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## Insights On The Behavior Of Nano-Copper In The Agroecosystem: Mycorrhizal Associations With Spearmint (mentha Spicata)

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INSIGHTS ON THE BEHAVIOR OF NANO-COPPER IN THE  
AGROECOSYSTEM: MYCORRHIZAL ASSOCIATIONS  
WITH SPEARMINT (*MENTHA SPICATA*)

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Suzanne Annette Apodaca

2022

## **Dedication**

To my parents. Thank you for helping me build a beautiful life. My achievements have been fueled by your hard work and sacrifices. I am so proud to be your daughter.

INSIGHTS ON THE BEHAVIOR OF NANO-COPPER IN THE  
AGROECOSYSTEM: MYCORRHIZAL ASSOCIATIONS  
WITH SPEARMINT (MENTHA SPICATA)

by

SUZANNE ANNETTE APODACA, M.S.

DISSERTATION

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## Abstract

Nanotechnology offers significant potential benefits to our society, including the agriculture sector. With the advancement of nano-enabled agrochemicals towards sustainable and efficient agricultural practices, it is essential to address environmental issues associated with the use of nanoscale materials. The same properties that give promise to applications of nanotechnology in modern agriculture could have unintended consequences on ecosystem dynamics. A point of concern for risk management is the impact of engineered nanomaterials (ENMs) on beneficial microbial communities, which support a variety of ecosystem services.

Use of copper (Cu) products in agriculture are based on their abundance, role as a micronutrient, and antimicrobial activity. As nanoparticles (NPs), their size allows for cost-effective application and easy absorption by plants. Although there are many benefits associated with Cu NPs in agrochemicals, in higher concentrations they may be toxic to the environment and co-existing organisms. Uptake routes of NPs may be intentional or unintentional (in the form of pollution). Therefore, understanding the fate, exposure, and toxicity of Cu NPs are important factors to consider for their safe use.

The most common mutualistic association between terrestrial plants and microbes are formed by arbuscular mycorrhizal (AM) fungi. These specialized fungi colonize the roots of the host plant, providing an array of benefits in exchange for key nutrients. Among these conferred benefits are enhanced stress tolerance, including amelioration of heavy metal toxicity. Despite this, fungi are known to be sensitive to abiotic stresses. Although there has been extensive research on plant stress responses, it often does not consider fungal symbiosis. Hence, there is a critical knowledge gap on the impact of Cu NPs to mycorrhizal symbiosis.

Spearmint (*Mentha spicata*) is characterized by its remarkable aroma and commercial value. The leaves and extracted essential oils are primarily used as a flavoring in foods and beverages, fragrance in perfumes and cosmetics, and antioxidative ingredient in traditional medicines. In addition to being widely cultivated for commercial production on a large scale, spearmint is also often grown in home gardens as a culinary herb. Several safety issues are brought forward with spearmint regarding its human uses and the intensive production systems used to meet growing demand.

This research was conducted to evaluate the impact of Cu-based NPs/compounds on spearmint plants symbiotically associated with AM fungi. Short term and long term effects of Cu(OH)<sub>2</sub> nanowires (*nCu*), Cu(OH)<sub>2</sub> pesticide (Kocide 3000)(*bCu*), and CuSO<sub>4</sub> solution (*iCu*) on AM fungi were investigated. The research was divided into three phases: Phase I assessed the acute toxicity of *nCu*, *bCu*, and *iCu* to AM fungi; Phase II determined the interactive effects of *nCu*, *bCu*, and *iCu* on agronomical and physiological parameters in mycorrhizal spearmint; and the transfer of nutrients from spearmint plants to AM fungi under exposure to *nCu*, *bCu*, and *iCu* was studied in Phase III.

In Phase I, toxicity testing was conducted with two separate bioassays. Endomycorrhizal spores (*Glomus etunicatum*) were cultivated in either agar or lysogeny broth growth medium containing 0 to 500 mg L<sup>-1</sup> of *nCu*, *bCu*, and *iCu*. Fungal growth was monitored over the span of 3 days through spore count (absorbance) and fungal biomass. Visual observations of fungal cultures were also made. The incidence of dark colored spores and mycelium expansion, especially in *iCu* treatments, indicated that Cu is likely being allocated to fungal structures as part of a metal detoxification strategy by AM fungi. However, based on our quantitative measurements, there were no significant changes in spore count or fungal biomass from exposure to Cu-based



NPs/compounds. This experiment demonstrated that AM fungi (*Glomus etunicatum*) can tolerate Cu concentrations of up to at least 500 mg L<sup>-1</sup> from *nCu*, *bCu*, and *iCu*.

In Phase II, spearmint plants inoculated with arbuscular mycorrhizal (AM) fungi were sprayed with 0.66 and 1.05 mg of *nCu*, *bCu*, and *iCu*. After a 50-day growth period spearmint plants were harvested for agronomic, biochemical, and elemental analysis. Accumulation of Cu was highest in the roots (149.40–427.03 mg kg<sup>-1</sup>). In general, Cu-based NPs/compounds were toxic, inhibiting plant growth and element accumulation. The root biomass and length were reduced by up to 8.38 g and 11.80 cm or 59.92 and 48.15%, respectively. Most alterations in element accumulation occurred in the leaves, with significant decreases in Mg ( $\leq 10.47$  mg kg<sup>-1</sup> or 22.59%), Mn ( $\leq 0.77$  mg kg<sup>-1</sup> or 39.77%), and Zn ( $\leq 0.20$  mg kg<sup>-1</sup> or 37.03%), from the respective controls. Mycorrhization alleviated Cu toxicity, most notably in the roots, where interactive effects were observed under application of AM fungi and Cu-based NPs/compounds for root biomass and length. An interactive effect found between the absence of AM fungi and presence of Cu-based NPs/compounds resulted in an influx of leaf Na content (0.70–1.13 mg kg<sup>-1</sup>, or 219.74–355.07%, increase from the corresponding control), which can be seen as an indicator of Cu-induced stress. Overall, bulk Cu-based compounds had the greatest impact over nano or ionic Cu-based compounds, with interactive effects in root accumulation of Cu (decrease by AM fungi + bulk Cu) and leaf accumulation of Mg and Mn (decrease by AM fungi + bulk Cu). This research confirms that Cu-based NPs/compounds can be phytotoxic to spearmint plants and that mycorrhizal symbiosis can alleviate Cu toxicity.

In Phase III, roots from 50-day-old mycorrhizal spearmint plants treated with 0.66 and 1.05 mg/Cu per pot of *nCu*, *bCu*, and *iCu* were isolated and analyzed. Protein, sugar, and starch contents were determined to further understand the transfer and regulation of nutrients from

spearmint plants to AM fungi. Mycorrhizal spearmint roots had higher amounts of protein than non-mycorrhizal spearmint roots, which is likely due to glomalin production by AM fungi. Furthermore, there was a negative dose-dependent relationship between protein concentration and Cu-based NPs/compounds. Although sugar accumulation was not impacted by Cu treatments, variations in sugar concentration suggest that sugar transport, metabolism, and storage may have been influenced by exposure to Cu-based NPs/compounds. Similar to results from Phase II, *b*Cu elicited a stronger response than *n*Cu or *i*Cu and differentially affected protein and sugar contents. Starch concentration was stable among all treatments. Findings from this study provide evidence that carbon exchange between spearmint plants and AM fungi is disrupted by Cu stress.

This dissertation provided valuable insight on the different interactions between Cu-based NPs/compounds, AM fungi, and spearmint plants. In summary, our results indicated that AM fungi are tolerant of high Cu concentrations and alleviated a wide spectrum of Cu-induced phytotoxicity in spearmint through heavy metal stress adaptations. However, based on our observations, exposure to Cu-based NPs/compounds could negatively influence the nutrient exchange from spearmint plants to AM fungi. Furthermore, bulk treatments elicited a stronger response in mycorrhizal spearmint than nano or ionic treatments. These findings demonstrate that plant-microbe interactions can improve crop productivity and provide environmental benefits, emphasizing the need for risk assessment of crop systems incorporating symbiotic microorganisms. Our research confirms that while Cu-based NPs/compounds do not significantly impede the benefits conferred to spearmint from AM fungi, providing evidence for their safe commercial use in complex environmental systems, further study on underlying mechanisms and bulk Cu-based compounds is also needed.

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## **Chapter 1: Introduction**

Nanoscience has become increasingly important in the 21<sup>st</sup> century. The merging of nanoscience and technology (also known as “nanotechnology”) can significantly improve, and even revolutionize, several major industries. Evolving applications of nanoscale technology are expected to have a massive bearing on the economy. By 2028, the global market of nanotechnology is projected to reach US\$ 290.93 billion (Emergen Research, 2021). With the advent of technological advances in nanoscale materials, it is paramount to not only harness their benefits, but also evaluate their environmental impact.

Environmental issues are inherently complex and require multidimensional analysis and solutions. To further complicate this, nanotechnology utilizes the unique physical, chemical, and biological properties expressed by structures at the nanoscale. There are two distinct facets of the environmental impact of nanotechnology: (1) innovations in nanotechnology to promote sustainable development, and (2) increased toxicological pollution from the novel properties of nanotechnological materials (Ibrahim et al., 2016). As it stands, the development of nanotechnology has outpaced risk assessment. Existing regulatory frameworks do not bestow special consideration to products containing nanomaterials (NMs). Research in areas of scientific uncertainty and appropriate legislation are needed to close the gap between risk and regulatory approaches to ensure the safe management of nanotechnologies (Roig, 2018).

### **1.1 BACKGROUND**

#### **1.1.1 Engineered nanomaterials**

Tiny matter in the nanoscale (with two dimensions between 1-100 nm) is referred to as nanoparticles (NPs)(Klaine et al., 2008). In comparison to their bulk counterpart materials, NPs

exhibit different physical and chemical properties. For example, the familiar lustrous appearance of metal gold is lost at the nanoscale and gold NPs can appear as either red or purple (Heiligtag & Niederberger, 2013). An explanation for this is found in the surface area to volume ratio – the smaller that an object is, the larger its surface area is compared to its volume (Figure 1.1) (Ramsden, 2016). Since atoms on the material surface are more reactive, a relative larger surface area equates to an overall more reactive material. Therefore, a higher surface area to volume ratio can have a significant effect on properties exhibited by NPs.

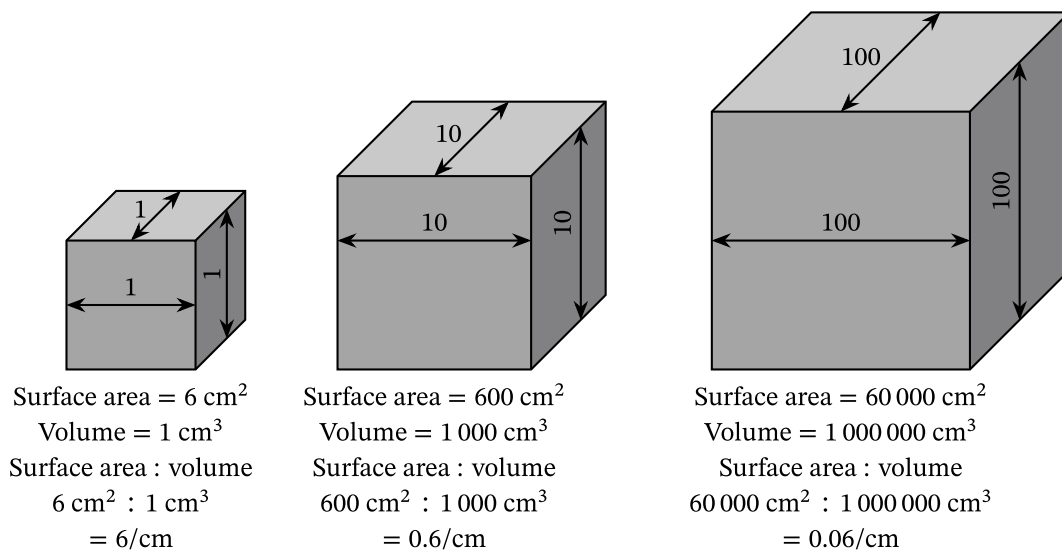


Figure 1.1: Illustration of how surface area to volume ratio is greater at smaller particle sizes.

Adapted by permission from Elsevier (Yokoyama et al., 2018).

NPs can be found in a multitude of conformations and amalgamations throughout the environment (Griffin et al., 2018). Based on their source, they are classified into three major groups: natural, incidental, and engineered. Naturally-occurring NPs are present in all of Earth's subsystems. For instance, mineral composites, volcanic ash, sea spray, and biological matter (e.g.,

viruses) all contain NPs. Both incidental and engineered nanomaterials (ENMs) are synthetic in origin, with the former being a byproduct of anthropogenic activity, and the latter being tailored for specific applications (Corredor, 2015).

### **1.1.2 Nanotechnology in agriculture**

ENMs are rapidly being exploited across several major industries such as medicine, biotechnology, electronics, materials science, and energy, among others (Mobasser & Firoozi, 2016). They also being used to optimize agricultural practices (Nicolopoulou-Stamati et al. 2016). Given that agriculture provides raw material for feed and food industries, effective and efficient practices must be developed to meet the demands of a growing population with a finite level of resources. Although agrochemicals (pesticides and fertilizers) have undeniably made major contributions to agricultural productivity, excessive use of these chemicals has also caused serious pollution to aquatic and terrestrial ecosystems (Jamala et al., 2013). This pollution has escalated disease burden – particularly to humans. In addition, high application rates of expensive agrochemicals are not economical. Nanotechnology has great potential as a tool for distributing more benign agrochemicals safely and productively (Prasad et al., 2017). Incorporating ENMs into agricultural practices can reduce the application of plant protection products through smart delivery systems, mediate genetic transformation in plants, minimize nutrient losses in fertilization, optimize water and energy management, and assess food safety and quality (Figure 1.2).

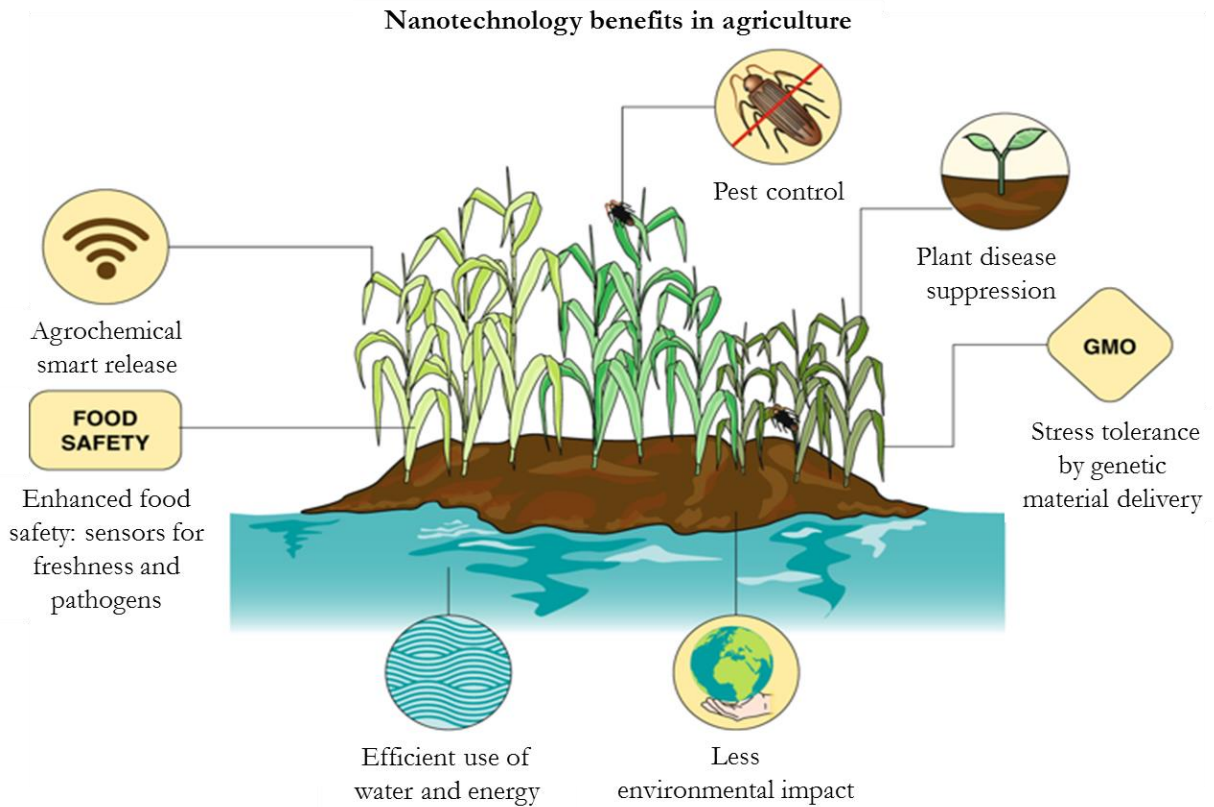


Figure 1.2: The application of nanotechnology in agriculture can provide an array of potential benefits. Nanotechnology devices and tools can increase crop quality and yield, reduce agronomic inputs, and enhance nutrient adsorption. Reprinted with permission from Springer Nature (White and Gardea-Torresdey 2018).

In the past decade, the number of publications associated with nano-enabled agriculture has risen nearly tenfold, indicating high activity for this field (“Nano for Agriculture, Not the Opposite,” 2020). The volume and variety of released ENMs are also expected to increase accordingly (Keller, Adeleye, Conway, Garner, Zhao, Cherr, Hong, Gardea-Torresdey, et al. 2017). Since agriculture fosters direct contact with environmental media, it is considered a major source of environmental release, with soil as the main repository. To enter the agricultural system, NPs are either directly applied as agrochemicals and biosolid fertilizers, or irrigated with water

from wastewater treatment plants (Kaphle et al., 2018). There is an intersect between agriculture and public health through human consumption. As a result, it is important to assess the ecotoxicological impacts of NPs on the considered agricultural field (European Public Health Alliance, 2016).

The transformations of ENMs applied to agricultural soils and their impacts in terrestrial environments have been extensively reviewed (Gardea-Torresdey et al., 2014). Both beneficial, neutral, and detrimental effects have been found on the agronomic traits, yield, and productivity of plants. In general, low concentrations (less than 50 mg kg<sup>-1</sup>) are stimulatory, while higher concentrations are damaging (Cota-Ruiz et al., 2018). The uptake processes of ENMs by plants are susceptible to natural factors such as soil dynamics, microorganisms, nutrients, and plant species. To illustrate, soil interacts with NPs to trigger aggregation and/or increased solubility, which diminishes their reactivity, enhances their stability, and potentiates ion release. This can induce accumulation of the component metal or carbon material in the fruit/grain of food crops. Furthermore, assimilation of NPs by plants is modified by the presence of microorganisms. Particularly those that establish symbiosis with plants, which alter the dynamics and availability of nutrients (Pérez-de-Luque, 2017). Comprehensive studies that incorporate realistic field conditions and comparisons to both conventional formulations and the pure active ingredient could help to evaluate the environmental tradeoffs of nanoagrochemicals.

### **1.1.3 Copper engineered nanomaterials**

The electrical, thermal, optical, and catalytic properties of copper (Cu) make it a primary industrial metal (Festa & Thiele, 2011). It is also a trace element that is integrated into macromolecules and enzymes, making it vital for the health of living organisms. Copper

nanoparticles (Cu NPs) exhibit unique properties from bulk-sized Cu particles, making them superior in function (Ponmurugan et al., 2016). Moreover, they are less expensive than other comparable metal NPs. Mineral nutrients can be delivered to target sites in plants by controlled release where, in this form, they are absorbed and assimilated by the plant faster than traditional fertilizers (Morales-Díaz et al., 2017). Although in its infancy, Cu NPs have been explored for the potential benefits that they may offer to the agricultural sector (Morales-Díaz et al., 2017; Ponmurugan et al., 2016). Several Cu-based nanopesticides and nanofertilizers are already commercialized. Their advanced microbial protection and targeted delivery could considerably improve crop quality and yield (Kah et al. 2018). Nano-enabled formulations were found to be 20-30% more effective than conventional pesticides or fertilizers, and several were even able to maintain the same productivity at lower applications rates. Since Cu is less bioavailable in soil, foliar application of Cu-based nanofertilizers is preferred to soil application (Dey et al., 2018). Foliar application also has the added advantages of immediate contact with the stomata and leaf epidermal cells, which are majorly involved in nutrient uptake, and reduced contribution of NPs into the environment. Due to these economic and environmental benefits, the distribution of Cu-based nanopesticides and nanofertilizers are expected to increase their share in the agricultural market (Keller, Adeleye, Conway, Garner, Zhao, Cherr, Hong, Gardea-Torresdey, et al., 2017).

The multimodal antimicrobial feature of Cu NPs is mediated by their size, which allows for close interaction with microbial membranes and metal ion release (Figure 1.3) (Ramyadevi et al., 2012). As the NPs begin to slowly oxidize in solution, they release Cu ions that generate reactive oxygen species (ROS)(Wei et al., 2010). These ROS degenerate the membrane-cell wall interface by disassembling membrane lipids. This allows for intracellular substances to leak out of cells from the perforated membranes, impairing their ability to sustain fundamental biochemical



processes. It has also been suggested that Cu ions can interact with biomolecules, like DNA and protein, distorting their structure and further disrupting biochemical development (Rai et al., 2018). Ultimately, these mechanisms induce microbial cell death.

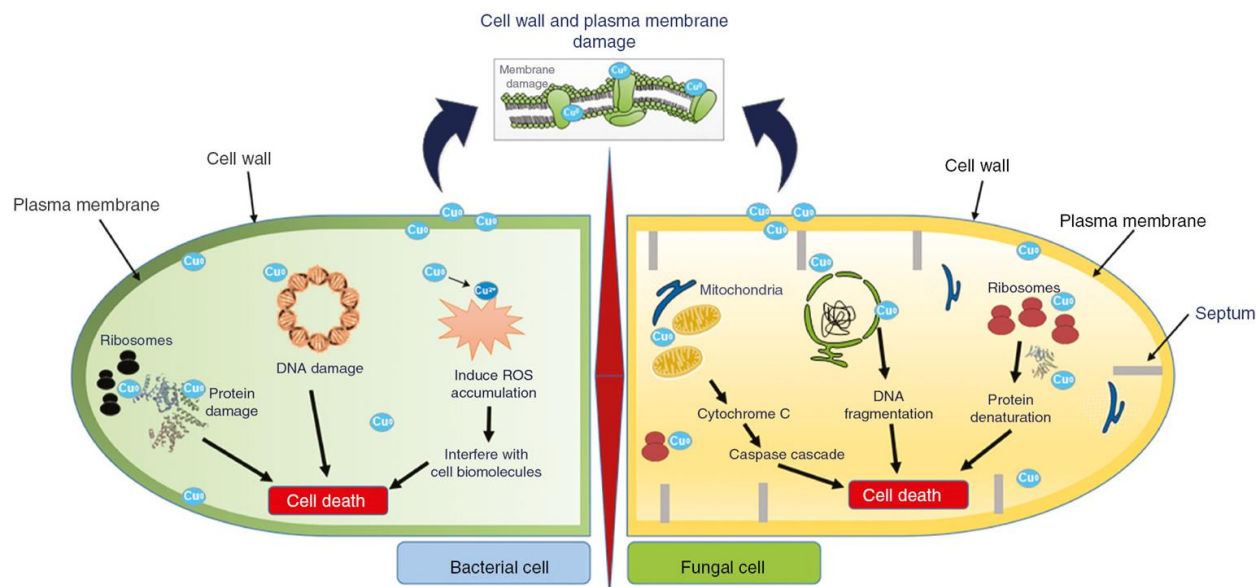


Figure 1.3: The antimicrobial mechanism of Cu NPs is a function of particle size and ion release. Reprinted by permission from Nanotechnology Reviews (Rai et al., 2018).

## 1.2 PROBLEM STATEMENT

With the advent of nanotechnology, the manufacture and application of ENMs has drastically increased. These ENMs can be indirectly or directly input into agricultural lands. Their interactions with the surrounding environment have been shown to be complex and dynamic. Currently, most studies on the consequences of ENMs focus on the agricultural soil environment and plant system separately. To understand agricultural ecosystems, it is vital to study integrated plant systems: the relationship between soils, microbes, and plants.

The dissemination of Cu-based nanopesticides and nanofertilizers is likely to increase due to the associated benefits of low cost, high efficiency, and broad-spectrum bioactivity. A fundamental question is whether concerns over the environmental impact of Cu-based NPs could simply be due to Cu itself since this metal in bulk form possesses antimicrobial properties and can be either toxic (in excess) or stimulatory.

The overall **goal** of the research:

- Evaluate the impact of Cu-based NPs/compounds on plant-fungal interactions.

### **1.3 PURPOSE AND SIGNIFICANCE**

#### **1.3.1 Ecological importance of fungi**

Microorganisms play a key role in maintaining soil health and crop productivity. Arbuscular mycorrhizal (AM) symbiosis is the association between specialized soil fungi and vascular plant roots (Gadkar et al. 2001). Dating back at least 460 million years ago, AM symbiosis affects roughly 90% of Earth's plant species and is considered an integral part of natural ecosystems. The mutually beneficial plant-fungi relationship is based on the exchange of nutrients: the fungi enhance mineral nutrient uptake by roots, while the plant supplies the fungi with photosynthetic carbon (Berruti et al., 2016). This affiliation can also enhance water absorption under drought conditions. Furthermore, the presence of AM fungi inhibits proliferation of some microorganisms (pathogen protection), while stimulating others (Berruti et al., 2016). Overall, crop performance is often improved when well-colonized by AM fungi.

While pesticides and fertilizers may protect plants from phytopathogens and nutrition deficiency, they may also have unintended consequences on non-target microorganisms by

increasing the soil concentration of heavy metals (Simonin et al., 2018). The role of agrochemicals on fungal community dynamics is essential since it inevitably affects plant health and production. When determining the interaction between AM symbiosis and heavy metals, two aspects must be considered: (1) the effect of heavy metals on AM fungi populations and their tolerance to heavy metals, and (2) the effect of AM fungi on the availability and transfer of heavy metals to the host plant (Figure 1.4) (Leyval et al., 1997). Through enhanced stress-tolerance, AM fungi can alleviate heavy metal toxicity to the host plant under suboptimal conditions.

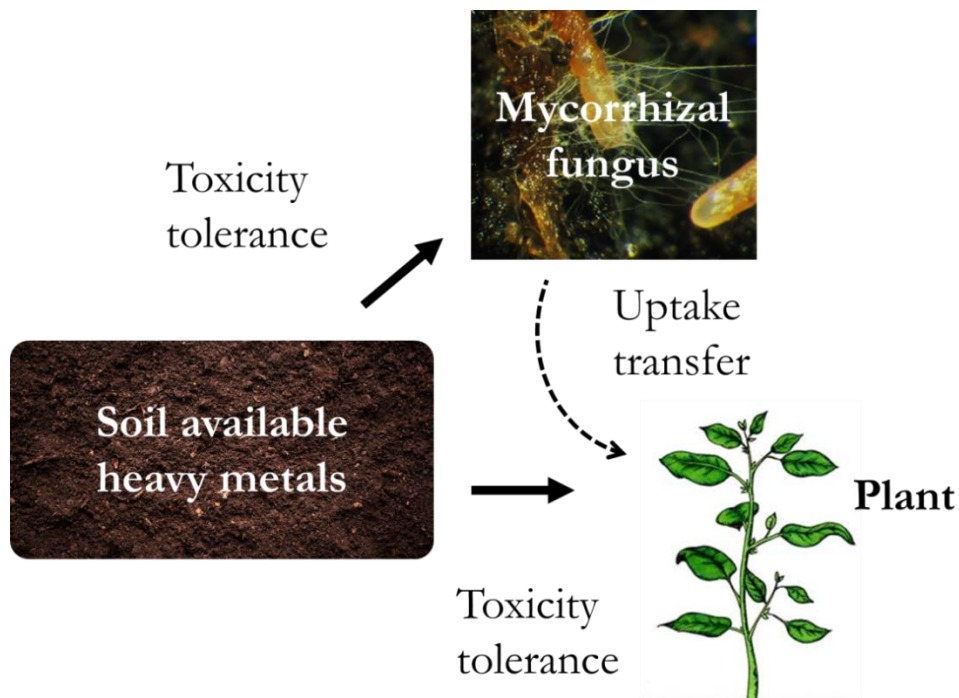


Figure 1.4: The relationship between heavy metals and plant-fungal symbiosis. The toxicity tolerance of AM fungi and plants to heavy metals depends on their bioavailability in soil and their uptake transfer at the root-soil interface. Adapted with permission from Springer (Leyval et al., 1997).

### 1.3.2 Spearmint as a plant model

Mint (*Mentha* species) is a versatile and robust herb with vigorous growth, making it a popular choice for home gardens (Chrysargyris, Solomou, et al., 2019). Its leaves are commonly used during preparation of beverages and foods throughout different cuisines. The strong flavor and aroma of mint brings economic value in the form of essential oils, which are used in a variety of consumer products including skin creams, ointments, oral care (e.g., toothpaste, mouthwash, chewing gum), cough drops, confectionary, and alcoholic liqueurs (Mahendran et al., 2021). Mint also has a long history of use in medical applications due to the presence of several bioactive ingredients in mint essential oils possessing antioxidant, anti-inflammatory, antimicrobial, and anticancer characteristics. Commercially, spearmint (*Mentha spicata*) is one of the two most important mint species (Cohen et al., 2020). The United States is the top producer of spearmint oil, followed by India and China. The result of human activities is that commercially cultivated plants, such as spearmint, are subjected to multiple stressors/stress conditions that may affect their growth, yield, and quality (Chrysargyris, Papakyriakou, et al., 2019). Furthermore, there are concerns regarding its human uses; especially since spearmint is often directly consumed.

## 1.4 RESEARCH QUESTIONS, OBJECTIVE, AND HYPOTHESES

The research was split into three phases. Combined, these studies provided insight on the impact of Cu-based NPs/compounds in mycorrhizal spearmint. **Phase I** served to observe the impact of Cu-based NPs/compounds on the growth of AM fungi, and determine ideal environmental conditions and treatment concentrations for future experiments. **Phase II** was based on the exposure of different Cu-based NPs/compounds to spearmint inoculated with AM fungi to

assess the combined effects on plant quality and yield. **Phase III** examined the transfer of nutrients from spearmint plants to AM fungi in the presence of Cu-based NPs/compounds.

The research work sought to answer the following **questions**:

- 1 What is the response of AM fungi to Cu-based NPs/compounds?
- 2 Do Cu-based NPs/compounds enhance or reduce spearmint plant benefits associated with mycorrhizal symbiosis?
- 3 Is the nutrient exchange in mycorrhizal symbiosis influenced by Cu-based NPs/compounds?

The **objectives** are to:

- 1 Determine the tolerance of AM fungi to Cu-based NPs/compounds.
- 2 Evaluate the interactive effects of Cu-based NPs/compounds and AM fungi on the physiological and biochemical parameters of spearmint plants.
- 3 Investigate whether the nutrient exchange between spearmint and AM fungi is regulated by Cu-based NPs/compounds.

This investigation will be performed under the working **hypotheses** that:

- 1 AM fungi will have an inverse dose-response relationship with Cu-based NPs/compounds.
- 2 There will be an advantageous synergistic effect on the yield and productivity of spearmint plants by Cu-based NPs/compounds and AM fungi.
- 3 Exposure to Cu-based NPs/compounds will disrupt nutrient uptake from spearmint plants by AM fungi.

## REFERENCES

- Berruti, A., Lumini, E., Balestrini, R., & Bianciotto, V. (2016). Arbuscular mycorrhizal fungi as natural biofertilizers: Let's benefit from past successes. *Frontiers in Microbiology*, 6(1559), 1–13.
- Chrysargyris, A., Papakyriakou, E., Petropoulos, S. A., & Tzortzakis, N. (2019). The combined and single effect of salinity and copper stress on growth and quality of *Mentha spicata* plants. *Journal of Hazardous Materials*, 368, 584–593.
- Chrysargyris, A., Solomou, M., Petropoulos, S. A., & Tzortzakis, N. (2019). Physiological and biochemical attributes of *Mentha spicata* when subjected to saline conditions and cation foliar application. *Journal of Plant Physiology*, 232, 27–38.
- Cohen, S. M., Eisenbrand, G., Fukushima, S., Gooderham, N. J., Guengerich, F. P., Hecht, S. S., Rietjens, I. M. C. M., Bastaki, M., Davidsen, J. M., Harman, C. L., McGowen, M. M., & Taylor, S. v. (2020). FEMA GRAS assessment of natural flavor complexes: Mint, buchu, dill and caraway derived flavoring ingredients. *Food and Chemical Toxicology*, 135.
- Corredor, C. (2015). *Methods to Assess Presence and Biological Impact of Engineered Nanoparticles*.
- Cota-Ruiz, K., Delgado-Rios, M., Martínez-Martínez, A., Núñez-Gastelum, J. A., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2018). Current Findings on Terrestrial Plants - Engineered Nanomaterial Interactions : Are Plants Capable of Phytoremediating Nanomaterials from ScienceDirect Current findings on terrestrial plants – Engineered nanomaterial interactions : Are plants capable of p. *Current Opinion in Environmental Science & Health*, 6(July), 9–15.

- Dey, J. K., Das, S., & Mawlong, L. G. (2018). *Nanotechnology and its importance in micronutrient fertilization*. 7(05), 2306–2325.
- Emergen Research. (2021). Nanotechnology Market Size, Share, Trends, By Type (Nanomaterials, Nanocomposites, Nano devices, Nano tools), By Industry (Food and Agriculture, Healthcare, Information and Technology, Environment, Energy, Cosmetics), and By Region, Forecast to 2028. In *Press Release*.
- European Public Health Alliance. (2016). Agriculture and Public Health. *Impacts and Pathways for Better Coherence*, May, 1–13.
- Festa, R. A., & Thiele, D. J. (2011). Copper: An essential metal in biology. *Current Biology*, 21(21). <https://doi.org/10.1016/j.cub.2011.09.040>.Copper
- Gardea-Torresdey, J. L., Rico, C. M., & White, J. C. (2014). Trophic Transfer, Transformation, and Impact of Engineered Nanomaterials in Terrestrial Environments. *Environmental Science & Technology*, 48, 2526–2540.
- Griffin, S., Masood, M. I., Nasim, M. J., Sarfraz, M., Ebokaiwe, A. P., Schäfer, K., Keck, C. M., & Jacob, C. (2018). Natural Nanoparticles: A Particular Matter Inspired by Nature. *Antioxidants*, 7(3), 1–21.
- Heiligtag, F. J., & Niederberger, M. (2013). The fascinating world of nanoparticle research. *Materials Today*, 16(7–8), 262–271.
- Ibrahim, R. K., Hayyan, M., Hayyan, A., & Ibrahim, S. (2016). Environmental application of nanotechnology: air, soil, and water. *Environmental Science and Pollution Research*, 23, 13754–13788.

- Jamala, G. Y., Ari, B. M., Tsunda, B. M., & Waindu, C. (2013). Assessment of Agro-Chemicals Utilization by Small-Scale Farmers in Guyuk , Adamawa State , Nigeria. *IOSR Journal of Agriculture and Veterinary Science*, 6(2), 51–59.
- Kaphle, A., Akhela, P. N. N., Hemant, U., & Daima, K. (2018). Nanomaterials for agriculture , food and environment: applications, toxicity and regulation. *Environmental Chemistry Letters*, 16, 43–58.
- Keller, A. A., Adeleye, A. S., Conway, J. R., Garner, K. L., Zhao, L., Cherr, G. N., Hong, J., Gardea-torresdey, J. L., & Godwin, H. A. (2017). Comparative environmental fate and toxicity of copper nanomaterials. *Nanoimpact*, 7, 28–40.
- Keller, A. A., Adeleye, A. S., Conway, J. R., Garner, K. L., Zhao, L., Cherr, G. N., Hong, J., Gardea-Torresdey, J. L., Godwin, H. A., Hanna, S., Ji, Z., Kaweeteerawat, C., Lin, S., Lenihan, H. S., Miller, R. J., Nel, A. E., Peralta-Videa, J. R., Walker, S. L., Taylor, A. A., ... Zuverza-Mena, N. (2017). Comparative environmental fate and toxicity of copper nanomaterials. *NanoImpact*, 7(May), 28–40.
- Klaine, S. J., Alvarez, P. J. J., Batley, G. E., Fernandez, T. F., Handy, R. D., Lyon, D. Y., Mahendra, S., McLaughlin, M. J., & Lead, J. R. (2008). Nanomaterials in the environment: Behavior, fate, bioavailability and effects. *Environmental Toxicology and Chemistry*, 27(9), 1825–1851.
- Leyval, C., Turnau, K., & Haselwandter, H. (1997). Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza*, 7, 139–153.



- Mahendran, G., Verma, S. K., & Rahman, L. U. (2021). The traditional uses, phytochemistry and pharmacology of spearmint (*Mentha spicata* L.): A review. In *Journal of Ethnopharmacology* (Vol. 278). Elsevier Ireland Ltd.
- Mobasser, S., & Firoozi, A. A. (2016). Review of Nanotechnology Applications in Science and Engineering. *Journal of Civil Engineering and Urbanism*, 6(4), 84–93.
- Morales-Díaz, A. B., Ortega-Ortíz, H., Juárez-Maldonado, A., Cadenas-Pliego, G., Gonzalez-Morales, S., & Benavides-Mendoza, A. (2017). Application of nanoelements in plant nutrition and its impact in ecosystems. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 8, 1–13.
- Nano for agriculture, not the opposite. (2020). In *Nature Nanotechnology* (Vol. 15, Issue 9, p. 725). Nature Research.
- Nicolopoulou-stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical Pesticides and Human Health : The Urgent Need for a New Concept in Agriculture. *Frontiers in Public Health*, 4(148), 1–8.
- Pérez-de-Luque, A. (2017). Interaction of Nanomaterials with Plants : What Do We Need for Real Applications in Agriculture? *Frontiers in Environmental Science*, 5(April), 1–7.
- Ponmurugan, P., Manjukurunambika, K., & Gnanamangai, B. M. (2016). Antifungal activity of biosynthesised copper nanoparticles evaluated against red root-rot disease in tea plants. *Journal of Experimental Nanoscience*, 11(13), 1019–1031.
- Prasad, R., Bhattacharyya, A., & Nguyen, Q. D. (2017). Nanotechnology in sustainable agriculture: Recent developments, challenges, and perspectives. *Frontiers in Microbiology*, 8(JUN), 1–13.

- Rai, M., Ingle, A. P., Pandit, R., Paralikar, P., Shende, S., Gupta, I., Biswas, J. K., & da Silva, S. S. (2018). Copper and copper nanoparticles: Role in management of insect-pests and pathogenic microbes. In *Nanotechnology Reviews* (Vol. 7, Issue 4, pp. 303–315). De Gruyter.
- Ramsden, J. J. (2016). *Chapter 2: The nanoscale* (Second). Elsevier Science.
- Ramyadevi, J., Jeyasubramanian, K., Marikani, A., Rajakumar, G., & Abdul, A. (2012). Synthesis and antimicrobial activity of copper nanoparticles. *Materials Letters*, *71*, 114–116.
- Roig, A. (2018). Nanotechnology Governance: from Risk Regulation to Informal Platforms. *Nanoethics*, *12*, 115–121.
- Simonin, M., Colman, B. P., Tang, W., Judy, J. D., Anderson, S. M., Bergemann, C. M., Rocca, J. D., Unrine, J. M., Cassar, N., & Bernhardt, E. S. (2018). Plant and microbial responses to repeated Cu(OH)<sub>2</sub> nanopesticide exposures under different fertilization levels in an agro-ecosystem. *Frontiers in Microbiology*, *9*(1769).
- Wei, Y., Chen, S., Kowalczyk, B., Huda, S., Gray, T. P., & Grzybowski, B. A. (2010). Synthesis of stable, low-dispersity copper nanoparticles and nanorods and their antifungal and catalytic properties. *Journal of Physical Chemistry C*, *114*, 15612–15616.
- White, J. C., & Gardea-torresdey, J. L. (2018). Achieving food security through the very small. *Nature Nanotechnology*, *13*, 627–629.
- Yokoyama, T., Masuda, H., Suzuki, M., Ehara, K., Nogi, K., Fuji, M., Fukui, T., Suzuki, H., Tatami, J., Hayashi, K., & Toda, K. (2018). Basic Properties and Measuring Methods of Nanoparticles. In *Nanoparticle Technology Handbook* (pp. 3–47). Elsevier.

## Chapter 2: Literature review

In this chapter, an assessment of recent published scientific research is provided. The literature review focused on three aspects: (1) effects of Cu NPs to fungi; (2) effects of Cu NPs to plants; and (3) effects of Cu NPs to plant-fungi symbiosis. Combined, these studies identify current knowledge on the effects of NPs on plants and associated microorganisms, as well as existing knowledge gaps on the impact of NPs on plant-microbe interactions.

### 2.1 FUNGI EXPOSED TO NPS

Most of the research on metallic NPs is based on (in descending order) Ag, ZnO, and Cu, and their antifungal efficacy towards plant pathogenic fungi. The consequences of these NPs span different exposure doses and periods in a variety of fungal species. In nearly every case, fungal activity was found to be inhibited in a concentration-dependent manner. For instance, colony formation of *Bipolaris sorokiniana* and *Magnaporthe grisea* decreased in as rapidly as 1 hour when concentration of Ag NPs increased from 25-500 ppm (Jo et al., 2009). Similarly, from a range of 10-100 ppm, the uppermost dose of Ag NPs were reported to have the highest inhibitory rate (17.6-100.0%) towards 18 pathogenic fungi on different agar media (Kim et al., 2012). As for ZnO NPs (5-25ppm), the maximum zone of inhibition was observed by 25  $\mu\text{g mL}^{-1}$  against *Pseudomonas aeruginosa* ( $22 \pm 1.8$  mm) and *Aspergillus flavus* ( $19 \pm 1.0$  mm)(Jayaseelan et al., 2012). In another study, the colony diameter of *Fusarium* species was inversely related with exposure of Cu NPs from 300-450 ppm (Viet et al., 2016). Inhibition efficiency by 450 ppm Cu NPs reached 67.3 and 93.9% at the end of 3 and 9 days, respectively. Kalatehjari et al. (2015) concluded that the antifungal efficacy of Cu NPs on *Saprolegnia* species had a positive correlation to both concentration (10-4000 ppm) and time of exposure (12-72 hours). Moreover, Cu NPs were

found to be superior to the commercially available fungicide (Bavistin) against *Phoma destructiva*, *Curvularia lunata*, *Alternaria alternata*, and *Fusarium oxysporum* (Kanhed et al., 2014).

In terms of symbiotic fungi, Prasad et al. (2010) exposed 10 mg 100 mL<sup>-1</sup> of Ag, TiO<sub>2</sub> and carbon nanotubes (CNTs) to *Piriformospora indica* at different stages of development. While the inclusion of NPs at a late growth stage emphasized their antimicrobial property, a stimulatory outcome was observed when NPs were incorporated as media ingredients. Fresh biomass was 62.4% higher in fungi treated with TiO<sub>2</sub>, whereas it was not statistically different from controls in CNT and Ag NP treatments. In general, *in vitro* studies based on the exposure of metal NPs to symbiotic fungi are limited.

## 2.2 TERRESTRIAL PLANTS EXPOSED TO NPS

As a micronutrient, a balance of Cu must be maintained to prevent deficiency or toxicity (copper poisoning). The response of terrestrial plants exposed to Cu NPs has been demonstrated to vary by NP properties (size/shape/stability/surface), exposure (concentration/method/time), and plant characteristics (species/age). Therefore, the effects of Cu NPs cannot be generalized. Positive, neutral, and negative results have been reported throughout literature. Several studies with similar NP type and/or plant species to the proposed research were summarized to illustrate the impact of Cu-based NPs on terrestrial plants (Table 2.1).

Table 2.1: Summaries of selected studies on the effects of Cu NPs to terrestrial plants.

Type	Concentration	Species	Measurements	Conclusion	Reference
Cu	20, 40, 60 mg kg <sup>-1</sup>	Sugarcane	Growth, micronutrients, chlorophyll, enzyme activity	No adverse results.	(Tamez et al., 2019)

Cu	50, 100, 200 mg kg <sup>-1</sup>		Oregano	Growth, micronutrients, macromolecules	Greater inhibition by bulk treatments than nano.	(Du et al., 2018)
Cu, CuO	20, 80 kg <sup>-1</sup>	mg	Cilantro	Growth, micronutrients, chlorophyll content	Nutrient element accumulation was depressed.  Plant size was reduced.	(Zuverza- mena et al., 2015)
Cu, Cu(OH) <sub>2</sub>	50, 10, 20 mg L <sup>-1</sup>		Lettuce, alfalfa	Growth, micronutrients, enzyme activity	Nutrient elements and enzymes were altered.  Less physiological impairments caused by nano and bulk treatments, compared to ionic.	(Hong et al., 2015)
Cu(OH) <sub>2</sub>	25, 75 L <sup>-1</sup>	mg	Alfalfa	Growth, micronutrients, macromolecules, gene expression	Less physiological impairments caused by nano and bulk treatments, compared to ionic.	(Cota-Ruiz et al., 2018)
Cu(OH) <sub>2</sub>	4.8 ppm		Basil	Plant growth, micronutrients, macromolecules, pigments, essential oils	Bulk treatment caused more biochemical alterations than nano or ionic.	(Tan et al., 2018)

### 2.3 STUDIES ON PLANT-FUNGAL SYMBIOSIS

NPs can alter plant growth by direct and indirect means, through effects of associated microbes (Olchowik et al., 2017). These growth alterations are often considered to be a result of the antimicrobial properties of NPs (Aggarwal et al., 2011). While there is a considerable amount of scientific research on the heavy metal resistance that AM fungi confer to plants, information on the interaction between AM fungi and metal NPs is limited. Fenugreek exposed to 125-500 µg g<sup>-1</sup> of ZnO NPs in the presence and absence of AM fungi (*Rhizobium melliloti*) had opposing results (Siani et al., 2017). Root/shoot biomass in non-AM fungi plants decreased by up to 50.0% from controls, while Zn uptake increased in a dose-dependent manner. In comparison, plants inoculated

with AM fungi had higher root/shoot biomass and Zn uptake was reduced by nearly half. This was due to the colonization of AM fungi promoting the secretion of a glycoprotein (referred to as glomalin) in the rhizosphere, which acted as a metal chelator that inhibited Zn uptake by roots, and subsequent translocation to shoots. The mediated low Zn uptake had a stimulatory effect on plant growth. Similarly, mycorrhizal fungus *Acaulospora mellea* played a protective role in maize treated with 500-3000 mg kg<sup>-1</sup> of ZnO NPs (Wang et al., 2017). Although Zn uptake and concentration in plants increased as a function of ZnO NP dose, uptake of other elements was inhibited by 44.7-89.0% from controls. The addition of AM fungi naturally ameliorated this toxicity by stimulating the accumulation of micronutrients (P, N, and K) in shoots, and enhancing the distribution of Zn to roots (restricting its translocation to shoots). Consequently, plant growth (root length, surface area, and volume) and nutritional value in mycorrhizal plants were improved. In another study, tomatoes inoculated with AM fungi and exposed to 12-36 mg kg<sup>-1</sup> of Ag NPs contained 14.0% less Ag in tissues than plants not associated with AM fungi (Noori et al., 2017). Furthermore, mycorrhizal colonization alleviated some of the negative effects on root dry weight from Ag NPs. Mixed results were found when Ag and FeO NPs were applied to mycorrhizal clover (Feng et al., 2013). Intermediate (0.1 mg kg<sup>-1</sup>) and high (1 mg kg<sup>-1</sup>) concentrations of Ag NPs did not trigger phytotoxicity, but root and shoot biomass were suppressed by 50.0 and 36.1%, respectively, at 0.01 mg kg<sup>-1</sup>. It is possible that a threshold exists for AM fungi to perform; hence, the low concentration did not activate AM fungi and its alleviation properties. All concentrations of FeO NPs significantly lowered mycorrhizal clover biomass and antioxidant enzymes by up to 34.0 and 50.0%, correspondingly. It was also observed that FeO NPs lowered extractable glomalin-related soil protein content (GRSP). This could have modified the ecological function of AM fungi,

hindering the ability of plant roots to acquire an adequate amount of nutrients. These findings suggest that the interaction between AM fungi and metal NPs are complex and not well understood.

Their ability to optimize the root system of plants and facilitate nutrient uptake delegates AM fungi as an attractive strategy for the phytoremediation heavy metals. We will limit the scope of our literature review on this topic to Cu-based compounds and edible plants. An experiment was conducted to evaluate the interaction between AM fungi and Cu in mint shrub (*Elsholtzia splendens*) (Jin et al., 2015). Exposure of 34 mg mL<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O to non-inoculated plants significantly delayed the onset dates (4 days), ending dates (4 days) and peak dates of flowering (2 days), and decreased flowering duration (6 days), compared to inoculated plants. An interactive effect was also found between AM fungi and Cu that stimulated inflorescence number and 1000-seed weight. Additionally, pepper plants grown in soil amended with 2, 4, and 8 mM of CuSO<sub>4</sub> and treated with AM fungi (*Funneliformis mosseae* or *Rhizophagus intraradices*) had higher total dry weight and leaf area than untreated plants (Ruscitti et al., 2017). In a separate study, AM fungi (*Glomus intraradices*) markedly increased the concentration of Cu in the roots of maize plants under Cu addition, while simultaneously improving the dry weight and P uptake (Physiology et al., 2017). At 200 mg kg<sup>-1</sup> Cu, the root-to-shoot ratio of inoculated plants was 44-48, in comparison to 12 for uninoculated plants. This retention of Cu in the plant roots could serve as a key mechanism for the alleviation of Cu stress by mycorrhizal associations.

It has been reported that AM fungi have a beneficial effect on mint plants in terms of plant growth, nutrient composition, and essential oil production. For instance, 65-day-old wild mint plants treated with *Gigaspora margarita* and *Glomus clarum* had increased dry matter production of shoots (334.0 and 330.0%), N content (143.0 and 123.0%), P content (139.0 and 142.0%), and K content (139.0 and 142.0%) compared to non-mycorrhizal plants (Freitas et al., 2006). Dry

weight, essential oil composition, and elemental concentration (N, P, K, Na, Ca, Mg, B, Zn, Mn, Fe, and Cu) were also 246.4-250.3%, 51.9-89.5%, and 80.5-701.2% higher, respectively, in mint plants inoculated with *Glomus etunicatum* (Karagiannidis et al., 2012). In Elgharably and Allam (2013), accumulation of micronutrients Cu, Fe, Mn, Cd, and Pb were enhanced by 10.3-133.3% in peppermint symbiotically associated with *Glomus geosporum* and grown for 90 days in soil. Moreover, application of monospecific inocula (*Glomus intaradicus*) and multi-indeginous inocula (*Glomus fasciculatum*, *G. etunicatum*, *G. claroides*, *G. microcarpum*, *G. austral*, *G. intaradicus*, *G. aggregatum*, *G. constrictum*, and *Gigaspora gigantea*) improved essential oil yield by 40.0-49.3% and 80.0-197.0%, correspondingly, from controls in 90-day-old wild mint varieties (Burni et al., 2013). Extramatrical hyphae increase the surface area of the root system, allowing exploration of the soil volume beyond the depletion zone, making the adsorption of water and diffusion of nutrients more efficient (Zhu et al., 2008). Overall, these results demonstrate the ability of AM symbiosis to aid in the reduction of agrochemical supplements and fertilizers, which can contribute to the advancement of sustainable commercial practices.



## REFERENCES

- Aggarwal, A., Kadian, N., Tanwar, A., Yadav, A., & Gupta, K. K. (2011). Role of arbuscular mycorrhizal fungi (AMF) in global sustainable development. *Journal of Applied and Natural Science*, 3(2), 340–351.
- Burni, T., Hussain, F., & Sharif, M. (2013). Effect of arbuscular mycorrhizal fungi on essential oils of two pharmaceutically important mentha species in marginal soils. *Pakistan Journal of Botany*, 45(1), 293–296.
- Cota-Ruiz, K., Hernandez-Viezcas, J. A., Varela-Ramirez, A., Valdés, C., Nunez-Gastelum, J. A., Martinez-Martinez, A., Delgado-Rios, M., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2018). Toxicity of copper hydroxide nanoparticles, bulk copper hydroxide, and ionic copper to alfalfa plants: A spectroscopic and gene expression. *Environmental Pollution*, 1–10.
- Du, W., Tan, W., Yin, Y., Ji, R., Peralta-Videa, J. R., Guo, H., & Gardea-Torresdey, J. L. (2018). Differential effects of copper nanoparticles/microparticles in agronomic and physiological parameters of oregano (*Origanum vulgare*). *Science of The Total Environment*, 618, 306–312.
- Elgharably, A., & Allam, N. (2013). Effect of arbuscular mycorrhiza on growth and metal uptake of basil and mint plants in wastewater irrigated soil. *Egyptian Journal of Soil Science*, 53(4), 613–625.
- Feng, Y., Cui, X., He, S., Dong, G., Chen, M., Wang, J., & Lin, X. (2013). The role of metal nanoparticles in influencing arbuscular mycorrhizal fungi effects on plant growth. *Environmental Science & Technology*, 47, 9496–9504.

- Freitas, M. S. M., Martins, M. A., & De Carvalho, A. J. C. (2006). Growth and mineral composition of mint in response to arbuscular mycorrhizal fungi inoculation and phosphorus fertilization. *Horticultura Brasileira*, *24*, 11–16.
- Hong, J., Rico, C. M., Zhao, L., Adeleye, A. S., Keller, A. A., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015). Toxic effects of copper-based nanoparticles or compounds to lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). *Environmental Science. Processes & Impacts*, *17*(1), 177–185.
- Jayaseelan, C., Rahuman, A. A., Kirthi, A. V., Marimuthu, S., Santhoshkumar, T., Bagavan, A., Gaurav, K., Karthik, L., & Rao, K. V. B. (2012). Novel microbial route to synthesize ZnO nanoparticles using *Aeromonas hydrophila* and their activity against pathogenic bacteria and fungi. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *90*, 78–84.
- Jin, Z., Li, J., & Li, Y. (2015). Interactive effects of arbuscular mycorrhizal fungi and copper stress on flowering phenology and reproduction of *Elsholtzia splendens*. *PLoS ONE*, *10*(12).
- Jo, Y., Kim, B. H., & Jung, G. (2009). Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi. *Plant Disease*, *93*, 1037–1043.
- Kalatehjari, P., Yousefian, M., & Khalilzadeh, M. A. (2015). Assessment of antifungal effects of copper nanoparticles on the growth of the fungus *Saprolegnia* sp. on white fish (*Rutilus frisii kutum*) eggs. *The Egyptian Journal of Aquatic Research*, *41*(4), 303–306.
- Kanhed, P., Birla, S., Gaikwad, S., Gade, A., Seabra, A. B., Rubilar, O., Duran, N., & Rai, M. (2014). In vitro antifungal efficacy of copper nanoparticles against selected crop pathogenic fungi. *Materials Letters*, *115*, 13–17.

- Karagiannidis, N., Thomidis, T., Panou-filotheou, E., & Karagiannidou, C. (2012). Response of three mint and two oregano species to *Glomus etunicatum* inoculation. *Australian Journal of Crop Science*, 6(1), 164–169.
- Kim, S. W., Jung, J. H., Lamsal, K., Kim, Y. S., Min, J. S., & Lee, Y. S. (2012). Antifungal effects of silver nanoparticles (AgNPs) against various plant pathogenic fungi. *Mycobiology*, 40(1), 53–58.
- Noori, A., White, J. C., & Newman, L. A. (2017). Mycorrhizal fungi influence on silver uptake and membrane protein gene expression following silver nanoparticle exposure. *Journal of Nanoparticle Research*, 19(66), 1–13.
- Olchowik, J., Bzdyk, R. M., Studnicki, M., Bederska-błaszczak, M., Urban, A., & Aleksandrowicz-trzeci, M. (2017). The Effect of silver and copper nanoparticles on the condition of english oak (*Quercus robur* L.) seedlings in a container nursery experiment. *Forests*, 8(310), 1–19.
- Physiology, E. P., Plata, L., & Aires, B. (2017). Improvement of copper stress tolerance in pepper plants (*Capsicum annuum* L.) by inoculation with arbuscular mycorrhizal fungi. *Theoretical and Experimental Plant Physiology*, 29(1), 37–49.
- Prasad, R., Jain, V. K., & Varma, A. (2010). Role of nanomaterials in symbiotic fungus growth enhancement. *Current Science*, 99(9), 1189–1191.
- Ruscitti, M., Arango, M., & Beltrano, J. (2017). Improvement of copper stress tolerance in pepper plants (*Capsicum annuum* L.) by inoculation with arbuscular mycorrhizal fungi. *Theoretical and Experimental Plant Physiology*, 29(1), 37–49.
- Siani, N. G., Fallah, S., Raj, L. R., & Rostamnejadi, A. (2017). Natural amelioration of Zinc oxide nanoparticle toxicity in fenugreek (*Trigonella foenum-gracum*) by arbuscular

- mycorrhizal (*Glomus intraradices*) secretion of glomalin. *Plant Physiology et Biochemistry*, *112*, 227–238.
- Tamez, C., Morelius, E. W., Hernandez-Viezcas, J. A., Peralta-Videa, J. R., & Gardea-Torresdey, J. (2019). Biochemical and physiological effects of copper compounds/nanoparticles on sugarcane (*Saccharum officinarum*). *Science of the Total Environment*, *649*, 554–562.
- Tan, W., Gao, Q., Deng, C., Wang, Y., Lee, W., Hernandez-viezcas, J. A., Peralta-idea, J. R., & Gardea-torresdey, J. L. (2018). *Foliar Exposure of Cu(OH)<sub>2</sub> nanopesticide to basil (Ocimum basilicum): Variety-dependent copper translocation and biochemical responses.*
- Viet, P. Van, Nguyen, H. T., Cao, T. M., & Hieu, L. Van. (2016). Fusarium antifungal activities of copper nanoparticles synthesized by a chemical reduction method. *Journal of Nanomaterials*, 2016.
- Wang, W., Wang, F., Li, S., & Liu, X. (2017). Arbuscular mycorrhizal symbiosis influences the biological effects of Nano-ZnO on maize. *Environmental Science*, *35*(8), 3135–3141.
- Zhu, H., Han, J., Xiao, J. Q., & Jin, Y. (2008). Uptake, translocation, and accumulation of manufactured iron oxide nanoparticles by pumpkin plants. *Journal of Environmental Monitoring*, *10*(6), 685–784.
- Zuverza-Mena, N., Medina-Velo, I. A., Barrios, A. C., Tan, W., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015). Copper nanoparticles/compounds impact agronomic and physiological parameters in cilantro (*Coriandrum sativum*). *Environmental Science: Processes & Impacts*, *17*, 1783–1793.

## Chapter 3: Tolerance of arbuscular mycorrhizal fungi (*Glomus etunicatum*) to Cu-based nanoparticles/compounds under *in vitro* conditions

### 3.1 INTRODUCTION

As an essential micronutrient, copper (Cu) is required for normal growth of fungi. However, when present in excess, it is toxic (Cornejo et al. 2013a). The release of Cu<sup>2+</sup> ions activate the formation of reactive oxygen species (ROS) and interactions with fungal cell components (cell wall, protein, enzymes, DNA), resulting in Cu toxicity (Martins et al. 2012). Cu-based fungicides are frequently used to combat plant fungal disease due to their low cost and antimicrobial property (Banik and Pérez-de-luque 2017). While the antimicrobial property of Cu makes it particularly effective against pathogenic fungi, there are concerns on its compatibility with symbiotic fungi.

Arbuscular mycorrhizal (AM) fungi are microorganisms that colonize the roots of the host plant to improve the supply of nutrients (Willis, Rodrigues, and Harris 2013). Secondary roles of AM fungi are: reduction in plant uptake of toxic heavy metals, increased plant disease resistance against rhizospheric pathogens, and improved plant water balance in drought stress conditions. As the most common symbiotic association of plants with microbes, the beneficial effects of AM fungi on plant growth and tolerance have been widely studied (Diagne et al. 2020). Although AM fungi are obligate symbionts that rely on the host plant for carbon substrates, they have been shown to survive without a host for a limited time (Hildebrandt, Janetta, and Bothe 2002).

The larger surface area-to-volume ratio of Cu nanoparticles (NP) is theorized to enhance their contact with fungi and ability to permeate cells (Cruz et al. 2021). Greater chemical and physical stability, and higher antimicrobial per unit mass have been shown in Cu-based NPs. Unfortunately, studies on the antimicrobial activity of Cu NPs have been mostly conducted mostly on pathogenic fungi (Rajput et al. 2019). The toxicity of nano and bulk CuO to yeast (*Saccharomyces cerevisiae*) and symbiotic bacteria (*Vibrio fischeri*) was studied by Hou et al.

(2017). It was concluded that CuO was more toxic as NPs than bulk particles. Reports on ZnO, Ag, and Fe<sub>3</sub>O NPs versus fungal symbiosis have indicated that higher concentrations of NPs reduced AM colonization, glomalin related soil proteins (GRSP), and alkaline phosphatase activity (Bhushan et al. 2020). Overall, understanding on the interaction of Cu NPs with symbiotic fungi is significantly lacking.

The main aim of this preliminary study was to investigate, under *in vitro* conditions, the tolerance of the mycorrhizal fungus *Glomus etunicatum* to three forms of Cu-based compounds (nano, bulk, and ionic). Two separate bioassays were conducted to evaluate the effects of Cu-based NPs/compounds on fungal growth. Results from this study will be used towards future investigation on the impact of Cu-based NPs/compounds on the interaction between AM fungi and plants.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Cu nanoparticles/compounds

In these studies, Cu(OH)<sub>2</sub> nanowires from US Research Nanomaterials served as *nCu*. A Cu(OH)<sub>2</sub> pesticide formulation known as Kocide 3000 (Dupont, Wilmington, DE) represented *bCu*. Characterization of these compounds (including particle size, morphology, hydrodynamic diameter, zeta potential, and Cu content) were supplied by US Research Nanomaterials and 81, which are shown in Table 3.1. The functional compound of Kocide 3000 is Cu (46.1%), followed by O, Na, Al, Si, S, and Cl (53.9%) – inactive ingredients. Reagent grade CuSO<sub>4</sub> salts (Sigma Aldrich) were used as ionic counterparts (*iCu*) to assess the effects of Cu dissolution.

Table 3.1: Physical properties of Cu NPs/compounds (Hong et al. 2015a).

Property	<i>nCu</i> <sup>a</sup>	<i>bCu</i> <sup>b</sup>
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Size (nm)	width, 50 nm; length, 3000-5000 nm*	$> 10^4$
Hydrodynamic diameter (nm)	50	$1532 \pm 580$
Zeta potential (mV)	$24.1 \pm 0.32$	$-40.9 \pm 2.7$
Cu content (wt%)	65.1	26.5
Other elements present	ND	C, O, Na, Al, Si, S, Cl
Morphology	Elongated	Spherical

<sup>a</sup> Refers to nano Cu form; <sup>b</sup> refers to bulk Cu form.

\*This is a nanowire; thus, the size is larger than 100 nm.

ND, non-defined.<sup>4</sup>

### 3.2.2 Suspension/solution and culture media preparation

Suspensions/solutions were prepared to have final concentrations ranging from 0 to 100 mg L<sup>-1</sup> (specified in the following sections). Dry compounds were weighed with respect to Cu content, immersed in Millipore water (MPW, 18 MΩ), and volume adjusted. To ensure homogeneity, suspensions/solutions were sonicated (Crest Ultrasonics, Trenton, NJ) for 30 minutes at 25°C with an intensity of 180 watts.

### 3.2.3 Cultivation of fungi

A potato dextrose agar (PDA) media was prepared according to the manufacturer's instructions and transferred into Petri dishes (100 mm x 10 mm). To counteract the potential aggregation of particles and limitations in bioavailability from incorporating the compounds in agar, suspensions/solutions of *n*Cu, *b*Cu, and *i*Cu at concentrations of 0, 5, 10, 20, 50, and 100 mg L<sup>-1</sup> were added onto the surface of solidified agar. Triplicate samples were used for all treatments. Spores were isolated from fungal colonies grown using dried whole inoculum of *Glomus etunicatum* (INVAM, West Virginia, VA), counted with a hemocytometer, used to make a spore suspension (~1,500 spores mL<sup>-1</sup>), and cultured onto Petri dishes. Petri dishes that were not exposed

to Cu-based NPs/compounds served as controls. Treatments were covered and allowed to incubate at 25°C. Absorbance (600 nm) was measured at 24-hourly intervals for 3 days using a spectrophotometer (NanoDrop One, Thermo Scientific). A calibration curve relating spore concentration to absorbance was generated and used to determine overall spore count.

A lysogeny broth (LB) was prepared according to the manufacturer's instructions. Treatments containing 0, 5, 50, and 500 mg L<sup>-1</sup> of *nCu*, *bCu*, and *iCu* were prepared as quadruplicates and transferred into Falcon tubes containing LB. Spores were isolated from fungal colonies grown using dried whole inoculum of *Glomus etunicatum* to create a spore suspension (~2,000 spores mL<sup>-1</sup>), which was then added to Falcon tubes. Falcon tubes were incubated for 3 days (under rotation) and fungal biomass was weighed.

### **3.2.4 Statistical analyses**

Statistical analyses were performed using the Statistical Package for Social Sciences 22.0 (SPSS, Chicago, IL). Differences among treatment means were calculated by one-way or two-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post-hoc test, based on a probability (*p* value) of 0.05.

## **3.3 RESULTS AND DISCUSSION**

### **3.3.1 Fungal cultures and spore count**

An image of *Glomus etunicatum* grown in petri dishes containing 0 to 100 mg L<sup>-1</sup> of *nCu*, *bCu*, and *iCu* after 3 days is shown in Figure 3.1. Strictly from a visual standpoint, it appears as though with increasing exposure dose of Cu-based NPs/compounds that fungal cultures spread out across a greater surface area. We can also see that *iCu* treatments seemed to have more widespread



fungal growth overall. Fungal cultures treated with *iCu* were darker in appearance, followed by *bCu*, and then *nCu*.

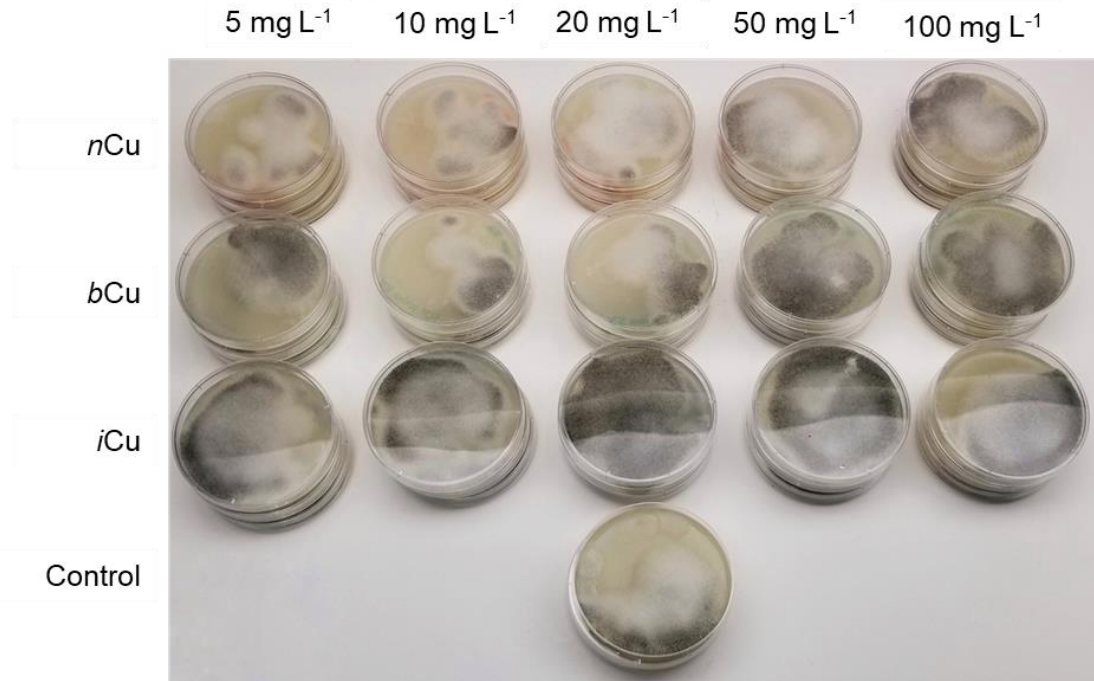


Figure 3.1: Photograph of petri dishes with *Glomus etunicatum* exposed to 0, 5, 10, 20, 50, and 100 mg L<sup>-1</sup> of *nCu*, *bCu*, and *iCu* after 3 days.

The spore count of *Glomus etunicatum* cultured in petri dishes with 0 to 100 mg L<sup>-1</sup> of *nCu*, *bCu*, and *iCu* at 1, 2, and 3 days are depicted in Figure 3.2A-C. From Day 1 to 3, spore count increased by 100-fold. Throughout all 3 days there were no statistically significant changes, but we note several observations. On Day 1, the addition of Cu-based NPs/compounds reduced spores by 24.46-84.40% from the control. By Day 2, spore count in Cu-based NPs/compounds treatments had stabilized to the point where most of them exceeded the number of spores in the control treatment (7.85-83.51%). Spore count returned to the initial pattern on Day 3, with Cu-based NPs/compounds treatments containing 25.07-68.38% less spores than the control. Despite these findings, the data were largely erratic with several high standard errors, so they are inconclusive.

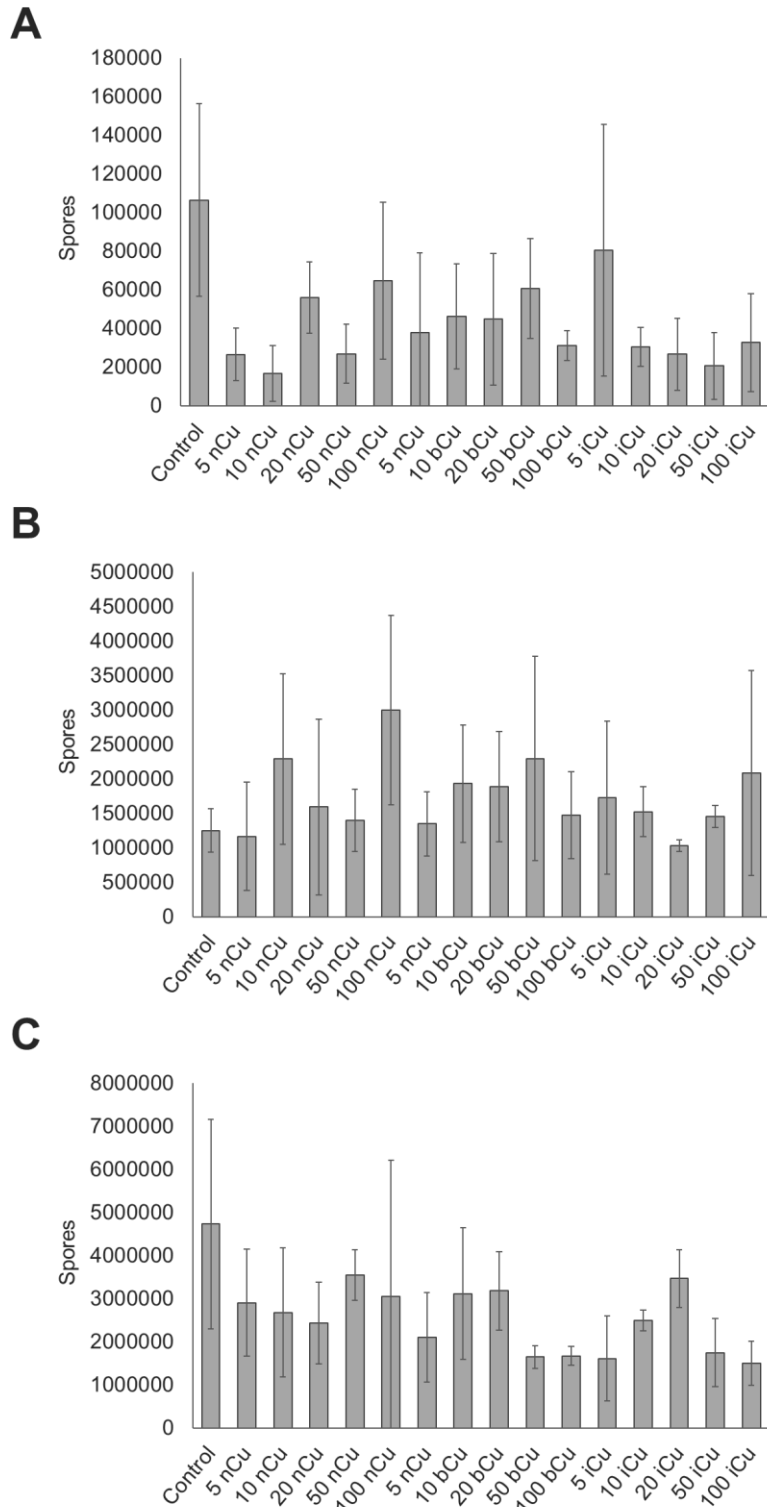


Figure 3.2A-C: Spore count of *Glomus etunicatum* grown in petri dishes treated with 0, 5, 10, 20, 50, and 100 mg L<sup>-1</sup> of nCu, bCu, and iCu after 1 (A), 2 (B), and 3 (C) days. Different letters represent statistically significant differences from the respective control ( $p \leq 0.05$ ).

Our visual observations in the petri dish experiment are similar to those reported by Cornejo et al. (2013), where green-blue AM fungal spores were found in monoxenic cultures of *Glomus intraradices* exposed to 0 to 500  $\mu\text{M}$  of  $\text{CuSO}_4$ . There was a positive correlation between percentage of green-blue spores and Cu concentration, with the number of green-blue spores increasing with time. The addition of acetic acid and ferrocyanide in collected and crushed green-blue spores caused a red precipitate to form, indicating the presence of Cu ions in the spore cytoplasm. Furthermore, green-blue spores were also detected in the extraradical and intraradical mycelium. This was attributed to heavy metals being strategically compartmentalized to specific AM fungal structures as part of a detoxification mechanism: the cytosol contains chelating agents, and the larger surface area in the external hyphae enhances adsorption capacity (Dhalaria et al. 2020a; 2020b; Gong and Tian 2019a). Although nano or bulk Cu forms were not considered, the prevalence of different colored spores and presumed hyphal expansion by ionic Cu are consistent with our findings. It is likely that Cu was more bioavailable to *Glomus etunicatum* as dissolved  $\text{CuSO}_4$  than other Cu-based NPs/compounds in our study, which would explain why *iCu* treatments contained the greatest number of dark spores over a larger surface area. Although it is unclear why *bCu* would contain more dark spores than *nCu*, as bulk sized particles are typically less bioavailable than their nano sized counterparts. The size, shape, and surface chemistry of NPs can affect their cellular uptake (Foroozandeh and Aziz 2018). Moreover, the experiment by Cornejo et al. (2013) lasted 15 days, where green-blue spores were not detected in lower Cu treatment concentrations until 5 days, while ours concluded at 3 days. Our interpretation of these results is limited to visual observations. Further experiments are needed to determine whether these factors are influencing NP uptake into AM fungal spores.

Although it is stated in the literature that spore count decreases in the presence of heavy metals, as previously mentioned, the spore count data are inconclusive (Ferrol, Tamayo, and Vargas 2016). There are two major conclusions that can be drawn from this experiment: (1) the overall increase in spore count from Day 1 to Day 3 indicates that the environmental conditions were suitable for growth of AM fungi, and (2) the concentration of Cu-based NPs/compounds was

not a main driver of the results. This information was used for the cell culture experiment, which elevated the Cu exposure concentration range.

### 3.3.2 Fungal biomass

Total mass of *Glomus etunicatum* grown in LB spiked with 0 to 100 mg L<sup>-1</sup> for 3 days is shown in Figure 3.3. There were no statistically significant differences. Average fungal biomass was similar for all samples, regardless of treatment (within 0.21 to 13.10% of each other).

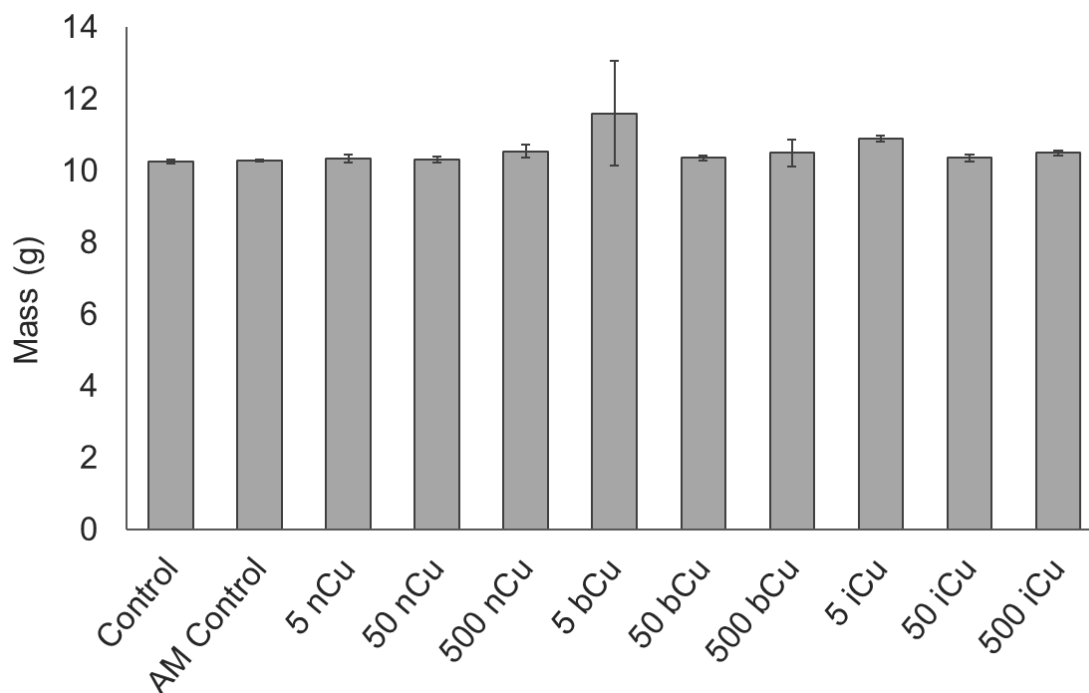


Figure 3.3: Mass of *Glomus etunicatum* cultivated in LB containing 0, 5, 50, and 500 mg L<sup>-1</sup> of *nCu*, *bCu*, and *iCu* after 3 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences from the respective control ( $p \leq 0.05$ ).

While we were unable to find a comparable study that measured AM fungal mass, there are several studies that evaluated the impact of heavy metals on spore density. For example, Yang, Song, et al. (2015) evaluated spore density in samples from AM fungi in uncontaminated and

contaminated heavy metals soils. No significant differences were found between spore density and Pb, Zn, Cu, or Cd concentrations. In contrast, spore density of *Glomus mosseae* and *Acaulospora laevis* was greatly affected by 0.5 to 1.5 mg L<sup>-1</sup> of Cu (Moussa and Abdelbagi 2014). The effects of heavy metals on AM fungi may depend on a myriad of environmental factors, including species.

### **3.4 CONCLUSIONS**

The purpose of this study was to determine the acute toxicity of Cu-based NPs/compounds to AM fungi (*Glomus etunicatum*). In the petri dish experiment, there were visual differences in color and distribution patterns of fungal cultures among the treatments after 3 days, which were likely due to heavy metal detoxification strategies being implemented by AM fungi. Although it appeared that spore count may have been impacted by Cu exposure, this data was inconclusive. In our cell culture experiment, fungal biomass was not significantly impacted by Cu-based NPs/compounds. Overall, we found that our environmental study conditions were favorable to growth of AM fungi, and that AM fungi can withstand high concentrations of Cu. These preliminary results will be used towards future experiments incorporating a host plant.

## REFERENCES

- Banik, S., & Pérez-De-Luque, A. (2017). In vitro effects of copper nanoparticles on plant pathogens, beneficial microbes and crop plants. *Spanish Journal of Agricultural Research*, 15(2).
- Bhushan, I., Kumar, V., Durgesh, S., & Tripathi, K. (2020). *Nanotechnology in the Life Sciences Nanomaterials and Environmental Biotechnology*. <http://www.springer.com/series/15921>
- Cornejo, P., Pérez-Tienda, J., Meier, S., Valderas, A., Borie, F., Azcón-Aguilar, C., & Ferrol, N. (2013a). Copper compartmentalization in spores as a survival strategy of arbuscular mycorrhizal fungi in Cu-polluted environments. *Soil Biology and Biochemistry*, 57, 925–928.
- Cornejo, P., Pérez-Tienda, J., Meier, S., Valderas, A., Borie, F., Azcón-Aguilar, C., & Ferrol, N. (2013b). Copper compartmentalization in spores as a survival strategy of arbuscular mycorrhizal fungi in Cu-polluted environments. *Soil Biology and Biochemistry*, 57, 925–928.
- Cruz, L. F., Cruz, J. C., Carrillo, D., Mtz-Enriquez, A. I., Lamelas, A., Ibarra-Juarez, L. A., & Pariona, N. (2021). In-vitro evaluation of copper nanoparticles as a potential control agent against the fungal symbionts of the invasive ambrosia beetle *Euwallacea fornicatus*. *Crop Protection*, 143.
- Dhalaria, R., Kumar, D., Kumar, H., Nepovimova, E., Kuca, K., Islam, M. T., & Verma, R. (2020a). Arbuscular mycorrhizal fungi as potential agents in ameliorating heavy metal stress in plants. In *Agronomy* (Vol. 10, Issue 6). MDPI AG.

- Dhalaria, R., Kumar, D., Kumar, H., Nepovimova, E., Kuca, K., Islam, M. T., & Verma, R. (2020b). Arbuscular mycorrhizal fungi as potential agents in ameliorating heavy metal stress in plants. In *Agronomy* (Vol. 10, Issue 6). MDPI AG.
- Diagne, N., Ngom, M., Djighaly, P. I., Fall, D., Hocher, V., & Svistoonoff, S. (2020). Roles of arbuscular mycorrhizal fungi on plant growth and performance: importance in biotic and abiotic stressed regulation. *Diversity*, *12*(10), 1–25.
- Ferrol, N., Tamayo, E., & Vargas, P. (2016). The heavy metal paradox in arbuscular mycorrhizas: from mechanisms to biotechnological applications. In *Journal of Experimental Botany* (Vol. 67, Issue 22, pp. 6253–6565). Oxford University Press.
- Foroozandeh, P., & Aziz, A. A. (2018). Insight into Cellular Uptake and Intracellular Trafficking of Nanoparticles. In *Nanoscale Research Letters* (Vol. 13). Springer New York LLC.
- Gong, X., & Tian, D. Q. (2019). Study on the effect mechanism of Arbuscular Mycorrhiza on the absorption of heavy metal elements in soil by plants. *IOP Conference Series: Earth and Environmental Science*, *267*(5).
- Hildebrandt, U., Janetta, K., & Bothe, H. (2002). Towards growth of arbuscular mycorrhizal fungi independent of a plant host. *Applied and Environmental Microbiology*, *68*(4), 1919–1924.
- Hong, J., Rico, C. M., Zhao, L., Adeleye, A. S., Keller, A. A., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015a). Toxic effects of copper-based nanoparticles or compounds to lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). *Environmental Science Process Impacts*, *17*(1), 177–185.
- Hong, J., Rico, C. M., Zhao, L., Adeleye, A. S., Keller, A. A., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015b). Toxic effects of copper-based nanoparticles or compounds to

- lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). *Environmental Science. Processes & Impacts*, 17(1), 177–185.
- Hou, J., Wang, X., Hayat, T., & Wang, X. (2017). Ecotoxicological effects and mechanism of CuO nanoparticles to individual organisms. *Environmental Pollution*, 221, 209–217.
- Martins, F., Soares, M. E., Oliveira, I., Pereira, J. A., de Lourdes Bastos, M., & Baptista, P. (2012). Tolerance and bioaccumulation of copper by the entomopathogen *Beauveria bassiana* (Bals.-Criv.) Vuill. Exposed to various copper-based fungicides. *Bulletin of Environmental Contamination and Toxicology*, 89(1), 53–60.
- Moussa, T. A., & Abdelbagi, I. (2014). Investigation the Effect of Arbuscular Mycorrhizal Fungi on the Tolerance of Maize Plant to Heavy Metals Stress. In *Life Science Journal* (Vol. 11, Issue 4).
- Rajput, V., Minkina, T., Ahmed, B., Sushkova, S., Singh, R., Soldatov, M., Laratte, B., Fedorenko, A., Mandzhieva, S., Blicharska, E., Musarat, J., Saquib, Q., Flieger, J., & Gorovtsov, A. (2019). Interaction of Copper-Based Nanoparticles to Soil, Terrestrial, and Aquatic Systems: Critical Review of the State of the Science and Future Perspectives. In *Reviews of Environmental Contamination and Toxicology* (Vol. 252, pp. 51–96).
- Willis, A., Rodrigues, B. F., & Harris, P. J. C. (2013). The Ecology of Arbuscular Mycorrhizal Fungi. *Critical Reviews in Plant Sciences*, 32(1), 1–20.
- Yang, Y., Song, Y., Scheller, H. v., Ghosh, A., Ban, Y., Chen, H., & Tang, M. (2015). Community structure of arbuscular mycorrhizal fungi associated with *Robinia pseudoacacia* in uncontaminated and heavy metal contaminated soils. *Soil Biology and Biochemistry*, 86, 146–158.



## Chapter 4: Arbuscular mycorrhizal fungi alleviate phytotoxic effects of copper-based nanoparticles/compounds in spearmint (*Mentha spicata*)<sup>1</sup>

### 4.1 INTRODUCTION

The unique physicochemical properties exhibited by nanoparticles (NPs), which measure between 1 to 100 nm in diameter, have led to their commercialization in the form of engineered nanoparticles (ENPs)(Singh, Handa, and Manchanda 2021). Large-scale and versatile applications of ENPs inevitably result in their incidental release into the environment.

Additionally, nanotechnology has been explored for its potential to provide effective solutions to several major agricultural problems, including sustainable production and food security (Usman et al. 2020; Zhao et al. 2020). Nanoparticulate formulations have shown promise in areas of fertilizer delivery, gene modification, and pest control. Entrance of ENPs into the agricultural system can occur indirectly, through consumer and industrial products, or directly, through nanoagrochemicals. Given that soil is considered a major sink for ENPs, there is a high likelihood for interactions with soil biota, prompting the need for risk assessment in complex plant ecosystems (Paramo et al. 2020).

Among the various metal NPs that have been exploited for their antimicrobial potential, copper (Cu) is cheaper and easier to produce (Ermini and Voliani 2021). Cu NPs may also dissolve faster than other comparable metals with the rapid release of Cu ions into the surroundings. Currently, the exact mechanism by which Cu NPs induce microbial death is debated, as well as the significance and extent of involvement of each proposed mechanism.

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<sup>1</sup> Reprinted from Suzanne A. Apodaca, Keni Cota-Ruiz, Jose A. Hernandez-Viezcas, and Jorge L. Gardea-Torresdey. 2022. "Arbuscular Mycorrhizal Fungi Alleviate Phytotoxic Effects of Copper-Based Nanoparticles/Compounds in Spearmint (*Mentha spicata*)."  
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There are three major suggested mechanisms: (1) physical interaction of Cu NPs with the cell or plasma membrane, triggering its degradation and leaving the microbe vulnerable to damage from Cu ions; (2) formation of reactive oxidative species (ROS) through reduction of Cu via Fenton reactions, which induce oxidative DNA damage, lipid peroxidation, and oxidation of proteins; and (3) release of Cu ions that rupture the membrane and infiltrate the cell, causing oxidative stress from endogenous ROS (Salah, Parkin, and Allan 2021). In agriculture, Cu-based NPs are employed as nanopesticides and nanofertilizers to combat plant disease and deliver nutrients. The inherent antimicrobial properties of Cu NPs could trigger undesired effects on non-target microbes and has become a topic of concern (D. Wang et al. 2022).

Mycorrhizal fungi are ubiquitous soil microorganisms that form symbiotic associations with ~340,000 land plants (Genre et al. 2020). Arbuscular mycorrhizal (AM) fungi colonize host plant roots through the formation of mycorrhizal structures (hyphae and arbuscules), generating a common mycorrhizal network that increases the surface area of the plant–fungal interface. This enlarged adsorptive area facilitates the uptake of mineral nutrients, including in non-ideal growth conditions (Riaz et al. 2021). AM fungi also provide tolerance to host plants against various environmental stressors such as heat, salinity, drought, and pollutants. For example, AM fungi bestow heavy metal resistance by secreting chemicals that can chelate heavy metals and bind them to the fungal cell wall, limiting their availability to the host plant. Given their ecological importance, it is essential to understand the response and feedback of AM fungi when evaluating the impact of ENPs in terrestrial ecosystems.

There is a considerable body of work on the effects of ENPs to plant species, with the consensus that phytotoxicity is largely dependent on parent material, concentration, and plant species (Dodds et al. 2021; Uddin, Desai, and Asmatulu 2020). Certain conditions even have the

potential to yield beneficial effects. The heavy metal resistance that AM fungi confer to host plants has also been widely discussed (Khalid et al. 2021). While several studies suggest that ENPs can be detrimental to symbiotic microbial communities (mainly bacteria), a knowledge gap remains concerning the potential consequences of ENPs to beneficial plant-fungal interactions (Ameen et al. 2021). Moreover, emerging research on the efficacy of nanopesticides and nanofertilizers for plant protection and fitness purposes demands a thorough understanding of NP-microbe interactions in agricultural systems.

Spearmint (*Mentha spicata*) is a medicinal and aromatic plant that is widely cultivated for its essential oils, which are used in a variety of commercial and medicinal products (L.-L. Zhang et al. 2022). In the United States 1.78 million pounds of spearmint were harvested in 2021 (National Agricultural Statistics Service 2022). Regarding heavy metals toxicity, it has been reported that Cu can alter spearmint growth and physiology. Exposure to 60  $\mu\text{M}$  of Cu decreased plant biomass, plant height, root fresh weight, and nutrient element concentrations (K, N, P, Zn) in plant tissues (Chrysargyris, Papakyriakou, et al. 2019b). With increasing demand for spearmint essential oils, there are concerns regarding its safety for human uses and the sustainability of commercial cultivation practices.

In this study, spearmint plants grown in potting mix (loam soil) were inoculated with AM fungi and sprayed with low and high concentrations of nano, bulk, and ionic Cu-based compounds. Spearmint was selected as the model plant for its widespread accessibility and multipurpose uses.  $\text{Cu}(\text{OH})_2$  NPs were chosen due to their growing application as a nanopesticide and nanofertilizer. It was hypothesized that AM fungi will counteract the phytotoxic effects of  $\text{Cu}(\text{OH})_2$  NPs and function to improve spearmint plant productivity. The objective of this study was to evaluate the interaction between Cu-based NPs in spearmint symbiotically associated with AM fungi.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Copper nanoparticles/compounds

Cu NPs in the form of  $\text{Cu}(\text{OH})_2$  nanowires were supplied by US Research Nanomaterials. The commercial pesticide Kocide 3000 (Dupont, Wilmington, DE) was used as bulk Cu material. Although Kocide 3000 is considered to be a mixture of particles in the nano and micron range, since it contains micron-sized metallic Cu it will be used as the comparison bulk material to  $\text{Cu}(\text{OH})_2$  nanowires (similar to other published experimental studies(Liao et al., 2019; Strayer-Scherer et al., 2018). The active ingredient of Kocide 3000 is  $\text{Cu}(\text{OH})_2$  (46.1%), followed by O, Na, Al, Si, S, and Cl (53.9%). Characterization of these compounds, including particle size, morphology, hydrodynamic diameter, zeta potential, and Cu content, were previously determined (Cota-Ruiz et al. 2020). The physicochemical properties are shown in Table S1. Reagent grade  $\text{CuSO}_4$  salt (Sigma Aldrich) was used as an ionic counterpart to assess the effects of Cu dissolution. Throughout this publication  $\text{Cu}(\text{OH})_2$  nanowires are denoted as *nCu*, Kocide3000 as *bCu*, and  $\text{CuSO}_4$  as *iCu*.

### 4.2.2 Suspension/solution preparation

Suspensions/solutions containing 0.66 and 1.05 mg of elemental Cu per pot (1.25 and 2 lbs/acre, respectively) were prepared in ultrapure water (MW, 18.2 M $\Omega$ ) using *nCu*, *bCu*, and *iCu*; per the manufacturer's instructions. Henceforth, the low concentration of this application rate will be referred to as "low" and the high concentration as "high." To ensure homogeneity, suspensions/solutions were sonicated (Crest Ultrasonics, Trenton, NJ) for 30 minutes at 25°C with an intensity of 180 watts.

### 4.2.3 Plant cultivation and treatment

Spearmint seeds were germinated in a seed-starting tray (KORAM). After 20 days, four seedlings were transplanted into plastic pots (12.5 cm diameter, 14 cm height) containing 420 g of sieved potting mix amended with commercial mycorrhizal inoculant (BioOrganics, New Hope, PA); in accordance with the manufacturer's instructions. Characterization of the potting mix (loam soil) can be found in Table S2. The inoculant was selected based on previous studies and is described as containing a wide range of mycorrhizal fungi that is appropriate for any combination of plant, soil, and climate (BEI Bio/Organics, La Pine, OR)(Elmer and Pignatello 2011). It is comprised of spores of nine different endomycorrhizal species, including: *Glomus aggregatum*, *G. etunicatum*, *G. clarum*, *G. deserticola*, *G. intraradices*, *G. monosporus*, *G. mosseae*, *Gigaspora margarita*, and *Paraglomus brasilianum*. Treatments were prepared as triplicates and all plants were kept in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) under the following pre-set conditions: 25/20 °C temperature, 14/10 h photoperiod, 65 ± 3% relative humidity, and illumination of 340 μmol m<sup>-2</sup> s<sup>-1</sup>. Plants were watered with equal amounts of MW, as necessary, to maintain soil moisture. On the 35<sup>th</sup> day (15 days post-transplantation and 15 days prior to harvest), plants were sprayed with 2 mL of *n*Cu, *b*Cu, and *i*Cu suspensions/solutions at low and high concentrations. Non-inoculated plants that were not exposed to Cu-based NPs/compounds served as controls.

### 4.2.4 Plant harvest and agronomic parameters measurement

After 50 days plants were severed and segregated into different tissues (roots, stems, and leaves), washed with water (to detach residual soil), and alternately rinsed with 0.01 M HNO<sub>3</sub> (EMD Millipore Life Science Research) and MW three times each (to remove adhered NPs on the

surface). Agronomic measurements, specifically biomass and length, of plant tissues were recorded. Observations indicating any visual differences among the treatments were noted during this time. Soil samples were also collected from each pot for elemental analysis.

#### **4.2.5 Determination of essential elements in plant tissue and soil**

Plant tissues were dried for 72 h at 65 °C and ground into a fine powder. Conventional heating from a DigiPREP Block Digestion System (SCP Science) with concentrated HNO<sub>3</sub> (SCP Science) was used to digest plant tissue and soil samples. Macro (Ca, Cu, K, Mg, P, and S) and microelements (B, Cu, Fe, Mn, Mo, Ni, and Zn) were measured in sample digests via inductively coupled plasma - optical emission spectrometry (ICP-OES)(Perkin Elmer). Instrument parameters were set as follows: nebulizer flow, 0.80 L min<sup>-1</sup>; power, 1400 W; peristaltic pump rate, 1.5 mL min<sup>-1</sup>; flush time, 20 s; delay time, 20 s; read time, 10 s; and wash time, 60 s. For validation of the digestion method, standard reference material 1570a (National Institute of Standards and Technology) was used. For quality control of the ICP OES data, a blank and spiked sample containing 10 mg L<sup>-1</sup> of the target analyte was analyzed every 25 samples.

#### **4.2.6 Chlorophyll content**

The chlorophyll content of spearmint leaves was measured at the time of harvest (50 days). A single-photon avalanche diode instrument (SPAD, Minolta Camera, Japan) was used to determine chlorophyll concentration. For each analysis three leaves were randomly selected and measured.

#### 4.2.7 Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Sciences 22.0 (SPSS, Chicago, IL). Differences among treatment means were calculated by one-way or two-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post-hoc test, based on a probability (p-value) of 0.05.

### 4.3 RESULTS AND DISCUSSION

#### 4.3.1 Root biomass impairments from bulk and ionic Cu-based compounds are alleviated by mycorrhizal fungi

The biomass of harvested spearmint plants with and without AM fungi (NM and M, respectively) exposed to Cu-based compounds is shown in Figure 4.1A-B and S1. Opposing trends in leaf biomass can be seen between NM and M treatments exposed to *b*Cu and *i*Cu, with lower yields in NM plants vs higher yields in M plants (Figure 4.1A). This was most pronounced in the ionic treatment, where NM plants exposed to a high dosage of *i*Cu were found to have 8.23 g (60.10%) less leaf biomass than counterpart M plants. Further analysis indicated an interactive effect ( $p \leq 0.05$ ) in M plants treated with *i*Cu. In shoots, no significant changes were observed (Figure S1). The greatest variation in tissue biomass was found in the roots (Figure 4.1B). Overall, a negative correlation between root biomass and application of Cu-based compounds was observed among all treatments (1.55-8.38 g or 11.10-59.92% difference from controls). In NM plants, *n*Cu had less severe of an effect and did not significantly deplete roots (1.55 g or 11.10% reduction from controls), compared to high concentrations of *b*Cu and all concentrations of *i*Cu (Figure 4.1B, clear bars). High *b*Cu and high/low *i*Cu treatments resulted in 7.08 g (50.63%) and 6.51-8.38 g (46.58-59.92%) less root biomass, respectively, than control

treatments. Yet in M plants, these same treatments were considerably less inhibitive towards root growth, with only a 3.34 g (20.98%) and 4.35-5.34 g (27.36-33.55%) decrease in root weight from the corresponding control (Figure 4.1B, striped bars). An interactive effect ( $p \leq 0.05$ ) between mycorrhization and Cu-based compounds (*bCu*, *iCu*) was detected.

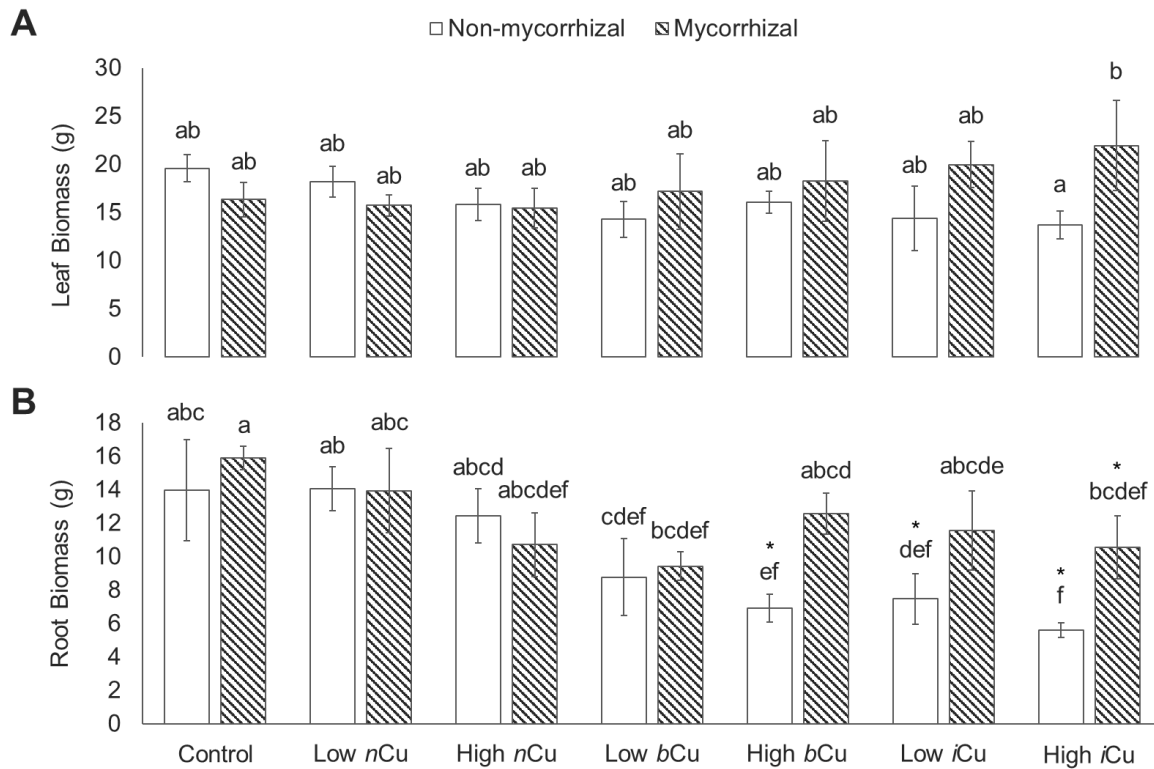


Figure 4.1A-B. Leaf (A) and root (B) biomass (g) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).



Although an essential trace element for plant metabolism, excess Cu has adverse impacts on plant health. Overproduction of oxyradicals and Cu-induced cell disturbances can disrupt biochemical reactions and physiological processes (Shabbir et al. 2020). One of the symptoms of Cu toxicity is compromised plant growth and development, including reduced plant biomass. There was a dose-dependent response on maize growth and yield to foliar Cu application, with doses higher than 100 g ha<sup>-1</sup> eliciting a toxic response (Barbosa et al. 2013). Soil and foliar application of Cu-based compounds (1000 and 2000 mg kg<sup>-1</sup>) to tomato plants inhibited dry root weight by 57.14-85.71% (Sonmez et al. 2006). In our experiment, exposure of Cu-based compounds to spearmint decreased its biomass, most notably in the roots. Our results also indicated that *n*Cu exhibited weaker toxicity than *b*Cu and *i*Cu treatments. Although NPs have been propositioned as potentially being more toxic due to their size, when using total metal concentration as the defining metric in toxicity, NPs are often less toxic than the dissolved ion (Notter, Mitrano, and Nowack 2014). It is not clear why *b*Cu exerted greater toxicity over *n*Cu. Commercial nanopesticides, including Kocide 3000, and micron-sized particles have been cited as being less toxic than their nano-sized counterparts (Keller, Adeleye, Conway, Garner, Zhao, Cherr, Hong, Gardea-Torresdey, et al. 2017b). It is possible that these differences were due to the method of exposure (foliar application). A wide range of factors may influence the final impact of foliarly administered NPs, such as plant species, applied concentration, environmental conditions, among others (Hong, Wang, Wagner, Gardea-Torresdey, et al. 2021). The presence of AM fungi could even be considered a potential environmental factor. Microscopy analysis is highly recommended to elucidate the adsorption and transport of different Cu forms in mycorrhizal spearmint.

Mycorrhization partially offset the loss in biomass production by Cu compounds. It has been well-established that symbioses with AM fungi can enhance stress tolerance of plants to a variety of stressors, including heavy metals (Gong and Tian 2019b; Dhalaria et al. 2020c). Various plant-fungal interaction mechanisms induce morphological, physiological, and/or biochemical alterations that enable the host plant to endure stressful conditions. For example, inoculation with AM fungi produced a positive response on the growth and development of common reed (Wu et al. 2020). Root growth was 107.8% higher in mycorrhizal plants treated with 1 mg L<sup>-1</sup> Cu. An even more pronounced mitigation of Cu stress was found in inoculated plants containing 5 mg L<sup>-1</sup> Cu. In addition, dry weight of shoots and leaves significantly increased with mycorrhization. Further analysis indicated that AM fungi promoted the use of photosynthetic resources and up-regulated the expression of transmembrane protein-pigment complexes involved in photosynthesis. Thus, growth enhancement by AM fungi was attributed to the alleviation of inhibited photosynthetic activities in response to Cu stress. Alternate proposed mechanisms for plant protection and growth stimulation by AM fungi were improved adsorption of environmental resources, interaction with indigenous microbes, and boosted secretion of glycoproteins into the rhizosphere. It is important to note that these observations were made under application of Cu in hydroponic solution. In our experiment, Cu NPs were administered foliarly to soil-grown plants. This method of application could have induced conditions that altered the interaction of spearmint and AM fungi (el Amerany et al. 2020). The basis of mycorrhizal symbiosis is the bi-directional movement of nutrients: the host plant supplies AM fungi with photosynthetic carbon, while AM fungi provides soil nutrients to the host plant (Wanxiao Wang et al. 2017a). It would be interesting to see whether the reduced spearmint

biomass limits the amount of carbohydrates delivered to AM fungi, which could play a role in its heavy metal modulating capabilities.

#### 4.3.2 Root length is differentially affected by Cu-based compounds and fungal inoculation

Differences in shoot length among NM and M spearmint plants treated with Cu compounds were minor (Figure S2). However, fungal inoculation significantly affected root length (Figure 2). Compared to NM control plants, roots of M control plants were 9.51 cm (38.80%) shorter. The addition of Cu compounds in NM plants decreased root length by up to 11.80 cm (48.15%), compared to respective controls (Figure 2, clear bars). Notably, this reduction was less severe in NM plants treated with a low concentration of *n*Cu (2.92 cm or 13.30%). In M plants, the addition of Cu did not significantly alter root length from the designated control (Figure 4.2, striped bars). The two-way ANOVA test showed combined effects between AM fungi and each individual form of Cu ( $p \leq 0.05$ ).

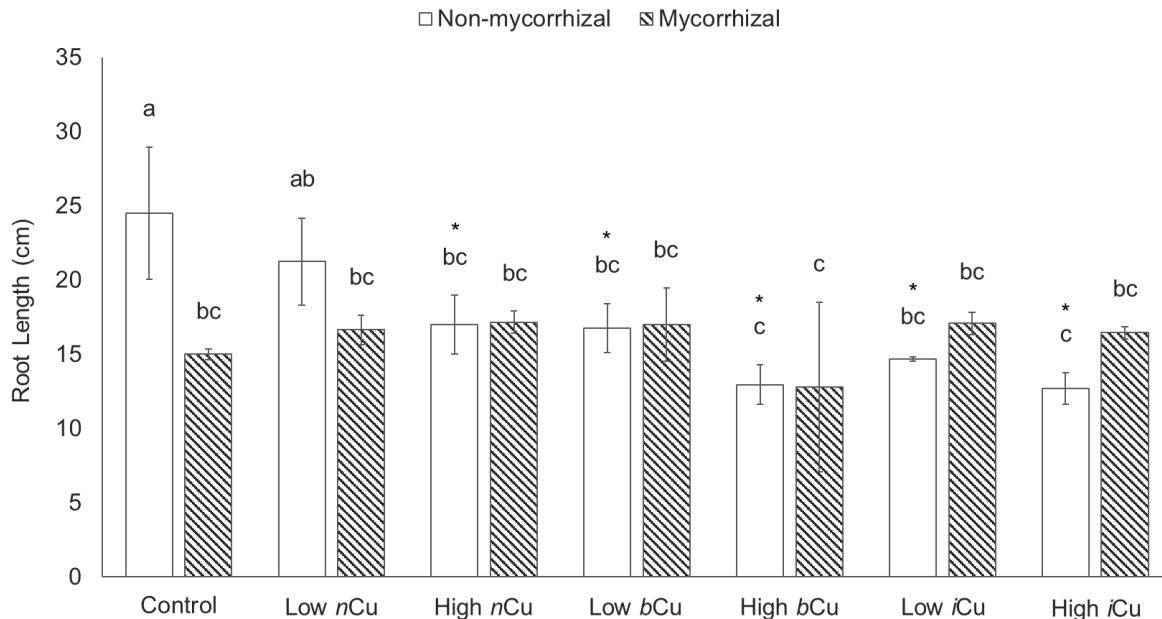


Figure 4.2. Root length (cm) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *n*Cu, *b*Cu, and *i*Cu that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

Although it seems counterintuitive for root length to be reduced by AM fungi, this has been reported in the literature. Apple trees treated with mycorrhizal inoculum had shorter roots compared to non-inoculated trees (Berdeni et al. 2018). Given that trees associated with AM fungi did not have diminished root biomass, it was concluded that inoculated trees had a coarser root system. The contrasting trends in root biomass and root length of inoculated plants in our experiment agrees with this concept – root biomass was greater throughout mycorrhizal plants, while root length was generally shorter in mycorrhizal plants.

Stunted root growth is another symptom of Cu toxicity and has been widely discussed. Wheat root length was significantly reduced from controls by 72% at 50 ppm Cu concentration (Gang, Vyas, and Vyas 2013). High levels of Cu (50 and 150  $\mu$ M) shortened root length of tomato and cucumber in accordance with increasing Cu levels (İşeri et al. 2011). In rice, treatment with 5  $\mu$ M of Cu impaired root length by 55.0%, compared to controls (Lin et al. 2013). In terms of spearmint growth parameters, toxicity was most prevalent in the roots, as as evidenced by our results on root biomass and length.

Similarly, it is well-established that mycorrhization can partially counterbalance heavy metal toxicity. Although we were unable to find studies demonstrating this effect on plant growth in terms of root length, several studies have reported this phenomenon with root dry weight. Non-

inoculated and inoculated *Dyosma versipellis* were exposed 200 and 400 mg kg<sup>-1</sup> of Cu ions (Luo et al. 2020). The addition of Cu decreased root dry weight by 26.13% and 76.19% from controls, respectively. Inoculation with AM fungi alleviated the damage from Cu stress, stimulating root dry weight by 16.98% and 22.22%, correspondingly, from Cu treatments. These results were attributed to the enlarged root mycelial system that emerged as a product of the fungal symbiosis, which ultimately reduced the adsorption of Cu via plant roots. The enhanced root density (increased biomass with shorter length) observed in our study illustrates the improved resistance that AM fungi impart onto the host plant to alleviate damage caused by Cu stress (Xiao et al. 2016).

### **4.3.3 Elemental allocation and composition is altered by Cu-based compounds with and without fungi**

#### **4.3.3.1 Cu accumulation and translocation**

The distribution of Cu throughout different parts of NM and M spearmint plants applied with Cu-based NPs/compounds are shown in Figures 4.3 and S3A-B. Concentrations of Cu in leaf tissues were low (< 0.50 mg kg<sup>-1</sup>) and several treatments had high error bars (Figure S3A). In shoots, Cu content was consistent among all treatments, except for the mycorrhizal high *n*Cu treatment, which had slightly more Cu than the non-mycorrhizal control (28.50 mg kg<sup>-1</sup>, or 19.89%)(Figure S3B). The highest amount of Cu was found in the roots (149.40-427.03 mg kg<sup>-1</sup>)(Figure 3). While Cu content was steady throughout NM plants (regardless of Cu treatment), the combination of AM fungi and Cu-based compounds impacted Cu translocation and accumulation. Overall, the addition of Cu-based compounds to M plants lowered the total accumulation of Cu in root tissues (58.80-277.63 mg kg<sup>-1</sup>, or 13.77-65.01%)(Figure 4.3, striped

bars). The most significant difference was found in the low *bCu* treatment, which had 277.63 mg kg<sup>-1</sup> (65.01%) less Cu than the respective control. Moreover, an interactive effect ( $p < 0.05$ ) was found between mycorrhization and *bCu*.

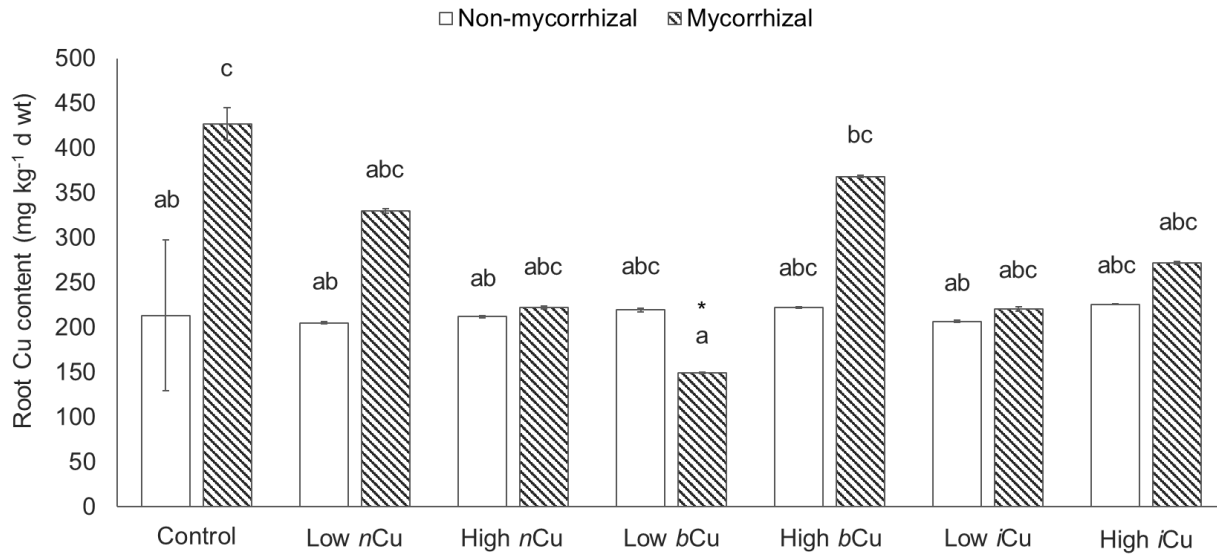


Figure 4.3. Root Cu content (mg kg<sup>-1</sup>) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

Concentration of Cu was up to four orders of magnitude higher in spearmint roots than other tissues. It has been shown that Cu exhibits downwards translocation via the phloem (Cao et al. 2020). It is also noted that foliar application of Cu can lead to accumulation in soil due to direct application, drift, or dripping from leaves (Ma et al. 2019). Thus, it is not surprising that Cu content was highest in the belowground tissues.

Plants pose flexible and adaptative strategies to maintain heavy metal homeostasis for optimum growth (Ferrol, Tamayo, and Vargas 2016). AM fungi contribute to heavy metal acquisition and performance depending on whether plants are in heavy metal deficient or toxic conditions. In nutrient deficient soils, root colonization by AM fungi often enhances nutrient uptake. The extensive mycorrhizal network produced by AM fungi expands root zone access into larger volumes of soil. This allows plants to absorb more nutrients, such as heavy metals, which may be poorly mobile in soil. However, in soils containing excess metals, colonized roots tend to accumulate lower amounts of heavy metals than non-colonized plants. This was demonstrated in an experiment where the effects of Zn on tomato plants inoculated with AM fungi were studied and Zn was applied at two extreme concentrations (0 and 100 mg kg<sup>-1</sup>)(Watts-Williams, Patti, and Cavagnaro 2013). Results showed that mycorrhizal tomato plants had lower tissue Zn concentration in deficient soil conditions and higher tissue Zn concentration in toxic soil conditions. This paradox may be attributed to: (1) heavy metal immobilization in the external fungal hyphae, and (2) alterations in metal solubility facilitated by variations in the soil medium of mycorrhizal treatments (Ferrol, Tamayo, and Vargas 2016). The decrease in root Cu uptake by mycorrhizal spearmint exposed to Cu-based compounds observed in our study could be a direct result of AM fungi modulating the plants' heavy metal tolerance mechanisms.

#### **4.3.3.2 Elemental concentrations**

Accumulation of essential elements in NM and M spearmint plant tissues and soil medium, with and without Cu compounds, can be found in the supporting information (Table S1). Element concentrations that were determined to have statistically significant results are depicted in Figures 4.4-4.6. The discussion below refers to the data from Figures 4.4-4.6.

The majority of changes in element accumulation occurred in spearmint leaves. Mycorrhization played a role in Mg and Mn accumulation, with Cu-based compounds triggering element restriction in inoculated plants (0.04-0.77 mg kg<sup>-1</sup>, or 2.49-39.77%, compared to controls)(Figure 4.4A-B). An interactive effect ( $p \leq 0.05$ ) was found by mycorrhization + *b*Cu for both Mg and Mn, which was largely driven by the high *b*Cu treatment. Concentrations of Mg and Mn in leaves of high *b*Cu treated M plants were both significantly lower than control M plants (10.47 and 0.77 mg kg<sup>-1</sup>, or 22.59% and 39.77%, correspondingly). There was a stark 0.77 mg kg<sup>-1</sup> (243.65%) difference in leaf Na between NM and M control spearmint plants, with the former having significantly less Na than all other treatments (0.63 mg kg<sup>-1</sup>) (Figure 4.4C). Accumulation of Na in NM spearmint leaves increased with the addition of Cu-based compounds (0.70 to 1.13 mg kg<sup>-1</sup>, or 219.74 to 355.07%), producing an interactive effect ( $p \leq 0.05$ ) between non-mycorrhizal status and Cu-based compounds. In contrast, the opposite trend was observed in M plants (up to 0.37 mg kg<sup>-1</sup>, or 33.61%, reduction). Application of Cu-based compounds reduced Zn uptake throughout all spearmint plants (0.012-0.20 mg kg<sup>-1</sup>, or 2.24-37.03%)(Figure 4.4D). Most notably, high *n*Cu (NM) contained 0.20 mg kg<sup>-1</sup> (37.03%) less Zn when compared to the respective control.



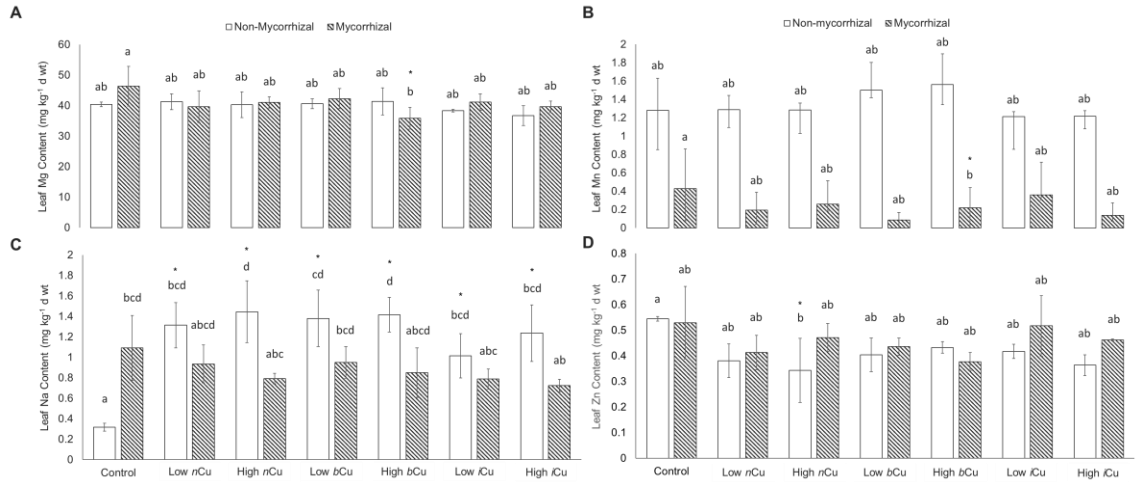


Figure 4.4A-D. Concentration ( $\text{mg kg}^{-1}$ ) of Mg (A), Mn (B), Na (C), and Zn (D) in leaves of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

There were some modifications in element content throughout spearmint shoots. Exposure to Cu-based compounds depleted Zn concentration in spearmint plants, with or without AM fungi, by  $3.16\text{--}22.46 \text{ mg kg}^{-1}$  (4.37–31.06%), respectively (Figure 4.5). This was most prevalent in the high *iCu* treatment, which had  $22.46 \text{ mg kg}^{-1}$  (31.06%) less Zn than the corresponding control.

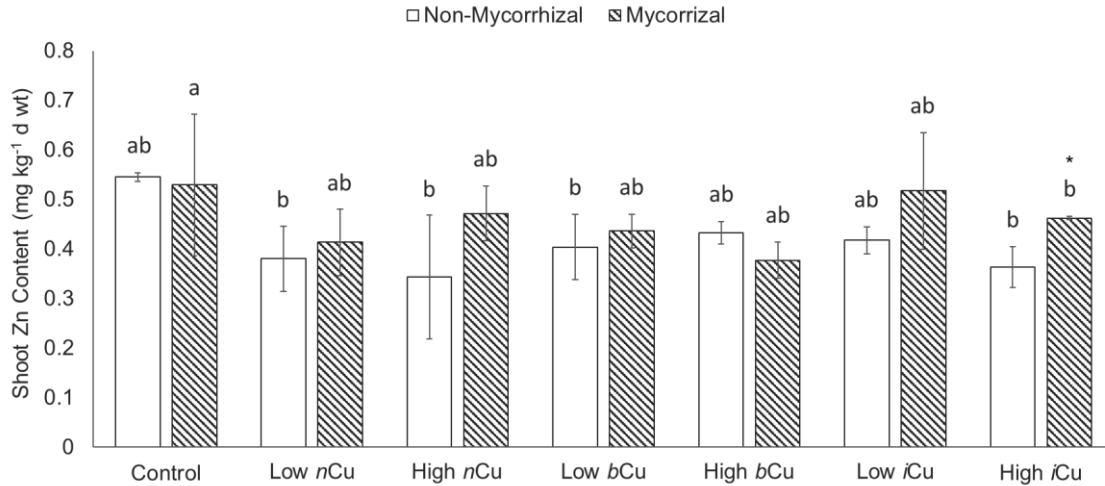


Figure 4.5. Concentration ( $\text{mg kg}^{-1}$ ) of Zn in shoots of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of  $n\text{Cu}$ ,  $b\text{Cu}$ , and  $i\text{Cu}$  that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

Belowground, nearly all statistically significant changes occurred in the cultivated soil. Although there were high error bars throughout the data, depletion of elements was highly prevalent throughout soil samples (Figure 4.6A-D). We will attempt to interpret these results to the best of our ability, focusing on general trends throughout the data. There was  $1250.83 \text{ mg kg}^{-1}$  (66.25%) less K in NM control samples than M control samples (Figure 4.6A). Accumulation of K throughout treatments containing Cu-based compounds was substantially lower than that of controls, regardless of inoculation status ( $1883.63\text{-}3150.47 \text{ mg kg}^{-1}$ , or 99.76-100.37%). Exposure to Cu-based compounds had a similar effect on Mg and P content: uptake was significantly reduced by  $3595.53\text{-}3302.34 \text{ mg kg}^{-1}$  (97.57-99.23%) and  $1204.67\text{-}1291.15 \text{ mg kg}^{-1}$  (93.07-99.80%), respectively (Figure 4.6B-C). While Cu-based compounds also had an

inhibitory effect on Na accumulation, the degree of inhibition varied (1274.94-4595.65 mg kg<sup>-1</sup>, or 35.27-95.24%, correspondingly)(Figure 4.6D).

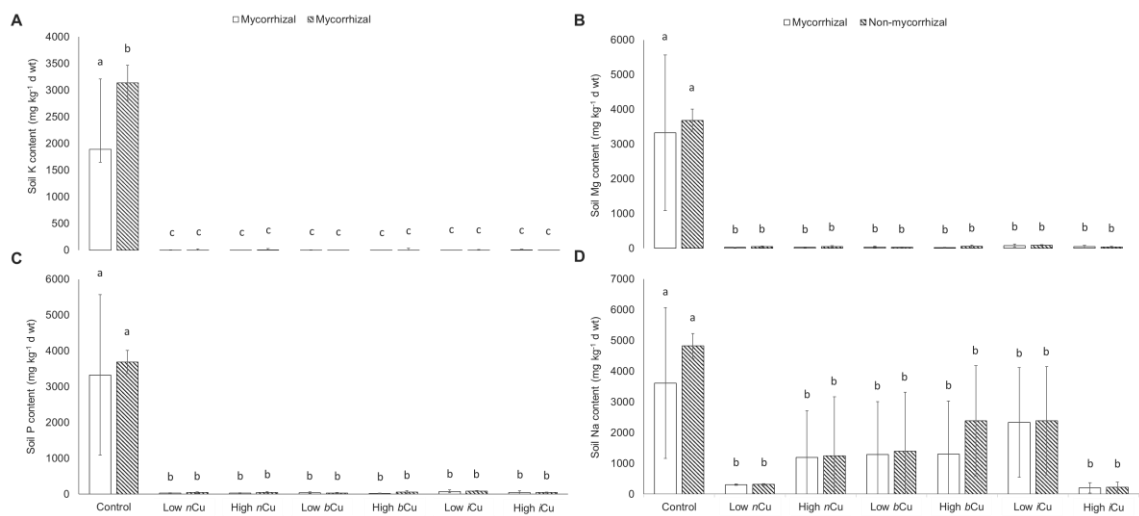


Figure 4.6A-D. Concentration (mg kg<sup>-1</sup>) of K (A), Mg (B), P (C), and Na (D) in soil of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

It has been established that Cu-based NPs can alter element accumulation in plants (Bonilla-Bird et al. 2020; Tamez et al. 2020; Dimkpa et al. 2019; Deng et al. 2022). Foliar application of NPs in the form of nanopesticides and nanofertilizers have been increasingly employed in agriculture. Leaves and roots vary in physiological function; therefore, absorption of NPs differs between these organs. Nutrient absorption occurs at a faster rate in plant stomata than root cells (Hong, Wang, Wagner, Gardea-torresdey, et al. 2021). As a result, foliar application of Cu formulations is seen as more efficient compared to soil amendment (El-

saadony et al. 2021). Exposure to any of the forms of Cu treatment elicited a toxic response in the form of reduced element accumulation (Mg, Mn, and Zn). It is not surprising that the highest degree of toxicity was observed in the leaves, where treatments were directly applied. The exception to this was Na content, which increased with Cu exposure. An influx of Na, especially in aerial parts of the plant, is linked with ionic stress (Assaha et al. 2017). Without the added protection from AM fungi, non-inoculated spearmint plants were more vulnerable to the stress effects from Cu ion release. The observed phytotoxicity on different spearmint parameters by Cu-based NPs/compounds throughout our study agrees with this assertion. The reduction in Mg and Mn by AM fungi + *b*Cu is somewhat inconsistent with the literature. While treatment with bulk Cu-based compounds have been reported to affect accumulation of Mg and Mn in aerial plant parts, the effect of mycorrhization on this relationship is unclear (Du et al. 2018b; Zuverza-Mena et al. 2015). Wheat and faba bean were grown in the presence or absence of AM fungi, where no statistically significant changes in nutrient uptake of Mg or Mn in above-ground tissues occurred (Ingraffia et al. 2019). It is important to note the differing response between wheat and faba bean in this experiment. Trends in Mg content were consistent among both plants (slight increase from respective controls ranging from 1.34 to 4.65%). However, trends in Mg content varied between the two plants. Concentration of Mn in mycorrhizal faba bean slightly decreased (3.84%) from controls, while it slightly increased in mycorrhizal wheat from controls (16.80%). In contrast, a different study reported that Mg shoot content was lower in mycorrhizal rye (14.72%) than that of its non-mycorrhizal counterpart (Schwalb et al. 2021). As previously stated, there is a need for additional research comparing different plant species, exposure methods, particle characteristics (type, concentration), and other environmental factors to fully understand the effects of Cu-based NPs/compounds on the modulatory mechanisms of

mycorrhizal fungi. Inoculation with AM fungi did not seem to affect nutrient acquisition through soil, suggesting that route of exposure is a critical factor when determining the response of Cu-based NPs/compounds to plant-fungal symbiosis (L. Wang et al. 2022).

#### **4.3.4 Chlorophyll concentration is unaffected by Cu exposure or mycorrhizae**

Leaf chlorophyll content is depicted in Figure S4. The amount of chlorophyll was consistent throughout all treatments. There was a slight decrease among *M* spearmint plants treated with both doses of *b*Cu and low dose of *i*Cu (5.70-9.10 SPAD, or 11.9-18.93%, from respective controls); however, this was not statistically significant.

Cu plays a role in several enzyme processes and is a critical element in chlorophyllin formation. The impacts of Cu application on chlorophyll biosynthesis reportedly vary. Foliar application of 0.06 mg mL<sup>-1</sup> of Cu NPs reduced total chlorophyll in wheat by 17.40%, compared to controls (Essa et al. 2020). Meanwhile, spraying 10 and 20 mg mL<sup>-1</sup> of Cu gave the highest chlorophyll content (35.22 SPAD) of any experimental treatment in faba beans (Alhasany, Noaema, and Alhmadi 2019). No significant changes from controls in total chlorophyll were observed in soil-grown cilantro treated with 20 and 80 mg kg<sup>-1</sup> of various nano and bulk-sized Cu-based compounds, including Cu(OH)<sub>2</sub> (Zuverza-Mena et al. 2015). Chlorophyll formation in plants under Cu exposure are environment-and-species-dependent. The effects of mycorrhization on chlorophyll production vary from positive to neutral (Zuccarini 2003; Lü and Wu 2017; Yang, Han, et al. 2015). Additional research is needed to draw more concrete conclusions on the effects of Cu-based NPs/compounds and mycorrhization on chlorophyll activity. However, a lack of statistical differences in chlorophyll content sheds some light on the photosynthetic characteristics in this plant-fungal system (H. Zhang et al. 2018). Gene expression studies could

provide further insight into whether plant photosynthetic mechanisms play a role in AM fungi improving spearmint resistance to Cu stress.

#### **4.4 CONCLUSIONS**

Beneficial services of AM fungi to host plants and Cu NP toxicity effects on plant growth and development have been extensively studied. However, the role of Cu NPs in mycorrhizal symbiosis is unclear. The results of our study demonstrate that Cu-based NPs/compounds are phytotoxic to spearmint. Root biomass, root length, and element allocation (Mg, Mn, Zn) were inhibited by Cu-based NPs/compounds. However, we observed that AM fungi can alleviate these phytotoxic effects. This alleviation was most notable in the roots, where there was a combined interactive effect between AM fungi and Cu-based NPs/compounds for root biomass and length. Bulk and ionic Cu-based compounds were overall more toxic than Cu NPs, with several combined interactive effects with AM fungi. Additional research on adsorption and transport processes is needed to clarify why these Cu forms elicited a greater response. As Cu NPs are increasingly used as nanopesticides and nanofertilizers they may affect plant growth and development through added exposure. Furthermore, the phytotoxicity of Cu NPs can be directly altered by AM fungi through plant-fungal interactions. Therefore, as an important component of plant systems, further studies focusing on the responses and feedback of AM fungi should be conducted for adequate risk assessment of Cu NPs in agricultural ecosystems.

## REFERENCES

- Alhasany, A. R., Noaema, A. H., & Alhmadi, H. B. (2019). The role of spraying copper and zinc on the growth and yield of *Vicia faba* L. *IOP Conference Series: Materials Science and Engineering*, 571(1).
- Ameen, F., Alsamhary, K., Alabdullatif, J. A., & ALNadhari, S. (2021). A review on metal-based nanoparticles and their toxicity to beneficial soil bacteria and fungi. In *Ecotoxicology and Environmental Safety* (Vol. 213). Academic Press.
- Assaha, D. V. M., Ueda, A., Saneoka, H., Al-Yahyai, R., & Yaish, M. W. (2017). The role of Na<sup>+</sup> and K<sup>+</sup> transporters in salt stress adaptation in glycophytes. In *Frontiers in Physiology* (Vol. 8, Issue JUL). Frontiers Media S.A.
- Barbosa, R. H., Tabaldi, L. A., Miyazaki, F. R., Pilecco, M., Kassab, S. O., & Bigaton, D. (2013). Foliar copper uptake by maize plants: effects on growth and yield . In *Ciência Rural* (Vol. 43, pp. 1561–1568).
- Berdeni, D., Cotton, T. E. A., Daniell, T. J., Bidartondo, M. I., Cameron, D. D., & Evans, K. L. (2018). The Effects of Arbuscular Mycorrhizal Fungal Colonisation on Nutrient Status, Growth, Productivity, and Canker Resistance of Apple (*Malus pumila*) . In *Frontiers in Microbiology* (Vol. 9, p. 1461).
- Bonilla-Bird, N. J., Ye, Y., Akter, T., Valdes-Bracamontes, C., Darrouzet-Nardi, A. J., Saupe, G. B., Flores-Marges, J. P., Ma, L., Hernandez-Viezcas, J. A., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2020). Effect of copper oxide nanoparticles on two varieties of sweetpotato plants. *Plant Physiology and Biochemistry*, 154, 277–286.

- Cao, Y., Ma, C., Chen, H., Zhang, J., White, J. C., Chen, G., & Xing, B. (2020). Xylem-based long-distance transport and phloem remobilization of copper in *Salix integra* Thunb. *Journal of Hazardous Materials*, 392.
- Chrysargyris, A., Papakyriakou, E., Petropoulos, S. A., & Tzortzakis, N. (2019). The combined and single effect of salinity and copper stress on growth and quality of *Mentha spicata* plants. *Journal of Hazardous Materials*, 368, 584–593.
- Cota-Ruiz, K., Ye, Y., Valdes, C., Deng, C., Wang, Y., Hernández-Viezcas, J. A., Duarte-Gardea, M., & Gardea-Torresdey, J. L. (2020). Copper nanowires as nanofertilizers for alfalfa plants: Understanding nano-bio systems interactions from microbial genomics, plant molecular responses and spectroscopic studies. *Science of the Total Environment*, 742.
- Deng, C., Wang, Y., Navarro, G., Sun, Y., Cota-Ruiz, K., Hernandez-Viezcas, J. A., Niu, G., Li, C., White, J. C., & Gardea-Torresdey, J. (2022). Copper oxide (CuO) nanoparticles affect yield, nutritional quality, and auxin associated gene expression in weedy and cultivated rice (*Oryza sativa* L.) grains. *Science of The Total Environment*, 810, 152260.
- Dhalaria, R., Kumar, D., Kumar, H., Nepovimova, E., Kuca, K., Islam, M. T., & Verma, R. (2020). Arbuscular mycorrhizal fungi as potential agents in ameliorating heavy metal stress in plants. In *Agronomy* (Vol. 10, Issue 6). MDPI AG.
- Dimkpa, C. O., Singh, U., Bindraban, P. S., Adisa, I. O., Elmer, W. H., Gardea-Torresdey, J. L., & White, J. C. (2019). Addition-omission of zinc, copper, and boron nano and bulk oxide particles demonstrate element and size -specific response of soybean to micronutrients exposure. *Science of the Total Environment*, 665, 606–616.



- Dodds, W. K., Guinnip, J. P., Schechner, A. E., Pfaff, P. J., & Smith, E. B. (2021). Fate and toxicity of engineered nanomaterials in the environment: A meta-analysis. *Science of the Total Environment*, 796.
- Du, W., Tan, W., Yin, Y., Ji, R., Peralta-Videa, J. R., Guo, H., & Gardea-Torresdey, J. L. (2018). Differential effects of copper nanoparticles/microparticles in agronomic and physiological parameters of oregano (*Origanum vulgare*). *Science of the Total Environment*, 618, 306–312.
- el Amerany, F., Meddich, A., Wahbi, S., Porzel, A., Taourirte, M., Rhazi, M., & Hause, B. (2020). Foliar application of chitosan increases tomato growth and influences mycorrhization and expression of endochitinase-encoding genes. *International Journal of Molecular Sciences*, 21(2).
- Elmer, W. H., & Pignatello, J. J. (2011). Effect of biochar amendments on mycorrhizal associations and fusarium crown and root rot of asparagus in replant soils. *Plant Disease*, 95(8), 960–966.
- El-saadony, M. T., Almoshadak, A. S., Shafi, M. E., Albaqami, N. M., Saad, A. M., El-tahan, A. M., Desoky, E. M., Elnahal, A. S. M., Almakas, A., El-mageed, T. A. A., Taha, A. E., Elrys, A. S., & Helmy, A. M. (2021). Saudi Journal of Biological Sciences Vital roles of sustainable nano-fertilizers in improving plant quality and quantity-an updated review. *Saudi Journal of Biological Sciences*.
- Ermini, M. L., & Voliani, V. (2021). Antimicrobial Nano-Agents: The Copper Age. In *ACS Nano* (Vol. 15, Issue 4, pp. 6008–6029). American Chemical Society.
- Essa, H. L., Abdelfattah, M. S., Marzouk, A. S., & Guirguis, H. A. (2020). Nano-formulations of Copper Species Coated with Sulfated Polysaccharide Extracts and Assessment of Their

- Phytotoxicity on Wheat ( *Triticum aestivum* L .) Seedlings in Seed Germination , Foliar and Soil Applications. *Applied Sciences*, 10(6302).
- Ferrol, N., Tamayo, E., & Vargas, P. (2016). The heavy metal paradox in arbuscular mycorrhizas: from mechanisms to biotechnological applications. In *Journal of Experimental Botany* (Vol. 67, Issue 22, pp. 6253–6565). Oxford University Press.
- Gang, A., Vyas, A., & Vyas, H. (2013). *Toxic effect of heavy metals on germination and seedling growth of wheat*.
- Genre, A., Lanfranco, L., Perotto, S., & Bonfante, P. (2020). Unique and common traits in mycorrhizal symbioses. In *Nature Reviews Microbiology* (Vol. 18, Issue 11, pp. 649–660). Nature Research.
- Gong, X., & Tian, D. Q. (2019). Study on the effect mechanism of Arbuscular Mycorrhiza on the absorption of heavy metal elements in soil by plants. *IOP Conference Series: Earth and Environmental Science*, 267(5).
- Hong, J., Wang, C., Wagner, D. C., Gardea-torresdey, J. L., He, F., & Rico, C. M. (2021). Environmental Science Nano absorption , transfer , and multiple impacts †. *Environmental Science Nano*, 8, 1196–1210.
- Hong, J., Wang, C., Wagner, D. C., Gardea-Torresdey, J. L., He, F., & Rico, C. M. (2021). Foliar application of nanoparticles: Mechanisms of absorption, transfer, and multiple impacts. In *Environmental Science: Nano* (Vol. 8, Issue 5, pp. 1196–1210). Royal Society of Chemistry.
- Ingraffia, R., Amato, G., Frenda, A. S., & Giambalvo, D. (2019). Impacts of arbuscular mycorrhizal fungi on nutrient uptake, N<sub>2</sub> fixation, N transfer, and growth in a wheat/faba bean intercropping system. *PLoS ONE*, 14(3).

- İşeri, Ö. D., Körpe, D. A., Yurtcu, E., Sahin, F. I., & Haberal, M. (2011). Copper-induced oxidative damage, antioxidant response and genotoxicity in *Lycopersicum esculentum* Mill. and *Cucumis sativus* L. *Plant Cell Reports*, *30*(9), 1713.
- Keller, A. A., Adeleye, A. S., Conway, J. R., Garner, K. L., Zhao, L., Cherr, G. N., Hong, J., Gardea-Torresdey, J. L., Godwin, H. A., Hanna, S., Ji, Z., Kaweeteerawat, C., Lin, S., Lenihan, H. S., Miller, R. J., Nel, A. E., Peralta-Videa, J. R., Walker, S. L., Taylor, A. A., ... Zuverza-Mena, N. (2017). Comparative environmental fate and toxicity of copper nanomaterials. *NanoImpact*, *7*, 28–40.
- Khalid, M., Ur-Rahman, S., Hassani, D., Hayat, K., Zhou, P., & Hui, N. (2021). Advances in fungal-assisted phytoremediation of heavy metals: A review. *Pedosphere*, *31*(3), 475–495.
- Liao, Y. Y., Strayer-Scherer, A. L., White, J., Mukherjee, A., de La Torre-Roche, R., Ritchie, L., Colee, J., Vallad, G. E., Freeman, J. H., Jones, J. B., & Paret, M. L. (2019). Nano-magnesium oxide: A novel bactericide against copper-tolerant *Xanthomonas perforans* causing tomato bacterial spot. *Phytopathology*, *109*(1), 52–62.  
<https://doi.org/10.1094/PHYTO-05-18-0152-R>
- Lin, C.-Y., Trinh, N. N., Fu, S.-F., Hsiung, Y.-C., Chia, L.-C., Lin, C.-W., & Huang, H.-J. (2013). Comparison of early transcriptome responses to copper and cadmium in rice roots. *Plant Molecular Biology*, *81*(4–5), 507–522.
- Lü, L., & Wu, Q. (2017). Research Article Mycorrhizas Promote Plant Growth , Root Morphology and Chlorophyll Production in White Clover. *Biotechnology*, *16*(1), 34–39.
- Luo, J., Li, X., Jin, Y., Traore, I., Dong, L., Yang, G., & Wang, Y. (2020). Effects of Arbuscular Mycorrhizal Fungi *Glomus mosseae* on the Growth and Medicinal Components of *Dioscorea*

- versipellis Under Copper Stress. *Bulletin of Environmental Contamination and Toxicology*, 0123456789.
- Ma, J.-Z., Zhang, M., Liu, Z.-G., Wang, M., Sun, Y., Zheng, W.-K., & Lu, H. (2019). Copper-based-zinc-boron foliar fertilizer improved yield, quality, physiological characteristics, and microelement concentration of celery (*Apium graveolens* L.). *Environmental Pollutants and Bioavailability*, 31(1), 261–271.
- National Agricultural Statistics Service. (2022). *Spearmint: Production by Year, US and Major States*. USDA.
- Notter, D. A., Mitrano, D. M., & Nowack, B. (2014). Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. *Environmental Toxicology and Chemistry*, 33(12), 2733–2739.
- Paramo, L. A., Feregrino-Pérez, A. A., Guevara, R., Mendoza, S., & Esquivel, K. (2020). Nanoparticles in agroindustry: Applications, toxicity, challenges, and trends. In *Nanomaterials* (Vol. 10, Issue 9, pp. 1–33). MDPI AG.
- Riaz, M., Kamran, M., Fang, Y., Wang, Q., Cao, H., Yang, G., Deng, L., Wang, Y., Zhou, Y., Anastopoulos, I., & Wang, X. (2021). Arbuscular mycorrhizal fungi-induced mitigation of heavy metal phytotoxicity in metal contaminated soils: A critical review. In *Journal of Hazardous Materials* (Vol. 402). Elsevier B.V.
- Salah, I., Parkin, I. P., & Allan, E. (2021). Copper as an antimicrobial agent: Recent advances. In *RSC Advances* (Vol. 11, Issue 30, pp. 18179–18186). Royal Society of Chemistry.
- Schwalb, S. A., Hemkemeyer, M., Watson, C., & Wichern, F. (2021). Mycorrhiza Reduces Phosphorus Uptake from Struvite in Rye (*Secale cereale* L.) Plants. *Journal of Soil Science and Plant Nutrition*.

- Shabbir, Z., Sardar, A., Shabbir, A., Abbas, G., Shamshad, S., Khalid, S., Natasha, Murtaza, G., Dumat, C., & Shahid, M. (2020). Copper uptake, essentiality, toxicity, detoxification and risk assessment in soil-plant environment. In *Chemosphere* (Vol. 259). Elsevier Ltd.
- Singh, R. P., Handa, R., & Manchanda, G. (2021). Nanoparticles in sustainable agriculture: An emerging opportunity. In *Journal of Controlled Release* (Vol. 329, pp. 1234–1248). Elsevier B.V.
- Sonmez, S., Kaplan, M., Sonmez, N. K., Kaya, H., & Uz, I. (2006). High level of copper application to soil and leaves reduce the growth and yield of tomato plants . In *Scientia Agricola* (Vol. 63, pp. 213–218).
- Strayer-Scherer, A., Liao, Y. Y., Young, M., Ritchie, L., Vallad, G. E., Santra, S., Freeman, J. H., Clark, D., Jones, J. B., & Paret, M. L. (2018). Advanced copper composites against copper-tolerant xanthomonas perforans and tomato bacterial spot. *Phytopathology*, 108(2), 196–205.
- Tamez, C., Molina-Hernandez, M., Medina-Velo, I. A., Cota-Ruiz, K., Hernandez-Viezcas, J. A., & Gardea-Torresdey, J. (2020). Long-term assessment of nano and bulk copper compound exposure in sugarcane (*Saccharum officinarum*). *Science of the Total Environment*, 718.
- Uddin, M. N., Desai, F., & Asmatulu, E. (2020). Engineered nanomaterials in the environment: bioaccumulation, biomagnification and biotransformation. In *Environmental Chemistry Letters* (Vol. 18, Issue 4, pp. 1073–1083). Springer.
- Usman, M., Farooq, M., Wakeel, A., Nawaz, A., Cheema, S. A., Rehman, H. ur, Ashraf, I., & Sanaullah, M. (2020). Nanotechnology in agriculture: Current status, challenges and future opportunities. In *Science of the Total Environment* (Vol. 721). Elsevier B.V.

- Wang, D., Saleh, N. B., Byro, A., Zepp, R., Sahle-Demessie, E., Luxton, T. P., Ho, K. T., Burgess, R. M., Flury, M., White, J. C., & Su, C. (2022). Nano-enabled pesticides for sustainable agriculture and global food security. *Nature Nanotechnology*, *17*(4), 347–360.
- Wang, L., Yang, D., Ma, F., Wang, G., & You, Y. (2022). Recent advances in responses of arbuscular mycorrhizal fungi - Plant symbiosis to engineered nanoparticles. In *Chemosphere* (Vol. 286). Elsevier Ltd.
- Wang, W., Shi, J., Xie, Q., Jiang, Y., Yu, N., & Wang, E. (2017). Nutrient Exchange and Regulation in Arbuscular Mycorrhizal Symbiosis. In *Molecular Plant* (Vol. 10, Issue 9, pp. 1147–1158). Cell Press.
- Watts-Williams, S. J., Patti, A. F., & Cavagnaro, T. R. (2013). Arbuscular mycorrhizas are beneficial under both deficient and toxic soil zinc conditions. *Plant and Soil*, *371*(1–2), 299–312.
- Wu, J.-T., Wang, L., Zhao, L., Huang, X.-C., & Ma, F. (2020). Arbuscular mycorrhizal fungi effect growth and photosynthesis of *Phragmites australis* (Cav.) Trin ex. Steudel under copper stress. *Plant Biology*, *22*(1), 62–69.
- Xiao, J. X., An, J., Chen, Y. Y., & Hu, C. Y. (2016). Improved growth and Cu tolerance of Cu excess- stressed white clover after inoculation with arbuscular mycorrhizal fungi. *Journal of Plant Nutrition*, *39*(2), 227–234.
- Yang, Y., Han, X., Liang, Y., Ghosh, A., Chen, J., & Tang, M. (2015). *The Combined Effects of Arbuscular Mycorrhizal Fungi (AMF) and Lead (Pb) Stress on Pb Accumulation , Plant Growth Parameters , Photosynthesis , and Antioxidant Enzymes in Robinia pseudoacacia L* . 1–24.

- Zhang, H., Xu, N., Li, X., Long, J., Sui, X., Wu, Y., Li, J., Wang, J., Zhong, H., & Sun, G. Y. (2018). Arbuscular mycorrhizal fungi (*Glomus mosseae*) improves growth, photosynthesis and protects photosystem II in leaves of *lolium perenne* L. in Cadmium contaminated soil. *Frontiers in Plant Science*, *9*.
- Zhang, L.-L., Chen, Y., Li, Z.-J., Li, X., & Fan, G. (2022). Bioactive properties of the aromatic molecules of spearmint (*Mentha spicata* L.) essential oil: a review. *Food & Function*, *13*(6), 3110–3132.
- Zhao, L., Lu, L., Wang, A., Zhang, H., Huang, M., Wu, H., Xing, B., Wang, Z., & Ji, R. (2020). Nano-Biotechnology in Agriculture: Use of Nanomaterials to Promote Plant Growth and Stress Tolerance. *Journal of Agricultural and Food Chemistry*, *68*(7), 1935–1947.
- Zuccarini, P. (2003). Mycorrhizal infection ameliorates chlorophyll content and nutrient uptake of lettuce exposed to saline irrigation. *Plant Soil Environment*, *53*(7), 283–289.
- Zuverza-Mena, N., Medina-Velo, I. A., Barrios, A. C., Tan, W., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015). Copper nanoparticles/compounds impact agronomic and physiological parameters in cilantro (*Coriandrum sativum*). *Environ. Sci.: Processes Impacts*, *17*(10), 1783–1793.

## **Chapter 5: Copper-based nanoparticles/compounds disrupt nutrient exchange between spearmint (*Mentha spicata*) and arbuscular mycorrhizal fungi**

### **5.1 INTRODUCTION**

Copper nanoparticles (Cu NPs) are widely incorporated into antimicrobial agents, electronics, batteries, catalysts, gas sensors, and heat transfer fluids, along with others (Fatima, Hashim, and Anees 2021). They are also used in agricultural applications such as fertilizers and pesticides. With growing demand for Cu-based NPs towards sustainable agriculture, there are emerging concerns on the environmental release of Cu NPs. The antimicrobial activity of Cu NPs for plant disease management is well-studied, but the mechanism of antimicrobial action by Cu NPs is not (Rai et al. 2018b). Furthermore, beneficial soil microbes may also be subject to this microbicidal activity. To ensure safe use of Cu NPs, it is paramount to conduct holistic risk assessments on the various dynamics of agricultural ecosystems.

As the most common symbiotic association between plants and microorganisms, the fitness benefits conferred by arbuscular mycorrhizal (AM) fungi have been widely reported. Colonization by AM fungi is predominantly based on the bidirectional nutrient exchange between the two organisms in the symbiotic relationship (Huan et al. 2019). The extensive network of mycelial hyphae produced by AM fungi increases the adsorptive area of the host plant's roots, thereby enhancing uptake of soil nutrients. In return, the host plant supplies photosynthetic carbon to AM fungi. Although carbohydrates are essential for fungal growth and development, AM fungi are not able to produce carbohydrates on their own (Wilkes 2021). Thus, establishment of close relationships with a host plant are important for their survival.

Fungal symbionts play an important role in plant abiotic stress tolerance (Bell et al. 2022). The application of AM fungi to mitigate crop losses is regularly proposed. While research on the



exposure of NPs to mycorrhizal symbiosis has progressively increased, these studies often used unrealistically high NP concentrations and/or soil-less media (Tian, Kah, and Kariman 2019). Recommended research priorities are as follows: (1) studies should be conducted under more realistic environmental conditions; (2) greater attention towards NP-based agrochemicals; and (3) further investigation on factors linked to functionality of symbioses, such as nutritional symbiotic benefits.

This study was a follow-up to our previous work investigating the impact of Cu-based NPs/compounds on the interactions between AM fungi and spearmint in terms of plant growth and development. Spearmint plants associated with AM fungi were cultivated in potting mix (loam soil) and treated with nano, bulk, and ionic Cu-based compounds. Roots were analyzed for macromolecular contents. The objective of this research was to understand the exchange of nutrients by mycorrhizal symbiosis in the presence of Cu-based NPs/compounds. In contrast to our previous work, analysis in this study focused on carbon flow in the rhizosphere.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Copper-based nanoparticles/compounds**

Cu(OH)<sub>2</sub> nanowires (*nCu*)(US Research Nanomaterials, Inc.), bulk Cu (*bCu*)(Kocide<sup>®</sup>3000), and ionic CuSO<sub>4</sub> (*iCu*)(Spectrum Chemical<sup>®</sup>) were used in this study. The characterizations of *nCu* and *bCu* were previously described and are shown in Table S1 (Cota-Ruiz et al. 2020). Solutions/suspensions of *nCu*, *bCu*, and *iCu* were bath sonicated for 30 min at 25°C (Crest Ultrasonics, Trenton, NJ Model 275 DA; 120 V, 3 A, 59/60 Hz), then they were used as foliar plant spray. Suspensions/solutions contained 0.658 (“high” concentration) and 1.05 (“low” concentration) mg/Cu per pot (1.25 and 2 lbs/acre, respectively).

### 5.2.2 Experimental design

Spearmint seeds were disinfected with 1% NaClO for 3 min, rinsed with deionized water (18.2 MΩ) 3 times, and left to air dry for 3 h. The seeds were germinated in a seed-starting tray (KORAM) and four 20-day old seedlings were transplanted into plastic pots (12.5 cm x 14 cm) containing 420 g of sieved commercial potting soil (Miracle-Gro®). Physical and chemical properties of the potting soil were previously determined and can be found in Table 2 (Barrios et al. 2015). Commercial AM fungi [powder inoculant, Bio-Organics™, La Pine, OR (*Glomus aggregatum*, *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. clarum*, *G. deserticola*, *G. monosporus*, *Gigaspora margarita*, and *Paraglomus brasilianum*), 10 cc (8 g) per plant] was blended into the potting soil. Treatments were prepared in triplicates (including a control treatment without mycorrhizal inoculant) and maintained in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) at 25±5 °C day/night temperature cycle, 65 ± 5% relative humidity, 14 h photoperiod with 340 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity. Plants were regularly watered with 100 mL of MPW at ~50% of their field capacity (every ~3 days). Fifteen days after transplantation (35 total days), plants were sprayed with 2 mL of *n*Cu, *b*Cu, and *i*Cu suspensions/solutions at low and high concentrations. Six plants in total (three replicates each with and without mycorrhizal inoculant) were not exposed to Cu-based compounds and set as controls. Plants were grown for another 15 days (50 total days) and then harvested. Root samples were collected and stored at either -80 °C or oven-dried at 70 °C for 72 h for further analysis. Since the focus of this study was to examine the underlying processes that facilitate mycorrhizal symbiosis, our samples consisted primarily of spearmint plant roots that were exposed to AM fungi.

### **5.2.3 Protein extraction and quantification**

Protein was extracted from root samples at 4 °C, using the PureLink™ Plant RNA Reagent (Invitrogen™). Proteins were precipitated and centrifuged following the addition of 5 M NaCl, in accordance with the manufacturer's instructions. To each protein sample, 150 µL of Tris-HCl (pH 7.5) buffer was added and then maintained at -20 °C. Protein quantification was conducted using Coomassie Protein Assay Reagent (Thermo Scientific Prod #1856209) following the manufacturer's recommendations. Absorbance was measured at 595 nm using a SpectraMax 190 Microplate Reader (Molecular Devices). Standard calibration curves were performed using bovine serum albumin (Thermo Scientific) with accepted R<sup>2</sup> values of 0.98 or greater.

### **5.2.4 Carbohydrates (sugar and starch) extraction and quantification**

Starch and sugar were measured according to the procedure described by (Verma S. and Dubey R.S. 2001) and is summarized here. Approximately 100 mg of dried root samples were combined with 10 cm<sup>3</sup> of 80% ethanol and bath sonicated at 80 °C for 30 min. Then, contents were centrifuged at 22,000 g for 20 min. Pooled sample supernatants (extracted three times) were reduced to 3 cm<sup>3</sup> via evaporation, upon which they were diluted up to 3 cm<sup>3</sup> with water. Total sugar concentration was estimated in ethanol. Leftover residues from sugar extractions were used for starch extractions. Residues were dried in an 70 °C oven for 24 h, combined with 2 cm<sup>3</sup> of water, and boiled in a water bath for 15 min. After cooling, 2 cm<sup>3</sup> of 9.2 M perchloric acid (PCA) was added. Contents were agitated for 15 min, brought up to 10 cm<sup>3</sup> with water, and centrifuged at 3,000 g for 20 min. Then, residues were twice extracted using 2 cm<sup>3</sup> of 4.6 M PCA. Pooled supernatants were diluted up to 50 cm<sup>3</sup> with water. Total sugars and starch were measured colorimetrically using a phenol-sulfuric acid method (Masuko et al. 2005). A calibration curve was constructed using standard glucose and corn starch.

### **5.2.5 Statistical analyses**

The Statistical Package for Social Sciences 22.0 (SPSS, Chicago, IL) was used for statistical analyses. Treatment means were compared using one-way analysis of variance (ANOVA) in conjunction with Tukey's honestly significant difference (HSD) post-hoc test. The significance level was set at 0.05.

## **5.3 RESULTS AND DISCUSSION**

### **5.3.1 Protein content**

Total protein content in roots of non-mycorrhizal (NM) and mycorrhizal (M) spearmint plants applied with Cu-based NPs/compounds are shown in Figure 5.1. While a statistical difference was not indicated, possibly due to a high standard deviation, M control roots contained 127.63% more average protein than NM control roots. The addition of Cu-based NPs/compounds generally reduced protein content in M roots (29.55-76.52% from M control roots). This effect was most prevalent in high treatments, which had 63.64-76.52% less protein than the M control. The exception to this negative trend was found in the low *b*Cu treatment, which had 19.71% increased protein than M control roots.

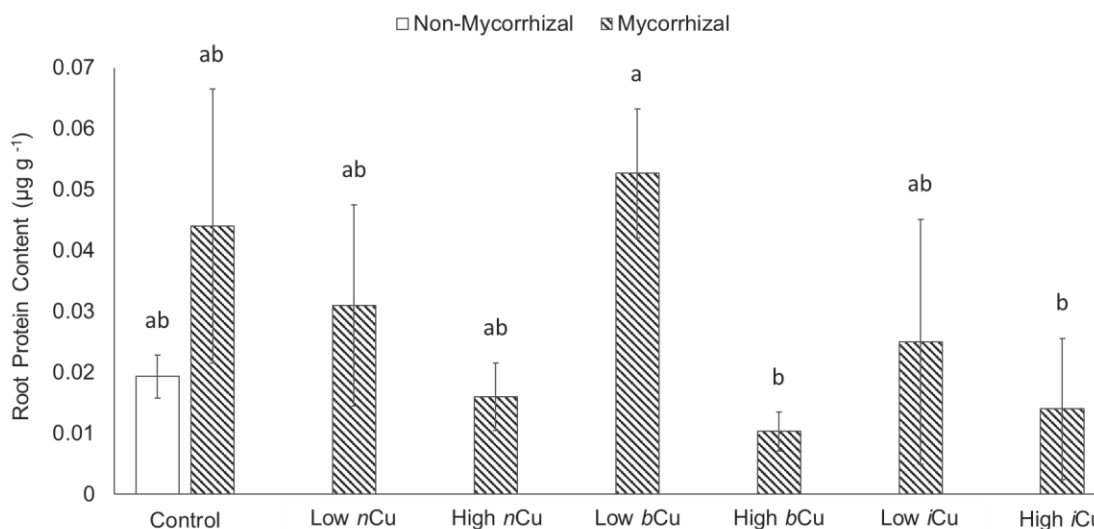


Figure 5.1: Root protein content ( $\mu\text{g g}^{-1}$ ) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences ( $p \leq 0.05$ ).

It is not surprising that M control roots contained greater amounts of protein than NM control roots. The basis of mycorrhizal symbiosis is that AM fungi enhance adsorption of water and nutrients to the host plant in exchange for carbon (Salvioli Di Fossalunga and Novero 2019). A large portion of this carbon is used to form glomalin, a glycoprotein unique to AM fungi (Gao, Wang, and Wu 2019). Glomalin is produced on the spores and hyphae of AM fungi and can be found throughout the roots and soil. An excess of protein indicates higher microbial activity and, in this case, successful colonization of spearmint roots by AM fungi.

The depletion in protein with exposure to Cu-based NPs/compounds is more complex. Glomalin possesses a strong ability to sequester heavy metals – one of several protective strategies utilized by AM fungi to reduce heavy metal toxicity (Dhalaria et al. 2020d). Several studies have reported that excess heavy metals can enhance glomalin production by AM fungi (Gujre et al. 2021; Herath BMMD et al. 2021). Despite this, the molecular mechanisms associated with

glomalin, including heavy metal immobilization, remain unclear due to a lack of biochemical characterization for glomalin (Vlček and Pohanka 2020; Eramma, Bhajantri, and B. 2021). For instance, mycorrhizal clover plants grown in sandy soil amended with  $3.2 \text{ mg kg}^{-1}$  of FeO NPs had significantly reduced glomalin related soil protein (Feng et al., 2013b). It was suggested that this could be due to the bioactive nature of Fe and its high affinity for organic compounds, which could have bound to glomalin. Although Cu also has a propensity to undergo complexation by organic matter, this is not completely comparable with our results, since Cu-based NPs/compounds were exposed through foliar application (Rieuwerts et al. 1998; Fernández and Brown 2013). We note that it is possible that there was some degree of incidental exposure of Cu-based NPs/compounds into the soil due to drip from plant leaves (Ma et al. 2019). Moreover, our analyses measured total protein content and did not discriminate between different protein types. High Cu levels have been shown to inhibit protein synthesis, which corresponds with our observations (Hippler et al. 2018; Yuan et al. 2023). Although most studies demonstrating reduced protein content by Cu exposure do not consider mycorrhization. Protein accumulation is not only affected by heavy metal stress, but also the presence of AM fungi. In Herrera et al. (2018), while protein content in orchid roots was inhibited by the presence of heavy metals, there was an over-expression of proteins related to copper transport found in root segments of non-mycorrhizal plants grown in heavy metal polluted soil compared to mycorrhizal root segments grown in the same soil treatment. This was thought to be due to the presence of AM fungi, which may reduce the availability of some metal concentrations. Further research on gene expression is needed to understand activation of plant defense mechanisms by AM fungi. Nevertheless, our results illustrate the intricacy of plant system dynamics. Studies on plant responses to metal NPs should not neglect soil microorganisms.

Regarding the elevated protein content in low *bCu*, we refer to our previous work in Chapter 4. Low *bCu* spearmint roots accumulated the least amount of Cu among all treatments (which was also significantly lower compared to high *bCu* spearmint roots). It is possible that the low *bCu* dose may not reach a high enough threshold to affect protein function and synthesis. Furthermore, a major conclusion from Chapter 4 was that *bCu* treatment elicited a stronger

response in mycorrhizal spearmint than *n*Cu or *i*Cu, with interactive effects in element accumulation. Results from these studies suggest that plant defense mechanisms imparted by mycorrhizal symbiosis may be more susceptible to Cu stress from bulk particles.

### 5.3.2 Sugar content

The sugar content of harvested NM and M spearmint plant roots, with and without Cu NPs/compounds, is shown in Figure 5.2. While there were no statistically significant differences among the treatments, it is important to note that M control roots contained 50.22% less sugar than NM control roots. The relatively high standard error of the NM control samples ( $21.27 \pm 11.69$  mg sugar g dry root<sup>-1</sup>) likely influenced the statistical analyses. Additionally, Cu-based NPs/compounds slightly stimulated sugar content in M roots (from the respective control) and were more comparable to sugar levels in NM control roots (within 0.07-28.35% average range).

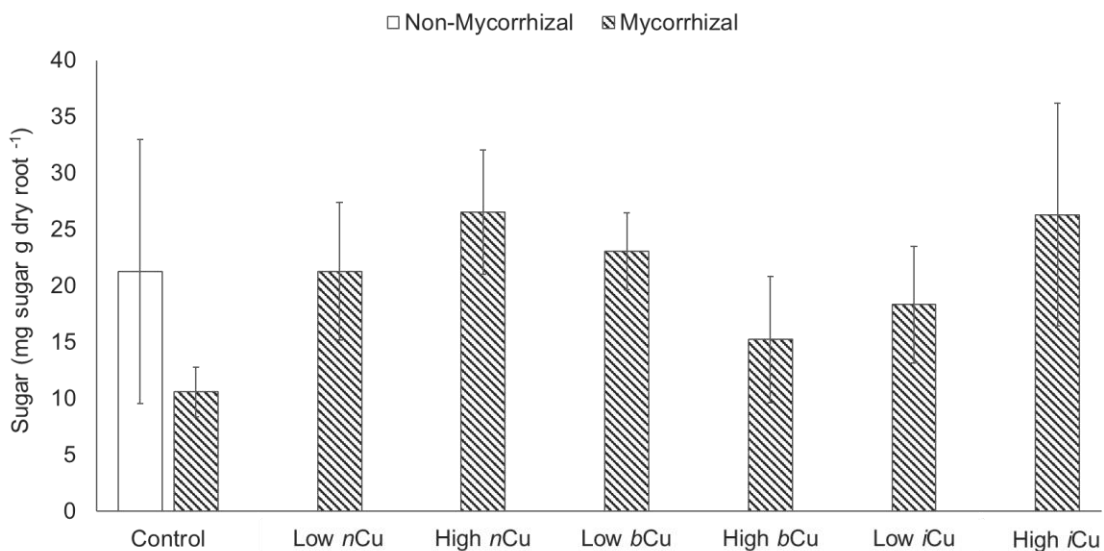


Figure 5.2: Root sugar content (mg sugar g dry root<sup>-1</sup>) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *n*Cu, *b*Cu,

and *i*Cu that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences ( $p \leq 0.05$ ).

Observations in sugar content of spearmint roots are consistent with those made in root protein. Fungal protein is produced from plant-derived carbon, with the bulk of this carbon in the form of sugars (Holátko et al. 2021). The least amount of sugar was detected in the M control treatment, indicating that sugar was allocated to mycorrhizal roots to support symbiotic functioning, including protein biosynthesis (Wanxiao Wang et al. 2017b). From the previous section, the dose-dependent reduction observed in root protein of M spearmint by Cu-based NPs/compounds provides insight into why root sugar was more comparable to NM controls. Exposure to Cu-based NPs/compounds had a similar dose-dependent effect on sugar concentration in M spearmint roots, but in the opposite direction (positive relationship). Moreover, application of Cu-based NPs/compounds could have caused sugars to accumulate, to cope with Cu-induced oxidative stress (Gramss 2012; Rosa et al. 2009). Interestingly, this effect was not seen in *b*Cu treatments. As previously stated, *b*Cu seems to elicit an abnormal response in mycorrhizal spearmint. Further study on the impact of Cu in bulk form to mycorrhizal plants could clarify these results. Although the observations in sugar content were not statistically significant, they raise the possibility that sugar transport, metabolism, and storage are influenced by AM fungi under Cu stress (Hennion et al. 2019).

### **5.3.3 Starch content**

Starch content throughout NM and M spearmint plant roots exposed to Cu-based NPs/compounds was relatively stable, with no statistically significant differences (Figure 5.3). There was a considerable 54.98% decline in high *i*Cu roots, compared to M control roots. While there was a slight decrease in low *i*Cu roots (15.28%, compared to M controls), treatment averages



had a high standard error ( $66.61 \pm 45.78$  mg starch g dry root<sup>-1</sup>), so we are not able to confirm a trend by *i*Cu.

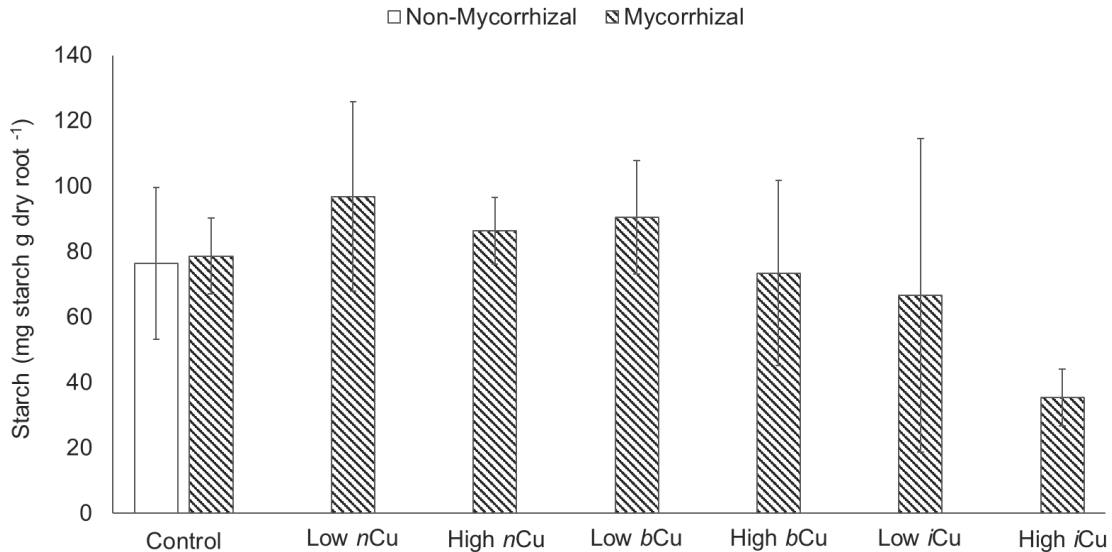


Figure 5.3: Root starch content (mg starch g dry root<sup>-1</sup>) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *n*Cu, *b*Cu, and *i*Cu that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences ( $p \leq 0.05$ ).

Previously, starch mobilization was thought to be correlated with sugar supply and an important factor in mycorrhizal symbiosis (Wanxiao Wang et al. 2017b). Studies by Gutjahr et al. (2009; 2011) showed that starch synthesis is not required for mycorrhizal colonization in *Lotus japonicus* and that carbohydrates can be delivered to AM fungi irrespective of starch accumulation. Our results indicating minimal response agree with these findings and suggest that starch accumulation is not a driving factor in the stress response of AM fungi to Cu-based NPs/compounds. It is possible that the high *i*Cu treatment degraded root starch accumulation, as is often the case with metal toxicity (Seneviratne et al. 2019). However, the elevated standard error

in the low *i*Cu treatment makes it difficult to determine whether this is a byproduct of the ionic Cu treatment itself or the high exposure dose.

#### **5.4 CONCLUSIONS**

This study sought to understand the underlying mechanisms that drive plant protection strategies by AM fungi. We observed that exposure to Cu-based NPs/compounds altered nutrient exchange and regulation in AM symbiosis. Protein metabolism in mycorrhizal spearmint was impacted by both experimental concentrations of Cu-based NPs/compounds. Roots of mycorrhizal spearmint treated with Cu-based NPs/compounds contained lower amounts of protein than controls (dose-dependent response). Meanwhile, Cu-based NPs/compounds did not inhibit sugar accumulation in mycorrhizal spearmint roots. Further work is needed to clarify whether sugar transport, metabolism, and storage is being affected through regulation of gene expression. Protein and sugar content were differentially affected by bulk Cu-based compounds, which is consistent with our previous study that bulk Cu-based compounds elicit a stronger response in mycorrhizal spearmint. No discernable impacts to starch content were detected. This research demonstrates the complexity of mycorrhizal interactions in the rhizosphere and how molecular mechanisms may be affected by abiotic stressors.

## REFERENCES

- Alhasany, A. R., Noaema, A. H., & Alhmadi, H. B. (2019). The role of spraying copper and zinc on the growth and yield of *Vicia faba* L. *IOP Conference Series: Materials Science and Engineering*, 571(1).
- Ameen, F., Alsamhary, K., Alabdullatif, J. A., & ALNadhari, S. (2021). A review on metal-based nanoparticles and their toxicity to beneficial soil bacteria and fungi. In *Ecotoxicology and Environmental Safety* (Vol. 213). Academic Press.
- Assaha, D. V. M., Ueda, A., Saneoka, H., Al-Yahyai, R., & Yaish, M. W. (2017). The role of Na<sup>+</sup> and K<sup>+</sup> transporters in salt stress adaptation in glycophytes. In *Frontiers in Physiology* (Vol. 8, Issue JUL). Frontiers Media S.A.
- Barbosa, R. H., Tabaldi, L. A., Miyazaki, F. R., Pilecco, M., Kassab, S. O., & Bigaton, D. (2013). Foliar copper uptake by maize plants: effects on growth and yield . In *Ciência Rural* (Vol. 43, pp. 1561–1568). cielo.
- Berdeni, D., Cotton, T. E. A., Daniell, T. J., Bidartondo, M. I., Cameron, D. D., & Evans, K. L. (2018). The Effects of Arbuscular Mycorrhizal Fungal Colonisation on Nutrient Status, Growth, Productivity, and Canker Resistance of Apple (*Malus pumila*) . In *Frontiers in Microbiology* (Vol. 9, p. 1461).
- Bonilla-Bird, N. J., Ye, Y., Akter, T., Valdes-Bracamontes, C., Darrouzet-Nardi, A. J., Saupe, G. B., Flores-Marges, J. P., Ma, L., Hernandez-Viezcas, J. A., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2020). Effect of copper oxide nanoparticles on two varieties of sweetpotato plants. *Plant Physiology and Biochemistry*, 154, 277–286.

- Cao, Y., Ma, C., Chen, H., Zhang, J., White, J. C., Chen, G., & Xing, B. (2020). Xylem-based long-distance transport and phloem remobilization of copper in *Salix integra* Thunb. *Journal of Hazardous Materials*, 392.
- Chrysargyris, A., Papakyriakou, E., Petropoulos, S. A., & Tzortzakis, N. (2019). The combined and single effect of salinity and copper stress on growth and quality of *Mentha spicata* plants. *Journal of Hazardous Materials*, 368, 584–593.  
<https://doi.org/10.1016/j.jhazmat.2019.01.058>
- Cota-Ruiz, K., Ye, Y., Valdes, C., Deng, C., Wang, Y., Hernández-Viezcas, J. A., Duarte-Gardea, M., & Gardea-Torresdey, J. L. (2020). Copper nanowires as nanofertilizers for alfalfa plants: Understanding nano-bio systems interactions from microbial genomics, plant molecular responses and spectroscopic studies. *Science of the Total Environment*, 742.
- Deng, C., Wang, Y., Navarro, G., Sun, Y., Cota-Ruiz, K., Hernandez-Viezcas, J. A., Niu, G., Li, C., White, J. C., & Gardea-Torresdey, J. (2022). Copper oxide (CuO) nanoparticles affect yield, nutritional quality, and auxin associated gene expression in weedy and cultivated rice (*Oryza sativa* L.) grains. *Science of The Total Environment*, 810, 152260.
- Dhalaria, R., Kumar, D., Kumar, H., Nepovimova, E., Kuca, K., Islam, M. T., & Verma, R. (2020). Arbuscular mycorrhizal fungi as potential agents in ameliorating heavy metal stress in plants. In *Agronomy* (Vol. 10, Issue 6). MDPI AG.
- Dimkpa, C. O., Singh, U., Bindraban, P. S., Adisa, I. O., Elmer, W. H., Gardea-Torresdey, J. L., & White, J. C. (2019). Addition-omission of zinc, copper, and boron nano and bulk oxide particles demonstrate element and size -specific response of soybean to micronutrients exposure. *Science of the Total Environment*, 665, 606–616.

- Dodds, W. K., Guinnip, J. P., Schechner, A. E., Pfaff, P. J., & Smith, E. B. (2021). Fate and toxicity of engineered nanomaterials in the environment: A meta-analysis. *Science of the Total Environment*, 796.
- Du, W., Tan, W., Yin, Y., Ji, R., Peralta-Videa, J. R., Guo, H., & Gardea-Torresdey, J. L. (2018). Differential effects of copper nanoparticles/microparticles in agronomic and physiological parameters of oregano (*Origanum vulgare*). *Science of the Total Environment*, 618, 306–312.
- el Amerany, F., Meddich, A., Wahbi, S., Porzel, A., Taourirte, M., Rhazi, M., & Hause, B. (2020). Foliar application of chitosan increases tomato growth and influences mycorrhization and expression of endochitinase-encoding genes. *International Journal of Molecular Sciences*, 21(2).
- Elmer, W. H., & Pignatello, J. J. (2011). Effect of biochar amendments on mycorrhizal associations and fusarium crown and root rot of asparagus in replant soils. *Plant Disease*, 95(8), 960–966.
- El-saadony, M. T., Almoshadak, A. S., Shafi, M. E., Albaqami, N. M., Saad, A. M., El-tahan, A. M., Desoky, E. M., Elnahal, A. S. M., Almakas, A., El-mageed, T. A. A., Taha, A. E., Elrys, A. S., & Helmy, A. M. (2021). Saudi Journal of Biological Sciences Vital roles of sustainable nano-fertilizers in improving plant quality and quantity-an updated review. *Saudi Journal of Biological Sciences*.
- Ermini, M. L., & Voliani, V. (2021). Antimicrobial Nano-Agents: The Copper Age. In *ACS Nano* (Vol. 15, Issue 4, pp. 6008–6029). American Chemical Society.
- Essa, H. L., Abdelfattah, M. S., Marzouk, A. S., & Guirguis, H. A. (2020). Nano-formulations of Copper Species Coated with Sulfated Polysaccharide Extracts and Assessment of Their

- Phytotoxicity on Wheat ( *Triticum aestivum* L .) Seedlings in Seed Germination , Foliar and Soil Applications. *Applied Sciences*, 10(6302).
- Ferrol, N., Tamayo, E., & Vargas, P. (2016). The heavy metal paradox in arbuscular mycorrhizas: from mechanisms to biotechnological applications. In *Journal of Experimental Botany* (Vol. 67, Issue 22, pp. 6253–6565). Oxford University Press.
- Gang, A., Vyas, A., & Vyas, H. (2013). *Toxic effect of heavy metals on germination and seedling growth of wheat*.
- Genre, A., Lanfranco, L., Perotto, S., & Bonfante, P. (2020). Unique and common traits in mycorrhizal symbioses. In *Nature Reviews Microbiology* (Vol. 18, Issue 11, pp. 649–660). Nature Research.
- Gong, X., & Tian, D. Q. (2019). Study on the effect mechanism of Arbuscular Mycorrhiza on the absorption of heavy metal elements in soil by plants. *IOP Conference Series: Earth and Environmental Science*, 267(5).
- Hong, J., Wang, C., Wagner, D. C., Gardea-torresdey, J. L., He, F., & Rico, C. M. (2021). Environmental Science Nano absorption , transfer , and multiple impacts †. *Environmental Science Nano*, 8, 1196–1210.
- Hong, J., Wang, C., Wagner, D. C., Gardea-Torresdey, J. L., He, F., & Rico, C. M. (2021). Foliar application of nanoparticles: Mechanisms of absorption, transfer, and multiple impacts. In *Environmental Science: Nano* (Vol. 8, Issue 5, pp. 1196–1210). Royal Society of Chemistry.
- Ingraffia, R., Amato, G., Frenda, A. S., & Giambalvo, D. (2019). Impacts of arbuscular mycorrhizal fungi on nutrient uptake, N<sub>2</sub> fixation, N transfer, and growth in a wheat/faba bean intercropping system. *PLoS ONE*, 14(3).

- İşeri, Ö. D., Körpe, D. A., Yurtcu, E., Sahin, F. I., & Haberal, M. (2011). Copper-induced oxidative damage, antioxidant response and genotoxicity in *Lycopersicum esculentum* Mill. and *Cucumis sativus* L. *Plant Cell Reports*, *30*(9), 1713.
- Keller, A. A., Adeleye, A. S., Conway, J. R., Garner, K. L., Zhao, L., Cherr, G. N., Hong, J., Gardea-Torresdey, J. L., Godwin, H. A., Hanna, S., Ji, Z., Kaweeteerawat, C., Lin, S., Lenihan, H. S., Miller, R. J., Nel, A. E., Peralta-Videa, J. R., Walker, S. L., Taylor, A. A., ... Zuverza-Mena, N. (2017). Comparative environmental fate and toxicity of copper nanomaterials. *NanoImpact*, *7*, 28–40.
- Khalid, M., Ur-Rahman, S., Hassani, D., Hayat, K., Zhou, P., & Hui, N. (2021). Advances in fungal-assisted phytoremediation of heavy metals: A review. *Pedosphere*, *31*(3), 475–495.
- Liao, Y. Y., Strayer-Scherer, A. L., White, J., Mukherjee, A., de La Torre-Roche, R., Ritchie, L., Colee, J., Vallad, G. E., Freeman, J. H., Jones, J. B., & Paret, M. L. (2019). Nano-magnesium oxide: A novel bactericide against copper-tolerant *Xanthomonas perforans* causing tomato bacterial spot. *Phytopathology*, *109*(1), 52–62.
- Lin, C.-Y., Trinh, N. N., Fu, S.-F., Hsiung, Y.-C., Chia, L.-C., Lin, C.-W., & Huang, H.-J. (2013). Comparison of early transcriptome responses to copper and cadmium in rice roots. *Plant Molecular Biology*, *81*(4–5), 507–522.
- Lü, L., & Wu, Q. (2017). Research Article Mycorrhizas Promote Plant Growth , Root Morphology and Chlorophyll Production in White Clover. *Biotechnology*, *16*(1), 34–39.
- Luo, J., Li, X., Jin, Y., Traore, I., Dong, L., Yang, G., & Wang, Y. (2020). Effects of Arbuscular Mycorrhizal Fungi *Glomus mosseae* on the Growth and Medicinal Components of *Dioscorea oppositifolia* Under Copper Stress. *Bulletin of Environmental Contamination and Toxicology*, *0123456789*.

- Ma, J.-Z., Zhang, M., Liu, Z.-G., Wang, M., Sun, Y., Zheng, W.-K., & Lu, H. (2019). Copper-based-zinc-boron foliar fertilizer improved yield, quality, physiological characteristics, and microelement concentration of celery (*Apium graveolens* L.). *Environmental Pollutants and Bioavailability*, *31*(1), 261–271.
- National Agricultural Statistics Service. (2022). *Spearmint: Production by Year, US and Major States*. USDA.
- Notter, D. A., Mitrano, D. M., & Nowack, B. (2014). Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. *Environmental Toxicology and Chemistry*, *33*(12), 2733–2739.
- Paramo, L. A., Feregrino-Pérez, A. A., Guevara, R., Mendoza, S., & Esquivel, K. (2020). Nanoparticles in agroindustry: Applications, toxicity, challenges, and trends. In *Nanomaterials* (Vol. 10, Issue 9, pp. 1–33). MDPI AG.
- Riaz, M., Kamran, M., Fang, Y., Wang, Q., Cao, H., Yang, G., Deng, L., Wang, Y., Zhou, Y., Anastopoulos, I., & Wang, X. (2021). Arbuscular mycorrhizal fungi-induced mitigation of heavy metal phytotoxicity in metal contaminated soils: A critical review. In *Journal of Hazardous Materials* (Vol. 402). Elsevier B.V.
- Salah, I., Parkin, I. P., & Allan, E. (2021). Copper as an antimicrobial agent: Recent advances. In *RSC Advances* (Vol. 11, Issue 30, pp. 18179–18186). Royal Society of Chemistry.
- Schwalb, S. A., Hemkemeyer, M., Watson, C., & Wichern, F. (2021). Mycorrhiza Reduces Phosphorus Uptake from Struvite in Rye (*Secale cereale* L.) Plants. *Journal of Soil Science and Plant Nutrition*.



- Shabbir, Z., Sardar, A., Shabbir, A., Abbas, G., Shamshad, S., Khalid, S., Natasha, Murtaza, G., Dumat, C., & Shahid, M. (2020). Copper uptake, essentiality, toxicity, detoxification and risk assessment in soil-plant environment. In *Chemosphere* (Vol. 259). Elsevier Ltd.
- Singh, R. P., Handa, R., & Manchanda, G. (2021). Nanoparticles in sustainable agriculture: An emerging opportunity. In *Journal of Controlled Release* (Vol. 329, pp. 1234–1248). Elsevier B.V.
- Sonmez, S., Kaplan, M., Sonmez, N. K., Kaya, H., & Uz, I. (2006). High level of copper application to soil and leaves reduce the growth and yield of tomato plants . In *Scientia Agricola* (Vol. 63, pp. 213–218). scielo.
- Strayer-Scherer, A., Liao, Y. Y., Young, M., Ritchie, L., Vallad, G. E., Santra, S., Freeman, J. H., Clark, D., Jones, J. B., & Paret, M. L. (2018). Advanced copper composites against copper-tolerant xanthomonas perforans and tomato bacterial spot. *Phytopathology*, 108(2), 196–205.
- Tamez, C., Molina-Hernandez, M., Medina-Velo, I. A., Cota-Ruiz, K., Hernandez-Viezcas, J. A., & Gardea-Torresdey, J. (2020). Long-term assessment of nano and bulk copper compound exposure in sugarcane (*Saccharum officinarum*). *Science of the Total Environment*, 718.
- Uddin, M. N., Desai, F., & Asmatulu, E. (2020). Engineered nanomaterials in the environment: bioaccumulation, biomagnification and biotransformation. In *Environmental Chemistry Letters* (Vol. 18, Issue 4, pp. 1073–1083). Springer.
- Usman, M., Farooq, M., Wakeel, A., Nawaz, A., Cheema, S. A., Rehman, H. ur, Ashraf, I., & Sanaullah, M. (2020). Nanotechnology in agriculture: Current status, challenges and future opportunities. In *Science of the Total Environment* (Vol. 721). Elsevier B.V.

- Wang, D., Saleh, N. B., Byro, A., Zepp, R., Sahle-Demessie, E., Luxton, T. P., Ho, K. T., Burgess, R. M., Flury, M., White, J. C., & Su, C. (2022). Nano-enabled pesticides for sustainable agriculture and global food security. *Nature Nanotechnology*, *17*(4), 347–360.
- Wang, L., Yang, D., Ma, F., Wang, G., & You, Y. (2022). Recent advances in responses of arbuscular mycorrhizal fungi - Plant symbiosis to engineered nanoparticles. In *Chemosphere* (Vol. 286). Elsevier Ltd.
- Wang, W., Shi, J., Xie, Q., Jiang, Y., Yu, N., & Wang, E. (2017). Nutrient Exchange and Regulation in Arbuscular Mycorrhizal Symbiosis. In *Molecular Plant* (Vol. 10, Issue 9, pp. 1147–1158). Cell Press.
- Watts-Williams, S. J., Patti, A. F., & Cavagnaro, T. R. (2013). Arbuscular mycorrhizas are beneficial under both deficient and toxic soil zinc conditions. *Plant and Soil*, *371*(1–2), 299–312.
- Wu, J.-T., Wang, L., Zhao, L., Huang, X.-C., & Ma, F. (2020). Arbuscular mycorrhizal fungi effect growth and photosynthesis of *Phragmites australis* (Cav.) Trin ex. Steudel under copper stress. *Plant Biology*, *22*(1), 62–69.
- Xiao, J. X., An, J., Chen, Y. Y., & Hu, C. Y. (2016). Improved growth and Cu tolerance of Cu excess- stressed white clover after inoculation with arbuscular mycorrhizal fungi. *Journal of Plant Nutrition*, *39*(2), 227–234.
- Yang, Y., Han, X., Liang, Y., Ghosh, A., Chen, J., & Tang, M. (2015). *The Combined Effects of Arbuscular Mycorrhizal Fungi (AMF) and Lead (Pb) Stress on Pb Accumulation, Plant Growth Parameters, Photosynthesis, and Antioxidant Enzymes in Robinia pseudoacacia L.* 1–24.

- Zhang, H., Xu, N., Li, X., Long, J., Sui, X., Wu, Y., Li, J., Wang, J., Zhong, H., & Sun, G. Y. (2018). Arbuscular mycorrhizal fungi (*Glomus mosseae*) improves growth, photosynthesis and protects photosystem II in leaves of *lolium perenne* L. in Cadmium contaminated soil. *Frontiers in Plant Science*, *9*.
- Zhang, L.-L., Chen, Y., Li, Z.-J., Li, X., & Fan, G. (2022). Bioactive properties of the aromatic molecules of spearmint (*Mentha spicata* L.) essential oil: a review. *Food & Function*, *13*(6), 3110–3132.
- Zhao, L., Lu, L., Wang, A., Zhang, H., Huang, M., Wu, H., Xing, B., Wang, Z., & Ji, R. (2020). Nano-Biotechnology in Agriculture: Use of Nanomaterials to Promote Plant Growth and Stress Tolerance. *Journal of Agricultural and Food Chemistry*, *68*(7), 1935–1947.
- Zuccarini, P. (2003). Mycorrhizal infection ameliorates chlorophyll content and nutrient uptake of lettuce exposed to saline irrigation. *Plant Soil Environment*, *53*(7), 283–289.
- Zuverza-Mena, N., Medina-Velo, I. A., Barrios, A. C., Tan, W., Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015). Copper nanoparticles/compounds impact agronomic and physiological parameters in cilantro (*Coriandrum sativum*). *Environ. Sci.: Processes Impacts*, *17*(10), 1783–1793.

## Chapter 6: Conclusions and future work

The main goal of this dissertation was to provide insight on the impact of Cu-based NPs/compounds to mycorrhizal spearmint. Our work was conducted in three phases to evaluate the interactions between Cu-based NPs/compounds, AM fungi, and spearmint plants. We found that AM fungi were able to alleviate Cu-induced phytotoxicity, offsetting impairments in spearmint plant growth and development. Although AM fungi appeared to be tolerant to high concentrations of Cu NPs/compounds from the *in vitro* bioassays, exposure of Cu-based NPs/compounds to mycorrhizal symbiosis disrupted the nutrient exchange between spearmint plants and AM fungi. Additionally, bulk Cu-based compounds were found to have stronger more varied effects in mycorrhizal spearmint. These results provide valuable information towards risk assessment of Cu-based NPs/compounds in agricultural ecosystems. Future studies on photosynthetic parameters, metabolomics, and gene expression would complement our work, offering insight on the underlying mechanisms of defense regulation in mycorrhizal symbiosis.

## Appendix

### 1 SUPPORTING INFORMATION FOR CHAPTER 3: ARBUSCULAR MYCORRHIZAL FUNGI ALLEVIATE PHYTOTOXIC EFFECTS OF COPPER-BASED NANOPARTICLES/COMPOUNDS IN SPEARMINT

Table S1. Physicochemical properties of Cu-based NPs/compounds (Cota-Ruiz et al., 2020).

Property	nCu <sup>a</sup>	bCu <sup>b</sup>
Size (nm)	width, 50 nm; length, 3000-5000 nm*	>10 <sup>4</sup>
Hydrodynamic diameter (nm)	50	1532 ± 580
Zeta potential (mV)	24.1 ± 0.32	-40.9 ± 2.7
Cu content (wt%)	65.1	26.5
Other elements present	ND	C, O, Na, Al, Si, S, Cl
Morphology	Elongated	Spherical

<sup>a</sup> Refers to nano Cu form; <sup>b</sup> refers to bulk Cu form.

\*This is a nanowire; thus, the size is larger than 100 nm.

ND, non-defined.<sup>4</sup>

Table S2. Potting mix composition (Barrios et al., 2015).

Characteristic	Measurement
pH	6.8 - 7.2
Forest products, compost, sphagnum peat moss, perlite, wetting agent and fertilizer (%)	50-60
Total nitrogen (N)* (%)	0.21
Ammoniacal nitrogen (%)	0.12
Nitrate nitrogen (%)	0.09
Available phosphate (P <sub>2</sub> O <sub>5</sub> ) (%)	0.07
Soluble potash (K <sub>2</sub> O)* (%)	0.14
Iron (Fe) (%)	0.1
Water soluble iron (Fe) (%)	0.1
Al (mg kg <sup>-1</sup> )	7551.28 ± 447.58
Ca (mg kg <sup>-1</sup> )	29570.39 ± 3406.41
Cu (mg kg <sup>-1</sup> )	30.52 ± 4.97
Fe (mg kg <sup>-1</sup> )	4653.38 ± 404.12
K	1868.65 ± 92.83
Mg	3110.12 ± 789.19
Mn	197.67 ± 12.08
P	1818.36 ± 261.48
Zn	44.22 ± 5.22

†Derived from: ammonium nitrate (polymer coated), ammonium phosphate, calcium phosphate, and potassium phosphate; and ammonium nitrate, ammonium phosphate, calcium phosphate, potassium sulfate, and ferrous sulfate.

\*A portion of the nitrogen, phosphate and potash has been coated to provide 0.15% coated slow release nitrogen (N), 0.03% coated slow release available phosphate (P<sub>2</sub>O<sub>5</sub>) and 0.08% coated slow release soluble potash (K<sub>2</sub>O)

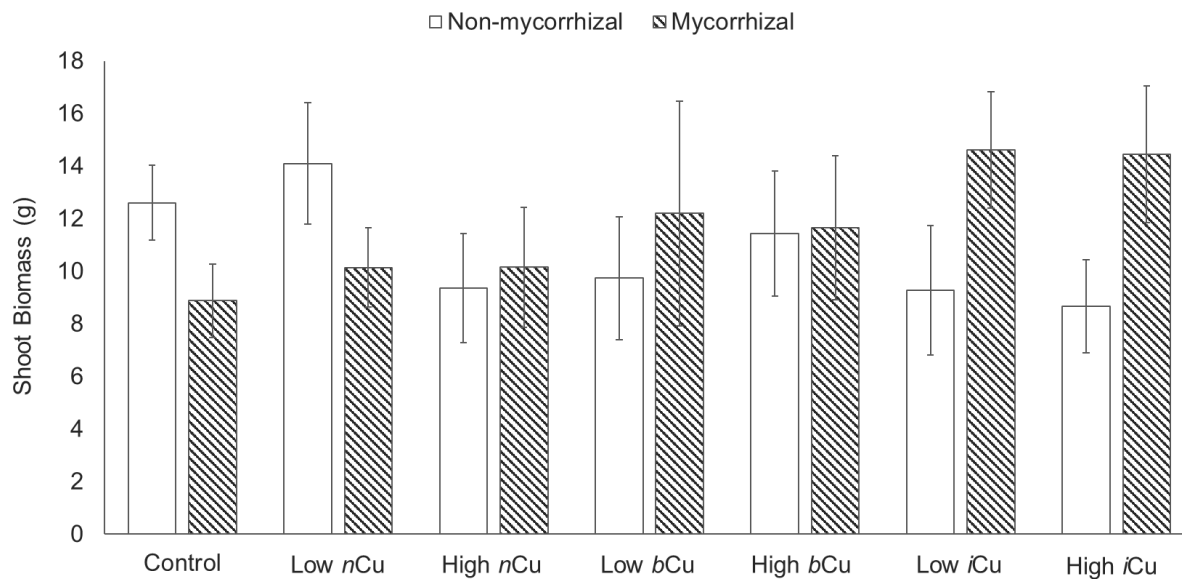


Figure S1. Shoot biomass (g) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

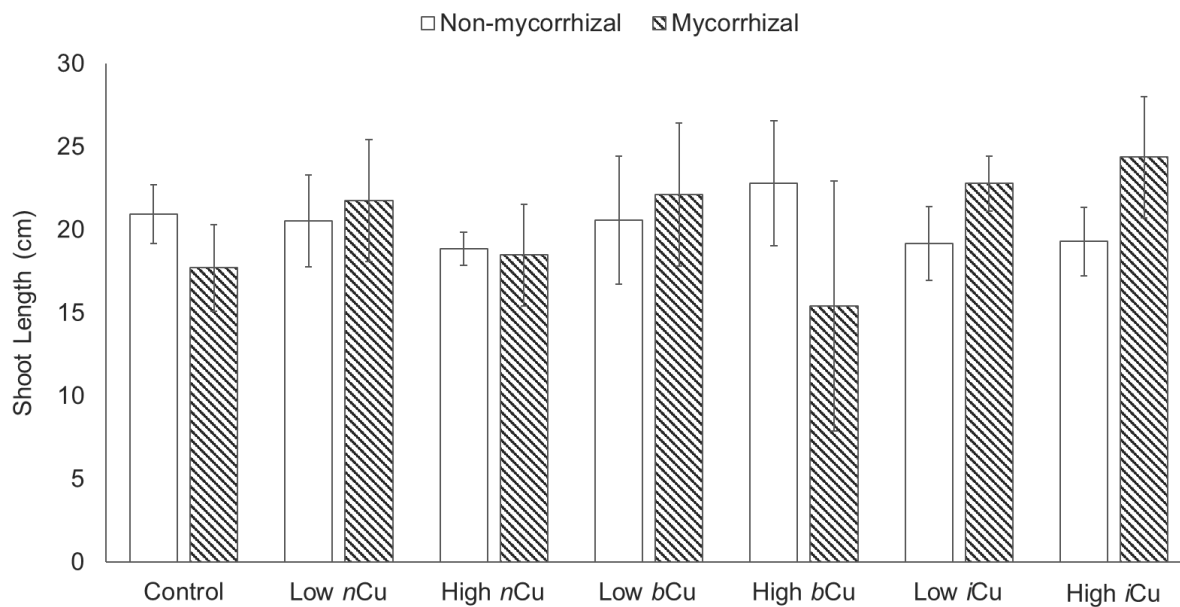


Figure S2. Shoot length (cm) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).



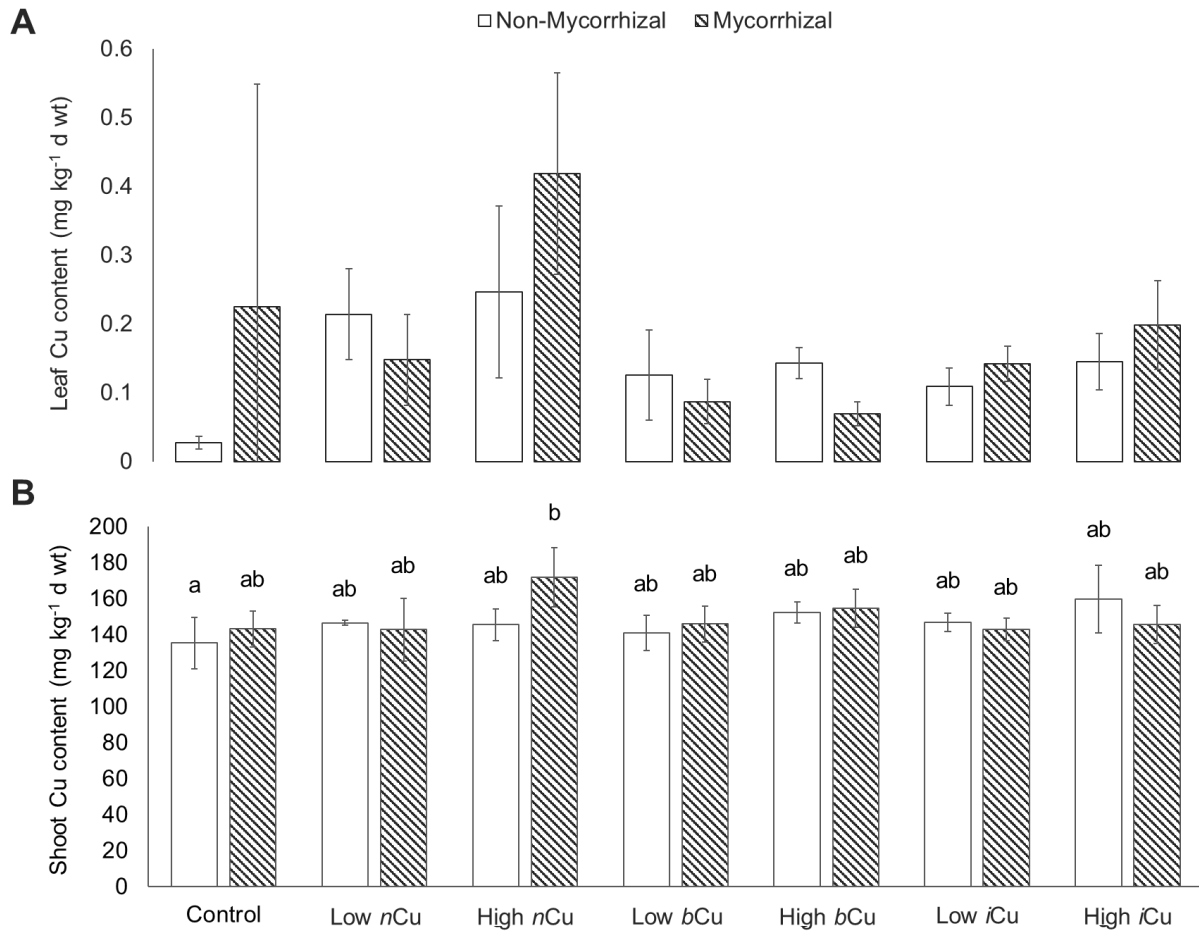


Figure S3A-B. Leaf (A) and shoot (B) Cu content (mg kg<sup>-1</sup>) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

Table S3. Elemental content in non-mycorrhizal (NM) and mycorrhizal (M) spearmint plant tissues treated with low and high concentrations of *n*Cu, *b*Cu, and *i*Cu. Data are means of three replicates  $\pm$  SE ( $n = 3$ ).

Tissue	Treatment	Ca		Cu		Fe	
Leaf	Control (NM)	79.38	$\pm$ 3.29	0.03	$\pm$ 0.01	0.40	$\pm$ 0.04
	Control (M)	100.48	$\pm$ 21.31	0.22	$\pm$ 0.32	0.44	$\pm$ 0.20
	Low <i>n</i> Cu (NM)	81.31	$\pm$ 11.72	0.21	$\pm$ 0.07	0.30	$\pm$ 0.05
	Low <i>n</i> Cu (M)	76.67	$\pm$ 5.40	0.15	$\pm$ 0.07	0.33	$\pm$ 0.11
	High <i>n</i> Cu (NM)	77.91	$\pm$ 9.72	0.25	$\pm$ 0.13	0.30	$\pm$ 0.04
	High <i>n</i> Cu (M)	81.06	$\pm$ 8.13	0.42	$\pm$ 0.15	0.30	$\pm$ 0.02
	Low <i>b</i> Cu (NM)	77.25	$\pm$ 4.64	0.13	$\pm$ 0.07	0.33	$\pm$ 0.03
	Low <i>b</i> Cu (M)	85.73	$\pm$ 12.13	0.09	$\pm$ 0.03	0.37	$\pm$ 0.05
	High <i>b</i> Cu (NM)	76.51	$\pm$ 8.47	0.14	$\pm$ 0.02	0.36	$\pm$ 0.06
	High <i>b</i> Cu (M)	71.71	$\pm$ 9.28	0.07	$\pm$ 0.02	0.37	$\pm$ 0.09
	Low <i>i</i> Cu (NM)	72.57	$\pm$ 1.72	0.11	$\pm$ 0.03	0.33	$\pm$ 0.03
	Low <i>i</i> Cu (M)	84.75	$\pm$ 10.18	0.14	$\pm$ 0.03	0.39	$\pm$ 0.05
	High <i>i</i> Cu (NM)	65.61	$\pm$ 4.17	0.14	$\pm$ 0.04	0.34	$\pm$ 0.06
	High <i>i</i> Cu (M)	73.28	$\pm$ 9.02	0.20	$\pm$ 0.06	0.34	$\pm$ 0.02
Shoot	Control (NM)	10393.14	$\pm$ 951.40	135.38	$\pm$ 14.40	90.45	$\pm$ 10.72
	Control (M)	12495.39	$\pm$ 1014.38	143.28	$\pm$ 10.01	101.95	$\pm$ 16.22
	Low <i>n</i> Cu (NM)	10751.46	$\pm$ 1091.95	146.56	$\pm$ 1.35	107.85	$\pm$ 43.36
	Low <i>n</i> Cu (M)	11794.36	$\pm$ 1341.55	142.85	$\pm$ 17.41	86.98	$\pm$ 3.65
	High <i>n</i> Cu (NM)	9523.09	$\pm$ 181.98	145.69	$\pm$ 8.79	81.41	$\pm$ 1.49
	High <i>n</i> Cu (M)	11176.26	$\pm$ 1051.41	171.77	$\pm$ 16.45	89.25	$\pm$ 8.62
	Low <i>b</i> Cu (NM)	10399.41	$\pm$ 1477.66	141.05	$\pm$ 9.62	83.08	$\pm$ 7.23
	Low <i>b</i> Cu (M)	11681.56	$\pm$ 238.51	145.94	$\pm$ 9.98	94.71	$\pm$ 16.86
	High <i>b</i> Cu (NM)	10920.24	$\pm$ 750.87	152.28	$\pm$ 5.87	86.78	$\pm$ 2.93
	High <i>b</i> Cu (M)	11539.32	$\pm$ 1084.32	154.81	$\pm$ 10.59	97.04	$\pm$ 8.01
	Low <i>i</i> Cu (NM)	8973.89	$\pm$ 988.64	146.87	$\pm$ 5.09	89.98	$\pm$ 0.13
	Low <i>i</i> Cu (M)	11724.11	$\pm$ 182.99	142.85	$\pm$ 6.22	90.83	$\pm$ 8.71
	High <i>i</i> Cu (NM)	10801.66	$\pm$ 1688.02	159.81	$\pm$ 18.92	93.01	$\pm$ 12.56
	High <i>i</i> Cu (M)	9855.76	$\pm$ 3339.83	145.74	$\pm$ 10.53	88.37	$\pm$ 7.71
Root	Control (NM)	4811.97	$\pm$ 1847.16	213.34	$\pm$ 84.17	381.49	$\pm$ 255.70
	Control (M)	6093.90	$\pm$ 292.07	427.03	$\pm$ 18.41	406.32	$\pm$ 177.35
	Low <i>n</i> Cu (NM)	4071.03	$\pm$ 179.41	205.08	$\pm$ 18.25	160.55	$\pm$ 16.77
	Low <i>n</i> Cu (M)	5258.16	$\pm$ 102.85	329.88	$\pm$ 96.56	283.61	$\pm$ 92.00
	High <i>n</i> Cu (NM)	4838.93	$\pm$ 1133.18	211.97	$\pm$ 44.41	187.00	$\pm$ 36.21
	High <i>n</i> Cu (M)	5144.00	$\pm$ 457.85	222.37	$\pm$ 36.85	232.63	$\pm$ 27.08
	Low <i>b</i> Cu (NM)	4048.61	$\pm$ 468.51	219.48	$\pm$ 28.66	156.56	$\pm$ 13.58
	Low <i>b</i> Cu (M)	3683.83	$\pm$ 3197.21	149.40	$\pm$ 133.57	199.61	$\pm$ 177.21

Tissue	Treatment	Ca		Cu		Fe	
	High <i>b</i> Cu (NM)	4216.85	± 638.67	222.56	± 32.36	170.95	± 11.68
	High <i>b</i> Cu (M)	5181.53	± 723.84	368.22	± 160.44	296.35	± 99.74
	Low <i>i</i> Cu (NM)	4179.04	± 471.72	206.84	± 18.57	161.76	± 1.69
	Low <i>i</i> Cu (M)	5463.52	± 653.97	220.47	± 20.70	319.51	± 41.83
	High <i>i</i> Cu (NM)	4723.68	± 364.40	225.86	± 21.93	218.20	± 79.40
	High <i>i</i> Cu (M)	5492.77	± 542.74	272.05	± 35.48	269.48	± 66.39
Soil	Control (NM)	13702.44	± 9332.01	7.00	± 4.61	3099.16	± 2118.84
	Control (M)	15489.49	± 1767.93	8.85	± 1.20	3602.90	± 306.28
	Low <i>n</i> Cu (NM)	60.16	± 74.73	3.97	± 0.92	249.29	± 17.74
	Low <i>n</i> Cu (M)	161.32	± 86.26	3.30	± 0.42	254.91	± 28.36
	High <i>n</i> Cu (NM)	3503.08	± 5922.16	4.05	± 1.84	974.71	± 1251.76
	High <i>n</i> Cu (M)	4087.01	± 6955.07	4.09	± 4.71	998.26	± 1518.95
	Low <i>b</i> Cu (NM)	4396.55	± 6162.85	5.78	± 0.45	1049.06	± 1409.03
	Low <i>b</i> Cu (M)	4097.22	± 7036.96	7.23	± 1.61	1189.64	± 1658.90
	High <i>b</i> Cu (NM)	3913.45	± 6547.56	4.48	± 1.34	1054.80	± 1406.53
	High <i>b</i> Cu (M)	9076.83	± 7017.20	7.39	± 0.22	2011.26	± 1531.55
	Low <i>i</i> Cu (NM)	8495.52	± 7388.91	5.95	± 0.31	1938.64	± 1488.38
	Low <i>i</i> Cu (M)	8262.09	± 6931.09	7.55	± 5.79	2014.73	± 1505.28
	High <i>i</i> Cu (NM)	65.12	± 15.99	2.43	± 3.36	167.23	± 134.24
	High <i>i</i> Cu (M)	143.01	± 21.68	1.85	± 2.57	191.99	± 123.19

Tissue	Treatment	K		Mg		Mn	
Leaf	Control (NM)	120.90	± 15.33	37.60	± 1.07	1.28	± 0.35
	Control (M)	108.36	± 9.54	46.21	± 8.84	1.93	± 0.43
	Low <i>n</i> Cu (NM)	106.26	± 14.72	40.50	± 3.17	1.29	± 0.16
	Low <i>n</i> Cu (M)	109.76	± 22.94	37.61	± 6.25	1.63	± 0.19
	High <i>n</i> Cu (NM)	102.27	± 11.88	40.17	± 5.91	1.29	± 0.07
	High <i>n</i> Cu (M)	106.55	± 8.77	39.44	± 2.44	1.88	± 0.26
	Low <i>b</i> Cu (NM)	108.29	± 2.75	39.71	± 2.11	1.50	± 0.30
	Low <i>b</i> Cu (M)	125.56	± 10.62	40.65	± 4.29	1.38	± 0.08
	High <i>b</i> Cu (NM)	124.69	± 16.04	40.23	± 5.30	1.56	± 0.34
	High <i>b</i> Cu (M)	115.64	± 11.26	32.97	± 3.95	1.16	± 0.22
	Low <i>i</i> Cu (NM)	124.16	± 9.44	36.06	± 0.27	1.21	± 0.05
	Low <i>i</i> Cu (M)	136.91	± 12.77	39.00	± 3.55	1.54	± 0.36
	High <i>i</i> Cu (NM)	124.71	± 8.73	34.22	± 4.03	1.22	± 0.06
	High <i>i</i> Cu (M)	129.07	± 2.87	36.75	± 1.58	1.47	± 0.14
Shoot	Control (NM)	56302.70	± 2830.84	2297.60	± 175.86	55.67	± 10.83
	Control (M)	53717.00	± 2781.34	2748.19	± 250.06	85.67	± 9.48
	Low <i>n</i> Cu (NM)	51087.28	± 3807.31	2520.73	± 159.96	72.60	± 15.63
	Low <i>n</i> Cu (M)	57529.71	± 4830.02	2805.29	± 350.92	80.77	± 10.76
	High <i>n</i> Cu (NM)	49210.99	± 3600.20	2042.36	± 244.51	57.27	± 7.51
	High <i>n</i> Cu (M)	55450.25	± 1709.39	2458.87	± 209.43	95.68	± 14.84
	Low <i>b</i> Cu (NM)	52690.51	± 6238.96	2233.80	± 433.27	81.90	± 30.60
	Low <i>b</i> Cu (M)	55392.59	± 6388.64	2495.33	± 257.41	81.75	± 8.82
	High <i>b</i> Cu (NM)	54473.52	± 4677.89	2636.33	± 219.06	87.89	± 9.77
	High <i>b</i> Cu (M)	52612.30	± 2954.91	2374.57	± 183.19	80.66	± 13.09
	Low <i>i</i> Cu (NM)	52298.84	± 5489.48	2136.82	± 152.38	69.12	± 6.24
	Low <i>i</i> Cu (M)	57398.47	± 213.21	2449.29	± 119.07	83.89	± 11.24
	High <i>i</i> Cu (NM)	55576.99	± 3502.93	2726.28	± 398.71	83.39	± 12.66
	High <i>i</i> Cu (M)	52359.01	± 10949.69	2450.16	± 809.30	84.90	± 36.92
Root	Control (NM)	46010.07	± 3337.49	3445.38	± 184.64	90.10	± 19.66
	Control (M)	48490.53	± 8817.27	3676.31	± 603.92	102.43	± 7.16
	Low <i>n</i> Cu (NM)	48448.99	± 4384.85	3402.53	± 134.40	70.71	± 13.61
	Low <i>n</i> Cu (M)	52241.50	± 1070.75	3629.02	± 162.44	93.98	± 7.93
	High <i>n</i> Cu (NM)	53762.13	± 1349.50	3375.57	± 656.04	70.12	± 7.66
	High <i>n</i> Cu (M)	54840.68	± 2988.24	3832.22	± 102.58	82.42	± 5.78
	Low <i>b</i> Cu (NM)	53001.61	± 3105.67	3645.91	± 339.94	80.18	± 17.17
	Low <i>b</i> Cu (M)	37117.19	± 32043.45	2222.08	± 1925.31	59.24	± 51.48
	High <i>b</i> Cu (NM)	53674.58	± 5226.21	3503.91	± 458.72	79.85	± 2.97
	High <i>b</i> Cu (M)	52884.53	± 4895.53	3554.64	± 560.54	89.53	± 16.78
	Low <i>i</i> Cu (NM)	49814.12	± 4029.48	3549.83	± 336.59	74.31	± 11.14
	Low <i>i</i> Cu (M)	51987.40	± 5621.72	3824.68	± 237.77	99.15	± 10.15
	High <i>i</i> Cu (NM)	47510.33	± 4683.60	3604.69	± 240.28	71.42	± 6.67
	High <i>i</i> Cu (M)	54794.68	± 3237.07	3752.43	± 355.06	88.11	± 18.64
Soil	Control (NM)	2167.45	± 1554.29	3327.88	± 2241.90	164.24	± 113.47

Tissue	Treatment	K		Mg		Mn	
	Control (M)	2263.91	± 1976.30	3684.98	± 327.34	198.95	± 2.62
	Low <i>n</i> Cu (NM)	23.42	± 29.80	31.14	± 5.11	52.26	± 2.23
	Low <i>n</i> Cu (M)	9.27	± 4.73	46.29	± 23.86	50.47	± 2.30
	High <i>n</i> Cu (NM)	651.89	± 1121.12	32.76	± 8.90	74.59	± 42.42
	High <i>n</i> Cu (M)	778.79	± 1301.08	53.11	± 26.14	70.36	± 81.99
	Low <i>b</i> Cu (NM)	832.63	± 1311.53	43.39	± 22.85	79.46	± 46.96
	Low <i>b</i> Cu (M)	771.61	± 1288.69	34.95	± 10.33	87.13	± 62.76
	High <i>b</i> Cu (NM)	798.44	± 1361.11	25.54	± 7.11	85.71	± 61.15
	High <i>b</i> Cu (M)	1628.41	± 1338.26	57.49	± 42.85	127.33	± 63.21
	Low <i>i</i> Cu (NM)	1733.85	± 1470.77	80.52	± 43.52	119.92	± 61.22
	Low <i>i</i> Cu (M)	1381.01	± 1189.34	89.44	± 15.58	123.27	± 62.32
	High <i>i</i> Cu (NM)	5.49	± 3.38	49.88	± 42.52	34.27	± 29.44
	High <i>i</i> Cu (M)	1.70	± 2.81	45.91	± 11.70	38.15	± 31.21

Tissue	Treatment	Na		Ni		P	
Leaf	Control (NM)	0.63	± 0.07	0.63	± 0.07	23.77	± 1.69
	Control (M)	2.20	± 0.69	2.20	± 0.69	25.95	± 3.44
	Low <i>n</i> Cu (NM)	2.43	± 0.39	2.43	± 0.39	17.37	± 2.32
	Low <i>n</i> Cu (M)	1.77	± 0.37	1.77	± 0.37	22.38	± 4.91
	High <i>n</i> Cu (NM)	2.75	± 0.61	2.75	± 0.61	17.17	± 0.54
	High <i>n</i> Cu (M)	1.52	± 0.12	1.52	± 0.12	19.98	± 0.69
	Low <i>b</i> Cu (NM)	2.58	± 0.58	2.58	± 0.58	20.62	± 0.49
	Low <i>b</i> Cu (M)	1.86	± 0.30	1.86	± 0.30	25.02	± 3.57
	High <i>b</i> Cu (NM)	2.74	± 0.39	2.74	± 0.39	21.44	± 2.36
	High <i>b</i> Cu (M)	1.65	± 0.46	1.65	± 0.46	20.79	± 1.89
	Low <i>i</i> Cu (NM)	1.91	± 0.37	1.91	± 0.37	20.36	± 2.81
	Low <i>i</i> Cu (M)	1.63	± 0.27	1.63	± 0.27	24.81	± 3.31
	High <i>i</i> Cu (NM)	2.38	± 0.55	2.38	± 0.55	19.69	± 0.78
	High <i>i</i> Cu (M)	1.45	± 0.11	1.45	± 0.11	22.14	± 2.31
Shoot	Control (NM)	700.26	± 190.87	700.26	± 190.87	3956.39	± 267.03
	Control (M)	565.83	± 44.85	565.83	± 44.85	4494.48	± 143.37
	Low <i>n</i> Cu (NM)	830.44	± 18.69	830.44	± 18.69	3436.89	± 122.49
	Low <i>n</i> Cu (M)	592.32	± 76.48	592.32	± 76.48	4143.60	± 240.95
	High <i>n</i> Cu (NM)	684.27	± 97.06	684.27	± 97.06	3316.41	± 88.27
	High <i>n</i> Cu (M)	556.73	± 89.89	556.73	± 89.89	3989.72	± 140.79
	Low <i>b</i> Cu (NM)	580.72	± 78.21	580.72	± 78.21	3748.08	± 282.33
	Low <i>b</i> Cu (M)	624.76	± 61.78	624.76	± 61.78	4235.22	± 107.88
	High <i>b</i> Cu (NM)	677.18	± 95.03	677.18	± 95.03	3417.20	± 106.53
	High <i>b</i> Cu (M)	709.95	± 159.68	709.95	± 159.68	4149.30	± 67.81
	Low <i>i</i> Cu (NM)	897.83	± 107.63	897.83	± 107.63	3679.58	± 100.43
	Low <i>i</i> Cu (M)	555.37	± 100.13	555.37	± 100.13	4335.46	± 231.50
	High <i>i</i> Cu (NM)	603.62	± 35.76	603.62	± 35.76	3800.01	± 272.51
	High <i>i</i> Cu (M)	660.77	± 132.14	660.77	± 132.14	3796.53	± 832.14
Root	Control (NM)	3116.60	± 695.72	2.92	± 2.09	6330.02	± 830.88
	Control (M)	2846.81	± 520.54	1.96	± 0.38	6807.91	± 1162.32
	Low <i>n</i> Cu (NM)	3818.28	± 403.99	1.26	± 0.12	6139.07	± 508.87
	Low <i>n</i> Cu (M)	2784.65	± 397.79	1.53	± 0.35	6828.43	± 671.71
	High <i>n</i> Cu (NM)	3606.21	± 348.45	1.40	± 0.20	6887.43	± 610.80
	High <i>n</i> Cu (M)	3278.96	± 544.37	1.43	± 0.02	7196.70	± 715.45
	Low <i>b</i> Cu (NM)	3381.07	± 519.92	1.18	± 0.04	5884.88	± 798.12
	Low <i>b</i> Cu (M)	2434.21	± 2147.25	1.32	± 0.83	4697.61	± 4142.59
	High <i>b</i> Cu (NM)	3523.84	± 837.48	1.23	± 0.18	6294.80	± 1093.20
	High <i>b</i> Cu (M)	2908.53	± 252.15	1.41	± 0.28	7557.68	± 1160.51
	Low <i>i</i> Cu (NM)	3097.16	± 235.10	1.23	± 0.19	5745.12	± 247.87
	Low <i>i</i> Cu (M)	2815.93	± 383.19	1.63	± 0.09	7347.90	± 662.13
	High <i>i</i> Cu (NM)	3950.67	± 563.12	1.42	± 0.40	6614.10	± 1159.77
	High <i>i</i> Cu (M)	3095.11	± 138.88	1.27	± 0.44	6959.51	± 745.60
Soil	Control (NM)	824.82	± 618.75	5.84	± 3.93	1294.39	± 964.33

<b>Tissue</b>	<b>Treatment</b>	<b>Na</b>		<b>Ni</b>		<b>P</b>	
	Control (M)	1084.85	± 90.12	5.83	± 0.74	1624.59	± 127.84
	Low <i>n</i> Cu (NM)	11.94	± 25.71	3.20	± 0.57	10.47	± 4.35
	Low <i>n</i> Cu (M)	-1.03	± 1.85	2.53	± 0.27	10.22	± 6.41
	High <i>n</i> Cu (NM)	174.35	± 307.40	3.35	± 1.24	3.96	± 7.40
	High <i>n</i> Cu (M)	217.69	± 339.46	3.41	± 2.95	10.80	± 6.55
	Low <i>b</i> Cu (NM)	244.01	± 373.93	4.43	± 0.95	3.24	± 4.60
	Low <i>b</i> Cu (M)	238.20	± 390.98	4.38	± 0.34	4.08	± 4.60
	High <i>b</i> Cu (NM)	212.41	± 369.97	3.37	± 0.96	9.49	± 6.83
	High <i>b</i> Cu (M)	450.64	± 362.84	4.98	± 0.46	23.31	± 22.93
	Low <i>i</i> Cu (NM)	473.20	± 379.76	4.55	± 0.21	89.71	± 90.73
	Low <i>i</i> Cu (M)	435.09	± 377.91	4.95	± 3.26	84.65	± 67.53
	High <i>i</i> Cu (NM)	-3.06	± 1.36	2.12	± 1.73	5.49	± 9.74
	High <i>i</i> Cu (M)	-2.37	± 1.95	2.39	± 1.57	12.41	± 7.56

<b>Tissues</b>	<b>Treatment</b>	<b>Si</b>		<b>Zn</b>	
Leaf	Control (NM)	1.39	± 0.08	0.55	± 0.09
	Control (M)	1.94	± 0.40	0.53	± 0.14
	Low <i>n</i> Cu (NM)	1.57	± 0.14	0.38	± 0.08
	Low <i>n</i> Cu (M)	1.43	± 0.12	0.41	± 0.07
	High <i>n</i> Cu (NM)	1.83	± 0.17	0.34	± 0.01
	High <i>n</i> Cu (M)	1.50	± 0.11	0.47	± 0.06
	Low <i>b</i> Cu (NM)	1.67	± 0.11	0.40	± 0.03
	Low <i>b</i> Cu (M)	1.62	± 0.19	0.44	± 0.03
	High <i>b</i> Cu (NM)	1.46	± 0.19	0.43	± 0.01
	High <i>b</i> Cu (M)	1.39	± 0.06	0.38	± 0.04
	Low <i>i</i> Cu (NM)	1.38	± 0.18	0.42	± 0.03
	Low <i>i</i> Cu (M)	1.62	± 0.24	0.52	± 0.12
	High <i>i</i> Cu (NM)	1.26	± 0.10	0.36	± 0.03
	High <i>i</i> Cu (M)	1.35	± 0.16	0.46	± 0.00
Shoot	Control (NM)	143.09	± 17.86	60.35	± 6.02
	Control (M)	157.13	± 16.15	72.30	± 5.96
	Low <i>n</i> Cu (NM)	149.37	± 10.48	48.94	± 3.53
	Low <i>n</i> Cu (M)	144.16	± 8.81	66.91	± 8.35
	High <i>n</i> Cu (NM)	123.58	± 3.83	48.21	± 1.80
	High <i>n</i> Cu (M)	144.49	± 11.08	69.14	± 14.34
	Low <i>b</i> Cu (NM)	128.10	± 17.75	49.11	± 6.47
	Low <i>b</i> Cu (M)	137.55	± 1.21	67.84	± 9.10
	High <i>b</i> Cu (NM)	125.90	± 5.95	54.42	± 10.04
	High <i>b</i> Cu (M)	148.35	± 11.82	61.88	± 7.77
	Low <i>i</i> Cu (NM)	124.04	± 16.07	51.85	± 2.02
	Low <i>i</i> Cu (M)	153.98	± 22.89	58.29	± 4.15
	High <i>i</i> Cu (NM)	144.98	± 22.34	49.43	± 1.51
	High <i>i</i> Cu (M)	143.74	± 48.84	49.84	± 9.50
Root	Control (NM)	480.95	± 332.91	86.12	± 16.34
	Control (M)	525.77	± 191.50	97.52	± 20.29
	Low <i>n</i> Cu (NM)	252.93	± 39.87	64.18	± 8.61
	Low <i>n</i> Cu (M)	438.52	± 141.76	81.96	± 18.06
	High <i>n</i> Cu (NM)	317.20	± 51.62	61.07	± 5.87
	High <i>n</i> Cu (M)	390.31	± 60.83	72.74	± 3.34
	Low <i>b</i> Cu (NM)	269.00	± 43.47	59.04	± 8.87
	Low <i>b</i> Cu (M)	283.24	± 249.82	50.27	± 45.98
	High <i>b</i> Cu (NM)	256.02	± 52.07	64.30	± 9.23
	High <i>b</i> Cu (M)	439.63	± 160.99	75.10	± 17.48
	Low <i>i</i> Cu (NM)	243.83	± 2.96	50.50	± 10.66
	Low <i>i</i> Cu (M)	506.68	± 83.00	65.03	± 11.52
	High <i>i</i> Cu (NM)	303.77	± 92.25	52.84	± 3.28
	High <i>i</i> Cu (M)	426.61	± 101.38	67.43	± 2.86
Soil	Control (NM)	3614.85	± 2454.53	19.38	± 13.81



Tissues	Treatment	Si		Zn	
	Control (M)	4825.24	± 404.22	23.33	± 2.78
	Low <i>n</i> Cu (NM)	309.84	± 20.01	12.29	± 2.55
	Low <i>n</i> Cu (M)	317.77	± 29.05	9.39	± 1.47
	High <i>n</i> Cu (NM)	1194.59	± 1526.16	12.70	± 4.65
	High <i>n</i> Cu (M)	1251.10	± 1924.52	12.46	± 10.47
	Low <i>b</i> Cu (NM)	1290.01	± 1719.60	16.61	± 1.28
	Low <i>b</i> Cu (M)	1397.78	± 1921.35	16.96	± 2.09
	High <i>b</i> Cu (NM)	1300.48	± 1733.15	14.31	± 4.32
	High <i>b</i> Cu (M)	2385.65	± 1801.53	18.53	± 1.43
	Low <i>i</i> Cu (NM)	2339.91	± 1785.21	18.58	± 2.15
	Low <i>i</i> Cu (M)	2387.00	± 1762.62	16.43	± 6.77
	High <i>i</i> Cu (NM)	205.96	± 170.10	8.52	± 6.67
	High <i>i</i> Cu (M)	229.59	± 170.27	9.23	± 7.06

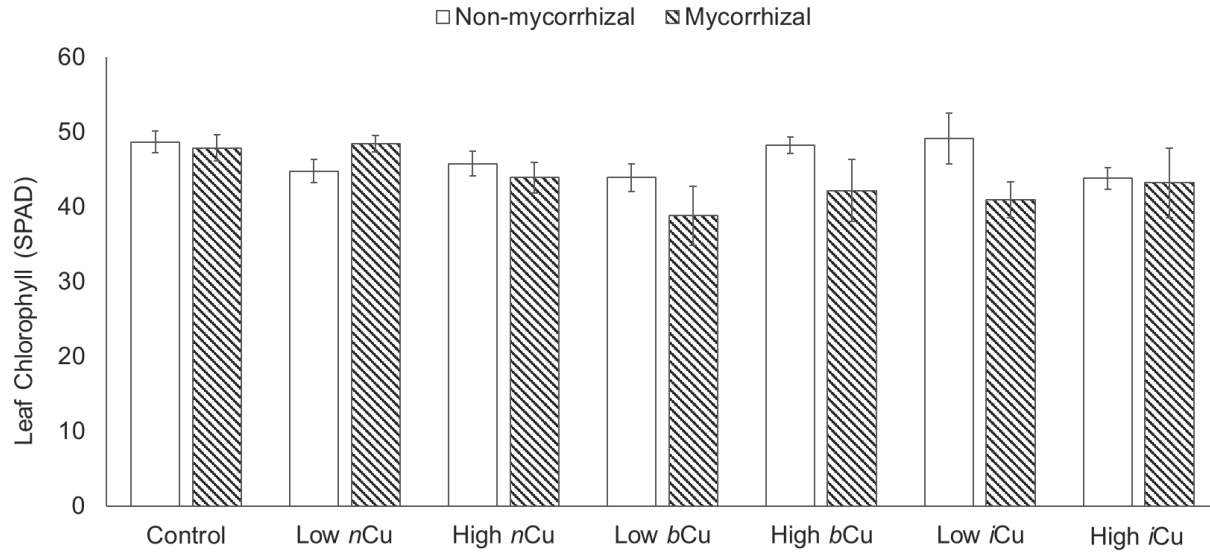


Figure S4. Leaf chlorophyll content (SPAD) of non-mycorrhizal (clear bars) and mycorrhizal (striped bars) spearmint plants sprayed with high and low concentrations of *nCu*, *bCu*, and *iCu* that were grown in soil for 50 days. Data are means of three replicates  $\pm$  SE ( $n = 3$ ). Different letters represent statistically significant differences, and asterisks (\*) indicate deviations from the respective control ( $p \leq 0.05$ ).

## REFERENCES

- Barrios, A. C., Rico, C. M., Trujillo-Reyes, J., Medina-Velo, I. a, Peralta-Videa, J. R., & Gardea-Torresdey, J. L. (2015). Effects of uncoated and citric acid coated cerium oxide nanoparticles, bulk cerium oxide, cerium acetate, and citric acid on tomato plants. *Science of The Total Environment*, 563–564, 956–964.  
<https://doi.org/http://dx.doi.org/10.1016/j.scitotenv.2015.11.143>
- Cota-Ruiz, K., Ye, Y., Valdes, C., Deng, C., Wang, Y., Hernández-Viezcas, J. A., Duarte-Gardea, M., & Gardea-Torresdey, J. L. (2020). Copper nanowires as nanofertilizers for alfalfa plants: Understanding nano-bio systems interactions from microbial genomics, plant molecular responses and spectroscopic studies. *Science of the Total Environment*, 742.  
<https://doi.org/10.1016/j.scitotenv.2020.140572>

## Vita

Suzanne Annette Apodaca was born in Chesapeake, Virginia. She earned her Bachelor of Science degree in Environmental Science from the University of Texas at El Paso (UTEP) in Fall 2013. During her undergraduate studies, in Summer 2012, she was an intern at Miller Electric for environmental health and safety. She conducted research from Fall 2012-2013 at UTEP with Dr. Wen-Yee Lee developing a method to detect Bisphenol A in milk using stir-bar sportive extraction coupled with gas chromatography-mass spectrometry. She also held an internship at Lawrence Berkeley National Lab in Spring 2014.

Suzanne earned her Master of Science degree in Environmental Science from UTEP in Fall 2016, under Dr. Jorge Gardea-Torresdey, with her thesis “Modulation of the physiological and biochemical effects of copper nanoparticles in kidney beans (*Phaseouls vulgaris*) treated by kinetin”. She went on to pursue her Doctor of Philosophy degree in Environmental Science and Engineering under the continued mentorship of Dr. Jorge Gardea-Torresdey. During her graduate studies, she was a Teaching Assistant for introductory environmental science and chemistry courses, and served on the executive committee (2017-2019) of the Graduate Student Assembly. At the time of this writing she has three first-author publications and two co-author publications.

Beginning in Summer 2018, Suzanne began working at the Texas Commission on Environmental Quality (TCEQ) as an intern/contractor. She was hired as a full-time Environmental Investigator by TCEQ in March 2019. In November 2019, Suzanne relocated to Dallas, Texas and joined the Environmental Protection Agency (EPA) as a Physical Scientist. She currently serves as the technical lead for ozone, nitrogen oxides, carbon monoxide, and volatile organic compounds in the ambient air monitoring group.

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