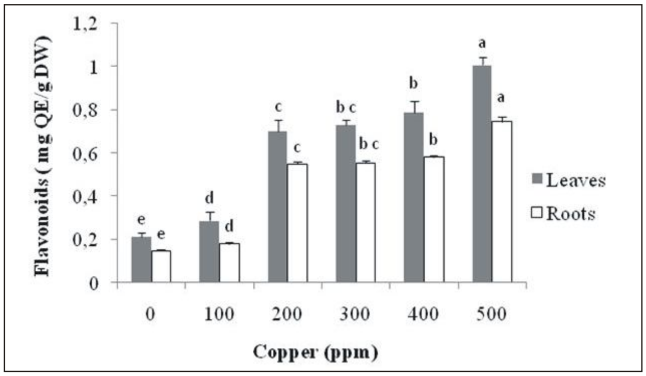

Fig. 5: Flavonoids content in roots and leaves of tomato plants exposed to different concentrations of copper.

- Polyphenols content in leaves and roots under zinc stress: The response pattern was different depending on the treatment concentration and the organ exposed to stress (Fig. 4). The synthesis of polyphenols compounds, under zinc exposure, continued to increase and reached a maximum level at 500 ppm of treatment. As is shown, a decrease of this parameter was clearly detectable with 400 ppm for leaves. Only In this particular dose, the accumulation was important in roots than leaves with values of (21.68, 18.33 mg GAE/g DW respectively). However, the effect remains to be superior to the control as observed. Compared to the untreated plants, the

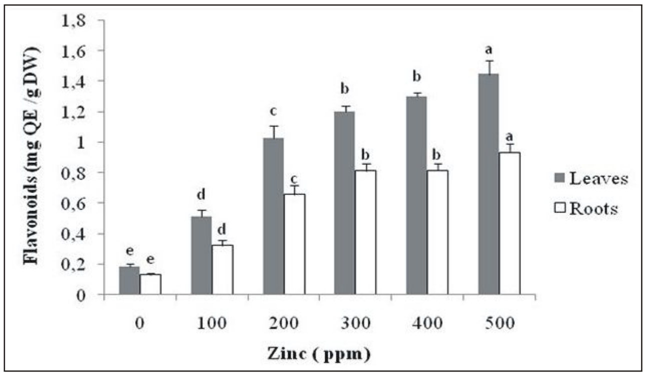

Fig. 6: Flavonoids content in roots and leaves of tomato plants exposed to different concentrations of zinc.

Table 1: Pearson correlation between proline, polyphenols and flavonoids in leaves and roots stressed by copper.

	Leaf proline	Root proline	Leaf polypenol	Root polypenol	Leaf flavonoids	Root flavonoids
Leaf proline	1.00	0.953**	0.824*	0.817*	0.836*	0.798
Root proline		1.00	0.758	0.757	0.827*	0.793
Leaf polypenol			1.00	0.99**	0.981**	0.978**
Root polypenol				1.00	0.977**	0.968**
Leaf flavonoids					1.00	0.997**
Root flavonoids						1.00

*Correlation is significant at the 0.05 level (2-tailed);
**Correlation is significant at the 0.01 level (2-tailed)

statistical test indicated a highly significant effect of zinc on the polyphenols profile for leaves and roots parts of the tomato plant.

Flavonoids content

- Flavonoids content in leaves and roots under copper stress: Under copper stress, an increasing trend of flavonoids content was detected in roots and leaves. Slightly superior results to the control are achieved with 100 ppm. However the effect becomes constant at 200 and 300 ppm, after that it continued to increase up from 400 ppm whereas the peak of the data occurs at 500 ppm. The simulation results indicate that flavonoids were accumulated in larger amounts in leaves compared to roots (Fig. 5). In comparison with control, the statistical analysis pointed out that copper stress causes a highly significant increase in flavonoids amount in leaves and roots.

- Flavonoids content in leaves and roots under zinc stress: As can be seen from fig. 6, there is a clear trend of increasing in flavonoids content in roots and leaves after zinc application. The great effect was clearly observed at 500 ppm (1.44, 0.93 mg QE /g DW) compared to the control (0.18, 0.13 mg QE /g DW) in roots and leaves respectively. On the other hand, the leaves exposed to zinc exhibited higher accumulation than roots along the treatments. Also there is an evident relationship between the doses and flavonoids content. A highly significant increase in flavonoids content was noted in leaves and roots as compared with control plants.

Table 2: Pearson correlation between proline, polyphenols and flavonoids in leaves and roots stressed by zinc.

	Leaf proline	Root proline	Leaf polypenol	Root polypenol	Leaf flavonoids	Root flavonoids
Leaf proline	1.00	0.919*	0.767	0.795	0.762	0.747
Root proline		1.00	0.786	0.956*	0.938**	0.934**
Leaf polypenol			1.00	0.711	0.673	0.699
Root polypenol				1.00	0.911*	0.916*
Leaf flavonoids					1.00	0.998**
Root flavonoids						1.00

*Correlation is significant at the 0.05 level (2-tailed);
**Correlation is significant at the 0.01 level (2-tailed)

Correlation analysis

Results presented in tables 1 and 2, showed a strong positive correlation between the accumulation of proline, polyphenols and flavonoids in leave and that in roots under copper and zinc stress. It is also indicated the presence of a high positive correlation between proline and polyphenols and flavonoids, also between polyphenols and flavonoids under both heavy metals.

Discussion

Proline

Accumulation of proline under stress in many plant species has been correlated with stress tolerance and its concentration has been shown to be generally higher in stress tolerant as *Atriplex lentiformis* than in stress-sensitive plants (Goni *et al.*, 2018; Eissa and Abeed, 2019). The accumulation was marked under heavy metals, in *Cinnamomum camphora* in response to Cu and Cd (Zhou *et al.*, 2019a), in spinach under copper (Gong *et al.*, 2019), in wheat under copper and pb (Jiang *et al.*, 2019), in *Astragalus tragacantha* under copper, zinc and arsenic (Salducci *et al.*, 2019). The accumulation of proline was also marked in tomato under different stresses, under heavy metals as Cd (Lima *et al.*, 2019), Salt stress (Siddiqui *et al.*, 2019 ; Poór *et al.*, 2019), drought stress (Chen *et al.*, 2018; Olivier and Nunes-nesi, 2018), Chilling tolerance (Aghdam *et al.*, 2018; Ghanbari and Sayyari, 2018), heat and drought (Zhou *et al.*, 2019b).

In the present study, the accumulation of proline was depending on heavy metal (copper or zinc) and its concentration and organ (leaves and roots). Various investigations have been conducted to identify the role of a particular amino acid(s) during Cu heavy metal stress (Kang *et al.*, 2017). The current study showed the accumulation of proline in leaves and roots of tomato under copper. Our results are consistent with the findings of Nazir e*t al.*, (2019), who suggested that Cu stress increased the level of proline in leaves of tomato in comparison to the control. Similar results were obtained in tomato leaves under copper stress by Kisa, (2019). Moreovre, in term of dose depending, our results concurred with those of

Hanafy *et al*., (2017), who indicated that proline content of tomato plants significantly increased gradually by increasing the Cu levels as compared with control plants, also that of Rizvi and Khan, (2018) in *Zea mays* under Cu and Pb, of Kalaikandhan *et al*., (2018) in *Sesuvium portulacastrum* under Cu and zinc, of Nannda and Agrawal, (2018) in *Cassia angustifolia* under copper. It has been reported that the content of proline in *Raphanus sativus* was elevated in plants treated with an excess of copper (100-250 mg kg^{-1}), whereas the lower level of copper treatment was (50 mg kg^{-1}) (Chrithuuthayam *et al*., 2018). In contrast to our results, Cu treatment caused a significant decrease in the proline content in *Egeria densa* and did not change its amount in *Ceratophyllum demersum* (Maleva *et al*., 2018).

On the other hand, this study indicated the accumulation of proline in leaves and roots of tomato under zinc. Our results are in agreement with the studies of Salimi *et al*., (2019), who showed that the increases in proline contents were provoked by applying the treatments of zinc (0, 50 and 100 mg L^{-1}) and obtained the content of (0.45, 0.54, 0.6, mg g^{-1} FW) respectively. The proline content of *Sesuvium portulacastrum* in shoot increased with increased concentration of zinc level (Kalaikandhan *et al*., 2018), these results collaborated with our findings. In addition, It is evident from the results that exposure to either metal (Co/Zn/Pb) in concentration resulted in a sharp rise in the proline content in the test plants (Menon *et al*., 2018). Subsequently there was a progressive increase in the proline content up to the 28th day in the order 50 ppm <100ppm < 300 ppm <500pm, these data are in line with our results and also has been confirmed by the findings of (Menon *et al*., 2018), who proposed that proline accumulation was of greater magnitude in the test plants especially at 500ppm concentration in all salt. Another study conducted by Kalaikandhan *et al*., (2018) demonstrate that minimum and maximum proline accumulation were recorded at 100 mg kg^{-1} and 600 mg kg^{-1} respectively, for zinc and copper level in *S. portulacastrum*. Similar observations were noted in our study with lower content at 100ppm and higher content at 500ppm for copper and zinc.

According to the current study, the accumulation of proline under copper and zinc stress was higher in leaves than roots. These results are in accordance with earlier studies conducted by Girilal *et al*., (2018), who proved that proline accumulated in all vegetative organs and in fruits when plants were subjected to stress, the highest concentration was found in growing leaves. Similar results were indicated by Alves *et al*., (2018) under salinity in tomato. It was reported by Saif and Khan, (2018) that proline accumulation followed the order: leaves > roots > shoots, these results differ from some published studies which suggested that proline content is higher in roots than leaves of tomato in control and salt stress (Horchani *et al*., 2010), similar results were noted by Ullah *et al*., (2019), also higher in roots than shoots in tomato plants (Natarajan *et al*., 2018). Root proline showed a strong linear relationship with endogenous Cu accumulated after exposure to the metal (Kebert *et al*., 2017). It has been reported that application of proline significantly increased the number of roots and root fresh weight (The *et al*., 2016).

Many studies have shown the reasons behind the accumulation of proline under heavy metals and environmental stress. Chandrakar *et al*., (2018) argued that proline has a role as protective agents, hence caused enhanced growth. Also, well documented in the literature that compatible osmolytes such as proline regulate the osmotic potential of cells exposed to abiotic stresses (Yadu *et al*., 2016; Chandrakar *et al*., 2017; Wiesenthal *et al*., 2019; Alyemeni *et al*., 2018). It has been observed that the net photosynthesis and transpiration were also decreased by the application of proline in both control and salt stressed plants (Orsini *et al*., 2018). On the other hand, proline mediates the elimination of ROS (Alyemeni *et al*., 2018; Alves *et al*., 2018) and directly scavenges OH radicals (Chandrakar *et al*., 2018; Per *et al*., 2017). It also plays important roles during stress as a metal chelator (Aslam *et al*., 2017). Proline protects folded protein structures against denaturation, stabilizes cell membranes by interacting with phospholipids, or serves as an energy and nitrogen source (Per *et al*., 2017; Arroussi *et al*., 2018) and protects the plant cells from the lipid peroxidation damage (Alves *et al*., 2018).

The exact mechanism of how proline accumulation helps the plant to cope up with heavy metal stress is difficult to elucidate. However, the available evidences suggest that proline acts by protecting the key enzymes from being inactivated by toxic metal ions (Menon *et al*., 2018). There was a direct correlation between enhanced proline content and activities of anabolic enzymes namely P5CS Pyrroline-5-carboxylate synthetase and GDH involved in its biosynthesis (Garg and Singh, 2018; Aswani *et al*., 2018). Proline dehydrogenase ProDH activity was induced by water deficit in both root types, exhibiting a higher activity in the primary or taproot tapR than in the fibR lateral or fibrous roots (fibR) (Castaneda *et al*., 2018). From our results, Aswani *et al*., (2018) suggested that proline metabolism can help to mediate inter-organelle interactions. Furthermore, proline-treated rice roots showed up-regulation and down-regulation of nine and eight proteins, respectively, when compared to those in the control (The *et al*., 2019).

Recent approaches have been used to regulate and enhance the accumulation of proline content under heavy metals, in order to improve plant tolerance. For example, Inoculation with plant growth promoting rhizobacteria (PGPR) (Bindu *et al*., 2018), inoculation with *Piriformospora indica* too significantly enhanced proline content as compared to Cu alone (Nanda and Agrawal, 2018), other applications; such as, exogenous glutathione (Hasanuzzaman *et al*., 2018), exogenous melatonin (Siddiqui *et al*., 2019), application of exopolysaccharide (Arroussi *et al*., 2018). Enhancement by phytohormones such as jasmonic acid and nitric oxide (Ahmad *et al*., 2018a), with *Pseudomonas aeruginosa* in tomato under heavy metal stress (Khanna *et al*., 2019).

Polypenols and flavonoids

The accumulation of total phenols and flavonoids content in different organs of tomato plant under heavy metal stress has been reported by many studies, in seedlings under Cd (Khanna *et al*., 2019), in fruits under selinium (Andrejiová *et al*., 2019), in roots and leaves under Cr and Pb (Ullah *et al*., 2019).

Focus on the results obtained in our study; we observed the accumulation of polyphenols and flavonoids in tissues of leaves and roots under copper and zinc treatment. This accumulation of antioxidant was depending on heavy metal (copper or zinc) and their concentration and organ (leaves and roots).

Copper stress induced the accumulation of polyphenols and flavonoids content in leaves and roots, these results corroborated the findings of Hanafy *et al*., (2017), who demonstrated that Cu stress caused a significant increase in phenols contents of tomato leaves under different concentration of Cu. Similar results were also obtained by Chrysargyris *et al*., (2019) in *Mentha spicata* under copper. According to Singh *et al*., (2018) and Chung *et al*., (2018), the application of CuO nanoparticles significantly enhanced the polyphenols and flavonoids content, these findings were also confirmed by (Pérez-Labrada *et al*., 2019) who found an increased the phenols (16%) in the leaves and phenols (7.8%) in the fruit compared with the control of tomato. Particularly relevant was the observation that flavonoids increased substantially in roots of *Solanum cheesmaniae* in response to excess Cu and decreased in shoots (Branco-Neves *et al*., 2017). Whereas, our findings contrast with (Kisa *et al*., 2019), who found that the applications of Cu, Cd and Pb significantly reduced the total phenolic content in tomato leaves..

Another effect in the present study was obtained by zinc application; the accumulation of polyphenols and flavonoids was also increased by zinc treatments in both organs. This is in good agreement with Ibiang *et al*., (2018), who reported that total polyphenols in fruits and shoot of tomato were significantly increased due to excess Zn. Furthermore, total phenolic compounds in roots and leaves were improved by the increasing gradient of Cd or Zn concentrations; total phenolic compounds significantly increased by 3.6-44.6% in the roots and by 0.4-126.6% in the leaves (Chen *et al*., 2019). In contrast to our results, the synthesis of antioxidants such as phenolic compounds declines as Cd and Zn leaf concentrations increase. These phenomena might be related to stress or the manifestation of a mechanism for tolerance to Cd and Zn accumulation (Sakurai *et al*., 2019). Also, it has been found that the contents of total phenol and flavonoids in the mature green fruits of tomato were reduced compared to control, following the n-ZnO nanoparticles treatment (Akanbi-Gada *et al*., 2019).

From this study, we can suggest that the accumulation of polyphenols and flavonoids content was higher in leaves than roots. These results were confirmed by (Ullah *et al*., 2019). Whereas, this level was higher in leaves than fruit (Pérez-Labrada *et al*., 2019) in tomato, in roots than shoot (Natarajan *et al*., 2018) in tomato, in fruits than shoots (Ibiang *et al*., 2018) in tomato treated by zinc. Singh *et al*., (2018) noted the correlation between these accumulations in different tissues; he demonstrated that the total phenol content in shoots showed a significant positive correlation with total phenol content in roots. It also showed a positive correlation with flavonoid content in shoots. Furthermore, the total phenol content in roots showed a positive correlation with flavonoid content in shoots. On the other hand, flavonoid content in roots and shoots also showed a positive correlation. (Singh *et al*., 2018). These findings confirm those obtained in this study.

Several researches have explained the cause and the main role of the elevated of these compounds under heavy metal stress, where they found that phenolic compounds became involved in one of the defensive systems that the plants used against Cd and Zn stress (Chen *et al*., 2019). Furthermore, these compounds provide defence against oxidative stress by acting as metal chelators and quenching of ROS (Ullah *et al*., 2019). Flavonoid and other phenolic compounds of plant origin have been reported as free radical scavengers (Singh *et al*., 2018), this is justified by total antioxidant activity in roots that showed significant positive correlation with total phenol, flavonoid (Singh *et al*., 2018). It has been indicated that Zn is a part of the antioxidant enzyme SOD (ZnSOD) thus enhances the activity of quenching the

ROS (Ahmad *et al*., 2018c). However, Zn concentrations had a moderate negative correlation with both polyphenol levels and radical scavenging activity (Sakurai *et al*., 2019). Phenolics possess hydroxyl and carboxyl groups and can bind to the metals. This may be the reason for the elevated level of secretion total phenolics in plants treated with silver nitrate (Girilal *et al*., 2018). An Other reason, An increase in soluble phenolic compounds such as intermediates in lignin biosynthesis increase cell wall endurance by the creation of physical barriers that protect cells against the harmful action of heavy metals, as well as influence the transition of metal ions within plant tissues since the lignification probably retains a substantial portion of metals into the cell wall fraction (Chen *et al*., 2019).

Regarding the effects obtained by heavy metals on the metabolism of the plant in this study, It has been reported that Plant phenolics are biosynthesized in plants from a biosynthetic intermediate, phenylalanine and shikimic acid through the shikimic acid pathway (Naikoo *et al*., 2019). Under stressful conditions, our findings were confirmed by Zaho *et al*., (2018), who indicated that Shikimate phenylpropanoid biosynthesis was perturbed by excess copper. Therefore, Cd and Zn treatments affected phenolic compounds metabolism in *Kandelia. obovata* (Chen *et al*., 2019). According to Soleimani *et al*., (2019), increasing phenols and flavonoids in tomatoes may arise from increasing PAL (phenylalanine ammonia-lyase) enzyme activity. This confirmed by (Aghdam *et al*., 2018). The decreases in the phenolics should be results of the decline in the activity of crucial enzymes involved in the biosynthesis of phenolic compounds under the heavy metal stress (Kisa *et al*., 2019). So, according to earlier strategies in term of plant tolerance against heavy metals, exogenous applications are used to improve antioxidant metabolism; such as, nitric oxide NO that increased the flavonoid and total phenol content in Cd-stressed tomato plants (Ahmad *et al*., 2018b). The same effect was observed under the application of jasmonic acid (Ahmad *et al*., 2018a) and exopolysaccharide treatment in tomato (Arroussi *et al*., 2018).

Conclusion

The application of copper and zinc on the tomato plant enhanced the accumulation of osmolytes such as proline and the antioxidants compound such as polyphenols and flavonoids. This accumulation increased significantly by the increase of doses for both heavy metals. The level of proline, polyphenol and flavonoids was higher in leaves than roots; this may be due to the phytoavailability of these heavy metals by the plant. A positive correlation was recorded between all these parameters. In conclusion, to cope against heavy metal stress, tomato induces the plant metabolism involved in abiotic stress tolerance. So, molecular researches are needed to better understand the heavy metal tolerance mechanism.

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