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Insights Into The Interaction Of Copper Oxide Nanopar-Ticles With Sweetpotato (ipomoea Batatas (l.) Lam.): The Role Of Lignin On Copper Uptake And Translocation

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INSIGHTS INTO THE INTERACTION OF COPPER OXIDE NANOPARTICLES WITH SWEETPOTATO (*IPOMOEA BATATAS* (L.) LAM.): THE ROLE OF LIGNIN ON COPPER UPTAKE AND TRANSLOCATION

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Dedication

I dedicated this great work

TO GOD

Sacred Heart of Jesus

for His blessings,

and especially

TO MY WIFE Fanny del Rosario Montenegro,

TO MY DAUGTHERS Elia Marina and Fanny Yahoska

TO MY SON Nestor Jesus,

TO MY FAMILIES; BONILLA-BIRD and MONTENEGRO-VALENZUELA

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Abstract

Sweetpotato [*Ipomoea batatas* (L.) Lam.] is widely cultivated in many countries worldwide. The world production of sweetpotato is around 127 billion tons and it is ranked seventh in the world as a staple food. After harvesting, sweetpotato storage roots are treated with fungicides to prolong shelf life. Lignin plays an important role in the conservation of the storage roots. One of the practices to activate the lignification pathway is called "curing process." The roots are placed under 80% relative humidity at a temperature of 29 ºC for a period of 7 to 15 days, followed by a cooling period at 10 - 18 ºC. Until recently, storage roots were protected against pathogens by using the fungicide dicloran, but foods with dicloran residues are no longer accepted in many markets including infant foods, organic foods, and exports. Thus, other fungicides, including copper, have been used. Thus far, only ionic copper compounds had been utilized to protect the plants against the fungus diseases in crop fields; therefore, new copper products—including the nano forms—may have great potential to be used to preserve the quality of the plants in open fields and the roots in shelves. Currently, there is a lack of information concerning the interaction of copper-based engineering nanomaterials (ENMs) with the skin components of the roots, the lignin content, and the interaction with the physiological process like chlorophyll content, photosynthesis, and carbohydrates production. The objectives of this study were: (1) to determine the effects of curing and lignin content of the periderm in the retention of copper ions/particles, and (2) to study the physiological effects of copper oxide (CuO) nanoparticles or compounds in sweetpotato plant growth and production. This study was performed in two phases. In phase I, commercial roots of Beauregard-14, low lignin content) and Covington (COV, high lignin content) were exposed to CuO nanoparticles (nCuO), bulk CuO (bCuO) and copper chloride ($CuCl_2$) at the concentration of 0-125 mg/L, before and after curing. After treatment, root tissues were analyzed by inductively

coupled plasma-optical emission spectroscopy (ICP-OES) or scanned with a two-photon microscope to study the possible penetration of Cu particles into the storage root tissues. At 25 mg/L, only bCuO showed higher Cu concentration in the periderm and cortex of B -14 (2,049 mg/kg and 76 mg/kg before curing; 6,769 mg/kg and 354 mg/kg after curing, respectively) and in cortex of COV (692 mg/kg before curing and 110 mg/kg after curing), compared with controls ($p \leq 0.05$). In medulla, the most internal tissue, only B-14 exposed to 125 mg/L bCuO showed significantly ($p \leq 0.05$) more Cu before curing (17 mg/kg) and after curing (28 mg/kg), compared with control ($p \leq 0.05$).

In Phase II, slips from the two varieties were planted in plastic pots containing 3 kg of soil each amended with the three copper compounds at 25, 75, and 125 mg/kg and cultivated under full sun exposure. Gas exchange parameters, root production, and nutritional composition of the roots were evaluated at the physiological maturity of the plants. After harvesting, roots were classified by size and analyzed for nutrient element contents or changes in macromolecules.

The interaction of copper compounds with photosynthesis showed that the higher variance was due to differences in lignin content between varieties. The ICP-OES data showed no increase of Cu in medulla, except for B-14 plants exposed to $CuCl_2$ at 125 mg/kg, which had 622% more Cu in medulla, compared with control ($p \leq 0.05$). The fresh weight of B-14 storage roots was higher in plants exposed to bCuO at 75 and 125 mg/kg, compared with nCuO at 125 mg/kg, but there were no differences with control. The root length was increased by nCuO at 25 mg/kg in COV, compared with the other treatments.

None of the treatments affected the protein content in storage roots. The sugar content was significantly increased by 75 mg/kg nCuO by 140%, respect to the control ($p \leq 0.05$). Starch was reduced by 71% in B-14 and by 80% in COV roots exposed to bCuO at 25 mg/kg). Additionally,

at 125 mg/kg, bCuO and $CuCl_2$ showed a reduced number of starch grains per sample, and most of them were misshaped. The prospective use of nCuO during root curing, plus the increase in sugar and root length on soil grown plants suggest that nCuO may represent a good alternative to protect sweetpotato plants during cultivation and increase the shelf life of the roots.

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Chapter 1. Introduction

1.1 COPPER-BASED ENGINEERED NANOMATERIALS IN AGRICULTURE

Nanotechnology is the newest field for the development of applied sciences, a level where the chemical elements have other properties in relation to their homologues (Whitesides. 2005; Roberts and Reigart 2013). Engineered nanomaterials (ENMs), the core of nanotechnology, are currently produced at high volume. Estimates indicate that the nanotechnology market will increase to $90.5 billion for the year 2021, while it was about $39.2 billion in 2016. Although Cu-based ENMs are not the most produced (200 ton/year in 2010; Keller et al. 2013), they are widely used in electricity, automobiles, and at various stages in the agricultural industry, among others (Peralta-Videa et al. 2016). The use of ENMs in agriculture is increasing. However, studies on the toxicological effects of the nanoparticles in higher plants and their transport in terrestrial ecosystems are limited (Peralta-Videa et al. 2011b). Studies have been made on the stress in the aerial part of plants caused by nanoparticles in the form of oxides and their aggregate forms (Chichiriccò et al. 2015). Stress due to copper nanoparticles was observed in stems and leaves of cilantro plants (Zuverza-Mena et al. 2015). In addition, Hong et al. (2015), found stress in leaves and stems of lettuce and alfalfa due the application of nano copper oxide (nCuO) and bulk copper oxide (bCuO). The toxicity of the nCuO in the green pea plant and their effects on the inhibition of the macro and micro elements have also been observed (Ochoa et al. 2017). In a research done in bell pepper (*Capsicum annuum* L.), it was reported that in soil amended with nCuO, bCuO, and ionic copper ($CuCl_2$) at 125, 250, and 500 mg/kg no significant affect were observed in stem development, plant biomass production, leaf chlorophyll content and fruit yield (Rawat et al. 2018). However, for the sweetpotato plant [*Ipomoea batatas* (L.) Lam.], similar research is scarce. The sweetpotato crop is widely grown in many tropical and subtropical countries (Larson et al.

2014). It ranks seventh in the world staple food production, and fifth for developing countries. The world production is around 105 million tons per year (International Potato Center 2015). The US production was around 23.8 million tons for the year 2016 (USDA-ERS 2017).

The sources of water are scarce, the groundwater quality is decaying, and no one can ensure the quality of the ground water. However, farmers are using wastewater for irrigation purposes with unknown impact on the crop, due the nanomaterials present in wastewater (Brar et al. 2010). It has been reported that farmers are using wastewater as an alternative to irrigate sweetpotato plants and to supply nutrients (Cao and Hu 2000; Antonious et al. 2011b). This suggest that sweetpotato roots could be exposed to different nanomaterials, including nCuO. Per the US Environmental Protection Agency (EPA) the sewage sludge could contain up to 4,300 mg Cu/ kg (EPA 1994).

In a recent review article, Du et al. (2017) mentioned that nanoparticles have two pathways for the uptake and translocation in plant tissues: (1) taken up by the stomata and transported downwards to the roots via phloem or (2) taken up by the periderm (apoplastic pathway) through the endodermis (symplastic pathway), and transported upwards toward the shoot meristems via xylem (Lin et al. 2009).The sweetpotato plants have different types of roots: storage, pencil, fibrous, and string roots (Huaman 1992). The storage roots, which are wrongly called "tubers", are subterranean organs that contain starch and sugars into the parenchyma cells located into the medulla (Edmunds et al. 2003). The periderm, the outermost tissue of the storage roots, is formed by three different tissues: the phellen, phellogen, and phelloderm (Figure 1). The periderm is the first barrier to stop water and nutrient loss. The periderm reaches its mature stage by lignification of the cell walls. Curing is a process implemented after root harvest that enhances the lignification of the periderm. Basically, the storage roots are placed under 80% relative humidity

at a temperature of 29 ºC for a period of 7 to 15 days, followed by a cooling period at 10 - 18 ºC (Lulai et al. 2001).

The periderm is the point of contact with the soil solution (York et al. 2016) and the pathogens like *Ceratocystus fimbriata* (Ellis and Halsted) Elliot and *Fusarium* spp that can survive 1-2 years in the soil (Sikora and Dangler 1995). Farmers use several fungicides to control fungi in crops (Loland and Singh 2004). For sweetpotato the most used products were dicloran (botran), azoxystrobin, thiabendazole (mefect), and fludioxonil. Botran was the most predominant fungicide used by farmers in the last 50 years (Christopher 2017). However, dicloran is not allowed in several products, which opened a door for the use of other fungicides. In addition, copper fungicides are recommended to be used in combination with biological control in organic production. This suggests that nCuO could have a great potential in sweetpotato production. So far copper-based nanoparticles have been used as fungicides to control fungi like *Phoma destructive*, *Curvularia lunata*, *Alternaria alternate*, and *Fusarium oxysporum*. The Cu-based nanoparticles were compared with commercial fungicides, such Kocide, used to control *Phytophthora infestans* and *Fusarium oxysporum* in tomato. It was observed that nCuO was more effective than the above mentioned commercial agrochemicals (Giannousi et al. 2013).

Despite papers describing the effects of Cu-based nanomaterials in plants, scarce information was found regarding sweetpotato. Bradfield et al. (2016) exposed sweetpotato plants to a series of copper products including nCuO. They found that ionic copper was the most toxic. In addition, they determined the Cu content in all root tissues but did not determine the effects of root layers in copper retention.

1.2 INTERACTION OF COPPER COMPOUNDS WITH LIGNIN

A few studies have shown the interaction of Cu with lignin. Ali et al. (2006) reported a research done with *Panax ginseng* roots with $CuSO_4$. The excess of Cu^{2+} affected the root growth and increased lignin content at 25 and 50 µM. In *Arabidopsis thaliana*, Cu^{2+} at 50 µM reduced the growth of the main root, increased the density of the lateral roots, along with lignin deposition in roots (Lequeux et al. 2010a). In addition, in radish (*Raphanus sativus*), Cu^{2+} at 1 µM increased the lignin and enzymes like peroxidases in seedling roots (Chen et al. 2002). The mechanisms of lignin increase, under Cu stress, is not well understood. Huang et al. (2018) suggest that the attraction would be attributed to ion exchange, surface adsorption, and complexation by lignin.

This research was performed in two phases:

Phase I. In this phase, the interaction of nCuO, bCuO, and $CuCl_2$ with lignin, during curing process, was investigated.

Phase II. In this phase, the soil was amended with the same products and the effects on plant growth and production were studied.

The objectives of this study were:

To determine the effects of curing process and lignin content in the periderm on the retention of Cu ions/particles.

To study the physiological effects of three types of copper in sweetpotato plant growth and production.

To determine the effects the three copper products in the nutritional components of storage roots.

Hypotheses

These studies were performed under the working hypotheses:

1. The lignin content modulates the retention of Cu storage roots.

2. The reaction of particulate materials with the lignin differs, compared to the ionic compound.
3. The nCuO improves the production of sweetpotato.

The research study was seeking to answer the following research questions:

1. Does lignin retain Cu in the surface of storage roots during curing?
2. Does the ionic compound induce phytotoxicity in sweetpotato plants?
3. Does particle size affect the interaction of CuO with the sweetpotato plants?

Chapter 2. Two-photon Microscopy and Spectroscopy Studies to Determine the Mechanism of Copper Oxide Nanoparticle Uptake by Sweetpotato Roots During Post-Harvest Treatment[1]

2.1 INTRODUCTION

The literature indicates a rapid increase in the use of nanomaterials (NMs) in agriculture industry. Currently, 9% of the nanoproducts targeted for agri/feed/food application are used in agriculture (Iavicoli et al 2017) Moreover, there are several NMs in development for application as nanopesticides or nanofertilizers. Nano-compounds of zinc, iron, and copper have been shown to have great potential as nanofertilizers.(Monreal et al. 2016) In addition, copper-based NMs, whose global production for 2010 was estimated at 200 tons,(Shelah et al. 2015) may have additional applications, since Cu has been approved as a pesticide in organic agriculture.(Cabús et al. 2017) The fast development of agriculture-oriented nanoproducts would reduce the amount of Cu used in the production and shelf preservation of several types of produce. So far, several Cu-based nanoparticles (NPs) have been tested to control fungus and bacteria, with promising results. CuO NPs, (nCuO), have proven to be efficacious of controlling coliform bacteria in ultra-filtrated cheese.(Dizaj et al. 2014) It was also reported that Cu-based NPs are able to control fungi at field level.(Giannousi et al. 2017) These researchers reported that nCuO, at 150 mg/l (15 g/hl), controlled about 60% of *Phytophthora infestans* in field grown tomato (*Solanum lycopersicum*) (Giannousi et al. 2017).

[1] Reprinted from Bonilla-Bird, N. J., A. Paez, A. Reyes, J. A. Hernandez-Viezcas, C. Li, J. R. Peralta-Videa, and J. L. Gardea-Torresdey. 2018. "Two-Photon Microscopy and Spectroscopy Studies to Determine the Mechanism of Copper Oxide Nanoparticle Uptake by Sweetpotato Roots during Postharvest Treatment." *Environmental Science and Technology* 52 (17): 9954–63. doi:10.1021/acs.est.8b02794.

Sweetpotato (*Ipomoea batatas* L. Lam.) ranks seventh in the world in staple food production and fifth in developing countries. The world production is around 105 million tons/year, while the production in the US is around 23.8 billion tons/year (FAO 2016). Sweetpotato plants have different types of roots including storage, pencil, fibrous, and string roots (Huaman 1992). Storage roots, the edible portion of sweet potato plants, are carefully cured and stored before they are exposed to the market. Curing implies the storage of roots under special temperature and ventilation for three to five days right after harvesting.(Edmunds et al. 2003) During the curing, the outermost parenchyma cells wounded at harvest desiccate, while the internal parenchyma cells suberize.(Walter et al. 1983) These changes heal wounds and protect the roots from future microbial infections throughout the storage period. Improper handling during a long curing period (one to four months)(Ray & Ravi 2005) may lead to a loss of up to 65% due to a reduction of freshness or fungal infestation. Several fungicides have been used to control sweetpotato fungal root rots, dicloran being the most used. However, foods with dicloran residues are no longer accepted in many markets including infant food, organic, and export (Brooke et al. 2009) Thus, other fungicides, including Cu have been tested (Eckert et al. 1988). However, until now, only ionic Cu has been utilized; thus, new Cu products, including nano forms, may have great potential in preserving the quality of sweet potato roots, before they reach the market.

To the authors' knowledge, only one publication has described the interaction of metal oxide NPs with sweetpotato.(Bradfield et al. 2016) These researchers cultivated sweetpotato slips in soil amended with either CeO_2, CuO, or ZnO NP (100, 500, or 1000 mg/kg of the respective metal) and evaluated treatment effects on storage root production, metal concentration, and possible dietary intake of Ce, Cu, or Zn. The authors reported that the peel retained more Ce than

Cu or Zn (Giannousi et al. 2013). However, there is no information about the role of the peel components on the retention mechanism.

Confocal microscopy has been used to observe the root uptake of nanoparticles.(WZhenyu et al. 2012). However, this technique requires the staining of non-fluorescent NPs. The Two-photon microscopy (TPM) offers the advantage of observing the uptake of NPs without staining. The capacity of TPM to obtain high depth of field images is greater than that of confocal microscopy. However, this is limited by the thickness of the samples(Feijó et al. 2004) because the quality of the image depends on the scattering and the absorption coefficients of the tissue, the type of fluorophore, and the objective lenses.(Nishikawa et al. 1997) CuO NPs may be good candidate to treat sweetpotato roots while they are cured. However, there is a lack of information concerning the penetration of the particles into the edible portion of the roots. It is hypothesized that the peel constitution will affect the retention of the NPs. Thus, the aim of this study was to determine the role of peel tissues and the curing process on the retention of CuO NPs and the possible penetration of NPs into the root.

Variation in the constitution of sweetpotato root peel may have differential effects on the interaction with metal oxide NPs. Thus, the aim of this study was to determine the role of peel tissues and the curing process on the retention of CuO NPs and the possible penetration of NPs into the root. The roots of two sweetpotato varieties, with different lignin content, were exposed to CuO NPs, bulk CuO, and $CuCl_2$. Subsequently, the samples of roots were screened with a two-photon microscopy and analyzed with inductively coupled plasma- optical emission spectroscopy to determine the retention of CuO and the absorption of Cu. Two-photon microscope has been used to track cell mobility in zebrafish (Iavicoli et al. 2017). 2-photon microscope, combined with

nanoparticle-assisted near-infrared has been used for *in vivo* image of fluorescent nanoparticles in mice ear. (Monreal et al. 2016).

To the authors' knowledge, this is the first time that a two-photon excited fluorescence and second-harmonic generation (SHG) microscope has been used to image metallic particles into the edible tissue.

2.2 MATERIALS AND METHODS

2.2.1 Characteristics of the Cu products and suspension/solution preparations

Characteristics of the Cu products and suspension/solution preparations. Commercial nano CuO (nCuO), bulk copper oxide, (bCuO), and reagent grade salt $CuCl_2$ (Sigma Aldrich, St. Louis, MO) were used in this research. These materials were supplied by the UC Center for Environmental Implications of Nanotechnology. The physicochemical properties of both the nCuO and bCuO were previously determined (Huaman et al. 1992; Iavicoli et al. 2017).

Table 2.1 shows major physicochemical properties of the particles used in this study. The nCuO had 50 nm, rhombus irregular, hydrodynamic diameter of 280 ± 15 nm and zeta potential of -34.4 ± 0.5 mV. bCuO had particles smaller than 1 µm with diverse morphology, hydrodynamic diameter of 376 ± 26 nm, and zeta potential of -42.7 ± 0.153 mV (Hong et al. 2015). Suspensions/solutions of Cu NP or compounds were prepared at 0, 25, 75, 125 mg/L in Millipore water (MPW) and homogenized by sonication in a water bath (Crest Ultrasonics, Trenton, N) at 25 ºC for 30 min and 180 watts. The 25 mg/L treatment was selected because the average Cu concentration in soil of the United States is 20 mg/kg,(Shacklette et al. 1984) while 75 mg/L was selected to mimic the highest- Cu concentration reported in agricultural soils amended with sewage

sludge (75 mg/kg) (Georgopoulos et al. 2001). The 125 mg/L treatment represents 83% of the CuO NPs used to control fungi at field level (Dizaj et al. 2014; Giannousi et al.2013).

Table 2.1. Major physicochemical properties of the particles used in this study.[1]

Property	*n*CuO	Bulk CuO	*n*Cu	Bulk Cu	Kocide 3000	CuPRO 2005
Primary particle size (nm)	$10^1 - 10^2$	$10^2 - 10^4$	$10^2 - 10^3$	$< 10^4$	$>10^4$	$10^4 - 10^6$
Hydrodynamic diameter (nm) [a]	280±15	376±26	2590±1138	4546±3940	1532±580	4779±4767
Zeta potential (mV) [a]	-34.4±0.5	-42.7±0.153±	-29.4±0.8	-35.4±1.27	-40.9±2.7	-47.8±1.1
Cu content (wt. %)	74.3	79.7	83.3	98.7	26.5	34.0
Other elements present	O, C	O	O, C	ND	C, O, Na, Al, Si, S, Cl	C, O, Na, Al, Si, P, Ca
Morphology	Rhombus, irregular	Prism, irregular	Irregular	Dendritic, plate-like, rhombus	Spherical	Spherical
Main Copper phase	CuO	CuO	Cu, Cu_2O	Cu	$Cu(OH)_2$	$Cu(OH)_2$
Crystal structure	Monoclinic	Monoclinic	Cubic	Cubic	Orthorhombic	Orthorhombic

[a]Measurement was done at pH 7

ND = Non-detect

[1]J. Hong, C. M. Rico, L. Zhao, A. S. Adeleye, A. A. Keller, J. R. Peralta-Videa, and J. L. Gardea-Torresdey, "Toxic effects of copper-based nanoparticles or compounds to lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*)," *Environ. Sci. Process. Impacts*, vol. 17, no. 1, pp. 177–185, 2015.

2.2.2 Preparation of root samples and Cu treatment applications

Storage roots of Covington and Beauregard-14 sweetpotato varieties varying in peel lignin content were used in this study. Beauregard-14 has 156 g/kg and Covington 178 g/kg lignin in the peel; they are significantly different at $p \leq 0.05$ (Bonilla-Bird 2015). Both varieties were procured from Mississippi State University, Pontotoc Experiment Station. Enough roots of both varieties were separated in two sets, one set was cured and the other one was uncured. For curing, roots were set at 29 ºC and 80% relative humidity for 10 days in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH). Uncured roots were kept in the laboratory at ~24 ºC and

47% relative humidity. At the end of the curing process, uncured and cured roots were individually tied up with a cotton thread and immersed in the Cu suspensions/solutions for 30 min under continuous stirring. The exposure time of 30 min was selected because the treatment period before storing in the curing facility is very short. Roots were kept in the laboratory for 72 h after treatment and processed for microscopy imaging and elemental analyses. Each Cu treatment had three replicates and one root was used as a replicate. Three replicate samples of uncured and cured roots were used as control (no Cu application).

2.2.3 Sample preparation for Cu determination

For element determination, roots were washed gently with Millipore water (MPW) to remove the debris adhered to the periderm of the storage roots. The washing procedure was also supposed to remove most of the CuO particles adhered to the roots surface. The roots were not washed with acid solution to avoid deterioration of the flesh tissue. After washing, the layers of tissues from each root were pulled apart with a bistouri (scalpel) in the following order: periderm, cortex, perimedulla, and medulla. Each tissue was put in a paper envelop, oven dried at 70 ºC for 72 h, and manually ground using a ceramic mortar and pestle. Dried samples of 0.2 g were acid digested in a block digestion system (DigiPREP MS, SCP Science. Canada) with three mL of 30% plasma pure HNO_3. Digests were adjusted to 50 mL with MPW and analyzed for Cu content by using an inductively coupled plasma-optical emission spectrometer (ICP-OES, Perkin-Elmer optima 4300 DV, Shelton, CT). To evaluate the digestion and analytical methods, (QC/QA), spiked samples and the standard reference material 1547 from the National Institute of Standards and Technology were treated and analyzed in a similar way as the root samples. The recovery rate was 99%.

2.2.4 Sample preparation for microscopy imaging

For microscopy observation with the 2-photon microscope, fresh transversal thin slides from plants exposed to bCuO and nCuO after curing were mounted in water-immersion objective lens (Olympus LUM Plan FLN) and observed in a nonlinear optical microscope, which can detect both 2-photon excited fluorescence. The light source is a femtosecond Ti: Sapphire laser (Spectra-Physics, Mai-Tai HP). Our experiment was set at the Mai Tai at 710 nm, 200, W. Its pulse duration was about 100 fs width at a repetition rate of 80 MHz. The wavelength of the laser was tuned from 690 to 1040 nm, with the maximum power up to 2.5 W. The fluorescence signal from the sample was deflected with the 665 nm long-pass dichroic mirror. A second long-pass dichroic beam splitter was used to separate the blue and green/red fluorescence signal. The blue signal was deflected by dichroic beam splitter and transmitted through a 417-477 nm band-pass filter. The green signal was transmitted through a 500-550 nm band-pass filter, while the red signal through a 570-616 nm band-pass filter. The outputs of the three signals were individually detected by photomultiplier tubes set at 0.9V, 2.20V, and 2.10 V for the red, green, and blue channels, respectively. The outputs of these three signals were fed into red/green/blue channels of a frame grabber installed on a computer. The images in X-Y plane were acquired through a home built software program (Acosta et al. 2014). Covington variety was selected because its higher lignin content in periderm could limit the Cu transport to cortex (Bonilla-Bird 2015).

2.2.5 Statistics analysis

The analysis of variance (ANOVA) of the interaction between the main factors (curing, varieties, treatments, and root tissues) and the Cu concentrations was performed using univariate analysis (SPSS 19.0 package, Chicago, IL). The Eta-squared (kind of t-test family similar to r^2) was used to assign the portion of variation to each one of the factors. The differences between

averages of triplicate data per treatment and control was calculated with the Tukey HSD multiple comparisons test. The data presented in tables and figures are means ± the standard error (SE).

2.3 RESULTS AND DISCUSSIONS

Figure 2.1 shows a description of the distribution of tissues in the sweetpotato storage root. As seen in this figure, the periderm has three layers of tissues, which contain lignin. Lignin is an organic polymer that increases during the curing process, which has been proven to bind metals (Ray et al. 2005).

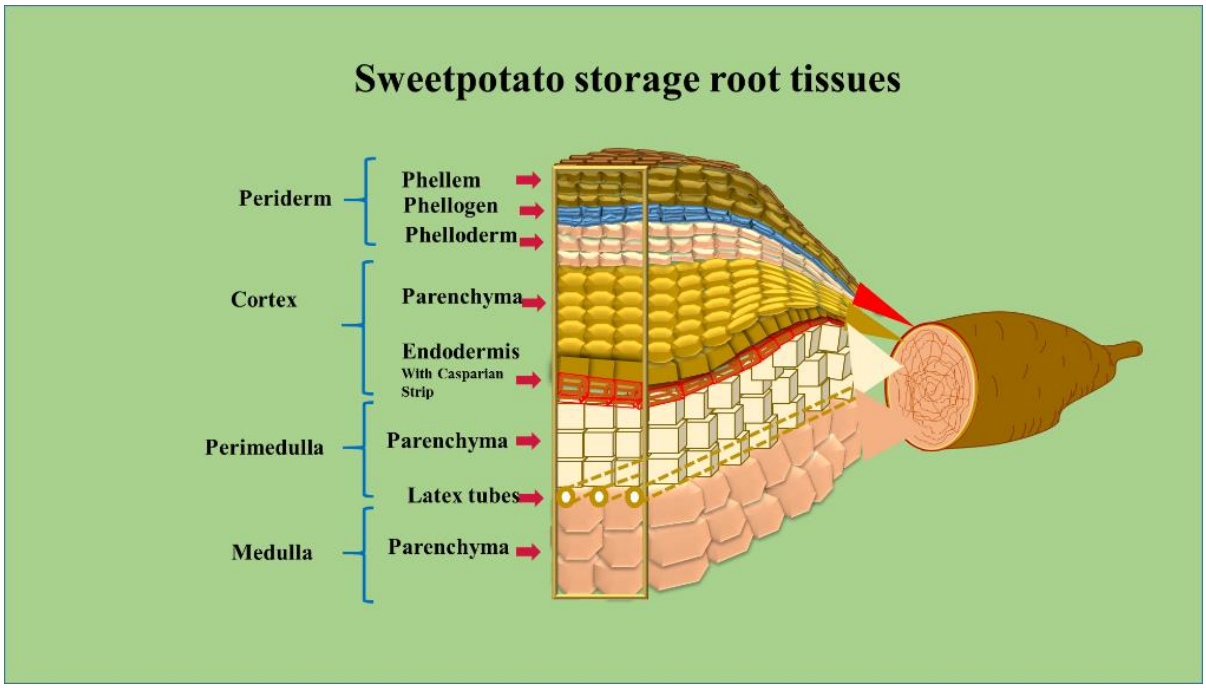

Figure 2.1. The positions of the tissues as seen in transverse section on sweetpotato storage root. The outermost the periderm, followed by cortex, perimedulla, and medulla or edible part.

2.3.1 Microscopy analysis

Figure 2.2 shows 2-photon microscopy images of the control (A) and the CuCl2 treated Covington variety roots (B). The samples were from roots exposed to the CuCl2 after curing. The figures show the periderm in the left part and the cortex in the right part. The autofluorescence shown in the control sample is due to the suberization of the cell walls, which gives firmness and resistance to excoriation to the roots (Shacklette et al, 1984). The insert in Figure 2B shows a lack of fluorescence from the $CuCl_2$ solution. Figure 2.3 shows the 2-photon images of Covington variety roots exposed after curing to 75 mg/L nCuO (A) or bCuO (B). The inserts in both figures show the fluorescence of the CuO suspensions. The areas in squares show CuO particles in the cortex tissues. The 2-photon microscope images of nano CuO and bulk CuO treated samples showed differences, compared with control. For instance, the root exposed to 75 mg/L of nCuO (Figure 2.3A and 2.4) shows fluorescent points inside of the cortex tissue, which is located below the periderm. Cell walls in the periderm show higher bright intensities than the inside cells. In the cortex tissue, fluorescence from several nanoparticles stuck in intercellular spaces were observed (arrow). Due to the fast flash moment of the excitation of the nanoparticles, only a few 2-photon fluorescences were obtained in the image. The internal part of the root cortex (Figure 2.3A) shows an increase in the number of 2-photon fluorescences from different bulk particles. Images of roots exposed to nCuO at 75 mg/L also showed 2-photon fluorescence (Figure 2.5), indicating that these particles also penetrated the tissues, the cortex tissue in (Figure 2.51-A and Figure 2.5-B). In addition Figure 2.5 (C) shows the surface graph to detect the nCuO between the cell walls. The fact that the 2-photon fluorescences were observed between the cells suggests the photochemical decomposition, which occurs when the particles are excited by the laser beam, allows their localization within the root tissues. The molecular constitution of each material has a different

reaction when they are impacted by the laser beam, creating varied ranges of fluorescence (Gerritsen et al. 1999). The signal/noise ratio for nCuO were 26/25, 21/168, and 27/122 for red, green, and blue, respectively. For bCuO were 22/27, 20/181, and 26/122 for red, green and blue, respectively.

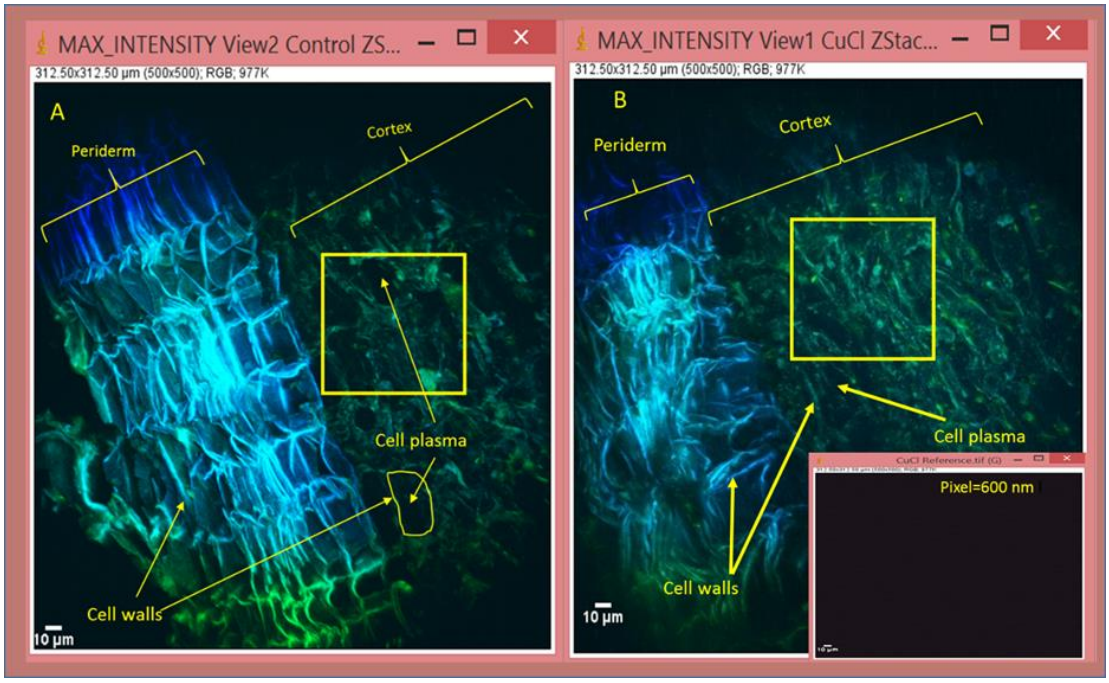

Figure 2.2. (A) Two-photon microscopy image of control root of the Covington variety showing the fluorescence of lignin in cell walls of periderm. The arrows are pointing the cell structures in the periderm and cortex. The opaque segment correspond to the cortex. (B) Two-photon microscopy image of the Covington variety root exposed after curing to 75 mg/L of copper chloride. The insert picture corresponding to the copper chloride in water, there was not observed fluorescence. The signals were obtained at a wavelength of 710 nm and a power of 200 mW.

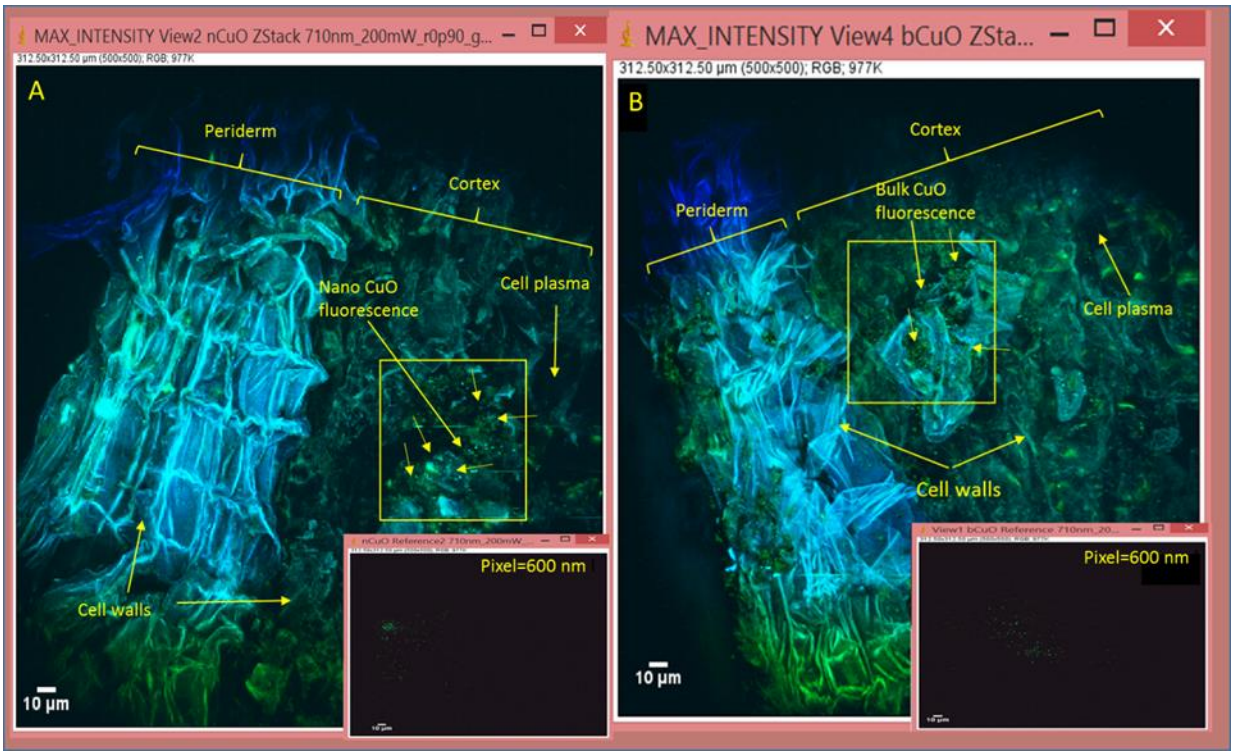

Figure 2.3. (A). Two-photon microscopy image of the Covington variety root exposed to 75 mg/L of nCuO nanoparticles showing the fluorescence of lignin in the periderm. The arrows are pointing out the fluorescence from the CuO nanoparticles inside the square in cortex. The insert corresponding to the nCuO solution in water, the fluorescence is from the nCuO particles (B) 2-photon microscopy image of the Covington variety root exposed to 75 mg/L of bCuO showing the fluorescence of lignin in the periderm and the fluorescence of the bCuO particles. The arrow is pointing out the fluorescence from bCuO particles inside the square in cortex. The insert corresponding to the bCuO solution in water, the fluorescence is from the bCuO particles. The fluorescence signals were obtained at a wavelength of 710 nm and a power of 200 mW.

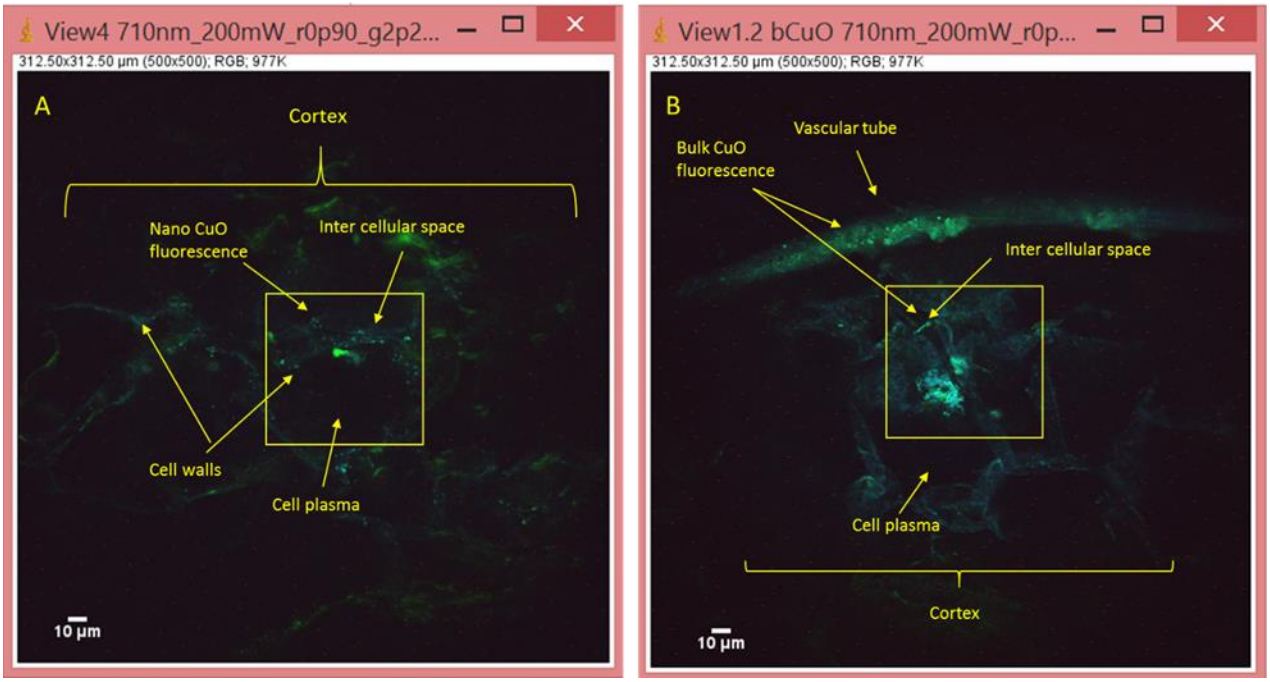

Figure 2.4. (A) Two-photon microscopy image of the Covington variety root exposed to 75 mg/L of nCuO particles showing the fluorescence (square) of the nCuO particles in the cortex tissue. The particles are located between the intercellular spaces (B) Image of the Covington variety root exposed to 75 mg/L of bCuO (square) of the bCuO particles inside of the vascular tissue. The square shows the bCuO particles between the cells walls. The signals were obtained at a wavelength of 710 nm and a power of 200 mW.

2.3.2 Effect of factors on Cu accumulation in roots

The statistical analysis of Cu concentration in roots showed effects of single factors variety, tissue, and Cu treatments (Table 2.2). In addition, the double, triple, and quadruple interactions between curing × tissue, curing × variety × Cu treatments, variety × tissue × Cu treatment, and curing × variety × tissue × Cu treatments were also statistically different. The Eta-squared statistics showed that the higher variance was due to the differences between tissues and the interaction

tissue × Cu treatment, with 50% and 18%, respectively ($p \leq 0.001$). Table 2.3, shows the Tukey test for the main factors curing, varieties, and tissues. As seen in this table, the curing, as a whole, had no statistical effects; however, there were effects for varieties and tissues. Beauregard-14, the variety with less lignin. had lower Cu concentration (720 ± 108 mg/kg), with differences statistically significant at $p \leq 0.05$, while Covington, the variety with higher lignin, had 1003 ± 148 mg/kg, with differences statistically significant at $p \leq 0.005$ (Bonilla-Bird 2015). It has been demonstrated that lignin binds metals through ion-exchange. The binding strength of Cu to lignin is one the strongest (Ray H. et al. 2002).

2.3.3 Copper concentration in individual tissues

The Cu concentration was analyzed from the outermost tissue, the periderm, to the innermost one, the medulla. Figure 2.5 shows the Cu concentration in cortex, and Figure 2.6 and 2.7 the periderm of uncured and cured storage roots of variety Beauregard-14. Before curing, at 25 mg/L, only roots exposed to bCuO had significantly more Cu, 2,049 mg/kg in the periderm (the most external layer). While at 75 and 125 mg/L, all treatments showed significantly more Cu. At 125 mg/L, the increases from bCuO and nCuO were 6,105 mg/kg and 2,635 mg/kg. After curing, similar results were observed at 25 mg/L, but not at 75 mg/L. and 125 mg/L. At 75 mg/L, there was no significant increase in Cu concentration, while at 125 mg/L, bCuO and nCuO showed 5,437 mg/kg and 4,147 mg/kg more Cu, respectively, compared with control. It is possible that the small differences in hydrodynamic diameter caused the similarity in Cu accumulation.

Table 2.2. UNIANOVA for the data of the four main components and the respective interactions in the experiment with sweetpotato roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L. (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	η^2 (b)	%
Corrected Model	1793405828.5[a]	159	11279281.9	23.35	0.00		
Intercept	356180365.0	1	356180364.9	737.43	0.00		
Curing	203209.0	1	203208.7	0.42	0.52 ns	0.00	0
Variety	9628379.6	1	9628379.7	19.93	0.00***	0.00	0
Tissue	972269596.8	3	324089865.6	670.99	0.00***	0.50	50
Treatment	126253724.5	9	14028191.6	29.04	0.00***	0.06	6
Curing * Variety	142739.9	1	142739.9	0.30	0.59 ns	0.00	0
Curing * Tissue	935420.7	3	311806.9	0.65	0.59*	0.00	0
Curing * Cu Treatment	48547153.6	9	5394128.2	11.17	0.00***	0.02	2
Variety * Tissue	28543572.4	3	9514524.1	19.70	0.00***	0.01	1
Variety * Cu Treatment	16759911.7	9	1862212.4	3.86	0.00***	0.01	1
Tissue * Cu Treatment	349163961.1	27	12931998.6	26.77	0.00***	0.18	18
Curing * Variety * Tissue	89101.2	3	29700.4	0.06	0.98 ns	0.00	0
Curing * Variety * Cu Treat	10997774.5	9	1221974.9	2.53	0.01**	0.01	1
Curing * Tissue * Cu Treatment	144141327.4	27	5338567.7	11.05	0.00***	0.07	7
Variety * Tissue * Cu Treatment	51912210.6	27	1922674.5	3.98	0.00***	0.03	3
Curing * Variety * Tissue * Cu Treatment	33817745.8	27	1252509.1	2.59	0.00***	0.02	2
Error	154560129.6	320	483000.4			0.07	7
Total	2304146323.0	480					
Corrected Total	1947965958.1	479					

*significant at p <0.05; **significant at p<0.005; *** significant at p<0.001; R Squared = .921; (Adjusted R Squared = .881); b. Eta square; % variation.

Table 2.3. UNIANOVA for the data of the four main components and the respective interactions in the experiment with sweetpotato roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L (n = 3).

Variety	Tissue	Before curing Cu (mg/kg)	After curing Cu (mg/kg)	Difference
Beauregard-14	Periderm	3823.51±1,309	3,955.90±421.5	-132.38
	Cortex	146.91±207.3	56.91±3.10	90
	Perimedulla	18.61±8.13	9.51±2.92	9.09
	Medulla	9.83±6.40	3.19±1.90	6.7*
Covington	Periderm	2635.03±211.6	2,888.40±771.12	-253.37
	Cortex	78.15±5.0	113.70±19.0	-35.52
	Perimedulla	9.32±4.35	21.40±15.83	-12.08
	Medulla	5.35±0.7	7.0±5.40	-1.57

*significant at $p \leq 0.05$.

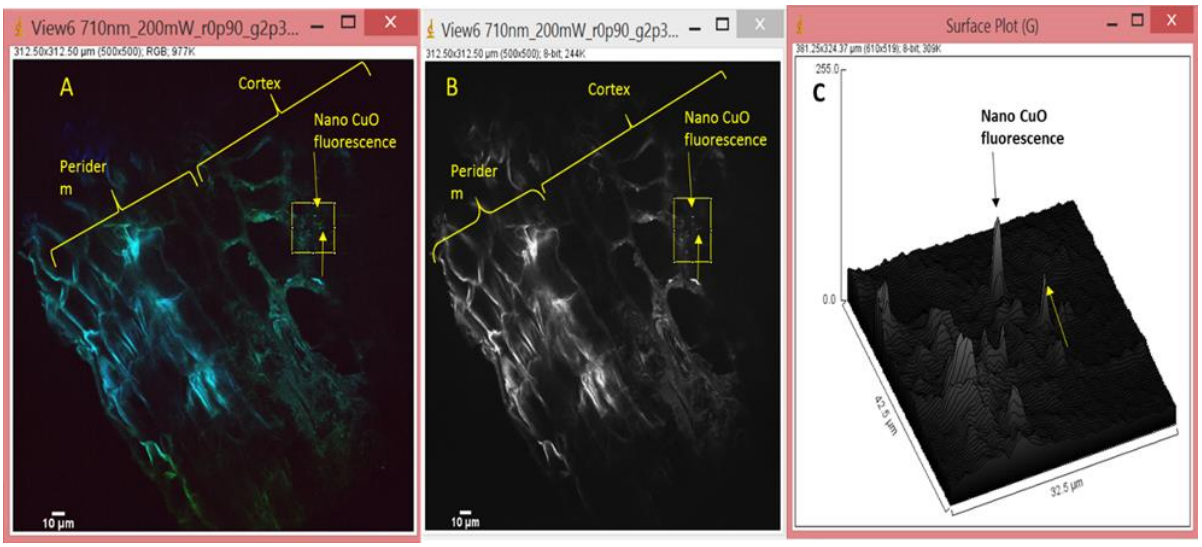

Figure 2.5. (A) - (B) Two-photon microscopy image of the Covington variety root exposed to 75 mg/L of nCuO showing the fluorescence of periderm cell walls. The arrow is pointing out at the fluorescence from several nCuO particles that penetrated into the cortex tissue. (C) Surface graphic taken at the depth of 255 µm. The arrow is indicating the localization of the nano particles. The white part correspond to the fluorescence from the cells walls of the cortex.

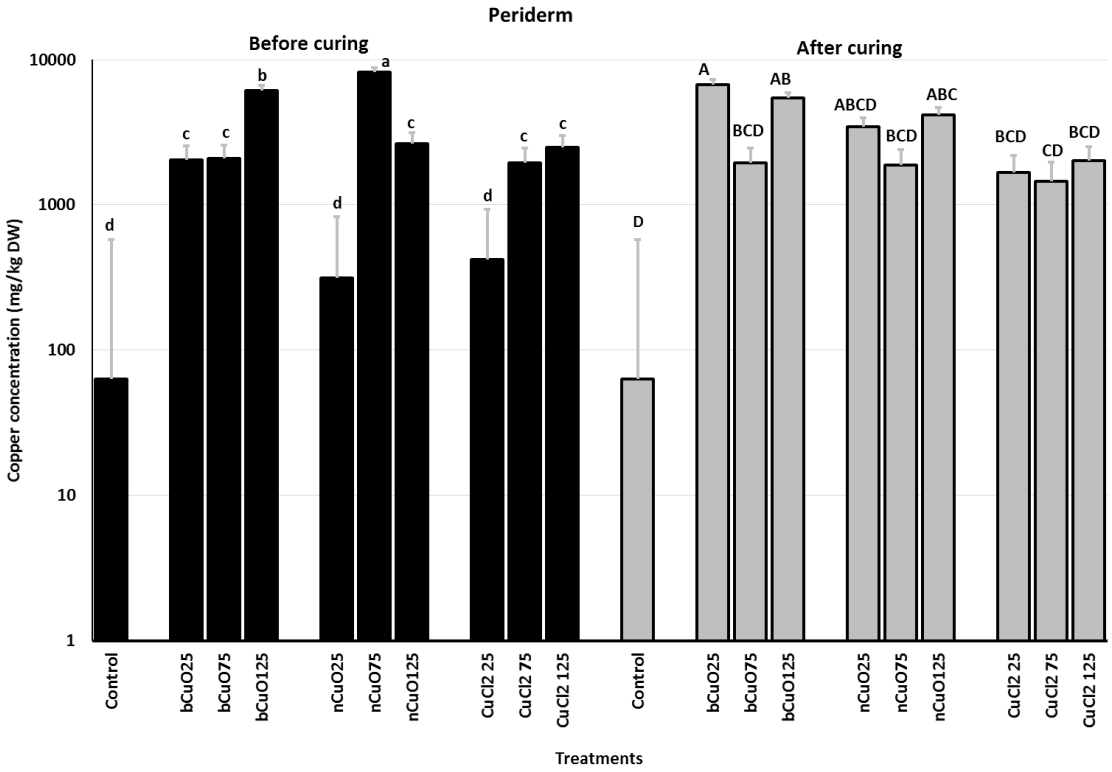

Figure 2.6. Copper concentration (mg/kg dwt) in periderm of the Beauregard-14 roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L, before and after curing. Data are average of three replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$, (n = 3).

Hong et al. (2014) demonstrated that the removal of CeO_2 NPs from cucumber leaves washed with water was lower, compared to the washing with $CaCl_2$ or HNO_3. As expected, less Cu was found in the cortex, the tissue beneath periderm (Table 2.3 and Table 2.4). As shown in Figure 2.7, variety Covington before curing, Cu concentration in cortex followed a similar trend than in periderm Figure 2.8. After curing, no effects were observed at 75 mg/L, while at 25 mg/L and 125 mg/L, bCuO increased (354 mg/kg and 144 mg/kg) and nCuO increased (142 mg/kg and 208 mg/kg) Cu in cortex ($p \leq 0.001$). The difference in Cu accumulation between uncured and

cured roots was attribute to the suberization and lignification of the internal parenchyma cells (Edmunds et al. 2010).

Table 2.4. Tukey HSD – Post Hoc test for the main factors. The sweetpotato roots were exposed to nCuO, bCuO, and CuCl2 at 0, 25, 75, and 125 mg/L (n=3).

Factors	Copper concentration (mg/kg)
1. Curing	
Before curing	841 ± 176
After curing	882 ± 186
2.Variety	
Beauregard-14	720 ± 108*
Covington	1003± 148**
3.Tissues	
Perimederm	3,326 ± 261***
Cortex	99.0 ±18**
Perimedulla	15.0±2**
Medulla	6.3±1**

*significant at p <0.05; **significant at p<0.005; *** significant at p<0.001.

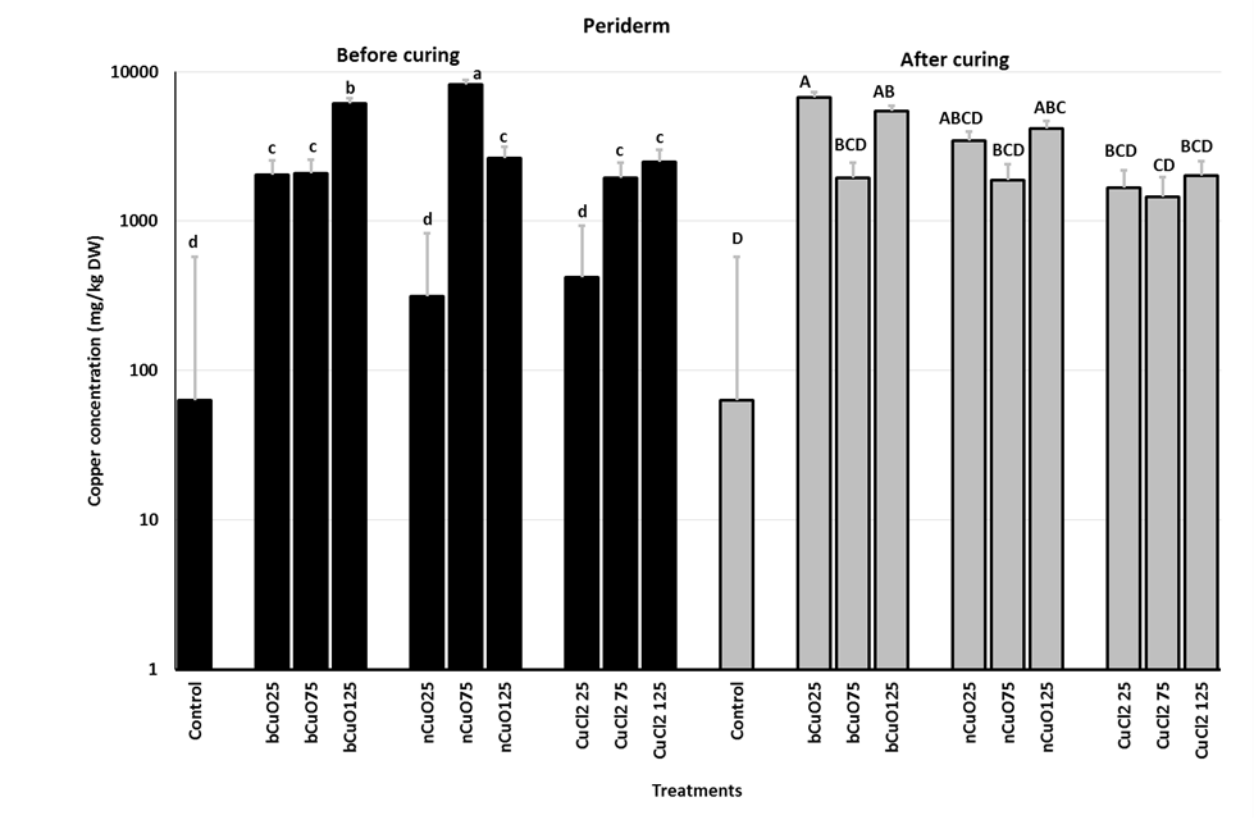

Figure 2.7. Copper concentration (mg/kg dwt) in periderm of the Covington roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L, before and after curing. Data are average of three replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$, (n = 3).

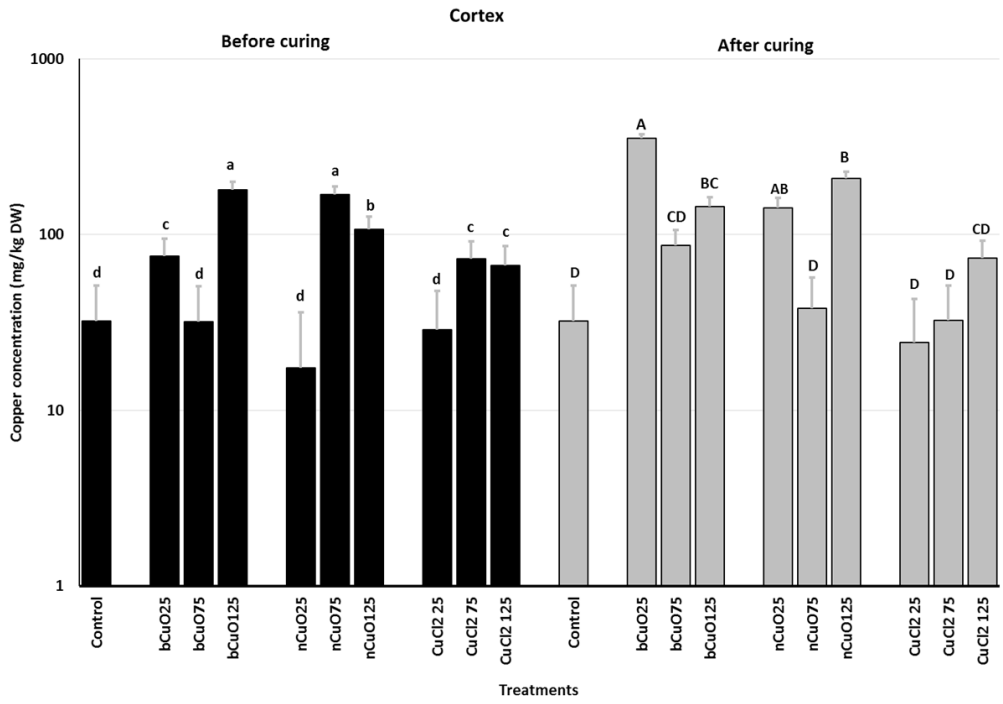

Figure 2.8. Copper concentration (mg/kg dwt) in cortex of the B-14 roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L, before and after curing. Data are average of three replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$, (n = 3).

Figure 2.9 shows data for cortex and Figure 2.10 shows data for periderm in Covington variety (higher lignin content). As seen in this figure, before curing, exposure to 25 mg/L of the three Cu-based compounds did not increase Cu in periderm, compared with control. While after curing, at 25 mg bCuO/L increased Cu in periderm by 8,311 mg/kg. In addition, before curing, at 75 mg/L, only nCuO increased Cu in periderm (7,803 mg/kg), and 125 mg/L, both bCuO and nCuO showed more Cu in periderm (7,052 mg/kg and 9,235 mg/kg). In cortex, before curing, all the treatments increased Cu. After curing, all bCuO concentrations increased Cu in periderm, and at 25 mg/L and 75 mg/L increased Cu in cortex- Figure 10- (110 mg/kg and 126 mg/kg,

respectively). On the other hand, at 75 and 125 mg/L, nCuO increased Cu in periderm (4,279 mg/kg and 7,055 mg/kg, respectively) and only at 125 mg/L, increase Cu in cortex (77 mg/kg). $CuCl_2$, at any concentrations, increased Cu in periderm and cortex, before and after curing. The fact that Cu concentration in cortex from particulate compounds were higher than in $CuCl_2$ treatments may be due to both the uptake of aggregates that contain a high proportion of Cu and to the high atomic percentage Cu in the CuO. The lack of Cu increase in uncured roots exposed to 25 mg/L could be due to the lower lignin content, (Edmunds et al. 2010), or the natural Cu accumulation from soil (Alifu et al. 2017), while after curing, the Cu increase was attributed to the increment in lignin, which occurs during the curing process (Edmunds et al. 2010).

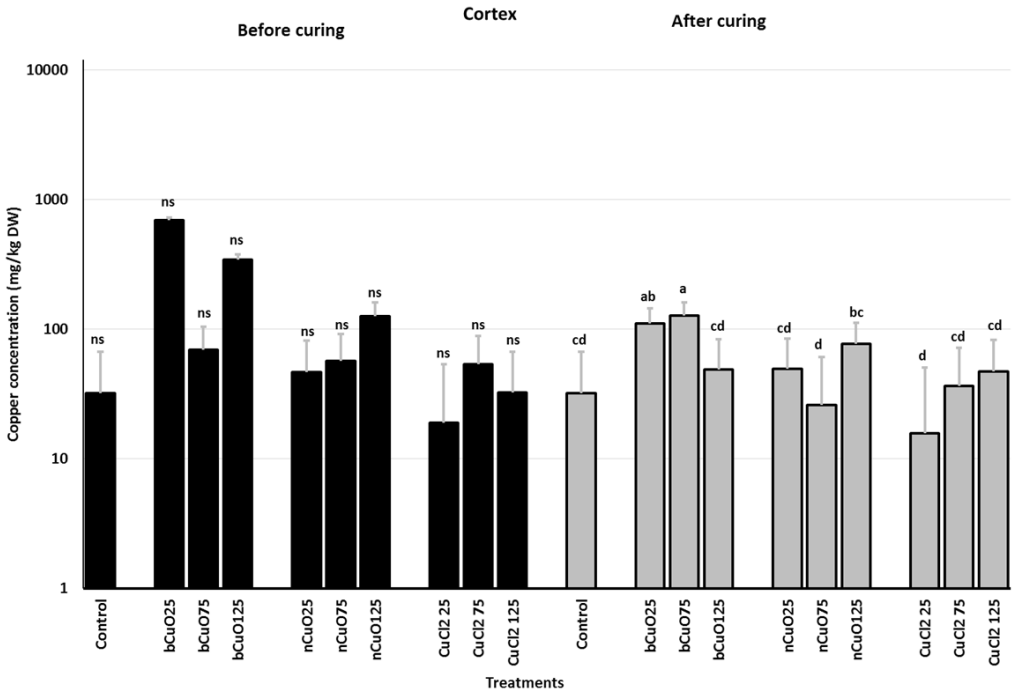

Figure 2.9. Copper concentration (mg/kg dwt) in cortex of the Covington roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L, before and after curing. Data are average of three replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$, (n = 3).

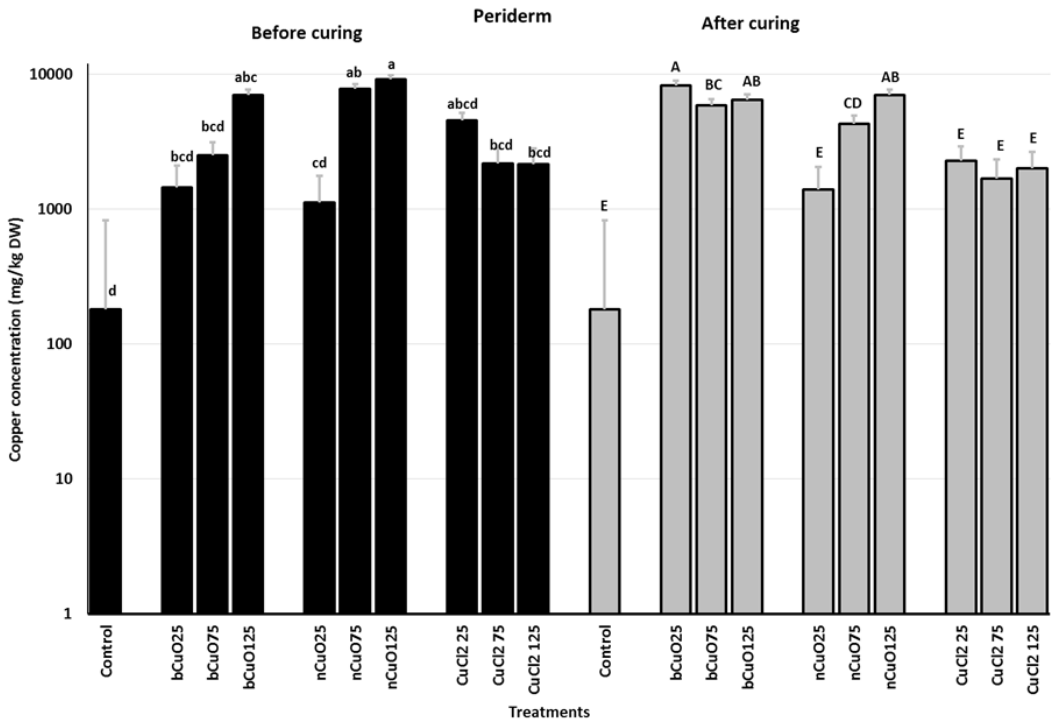

Figure 2.10. Copper concentration (mg/kg dwt) in periderm of the Covington roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L, before and after curing. Data are average of three replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$, (n = 3).

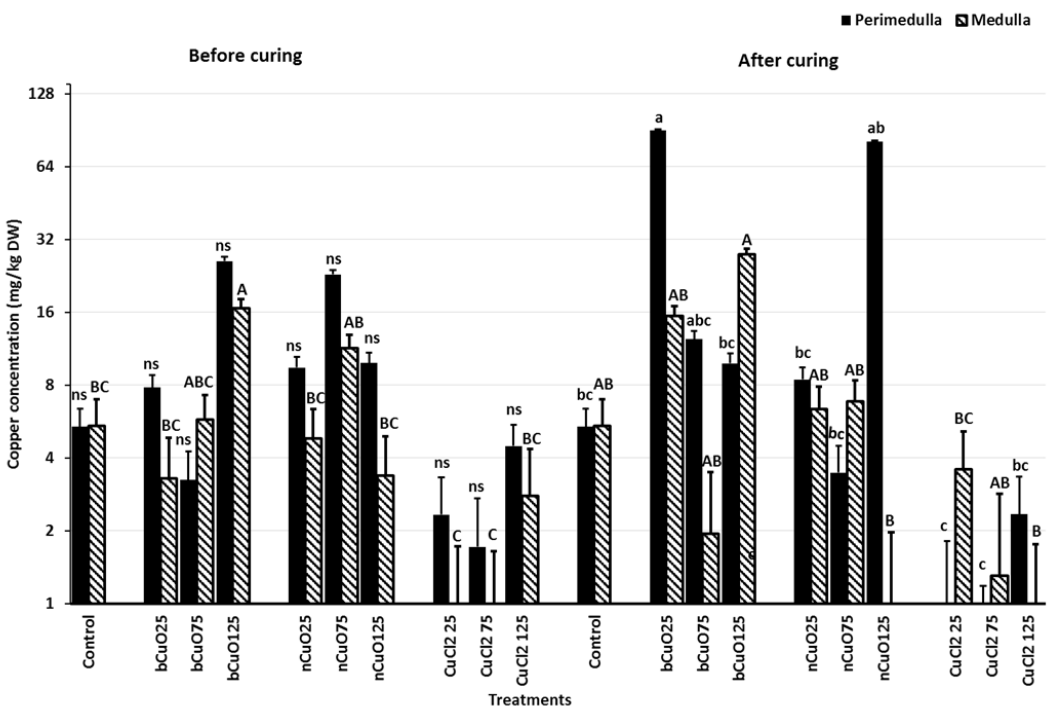

Figure 2.11. Copper concentration (mg/kg dwt) in perimedulla and medulla of B-14 roots exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L, before and after curing. Data are average of three replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$, ($n = 3$).

There were not much differences in the most internal tissues, perimedulla and medulla (Figure 2.11). Before curing, the perimedulla of Covington and medulla of Beauregard-14 showed significantly higher Cu concentration, respect to control, in roots exposed to 125 mg/L of bCuO. After curing, none of the treatments increased Cu in perimedulla or medulla of both varieties, except bCuO, which at 25 mg/L increased Cu in perimedulla of Beauregard-14 ($p \leq 0.001$). The fact that only bCuO showed significantly more Cu in the internal tissues might be a results of the lower dissolution of bCuO, compared with nCuO (Gerritsen et al. 1999), bCuO aggregates could remain in the tissues resulting in high total Cu concentration. Thus, some of the bCuO particles

that penetrated into cortex dissolved, allowing the movement of the Cu ions into deeper tissues. In addition, it is assumed that Cu did not increase in perimedulla and medulla of cured roots because Cu was retained in suberized and lignified external tissues. Kulik et al. demonstrated that lignin in decay of wood strongly bind Cu through the COO- binding group (Crist et al. 2002). Unfortunately, there are no previous reports about the Cu uptake by non-growing plant/tissue exposed to nCuO; thus, it is difficult to make comparisons." The uptake of Cu by the roots of growing plants exposed to nCuO, bCuO, and $CuCl_2$ has shown different results. For instance, Rawat et al. (2018) reported that the roots of bell pepper cultivated in soil amended with 250 mg/kg of bCuO, nCuO, or $CuCl_2$ had similar Cu concentrations. Conversely, the roots of rice (*Oryza sativa* L.) plants grown in soil amended with 1000 mg/kg of nCuO had significantly more Cu than the roots of plants exposed to the same concentration of bCuO (Peng et al. 2017). This suggest that the Cu uptake of non-growing tissues cannot be compared with that of growing tissues.

A comparison between Covington and Beauregard-14 shows some differences in Cu accumulation (Table 2.2). The data suggests and interaction variety × treatment × tissues (Table 2.3). In Beauregard-14, the variety with less lignin content (156 g/kg), before curing, all the compounds, at the highest concentration, showed significant Cu concentration in periderm and cortex ,while after curing, at 125 mg/L, both bCuO and nCuO, showed significantly more Cu in periderm and cortex, compared with control. However, in uncured roots of Covington, the variety with higher lignin content (178 g/kg), both bCuO and nCuO, at 125 mg/L, significantly increased Cu in the periderm ($p \leq 0.001$), but had no effect in cortex. While in cure roots, the same compounds, at the same concentration, increased Cu in periderm but not in cortex. A comparison of the total Cu contraptions, before and after curing (Table 2.3), shows that in Beauregard-14, only the periderm showed more Cu after curing than before curing, while converse data was found in

cortex, perimedulla and medulla. The difference between uncured and cured roots was statistically significant ($p \leq 0.05$) only in medulla (Table 2.3); the data showed that the difference was caused by bCuO (Figure 2.12).

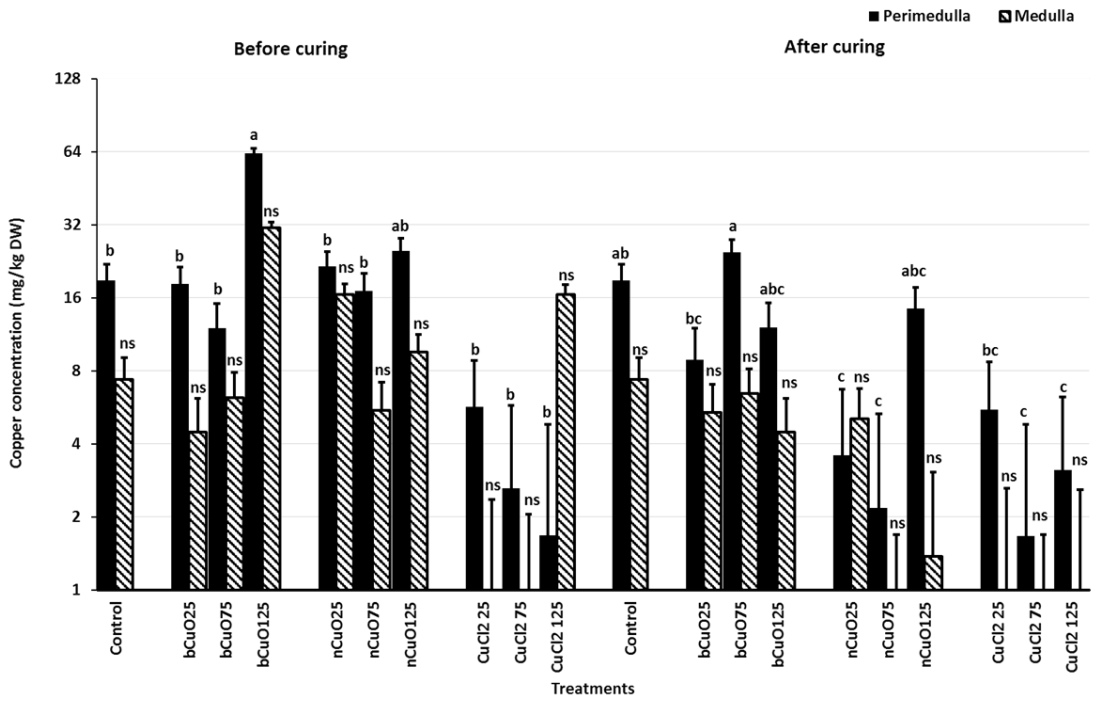

Figure 2.12. Copper concentration (mg/kg dwt) in perimedulla and medulla of Covington roots exposed to nCuO, bCuO, and CuCl2 at 0, 25, 75, and 125 mg/L, before and after curing. Data are average of three replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$, (n = 3).

On the other hand, in Covington, the Cu concentrations in all tissues where higher after curing; however, the differences did not show statistical significance. The data suggest that the curing, lignin content, tissue, and compound influence the Cu uptake in sweetpotato roots. Suberization during curing increases lignin,(Walter et al. 1983) which has shown to bind Cu

ions; (Dragović et al. 2008) this could explain the reduction of Cu concentration in the ionic treatment. On the other hand, Figures 2.3-2.4-2.5 show that bCuO and nCuO penetrated the tissues through the apoplast, which explains the Cu increase in roots treated with such compounds.

2.4 CONCLUSIONS

This research has shown, for the first time, the use of the 2-photon microscope for the observation of fluorescence from particulate CuO in sweetpotato root tissues. The fluorescence of the particles was observed in the periderm and cortex. The data showed that the nCuO and bCuO are able to penetrate the tissues through the fluid uptake into the xylem of the sweetpotato roots. Results also showed that lignin content has a strong effect in retaining the NPs in the exterior layers. As a whole, the curing process seems to have no effect in the absorption of the copper products and the copper content in internal tissues, except for bCuO treatment that at 25 mg/L caused and effect in Beauregard-14, the variety with less lignin content. The Cu concentration in internal tissues of roots treated with nCuO was similar than control roots, which suggests there should be no risk of Cu contamination in peeled roots. This also suggests that CuO nanoparticles may be used as an alternative to preserve and increase the shelf life of sweetpotato roots before they reach the market, without an overload of the copper content.

Chapter 3. Root Lignin Modulates the Response of Sweetpotato Plants to CuO Nanoparticles, Bulk Copper Oxide (bCuO), and Ionic Copper ($CuCl_2$) Exposure

3.1 INTRODUCTION

For several years, copper (Cu) has been used in agriculture as a fertilizer and as a fungicide (Zhu et al., 2012). Currently, the use of traditional Cu-based agricultural products tends to be displaced by Cu-based nanomaterials (Kah, 2015). In addition, Cu-based nanomaterial have been found to replace traditional bulk Cu products in a series of applications due to particular properties imparted by the small size and surface reactivity (Peralta-Videa et al., 2016). These sort of applications have increased their production, which by 2010 was estimated at 200 tons/year, 18% of which ended up in the soil and 6% in bodies of water (Keller et al., 2013). Although recent literature has shown the effects of nanoparticulate Cu in plants, the information about its agronomical impacts and physiological processes in crop plants are limited (Du et al., 2017).

There are a few studies about the effects of nano copper oxide (nCuO) on the physiological and biochemical processes of crop plants (Rawat et al., 2018). A variety of responses have been reported. These have been linked to the exposure concentration and plant species. In soybean and chickpea seeds exposed to nCuO, the maximum development of embryo was found at 60 and 100 mg/L, respectively (Adhikari et al. 2012). Adhikari et al. (2012) also reported that nCuO reduced root elongation in rice and maize by 50% when the exposure concentrations were 30 and 60 mg/L and by 96 and 97% at 2000 mg/L, respectively. In spring barley (*Hordeum sativum distichum*) on the other hand, a concentration as low as 10 mg nCuO/L damaged roots, reduced stomatal density, and changed cell wall integrity of epidermis and endodermis (Adhikari et al., 2012). In Indian

mustard (*Brassica juncea* L.), nCuO (20-500 mg/L) reduced root and shoot biomass and increased lignification in roots and shoots (Nair and Chung, 2015). In onion (*Allium cepa*), 80 mg nCuO/L caused the death of roots (Deng et al. 2016). On the other hand, Lin et al. (2005) reported that Cu ion increased lignin concentration in soybean roots, which impart benefits to some plants like sweetpotato.

Sweetpotato [*Ipomoea batatas* (L.) Lam.] is one of the most important staple crops in the world after wheat, rice, maize, potato, barley, and cassava (Sun et al. 2014). The world production is around 105 million tons/year, 23% of which is produced in the United States (FAO, 2016). The nutritional value of sweetpotato is given by pro-vitamin A, vitamin C, digestible fiber, and carbohydrates. In addition, it is a good source of Zn and Fe, which relates to important nutrition deficiencies in humans (Laurie, 2015). Furthermore, sweetpotato green extract is an excellent source of anthocyanin and phenolic acids, that catalyze significant production of antiproliferative prostate cancer cells (Karna et al. 2011).

Sweetpotato root definition and development are key factors for final crop yield. For a better development, the plants have to produce more storage roots than pencil roots. Pencil roots are lateral roots, where secondary growth is blocked by growth regulator like IAA and GA (Villordon et al. 2012; Firon et al. 2013). Storage roots develop lignin, an organic polymer that gives protection against excoriation and loss of the skin, as well as protection against the penetration of pathogen (Villavicencio et al. 2007). There are commercial varieties differing in lignin content, such as B-14 with 142.5 g/kg and COV with 156.1 g/kg (Bonilla-Bird, et al. 2015).

To the author's knowledge, only two studies have shown the effects of nanoparticles on sweetpotato. Bradfield et al. (2017) evaluated the element accumulation in sweetpotato roots exposed to nanoparticulate Zn, Cu, or Ce. The other study showed the accumulation of Cu in

storage roots exposed to different nCuO concentrations (Bonilla-Bird et al. 2018). The current study shows the effects of nCuO, bCuO and $CuCl_2$ on plant development and root production on B-14 and COV varieties of sweetpotato. The plants were cultivated under full sun exposure and evaluated for differences in gas exchange, root production, and elemental content in storage roots.

3.2 MATERIAL AND METHODS

3.2.1 Plant material

Sweetpotato fresh slips (± 30 cm length) of COV and B-14 varieties were procured from Mississippi State University, Pontotoc Ridge-Flatwoods Branch Experiment Station. After arriving, the slips were planted in temporary pots "to heel" (a term that means to keep the slips alive) and produce roots before transplanting into experimental pots containing Miracle-Gro® potting soil. The slips were kept in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14 h photoperiod (at 340 µmole $m^{-2}s^{-1}$) 28/20 ºC day/night temperature and 70-80% relative humidity. A set of three pots per treatment were used to plant one slip per pot. Each pot had an opening width of 27 cm and 23 cm height, with a capacity of three kilograms of soil. The slips were planted at a depth of 10 cm from the soil surface, leaving 2/3 of length of the distal end of the scion upward. A summary description of the Miracle-Gro® soil is shown in Table 3.1 (Barrios et al., 2016).

Table 3.1. Soil composition in potting Mix used as the medium amended with copper compounds for sweetpotato plants (Barrios et al. 2016).

Miracle-Gro® Potting Mix	%	Concentration (mg/kg)	Element
Forest products, compost, sphagnum peat moss, perlite, wetting agent and fertilizer	50-60	7551.28 ± 447.58	**Al**
Total nitrogen (N)*	0.21	29570.39 ± 3406.41	**Ca**
ammoniacal nitrogen	0.12	30.52 ± 4.97	**Cu**
nitrate nitrogen	0.09	4653.38 ± 404.12	**Fe**
Available phosphate (P_2O_5)	0.07	1868.65 ± 92.83	**K**
Soluble potash (K_2O)*	0.14	3110.12 ±789.19	**Mg**
Iron (Fe)	0.1	197.67 ± 12.08	**Mn**
water soluble iron (Fe)	0.1	1818.36 ± 261.48	**P**
		44.22 ± 5.22	**Zn**

Derived from: polymer coated: ammonium nitrate, 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 (P2O5) and 0.08% coated slow release soluble potash (K2O). Soil pH= 6.8-7.2

3.2.2 Characteristics of the Cu products and suspension/solution preparations

Characteristics of the copper compounds used in this experiment were described in chapter 2. Suspensions/solutions of nCuO, bCuO and $CuCl_2$ were prepared in Millipore water (MPW) and homogenized by sonication in a water bath (Crest Ultrasonics, Trenton, N) at 25 ºC for 30 min and 180 watts. Enough volume of the sonicated suspension/solution was mixed with three kilograms of commercial potting mix (MiracleGro® with micromax, Marysville, OH, USA) to have 0 (control), 25 (low), 75 (medium), and 125 (high) mg Cu per kg of soil media. The 25 mg/kg treatment was selected because the average Cu concentration in soil of the United States is 20 mg/kg (Shacklette and Boerngen, 1984), while 75 mg/kg was selected to mimic the highest Cu concentration reported in agricultural soils amended with sewage sludge (75 mg/kg) (Lioy et al. 2001). The 125 mg/L treatment represents 83% of the CuO NPs used to control fungi at field levels (Giannousi et al., 2013).

Plants were cultivated under full sun and irrigated four days a week to maintain 80% of field capacity moisture regime. The water requirements for sweet potato plants are around 40-45 mm/week, depending on the soil type and external temperature. We were using approximately 640 mm of water during all season. No fertilizer was added; no pest control was needed (Ekanayake and Collins, 2004).

3.2.3 Gas exchange and chlorophyll measurement

Gas exchange measurements were conducted during the middle phase of the growth cycle, at the peak of leaf development (Li-Cor-6400, Li-Cor Lincoln, Nebraska). Photosynthetic rate (Photo), conductance of H_2O (Cond), intercellular CO_2 concentration (Ci), and ambient CO_2 (Amb) were measured to obtain Ci: Amb ratio. Transpiration rate (Trmmol), and vapor pressure deficits were both determined based on leaf temperature (VpdL). The ratios of photosynthesis and transpiration rates were further used to calculate Water Efficiency (WE) (Von Caemmerer et al. 2004). In addition, relative chlorophyll content was determined by a hand held single photon avalanche diode (SPAD-502, Minolta Camera, Japan). The SPAD meter measures the absorption of light by the leaf with a spectral range between the red (650 nm) and near infra-red (940 nm) regions of the electromagnetic spectrum. Combining the absorption wavelength values, the meter computes a numerical SPAD value, which is directly proportional to the quantity of chlorophyll in the leaf (Spectrum Technologies 2009). In addition, SPAD-502 was tested against the spectrophotometer and dimethyl formamide in leaf tissue extract, a method used to determine instrumental accuracy, proposed for *Arabidopsis thaliana* leaves, the data from SPAD-502 was closely correlated with direct photometric measurements from the extracted chlorophyll (Ling et al. 2011).

3.2.4 Measurements at harvest

At the end of life cycle, 140 days after planting, three replicate/plants for each treatment were harvested. The plants with their respective storage, pencil, and adsorption roots were harvested gently to avoid damage to the periderm of storage roots. The aerial parts were oven dried at a temperature of 70 ºC for 72 h. Dried samples were weighed and separated for further analyses. Roots were gently washed with millipore water (MPW) to remove soil particles from periderm. Storage and pencil roots were counted, measured longitudinally and transversally, and weighed. The US Department of Agriculture standard grades were used to categorize the storage roots (Table 3.2) (Clark et al., 2010).

Table 3.2. U.S. standard classification guide for commercialization of sweetpotato storage roots (Clark et al. 2010).

Category	Length cm	Diameter cm	Weigth gr
U.S. No.1	7.6 - 23.0	4.5 - 8.25	567
U.S.Commercial	7.6 - 23.0	< 9	< 570
Canner	4.4 - 17.5	2.2 - 4.5	

3.2.5 Sample preparation for ICP-OES

For ICP-OES analyses, root tissues were separated with a scalpel in the following order: periderm, cortex, perimedulla, and medulla. Each tissue was set in a paper envelope, oven dried at 70 ºC for 72 h, and mechanically ground using a blender. Fine powdered samples of 0.2 g were acid digested with 3 mL of 30% plasma-pure HNO_3 and set in a digestion device for 45 min at 115 ºC (Digiprep MS, SCP Science, Canada). Digests were adjusted to 50 mL with MPW, followed by Cu and essential element concentration determination using an inductively coupled plasma-optical

emission spectrometer (ICP-OES, Perkin-Elmer optima 4300 DV, Shelton, CT). To validate the digestion and analytical methods (QC/QA), spiked samples and the standard reference material 1547 from the National Institute of Standards and Technology (NIST) were treated and analyzed in an analogous manner as all plant samples. The recovery rate was 96% for the NIST standard used.

3.2.6 Crude protein analysis

Total nitrogen content was used to determine crude protein. Initially, samples were oven dried for five days at 60 ^{o}C until constant weight was achieved. Dried samples were then powdered and sieved in a nylon mesh, and were stored in paper envelopes until assayed. Nitrogen content was determined by the Total Kjeldahl Nitrogen (TKN) using an extraction and distillation unit (Labconco, Kansas City, MO; AOAC2000) and expressed as % N (Bremner, 1996). A sample of 0.1 g of dry material, a K_2SO_4 –catalyst mixture (~1.5 g) and 6 mL of concentrated sulfuric acid (H_2SO_4) were added in a 250 mL Kjeldahl digestion flask and heated at 175^{o} C for 1 h. After that, the samples were heated again at 375^{o} C for 2 h to ensure complete digestion, cooled, 20 mL of distilled water was added and shaken using an automatic stirrer. The distilled sample was collected, 40 mL of 10 N sodium hydroxide (NaOH) was added, and placed in the distillation unit until 50 mL was collected; Then, 10 mL boric acid (BH_3O_3) containing a colorant reagent was added and adjusted to pH 5.0; the distilled sample was titrated with 0.05 N H_2SO_4 (Bremner, 1996), until the green color changed to rose color. The crude protein content was calculated as % N x 6.25 (Allen, 2002).

3.2.7 Sugar determination

Total sugar was determined following the method of Dubois et al. (1956). Sugar extraction was done from a sample of 100 mg of oven dried sweetpotato storage root samples homogenized in 10 mL of 80% ethanol, boiled in a water bath at 80 ºC for 30 min, and centrifuged at 5000 rpm for 20 min (Eppendorf AG bench centrifuge 5417 R, Hamburg, Germany). The extraction was repeated three times per each sample, then the supernatants were collected together, the volume was reduced to 3 mL through evaporation, and diluted up to 25 mL with MPW. The dry residue was saved for later starch analysis. In a test tube, 100 µL of the extract was diluted to 1 mL, and 1 mL of 5% phenol + 5 mL 96% H_2SO_4 were added, mixed, and let it cool down to room temperature for 30 min. Glucose standards (Sigma-Aldrich, 99.9% pure) and water (blank) were treated with the same protocol to obtain the calibration curve at concentrations of 0.02, 0.04, 0.06, 0.08 and 0.1 g/mL. The absorbance of the samples was recorded using a UV–Vis spectrometer (Perkin-Elmer Lambda 14 UV/Vis Spectrometer, Uberlinger, Germany) at 490 nm, and total sugar was quantified from the standard calibration curve. Water (blank) were treated with the same protocol to obtain the calibration curve at concentrations of 0.02, 0.04, 0.06, 0.08 and 0.1 g/mL. The samples were analyzed in a 96 well microplate by a phenol-sulfuric acid method (Masuko et al. 2005). The absorbance of the samples was recorded using the UV–Vis spectrometer at 490 nm, and total sugar was quantified from the standard calibration curve.

3.2.8 Starch determination

Starch content was analyzed according to Verma and Dubey (2001). The dry filtrate from sugar determination was diluted with 2 mL of MPW, boiled in a water bath for 15 min, and cooled to room temperature. At that point, 2 mL of 96% H_2SO_4 was added, stirred for 15 min, and diluted to 10 mL with MPW. Diluted samples were centrifuged for 20 min at 5000 rpm. After that, the

supernatant was collected. A second extraction was performed with 50% H_2SO_4, and the supernatants were collected and diluted to 40 mL with MPW. The starch content was estimated in the same way as the method for total sugars (Dubois et al., 1956). One hundred (100) µL of the extract was taken from each sample. After reaction, it was reading 490 nm using a calibration curve of potato starch.

3.2.9 Microscopy study of sweetpotato samples

Dry samples from the medulla of the storage roots were ground, coated with gold for a minute (ca. 1 nm gold layer) by using a Sputter Coater (Hitachi E-1010, Japan), after that, observed by scanning electron microscopy (SEM; Philips SEM-505), Netherlands) with Energy Dispersive Spectrometer (EDS) analysis. All samples where for SEM were prepared following the standard procedures (Kopek et al. 2017).

3.2.10 Statistics analysis

Triplicate data samples were analyzed per treatment. Multivariate Data analysis was performed, followed by One-way ANOVA to determine significant differences among each factor/treatment. Coefficient of Variance (CV) was conducted for Chlorophyll content; the Tukey Honestly Significant Difference and Waller-Duncan were used to determine multiple comparison tests (SPSS 19.0 package, Chicago, IL, and SAS/STAT 14.2. SAS Institute In. Cary, North Carolina, U.S.).

3.3 RESULTS AND DISCUSSIONS

3.3.1 Gas exchange and chlorophyll measurement

Table 3.3 shows the multivariate analysis for gas exchange components of the photosynthesis in sweetpotato plants. Most of the factors had different significance including $p \leq 0.05$; 0.01; 0.001 for photosynthesis rate (Photo), conductance (Cond), intercellular CO_2 (Ci), transpiration rate (Trmmol), ratio of Ci/Amb (Amb: ambient CO_2), vapor pressure deficit (VpdL), and water efficiency (WE). The Partial Eta-squared statistics showed that the higher variance was due to the difference between lignin content in the varieties [24%, 19%, 37%, and 12% for Photo, Cond, Trmmol and WE, respectively ($p \leq 0.001$)]. However, the copper treatments significantly affected all parameters related to photosynthesis, where the higher variances were 21.5% and 24.5% for Ci and VpdL, respectively ($p \leq 0.001$). Furthermore, the interaction between the varieties × treatments was significant for Ci (12%) and WE (17%). Overall, conductance and photosynthesis had the lowest variation associated with this interaction, with a variance of 5.4 % and 5.7%, respectively ($p \leq 0.001$).

Table 3.3. Statistical descriptive data using UNIANOVA test to compare the two main components (variety and treatment) with their respective interactions with sweetpotato plants exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/L, (n = 10).

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	% Variation
Variety	Photosynthesis (Photo)	1570.962	1	1570.962	104.993	.000	.247	24.7
	Conductance (Cond)	.887	1	.887	76.561	.000	.193	19.3
	Intercellular CO_2 (Ci)	975.459	1	975.459	2.069	.151	.006	0.6
	Ratio Ci/CO_2 Ambient(Amb)	.033	1	.033	8.784	.003	.027	2.7
	Transpiration rate(Trmmol)	288.142	1	288.142	190.057	.000	.373	37.3
	Vapor pressure deficit (VpdL)	.014	1	.014	.205	.651	.001	0.1
	Water efficiency (WE)	6.824	1	6.824	46.385	.000	.127	12.7
Treatments	Photo	578.295	9	64.255	4.294	.000	.108	10.8
	Cond	.588	9	.065	5.640	.000	.137	13.7
	Ci	41267.531	9	4585.281	9.726	.000	.215	21.5
	Ci/Amb	.280	9	.031	8.410	.000	.191	19.1
	Trmmol	55.276	9	6.142	4.051	.000	.102	10.2
	VpdL	7.193	9	.799	11.508	.000	.245	24.5
	WE	5.574	9	.619	4.210	.000	.106	10.6
Variety* Treatments	Photo	288.320	9	32.036	2.141	.026	.057	5.7
	Cond	.212	9	.024	2.036	.035	.054	5.4
	Ci	20206.965	9	2245.218	4.762	.000	.118	11.8
	Ci/Amb	.130	9	.014	3.894	.000	.099	9.9
	Trmmol	36.283	9	4.031	2.659	.005	.070	7.0
	VpdL	2.030	9	.226	3.247	.001	.084	8.4
	WE	9.743	9	1.083	7.358	.000	.171	17.1

3.3.2 Gas exchange and chlorophyll measurement

Effect of treatments on chlorophyll content

Figure 3.1 shows the SPAD data of chlorophyll content for both the COV and B-14 varieties. As seen in this figure, there were no effects on B-14; however, COV presented statistical differences for nCuO at 75 and $CuCl_2$ at 125 mg/kg. At such concentrations, the SPAD value for nCuO was 25% higher than control, while $CuCl_2$ at 125 mg/kg significantly reduced the

chlorophyll content by 17%, compared with control ($p \leq 0.05$). The fact that these copper compounds had an effect on chlorophyll content on COV was attributed to the interaction of the compounds with the high root lignin content of this variety; at 75 mg/kg, nCuO could release enough Cu to act as chlorophyll promoter. On the other hand, the toxicity of $CuCl_2$ at 125 mg/kg could be due to the rapid absorption of Cu by the roots and subsequent transport to the leaves (Table 3.4).

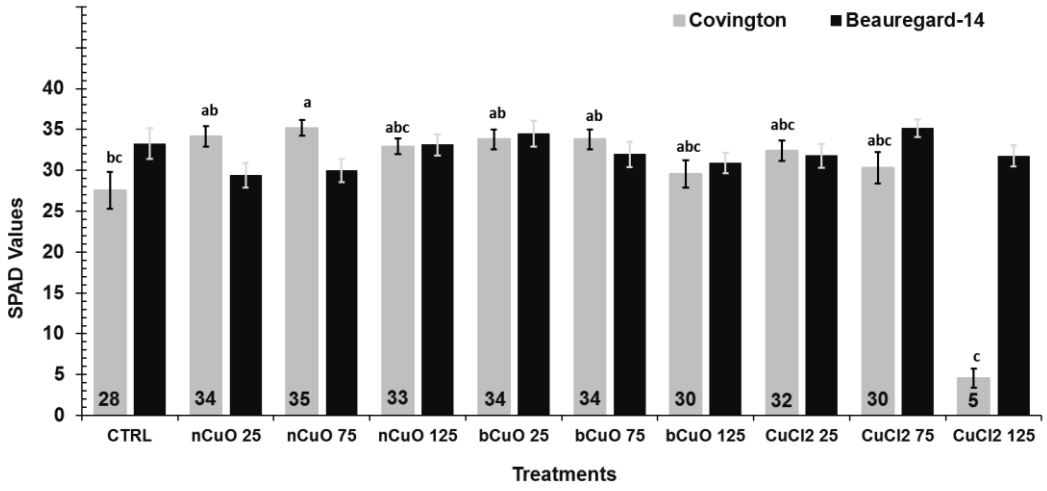

Figure 3.1. Measurement of the chlorophyll content in sweetpotato plants growing in soil amended with nCuO, bCuO, and CuCl2 at concentration of 25, 75, and 125 mg/kg. Data are average of six replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$.

Table 3.4. Copper uptake by sweetpotato plants exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/kg, $p \leq 0.05$ (n = 3).

Varieties	B-14 Cu (mg/kg)	Covington Cu (mg/kg)	B-14 Cu (mg/kg)	Covington Cu (mg/kg)
Treatments	**Periderm**		**Perimedulla**	
Control	18.5±1.3	43.4±9.1	6.2±2.4	1.0±0.2
nCuO25	52.6±8.3	45.2±10.0	2.6±0.1	2.0±0.2
nCuO75	100.6±20.0	74.8±15.2	4.0±1.9	3.6±0.2
nCuO125	54.4±14.3	42.3±16.3	6.4±0.7	4.3±0.5
bCuO25	193.5±22.5	91.5±25.9	4.2±0.2	2.2±0.4
bCuO75	70.2±12.0	65.2±14.9	5.0±0.3	2.4±0.1
bCuO125	27.0±4.6	136.8±35.8	6.1±0.6	11.9±9.4
$CuCl_2$ 25	53.3±17.6	22.5±4.3	2.1±0.5	2.0±1.1
$CuCl_2$ 75	66.6±41.3	73.8±45.2	3.8±1.2	5.0±1.4
$CuCl_2$ 125	18.5±2.2	60.6±15.0	5.4±0.5	7.2±0.4
	Cortex		**Medulla**	
Control	5.1±0.2	1.0±0.1	1.8±0.5b	2.0±0.4
nCuO25	3.6±0.7	2.5±0.47	2.3± 0.6ab	2.6±0.58
nCuO75	4.2±1.5	4.5±0.9	5.5± 3.2ab	3.5±0.3
nCuO125	30.9±3.5	4.5±0.2	10.1± 3.6ab	3.9±1.3
bCuO25	4.0±0.2	2.4±0.1	3.7± 0.7ab	2.9±1.2
bCuO75	6.0±1.4	3.3±0.6	3.4± 0.7ab	3.5±0.7
bCuO125	6.2±0.5	6.9±2.0	3.5± 0.5ab	5.3±1.6
$CuCl_2$ 25	2.8±0.5	1.4±0.1	1.4± 0.3b	1.4±0.1
$CuCl_2$ 75	2.8±0.5	3.7±1.4	9.1± 0.6ab	6.0±2.2
$CuCl_2$ 125	6.4±0.6	3.8±0.3	13.0±4.5a	5.9±0.3
	Stem		**Leaves**	
Control	1.6±0.3b	2.3±0.6	7.8±1.0	5.6±2.9b
nCuO25	3.5±0.2ab	3.1±0.1	13.8±5.1	22.9±11.3ab
nCuO75	5.9±1.0a	12.9±9.1	16.9±6.5	9.5±1.3b
nCuO125	4.2±1.7ab	4.0±1.0	7.1±0.3	10.2±2.5ab
bCuO25	4.3±0.6ab	3.1±0.7	9.2±2.6	6.7±1.3b
bCuO75	4.4±0.4ab	4.6±0.2	8.3±0.6	25.6±6.9ab
bCuO125	6.3±0.3a	4.6±0.9	14.8±0.4	23.0±6.5ab
$CuCl_2$ 25	2.8±0.2ab	1.7±0.3	18.6±5.2	12.1±7.6ab
$CuCl_2$ 75	3.7±0.4ab	4.2±2.0	10.0±2.8	70.9±24.7ab
$CuCl_2$ 125	4.8±0.6ab	5.3±0.2	11.1±2.1	93.0±26.0a

Several papers have shown that Cu is toxic to chloroplast membranes, they are fragile to Cl^-, which affects the photosynthetic rate as a consequence of the chlorophyll breakdown caused by a direct damage in photosystem II (Dimkpa et al. 2012; Hajrasuliha, 1980). It is well known that Cu^{2+} toxicity increased the reactive oxygen species (ROS) affecting the photosynthesis (Nikookar, et al. 2005). The thylakoid membrane in chloroplast is affected by the Cu toxicity

(Burkhead et al. 2009). Moreover, bCuO and nCuO showed slower release of Cu^{2+} to the soil solution (compared to $CuCl_2$), which is cofactor of enzyme activity and electron transfer for different physiological process like respiration, photosynthesis, cell wall uptake, lignification, and the scavenging of reactive oxygen radicals (Lequeux et al., 2010). On the other hand, Cu inhibits the integration of chlorophyll molecule to the photosynthesis system when it is in excess (Caspi, et al. 1999). That would be the reason for the effect of the high Cu concentration at 125 mg/kg of $CuCl_2$. Nevertheless, Zuverza-Mena et al. (2015) found, in cilantro plants, that the concentration of 20 mg/kg of bCuO reduced the chlorophyll content by 26.4%, compared with 20 and 80 mg/kg of nCuO and $CuCl_2$, respectively, except with the 80 mg/kg treatment of bCuO, where the reduction was about 16.5%. Rawat et al. (2018) cutilvated bell pepper in soil amended with copper compounds at concentrations of 62.5, 125, 250, and 500 mg/kg of nCuO,bCuO, and $CuCl_2$ and found no effect on chlorophyll content.

Figure 3.2, transversal view of the leaf structure, and its tissues were the photosynthesis takes place and other physiological process.

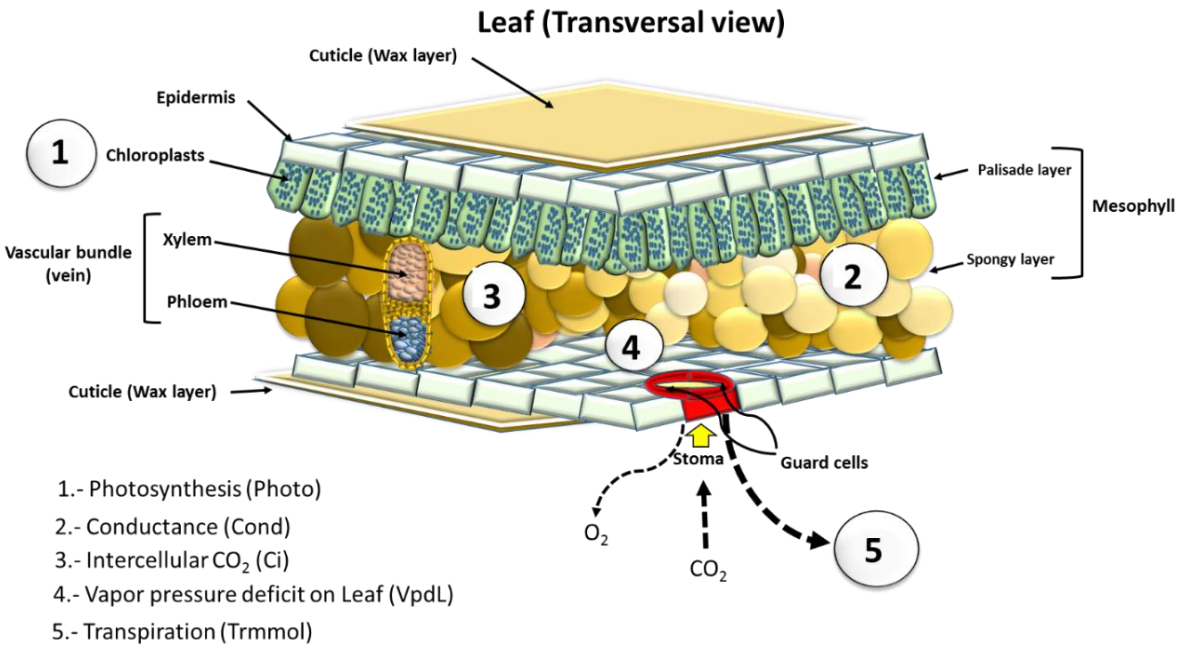

Figure 3.2. Transversal view of the leaf structure, and its tissues were the photosynthesis takes place and interact with the stress caused by the copper compounds.

The intercellular CO_2 was affected by the copper treatments (Figure 3.3). B-14 variety showed a significant increase in the intercellular CO_2 in plants exposed to 25 mg/kg of $CuCl_2$, 22% higher than 75 mg/kg of bCuO ($p \leq 0.05$). In addition, at 25 mg/kg, $CuCl_2$ affected B-14 variety, that decreased Zn and Fe in the leaves by 63% and 28%, respect to control ($p \leq 0.05$). On the other hand, in COV variety exposed to $CuCl_2$ at 125 mg/kg, CO_2 was 21 % higher than 125 mg/kg bCuO ($p \leq 0.05$). The effect of the high lignin content in the roots of the COV variety, plus the high concentration of copper treatments in the soil, had a complementary effect in the thickness and stiffening of the cell walls in roots and stems. In an early study, the exposure to nCuO enhanced the lignin biosynthesis. In soybean seedlings, nCuO at 100, 200, 300, 400, and 500 mg/L increased the lignin content in the roots (Nair and Chung 2014). Differences in cellular anatomical features may restrict the diffusion of CO_2, affecting photosynthesis. Mesophyll conductance depends on

membrane permeability and cytosol conductance, which may be limited by the cell wall thickness (Tomás et al. 2013).

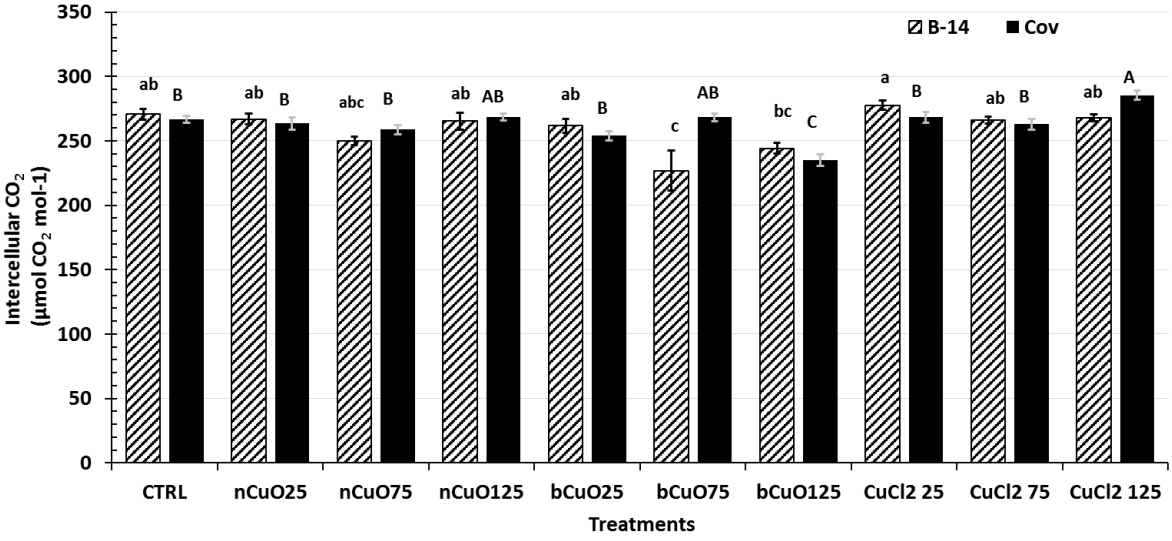

Figure 3.3. Measurement of the Intercellular CO2 in sweetpotato plants growing in soil amended with nCuO, bCuO, and CuCl2 at concentration of 25, 75, and 125 mg/kg. Data are average of six replicates ± SE. Different letters stand for statistical differences at p ≤ 0.05.

The transpiration rate (Trmmol) (Figure 3.4 A) significantly increased (35%), compared to the control in B-14 variety exposed to 25 mg/kg nCuO. However, at 125 mg/kg, both the nCuO and bCuO significantly decreased Trmmol by 4% and 7%, respectively, compared with control ($p \leq 0.05$). The soil amended with the treatments at high concentration, like 125 mg/kg of nCuO and bCuO, stimulated the lignin accumulation in the cell walls of the leaves, which were thickened. This gives less spaces into the mesophyll intercellular spaces. On the other hand, COV variety decreased significantly its transpiration rate by 6% under 25 mg/kg $CuCl_2$ exposure, with regard

to the control, while the other treatments remained consistent. COV has high lignin content, which is able to retain Cu. Adams et al. (2017) reported that wheat seedlings exposed to nCuO and $CuSO_4$ at 30 -300 mg/kg and 4.5 -6 mg/kg, respectively, were irregular in shape and had curved cell walls.

Vapor pressure (VpdL) data is shown in Figure 3.4 B. As seen in this figure, B-14 showed 20% higher VpdL under exposure to nCuO at 75 mg/kg or bCuO at 75 and 125 mg/kg, with respect to 125 mg/kg $CuCl_2$. In COV, 125 mg/kg bCuO increased VpdL by 15%, compared with control. However, $CuCl_2$ at 125 mg/kg decreased VpdL by 20%, compared with control ($p \leq 0.05$). The higher lignin content in COV, plus the slow release Cu ions at 125 mg/kg bCuO, had a positive effect on VpdL. The VpdL and Trmmol were inversely affected by nCuO and bCuO at 25 and 125 mg/kg in B-14 variety. In this variety, the photosynthesis rate increased by 40% under exposure to 25 mg/kg nCuO ($p \leq 0.05$), which gave high transpiration rate and lower vapor pressure in the leaves (Fig. 3.4 A-B). On the other hand, bCuO at 125 mg/kg increased VpdL and reduced Trmmol in B-14, but did not affect the photosynthesis, respect to control. High VpdL affects photosynthesis, the activity of rubisco-activase, the supply of ATP, and the assimilation of CO_2 (Shirke and Pathre, 2004). In addition, changes in VpdL affected sucrose metabolism by decreasing the cycle of sucrose-phosphate synthase (SPS) in the leguminous tree *Prosopis juliflor* (Shirke and Pathre, 2004). This could be the reason for the effects observed in water efficiency (Figure 3.4A) and photosynthesis (Figure 3.4B) of sweetpotato.

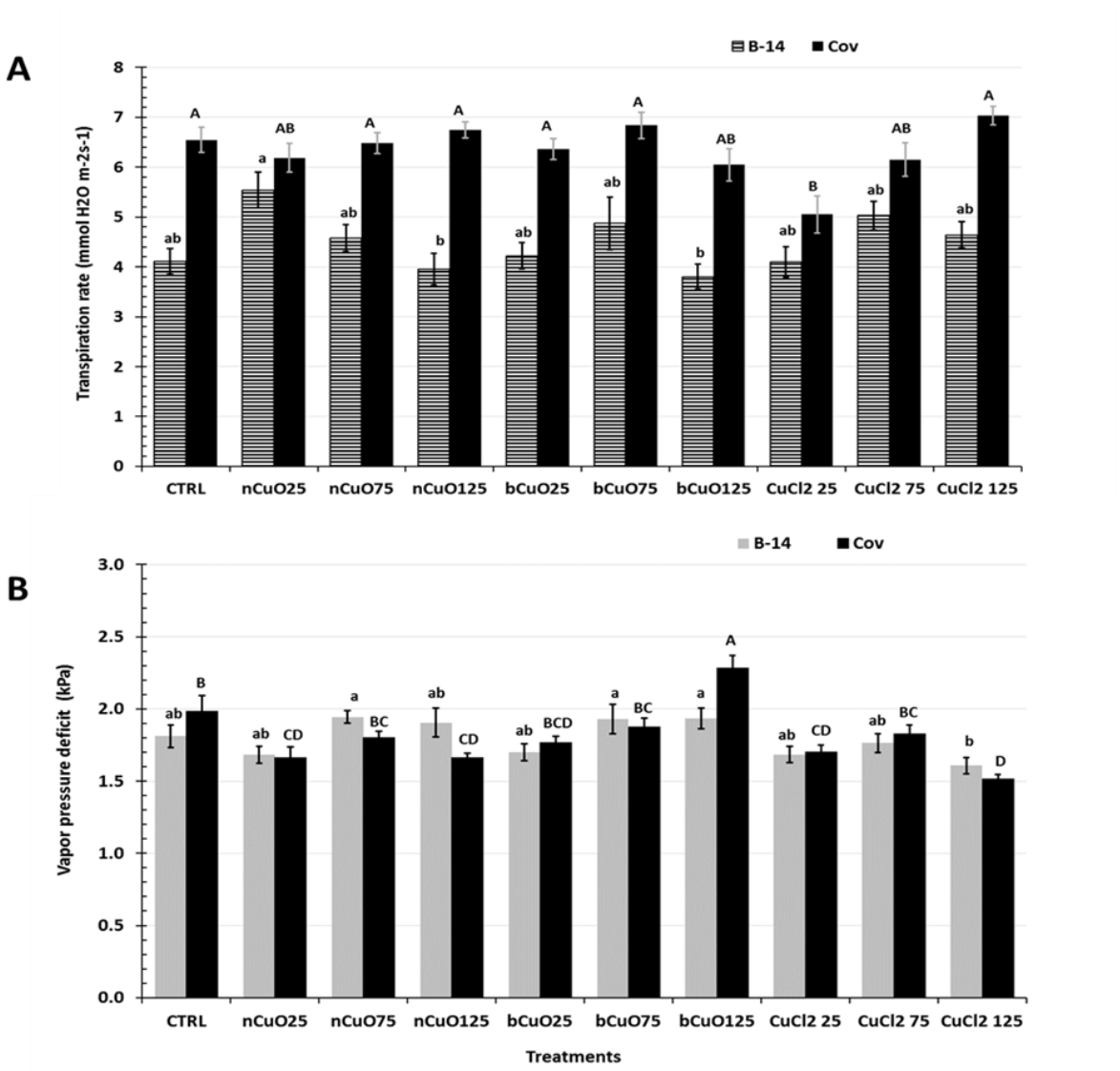

Figure 3.4. Measurement of the transpiration (A) rate and vapor pressure (B) in sweetpotato plants growing in soil amended with nCuO, bCuO, and $CuCl_2$ at concentration of 25, 75, and 125 mg/kg. Data are average of six replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$.

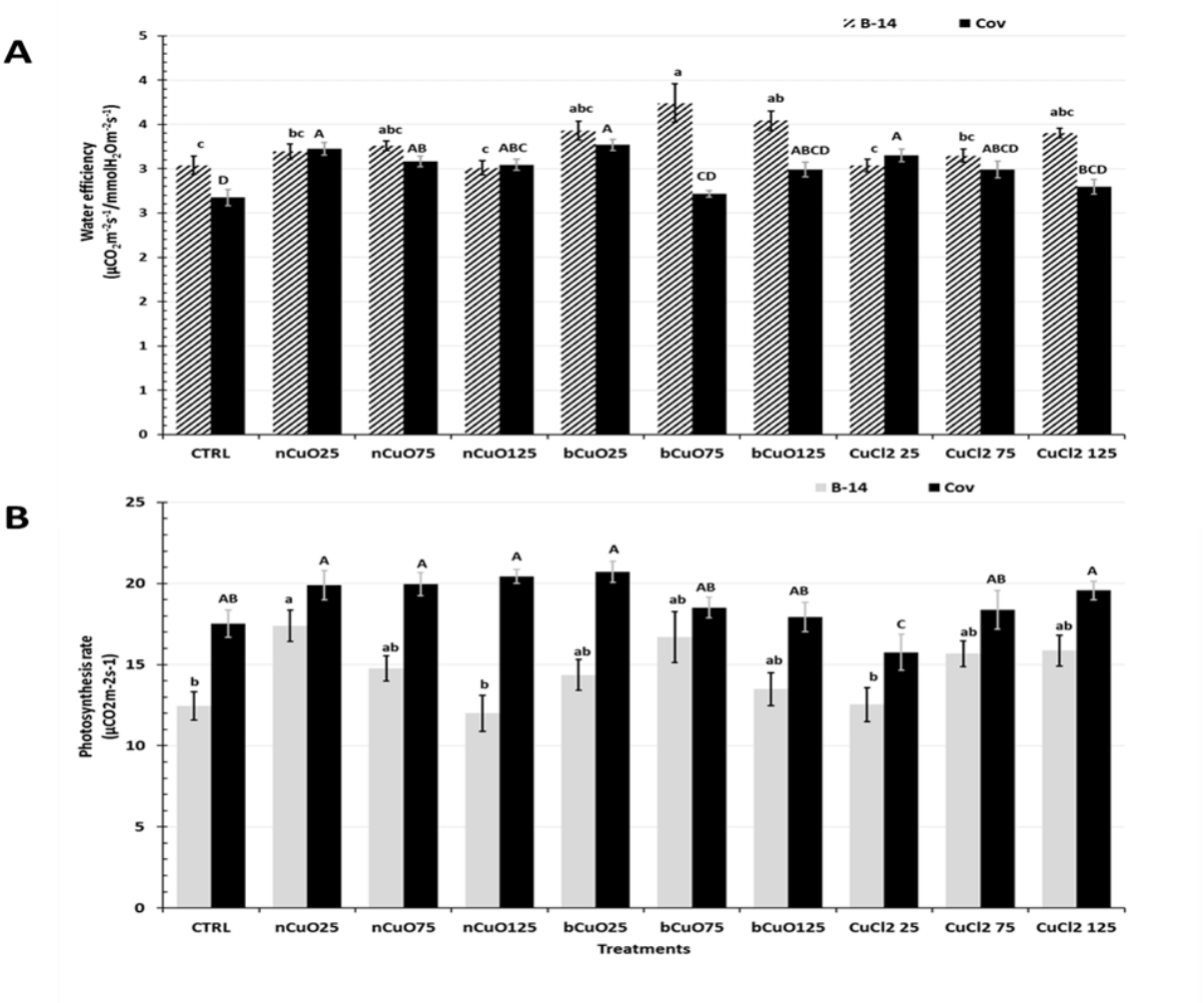

Figure 3.5. Measurement of the water efficiency (A) and photosynthesis (B) in sweetpotato plants growing in soil amended with nCuO, bCuO, and $CuCl_2$ at concentration of 25, 75, and 125 mg/kg. Data are average of six replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$.

Data on water efficiency (WE) are shown in Figure 3.5-A. This figure shows that bCuO at 75 mg/kg increased WE by 23%, respect to control, in B-14. It was observed that 75 mg/kg bCuO in B-14 produced lower intercellular CO_2, high VpdL, low Trmmol, and intermediate photosynthesis rate, but none of the other treatments affected WE in this variety. In COV however,

exposure to 25 mg/kg of nCuO, bCuO, or $CuCl_2$ increased WE by 21%, 22%, and 17%, respectively, compared with control ($p \leq 0.05$).

Photosynthesis was also differentially affected by the Cu treatments in both varieties. In B-14, 25 mg/kg of nCuO enhanced the photosynthesis (40%), transpiration rate (35%), and WE (7%), over the control ($p \leq 0.05$). Nevertheless, in COV, none of the treatments affected chlorophyll (Fig 3.4) and photosynthesis (Fig 3.5-B). Differences between varieties were attributed to variations in lignin content and the low copper concentration from, 25 and 75 mg/kg of nCuO and bCuO, which increased the chlorophyll content and photosynthesis in B-14. Copper released to the soil solution from these treatments increased lignin content in the roots, which reduced Cu translocation to the leaves. Lignin acts as a shell to protect and stabilized the physiological and biochemical process in sweetpotato plants. In a research performed in transgenic tobacco (*Nicotiana tabacum*), it was found that high lignin content enhanced the photosynthesis rate and biomass production (Biemelt et al. 2004). However, at 25 mg/kg $CuCl_2$, the photosynthetic rate was 10% less than the control in COV variety. Similar results were observed in a previous study with bell pepper, where 62.5 mg/kg $CuCl_2$ reduced photosynthesis by 38% (Rawat et al. 2018).

Photosynthesis can be limited by biochemical processes and diffusion of essential gases such as CO_2. In the present study, it was observed that the highest photosynthetic rate occurred in COV variety, which had a high lignin content. Contrarily, in B-14, it was observed that the treatment with 75 mg/kg bCuO had the lowest intercellular CO_2 (17%), highest VpdL (6%), water efficiency (23%), and photosynthesis rate (37%), respect to the control ($p \leq 0.05$). Figure 3.2 shows the relationship between the interstitial cellular spaces, the thickness of the cell walls, and the capacity of CO_2, where the mesophyll tissue resides and affects the mobility of rubisco through the tissues.

The lignin acts as natural barrier as it gets deposited in the cells walls. The stress caused by the copper treatment in B-14, which has less lignin in the roots, enhanced the lignin production, which could be a limiting factor for the translocation of nutrients from the roots to the leaves, affecting the photosynthesis. Presently, there are two known pathways that lead to rubisco; from the sub-stomatal cavities to the carboxylation sites in the liquid phase (cytosol), or through the intercellular airspaces. In the interstitial space between the cell membrane and cell wall, CO_2 moves at a faster rate than in the cytosol due to the differing nature of each medium. In the cytosol, CO_2 moves through liquid fluid to reach the chloroplast, where it gets captured for photosynthesis processes (Tosens et al. 2012). However, photosynthesis is limited by mesophyll conductance, which occurs at a low rate, thus the structural cell wall plays a key role in biological processes. A major portion of the mesophyll surface is exposed to CO_2, resulting in CO_2 diffusion, which further enhances photosynthesis efficiency. Consequently, mesophyll cell wall thickness and chloroplast membrane envelopes are the greatest limiting factors in mesophyll conductance and photosynthesis (Terashima et al. 2011).

3.3.3 Copper uptake

Table 3.2 shows the copper concentrations in tissues of the sweetpotato plants. As seen in this table, only B-14 showed differences in Cu concentration in tissues. In this variety, plants exposed to $CuCl_2$ at 125 mg/kg had 622% more Cu in medulla (the central part of the storage root), compared with control. In addition, plants exposed to 75 mg/kg nCuO and 125 mg/kg bCuO had more Cu in stems (269% and 294%, respectively), compared with control. None of the other tissues in B-14 or COV showed changes in Cu concentration.

3.3.4 Biomass production

The leaves biomass production of B-14 and COV is shown in Figure 3.6-A. As seen in the figure, none of the treatments impacted the biomass production in leaves of B-14. In COV however, nCuO at 25 and 75 mg/kg significantly increased leaf biomass (about 68%), compared with control ($p \leq 0.05$). Contrary results were observed with 25 mg/kg $CuCl_2$, which reduced the leaf biomass by 60%, compared with control. In COV, most of the Cu was retained in roots. At the low concentration of the ionic form, 25 mg/kg, Cu was available for translocation and fast reached the leaves, where it caused stress and damage in the photosynthetic system. That stress activated the defense mechanism enhancing the production of other metabolites include lignin, which stops cell enlargement due to the thickening of the cell walls. The response of plants against the biotic and abiotic stress is to activate the antioxidant system. It could be an enzymatic activation or non-enzymatic like polyamines (PAs), whose role in plant defense is key to reduce the oxidative stress and stabilize physiological processes under stress. (Bernard, et al. 2015). Additionally, none of the treatments affected the stem biomass production (Fig 3.6-B). Converse results have been reported about the effects of nCuO on biomass production. Du et al. (2018) reported that concentrations of 50-200 mg/kg of nCuO and bCuO, reduced shoot biomass (21.6 to 58.5%) in oregano plants. However, Zuverza-Mena et al. (2015) reported that nCuO or bCuO (20 and 80 mg/kg) did not reduce biomass in cilantro. Lequeux et al. (2010) mention that Cu compounds affect the biomass production in most plant species.

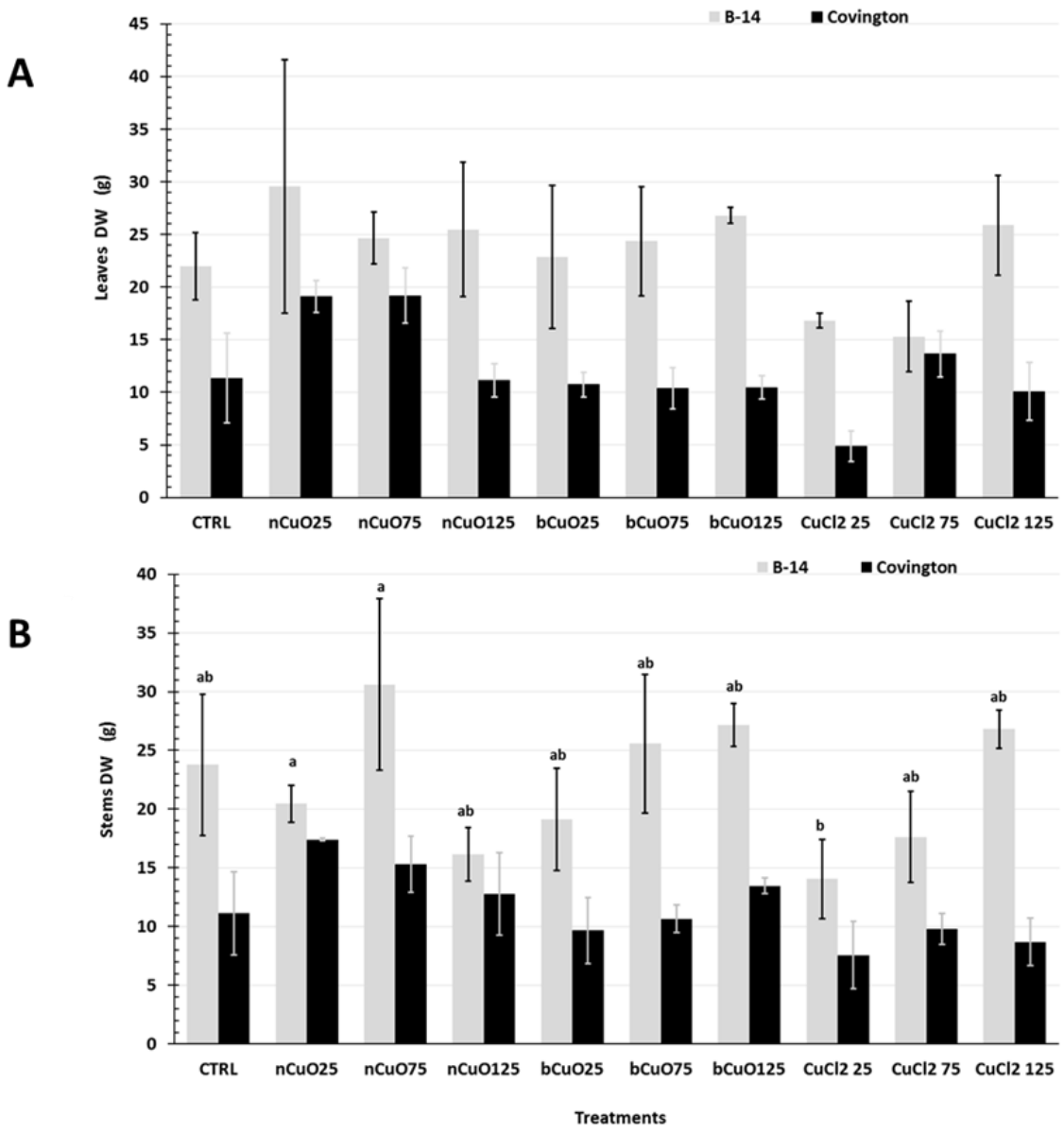

Figure 3.6. Measurement of the stems DW (A) and leaves DW (B) in sweetpotato plants growing in soil amended with nCuO, bCuO, and $CuCl_2$ at concentration of 25, 75, and 125 mg/kg. Data are average of six replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$.

3.3.4 Storage root production

Table 3.5 shows the effect of copper treatments on the weight of sweetpotato roots (storage and pencil); there were no significant effects in pencil roots weight and the ratio of pencil roots/storage roots. The B-14 variety, which has lower lignin content, showed significant differences, compared with the control (Table 3.5). The highest average was obtained under exposure to 75 mg/kg bCuO, and 125 mg/kg bCuO, 637.3 g and 619.0 g, respectively ($p \leq 0.05$).

Table 3.5. Root production in sweetpotato plants exposed to nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/kg, $p \leq 0.05$ (n = 3).

Treatment	Beauregard-14							
	Root fresh weight (g)		Total weight (g)	No. Storage roots	No. Pencil roots	Lenght of storage roots (cm)	Diameter of storage roots (cm)	Ratio no.pencil / no.storage
	Storage roots	Pencil roots						
CTRL	457.3 ±16.4 abc	28.0±3.1	485.3	9.7±1.8	7.0±1.2	14.7±1.3	4.6±0.7	0.72
nCuO25	414.5±11.5 abc	29.0±12.1	443.5	9.5±4.5	13.5±1.5	12.0±0.8	4.3±0.4	1.42
nCuO75	514.0±23.4 abc	36.7±10.1	550.7	7.7±2.0	8.0±2.1	13.4±1.3	3.6±0.3	1.04
nCuO125	265.0±8.0 c	50.3±6.7	315.3	4.5±0.5	11.3±1.8	7.37±0.9	2.8±0.3	2.51
bCuO25	269.0±64.3 bc	27.5±2.9	333.3	8.7±1.2	6.3±2.0	10.0±0.8	6.6±2.3	0.72
bCuO75	637.3±30.5 a*	24.6±8.4	661.9	6.3±0.3	7.3±1.2	15.0±1.3	4.9±0.2	1.16
bCuO125	619.0±78.5 ab*	37.2±2.4	656.2	11.7±5.6	7.7±0.3	14.6±1.2	5.3±0.4	0.66
$CuCl_2$ 25	280.6±5.3 bc	26.3±8.7	306.9	8.3±0.8	6.7±1.2	12.8±1.2	3.3±0.3	0.81
$CuCl_2$ 75	407.3±76.3 abc	46.2±3.8	453.5	4.3±1.0	8.3±0.9	13.5±2.2	4.3±0.2	1.93
$CuCl_2$ 125	507.0±49.7 abc	30.0±9.6	537.0	6.6±1.4	8.3±1.5	12.3±0.8	3.9±0.3	1.26
Covington								
CTRL	436.2±41.9	42.5±8.1	478.7	5.3±0.3	7.5±2.3	14.6±0.8b	4.4±0.5	1.42
nCuO25	464.7±54.0	61.5±31.0	526.2	2.5±0.5	10.5±1.5	20.7±2.0a	4.6±0.2	4.20
nCuO75	472.1±75.0	33.0±9.1	505.1	5.7±1.3	8.0±3.5	16.0±2.5b	4.6±0.3	1.40
nCuO125	428.0±69.5	15.7±0.9	497.5	5.0±0.5	6.3±1.3	15.0±1.8b	4.1±0.2	1.26
bCuO25	424.3±77.6	26.0±7.9	450.3	4.0±1.1	6.0±2.1	17.7±2.8b	4.7±0.3	1.50
bCuO75	563.5±60.3*	66.3±12.1	629.8	4.7±1.2	9.3±3.0	15.2±1.3b	4.9±0.2	1.98
bCuO125	565.5±47.3*	54.0±15.3	619.5	2.3±0.6	11.3±1.5	15.1±2.5b	5.4±0.8	4.91
$CuCl_2$ 25	289.8±53.3	43.6±14.2	333.4	6.3±4.3	7.0±4.0	12.0±1.0b	3.8±0.3	1.11
$CuCl_2$ 75	399.8±47.6	66.0±11.3	465.8	3.0±1.5	10.0±4.0	15.3±2.1b	3.9±0.4	3.33
$CuCl_2$ 125	429.5±44.7	61.0±13.4	490.5	2.6±0.6	6.0±1.0	13.4±1.7b	3.8±0.4	2.31

*U.S. No.1 according to commercial U.S. classification

The storage root characteristics are highlighted in Table 3.5. As seen in this table, roots of COV variety had a significant increase in length (41%, compared with the control) at 25 mg/kg nCuO.

In wheat (*Triticum aestivum* L.), exposure to 10 -300 mg/kg nCuO caused changes in the roots architecture due to hormonal imbalance (auxins). In addition, it caused the activation of some defense and detoxification mechanisms, which affected the growth of the principal root and promoted the lateral roots growth. The root relative growth decreased from day two to day 10, where the root growth completely stopped and the root morphology changed (Adams et al., 2017; Zhang et al. 2018).

3.3.5 Macronutrient accumulation in storage roots

Among all macroelements analyzed, only P and Mg showed changes in storage roots. Table 3.6 shows the distribution of P in root tissues of B-14 and COV cultivated in soil amended with nCuO and their analogues during the whole life cycle. Phosphorus concentration was lower than the control (9%) in the periderm of B-14 under exposure to 125 mg/kg $CuCl_2$ **(**$p \leq 0.05$). However, in the inner most tissues, such as the cortex, P increased, respect to the control, in B-14 plants exposed to 125 mg/kg nCuO and 25 mg/kg $CuCl_2$ (11,810.2 ± 641; 206%) mg/kg and 12,395.3 ± 732; (216%) mg/kg, respectively **(**$p \leq 0.05$). The periderm has the endodermis, a single cell layer, which shows a thickness in its radial and transverse cell walls. This ring is made of impermeable substances such as lignin and suberin (Casparian strip) that stop the transport of compounds (Naseer et al. 2012). In COV, P concentration did not present significant differences in periderm and cortex, respect to the control, but in medulla, P increased at 75 mg/kg of $CuCl_2$, as much as 13,283.4 ± 1,440.0 mg/kg. The stress inflected by the 75 mg/kg $CuCl_2$ **(**$p \leq 0.05$) either as Cl^- or Cu^{2+} caused high demand of P. However, this was not observed in B-14. It has been reported that

Cl^- reduced the uptake of some ions due to a competitive effect or antagonism with P, K, Cu, Mn, Fe, and Zn (Chen et al. 2010). The high concentration of 125 mg/kg of $CuCl_2$ inhibited the P accumulation in the periderm of the storage roots due to the competition between Cl^- and P. Previous studies have shown that lettuce and alfalfa crops significantly reduced the P uptake by the root exposed to 5, 10, and 20 mg/L of nCuO, bCuO, and $CuCl_2$ (Hong et al. 2015). In addition, Zuverza-Mena et al. (2015), reported that 20 and 80 mg of nCuO, bCuO, or $CuCl_2$ reduced P accumulation. Likewise, Rawat et al. (2018) reported that in bell pepper, 500 mg/kg of bCuO reduced the root absorption of P by 36%, respect to the control.

Magnesium accumulation increased significantly by 140% in B-14 roots exposed to 125 mg/kg nCuO **(**$p \leq 0.05$), but only in the most external tissue, the periderm. None of the other treatments had a significant effect, compared with control. In cortex of B-14, Mg content was increased by all treatment concentrations, except with the 25 mg/kg of nCuO treatment, which had 9.2 mg/kg (60% less), respect to the control. No differences were observed in COV, which would be an effect of the high lignin content in the storage roots. Zuverza-Mena et al. (2015) reported a significant reduction in Mg content in cilantro roots of plants exposed to 20 and 80 mg/kg nCuO, bCuO, and $CuCl_2$. Likewise, Trujillo-Reyes et al. (2014) found that Mg was reduced in lettuce roots exposed to core-shell nanoscale materials of Cu/CuO at 10 and 20 mg/L.

Table 3.6. Distribution of the phosphorus and magnesium into the tissues of the sweetpotato storage roots in varieties Beauregard-14 and COV. Roots were exposed to soil amended with nano copper compounds and their analogues during all life cycle exposed to soil amended with copper compounds as nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/kg ($p \leq 0.05$;= 3).

Variety		Beauregard-14	Covington	Beauregard-14	Covington
		Macronutrient			
Tissue	Tratments	P (mg/kg)		Mg (mg/kg)	
Periderm	Control	7,101.4± 314.9a	1,398.9± 297.0	54.0± 3.6b	61.3± 5.0
	nCuO 25	5,551.1± 568.6a	1,100.2± 664.9	52.2± 9.3b	53.6± 9.9
	nCuO 75	6,429.6± 325.5a	2,853.0± 2025.8	69.5± 4.4b	57.3± 3.7
	nCuO 125	7,063.9± 867.6a	1,787.5± 571.8	77.3± 12.7a	49.2± 4.6
	bCuO 25	4,443.7± 391.3ab	1,713.3± 426.2	56.1± 2.6b	68.9± 6.4
	bCuO75	5,471.8± 293.0a	344.7± 165.4	61.9± 3.3b	57.2± 1.2
	bCuO125	6,812.2± 147.3a	3,268.5± 1252.7	61.5± 0.4b	46.1± 7.0
	$CuCl_2$ 25	6,077.1± 791.3a	907.5± 214.5	68.9± 8.8b	50.0± 3.5
	$CuCl_2$ 75	5,552.6± 982.0a	1,230.7± 689.8	60.1± 7.3b	65.3± 0.3
	$CuCl_2$ 125	600.7± 201.0b	2,240.5± 806.5	73.9± 5.6b	52.4± 3.2
Cortex	Control	5,732.0±739.6bc	7,220.6±845.8	15.2±1.3bc	36.5±4.4
	nCuO 25	4,373.1±592.1c	9,390.4±2069.3	9.2±0.8c	28.6±2.5
	nCuO 75	11,006.2±2070.4ab	9,794.0±935.6	43.0±6.2a	36.4±2.3
	nCuO 125	11,810.2±641.8a	10,707.6±744.5	46.8±8.0a	39.7±1.1
	bCuO 25	9,329.7±1365.4abc	10,298.9±1741.2	37.9±4.3ab	32.2±3.2
	bCuO75	10,586.4±1092.4ab	9,695.8±707.2	50.7±4.1a	34.9±2.2
	bCuO125	11,041.8±1120.4ab	12,284.2±1953.8	53.6±2.5a	57.1±3.3
	$CuCl_2$ 25	12,395.3±732.3a	8,098.7±639.4	48.5±2.8a	35.2±1.5
	$CuCl_2$ 75	11,583.1±1162.2ab	10,690.9±225.1	41.7±3.2a	36.0±3.6
	$CuCl_2$ 125	9,649.0±1508.1abc	11,475.0±983.7	47.8±6.7a	36.9±1.9
Medulla	Control	7,328.0± 2180.7	7,001.2± 516.6b	45.0±4.4	34.8±2.6
	nCuO 25	7,567.1± 1570.7	8,351.1± 1020.8ab	47.1±5.2	44.3±3.5
	nCuO 75	8,218.9± 2884.0	11,050.6± 759.7ab	44.1±10	54.3±12.0
	nCuO 125	12,455.4± 1809.2	8,067.4± 421.6ab	44.4±8.0	48.1±6.6
	bCuO 25	8,659.9± 1165.8	6,747.5± 1694.0b	39.1±5.4	36.7±10.0
	bCuO75	6,269.2± 499.0	7,852.0± 630.1ab	37.6±1.3	47.2±9.5
	bCuO125	5,226.7± 849.4	8,376.0± 468.4ab	31.2±2.4	43.8±9.0
	$CuCl_2$ 25	6,316.3±1772.3	6,750.3± 509.4b	34.9±8.5	31.0±3.3
	$CuCl_2$ 75	6,579.6± 183.0	13,283.4± 1440.5a	45.0±4.2	63.0±12.16
	$CuCl_2$ 125	9,870.7± 1217.5	10,837.6± 2632.7ab	63.7±14.7	57.8±18.3

3.3.6 Micronutrient accumulation in storage roots

Only three of the micronutrients analyzed (Cu, Zn, and Mn) showed significantly differences in roots of pants exposed the Cu compounds (Tables 3.4 -3.7).

Cu accumulation- The Cu concentration was enhanced by 1000 %, respect to the control, in the periderm of B-14 (Table 3.2) with the treatment of 25 mg/kg bCuO (193.5 ± 22.5 mg/kg) ($p \leq 0.05$). In the innermost tissues of the storage roots, the medulla (Table 3.5), it was observed that $CuCl_2$ at 125 mg/kg increased Cu by 722%, respect to control ($p \leq 0.05$). Similar results were obtained by Bradfield et al. (2017), who exposed the Georgia Jet variety to 100 mg/kg nCuO and found that the Cu concentration increased from 5 to 16 mg/kg. The concentrations used in the present study were slightly higher than that used by Bradfield et al. (2017).

Zinc accumulation- The Zn concentration increased in the periderm of B-14 as much as 172%, respect to the control (0.31 ± 0.03 mg/kg) at 125 mg/kg $CuCl_2$ ($p \leq 0.05$). In addition, Zn increased by 175%, respect to the control, in the cortex of B-14 (0.35 ± 0.08 mg/kg) plants exposed to 125 mg/kg nCuO, which was supposed to be due to the apoplastic translocation of the Zn. Contrarily, Zn was reduced by 70%, respect to control, in the cortex of B-14 roots exposed to nCuO at 25 mg/kg ($p \leq 0.05$). On the other hand, 75 mg/kg $CuCl_2$ increased Zn by 185%, compared with control, in the periderm of COV ($p \leq 0.05$). Varied responses were observed in cortex samples, which could be due to the high lignin content of the COV variety. Lequeux et al. (2010), working with *Arabidopsis thaliana*, found that $CuSO_4$ at concentrations of 2.5 and 5 μM resulted in 371.7 ± 31.9 mg/g and 281.5 ± 11.0 mg/g DW, respectively in roots.

Manganese accumulation- In COV variety, Mn was enhanced in cortex, by 1,206% and 1,310 %, in plants exposed to 125 mg/kg bCuO and 125 mg/kg $CuCl_2$, respectively, compared to control ($p \leq 0.05$). No variations were found in B-14. Similar to the other variables, the differences

between varieties were attributed to the lignin content, which may cause a strong interaction with metallic elements.

Nickel accumulation- As with other elements, the nickel absorption was different between the two varieties. In B-14 (Table 3.7), Ni increased by 240% in medulla of roots exposed to 75 mg/kg $CuCl_2$, but decreased by 38% and 59% in roots exposed to 25 mg/kg nCuO or 25 mg/kg $CuCl_2$, respectively ($p \leq 0.05$). The reduction in Ni absorption on B-14 could be explain by the increased Zn uptake by roots exposed to nCuO and $CuCl_2$. Previous studies have shown that Ni and Zn are transported by the same carrier (Cataldo et al., 1978); thus, by simple interference, an increase in Zn resulted in a reduction of Ni. Medina-Velo et al. (2018) reported that ZnO NPs significantly reduced Ni uptake in kidney bean plants. In soybean seedlings (*Glycine max* cv. Williams), the presence of Cu, Zn, and Fe inhibited the transport of Ni from the soil solution to the roots; thus, its absorption by shoot tissues (Cataldo et al., 1978).

Table 3.7. Distribution of the zinc, manganese, and nickel into the storage root tissues of the sweetpotato storage roots in varieties Beauregard-14 and COV. Roots were exposed to soil amended with nano copper compounds and their analogues during all life cycle exposed to soil amended with copper compounds as nCuO, bCuO, and $CuCl_2$ at 0, 25, 75, and 125 mg/kg (*p* ≤ 0.05; n = 3).

			Treatments									
Tissue	**Micronutrient**	**Variety**	**Control**	**nCuO 25**	**nCuO 75**	**nCuO 125**	**bCuO 25**	**bCuO75**	**bCuO125**	**$CuCl_2$ 25**	**$CuCl_2$ 75**	**$CuCl_2$ 125**
Periderm	**Zn (mg/kg)**	B-14	0.18± 0.01b	0.22± 0.04ab	0.30± 0.05ab	0.25± 0.04ab	0.19± 0.02ab	0.23± 0.02ab	0.16±0.01b	0.27±0.06ab	0.24±0.03ab	0.31±0.03a
		COV	0.20±0.02b	0.26±0.01ab	0.22±0.03ab	0.22±0.03ab	0.25±0.01ab	0.23±0.02ab	0.26±0.02ab	0.19±0.01b	0.37±0.04a	0.22±0.05ab
Cortex	**Zn (mg/kg)**	B-14	0.20±0.01ab	0.14±0.01b	0.25±0.05ab	0.35±0.08a	0.19±0.03ab	0.28±0.03ab	0.26±0.03ab	0.23±0.02ab	0.26±0.04ab	0.33±0.07ab
		COV	0.24±0.02	0.25±0.02	0.35±0.02	0.28±0.04	0.28±0.03	0.23±0.01	0.40±0.03	0.29±0.01	0.30±0.02	0.22±0.06
	Mn (mg/kg)	B-14	0.18±0.04	0.06±0.02	0.75±0.04	0.41±0.05	0.36±0.03	0.30±0.04	0.33±0.04	0.55±0.1	0.31±0.02	0.38±0.07
		COV	0.29±0.08b	1.24±0.7b	2.5±0.7ab	2.5±0.4ab	1.7±0.4b	1.7±0.5b	3.5±0.7a	1.0±0.4b	0.6±0.4 b	3.8±0.3 a
Medulla	**Zn (mg/kg)**	B-14	4.56± 0.27	0.26± 0.05	0.24± 0.09	0.32± 0.06	0.23± 0.03	0.18± 0.02	0.17± 0.03	3.0± 0.06	0.30± 0.03	0.42± 0.08
		COV	0.20± 0.02b	0.27± 0.03ab	0.29± 0.04ab	0.20± 0.01ab	0.19± 0.04b	0.20± 0.01ab	0.22± 0.01ab	0.14± 0.01b	0.35± 0.04a	0.24± 0.09ab
	Ni (mg/kg)	B-14	28.4± 8.2 abc	11.0± 5.8 c	29.7± 8.7 abc	36.3± 7.4 abc	28.0± 5.3 ab	24.8± 4.5 abc	24.0± 9.4 bc	16.8± 7.0 c	68.3± 7.5 a	61.6± 3.7 ab
		COV	89.8± 8.0	30.1± 7.7	15.2± 1.9	36.1± 8.8	93.7± 9.6	29.1± 6.9	51.8± 18.5	25.8± 1.9	52.9± 11.6	103.2± 7.3

3.3.7 Protein analysis

Figure 3.7 shows the interaction of the copper treatments and the percentage of crude protein content in the sweetpotato storage roots. There were no statistically significant differences in protein content from the treatments respect to the control. This suggests that the interaction of Cu-based NPs, or compounds, with differences in lignin content, at least in a root crop like sweetpotato, does not affect the production of biopolymers, like proteins. However, in other types of crops, like bean producers, Cu-based NPs, or compounds, have shown significant interaction with protein production. Apodaca et al. (2018) found that bCu NPs (50 and 100 mg/kg) increased the protein content by 11% and 12%, respectively, in kidney bean grains ($p \leq 0.05$). Conversely, Ochoa et al. (2018) reported no significant changes in protein content in green pea exposed to 50 and 100 mg/kg of nCuO, bCuO, and $CuCl_2$.

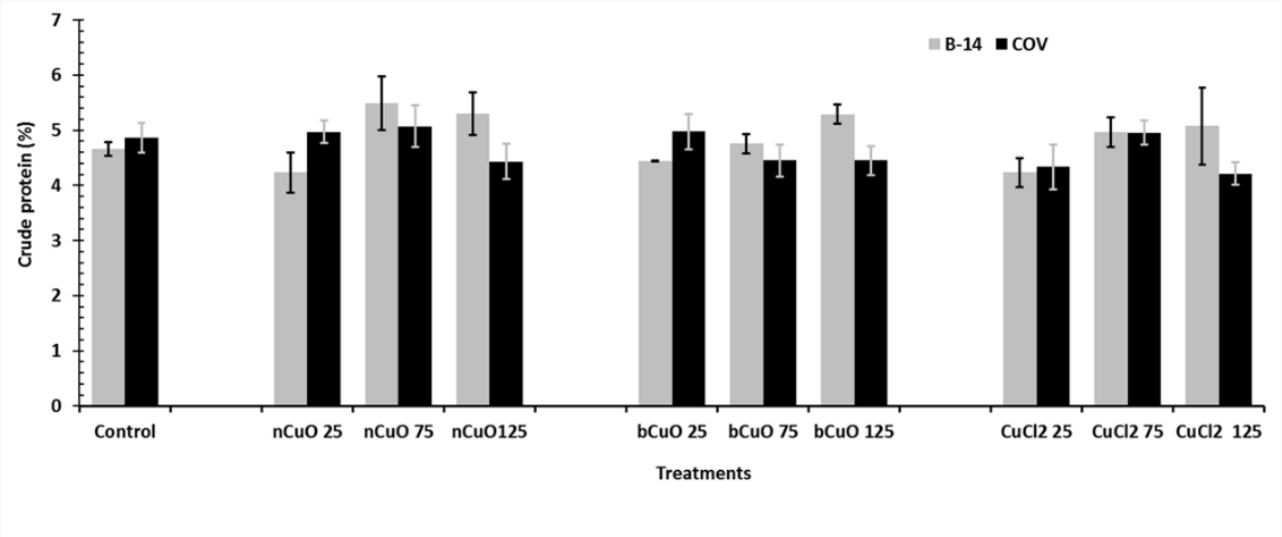

Figure 3.7. Measurement of the crude protein in the edible part of sweetpotato storage roots developed in soil amended with nCuO, bCuO, and $CuCl_2$ at concentration of 25, 75, and 125 mg/kg. Data are average of six replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$ (n=3).

3.3.8 Sugar and starch concentrations

Figure 3.8-A shows the concentration of sugars in sweetpotato storage roots of B-14 and COV varieties. As seen in this figure, lignin had an effect in the response of these varieties to Cu-based compounds' exposure. No significant differences were observed on COV, the variety with higher lignin content. However, B-14 roots exposed to 75 mg/kg nCuO, significantly increased the sugar content by 140%, respect to the control ($p \leq 0.05$). Data on starch content showed reduction in B-14 and COV, mainly under exposure to bCuO (25 and 125 mg/kg) and $CuCl_2$, also at 25 and 125 mg/kg (Figure 3.8-B). Reductions with bCuO in B-14 were about 71%, 21%, and 36% for bCuO at 25 and 125 mg/kg, and $CuCl_2$ at 25 mg/kg. For COV, the reductions were about 80%, 67%, 80%, and 60% with bCuO at 25 and 125 mg/kg, 25 and 125 mg/kg of $CuCl_2$, respectively.

Additionally, Figure 3.9 shows some changes in the starch grains. As seen in Figure 3.8A and 3.8B, control roots had round starch grains, while nCuO at 125 mg/kg showed mainly round grains, but some of them were a little bit curved (Figure 3.8C and 3.8D). On the other hand, at 125 mg/kg, bCuO and $CuCl_2$ both showed a reduced number of grains per sample, and most of them were misshaped.

Panou-Filotheou et al. (2001) showed that Cu avoided the formation of starch in oregano. Alaoui-Sossé et al. (2004) reported no changes in sugar and starch in cucumber exposed to Cu. Varied results have been published regarding the effects of nanoparticles in plant macromolecules. Ochoa et al. (2018) reported that nCuO, bCuO, and $CuCl_2$ at 50 and 100 mg/kg did not affect sugar content in green pea seeds. Rico et al. (2013) mentioned that in rice, CeO_2 NPs did not affect sugar content but reduced starch content. Du et al. (2015) also reported that $nCeO_2$ changed the number and size of starch granules in endosperm cells of wheat. Du et al. (2018) also reported that nCu and µCu reduced sugar and starch content in oregano leaves. The significant increase in sugar ($p \leq 0.05$) and a numerical increase in starch in roots of the variety with low lignin content suggest that nCuO at 75 mg/kg, has great potential as nanofertilizer for sweetpotato varieties with low lignin content.

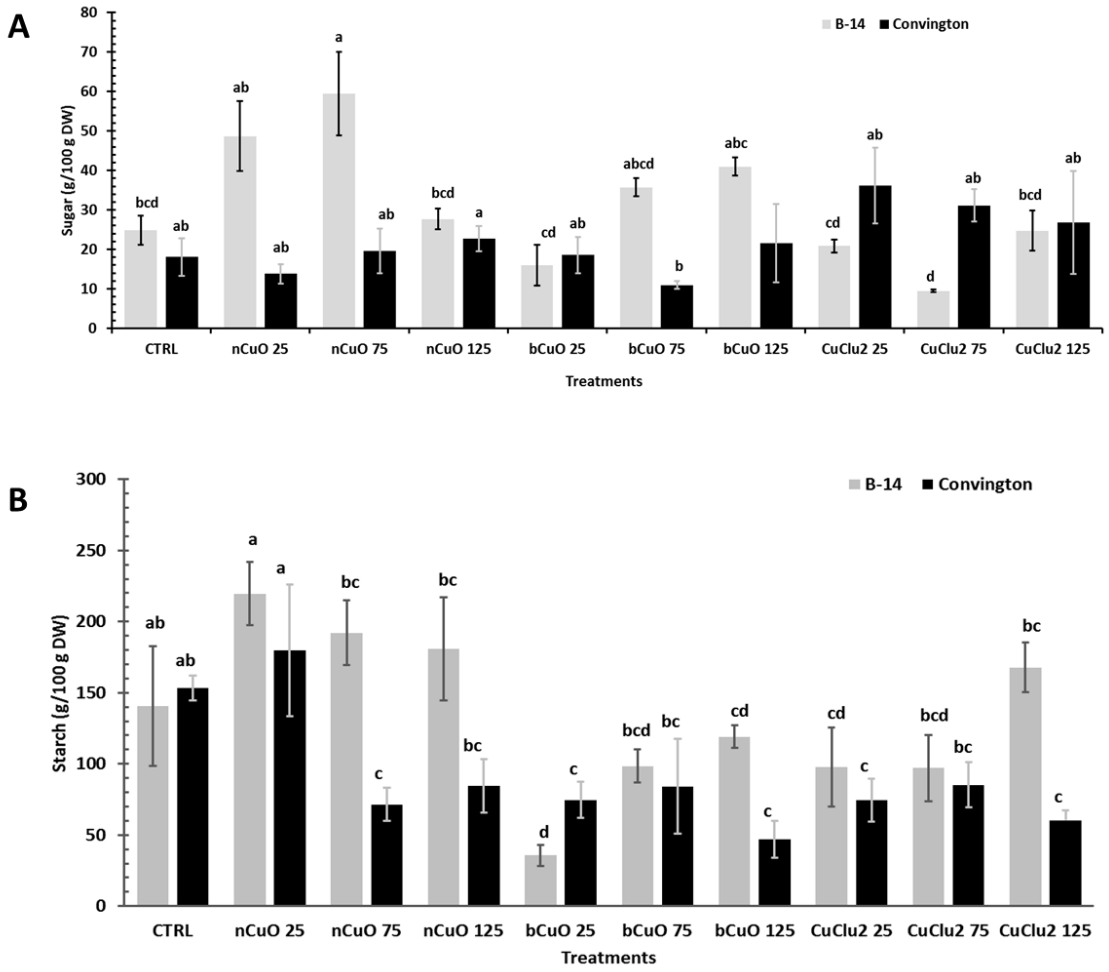

Figure 3.8. Measurement of the sugar (A) and starch (B) in the edible part of sweetpotato storage roots developed in soil amended with nCuO, bCuO, and $CuCl_2$ at concentration of 25, 75, and 125 mg/kg. Data are average of six replicates ± SE. Different letters stand for statistical differences at $p \leq 0.05$ (n=3).

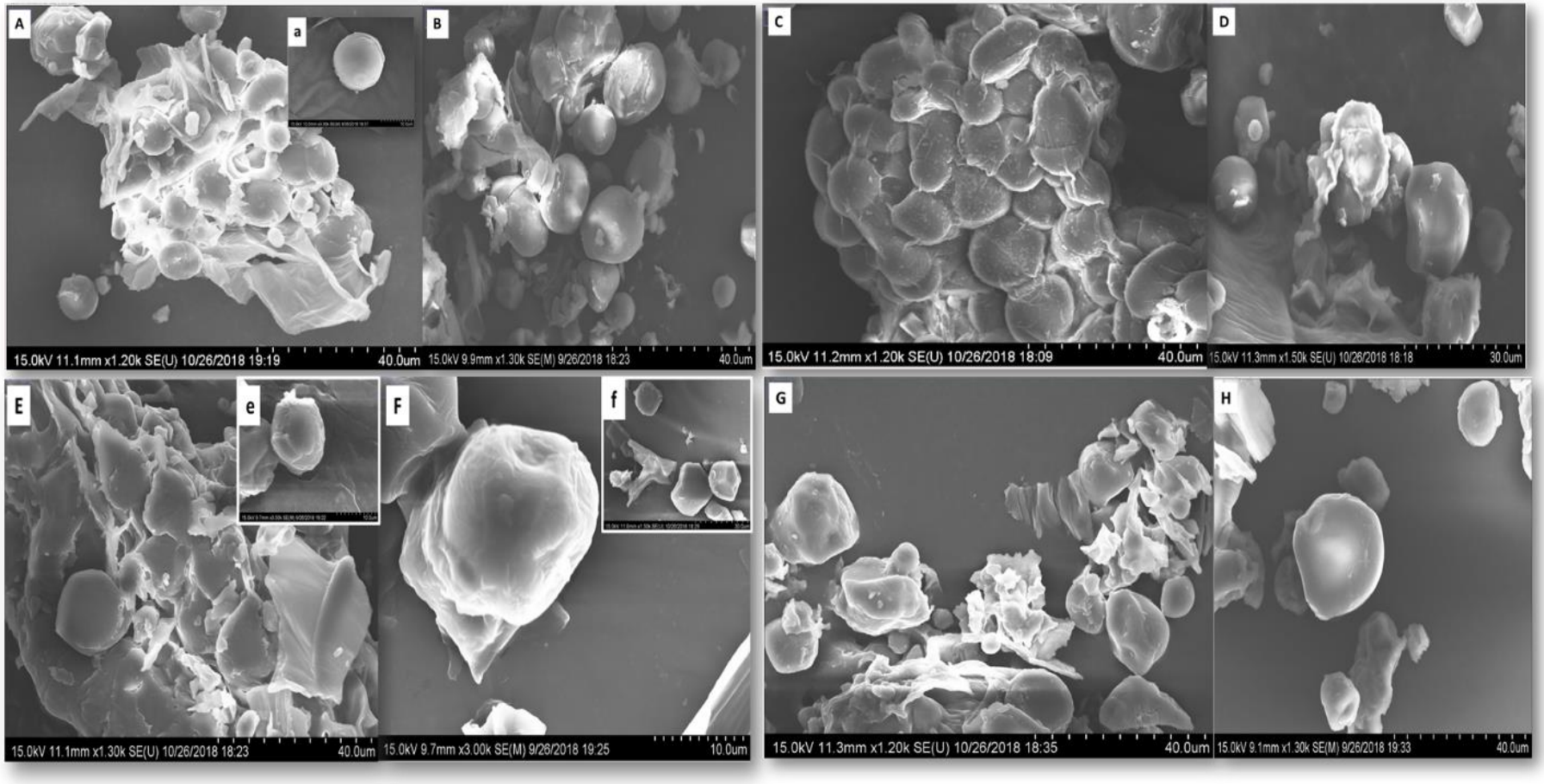

Figure 3.9. TEM micrographs of the starch grains evaluated in this research from variety (Covington). (A-B) Control, (a) insert amplification from one starch grain. (C-D) Starch grains under treatment with 125 mg nCuO/kg. (D) Close up of the starch grains. (E) Starch grains under treatment with 125 mg bCuO/kg. (F) Close up of the starch grain. (G) Starch grains under treatment with 125 mg/kg $CuCl_2$.

3.4 CONCLUSIONS

In summary, this study demonstrated that lignin content modulated the response of sweetpotato plants to CuO and $CuCl_2$ exposure. The chlorophyll content was increased at 75 mg/kg nCuO in COV (high lignin content), while the intercellular CO_2 was reduced in B-14 (low lignin content) under exposure to bCuO at 75 mg/kg. The photosynthesis rate was increased in COV, while water efficiency was increased in B-14. Most of the macro and micronutrients were affected in B-14, but not in COV. Macronutrients, such as P, increased in the cortex of B-14 under exposure to 125 mg/kg of nCuO and 25 mg/kg $CuCl_2$. In addition, 75 mg/kg of $CuCl_2$ increased the P concentration in medulla of COV. In B-14, Mg increased at periderm level under exposure to 125 mg of nCuO/kg ($p \leq 0.05$). Finally, copper compounds, in all forms and concentrations, did not affect the protein content. However, the sugar content was increased in B-14 at 25 mg nCuO/kg and 75 mg nCuO/kg, by 96% and 140%, respectively, respect to control. Contrarily, at all concentrations bCuO and $CuCl_2$ decreased the sugar content in COV and B-14. The starch content was affected the most by bCuO and $CuCl_2$ at all concentrations, respect to the control, in both varieties ($p \leq 0.05$). Electron microscope images showed that at 125 mg/kg, bCuO and $CuCl_2$ produced changes in the shape and morphology of starch grains in storage roots.

Chapter 4. Conclusions

The present doctoral research was conducted to understand the interaction of nano copper oxide (nCuO) with the surface tissues of storage roots and their effects in the development and yield of the sweetpotato (*Ipomoea batatas* L. Lam.) plants. This study sheds light on the potential use of nCuO on sweetpotato plant cultivation and storage root treatment. The responses from nCuO were compared to those of bulk CuO and $CuCl_2$.

In this research, the objective of Phase I was to determine whether the lignin content in the surface of storage roots and the curing process had effects on the penetration of CuO particles/ions into the root tissues. Beauregard-14 (B-14, low lignin) and Covington (COV, high lignin), were exposed to the Cu products before and after curing. Images from the 2-photon microscope showed the fluorescence of nCuO/bCuO in the periderm and cortex. The ICP-OES data showed significantly higher Cu concentrations in the surface of roots exposed to the Cu compounds after curing. Moreover, the variety with higher lignin content had more Cu, suggesting a strong effect of lignin in Cu retention. The presence of Cu in external layers suggested that CuO nanoparticles can be used as an alternative to preserve and increase the shelf life of sweetpotato roots before they reach the market.

The objectives of Phase II were to study the physiological effects of the three types of copper compounds in sweetpotato plant growth and production. Results demonstrated that the root lignin content modulates the response of the plant to the copper products. For instance, the chlorophyll content increased in COV exposed to nCuO at 75 mg/kg, compared to control. The nCuO improved water efficiency in the variety with less lignin but converse results were found in the photosynthesis rate. The lignin content also controlled the effects of copper compounds on root production. While no significant effect was found in the root weight, nCuO at 25 mg/kg

significantly increased root length in COV. On the other hand, imbalances in macronutrients P and Mg were mainly observed in B-14. While a variety of responses were observed in the absorption of Ni, Mn, and Zn, which were the only three microelements affected by nCuO at 25 mg/kg. Finally, none of the treatments affected the protein content, while the sugar content was significantly increased in Beauregard-14 exposed to 75 mg/kg nCuO.

It is worth noting that bCuO and $CuCl_2$, at 125 mg/kg, reduced the number of starch grains and misshaped their morphology, unlike the nCuO, which showed no difference, compared with control. In summary, the results of this research suggest that nCuO are a good alternative to protect sweetpotato plants during cultivation and increase the shelf life of the roots.

References

Adams, J. et al., 2017. Cu from dissolution of CuO nanoparticles signals changes in root morphology. *Plant Physiology and Biochemistry*, 110, pp.108–117. Available at: http://dx.doi.org/10.1016/j.plaphy.2016.08.005.

Adeleye, A.S. et al., 2014. Influence of extracellular polymeric substances on the long-term fate, dissolution, and speciation of copper-based nanoparticles. *Environmental Science and Technology*, 48(21), pp.12561–12568.

Adhikari, T. et al., 2012. Effect of Copper Oxide Nano Particle on Seed Germination of Selected Crops. *Journal of Agricultural Science and Technology A Journal of Agricultural Science and Technology*, 2(May 2014), pp.815–823. Available at: https://www.researchgate.net/publication/262178472.

Alaoui-Sossé, B. et al., 2004. Effect of copper on growth in cucumber plants (Cucumis sativus) and its relationships with carbohydrate accumulation and changes in ion contents. *Plant Science*, 166(5), pp.1213–1218.

Ali, M.B. et al., 2006. Phenolics metabolism and lignin synthesis in root suspension cultures of Panax ginseng in response to copper stress. *Plant Science*, 171(1), pp.147–154.

Apodaca, S.A. et al., 2018. Different forms of copper and kinetin impacted element accumulation and macromolecule contents in kidney bean (Phaseolus vulgaris) seeds. *Science of the Total Environment*, 636, pp.1534–1540. Available at: https://doi.org/10.1016/j.scitotenv.2018.04.360.

Barrios, A.C. et al., 2016. 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, pp.956–964. Available at:

http://dx.doi.org/10.1016/j.scitotenv.2015.11.143.

Bernard, F., Navab Moghadam, N. & Mirzajani, F., 2015. The effect of colloidal silver nanoparticles on the level of lignification and hyperhydricity syndrome in Thymus daenensis vitro shoots: a possible involvement of bonded polyamines. *In Vitro Cellular and Developmental Biology - Plant*, 51(5), pp.546–553.

Biemelt, S., Tschiersch, H. & Sonnewald, U., 2004. Impact of Altered Gibberellin Metabolism on Biomass Accumulation, Lignin Biosynthesis, and Photosynthesis in Transgenic Tobacco Plants. *Plant Physiology*, 135(1), pp.254–265. Available at: http://www.plantphysiol.org/cgi/doi/10.1104/pp.103.036988.

Bonilla-Bird, N.J., 2015. Morphological and Physiological Characterization of Sweetpotato Roots After Skinning. (PhD Dissertation, Mississippi State University). , 1(December), pp.1–86. Available at: https://search.proquest.com/docview/1747435216?pq-origsite=gscholar.

Bradfield, Scott J.; Kumar, Pawan.; White, Jason C.; Ebbs, S.D., 2016. Zinc, Copper, or Cerium Accumulation from Metal Oxide Nanoparticles or Ions in Sweet Potato: Yield Effects and Projected Dietary Intake From Consumption. *Plant Physiology and Biochemistry*, XXX, pp.1–10.

Brooke, A.E.; Holmes, G.J., 2009. Evaluation of Alternative Decay Control Products for Control of Postharvest Rhizopus Soft Rot of Sweetpotatoes Plant Health Progress. , (December 2008).

Burkhead, J.L. et al., 2009. Tansley review. Copper homeostasis. *New Phytologist*, 182(4), pp.799–816. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19402880.

Cabús, A.; Pellini, M.; Zanzotti, R.; Devigili, L.; Maines, R.; Giovannini, O.; Mattedi, L.

Mescalchin, E., 2017. Efficacy of reduced copper dosages against Plasmopara viticola in organic agriculture. *Crop Protection*, 96, pp.103–108. Available at: http://dx.doi.org/10.1016/j.cropro.2017.02.002.

Von Caemmerer, S. et al., 2004. Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. *Journal of Experimental Botany*, 55(400), pp.1157–1166.

Caspi, V., Droppa, M. & Raskin, V.I., 1999. The effect of copper on chlorophyll organization during greening of barley leaves. *Photosynthesis Research*, pp.165–174.

Cataldo, D. a. et al., 1978. Nickel in Plants. *Plant physiology*, 62(4), pp.566–570.

Chen, E.L. et al., 2002. Effect of copper on peroxidase activity and lignin content in Raphanus sativus. *Plant Physiology and Biochemistry*, 40(5), pp.439–444.

Chen, W. et al., 2010. Chlorine nutrition of higher plants: Progress and perspectives. *Journal of Plant Nutrition*, 33(7), pp.943–952.

Clark, C.A. et al., 2010. Performance of sweetpotato foundation seed after incorporation into commercial operations in Louisiana. *HortTechnology*, 20(6), pp.977–982.

Deng, F., Wang, S. & Xin, H., 2016. Toxicity of CuO Nanoparticles to Structure and Metabolic Activity of Allium cepa Root Tips. *Bulletin of Environmental Contamination and Toxicology*, 97(5), pp.702–708. Available at: http://dx.doi.org/10.1007/s00128-016-1934-0.

Dimkpa, C.O. et al., 2012. CuO and ZnO nanoparticles: Phytotoxicity, metal speciation, and induction of oxidative stress in sand-grown wheat. *Journal of Nanoparticle Research*, 14, pp.1–15.

Dizaj, S.; Lotfipour, F.; Barzegar-Jalali, M.; Zarrintan, M;. Adibkia, K., 2014. Antimicrobial Activity of the Metals and Metal Oxide Nanoparticles. *Materials Science and Engineering*

C, 44, pp.278–284.

Dragović, S.; Mihailović, N.; Gajić, B., 2008. Heavy Metals in Soils: Distribution, Relationship with Soil Characteristics and Radionuclides and Multivariate Assessment of Contamination Sources. *Chemosphere*, 72(3), pp.491–495.

Du, W. et al., 2017. Interaction of metal oxide nanoparticles with higher terrestrial plants: Physiological and biochemical aspects. *Plant Physiology and Biochemistry*, 110, pp.210–225. Available at: http://dx.doi.org/10.1016/j.plaphy.2016.04.024.

E. M.R. Lioy, P.J.G.P.G.R.A.Y.-L.J.O. et al., 2001. Environmental Copper: its Dynamics and Human Exposure Issues. *Journal of Toxicology and Environmental Health*, 4(4), pp.341–394. Available at: hemport.cas.org/cgi-bin/sdcgi?APP=ftslink&action=reflink&origin=rsc&version=1.0&coi=1:CAS:528:DC%2BD3MXotVSltrk%3D&md5=69742cf3e00b168868c7780579a02485.

Eckert, J.W.; Ogawa, J.M., 1988. The Chemical Control of Postharvest Diseases: Subtropical and Tropical Fruits. *Annual Review of Phytopathology*, 26(1), pp.433–69. Available at: http://dx.doi.org/10.1146/annurev.py.23.090185.002225.

Edmunds, B.; Boyette, M.; Clark, C.; Ferrin, D.; Smith, T.; Holmes, G., 2003. *Postharvest Handling of Sweetpotatoes*, North Caroline.

Ekanayake, I.J. & Collins, W., 2004. Effect of irrigation on sweet potato root carbohydrates and nitrogenous compounds. *Journal of Food Agriculture & Environment*, 2(1), pp.243–248.

FAO, 2016. FAOSTAT CROPS. Available at: http://www.fao.org/faostat/en/#data/QC.

Feijó, J.A.; Moreno, N., 2004. Imaging Plant Cells by Two-photon Excitation. *Protoplasma*, 223(1), pp.1–32.

Firon, N. et al., 2013. Transcriptional profiling of sweetpotato (Ipomoea batatas) roots indicates

down-regulation of lignin biosynthesis and up-regulation of starch biosynthesis at an early stage of storage root formation. *BMC Genomics*, 14(1).

George, W., 2005. Nanoscience, Nanotechnology, and Chemistry. *small*, 1(2), pp.172–179.

Georgopoulos, P. G.; Roy, A.; Yonone-Lioy, M. J.; Opiekun, R. E.; Lioy, P.J., 2001. Environmental copper: Its dynamics and human exposure issues. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews*, 4(4), pp.341–394.

Gerritsen, H. C.; De Grauw, C.J., 1999. Imaging of Optically Thick Specimen Using Two-photon Excitation Microscopy. *Microscopy Research and Technique*, 47(3), pp.206–209.

Giannousi, K.;Avramidis, I.; Dendrinou-Samara, C., 2013. Synthesis, Characterization and Evaluation of Copper Based Nanoparticles as Agrochemicals Against Phytophthora Infestans. *RSC Advances*, 3(44), p.21743. Available at: http://xlink.rsc.org/?DOI=c3ra42118j.

Giannousi, K.;Pantazaki, A.;Dendrinou-Samara, C., 2017. Copper-Based Nanoparticles as Antimicrobials. *Nanostructures for Antimicrobial Therapy*, pp.515–529.

Hajrasuliha, S., 1980. Accumulation and toxicity of chloride in bean plants. *Plant and Soil*, 55(1), pp.133–138.

Hong, J. et al., 2015. Toxic Effects of Copper-based Nanoparticles or Compounds to Lettuce (Lactuca sativa) and Alfalfa (Medicago sativa). *Environ Sci Process Impacts*, 23(17), pp.177–185.

Huaman, Z., 1992. Systematic Botany and Morphology of the Sweetpotato Plant. *Technical-Information-Bulletin---International-Potato-Center*, pp.1–22.

Huang, Y. et al., 2018. Quantitative analysis of changes in amino acids levels for cucumber (Cucumis sativus) exposed to nano copper. *NanoImpact*, 12, 2018, pp.9–17. Available at:

https://doi.org/10.1016/j.impact.2018.08.008.

Iavicoli, I.; Leso, V.; Beezhold,; D.H. Shvedova, A., 2017. Nanotechnology in Agriculture: Opportunities, Toxicological Implications, and Occupational Risks. *Toxicology and Applied Pharmacology*, 329, pp.96–111. Available at: http://dx.doi.org/10.1016/j.taap.2017.05.025.

Kah, M., 2015. Nanopesticides and Nanofertilizers: Emerging Contaminants or Opportunities for Risk Mitigation? *Frontiers in Chemistry*, 3(November), pp.1–6. Available at: http://journal.frontiersin.org/Article/10.3389/fchem.2015.00064/abstract.

Karna, P. et al., 2011. Polyphenol-rich sweet potato greens extract inhibits proliferation and induces apoptosis in prostate cancer cells in vitro and in vivo. *Carcinogenesis*, 32(12), pp.1872–1880.

Keller, A.A. et al., 2013. Global life cycle releases of engineered nanomaterials. *Journal of Nanoparticle Research*, 15(6), pp.1–17.

Kopek, B.G. et al., 2017. HHS Public Access. , 61(5), pp.223–235.

Laurie, S.F.M.A.P.B.A., 2015. Biofortification of sweet potato for food and nutrition. *Food Research International; 2015*, 76, pp.962–970.

Lequeux, H. et al., 2010a. Response to copper excess in Arabidopsis thaliana: Impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. *Plant Physiology and Biochemistry*, 48, pp.673–682.

Lequeux, H. et al., 2010b. Response to copper excess in Arabidopsis thaliana: Impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. *Plant Physiology and Biochemistry*, 48(8), pp.673–682.

Lin, S. et al., 2009. Uptake, translocation, and transmission of carbon nanomaterials in rice plants. *Small*, 5(10), pp.1128–1132.

Ling, Q., Huang, W. & Jarvis, P., 2011. Use of a SPAD-502 meter to measure leaf chlorophyll concentration in Arabidopsis thaliana. *Photosynthesis Research*, 107(2), pp.209–214.

Masuko, T. et al., 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Analytical Biochemistry*, 339(1), pp.69–72.

Medina-Velo, I.A. et al., 2018. Minimal Transgenerational Effect of ZnO Nanomaterials on the Physiology and Nutrient Profile of Phaseolus vulgaris. *ACS Sustainable Chemistry and Engineering*, 6(6), pp.7924–7930.

Monreal, C. M.;Derosa, M.; Mallubhotla, S. C.; Bindraban, P. S.; Dimkpa, C., 2016. Nanotechnologies for Increasing the Crop use Efficiency of Fertilizer-Micronutrients. *Biology and Fertility of Soils*, 52(3), pp.423–437.

Nair, P.M.G. & Chung, I.M., 2014. A Mechanistic Study on the Toxic Effect of Copper Oxide Nanoparticles in Soybean (Glycine max L.) Root Development and Lignification of Root Cells. *Biological Trace Element Research*, 162, pp.342–352.

Nair, P.M.G. & Chung, I.M., 2015. Study on the correlation between copper oxide nanoparticles induced growth suppression and enhanced lignification in Indian mustard (Brassica juncea L.). *Ecotoxicology and Environmental Safety*, 113, pp.302–13. Available at: http://www.sciencedirect.com/science/article/pii/S0147651314005685 [Accessed November 20, 2015].

Naseer, S. et al., 2012. Casparian strip diffusion barrier in Arabidopsis is made of a lignin polymer without suberin. *Proceedings of the National Academy of Sciences*, 109(25), pp.10101–10106. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1205726109.

Nikookar, K., Moradshahi, A. & Hosseini, L., 2005. Physiological responses of Dunaliella salina and Dunaliella tertiolecta to copper toxicity. *Biomolecular Engineering*, 22(4), pp.141–146.

Nishikawa, T.; Sook, In.;Lee, M.; Shiraishi, N.; Ishikawa, T.; Ohta, Y.; Nishikimi, M., 1997. Identification of S100b Protein as Copper-binding Protein and Its Suppression of Copper-induced Cell Damage*. *THE JOURNAL OF BIOLOGICAL CHEMISTRY*, 37(12), pp.23037–23041.

Ochoa, L. et al., 2018. Copper oxide nanoparticles and bulk copper oxide, combined with indole-3-acetic acid, alter aluminum, boron, and iron in Pisum sativum seeds. *Science of the Total Environment*, 634, pp.1238–1245. Available at: https://doi.org/10.1016/j.scitotenv.2018.04.003.

Panou-Filotheou, H., Bosabalidis, A.M. & Karataglis, S., 2001. Effects of copper toxicity on leaves of oregano (Origanum vulgare subsp. hirtum). *Annals of Botany*, 88(2), pp.207–214.

Peng, Cheng.;Xu, Chen.;Liu, Qinglin.;Sun, Lijuan.;Luo, Yongming.;Shi, J., 2017. Fate and Transformation of CuO Nanoparticles in the Soil-Rice System during the Life Cycle of Rice Plants. *Environmental Science and Technology*, 51(9), pp.4907–4917.

Rawat, S. et al., 2018. Impacts of Copper Oxide Nanoparticles on Bell Pepper (Capsicum annum L.) Plants: a Full Life Cycle Study. *Environmental Science: Nano*, 5, pp.83–95. Available at: http://xlink.rsc.org/?DOI=C7EN00697G.

Ray H., D.R.C. et al., 2002. Heavy metal uptake by lignin: Comparison of biotic ligand models with an ion-exchange process. *Environmental Science and Technology*, 36(7), pp.1485–1490.

Ray, R.C. et al., 2005. Post harvest spoilage of sweetpotato in tropics and control measures. *Critical Reviews in Food Science and Nutrition*, 45(7–8), pp.623–644.

Ray, R.C. & Ravi, V., 2005. Post harvest spoilage of sweetpotato in tropics and control measures. *Critical Reviews in Food Science and Nutrition*.

Roberts, J.R. & Reigart, J.R., 2013. *Recognition and Management of Shock*, Washington,DC. Available at: http://linkinghub.elsevier.com/retrieve/pii/B9781455705542000071.

Shacklette, H.T. & Boerngen, J.G., 1984. Element concentrations in soils and other surfical materials of the conterminous United States. *U.S. Geological Survey Professional Paper 1270*, I, pp.1–63. Available at: q:/Euras/Literature/pdf database/Ti-193 Shacklette and Boerngen 1984.pdf.

Shelah, M.; Johnson, P.;Holdridge, G., 2015. Quantifying Exposure to Engineered Nanomaterials (QEEN) from Manufactured Products. Addressing Environmental, Health, and Safety Implications. In G. H. Morita, Shelah, Pat Johnson, ed. Arlington, VA: National Nanotechnology Coordination Office, p. 162.

Shirke, P.A. & Pathre, U. V., 2004. Influence of leaf-to-air vapour pressure deficit (VPD) on the biochemistry and physiology of photosynthesis in Prosopis juliflora. *Journal of Experimental Botany*, 55(405), pp.2111–2120.

Spectrum Technologies, I., 2009. *SPAD 502 Plus Chlorophyll Meter Product Manual*, Available at: http://www.specmeters.com/assets/1/22/2900P_SPAD_502.pdf.

Sun, H. et al., 2014. Sweet potato (Ipomoea batatas L.) leaves as nutritional and functional foods. *Food Chemistry*, 156, pp.380–389.

Terashima, I. et al., 2011. Leaf Functional Anatomy in Relation to Photosynthesis. *Plant Physiology*, 155(1), pp.108–116. Available at: http://www.plantphysiol.org/cgi/doi/10.1104/pp.110.165472.

Tomás, M. et al., 2013. Importance of leaf anatomy in determining mesophyll diffusion conductance to CO2across species: Quantitative limitations and scaling up by models. *Journal of Experimental Botany*, 64(8), pp.2269–2281.

Tosens, T. et al., 2012. Anatomical basis of variation in mesophyll resistance in eastern Australian sclerophylls: News of a long and winding path. *Journal of Experimental Botany*.

Trujillo-Reyes, J. et al., 2014. Exposure studies of core-shell Fe/Fe3O4 and Cu/CuO NPs to lettuce (Lactuca sativa) plants: Are they a potential physiological and nutritional hazard? *Journal of Hazardous Materials*, 267, pp.255–263.

Villavicencio, L.E. et al., 2007. Temperature Effect on Skin Adhesion, Cell Wall Enzyme Activity, Lignin Content, Anthocyanins, Growth Parameters, and Periderm Histochemistry of Sweetpotato. *Journal of the American Society for Horticultural Science*, 132(5), pp.729–738.

Villordon, A. et al., 2012. Characterization of lateral root development at the onset of storage root initiation in "Beauregard" sweetpotato adventitious roots. *HortScience*, 47(7), pp.961–968.

Walter, W.E. Jr.; Schadel, W.E., 1983. Structure and Composition of Normal Skin (Periderm) and Wound Tissue from Cured Sweet Potatoes. *J Am. Soc. Hort. Sci.*, 108(6), pp.909–914.

WZhenyu, W.; Xiaoyan, X.; Jian, Z. Xiaoyun, L. Wenqiang.; White, J.C.; Baoshan, X., 2012. Xylem- and Phloem-based Transport of CuO Nanoparticles in Maize (Zea mays L.). *Environmental Science and Technology*, 46, pp.4434–4441.

Zhang, Z. et al., 2018. Impact of copper nanoparticles and ionic copper exposure on wheat (Triticum aestivum L.) root morphology and antioxidant response. *Environmental Pollution*, 239, pp.689–697. Available at: https://doi.org/10.1016/j.envpol.2018.04.066.

Zhao, L. et al., 2012. Effect of surface coating and organic matter on the uptake of CeO 2 NPs by corn plants grown in soil: Insight into the uptake mechanism. *Journal of Hazardous Materials*, 225–226, pp.131–138. Available at:

http://dx.doi.org/10.1016/j.jhazmat.2012.05.008.

Zhu, Q., Zhang, M. & Ma, Q., 2012. Copper-based foliar fertilizer and controlled release urea improved soil chemical properties, plant growth and yield of tomato. *Scientia Horticulturae*, 143, pp.109–114. Available at: http://dx.doi.org/10.1016/j.scienta.2012.06.008.

Curriculum Vita

Nestor Javier Bonilla Bird was born on January 1, 1959 in Managua, Nicaragua. He earned his Bachelor of Science degree in Agronomy Engineering from the Universidad Nacional Agraria (UNA) de Nicaragua in 1984. He became a lecturer at the UNA in Nicaragua, in Botany, Seed production. In 1984, he traveled to Roma, Italy to get a training in seed quality control at the Food and Agriculture Organization (FAO) of the United Nations, and at the COOS-82 Seed Company in Verona, Italy for five months. Back his home country, joined the Genetic Resources Program at UNA-Nicaragua working in research with germplasm seed banks, and given a lectures in seed production. In 1988, He awarded a scholarship from the Japan International Cooperation Agency at the Tsukuba International Agricultural Training Center to specialize in Seed production and Quality control in vegetable seed production. In 1991, he was awarded with the LASPAU-FULBRIGTH scholarship to attend his master studies at Mississippi State University. In 1997, he joined the National seed Program (PROMESA-USAID-DAI) founding by the USAID, promoting the use of new grain crop varieties. From 2002, to 2005, he started to work with the INTA and after with the Agriculture Ministry of Nicaragua, and the Inter-American Development Bank (IDB). In 2007, he started working with the Inter-American Institute for Cooperation on Agriculture (IICA) project Red SICTA. In 2010, he started his doctorate studies in agriculture at Mississippi State University. In 2014, he was working for the INGAL- European project in Nicaragua. In 2015, he started his doctorate studies at the University of Texas at El Paso in the Environment Science Engineering Interdisciplinary PhD program, and work as Teaching Assistant at the Chemistry department.

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