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Investigation Into The Effects Of Copper Based Nanoparticles On Sugarcane (saccharum Officinarium) And Zucchini (cucurbita Pepo)

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INVESTIGATION INTO THE EFFECTS OF COPPER BASED NANOPARTICLES ON SUGARCANE (*SACCHARUM OFFICINARUM*) AND ZUCCHINI (*CUCURBITA PEPO*)

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Dedication

To Mom and Dad, I think I'm finally done with school.

"Science is a way of thinking much more than it is a body of knowledge."
– Carl Sagan

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by

CARLOS TAMEZ JR, MS

DISSERTATION

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Abstract

The widespread use of copper-based nanomaterials has been accompanied by an increasing interest to understand their potential risks. Due to high surface area to volume properties, nanomaterials are more reactive than their bulk counterparts. Copper nanoparticles are used in numerous products, and could enter the environment through their synthesis or incidental use. It is essential to understand the effects of nanoparticles on edible crops by performing both short-term and long-term experiments at relevant exposure concentrations. In order to evaluate biochemical and physiological effects crops, sugarcane and zucchini, were grown in soil amended with: Kocide 3000 (copper-based fungicide), nano-sized CuO (nCuO), a bulk micron-sized CuO (bCuO), copper nanoparticles (Cu NP), and $CuCl_2$. Briefly, our results show immature sugarcane plants increased their activity of stress enzymes ascorbic peroxidase (APX) and catalase (CAT), at varying concentrations. Concentrations of Cu in roots increased with treatment, with minimal translocation into aerial tissues. Mature sugarcane plants showed no changes in Cu concentrations in root and leaf tissues, but superoxide dismutase activity in sugarcane roots treated with Cu NP and $CuCl_2$ decreased by 55%. Zucchini grown for 3 weeks saw Cu concentrations in root, stem, and leaf tissues increase with rising treatment. APX activity in zucchini roots treated with Kocide, nCuO, and bCuO decreased 45%, while CAT activity in roots treated with Cu NP decreased 77%. Similarly, mature zucchini showed Cu increases in root, leaf, and flower tissues, with all applied treatments. CAT activity in roots increased only in plants treated with Cu NP at 400 mg kg^{-1}. In all studies, plant growth was unaffected by the applied treatments. Based on the results observed, sugarcane and zucchini displayed minimal negative effects upon exposure to copper-base nanoparticles at the tested concentrations.

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Chapter 1: Introduction

Nanotechnology is changing every aspect of science. Old technologies and ways of thought have been reinvigorated, thanks in part to the embrace of nanotechnology. Within that emerging world engineered nanoparticles have found a highly productive niche (Behzadnasab et al., 2011; Espitia et al., 2012; Horcajada et al., 2010; Hua et al., 2012; Kim et al., 2012; Koponen et al., 2011; Krishnaraj et al., 2010; Longano et al., 2012; Veiseh et al., 2010). Engineered nanoparticles are materials with at least two dimensions less than 100 nm (Bhatt and Tripathi, 2011). As the dimensions of a particle decrease, the surface area increases; an increased surface area allows a material to be more reactive, or otherwise display properties not found in their larger sized counterparts (Ma et al., 2010). Conventionally engineered nanoparticles are separate into two distinct categories, organic and inorganic (Peralta-Videa et al., 2011). Organic nanoparticles include: Carbon Nanotubes (both single walled and multi-walled) and fullerenes. Inorganic nanoparticles are further divided into: metals, metal oxides, and quantum dots.

Currently engineered nanoparticles such as: TiO_2, ZnO, CuO, CeO_2, Ag, and Au are found in a wide variety of commercial and industrial products. Sunscreens, food packaging, automotive paint coatings, and electronics are just a few of the everyday products that contain these vital materials. Of these metal-based nanoparticles only Ag, TiO_2 and ZnO have applications approved by the FDA (anti-microbial bandages and sunscreens, respectively) (Sadrieh, 2005).

The ubiquity of nanoparticles poses distinct environmental challenges, especially at the massive quantities being produced. Keller et al. estimates the production of nano-copper and nano-copper oxides at 200 metric tons in 2010 (Keller et al., 2013). The fate of these manufactured materials was traced through the environment and was shown that approximately 25% of these materials were located in the soil, air, or bodies of water. The contamination of

soil and water with nanoparticles leaves plants, wildlife, and humans at risk of exposure with potentially dangerous side effects (Baek and An, 2011; Buffet et al., 2013, 2011; Gomes et al., 2011; Manusadžianas et al., 2012; Saison et al., 2010; Shaw et al., 2012). In addition to contamination via waste disposal, there is also an increased demand for authorization to apply copper-based nanoparticles onto agricultural lands and crops (Naderi and Danesh-Shahraki, 2013). These demands bring urgency to the need to study copper-based nanoparticles such as: Copper metal nanoparticles (Cu NPs), nano-sized CuO (nCuO), a bulk micron-sized CuO (bCuO), and the commercially available fungicide Kocide 3000 ($Cu(OH)_2$). Currently there is not sufficient evidence to determine the implications of these nanoparticle's application onto food crops over the entire life cycle of the treated plants.

Interactions between nanoparticles and plants have been well documented, but the research area has a sizeable knowledge gap when it comes to copper-based nanoparticles(Clément et al., 2013a; Hong et al., 2014; Lee et al., 2012; Schwabe et al., 2013; Wang et al., 2015). Previously conducted studies using copper metal nanoparticles (Cu NPs) in hydroponic environments have shown a 77% reduction in root emergence and a 90% decrease in the biomass of squash (Stampoulis et al., 2009). A similar study also found that the growth and transpiration of squash was reduced by 60-70% in the presence of Cu NPs (Musante and White, 2010). Both of these studies found similar albeit, less toxic effects when non-nanosized (bulk) copper was exposed to the plants. Cucumber seedlings grown in suspensions of CuO, Cu metal, ZnO, or Zn metal NPs saw a significant decrease in biomass (Kim et al., 2012). Plants exposed to Zn or ZnO NPs experienced a 56% and 42% decrease in biomass, respectively. Alternatively, cucumber seedlings exposed to nCuO experienced a higher decrease in biomass, 75%, compared to 33% in plants exposed to Cu NPs. Atha et al. found significant amounts of DNA damage to radish sprouts grown in the presence of nano-sized copper oxide (nCuO). This same study also found decreased growth in radish, perennial ryegrass, and annual ryegrass (Atha et al., 2012).

Hydroponic studies have also been published using core/shell nanoparticles. Core/shell nanoparticles are materials that combine a metal core with a metal oxide shell, or vice versa. Trujillo-Reyes et al. sprouted lettuce in suspensions of Cu/CuO NPs and found diminished chlorophyll levels, up to 14%, as well as lower biomass and up to 50% shorter roots (Trujillo-Reyes et al., 2014). These results were mirrored in lettuce plants grown with copper ions from $CuSO_4$. Numerous hydroponic studies have been published using copper-based nanoparticles on different plants, ranging from aquatic weeds to maize, all obtaining similar results of diminished biomass, increased oxidative stress, and reduced chlorophyll content and photosynthetic activity (Hong et al., 2015; Johnson et al., 2011; Lee et al., 2013; Nekrasova et al., 2011; Perreault et al., 2010; Shaw and Hossain, 2013; Shi et al., 2011; Wang et al., 2012).

Servin et al. have demonstrated the uptake and translocation of intact nanoparticles, using hydroponically grown cucumber exposed to nano-sized TiO_2 (Servin et al., 2012). Using synchrotron and micro X-ray absorption near edge structure (micro-XANES) spectroscopy, Servin was able to confirm the chemical speciation of the translocated titanium to be consistent with TiO_2 nanoparticles. Translocation of nCuO has also been demonstrated using maize grown in a suspension of nanoparticles. Transmission Electron Microscopy (TEM) images confirmed transport of nCuO from root to shoot in the plant phloem (Wang et al., 2012). Other studies completed with wheat grown in sterilized sand have further confirmed the uptake of nCuO into plant shoots (Dimkpa et al., 2013, 2012).

From the literary search conducted here, few studies were performed using soil as the growth medium. In one report lettuce was sprouted in soil amended with Cu NPs; the authors found no difference in shoot-root ratios, when planted immediately after exposure (Shah and Belozerova, 2009). After a 15-day incubation period, higher concentrations of Cu NPs produced seedlings with high shoot/root ratios, indicating a reduction in root growth. Ebbs et al. looked at the effects of nCuO exposure to carrots grown in sand (Ebbs et al., 2016). Following 16 weeks of growth, the treated carrots showed no effects on biomass or root/shoot ratios when

compared to controls. The authors also found higher copper concentrations in the aerial tissues as opposed to the edible tap root.

It is clear that the vast majority of studies on the interactions of copper nanoparticles and plants are lacking in two serious ways: (1) The studies focus on plants grown in either a nutrient solution suspension or in soil medium that has been washed and/or sterilized. (2) The studies monitor only the effects that manifest during the early growth stages. This illustrates the need for experiments to be completed under more realistic conditions, using natural soil that has not been sterilized, with the effects of nanoparticle exposure going beyond the developmental stages and through the entire life cycle. It is also apparent for the literary search conducted, that more exploration of copper-based nanoparticle interaction with food crops is needed.

Sugarcane (*Saccharum officinarum*) is a perennial grass with a life cycle of approximately 12 to 20 months, and is grown predominately in tropical to subtropical regions. According to the FAO cane sugar accounts for 70% of the world's sugar supply, over 1.9×10^{12} kg, making it the top commodity in 2016 (Food and Agriculture Organization, 2016) . The United States is the 10^{th} largest grower of sugarcane, approximately 2.8×10^{10} kg, with the majority of cultivation occurring in: Florida, Louisiana, Texas, and Hawaii. Although the US is one of the top 10 producers of sugar cane, the vast majority of cane production occurs in newly industrialized countries such as: Brazil, China, India, and Mexico. These four countries alone account for 68% of total production, making sugarcane an important crop on both the national and global stages.

In 2017 the average American consumed nearly 18 kg of refined cane or beet sugar (USDA ERS, 2018). The potential for the translocation of copper nanoparticles into the aerial tissues of the sugarcane plant could present two potential problems: (1) the presence of copper nanoparticles in the edible tissues of sugarcane provides a direct route into the food chain, and (2) nanoparticles accumulated in the leaf tissue of sugarcane could be redistributed in the surrounding environment. In many parts of the world the edible parts of sugarcane are directly consumed, either by chewing of the stalk to extract the sugary juices or by pressing the stalk to

make sugarcane drinks. Mechanized harvesting of sugarcane requires the removal of all leaf tissue; setting an entire field on fire prior to harvest does this. The incineration of leaf tissue with copper nanoparticles can contaminate the surrounding air, exposing field workers and surrounding communities.

Grey Zucchini is a variety of summer squash belonging to the species *Cucurbita pepo*. Domestic cultivation of all squash varieties in the US is 2.6×10^{11} kg, representing a product value of 1.92 million dollars (USDA NASS, 2015). Zucchini is consumed worldwide and can be a significant source of minerals (e.g. magnesium, potassium, and phosphorus) and vitamin C (USDA ARS, 2015). Because there are many varieties of zucchini within the *C. pepo* species, Grey Zucchini could possibly serve as a model for nanoparticle exposure. If it holds true that closely related plant species would show similar effects, then it can be expected that zucchini will behave similar to the closely related cucumber, which has already demonstrated uptake and translocation of metal oxide nanoparticles (Servin et al., 2012).

This study aims to understand the effects and implications of copper-based nanoparticle exposure to both sugarcane and zucchini. To the best of the author's knowledge research into the effects of nanoparticle exposure to sugarcane is completely lacking, and more investigation with zucchini done under soil-based conditions is warranted. Understanding the effects of copper nanoparticle can be carried out by conducting both short term (over a few weeks) and complete life cycle studies on sugarcane and zucchini exposed to varying concentrations of: Kocide 3000 nCuO, bCuO, and $CuCl_2$. Overall plant health will be evaluated by determining nutrient content, via inductively coupled plasma optical emission spectroscopy (ICP-OES), monitoring plant growth, and biochemical analysis for chlorophyll content and enzymatic activity. The uptake of Cu into the root, stem and leaf tissues can be established using ICP-OES.

Research Objectives

1. Investigate the potential detrimental effects of Cu NPs, nCuO, bCuO, Kocide 3000, and $CuCl_2$ exposure on sugarcane and zucchini.
2. Determine if nanoparticles will translocate to the edible tissues of sugarcane or zucchini.

Specific Aims

- To measure physical changes (plant height, root length, and tissue mass) in sugarcane and zucchini exposed to copper-based nanoparticles.

- Determine biological effects of nanoparticle exposure by measuring biochemical indications of plant health such as: photosynthetic activity, oxidative stress indicators, and nutrient levels.

- Quantify uptake of copper in treated plants in comparison to control plants, confirm that copper speciation is consistent with that of the appropriate nanoparticle.

- Evaluate fruit quality, measure translocation of copper, and determine any correlation between quantity/quality and nanoparticle treatment.

Hypothesis

1. Plants exposed to copper treatments will be less productive than control plants.

2. Nanoparticles will be translocated from the roots to the aerial plant tissues, and possibly edible portions.

3. nCuO will be more toxic than bCuO.

4. Fruit production and quality will be negatively affected when exposed to nanoparticle treatments.

Chapter 2: Biochemical and physiological effects of copper compounds/nanoparticles on sugarcane (*Saccharum officinarum*)[1]

1. Introduction

The unique properties of engineered nanomaterials has led to its widespread applications in sectors ranging from medicine and energy to cosmetics and textiles (Jeevanandam et al., 2018). Apart from their increased surface area, copper-based nanoparticles (NPs) have unique electrical, chemical and thermal properties that makes them attractive for commercial purposes (Dang et al., 2011; Kim et al., 2009; Magdassi et al., 2010). Keller et al. estimates the production of nano-copper and nano-copper oxides at 200 metric tons in 2010 (Keller et al., 2013). The fate of these manufactured materials was traced through the environment and was shown that approximately 25% of these materials were located in the soil, air, or bodies of water. The contamination of soil and water with nanoparticles leaves plants, wildlife, and humans at risk of exposure with potentially dangerous side effects (Baek and An, 2011; Buffet et al., 2013, 2011; Gomes et al., 2011; Manusadžianas et al., 2012; Saison et al., 2010; Shaw et al., 2012). In addition to contamination via waste disposal, there is also an increased demand for authorization to apply copper-based nanoparticles onto agricultural lands and crops (Naderi and Danesh-Shahraki, 2013). These demands highlight the urgency to study copper-based nanoparticles such as: Copper metal nanoparticles (Cu NPs), nano-sized CuO (nCuO), a bulk micron-sized CuO (bCuO), and commercially available Kocide 3000 ($Cu(OH)_2$).

Interactions between nanoparticles and plants have been well documented, but the research area has a sizeable knowledge gap when it comes to copper-based nanoparticles (Clément et al., 2013b; Hong et al., 2014; Lee et al., 2012; Schwabe et al., 2013; Wang et al., 2015). Studies have been published showing the effects of Cu NP and nCuO exposure on lettuce, cucumber, wheat, rice, alfalfa, cilantro, maize, and yellow squash (Dimkpa et al., 2013,

[1]Reprinted from Tamez, C., Morelius, E.W., Hernandez-Viezcas, J.A., Peralta-Videa, J.R., Gardea-Torresdey, J., 2019. Biochemical and physiological effects of copper compounds/nanoparticles on sugarcane (Saccharum officinarum). Sci. Total Environ. 649, 554–562.

2012; Hong et al., 2015; Kim et al., 2012; Musante and White, 2010; Shaw and Hossain, 2013; Trujillo-Reyes et al., 2014; Wang et al., 2012). Published results indicated that nanoparticle exposure could lead to decreased biomass, reduced chlorophyll content, shorter roots and shoots, lower germination rates, and increased stress indicators. While investigations into the effects of copper nanoparticles on plants have been published, they are merely the building blocks of a much larger picture. The majority of the current research has been conducted only on small time scale and in hydroponic solutions; more work is needed under longer time frames and in natural soil conditions.

Sugarcane (*Saccharum officinarum*) is a perennial grass with a life cycle of approximately 12 to 20 months, and is grown predominately in tropical to subtropical regions. According to the FAO sugarcane accounts for 70% of the world's sugar supply, over 1.9×10^{12} kg, making it a top commodity in 2016 (Food and Argiculture Organization, 2016). Sugarcane's widespread use makes it a concern for nanoparticle contamination of the human food chain. As far as the authors are aware, no research has been published on the effects of copper nanoparticles on sugarcane. In order to investigate the potential for copper nanoparticle translocation a year-long experiment exposing sugarcane to Kocide 3000, Cu NP, bCuO, and $CuCl_2$ was conducted. Results show that stress indicators such as catalase and ascorbic peroxidase enzymatic activity were activated and Chlorophyll A content was higher in plants treated with Kocide 3000 at 20 and 60 mg kg^{-1}, bCuO at 20 mg kg^{-1}, and $CuCl_2$ at 20 and 60 mg kg^{-1}. Dose dependent increases in copper concentrations in sugarcane root tissues were also found in all treatments.

2. Methods

2.1. Nanoparticles

Nanoparticles were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). Particle sizes were previously determined by Hong et al. to range from 10 to 100 nm (bCuO), 100 to 1000 nm (Cu NP), and 10 μm (Kocide 3000). $CuCl_2$ salt was also used to serve as an ionic control.

2.2. Growth Conditions

Sugarcane stalks were obtained from Rio Farms, Inc., and were cut to give single sett pieces. Setts were sprouted in Miracle-Gro potting mix for 31 days in an environmental growth chamber (settings). After this initial period individual sprouts were transferred to 20 cm pots containing 3 kg of 1:1 (v/v) mixture of natural soil, previously characterized by Zhao et al. as loamy sand (3.7% clay, 12.2% silt, 84.1% sand, and 0.04% organic matter, pH 7.9) and Miracle-Gro potting mix. After a 24-hour settling period an environmental growth chamber, pots were transfer to a greenhouse with a 14 h photoperiod, light intensity of at least 340 μmol m^{-2} s^{-1}, 25/20 °C day/night temperature, and 70% relative humidity. Prior to use air dried soil was amended to contain 20, 40, or 60 mg kg^{-1} of Cu, using Cu NPs, bCuO, Kocide 3000, or $CuCl_2$.

2.3. Physiological Measurement

At the end of the experiment physical measurements were taken. Plant height was measured as the distance between the base of the plant (ground level) and the tip of the longest leaf. Stem height was measured from plant base to the base of the highest unfurled leaf. Stem diameter was measured at the base of the plant using digital calipers. While sugarcane progresses through its lifecycle additional secondary shoots began to grow, as a sign of plant growth the number of secondary shoots and the length of the largest shoot were recorded.

2.4. Enzymatic Activity Assays

Catalase (CAT) and ascorbic peroxides (APX) activity was measured by methods established by Murgia et al. and Gallego et al. In short, leaf tissue was homogenized in 25 mM KH_2PO_4 buffer. Supernatant will be decanted and measured using a Varian Cary 50 UV-Vis spectrophotometer in kinetics mode. CAT is will be measured at 240 nm for 3 minutes, APX will be measured at 265 nm for 2 min.

2.5. Chlorophyll Content

Chlorophyll content in leaf tissues was determined at the time of harvest by following procedures established by Kumar et al. Briefly, ~0.2 g of tissue was homogenized with 5 mL of

acetone, supernatant collected. Chlorophyll concentration in the resulting extract was then measured using a Varian Carey 50 UV-Visible Spectrophotometer at 646 nm and 663 nm wavelengths.

2.6. Copper Uptake and Nutrient Content

Copper uptake and nutrient content will be determined by separating plant tissues into root, stem, and leaf after harvesting. Tissue samples will be oven dried at 70°C for 72 hours, after which they will be homogenized using a household coffee grinder. Homogenized samples will be weighed to ~0.2 g and digested with concentrated plasma pure HNO_3 according to EPA Method 3051A or 3050B, using either microwave assisted digestion or hot block digestion, and diluted to 50 mL. Following digestion, macronutrients (Mg, Ca, S, K, and P) and micronutrients (Cu, Mo, B, Mn, and Fe) will be analyzed via either Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) or Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) using a Perkin Elmer Optima 4300 or a Perkin Elmer ELAN DRC II, respectively.

2.7. Statistical Analysis

Experiments will be completed in triplicate, with findings reported as averages of replicates ± standard error. Statistical significance was determined by conducting one-way analysis of variance (ANOVA) tests, with a p-value of 0.05, followed by Tukey Honest Significant Difference (Tukey HSD) post-hoc tests using R statistical software.

3. Results and Discussion

3.1. Physiological Measurements

In order to evaluate the effects of copper-based compounds and nanoparticles on the overall growth of sugarcane, several growth parameters were measured at the conclusion of the experiment. Figures 1 and 2 show the results of measurements taken for: plant height, plant stem diameter, number of secondary shoots, and secondary shoot height. Figure 1A shows the average plant height of treated sugarcane plants compared to control. Statistical analysis

revealed no significant differences between the heights of sugarcane treated with copper compounds or nanoparticles, when compared to control plants. These results are similar to those obtained by Dimkpa et al., where wheat exposed to bCuO showed no change in shoot height. However, a decrease in shoot length was observed in plants treated with nCuO. Similarly, lettuce grown in soil with copper nanoparticles for 15 days exhibited no change in shoot/root ratios, but high concentrations (0.066% (*w/w*)) of copper nanoparticles did increase shoot/root ratios after 15 days (Shah and Belozerova, 2009). Growth suppression has also been reported in duckweed, maize, lettuce, and alfalfa exposed to nCuO (Hong et al., 2015; Shi et al., 2011; Wang et al., 2012).

Figure 1B shows that no statistically significant differences were found between the stalk diameters of treated and control sugarcane. In a study that looked at sugarcane grown in soil modified with 61.65 mg kg^{-1} $CuSO_4$ found an average stalk diameter of 16.25 mm (Su et al., 2015). This diameter is larger than the 9.65 mm average diameter of sugarcane treated with $CuCl_2$ in this study. However, the current study found taller sugarcane, 1.33 m compared to 1.21 m, when plants were treated with ionic copper at approximately 60 mg kg^{-1}.

Figure 2A illustrates the average number of secondary shoots for each treatment compared to the control sugarcane. As seen in the graph, there was large variability in the number of secondary shoots in all of the sugarcane grown; this could be due to simple variability among different plants. There was however, significantly shorter secondary shoots (Figure 2B) in sugarcane grown with Kocide 3000 at 40 mg kg^{-1} and with bCuO at 60 mg kg^{-1}.

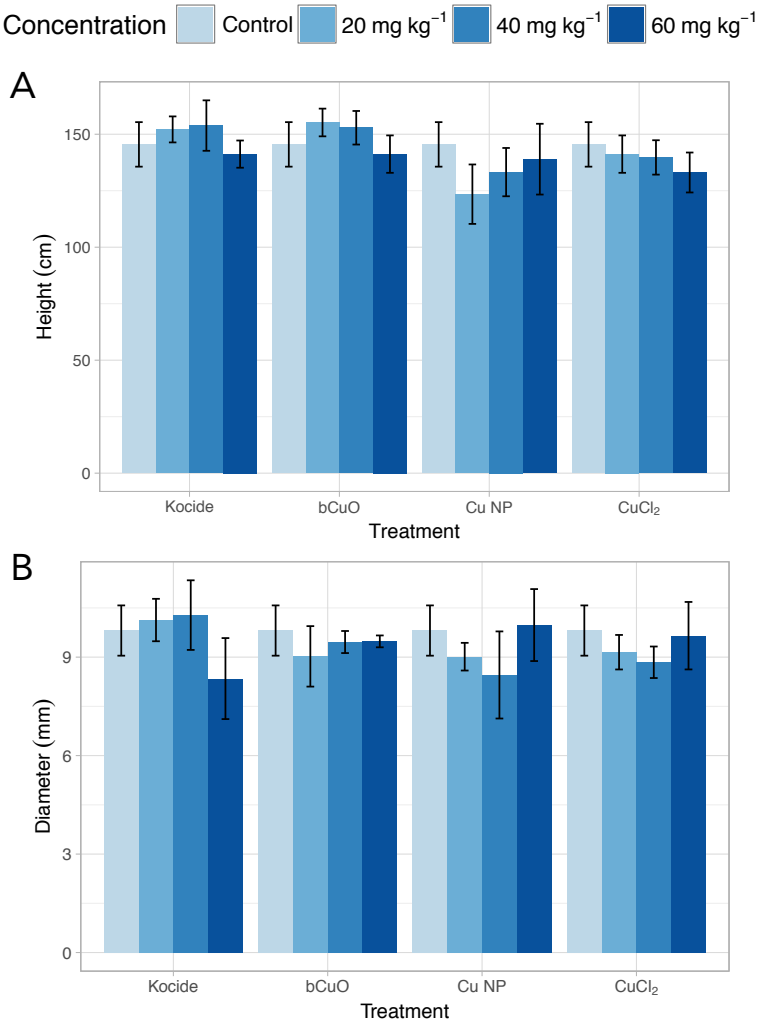

Figure 1. Physiological measurements of sugarcane exposed to Kocide 3000, bCuO, Cu NP, and $CuCl_2$. A) plant height and B) stalk diameter at the base. Values displayed as mean ± SE of 3 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control, ($p \leq 0.05$).

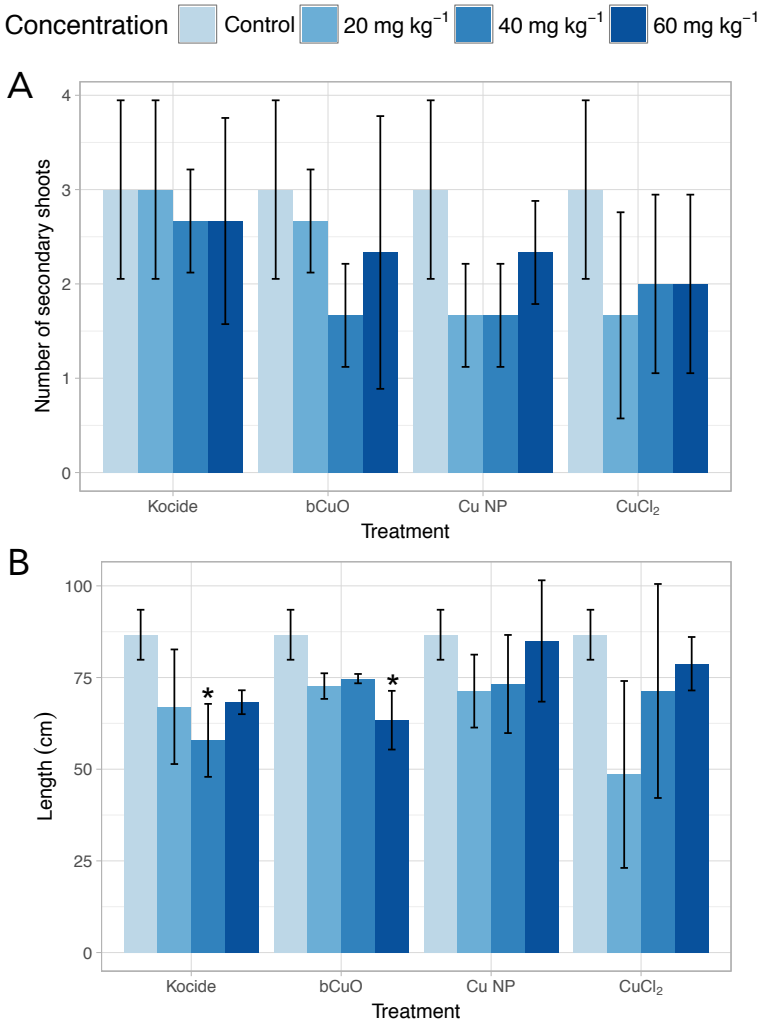

Figure 2. Physiological measurements of sugarcane exposed to Kocide 3000, bCuO, Cu NP, and $CuCl_2$. C) Number of secondary shoots, and D) Length of longest secondary shoot. Values displayed as mean ± SE of 3 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control, ($p \leq 0.05$).

3.2. Catalase and Ascorbate Peroxidase Activity

Catalase (CAT) and Ascorbate Peroxidase (APX) activity provide a glimpse into oxidative stress being placed on a biological system. Both of these enzymes scavenge H_2O_2 generated by superoxide dismutase and produced water, Figures 3 and 4 show the activity of CAT and APX, respectively. Sugarcane treated with bCuO at 20 mg kg^{-1} showed nearly twice the CAT activity (Figure 3) compared to control plants. This activity decreased by more than half of the control CAT activity when treatment was increased to 60 mg kg^{-1}. A similar pattern was observed in the

$CuCl_2$ treatments, CAT activity increased from 6.9 mmol min^{-1} mg^{-1} in the control to 13.8 mmol min^{-1} mg^{-1} in the 20 mg kg^{-1} treatment, followed by a sharp decrease at 40 mg kg^{-1}. Significantly lower catalase activity was also observed in sugarcane treated with Cu NPs at 60 mg kg^{-1}. Song et al. found increases in CAT activity in duckweed treated with nCuO at concentrations above 10 mg L^{-1} and in bulk CuO at concentrations above 100 mg L^{-1}. Similarly oxidative stress, as measured through catalase activity, increased in studies done on aquatic weeds, lettuce, and alfalfa exposed to nCuO (Hong et al., 2015; Shi et al., 2011). Moreover, ionic copper suppressed catalase activity by up to 35% in peas (Chaoui and El Ferjani, 2005).

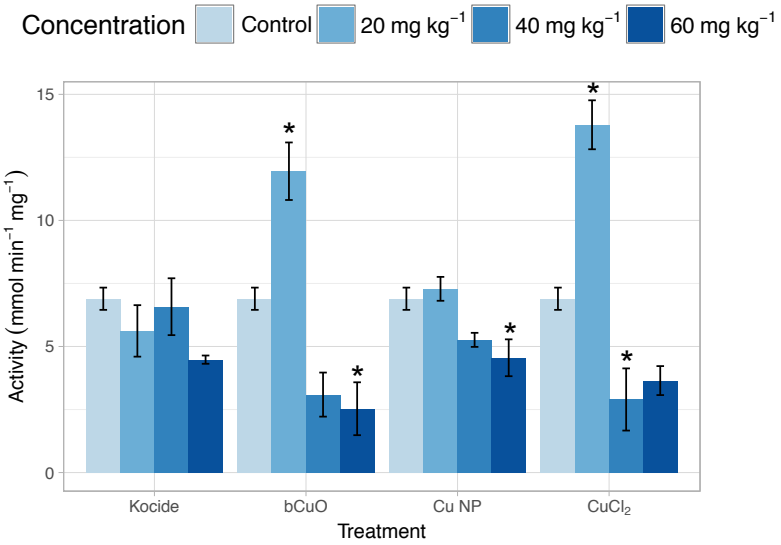

Figure 3. Catalase (CAT) activity in leaves of sugarcane exposed to Kocide 3000, bCuO, Cu NP, and $CuCl_2$. Values displayed as mean ± SE of 3 replicates per treatment. Asterisks (*) indicated a statistically significant difference from control, ($p \leq 0.05$).

Sugarcane treated with Kocide 3000 exhibited a trend of increased APX activity (Figure 4), with increasing concentration of Kocide. The increased levels, however, were not statistically significant when compared to control plants. Significantly higher APX activity was observed in sugarcane treated with Cu NP at 60 mg kg^{-1} and with $CuCl_2$ at 20 mg kg^{-1} and 60 mg kg^{-1}, indicating a higher level of oxidative stress being placed on the plant. However, when sugarcane was treated with $CuCl_2$ at 40 mg kg^{-1} APX activity returned to control levels, while as mentioned above, CAT activity decreased to below the activity of controls. In comparison, APX activity in

rice germinated in nCuO increased as it did in alfalfa and lettuce (Hong et al., 2015; Shaw and Hossain, 2013).

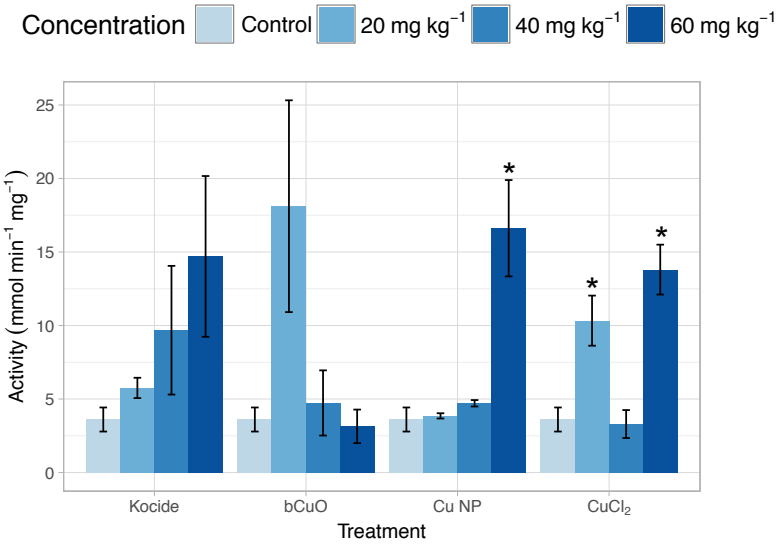

Figure 4. Ascorbate Peroxidase (APX) activity in leaves of sugarcane exposed to Kocide 3000, bCuO, Cu NP, and $CuCl_2$. Values displayed as mean ± SE of 3 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control, ($p \leq 0.05$).

Exposure of sugarcane plants to increasing levels of Cu NP, bCuO, and $CuCl_2$ would lead to the expectation of increasing levels of stress indicators (CAT and APX), however that is not the trend observed in this work. Recent work conducted by Pham et al. investigates the reduction of various oxidation states of copper with H_2O_2 under circumneutral pH conditions (Pham et al., 2013). The presence of additional copper in the cellular tissues can provide an additional H_2O_2 scavenger, which would reduce the activity of biologically produced scavengers. Though, when sugarcane is exposed to Cu NP at 60 mg kg^{-1} we observe both a decrease in CAT activity and an increase in APX activity. Looking at copper uptake in the leaf tissues, there is a much higher uptake at this treatment concentration (Figure 6A). This increased copper uptake could further scavenge H_2O_2 from the plant's normal ROS defense system. It has been reported that APX has a much higher binding affinity for H_2O_2 than CAT, K_m = 100 μM for APX compared to K_m = 40 to 600 mM for CAT (Su et al., 2015). This extreme difference in affinity could push for a cellular preference for APX to scavenge H_2O_2 at low concentrations.

3.3. Chlorophyll Content

The results of the chlorophyll analysis are shown in Figures 5 A–C. As seen in Figure 5A, there was a significant increase in chlorophyll A concentration in sugarcane treated with Kocide, bCuO, and $CuCl_2$ of at least 80% over the control sugarcane. Sugarcane treated with Kocide at 20 and 60 mg kg^{-1} had chlorophyll A concentrations increase from 182 mg g^{-1} in the control plants to 360 mg g^{-1} and 330 mg $^{-1}$, respectively. Chlorophyll A levels also increased when sugarcane was treated with $CuCl_2$ at 20 and 60 mg kg^{-1}, up to 395 mg g^{-1} and 325 mg g^{-1}. Sugarcane exposed to bCuO at 20 mg kg^{-1} increased to 336 mg g^{-1}, however, higher exposure levels did not yield chlorophyll levels significantly different from controls.

Chlorophyll B (Figure 5B) concentrations experienced a significant difference from controls only in sugarcane treated with Cu NP at 40 mg kg^{-1}. Concentrations decreased from 206 mg g^{-1} to 118 mg g^{-1}, a difference of 43%. When chlorophyll A and B concentrations are combined to give total chlorophyll (Figure 5C), no significant differences were observed. Previous studies that measured chlorophyll content of plants treated with nCuO obtained opposite results. These studies showed decreases in total chlorophyll in lettuce, duckweed, and aquatic weeds (Nekrasova et al., 2011; Shi et al., 2011; Trujillo Reyes et al., 2014). Song et al. found nCuO to suppress chlorophyll production at lower concentrations than bCuO, 100 mg L^{-1} compared to 150 mg L^{-1}. Green algae chlorophyll decreased after exposure to core shell CuO at 0.01 g L^{-1} and 0.02 g L^{-1} (Saison et al., 2010).

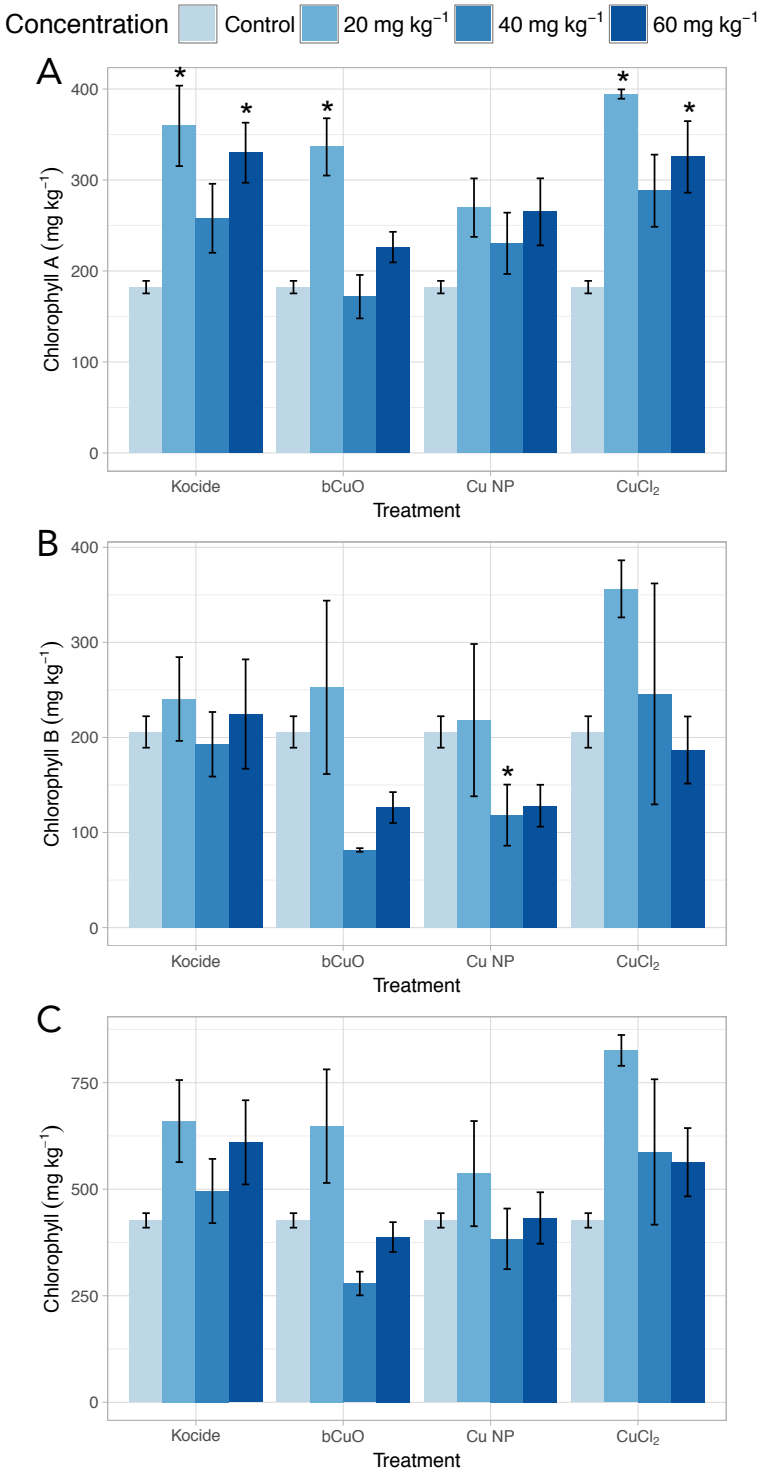

Figure 5. Chlorophyll content in sugarcane treated with Kocide 3000, bCuO, Cu NP, and $CuCl_2$ at 20, 40, and 60 mg kg^{-1}. A) chlorophyll A, B) chlorophyll B, and C) total chlorophyll. Values displayed as mean ± SE of 3 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control, ($p \leq 0.05$).

3.4. Copper Uptake

The uptake of copper into the leaf, stem, and root tissues of sugarcane are shown in Figures 6 A–C, respectively. In leaf tissues (Figure 6A) only sugarcane treated with Cu NP at 60 mg kg^{-1} showed significantly higher levels of copper, 9.6 mg kg^{-1}, compared to controls at 5.5 mg kg^{-1}. Although copper concentrations in the leaf tissues of other treatments varied, sometimes in a dosage dependent manner, as was the case with Kocide and Cu NP treatments, the results remain statistically similar to controls.

No discernable pattern was observed in the copper concentrations of sugarcane stem tissues (Figure 6B). However, there were significantly higher levels of copper in sugarcane treated with bCuO at 40 mg kg^{-1}, 8.2 mg kg^{-1}, compared to 5.6 mg kg^{-1} in control plants.

Copper uptake into root tissues is presented in Figure 6C. Dosage dependent increases in copper concentrations were observed in sugarcane exposed to Kocide, Cu NP, and bCuO. Copper concentrations in control sugarcane plants were 21.1 mg kg^{-1}, however, when sugarcane was exposed to Kocide at 20, 40, or 60 mg kg^{-1} copper concentrations increased to 31 mg kg^{-1}, 48.4 mg kg^{-1}, 62.5 mg kg^{-1}, respectively. Sugarcane exposed to Cu NP similarly increased to 37.8 mg kg^{-1}, 53.7 mg kg^{-1}, and 77.8 mg kg^{-1} for 20, 40, and 60 mg kg^{-1} treatments, respectively. bCuO treatments of 20, 40, and 60 mg kg^{-1} saw root copper concentrations increase to 33.1 mg kg^{-1}, 55.8 mg kg^{-1}, and 71.9 mg kg^{-1}. Sugarcane treated with $CuCl_2$ did not follow this same dosage dependent increase in copper concentrations. Instead, copper concentrations in root tissues rose to 43.9 mg kg^{-1} when treated at 20 mg kg^{-1}, but fell to 37.4 when treated with 40 mg kg^{-1}. Copper levels in sugarcane treated with $CuCl_2$ at 60 mg kg^{-1} increased to 64.8 mg kg^{-1}. Upon application of copper-based compounds or nanoparticles, copper levels in root tissues increased from at least 46% (Kocide 20 mg kg^{-1}) to as much as 267% (Cu NP 60 mg kg^{-1}).

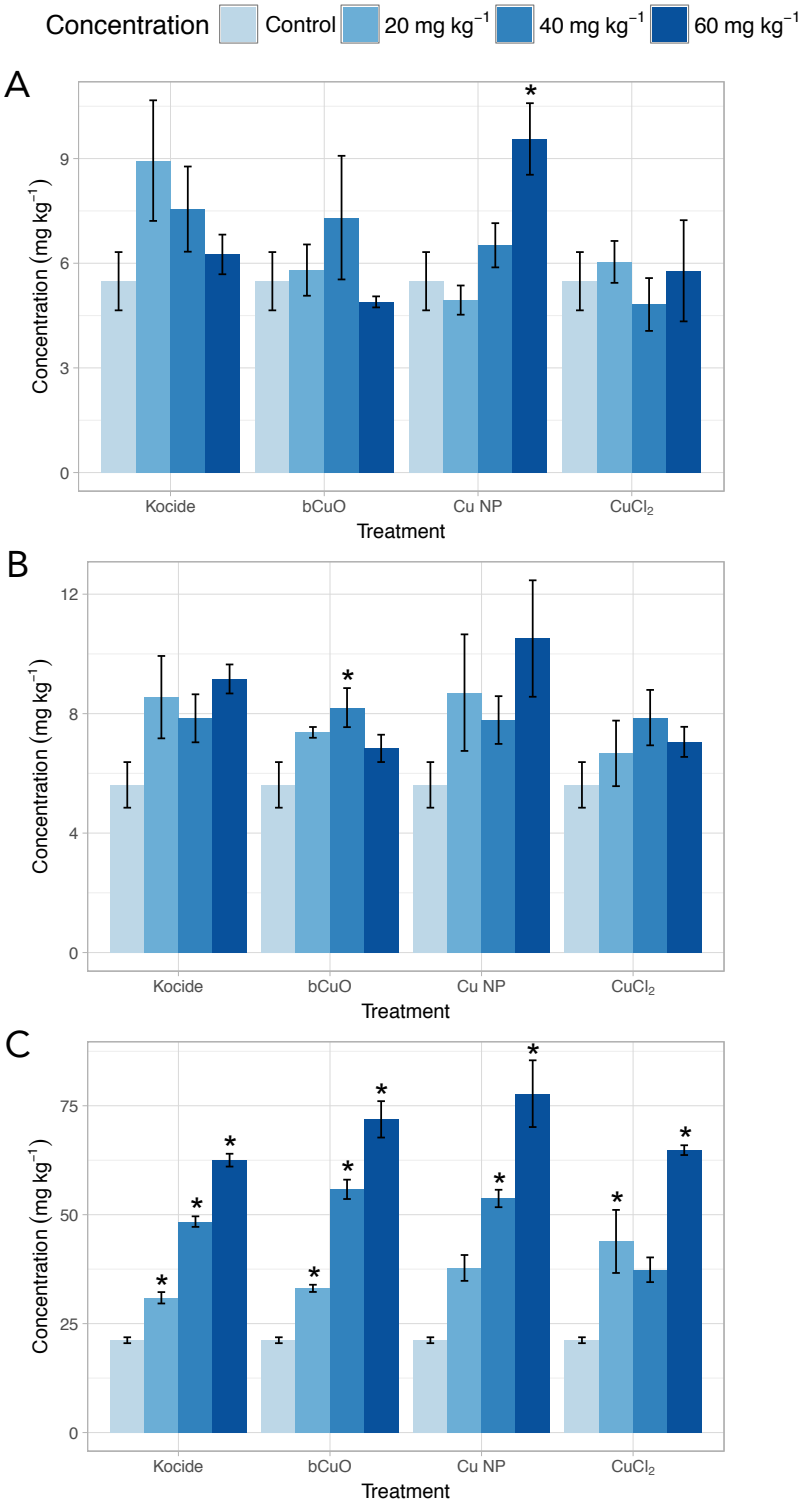

Figure 6. Copper concentration in A) leaf, B) stem, and C) root of sugarcane exposed to Kocide 3000, bCuO, Cu NP, and $CuCl_2$ at 20, 40, and 60 mg kg^{-1}. Values displayed as mean ± SE of 3 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control, ($p \leq 0.05$).

Similar copper levels were obtained in leaf and stalk tissues analyzed from sugarcane grown in soil amended with sewage sludge, however, soil copper concentrations ranged only from 6 to 10 mg kg^{-1} (Nogueira et al., 2013). Low stalk copper concentrations were also reported in a 10-month study with soil amended with 61.65 mg kg^{-1} ionic copper (Su et al., 2015). Results were drastically different when experiments were conducted under hydroponic conditions rather than soil. Sugarcane grown in solutions treated to 500 μM of ionic copper (31.75 mg kg^{-1} equivalent) had accumulated up to 389 mg kg^{-1} Cu in shoot tissues and 6465 mg kg^{-1} in root tissues after only 11 days of exposure (Sereno et al., 2007). This same study found ionic copper levels of 250 μM and 500 μM were lethal to sugarcane. Segura-Muñoz et al. found sugarcane grown at a landfill site contaminated with heavy metals had root and stem copper concentrations nearly four times higher than sugarcane from control sites.

Previous research has found that copper concentrations in sampled tissues increased regardless of copper compound treatment (Hong et al., 2015). A different study conducted on mung bean and wheat grown in an agar media found that copper concentration in plant parts increased with increasing treatment (Lee et al., 2008). Similarly, ionic treatments have produced higher metal concentrations in carrot, leading to the conclusion that the nanoparticles tested were no more toxic than their ionic counterparts (Ebbs et al., 2016). Dimkpa et al. has demonstrated that copper uptake by wheat grown in the presence of nCuO did originate from the applied treatments (Dimkpa et al., 2012).

3.5. Nutrient Content

3.5.1. Macronutrients

The results from the elemental analysis for macronutrients on the sugarcane leaf and root are shown in Table 1. Magnesium levels in leaves and roots treat with Cu NP were significantly different from control plant tissues. Concentrations in leaf tissues steadily decreased from 2520 mg kg^{-1} at 20 mg kg^{-1}, down to 1960 mg kg^{-1}, at 60 mg kg^{-1}. Concentrations in the roots, however, showed the opposite trend. Magnesium levels gradually increased from 1808 mg kg^{-}

^{1} at 20 mg kg^{-1}, to 2475 mg kg^{-1} at 60 mg kg^{-1}, while remaining below the concentration found in control root tissues. Plants treated with either Cu NP or bCuO showed a similar trend of decreasing phosphorous concentration with increasing treatment dosage. Phosphorous concentrations in sugarcane grown in soil amended with Cu NP decreased from control levels of 799 mg kg^{-1} to 558 mg kg^{-1} at 20 mg kg^{-1} of treatment, and 326 mg kg^{-1} at 60 mg kg^{-1}. bCuO treated sugarcane showed phosphorous levels of 521 mg kg^{-1} at 20 mg kg^{-1}, which decreased to 351 mg kg^{-1} when the dosage was increased to 60 mg kg^{-1}. Sugarcane grown in soil treated with Cu NP at all concentrations showed significantly lower potassium levels compared to controls. There was no dosage dependent response observed, instead potassium decreased from 4333 mg kg^{-1} at the 20 mg kg^{-1} treatment level, to 3390 mg kg^{-1} at 40 mg kg^{-1}. However, when Cu NP treatment was further increased to 60 mg kg^{-1} potassium levels increased to 4357 mg kg^{-1}.

3.5.2. Micronutrients

The results of the elemental analysis for micronutrient content in the leaf and root tissues of sugarcane are show in Table 1. Sugarcane treated with Cu NP and bCuO exhibited significantly higher concentrations of Fe in the root tissues. Trujillo-Reyes et al. also obtained these results in hydroponically grown lettuce, both ionic copper and copper nanoparticles increased Fe in root tissues. Work conducted by Deák et al. suggests that increased plant stress possibly caused by nanoparticle treatment increases the production of ferritin, a protein that binds iron. Further, it has also been established that grasses can excrete iron-chelating compounds, enabling sugarcane to uptake insoluble iron (III) without external reduction (Segura-Muñoz et al., 2006). This could further combat oxidative stress by increasing ferritin production. Sugarcane treated with Cu NP at 40 mg kg^{-1} and 60 mg kg^{-1} contained at least 73% more iron than the control plants, 3851 mg kg^{-1} and 3628 mg kg^{-1}, respectively compared to 2092 mg kg^{-1} in controls. Similarly, sugarcane treated with bCuO at 20 mg kg^{-1} and 40 mg kg^{-1} increased Fe content by at least 70%, up to 3616 mg kg^{-1} and 3768 mg kg^{-1}, respectively, over control iron

levels. Manganese concentrations in roots treated with bCuO were significantly higher than controls. While higher than controls, increased levels of treatment produced decreased manganese levels, from 167 mg kg^{-1} at 20 mg kg^{-1} of treatment down to 131 mg kg^{-1} at 60 mg kg^{-1}. Sugarcane treated with Cu NP at 40 mg kg^{-1} also showed significantly higher manganese levels, up to 170 mg kg^{-1} compared to 105 mg kg^{-1} in control plants.

Table 1. Altered nutrient content of sugarcane tissues exposed to Kocide 3000, bCuO, Cu NP, and $CuCl_2$. Values displayed as mean ± SE of 3 replicates per treatment. Asterisk (*), significantly different from control, ($p \leq 0.05$).

Tissue	Nutrient	Treatment	Concentration (mg/kg dry tissue)		
			20 mg/kg	40 mg/kg	60 mg/kg
Root	Fe	Control	2092 ± 194	2092 ± 194	2092 ± 194
		Kocide 3000	2915 ± 910	2748 ± 484	2401 ± 215
		bCuO	3616 ± 371 *	3768 ± 184 *	3124 ± 149
		Cu NP	1886 ± 89	3851 ± 279 *	3628 ± 451 *
		$CuCl_2$	2586 ± 138	2025 ± 224	3115 ± 551
	Mn	Control	105 ± 5	105 ± 5	105 ± 5
		Kocide 3000	106 ± 25	102 ± 11	85 ± 5
		bCuO	167 ± 8 *	158 ± 3 *	131 ± 2 *
		Cu NP	104 ± 9	170 ± 17 *	156 ± 19
		$CuCl_2$	125 ± 13	86 ± 8	117 ± 21
	Mg	Control	2920 ± 141	2920 ± 141	2920 ± 141
		Kocide 3000	2485 ± 138	2360 ± 43	2718 ± 221
		bCuO	2761 ± 68	2479 ± 200	2851 ± 45
		Cu NP	1808 ± 72 *	2295 ± 96	2475 ± 141 *
		$CuCl_2$	2935 ± 132	2227 ± 314	2690 ± 269
	P	Control	799 ± 76	799 ± 76	799 ± 76
		Kocide 3000	623 ± 108	526 ± 53	445 ± 29 *
		bCuO	522 ± 19 *	426 ± 64 *	351 ± 61 *
		Cu NP	559 ± 34 *	329 ± 47 *	327 ± 35 *
		$CuCl_2$	594 ± 48	575 ± 91	381 ± 23 *
	K	Control	5941 ± 223	5941 ± 223	5941 ± 223
		Kocide 3000	4816 ± 856	5142 ± 459	6821 ± 581
		bCuO	4393 ± 470	3281 ± 399	4555 ± 669
		Cu NP	4333 ± 395 *	3390 ± 389 *	4358 ± 325 *
		$CuCl_2$	6130 ± 639	5987 ± 980	5396 ± 473
Leaf	Mg	Control	3003 ± 185	3003 ± 185	3003 ± 185
		Kocide 3000	2751 ± 99	2818 ± 251	3006 ± 229
		bCuO	2188 ± 57	2511 ± 165	2357 ± 202
		Cu NP	2510 ± 67 *	2249 ± 49 *	1960 ± 119 *
		$CuCl_2$	2406 ± 234	2700 ± 154	3256 ± 154

4. Conclusion

Mesocosm experiments were conducted with sugarcane grown in soil amended with either: Kocide 3000, copper metal nanoparticles (Cu NP), micron-sized CuO (bCuO), or $CuCl_2$. In nearly all treatments, copper concentrations in the root tissues increased in a dose dependent manner. However, there was no evidence of copper translocation to aerial tissues, as measured by ICP-MS. None of the copper-based treatments affected the growth or total chlorophyll production. Catalase activity was increased in sugarcane treated with bCuO and $CuCl_2$ at 20 mg kg^{-1}, but decreased with $CuCl_2$ at 40 mg kg^{-1} and with Cu NP and bCuO at 60 mg kg^{-1}. Ascorbate Peroxidase activity also increased in the Cu NP 60 mg kg^{-1} and the $CuCl_2$ 20 and 60 mg kg^{-1} treatments. Although this study was conducted over an entire year, the sugarcane did not complete its lifecycle; therefore, it is imperative that additional studies be conducted. If the sugarcane is allowed to mature, it is possible that the increased biomass production could facilitate nanoparticle translocation.

Chapter 3: Uptake, transport, and effects of nano-copper exposure in zucchini (*Cucurbita pepo*)[2]

1. Introduction

Copper is frequently used in nanomaterials, due to its catalytic and antimicrobial properties, and is found in a wide assortment of products (Anjum et al., 2015). The widespread manufacture and use of nano-copper provides a route to environmental contamination. The exposure risk of nano-sized materials is greater due to their greater reactivity, stemming from a greater surface area to volume ratio. It has been reported that 18% of all manufactured copper is released into soils, possibly polluting agricultural land (Keller et al., 2013). The contamination of agricultural soils poses a food safety issue, especially for widely grown and consumed crops, such as zucchini (*Cucurbita pepo*).

The interactions between plants and copper nanomaterials have been the focus of numerous publications. Studies conducted under hydroponic conditions, with amendments of nano/bulk Cu, nano/bulk CuO, $Cu(OH)_2$, or $CuCl_2$ at up to 1000 mg kg^{-1}, have reported reduced biomass, diminished root length, stunted growth, and lower chlorophyll levels (Hong et al., 2015; Musante and White, 2010; Stampoulis et al., 2009; Trujillo-Reyes et al., 2014). For example, zucchini exposure to nCu at 250 and 750 mg L^{-1}, reduced biomass by nearly 50% (Hawthorne et al., 2012). Similarly, copper nanomaterials have also been shown to reduce germination of rice and wheat by up to 20% (Shaw and Hossain, 2013; Yasmeen et al., 2015). The bulk of the results from these hydroponic studies indicate that copper nanomaterial exposure results in detrimental consequences.

An area of concern is the bioaccumulation of potentially toxic levels of heavy metals that may find their way into the human food supply. Studies have shown the application of copper nanoparticles by either a suspension solution or foliar spray, cause a significant increase in

[2] Reprinted from Tamez, C., Hernandez-Molina, M., Hernandez-Viezcas, J.A., Gardea-Torresdey, J.L., 2019. Uptake, transport, and effects of nano-copper exposure in zucchini (Cucurbita pepo). Sci. Total Environ. 665, 100–106.

copper concentration in the exposed tissues (Adhikari et al., 2016). Other reports have similarly shown the accumulation of metals from metallic nanomaterials in exposed tissues and their translocation to the aerial portions of the plant (Barrios et al., 2016; López-Moreno et al., 2016; Medina-Velo et al., 2017; Tassi et al., 2017). The effects of nano-copper compounds have been investigated using nutrient solutions, but the complex nature of soil based studies have shown contrasting results (Apodaca et al., 2017; Da Costa and Sharma, 2016; Hawthorne et al., 2012; Musante and White, 2010; Ochoa et al., 2017). This trend highlights the importance of the growth medium to the uptake and effects of copper nanomaterial exposure. The potential for nanomaterial translocation into aerial tissues necessitates the further study of agricultural crops. Production of all cultivars of *Cucurbita pepo* in 2017 was nearly 27.5 million tonnes, illustrating the immediate need investigate this species (Food and Argiculture Organization, 2017). To the best of the authors' knowledge, no work has been published on the effects of nano-copper exposure in zucchini grown in a complex soil medium.

The goal of this study is to ascertain the translocation potential and short-term exposure effects of copper-based nanoparticles and compounds in a complex soil medium. To accomplish this, zucchini was grown for 3 weeks in soil amended with either the commercially available fungicide Kocide 3000 ($Cu(OH)_2$), nano-sized CuO (nCuO), micron-sized CuO (bCuO), or nano-sized Cu metal (Cu NP). Plant growth, biomass, chlorophyll content, micro and macro nutrient concentration, and the quantification of enzymatic activity related to reactive oxygen species production were chosen as the end points to establish the effects of nano-copper exposure.

2. Methods

2.1. Copper Nanomaterials/Compounds

The nanomaterials and compounds used in this study were obtained in collaboration with the University of California Center for Environmental Implications of Nanotechnology. The copper nanomaterials/compounds were used without modification, and had been previously

characterized to be of the following sizes: 10 μm (Kocide 3000), 10–100 nm (nCuO), 0.1–10 μm (bCuO), and 100–1000 nm (Cu NP) (Hong et al., 2015).

2.2. Preparation of soil medium

In order to evaluate the effects of nano-copper exposure to grey zucchini, a 1:2 (v/v) mixture of Miracle-Gro brand potting with native El Paso soil, previously characterized as loamy sand with pH 7.9 (Zhao et al., 2015), was amended with different copper compounds: Kocide 3000 (a commercially available $Cu(OH)_2$ fungicide), a nano-sized CuO (nCuO), a micron-sized CuO (bCuO), or a nano-sized Cu metal (Cu NP). The soils were amended, to a concentration of 50 or 200 mg kg^{-1} (Cu content), by the addition of the compounds in powder form to 200 g of soil medium with the addition of a small amount of deionized water, and mixed until homogeneity was achieved. After a 24-h settling period, 3 seeds were placed in each 0.5 L pot, with 4 pots per treatment. All pots were then transferred to environmentally controlled growth chambers for 3 weeks. Environmental growth chambers were operated on a 25/20°C day/night temperature cycle, 65–70% relative humidity, and a 14 h photoperiod with a light intensity 340 μmol m^{-2} s^{-1}.

2.3. Tissue Harvest and collection

At the end of the 3-week growing period total plant length was recorded and plants were then separated into roots, stems, and leaves. After root lengths were measured, each group of tissues were washed with 5% nitric acid and weighed. Fresh tissues (roots and fully expanded leaves) were set aside for biochemical analysis and remaining tissues were oven-dried at 70°C for 72 h.

2.4. Chlorophyll Content

To measure the concentration of chlorophyll, pigments were extracted by grinding 0.1 g of fresh leaf tissue in a mortar with 80% acetone. The extract was then analyzed by measuring

the absorption of light at 663 nm and 646 nm, for chlorophyll A and B, respectively. Concentrations were calculated following the equations proposed by Porra et al., 1989.

2.5. Enzymatic Activity Assays

To assess the amount of stress experienced by the plant the activity of ascorbic peroxidase (APX) and catalase (CAT) were measured. Briefly, 0.2 g of fresh root or leaf tissue was ground with potassium phosphate buffer and the resulting extract was stored at -80°C until analysis. The APX assay was carried out by mixing sample extract with 25mM Ascorbate, 17 mM H_2O_2, and 0.1 M potassium phosphate buffer. APX activity was determined by measuring the decrease in absorption at 265 nm for 2 min. CAT activity was measured by the decrease in absorption at 240 nm for 3 min upon the addition of 10 mM H_2O_2 to the sample extract (Gallego et al., 1996; Murgia et al., 2004).

2.6. Copper uptake and Nutrient Content

Elemental analysis on the root, stem, and leaf tissues was performed using inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin Elmer Optima 4300 DV) and ICP mass spectroscopy (ICP-MS, Perkin Elmer ELAN DRC II). Approximately 0.2 g of dried tissues were mixed with 5 mL of trace-metal free nitric acid (HNO_3) and placed in a digestion block for 45 min at 115°C. Aqueous samples were then diluted to 50 mL with DI water (18 mΩ). For quality control Standard Reference Material 1570a was processed alongside sample tissues.

2.7. Statistical Analysis

All experiments were performed in quadruplicate. Comparisons between treated plants and controls were done with 1-way analysis of variance (ANOVA) significance tests ($p \leq 0.05$), followed by a Tukey HSD post hoc analysis when necessary. Data were transformed as needed to allow for normality. For cases where data was not normally distributed non-parametric analysis were employed. Statistical tests were performed using R statistical software (version 3.1.2) and RStudio (version 1.0.136).

3. Results and Discussion

3.1. Copper Uptake and Translocation

Cu was translocated from the root tissues into the aerial portions of the zucchini plants (Figure 7), with nearly all treatments showing significantly higher Cu concentrations when compared to controls. Exceptions to this trend were roots exposed to Kocide 3000 at 50 mg kg^{-1} and nCuO at 50 mg kg^{-1}, stems from plants treated with bCuO at 50 mg kg^{-1}, and the leaves of plants exposed to Cu NP at 50 mg kg^{-1}. The root tissues of exposed plants showed the highest concentration of Cu, with Kocide 3000 containing 309 mg Cu per kg dry tissue. These results are consistent with prior studies that showed accumulation of Cu occurring mostly in the root tissues (Peng et al., 2015; Trujillo-Reyes et al., 2014; Zuverza-Mena et al., 2015). In hydroponically grown cucumbers plants exposed to Cu NPs, researchers found at least double the Cu concentrations in root and stem tissues, compared to controls, after only 7 days of exposure (Zhao et al., 2016). A 5.5-fold Cu increase was reported in rice leaves treated with a solution of nCuO (Da Costa and Sharma, 2016). Insights into the mechanisms of nano-copper uptake and translocation are provided by plants treated with Kocide 3000 and nCuO at 50 mg kg^{-1}. In these treatments, low accumulation of Cu in the roots was paired with significantly higher aerial tissue concentrations, highlighting that at low soil concentrations small size and higher solubility play an important role in Cu translocation. Additionally, previous research has demonstrated the accumulation of Cu from nano-based treatments to the edible tissues of root vegetables or their migration to aerial edible seeds/grains (Bradfield et al., 2017; Sathiyabama and Manikandan, 2018). Our results corroborate Cu translocation in soil-grown zucchini and suggest the need to investigate the accumulation of Cu in edible tissues.

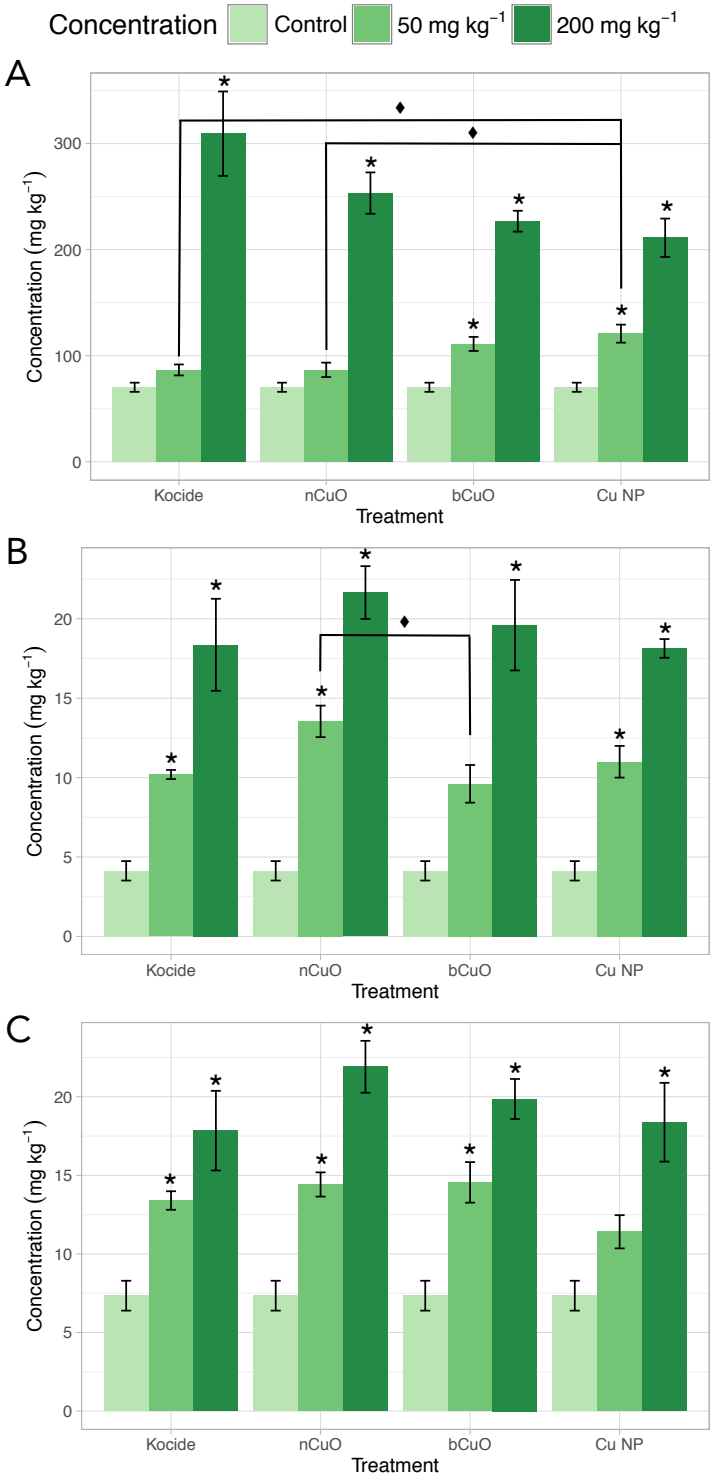

Figure 7. Copper concentration in A) the root, B) the stem, and C) the leaf tissues of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 50, and 200 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$). Diamond (♦) indicates a statistically significant difference between treatments ($p \leq 0.05$).

3.2. Effects of Nano-copper on Plant Growth

All of the treatments and concentration levels tested show no significant changes in the root or total plant length (Fig. 8A–B). Although there is a trend of decreased fresh root mass when compared to control (Fig. 9A), these changes were not significant. Leaf and stem biomass were also not significantly different from control plants, but unlike root tissues there were no visible trends (Fig. 9B–C). These results are considerably different from similar studies conducted under hydroponic conditions. When yellow squash was grown in solution, exposed with up to 500 mg kg^{-1} concentrations of Cu NP or its micron-sized counterpart plant biomasses decreased by as much as 99% and 74%, respectively (Musante and White, 2010). Rice seedlings germinated in the presence of nCuO for 7 days produced significantly stunted roots compared to controls (Shaw and Hossain, 2013). Although these studies differ with the results presented here, a study conducted with soil amended with bCuO or Cu NP produced cilantro plants with 11% and 12.4% smaller shoots, respectively (Zuverza-Mena et al., 2015). The varying results indicate that effects caused by nano copper compounds are influenced by growth medium, plant species, and exposure time.

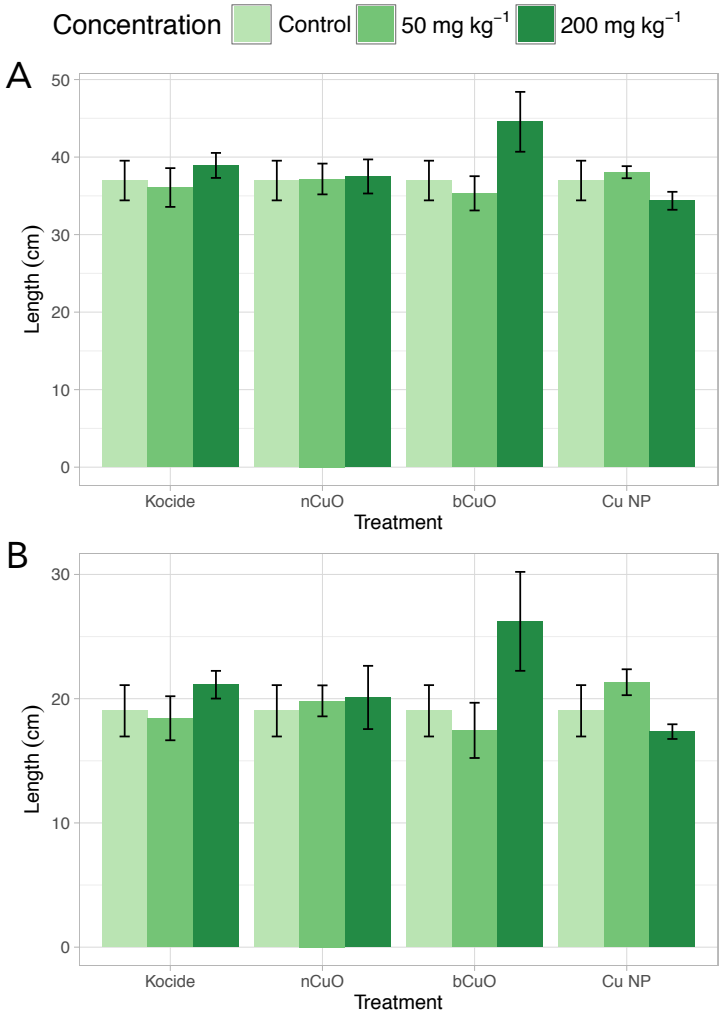

Figure 8. Total plant length (A) and root length (B) of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 50, and 200 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

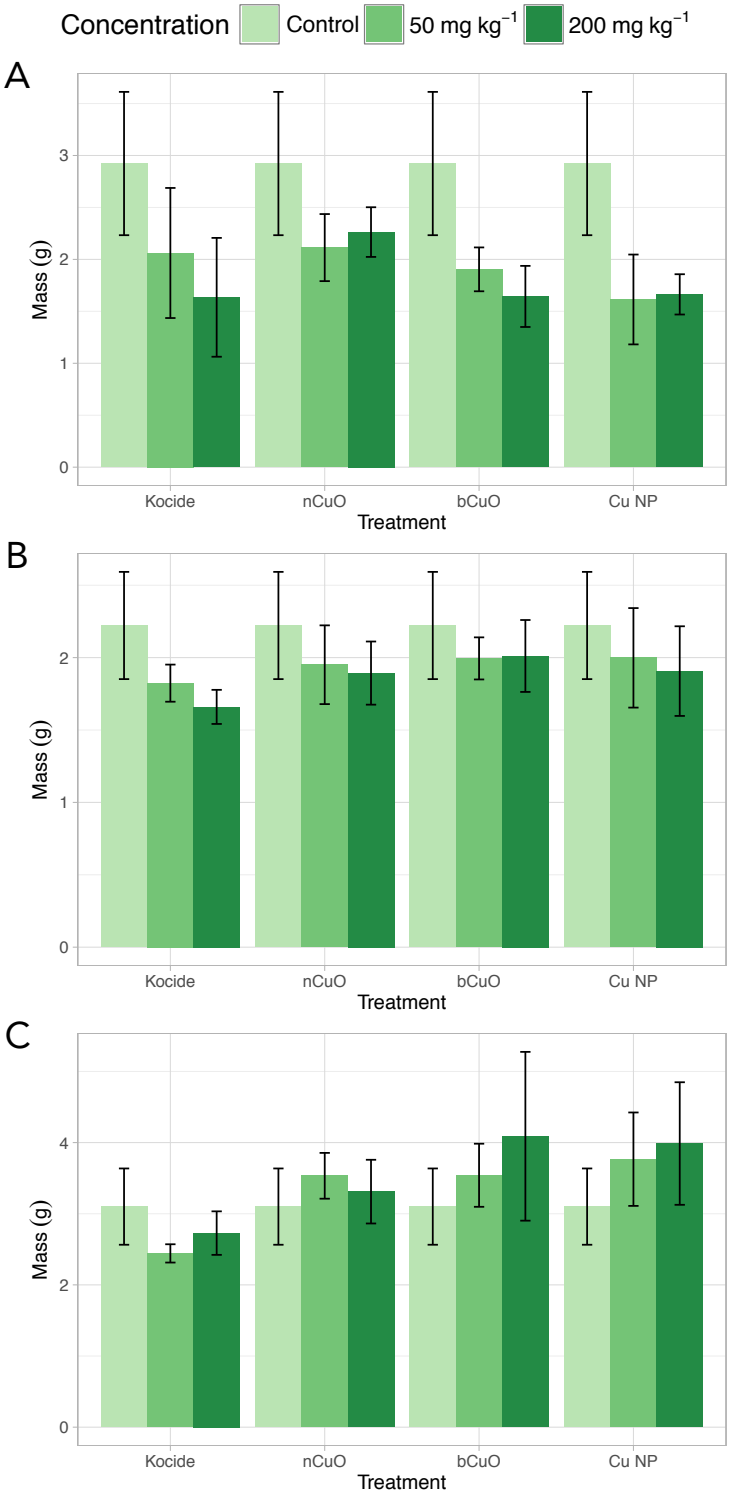

Figure 9. Fresh tissue mass of A) root, B) stem, and C) leaves of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 50, and 200 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.3. Chlorophyll Content

Chlorophyll is a reliable method for gauging the overall health of the photosynthetic process (Saison et al., 2010). Plants treated with 50 mg kg^{-1} of nCuO and 200 mg kg^{-1} of bCuO, showed a significant reduction in chlorophyll A of 8.1% and 12.6%, respectively (Figure 10A). Chlorophyll B and total chlorophyll content remained unchanged, compared to controls (Figure 10B and C). Previous work with soybean has shown chlorophyll content to remain unaffected upon exposure to nCuO, until concentrations as high as 400 mg L^{-1} (Zuverza-Mena et al., 2017). Similarly, other metal oxide nanoparticles (e.g., TiO_2, ZnO, CeO_2) have been shown to reduce chlorophyll levels in green peas, corn, rice, and tomato (Du et al., 2017). However, it has also been demonstrated that there are no measurable differences in chlorophyll content in mature bell pepper treated with nCuO or bCuO, or in oregano treated with Cu NP (Du et al., 2018; Rawat et al., 2018). The results in this study point to no significant alteration in the production of chlorophyll upon exposure to any of the copper compounds used at the test concentrations.

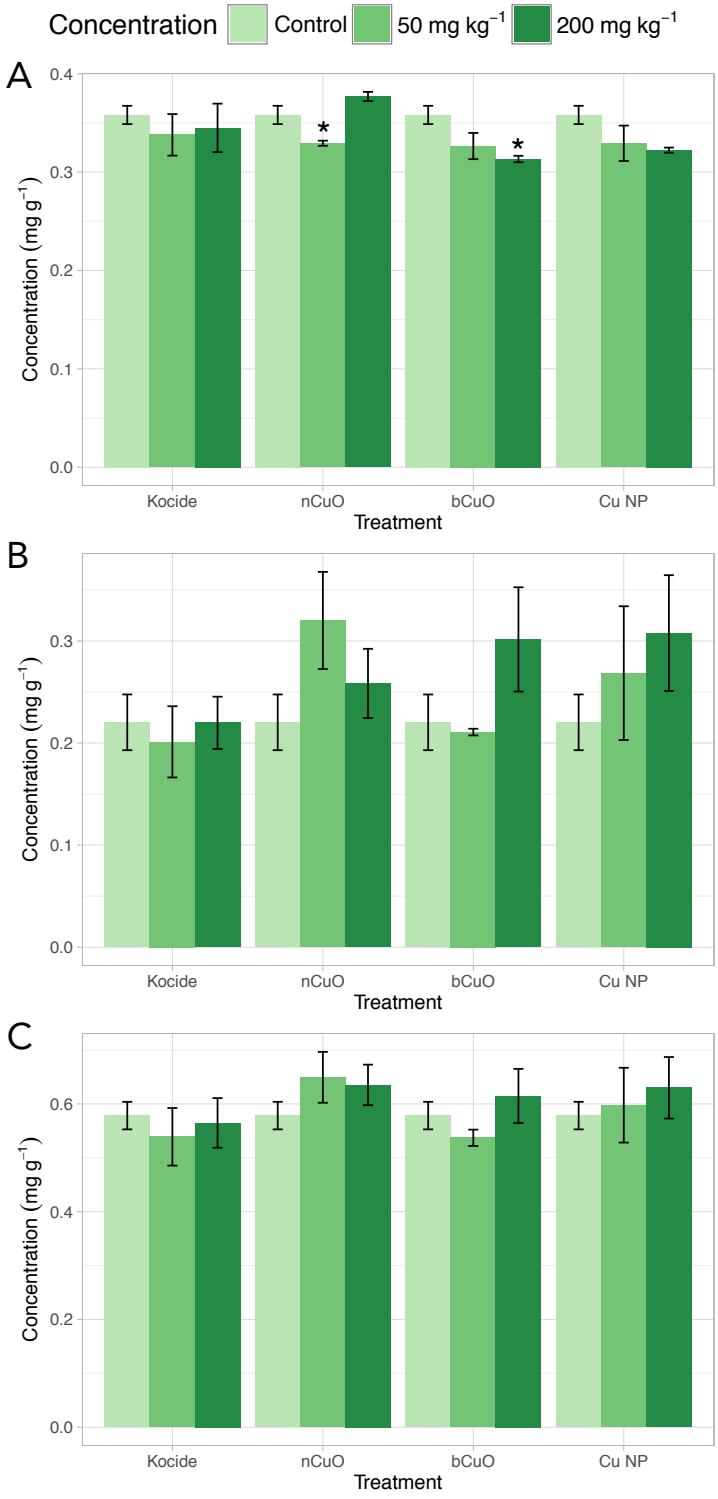

Figure 10. Chlorophyll content of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 50, and 200 mg kg^{-1}. A) Chlorophyll A, B) chlorophyll B, C) total chlorophyll. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$)

3.4. Antioxidant Enzyme Activity

Increases in APX and CAT activities are indicative of plant stress levels, by means of their ability to eliminate H_2O_2. Reactive oxygen species, such as H_2O_2, are produced in excess when plants face adverse growing conditions. Both APX and CAT scavenge H_2O_2, but their process and location in the plant cell differ (Inzé and Montagu, 1995). The presence of excess copper in plant tissues can lead to the formation of reactive oxygen species via Fenton-like reactions (Anjum et al., 2015). Notwithstanding, APX levels in root tissues of treated plants were lower than controls (Figure 11A). Plants treated with Kocide 3000 at 50 mg kg^{-1} and 200 mg kg^{-1} had 49% and 45% lower APX activity, while nCuO at 50 mg kg^{-1} and 200 mg kg^{-1}, and bCuO at 200 mg kg^{-1} showed 56%, 49%, and 57% less enzyme activity, respectively. The APX levels in leaf tissues remained unchanged compared to controls (Figure 11B). Decreases in root APX activity were observed in lettuce treated with core-shell Cu/CuO NPs at 10 and 20 mg L^{-1} (Trujillo-Reyes et al., 2014). These results draw a distinction from hydroponic studies on lettuce and alfalfa using nano-copper compounds showed contrasting results to the ones reported here. Lettuce exposed to nCuO, bCuO, Cu NP, and Kocide 3000 showed significant increases in APX activity, compared to control plants (Hong et al., 2015). Similar results were seen in alfalfa roots grown under the same conditions, with the exception of Kocide 3000 where no changes in activity were observed. Increases in oxidative stress have also been observed in rice seedlings, with exposure levels of up to 1.5 mM for 14 days resulted in a 1.3 fold rise in APX activity (Shaw and Hossain, 2013). In a separate study, rice exposed to a range of concentrations of nCuO (2.5 mg L^{-1} to 1000 mg L^{-1}) revealed significantly higher APX activity at all both treatment levels (Da Costa and Sharma, 2016). The reduction in APX activity, in plants treated at the 100 mg kg^{-1} level, can be attributed to the increased copper tissue concentrations. It has been reported that copper can undergo redox reactions with H_2O_2 (Pham et al., 2013). It is possible that the higher copper tissue concentrations could serve as a H_2O_2 scavenger, reducing the need of an enzymatic pathway. However, plants that did not show significant increases in root Cu concentrations likewise showed reduced APX activity. Zucchini treated with Kocide 3000 and nCuO at 50 mg

kg^{-1} experienced reduced Zn accumulation in root tissues, and previous work has observed decreased APX activity in Zn deficient tobacco (Yu et al., 1998).The results presented here point to Cu and Zn playing a role in APX activity regulation.

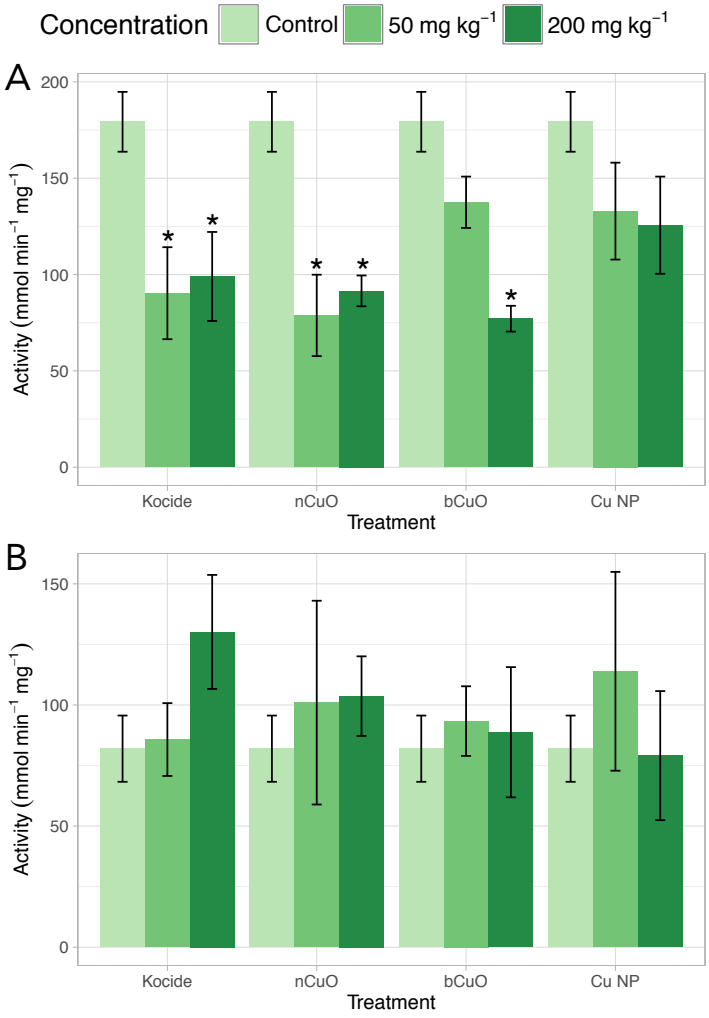

Figure 11. APX activity in A) root and B) leaves of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 50, and 200 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

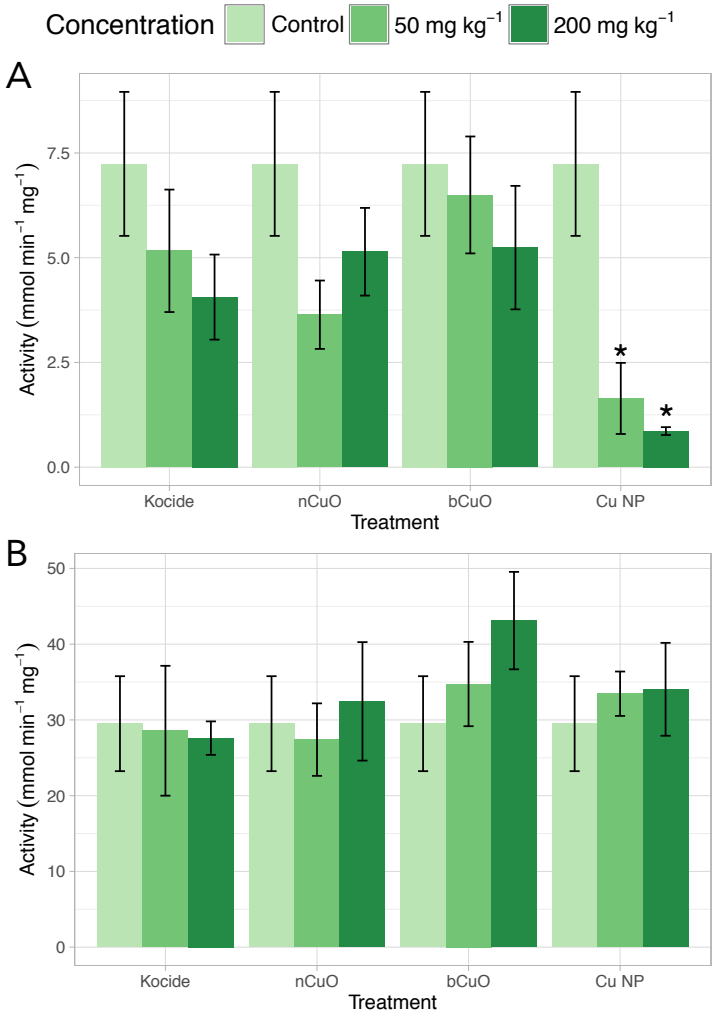

Figure 12. CAT activity in A) root and B) leaves of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 50, and 200 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

CAT activity in both tissues remained at control levels for all treatments (Figure 12A and B), except for the roots of plants grown in soil amended with Cu NP at both 50 mg kg^{-1} and 200 mg kg^{-1}, which contained 77% and 80% less enzyme activity, respectively, compared to control plants. In previously conducted studies there were no significant differences in CAT activity in both the roots and leaves of kidney beans, or in the roots of green peas when treated with nano/micron sized copper based compounds (Apodaca et al., 2017; Ochoa et al., 2017). However, lettuce exposed to Cu/CuO nanoparticles experienced a nearly 60% increase in root CAT activity and a 129% increase in leaf CAT activity (Trujillo-Reyes et al., 2014). The response

of CAT could depend on a multitude of factors, and not simply on the exposure on nanomaterials. This is evident in a study on wheat seeds pretreated with colloidal copper solutions that produced seedlings with no change in CAT activity, however if the seedlings were subjected to drought conditions, CAT activity increased by 21% over the non-treated drought control (Taran et al., 2017).

It has been reported that Cu deficiencies can cause elevated ROS generation and nano based copper treatments could be alleviating Cu deficiencies in the growing medium, thereby reducing plant stress levels (Adhikari et al., 2016). Both Cu deficiency and Cu excess can cause damage to vital plant cell processes, such as damage to membrane lipids and increased electrical conductivity (Blasco et al., 2018). However, this is not the trend observed in this study. Only plants treated with Cu NP at 50 mg kg^{-1} and 100 mg kg^{-1} exhibited reduced CAT activity. Micronutrient data (Tables 2–4) shows that these plants also contained reduced boron root concentrations, which has been shown to reduced CAT activity (Han et al., 2008).

3.5. Nutrient Content

The applied Cu treatments altered the accumulation of certain micro and macronutrients (Tables 2–4). Concentrations of Mn, Mg, and Ca were not significantly different from control levels and their data is omitted from this article. In root tissues Zn concentrations decreased from 46% to 60% upon application of nCuO or bCuO at both 50 mg kg^{-1} and 200 mg kg^{-1} treatment levels, as well as with Kocide 3000 at 50 mg kg^{-1}. These results are in agreement with reports that Cu can inhibit the accumulation of Zn, however it remains unclear as to why Zn depression is not observed in the aerial tissues (Hafeez et al., 2013). Zn plays a critical role as a cofactor of numerous plant enzymes and transcription regulation (Sharma, 2007). Similarly, B concentrations in plants treated with Cu NP at 50 mg kg^{-1} and 200 mg kg^{-1} were reduced by 65% and 61%, respectively. Studies have suggested that B plays a role in the plasma membrane and related transport processes (Goldbach and Wimmer, 2007). Such a decrease in B may begin to affect critical plant systems, however this treatment was marked by a significant decrease in

stress enzymatic activity. B concentrations in the root tissues remained above 20 mg kg^{-1}, and it may be that threshold for B is lower than this and no biological effects occurred. Mo concentrations in roots of plants treated with Kocide 3000 and nCuO at 200 mg kg^{-1} increased by over 91%. The excess Mo in the roots was mirrored in the stems, although to a lesser extent, where levels were 38% higher than control plant stems. Application of Kocide 3000 at 200 mg kg^{-1} to zucchini increased Fe root concentration by 62%, when compared to controls. The stems of nCuO treated plants contained 25% to 32% more Fe, however increased treatment concentration did not equate to more Fe accumulation.

Leaves of plants treated with nCuO at 200 mg kg^{-1} and Cu NP at 50 mg kg^{-1} contained 31% and 75% higher levels of Zn and Mo, respectively. Only leaf tissues demonstrated changes in macronutrients concentration. S content increased upon exposure to Kocide 3000 (60%), nCuO (69%), and Cu NP (56%) at the 200 mg kg^{-1} treatment level. The 200 mg kg^{-1} treatment levels of Kocide 3000 also raised P levels, by nearly 17%. It is well documented that the application of Cu, in nano-form or otherwise, alters nutrient accumulation in plants (Du et al., 2018; Hong et al., 2015; Ochoa et al., 2017; Rawat et al., 2018). In this study, the changes in nutrients of treated plants did not cause any measurable negative effects.

Table 2. Altered root nutrient content of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP. Values displayed as mean ± SE of 4 replicates per treatment. Asterisk (*), significantly different from control, ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)	
		50 mg/kg	200 mg/kg
Zn	Control	65 ± 16	65 ± 16
	Kocide 3000	29 ± 2 *	40 ± 8
	nCuO	26 ± 2 *	32 ± 6 *
	bCuO	35 ± 3 *	33 ± 1 *
	Cu NP	52 ± 11	48 ± 3
Mo	Control	0.7 ± 0.1	0.7 ± 0.1
	Kocide 3000	1.1 ± 0.12	1.4 ± 0.25 *
	nCuO	1.1 ± 0.07	1.4 ± 0.22 *
	bCuO	0.6 ± 0.06	0.6 ± 0.04
	Cu NP	0.7 ± 0.08	0.7 ± 0.03
Fe	Control	811 ± 120	811 ± 120
	Kocide 3000	491 ± 17	1315 ± 126 *
	nCuO	1008 ± 113	1002 ± 104
	bCuO	1203 ± 297	853 ± 36
	Cu NP	988 ± 91	822 ± 51
B	Control	72 ± 15	72 ± 15
	Kocide 3000	80 ± 20	56 ± 13
	nCuO	42 ± 7	41 ± 13
	bCuO	36 ± 7	37 ± 6
	Cu NP	25 ± 5 *	28 ± 5 *

Table 3. Altered stem nutrient content of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP. Values displayed as mean ± SE of 4 replicates per treatment. Asterisk (*), significantly different from control, ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)	
		50 mg/kg	200 mg/kg
Mo	Control	0.37 ± 0.03	0.37 ± 0.03
	Kocide 3000	0.39 ± 0.03	0.54 ± 0.09
	nCuO	0.38 ± 0	0.51 ± 0.03 *
	bCuO	0.36 ± 0.01	0.38 ± 0.02
	Cu NP	0.45 ± 0.03	0.46 ± 0.06
Fe	Control	82 ± 3	82 ± 3
	Kocide 3000	78 ± 3	102 ± 15
	nCuO	103 ± 6 *	109 ± 4 *
	bCuO	104 ± 6	92 ± 10
	Cu NP	97 ± 10	95 ± 5

Table 4. Altered leaf nutrient content of zucchini exposed to Kocide 3000, nCuO, bCuO, and Cu NP. Values displayed as mean ± SE of 4 replicates per treatment. Asterisk (*), significantly different from control, ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)	
		50 mg/kg	200 mg/kg
Zn	Control	32 ± 1	32 ± 1
	Kocide 3000	35 ± 1	41 ± 6
	nCuO	34 ± 2	45 ± 3 *
	bCuO	34 ± 2	34 ± 2
	Cu NP	34 ± 3	35 ± 2
Mo	Control	0.4 ± 0.05	0.4 ± 0.05
	Kocide 3000	0.39 ± 0.03	0.53 ± 0.08
	nCuO	0.37 ± 0.03	0.51 ± 0.06
	bCuO	0.38 ± 0.05	0.37 ± 0.02
	Cu NP	0.7 ± 0.09 *	0.61 ± 0.05
S	Control	2312 ± 148	2312 ± 148
	Kocide 3000	2575 ± 136	3699 ± 480 *
	nCuO	2934 ± 158	3902 ± 388 *
	bCuO	2745 ± 223	3175 ± 314
	Cu NP	3257 ± 407	3613 ± 356 *
P	Control	2654 ± 212	2654 ± 212
	Kocide 3000	2345 ± 141	3093 ± 377 *
	nCuO	2700 ± 124	3463 ± 258
	bCuO	2877 ± 126	3050 ± 216
	Cu NP	3459 ± 729	3346 ± 463

4. Conclusion

The application of Kocide 3000, nCuO, bCuO, or Cu NP to zucchini for 3 weeks, showed an increase in Cu concentration in all tissues. The transport of Cu observed here present the case for possible contamination of edible tissues. Despite the increase of Cu concentrations, no harmful effects were observed with respect to plant growth or chlorophyll production. In fact, the activity of enzymes associated with the sequestration of ROS decreased in root tissues. The decrease in CAT and APX activity suggests that the treatments provided a copper supplement that reduced stress and decreased the availability of ROS. This study suggests that copper exposure at relatively low concentrations under soil conditions may have beneficial effects, as exposed plants showed minimal adverse effects.

Chapter 4: Long-term effects of nano-copper exposure in sugarcane (*Saccharum officinarum*): Uptake, transport, and effects

1. Introduction

The fate of nanomaterials released into the environment, incidentally or otherwise, remains the subject of numerous studies. Currently, copper-based nanomaterials are being employed in a wide variety of consumer products and industrial processes (Dang et al., 2011; Kim et al., 2009). One area of concern is the contamination of agricultural soils, it has been shown that nanomaterials located in the soil can penetrate plant roots and translocate to aerial tissues (Servin et al., 2012). Studies into the exposure effects of copper nanomaterials to agricultural plants are widely reported, but results are often conflicting. Initial studies have shown that copper nanomaterial exposure can reduce germination rates, decrease plant growth, increase oxidative stress, and strain photosynthetic processes. Copper-based nanomaterials reduced the root length of both lettuce and alfalfa by 49% (Hong et al., 2015). Cilantro grown in soil containing nCuO or bCuO suffered reduced germination rates by 50% and 40%, respectively (Zuverza-Mena et al., 2015). Similarly, rice grown with nCuO at concentrations up to 1000 mg kg^{-1} showed decreases in biomass and germination (Da Costa and Sharma, 2016).

Lately, inquiries into these nanomaterials and their possible beneficial agricultural applications are showing promise. Recent studies have found copper-based nanomaterials, such as CuO can reduce the effects of drought stress (Dimkpa et al., 2017). Coated copper nanomaterials have also been shown to suppress plant diseases and increase yield (Sathiyabama and Manikandan, 2018). These studies remain overshadowed by the body of studies highlighting the negative outcomes of nanomaterial exposure. An important caveat of early studies was the implementation of short-term, high concentration experimental models that can force negative endpoint results. Recent research on the effects of exposure over the entire life-cycle of plants

is beginning to paint a different picture, with most growth-related effects diminishing or completely absent (Apodaca et al., 2017; Ochoa et al., 2017; Rawat et al., 2018).

New evidence highlights the need for continued research into the long-term effects of cooper nanomaterial exposure. Copper nanomaterial interactions with agricultural crops, such as sugarcane, are only now beginning to be explored in the long-term. Previous work with sugarcane showed an increase in oxidative stress and chlorophyll content, but no translocation of copper from roots into the aerial tissues (Tamez et al., 2019b). However, although this was conducted over nearly 11 months, growth conditions did not allow plants to reach maturity. It is possible that a study on plants reaching full maturity may alter these results. In order to answer this question, sugarcane plants were grown until maturity and edible portions can be harvested. After harvest, the translocation and effects of copper compounds/nanomaterials were measured including their effects on sugarcane juice production.

2. Methods

2.1. Nanomaterials/Compounds

All of the copper-based nanomaterials and compounds used in this study were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). Materials were used as received and had been previously characterized to ensure appropriate size, Kocide 3000 (10 μm), nCuO (10-100 nm), bCuO (100 nm to 10 μm), and Cu NP (100-1000 nm) (Hong et al., 2015). $CuCl_2$ served as a source of ionic Cu.

2.2. Study design

In order to facilitate the growth of the sugarcane to maturity, this study was conducted in a large-scale mesocosm format. After an initial sprouting in an environmental growth chamber for a period of 45-days, sugarcane sprouts were transplanted to #10 size large nursery pots filled with 15 kg of a 50:50 mixture of Garden Time brand top soil and Berger BM7 potting mix amended with 20, 40, or 60 mg kg^{-1} of one of the following compounds: Kocide 3000, nCuO,

bCuO, Cu NP, or $CuCl_2$. Treatments were applied as approximately 2 L suspensions that underwent direct sonication for 15 min to prevent agglomeration. Suspensions were added to the pre-weighed soil and mixed until homogenous. After 24 h sugarcane sprouts were transplanted into the treated pots and placed in a greenhouse.

2.3. Chlorophyll Content

After 200 days of exposure, relative chlorophyll content was determined using a single-photon avalanche diode (SPAD) chlorophyll meter. Chlorophyll content was determined by compiling 10 readings per plant into a single measurement for a replicate, with each treatment group consisting of 4 replicates.

2.4. Tissue Collection

In order to perform elemental and enzymatic analysis, representative samples of root and leaf tissues were collected and thoroughly washed to remove any contaminates. A sample of root tissues were immediately frozen using dry ice (solid CO_2), then stored at -80°C for enzymatic activity analysis. Remaining root and leaf tissues were oven dried at 70°C for 72 h. Oven-dried samples were subsequently homogenized using a household coffee grinder.

2.5. Sugarcane Juice Analysis

The edible portion of the sugarcane plant is the juice contained within the stalk. To determine the effect of copper nanomaterial/compounds on sugarcane juice quality, 2-4 sett pieces were manually pressed and °Brix measurements were taken using a Brix refractometer to determine sugar content. °Brix was chosen as a metric for sugar content due to the ease of performing the analysis, its wide spread acceptance in the sugar industry, and its correlation to the commercial cane sugar (CCS) metric (Mat Nawi et al., 2013). Aliquots of sugarcane juice were also set aside for elemental analysis. Juice yield was determined by dividing the volume of expressed juice by the mass of pressed stalk.

2.6. Enzymatic Activity Assays

As an indicator of the oxidative stress experienced by sugarcane, ascorbic peroxidase (APX) and super oxide dismutase (SOD) activities were measured. Enzyme assay substrates were prepared by homogenizing 0.6 g of root tissue in 25 mM potassium phosphate buffer (KH2PO4). After centrifugation, the supernatant was collected and samples were stored at -80°C until assays were performed. APX activity was evaluated by measuring the decomposition of H_2O_2 following methods previously established (Gallego et al., 1996; Murgia et al., 2004). SOD activity was determined by measuring the reduction in the absorbance of nitroblue tetrazolium (NBT). In short, 50 µL of enzyme extract was combined with NBT, L-methionine, EDTA, riboflavin, and potassium phosphate buffer. The mixture was then illuminated for 15 min with a compact fluorescent light and absorbance measurements were made. Inhibition was determined by comparing the absorbance of NBT in the enzyme extract samples to a control containing only the assay reagents. Units of SOD activity were defined as the amount of activity that lead to a 50% reduction in NBT concentration (Medina-Velo et al., 2018).

2.7. Copper Uptake and Nutrient Content

Copper uptake, translocation, macro (Ca, Mg, P, K, and S), and micro (Fe, Zn, B, Mo, and Mn) nutrient content were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES). Aqueous tissue samples were prepared by homogenizing previously oven-dried plant samples in a household coffee grinder, then 0.2 g of sample were hot block acid digested following EPA method 3050B with 5 mL of plasma pure nitric acid (HNO_3) at 115°C for 45 min. Sugarcane juice samples were similarly prepared for elemental analysis, using 0.5 g of juice and 3 mL of plasma pure nitric acid (HNO_3). Following digestion, samples were diluted to an appropriate volume with Millipore water.

2.8. Statistics

Experiments were performed in quadruplicate. Statistical differences between test plants and controls were determined with 1-way analysis of variance (ANOVA) significance tests

(p=0.05), followed by a Tukey's HSD post hoc analysis. To ensure normality, data were transformed when necessary. Non-parametric tests were employed when normality could not be achieved. All statistical tests were performed using R statistical software.

3. Results and Discussion

3.1. Copper Uptake and Translocation

Copper translocation has been well documented and research has shown increased root copper concentrations that moved into aerial tissues as well as the translocation of copper, applied to foliage, travel down to roots, indicating the ease of Cu transport (Da Costa and Sharma, 2016; Du et al., 2018; Elmer and White, 2016; Hong et al., 2015). In this study, roots of sugarcane grown in soils amended with either Kocide 3000, nCuO, or Cu NP at 60 mg kg^{-1} contained significantly higher copper concentrations, than control plants (Figure 13A). Sugarcane roots grown in soils treated with nCuO displayed an increase in Cu concentration from 37.5 mg kg^{-1} to 54.8 mg kg^{-1}, while plants exposed to either Kocide 3000 or Cu NP increased by 146% and 191%, respectively. Similarly, Cu content in leaf tissues of treated plants was affected by the applied treatments (Figure 13B). The leaves of sugarcane plants exposed to 60 mg kg^{-1} levels of Kocide 3000, nCuO, and bCuO showed Cu concentrations 98%, 90%, and 75% above controls. Meanwhile, 20, 40, and 60 mg kg^{-1} treatments of both Cu NP and $CuCl_2$ showed a significant increase, from 2.4 mg Cu kg^{-1} leaf tissue (dry mass) to a range of 3.9 to 4.6 mg Cu kg^{-1} leaf tissue (dry mass). Previous work assessing the speciation of tissue Cu found plants exposed to weathered nCuO contained predominately complexed Cu, while plants exposed to unweathered nanoparticles contained mostly nCuO (Servin et al., 2017). The trend of increased leaf Cu with increasing applied treatment supports the idea of the internalized Cu in all tissues being in the same form. However, the inconsistencies in root Cu throughout treatments suggests the source of Cu and concentration play a role in its transport to aerial tissues.

Mature bell peppers grown in soil treated with nCuO, bCuO, and $CuCl_2$ showed increases in root copper by as much as 196% (Rawat et al., 2018). Likewise, green peas grown for 90 days in soil amended with nCuO up to 100 mg kg^{-1} showed a 130% increase in root cooper concentrations (Ochoa et al., 2017). In short-term studies, exposure of plants to nano-sized copper has shown consistent uptake and translocation. For example, Arabidopsis thaliana cultivated in the presence of nCuO at 20 and 50 mg kg^{-1} showed an increase in root and shoot copper, up from 0.10 mg kg^{-1} to 1.08 and 2.22 mg kg^{-1}, respectively (Zhao et al., 2018).

Predictable changes in root copper concentrations were observed in a previous sugarcane study (Tamez et al., 2019b), and although the total amount of exposure in this study was higher, allowing the sugarcane plants to increase biomass and reach maturity has lessened the accumulation of copper in the root tissues. Sugarcane has been the subject of numerous phytoremediation studies due to its high biomass production and studies of its exposure to ionic forms of copper within the same range produced contrasting results (Yadav et al., 2010). Ionic copper concentrations of up to 130 mg kg^{-1} caused a 240% increase in root copper, up from 52 mg kg^{-1} to 178 mg kg^{-1} (Jain et al., 2008). This rise in root copper concentration lead to observable physiological differences: diminished plant height, reduced leaf area, and stunted root growth. This evaluation, however, was conducted on 50-day-old plants, highlighting sugarcane's ability to adjust to abiotic stressors, like excess copper exposure.

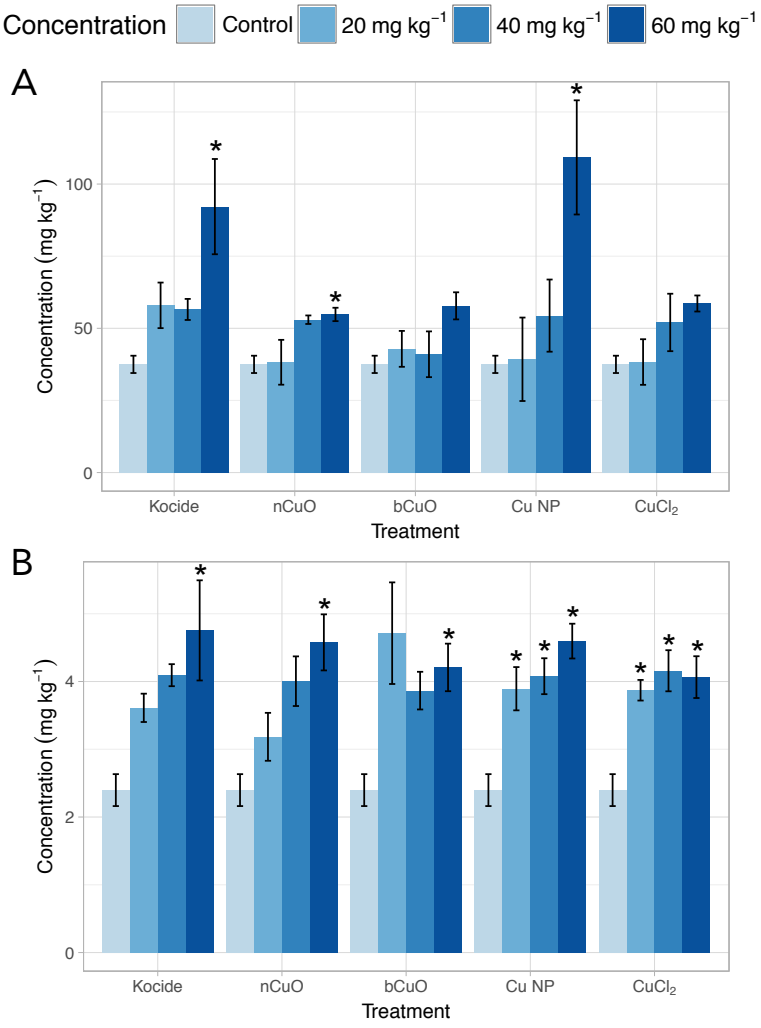

Figure 13. Copper concentration in A) the root and B) the leaf tissues of sugarcane exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 20, 40, and 60 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.2. Chlorophyll Content

Chlorophyll is one of the primary photosynthetic pigments; any alterations to its concentration can affect the photosynthetic process, thereby reducing the plants ability to produce and store sugar, affecting crop yield. Chlorophyll content in the leaves treated with any of the copper nanomaterials/compounds showed no statistical difference from control plants (Figure 14). In general, these results are similar to other mature studies, but contrast with immature studies. For example, a full life-cycle study of bell pepper exposed to nCuO and bCuO at concentrations up to 500 mg kg^{-1} showed no significant change in chlorophyll content (Rawat et al., 2018).

In addition, immature sugarcane exposed to the same levels of copper nanomaterials, showed significant increases in chlorophyll A content, but not in the total amount of chlorophyll (Tamez et al., 2019). Fluctuations in copper concentrations can have a dramatic effect on photosynthesis, especially due to its role in the electron transport train (Vinit-Dunand et al., 2002). Although there were significant changes to leaf Cu concentrations they were not sufficient to affect chlorophyll content.

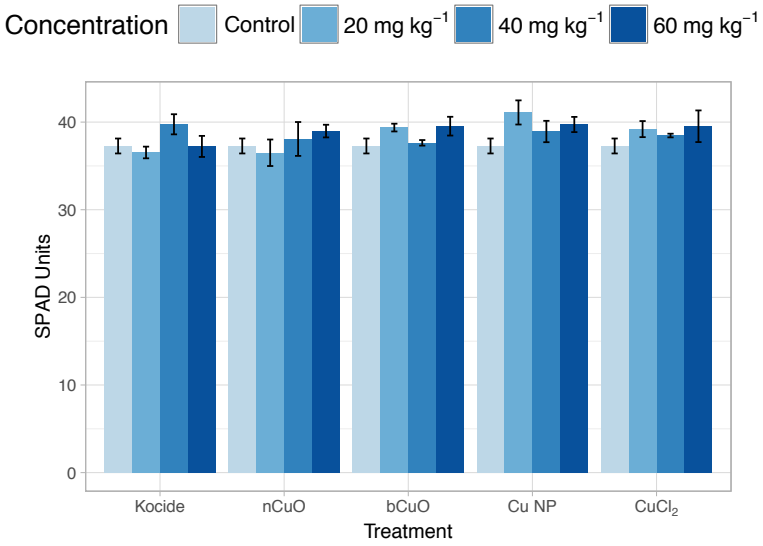

Figure 14. Chlorophyll content of sugarcane exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 20, 40, and 60 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.3. Ascorbate Peroxidase and Superoxide Dismutase Activity

Exposure to metal nanomaterials, such as nCuO has been associated with plant stress, which causes increased production of reactive oxygen species (ROS) (Hossain et al., 2015). The activities of both ascorbic peroxidase (APX) and superoxide dismutase (SOD) are reflective of this rise in stress. The activity of APX in sugarcane roots was down-regulated upon exposure to Kocide 3000, nCuO, Cu NP, and $CuCl_2$ (Figure 15A). Sugarcane treated with Kocide 3000 at 60 mg kg^{-1}, nCuO and Cu NP at 40 mg kg^{-1} exhibited APX enzyme activity reduced by 65–86%. Additionally, $CuCl_2$ at 20, 40, and 60 mg kg^{-1} experienced a 92%, 65%, and 80% decrease in APX activity, respectively, when compared to controls. Germination studies conducted on rice plants exposed to 1.0 and 1.5 mM solutions of CuO showed a 30% increase in APX activity after 14 d, compared to controls (Shaw and Hossain, 2013). Similarly, APX activity in cowpea roots increased 2-fold, upon exposure to Cu NP at concentrations up to 1000 mg kg^{-1} (Ogunkunle et al., 2018).

SOD activity (Figure 15B) in sugarcane roots treated with Cu NP at 40 and 60 mg kg^{-1} and $CuCl_2$ at 60 mg kg^{-1} showed a significant decrease of 58%, 60%, and 55%, respectively. Reductions in SOD activities of 67% and 57% were observed at 60 mg kg^{-1} of nCuO and bCuO, and an activity drop of 50% was observed in sugarcane treated with nCuO and bCuO at 20 mg kg^{-1}. Recent work with mature cowpeas has shown similar results, treatments of 1000 mg kg^{-1} of Cu NP exhibited a nearly 65% reduction in root SOD activity (Ogunkunle et al., 2018). However, 14 d rice seedlings exposed to nCuO showed an increase in SOD activity only in the highest treatment of 1.5 mM (Shaw and Hossain, 2013). Similarly, maize sprouts treated with 0.02 mg kg^{-1} showed an increase in SOD activity (Adhikari et al., 2016). The contrast in the results obtained from immature and mature studies shows that older plants may be better equipped to mitigate the stress imposed by copper nanomaterial exposure. SOD catalyzes the dismutation of superoxide radicals to H_2O_2 and O_2, the H_2O_2 is then scavenged by APX. Decreases in SOD/APX activity would indicate that Cu treatments are alleviating some environmental stress, however, increased root Cu matched with reduced APX or SOD activity in

plants treated with Kocide 3000, nCuO, and Cu NP at 60 mg kg^{-1}. Further, the mechanism of this stress relief remains unclear, though, as not all treatments with decreases in SOD corresponded to decreases in APX activity. Micronutrient deficiencies have been shown to reduce SOD and APX activity (Yu et al., 1998); in this study plants with reduced SOD or APX activity also displayed root decreases in B, Fe, Mo, Mn, or Zn (Table 5) indicating alterations to critical plant processes, inhibiting the ability to properly respond to stressors (Hajiboland, 2011).

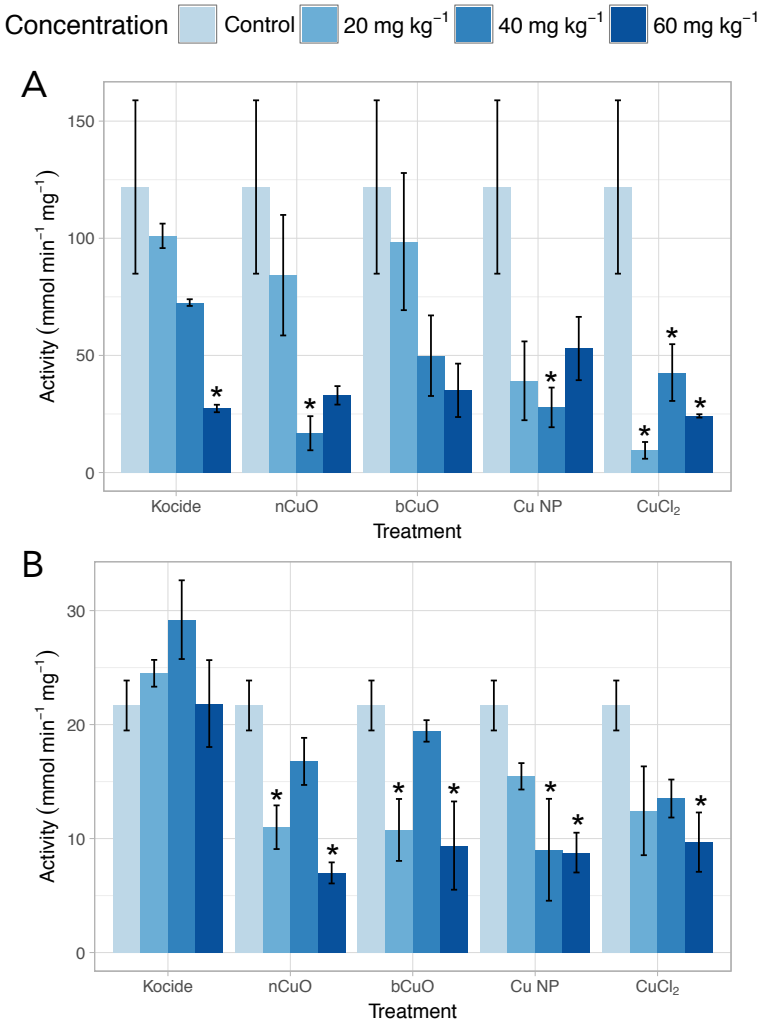

Figure 15. A) APX activity and B) SOD activity in the roots of sugarcane exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 20, 40, and 60 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.4. Nutrient Content

In order to assess sugarcane's ability to gather necessary minerals, micronutrients (Fe, B, Mn, Zn, and Mo) and macronutrients (P, K, Ca, S, and Mg) were measured (Tables 5 and 6). Generally, the applied treatments down-regulated the acquisition of nutrients in root tissues. All treatments, at varying concentrations, caused statistically significant changes in sugarcane roots. Root Fe levels were significantly reduced from 309 mg kg^{-1}, down to 109 and 113 mg kg^{-1} with nCuO at 20 and 60 mg kg^{-1}. Molybdenum accumulation in the roots decreased by approximately 27–36% when treated with nCuO, bCuO, or Cu NP at 20 mg kg^{-1} and Cu NP at 40 mg kg^{-1}. All concentration levels of nCuO lessened Zn from 32 mg kg^{-1} down to 20, 18, and 13 mg kg^{-1} for 20, 40, and 60 mg kg^{-1} treatments, respectively. There was a 57% decrease in Mn in sugarcane roots treated with nCuO at 20 mg kg^{-1}. Finally, there was nearly 25% decline in B uptake with sugarcane was treated with either nCuO, Cu NP, and $CuCl_2$ at 60 mg kg^{-1} and 43% with 20 mg kg^{-1} of nCuO. Comparably, there was a general decline in macronutrient accumulation in the roots. Phosphorous content was decreased by up to 24% in sugarcane treated with Kocide 3000 at 60 mg kg^{-1}, nCuO at 60 mg kg^{-1}, and $CuCl_2$ at 40 mg kg^{-1}. In contrast, S increased by over 56% in Cu NP and $CuCl_2$ at all concentrations, Kocide 3000 and bCuO at 20 mg kg^{-1}, and with nCuO and bCuO at 60 mg kg^{-1}.

In leaves Zn accumulation increased from 14 mg kg^{-1} to between 20 and 24 mg kg^{-1} when exposed to differing concentrations of nCuO, bCuO, Cu NP, and $CuCl_2$. In addition, leaf Fe accumulation was up-regulated by 70% upon treatment with Cu NP at 40 mg kg^{-1}. Macronutrient content in the leaves experienced little change, except for a 48% decrease in Mg concentration in the $CuCl_2$ 40 mg kg^{-1} treatment level. Changes to micronutrient minerals like Zn, Fe, and Mn play a critical role in the synthesis and activation of ROS scavenging enzymes, their reduction would prevent adequate response to stress (Hajiboland, 2011).

Table 5. Altered nutrient content of sugarcane roots exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$. Values displayed as mean ± SE of 4 replicates per treatment. Asterisk (*) indicate a statistically significant difference from control ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)		
		20 mg/kg	40 mg/kg	60 mg/kg
B	Control	1656 ± 71	1656 ± 71	1656 ± 71
	Kocide 3000	1863 ± 284	1543 ± 267	1361 ± 203
	nCuO	941 ± 150 *	1449 ± 106	1263 ± 63 *
	bCuO	1318 ± 157	1087 ± 330	1846 ± 266
	Cu NP	1290 ± 144	1440 ± 57	1245 ± 115 *
	$CuCl_2$	1358 ± 148	1632 ± 47	1215 ± 125 *
Fe	Control	309 ± 42	309 ± 42	309 ± 42
	Kocide 3000	740 ± 363	317 ± 131	265 ± 45
	nCuO	109 ± 13 *	126 ± 27	113 ± 22 *
	bCuO	213 ± 64	180 ± 65	214 ± 64
	Cu NP	335 ± 75	115 ± 37	371 ± 100
	$CuCl_2$	400 ± 137	383 ± 122	140 ± 5
Mo	Control	5.6 ± 0.1	5.6 ± 0.1	5.6 ± 0.1
	Kocide 3000	5.5 ± 0.7	3.9 ± 0.6	4.6 ± 0.8
	nCuO	3.6 ± 0.5 *	4 ± 0.8	5.2 ± 0.4
	bCuO	4 ± 0.3 *	4.1 ± 0.9	4.9 ± 0.6
	Cu NP	3.7 ± 0.3 *	4.1 ± 0.6 *	4.8 ± 0.3
	$CuCl_2$	4.4 ± 0.5	5.2 ± 0.6	4.5 ± 0.4
Zn	Control	32 ± 3	32 ± 3	32 ± 3
	Kocide 3000	40 ± 5	20 ± 9	26 ± 7
	nCuO	20 ± 4 *	18 ± 2 *	13 ± 1 *
	bCuO	23 ± 2	18 ± 4	32 ± 6
	Cu NP	23 ± 1	27 ± 2	26 ± 7
	$CuCl_2$	26 ± 2	23 ± 3	31 ± 3
Mn	Control	185 ± 26	185 ± 26	185 ± 26
	Kocide 3000	342 ± 145	178 ± 65	150 ± 32
	nCuO	79 ± 9 *	83 ± 17	123 ± 31
	bCuO	112 ± 8	131 ± 44	151 ± 39
	Cu NP	190 ± 47	127 ± 33	160 ± 0
	$CuCl_2$	191 ± 67	182 ± 54	87 ± 7
	Kocide 3000	788 ± 57	595 ± 9	574 ± 13 *
	nCuO	576 ± 31	614 ± 81	534 ± 44 *
	bCuO	678 ± 75	652 ± 67	729 ± 32
	Cu NP	715 ± 48	689 ± 70	688 ± 29
	$CuCl_2$	701 ± 16	786 ± 28 *	647 ± 18
S	Control	2871 ± 176	2871 ± 176	2871 ± 176
	Kocide 3000	4469 ± 183 *	3666 ± 221	3715 ± 323
	nCuO	2994 ± 279	3546 ± 293	4475 ± 457 *
	bCuO	4778 ± 480 *	4100 ± 621	4417 ± 253 *
	Cu NP	4917 ± 248 *	4779 ± 508 *	6499 ± 363 *
	$CuCl_2$	4986 ± 677 *	5520 ± 807 *	4888 ± 317 *

Table 6. Altered nutrient content of sugarcane leaves exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$. Values displayed as mean ± SE of 4 replicates per treatment. Asterisk (*) indicate a statistically significant difference from control ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)		
		20 mg/kg	40 mg/kg	60 mg/kg
Fe	Control	27 ± 4	27 ± 4	27 ± 4
	Kocide 3000	32 ± 1	35 ± 3	39 ± 11
	nCuO	30 ± 4	28 ± 2	35 ± 2
	bCuO	27 ± 3	28 ± 1	32 ± 4
	Cu NP	32 ± 2	46 ± 2 *	37 ± 1
	$CuCl_2$	42 ± 4	34 ± 4	36 ± 4
Zn	Control	14 ± 1	14 ± 1	14 ± 1
	Kocide 3000	14 ± 2	17 ± 1	18 ± 0
	nCuO	17 ± 1	18 ± 1	21 ± 0 *
	bCuO	24 ± 2 *	23 ± 2 *	17 ± 2
	Cu NP	16 ± 1	20 ± 2 *	21 ± 1 *
	$CuCl_2$	18 ± 2	20 ± 1 *	19 ± 1
Mg	Control	3715 ± 541	3715 ± 541	3715 ± 541
	Kocide 3000	2884 ± 88	3315 ± 319	2713 ± 304
	nCuO	3340 ± 387	3468 ± 385	3348 ± 317
	bCuO	4205 ± 414	2879 ± 446	3188 ± 207
	Cu NP	3244 ± 143	3178 ± 150	3978 ± 385
	$CuCl_2$	3040 ± 136	1940 ± 180 *	3105 ± 171

3.5. Sugarcane Juice Yield and Quality

A main concern on the exposure of sugarcane to copper nanomaterials is the effect on sugar juice production and juice quality. Sugarcane juice yield (Figure 16A), as obtained by hand-pressing, showed no difference when compared to control, expect for plants treated with Kocide 3000 at 60 mg kg^{-1}. Plants in this treatment produced 31% less juice than controls. Brix measurements of the obtained juice showed no changes in the total dissolved solids (TDS), signifying no difference in the sugar quality of the juice (Figure 16B).

Considering that fresh sugarcane juice is widely consumed, increases in copper concentrations could pose a potential food safety risk. When compared to controls (Fig. 16C) all treatments produced juice with elevated copper. All treatment levels of Cu NP produced cane juice with at least 75% more Cu than controls. Juice from sugarcane grown in soil amended with Kocide 3000 and nCuO at 20 and 60 mg kg^{-1} showed an increase in Cu of 75% and 58%, respectively, while bCuO at 20 mg kg^{-1} increased by 112%. Sugarcane treated with $CuCl_2$ at 40

mg kg^{-1} produced cane juice with the highest Cu content of 0.43 mg Cu kg^{-1} juice. To the best of the author's knowledge no work has been published on the effects of copper nanomaterial exposure on sugarcane juice quality, production, or changes to Cu content. It is clear, in the work presented here, that copper accumulation is occurring in the edible products. However, the increased Cu concentrations observed here would not pose a food safety risk, based on recommend Cu intake values for adults (Institute of Medicine Panel on Micronutrients, 2001).

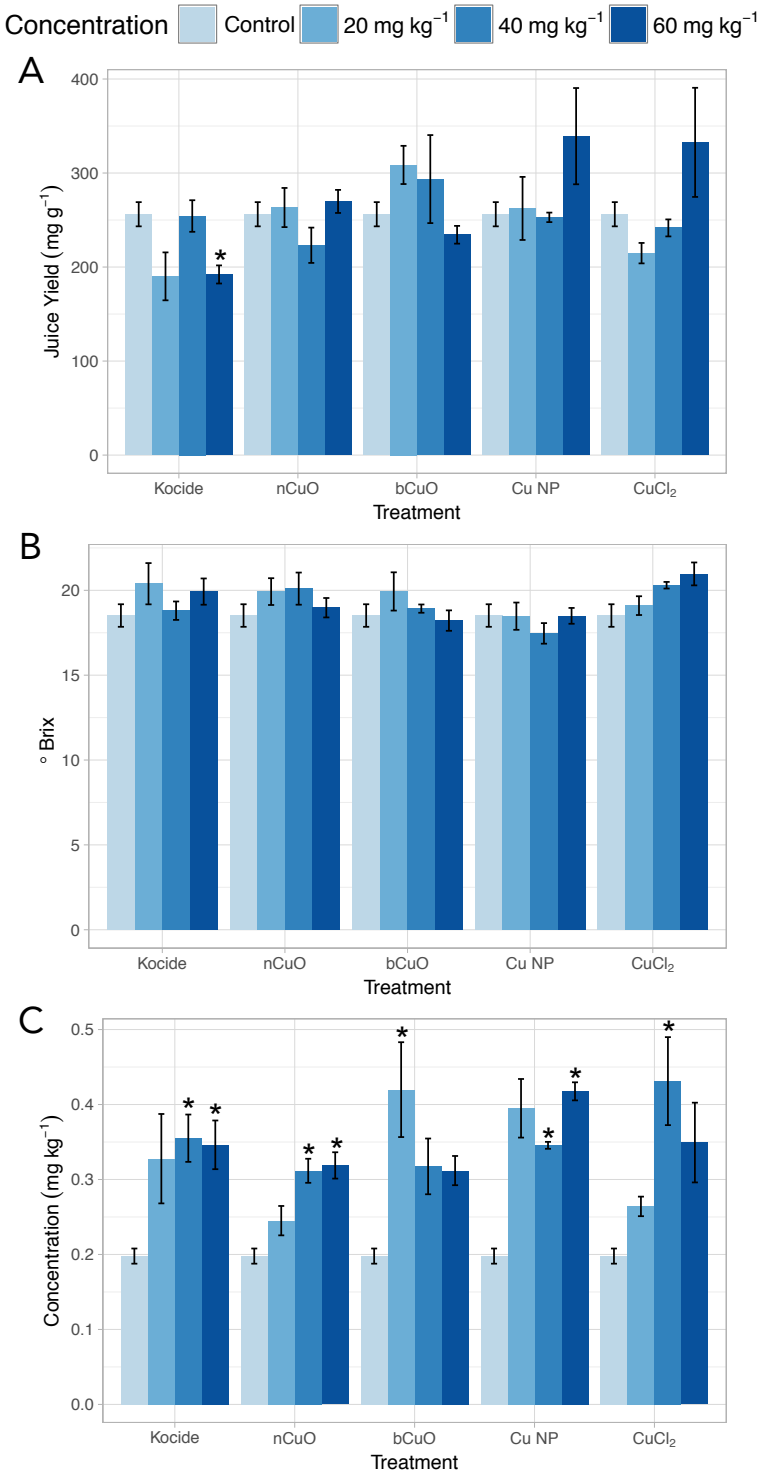

Figure 16. A) Juice yield, B) juice quality (measured as °Brix), and C) copper content of juice from sugarcane exposed to Kocide 3000, nCuO, bCuO, and Cu NP at 0, 20, 40, and 60 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

4. Conclusion

A large-scale mesocosm study was conducted to grow sugarcane to maturity in soil amended with either: Kocide 3000, nano-sized copper oxide (nCuO), micron-sized copper oxide (bCuO), copper metal nanoparticles (Cu NP), and $CuCl_2$. Elemental analysis revealed increases in copper concentrations in the roots of plants exposed to Kocide 3000, nCuO, and Cu NP at 60 mg kg^{-1}. Increases in Cu were measured in the leaves of sugarcane treated with Kocide 3000, nCuO, and bCuO at 60 mg kg^{-1}, as well as plants treated with Cu NP and $CuCl_2$. Additionally, there was accumulation of copper in the pressed cane juice collected from all treated plants, but this did not adversely affect the °Brix, which was used as an indicator of sugar content. Sugarcane treated with varying concentrations of nCuO, bCuO, Cu NP, or $CuCl_2$ showed a significant decrease of 50% or more in SOD activity. Similarly, APX activity decreased by 65% or greater in sugarcane plats that received Kocide 3000, nCuO, Cu NP, or $CuCl_2$ at various concentrations. The results obtain in this study are different from those obtained in a previous immature sugarcane study. It is evident from the results presented here, that sugarcane grown to maturity reduces the impacts of copper nanomaterial exposure. Increased biomass allows for better distribution of the higher copper levels in the soil, decreasing the need for a physiological response.

Chapter 5: Effects of nano-copper exposure in zucchini (*Cucurbita pepo*) grown to maturity

1. Introduction

The potential benefits from the application of nanotechnology are undeniable, however, there are still questions about the implications of their exposure to the environment. Nano-copper compounds can be found in a wide variety of consumer products and are widely used in industry due to their increased reactivity (Anjum et al., 2015). The fate of 95% these materials, once they have been consumed, is accumulation in soils or sediments (Keller et al., 2017) A primary concern that motivates researchers is the effects these compounds may have on plants, especially those that are agriculturally relevant, such as zucchini (*Cucurbita pepo*).

Published research on the interactions of nano-copper with plants have shown a variety of effects. Plants grown under hydroponic conditions and exposed to different nano-copper compounds have negatively affected. Mustard, squash, mung bean and lettuce exposed to nano-sized CuO or nano-sized Cu metal suffered from reduced growth, lower biomass, and a reduction in photosynthetic pigments (Zuverza-Mena et al., 2017). Additionally, exposure to nano-sized CuO has been shown to reduce the germination of cilantro and rice by up to 50% (Shaw and Hossain, 2013; Zuverza-Mena et al., 2015). Nano-copper exposure has also been shown to induce the generation of reactive oxygen species (ROS), usually measured through the activity of ROS sequestering enzymes. Lettuce grown in solution treated with 10 mg L^{-1} of nano-sized Cu/CuO nearly doubled catalase (CAT) activity while ascorbate peroxidase (APX) activity was reduced by 50% (Trujillo-Reyes et al., 2014).

Hydroponic studies are often contrasted by long-term soil-based studies showing little to no negative effects. For example, bell peppers grown to maturity display no signs of stunted growth or reduced chlorophyll production (Rawat et al., 2018). Nano and bulk-sized Cu metal at concentrations up to 200 mg kg^{-1} also did not alter oregano plant length or chlorophyll production (Du et al., 2018). When grown to maturity, green peas and kidney beans have not

shown indications of increased oxidative stress, as measured by CAT activity (Apodaca et al., 2017; Ochoa et al., 2017). Studies have demonstrated the potential for translocation of metal-based nanomaterials to edible tissues, including bioaccumulation in a food chain (Hawthorne et al., 2012; Majumdar et al., 2016; Servin et al., 2017). The differences in exposure effects between short-term hydroponic studies and long-term soil-based studies warrants increased research to ensure toxicity judgements are made with sufficient evidence. To the best of the authors' knowledge no work has been published on the effects of nano-copper exposure in zucchini grown to maturity.

The work presented here seeks to elucidate the effects of nano-copper long-term exposure over the life-span of the plant. In order to accomplish this zucchini (*Cucurbita pepo*) was grown to maturity in soil amended with either the commercially available fungicide Kocide 3000 ($Cu(OH)_2$), nano-sized CuO (nCuO), bulk CuO (bCuO), nano-sized metal Cu (Cu NP), or $CuCl_2$. Indicators of plant health, such as plant growth and chlorophyll content were monitored. The effects of nano-copper exposure on the production of reactive oxygen species (ROS) was evaluated by determining the activity of catalase and ascrobate peroxidase. Cu uptake and translocation were measured to determine the potential for edible tissue contamination.

2. Methods

2.1. Nanomaterials

Copper-based nanomaterials and compounds used in this study were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). Materials were used as received and had been previously characterized to ensure appropriate size, Kocide 3000 (10 μm), nCuO (10-100 nm), bCuO (100 nm to 10 μm), and Cu NP (100-1000 nm) (Hong et al., 2015). $CuCl_2$ served as a source of ionic Cu.

2.2. Study Design

In order to evaluate the effects of nano-copper exposure to grey zucchini, 3 kg of a 1:2 (v/v) mixture of Miracle-Gro brand potting and native El Paso soil, previously characterized as loamy sand with pH 7.9 (Zhao et al., 2015), was amended with different copper compounds: Kocide 3000, nCuO, bCuO, Cu NP, or $CuCl_2$. Soils were treated to a concentration of 50, 100, 200 or 400 mg kg^{-1} (Cu content). Treatments were applied as suspensions that underwent direct sonication for 15 min to prevent agglomeration of nanomaterials. Suspensions were added to the pre-weighed soil and mixed until homogenous, then soils were placed into 20 cm pots. After a 24-h settling period, 3 seeds were placed in each pot, with 4 replicate pots per treatment. All pots were then transferred to a greenhouse for the duration of the experiment.

2.3. Tissue Collection

After 80 d of exposure the experiment was ended and tissue samples were collected. Aerial plant length was recorded, then plants were then separated into roots, stems, and leaves. After root lengths were measured, tissues were washed with 5% nitric acid and weighed. Fresh tissues (roots and fully expanded leaves) were set aside for biochemical analysis and remaining tissues were oven-dried at 70°C for 72 h.

2.4. Chlorophyll Content

To assess the effects of Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ exposure to photosynthetic activity the concentration of chlorophyll was measured. Briefly, chlorophyll pigments were extracted by homogenizing 0.1 g of fresh leaf tissue in a mortar with 5 mL of 80% acetone. The extract was then analyzed by measuring the absorption of light at 663 nm (chlorophyll A) and 646 nm (Chlorophyll B). Concentrations were calculated following the equations proposed by Porra et al., 1989.

2.5. Enzymatic Activity Assays

The formation of reactive oxygen species is an indication of plant stressors. To assess the amount of stress experienced by the plant upon exposure to Kocide 3000, nCuO, bCuO,

Cu NP, and $CuCl_2$ the activity of ascorbic peroxidase (APX) and catalase (CAT) were measured. Briefly, 0.2 g of fresh root tissue was ground with potassium phosphate (K_2HPO_4) buffer and the resulting extract was stored in at -80°C until analysis. The APX assay was carried out by mixing sample extract with 25mM Ascorbate, 17 mM H_2O_2, and 0.1 M potassium phosphate (K_2HPO_4) buffer. APX activity was determined by measuring the decrease in absorption at 265 nm for 2 min. CAT activity was measured by the decrease in absorption at 240 nm for 3 min upon the addition of 10 mM H_2O_2 to the sample extract (Gallego et al., 1996; Murgia et al., 2004).

2.6. Copper Uptake and Nutrient Content

In order to monitor the uptake and distribution of Cu, macro-nutrients (Ca, Mg, P, K, and S), and micro-nutrients (Fe, Zn, B, Mo, and Mn) elemental analysis was performed using inductively coupled plasma optical emission spectroscopy (ICP-OES). Aqueous tissue samples were prepared by homogenizing oven-dried root, stem, and leaf samples in a household coffee grinder, then 0.2 g of sample were acid digested in a hot block following EPA method 3050B using 4 mL of plasma pure nitric acid (HNO_3) at 115°C for 45 min. Dried flower samples were similarly digested, using approximately 0.2 g of whole tissue. Following digestion, samples were diluted to an appropriate volume with Millipore water.

2.7. Statistics

All experiments were performed in quadruplicate. One-way analysis of variance (ANOVA) significance tests were performed to determine differences between treated and control plants using a p-value of 0.05. ANOVA tests were followed by a Tukey's HSD post hoc analysis. Data were transformed when necessary, to ensure normality. Non-parametric Kruskal-Wallace tests were employed when normality could not be achieved. All statistical tests were performed using R statistical software.

3. Results and Discussion

3.1. Copper Uptake and Translocation

In this study significant uptake and translocation of Cu was observed in zucchini roots, leaves and flowers. Zucchini root (Figure 17A) tissues exposed Kocide 3000, nCuO, Cu NP, and $CuCl_2$ at concentrations of 100–400 mg kg^{-1} and bCuO at 200 and 400 mg kg^{-1} contained at least 85% more Cu than control plants. Nearly all treatments, expect $CuCl_2$ at 50 mg kg^{-1}, increased Cu in leaf tissues from 6.2 mg kg^{-1} to between 12.8 to 26.7 mg kg^{-1} (Figure 17C). The male flowers of the zucchini plant experienced the greatest accumulation of Cu upon exposure to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ across the entire treatment range (Figure 17D). Cu concentrations in flower tissues increased between 221% to 477% from control levels of 9.6 mg kg^{-1}. Similar to previous studies involving plant exposure to nano-copper compounds Cu concentrations were significantly higher in nearly all tissues of treated zucchini (Peng et al., 2015; Zhao et al., 2016). Regardless growth medium it has been consistently shown the highest accumulation of Cu occurs in the roots of treated plants (Apodaca et al., 2017; Ochoa et al., 2017; Rawat et al., 2018; Trujillo-Reyes et al., 2014). The main concern with zucchini exposure to nano-copper compounds is the potential for edible tissues to contain nano-copper or elevated copper concentrations. Albeit that zucchini fruit were not obtained in this study, the greatest change in copper concentrations were observed in the flowers collected and zucchini flowers are often consumed as a garnish in soup dishes. Additionally, evidence has shown nano-copper exposure induces Cu accumulation in the edible tissues of lettuce, carrots and sweet potato exposed to nCuO (Bradfield et al., 2017; Ebbs et al., 2016; Trujillo-Reyes et al., 2014). In this study, Cu was easily acquired by zucchini, regardless of source, and translocated to aerial tissues with the only exception being that no Cu accumulation occurred in stem tissues, except in zucchini treated with nCuO at 400 mg kg^{-1}. Further, it is clear that the edible male flowers of zucchini are serving as a Cu sink, leaving open the possibility of Cu translocation to zucchini fruit.

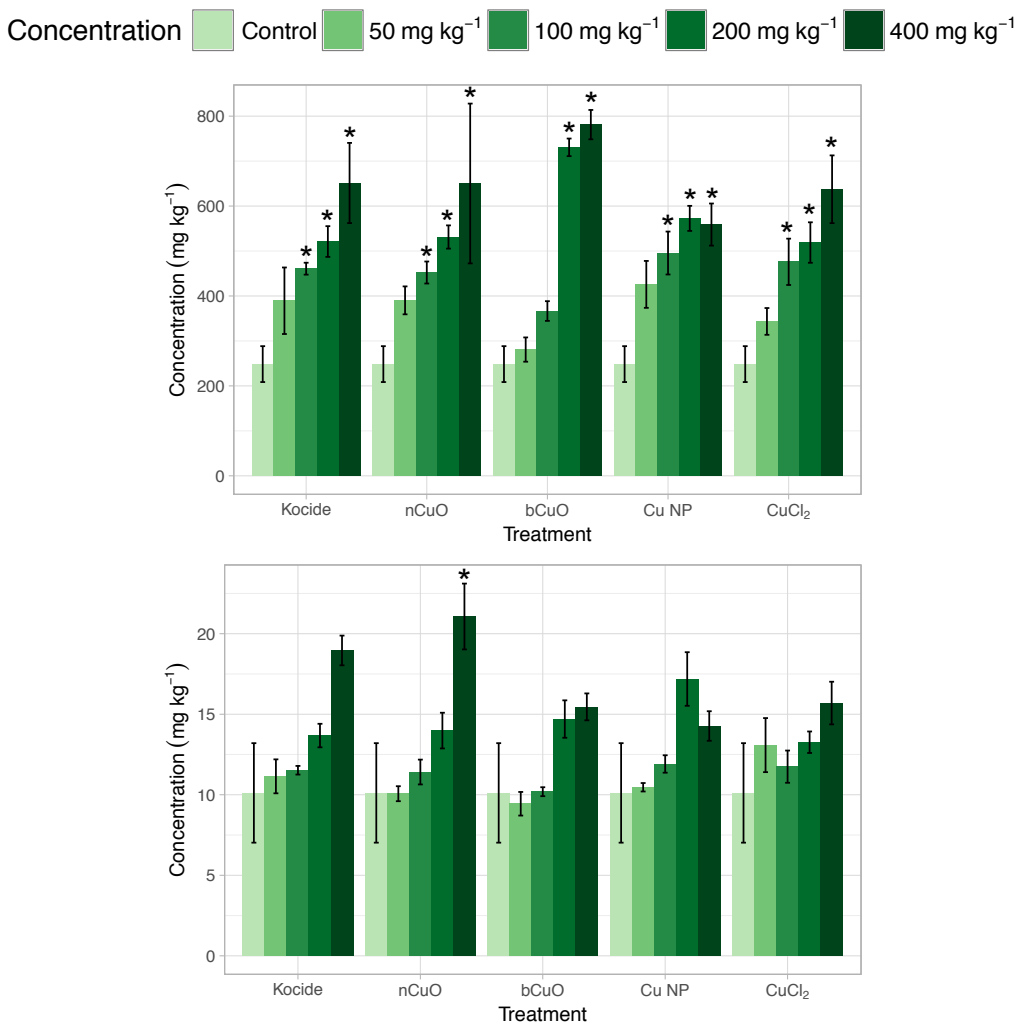

Figure 17. Copper concentration in A) the roots and B) the stems of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

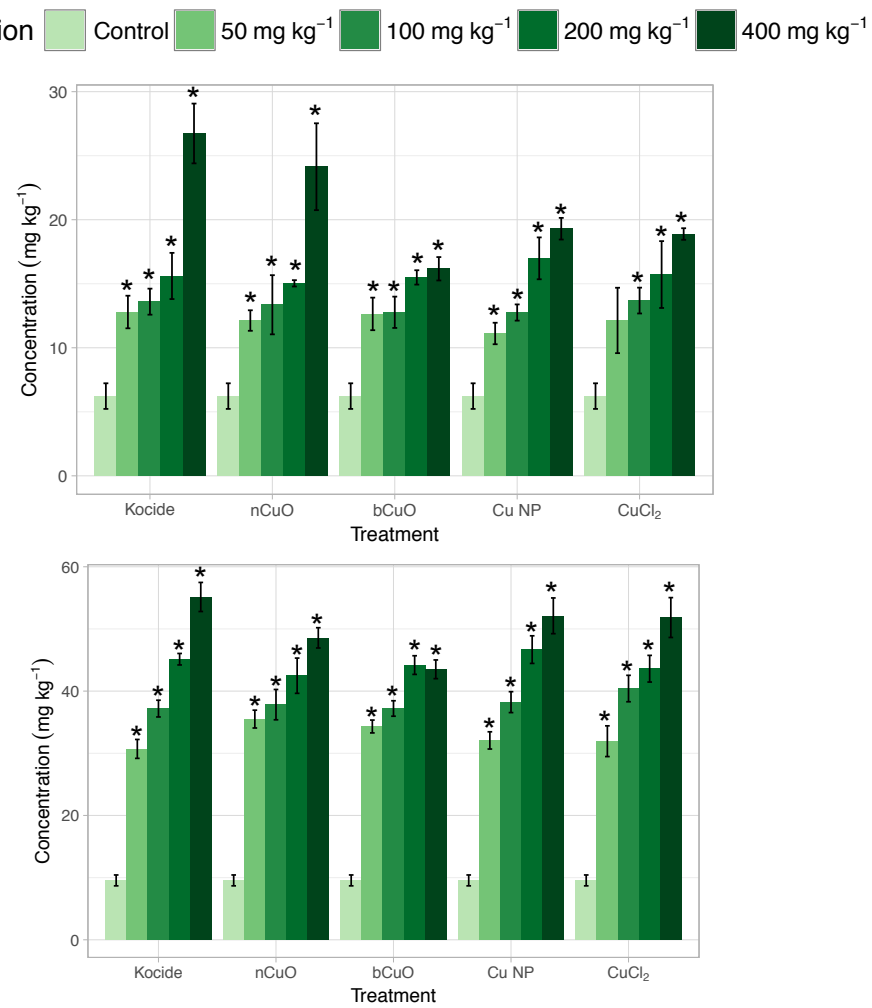

Figure 18. Copper concentration in the A) leaves and B) flowers of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.2. Effects of Nano-copper on growth

Plant and root length were monitored to assess changes in plant growth due to exposure to either Kocide 3000, nCuO, bCuO, Cu NP, or $CuCl_2$. None of the applied treatments produced plants that grew significantly different than controls (Figure 19A and B). These results are similar to a previous 3 week study that found no growth affects after treatments up to 200 mg kg^{-1} (Tamez et al., 2019a). Short-term studies conducted in agar or under hydroponic conditions with nano-copper compounds with maximum concentrations ranging from 20 to 1000 mg L^{-1} showed reduced root elongation, shoot growth, and diminished biomass (Zuverza-Mena et al., 2017). The results presented here are supported by other long-term studies with bell pepper and green peas that found no significant changes in plant growth with exposure to nano-copper compounds at concentrations up to 500 mg kg^{-1} (Ochoa et al., 2017; Rawat et al., 2018).

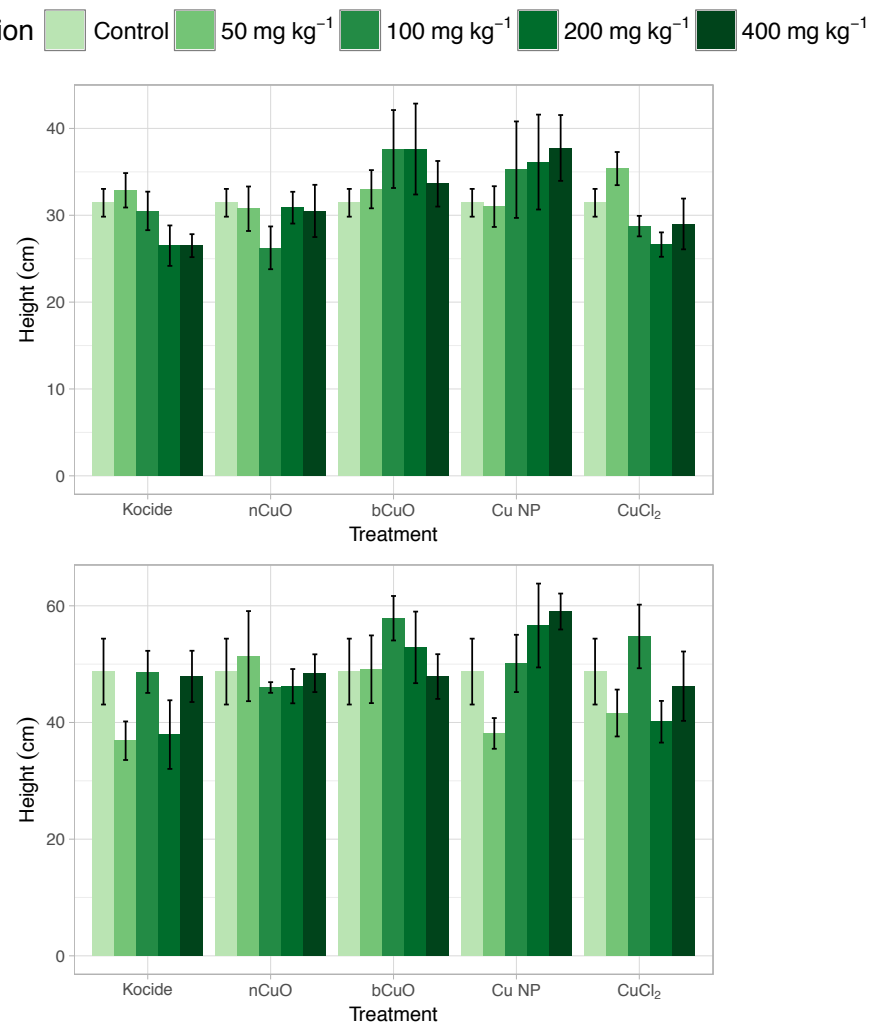

Figure 19. Plant length (A) and root length (B) of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.3. Chlorophyll content

Photosynthetic pigments are a crucial part of proper plant function, changes in their production could affect photosynthesis, which could in turn affect other plant processes (Saison et al., 2010). Upon exposure to the tested nano-copper compounds, only zucchini grown in soils amended with $CuCl_2$ at 50 and 400 mg kg^{-1} contained significantly less chlorophyll A and B than control plants (Figure 20A and B). All remaining plants contained chlorophyll consistent with control levels, although chlorophyll B concentrations did decrease in some cases by up to 52% these changes were not significant. Publish research has shown metal oxide nanomaterial exposure can lead to decreased chlorophyll production in hydroponically-grown plants such as rice, green peas, soybean, and radish (Du et al., 2017). However, when compared to studies conducted with nano-copper compounds under similar conditions (soil medium, moderate concentrations) no changes to chlorophyll production were measured in bell pepper, oregano, cilantro, or kidney beans (Apodaca et al., 2017; Du et al., 2018; Rawat et al., 2018; Zuverza-Mena et al., 2015). An exception to this trend was soybean that began to show reduced chlorophyll with exposure to 400 mg kg^{-1} of nCuO (Zuverza-Mena et al., 2017).

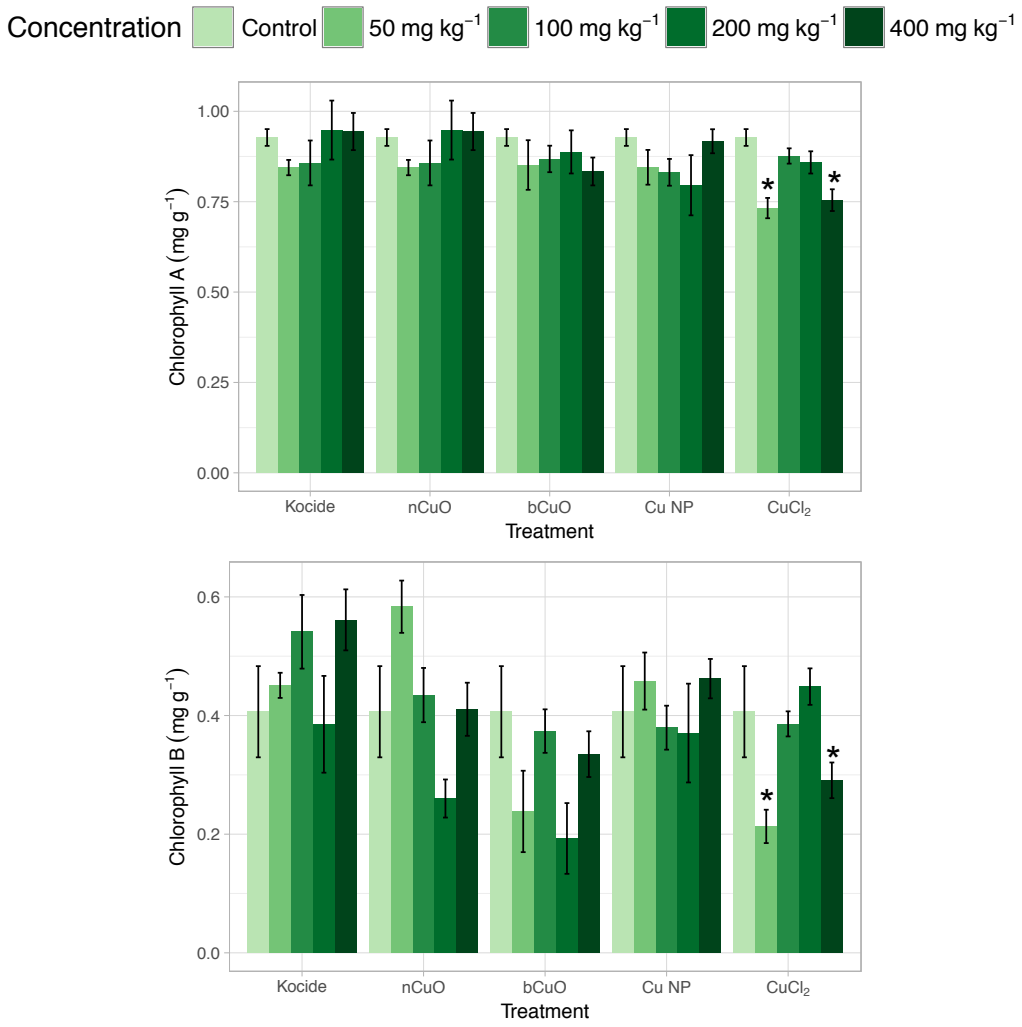

Figure 20. Chlorophyll content of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. A) Chlorophyll A and B) chlorophyll B. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.4. Ascorbate Peroxidase and Catalase Activity

It has long been established that plants produce reactive oxygen species (ROS) in response to abiotic stressors (Mittler, 2002). The activity of ascorbate peroxidase (APX) and catalase (CAT) were measured to determine whether zucchini grown in soils amended with nano-copper compounds elicit a stress response (Figure 21A and B). APX activity increased by 112% and 70% only in zucchini grown in soils treated with bCuO and Cu NP at 100 mg kg^{-1}. These results are distinct from previous studies on rice, lettuce, and alfalfa where APX activity increased when plants were grown exposed to either Kocide 3000, nCuO, Cu NP or $CuCl_2$ (Hong

et al., 2015; Shaw and Hossain, 2013). The absence of APX activity induction was also observed in a study conducted on lettuce with Cu/CuO nanoparticles, supporting the lack of toxic effect.

Further, CAT activity remained similar to control levels, except for an increase of 81% and 109% at the 400 mg kg^{-1} application of Cu NP and $CuCl_2$, respectively. Kidney beans and green peas grown in soil amended with Cu NP or nCuO, respectively, at concentrations up to 100 mg kg^{-1} experienced no change in CAT activity compared to controls (Apodaca et al., 2017; Ochoa et al., 2017). In short-term studies, especially those conducted hydroponically, have consistently shown CAT activity to increase upon nano-copper exposure, highlighting the importance of the growth medium (Dimkpa et al., 2012; Trujillo-Reyes et al., 2014). The results presented here are contrary to a previous 3-week zucchini study, in which both APX and CAT activity were observed to decrease upon exposure to the same nano-copper compounds at 50 and 200 mg kg^{-1}. Although it has been reported that excess tissue Cu can lead to the formation of H_2O_2 via Fenton like reactions (Anjum et al., 2011), increased APX and CAT activities were not isolated to roots with the highest Cu concentrations, eliminating this possibility. In contrast to most of the available published research, plants grown in a natural soil medium for a longer period of time are better equipped to handle the stresses of nano-copper experienced in earlier studies.

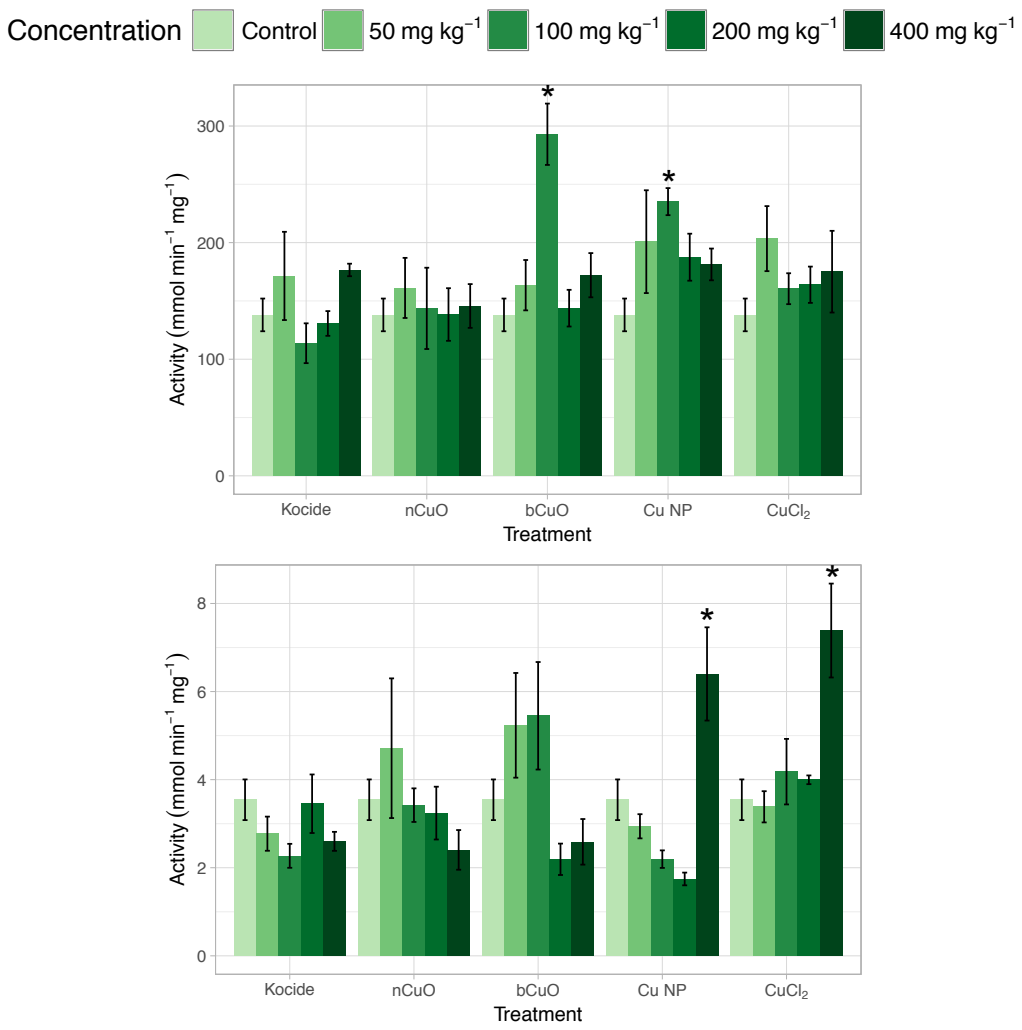

Figure 21. A) Ascorbate peroxidase and B) catalase activity for zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

3.5. Nutrient Content

In general, zucchini exposure to Kocide 3000, nCuO, bCuO, Cu NP, or $CuCl_2$ caused changes in nutrient acquisition, though not all were significant. Roots of zucchini plants exposed to Kocide 3000 at 400 mg kg^{-1}, nCuO at 200 and 400 mg kg^{-1}, and Cu NP at 200 mg kg^{-1} contained between 30 to 40% less P than controls (Table 7). Zucchini treated with Cu NP at 100 mg kg^{-1} produced roots with 22% Ca and 66% Mn deficiency. Fe decreased by 34 and 40% in the stems of plants treated with nCuO at 50 and 100 mg kg^{-1}, respectively (Table 8). Simultaneously, stem Fe was reduced, on average, by 28% in zucchini treat with $CuCl_2$ at 100 and 400 mg kg^{-1}. Stem P decreased by approximately 31% in plants exposed to Kocide 3000 at 50 mg kg^{-1} and nCuO at 50, 200, and 400 mg kg^{-1}. Concurrently, P was diminished by 37 to 53% in plants treated with Cu NP and by 35 to 62% in the $CuCl_2$ treatments. Zucchini treated with either Kocide 3000 or nCuO at 50, 200, or 400 mg kg^{-1} reduced Mn by up to 56%.

Leaf tissues experienced similar changes to nutrient content as those seen in the stems (Table 9). Fe content in the leaves of plants treated with 400 mg kg^{-1} of both bCuO and $CuCl_2$ decreased by nearly 40%; when plants were treated with 100 mg kg^{-1} of $CuCl_2$ the decrease was only 30%. There were significant reductions in P of at least 25% in zucchini treated with Kocide 3000 at 100 or 200 mg kg^{-1} and with nCuO, bCuO, Cu NP, or $CuCl_2$ at any treatment level. A similar pattern was observed with respect to Mn concentrations, with all treatment levels of nCuO, bCuO, Cu NP, and CuC_2 experiencing a decrease of approximately 20% or greater. Plants treated with Kocide 3000 at 50, 200, and 400 mg kg^{-1} also contained significantly lower Mn, between 30 and 44%, compared to controls. Zucchini flowers underwent the fewest changes to nutrient content where Fe concentrations were depressed up to 22% in plants treated with nCuO at any concentration or with Kocide 3000 at 200 and 400 mg kg^{-1}(Table 10). Additionally, Mn was reduced by less than 20% with Kocide 3000 at 200 mg kg^{-1}, nCuO at 100 and 400 mg kg^{-1}, and with Cu NP at 400 mg kg^{-1}. Changes in P content were limited to a 19% decrease in flowers from plants treated with Cu NP at 200 mg kg^{-1}.

Table 7. Altered root nutrient content of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)			
		50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Mn	Control	303 ± 36	303 ± 36	303 ± 36	303 ± 36
	Kocide 3000	356 ± 96	381 ± 15	212 ± 82	285 ± 87
	nCuO	409 ± 86	497 ± 100	362 ± 56	268 ± 91
	bCuO	312 ± 72	315 ± 23	440 ± 32	456 ± 34
	Cu NP	419 ± 27	505 ± 65 *	372 ± 54	304 ± 46
	$CuCl_2$	373 ± 48	394 ± 45	337 ± 24	290 ± 22
P	Control	4946 ± 269	4946 ± 269	4946 ± 269	4946 ± 269
	Kocide 3000	3777 ± 346	4726 ± 482	2573 ± 906	3286 ± 584 *
	nCuO	4162 ± 317	3450 ± 182	3376 ± 438 *	2880 ± 687 *
	bCuO	4915 ± 629	4715 ± 379	3466 ± 277	3831 ± 291
	Cu NP	3532 ± 430	3594 ± 347	2987 ± 356 *	4635 ± 784
	$CuCl_2$	5176 ± 513	4613 ± 637	4793 ± 443	4299 ± 237
Ca	Control	10728 ± 447	10728 ± 447	10728 ± 447	10728 ± 447
	Kocide 3000	10810 ± 356	10988 ± 389	8807 ± 3023	9964 ± 800
	nCuO	12329 ± 1051	12010 ± 839	11305 ± 349	11314 ± 824
	bCuO	12056 ± 2503	12416 ± 837	12883 ± 297	12567 ± 484
	Cu NP	12110 ± 631	12987 ± 682 *	10499 ± 448	11042 ± 213
	$CuCl_2$	11347 ± 429	11214 ± 371	11487 ± 508	9929 ± 461

Table 8. Altered stem nutrient content of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)			
		50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Fe	Control	52 ± 3	52 ± 3	52 ± 3	52 ± 3
	Kocide 3000	31 ± 2	40 ± 6	36 ± 6	40 ± 13
	nCuO	34 ± 3 *	31 ± 3 *	44 ± 5	45 ± 8
	bCuO	40 ± 4	45 ± 8	49 ± 5	48 ± 4
	Cu NP	57 ± 10	43 ± 8	50 ± 5	50 ± 14
	$CuCl_2$	62 ± 2	38 ± 2 *	42 ± 2	37 ± 4 *
Mn	Control	175 ± 11	175 ± 11	175 ± 11	175 ± 11
	Kocide 3000	120 ± 8 *	140 ± 10	89 ± 13 *	78 ± 16 *
	nCuO	119 ± 2 *	143 ± 12	118 ± 12 *	85 ± 12 *
	bCuO	146 ± 6	136 ± 13	120 ± 6 *	110 ± 14 *
	Cu NP	137 ± 12	113 ± 4 *	109 ± 9 *	101 ± 4 *
	$CuCl_2$	137 ± 14	112 ± 8 *	111 ± 1 *	96 ± 5 *
P	Control	4856 ± 351	4856 ± 351	4856 ± 351	4856 ± 351
	Kocide 3000	3517 ± 161 *	3857 ± 146	3886 ± 150	4164 ± 394
	nCuO	3399 ± 538 *	3582 ± 303	3166 ± 123 *	3292 ± 286 *
	bCuO	3045 ± 316 *	2999 ± 303 *	3629 ± 408	2660 ± 49 *
	Cu NP	2764 ± 221 *	3077 ± 117 *	3175 ± 216 *	2283 ± 249 *
	$CuCl_2$	3172 ± 185 *	2256 ± 187 *	2280 ± 273 *	1859 ± 199 *

Table 9. Altered leaf nutrient content of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)			
		50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Fe	Control	134 ± 8	134 ± 8	134 ± 8	134 ± 8
	Kocide 3000	114 ± 9	122 ± 18	106 ± 10	115 ± 21
	nCuO	105 ± 11	112 ± 19	88 ± 3	135 ± 45
	bCuO	109 ± 15	99 ± 6	102 ± 6	83 ± 3 *
	Cu NP	119 ± 20	92 ± 10	108 ± 32	83 ± 2
	$CuCl_2$	104 ± 9	94 ± 12 *	97 ± 14	81 ± 3 *
Mn	Control	285 ± 16	285 ± 16	285 ± 16	285 ± 16
	Kocide 3000	200 ± 7 *	224 ± 18	160 ± 15 *	184 ± 14 *
	nCuO	197 ± 7 *	222 ± 8 *	193 ± 11 *	171 ± 15 *
	bCuO	234 ± 16 *	212 ± 11 *	202 ± 10 *	193 ± 22 *
	Cu NP	201 ± 5 *	204 ± 17 *	194 ± 11 *	186 ± 5 *
	$CuCl_2$	222 ± 14 *	198 ± 6 *	192 ± 11 *	168 ± 6 *
P	Control	2285 ± 113	2285 ± 113	2285 ± 113	2285 ± 113
	Kocide 3000	1976 ± 76	1611 ± 85 *	1707 ± 173 *	2098 ± 78
	nCuO	1746 ± 139 *	1486 ± 137 *	1432 ± 60 *	1542 ± 95 *
	bCuO	1512 ± 138 *	1494 ± 70 *	1584 ± 151 *	1301 ± 82 *
	Cu NP	1369 ± 165 *	1311 ± 135 *	1511 ± 174 *	1246 ± 57 *
	$CuCl_2$	1308 ± 172 *	1224 ± 91 *	1176 ± 108 *	917 ± 41 *
Ca	Control	68448 ± 1881	68448 ± 1881	68448 ± 1881	68448 ± 1881
	Kocide 3000	64416 ± 3544	62473 ± 1826	64932 ± 3745	58378 ± 780 *
	nCuO	64294 ± 2927	67323 ± 4547	68713 ± 2631	65719 ± 2808
	bCuO	66329 ± 1731	66524 ± 3190	64073 ± 492	63685 ± 1451
	Cu NP	67705 ± 2596	66447 ± 5087	58590 ± 1755	57143 ± 1990
	$CuCl_2$	67103 ± 3497	65941 ± 2144	65091 ± 4347	63148 ± 5065 *

Nano-copper treatment significantly reduced P content in the stems and leaves of treated plants. P plays a crucial role in cellular processes, and is a principal component of energy storage compounds such as ATP. Deficiencies in P can cause a reduction photosynthetic activity (Maathuis, 2009), however, there were only significant reductions in chlorophyll in plants treated with $CuCl_2$ at 50 and 400 mg kg^{-1}. Similarly, Mn decreases are associated with diminished photosynthetic activity and increase oxidative stress (Hajiboland, 2011). The absence of these effects suggests that the changes in P and Mn were statistically significant but not necessarily biologically significant.

Table 10. Altered flower nutrient content of zucchini exposed to Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ at 0, 50, 100, 200 and 400 mg kg^{-1}. Values displayed as mean ± SE of 4 replicates per treatment. Asterisks (*) indicate a statistically significant difference from control ($p \leq 0.05$).

Nutrient	Treatment	Concentration (mg/kg dry tissue)			
		50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Fe	Control	113 ± 1	113 ± 1	113 ± 1	113 ± 1
	Kocide 3000	96 ± 5	96 ± 10	88 ± 7 *	88 ± 7 *
	nCuO	99 ± 4 *	88 ± 3 *	91 ± 5 *	92 ± 4 *
	bCuO	128 ± 2	102 ± 11	115 ± 26	97 ± 6
	Cu NP	112 ± 5	106 ± 10	107 ± 10	101 ± 4
	$CuCl_2$	106 ± 9	108 ± 4	118 ± 6	104 ± 10
Mn	Control	241 ± 6	241 ± 6	241 ± 6	241 ± 6
	Kocide 3000	212 ± 5	221 ± 12	199 ± 7 *	206 ± 19
	nCuO	218 ± 4	211 ± 4 *	224 ± 10	195 ± 10 *
	bCuO	255 ± 10	226 ± 6	221 ± 12	214 ± 7
	Cu NP	233 ± 3	236 ± 3	245 ± 13	208 ± 12 *
	$CuCl_2$	244 ± 19	221 ± 8	223 ± 8	208 ± 4
P	Control	7800 ± 292	7800 ± 292	7800 ± 292	7800 ± 292
	Kocide 3000	6672 ± 312	6731 ± 123	6325 ± 239	6543 ± 307
	nCuO	7665 ± 325	6750 ± 745	6632 ± 335	7073 ± 441
	bCuO	6273 ± 266	6408 ± 480	6728 ± 576	6986 ± 558
	Cu NP	6503 ± 235	6622 ± 250	6332 ± 526 *	6720 ± 197
	$CuCl_2$	6569 ± 238	7188 ± 505	6367 ± 323	6392 ± 394

4. Conclusion

In order to evaluate the long-term toxicity of nano-copper compounds zucchini was grown in soil amended with Kocide 3000, nCuO, bCuO, Cu NP, or $CuCl_2$. Analysis of tissues revealed all nano-compounds tested raised Cu concentrations in all tissues except for stems, which only showed an increase upon exposure to nCuO at 400 mg kg^{-1}. The high amount of Cu translocation reinforces the possibility of food chain contamination. This is especially true with zucchini flowers which are widely consumed and showed a Cu increase of up to 446%. Despite higher Cu concentrations in the tissues plant growth or chlorophyll production were not impacted. Additionally, only plants treated with bCuO and Cu NP at 100 mg kg^{-1} and Cu NP and $CuCl_2$ at 400 mg kg^{-1} showed higher APX or CAT activity, respectively. This evidence gathered in this study suggests that long-term exposure to nano-copper compounds does not produce the same toxic effects seen in short-term studies.

Chapter 6: Conclusions

The doctoral research presented here was conducted in order to determine the effects of the exposure of Kocide 3000, nCuO, bCuO, Cu NP, and $CuCl_2$ on two different agriculturally relevant crops, sugarcane (*Saccharum officinarum*) and zucchini (*Cucurbita pepo*). The data collected provides significant information on the uptake and translocation of Cu, changes in chlorophyll production, the activity of stress related enzymes, and alterations to nutrient content. These studies were performed on both during the early life of the plant and until maturity was reached.

Previously published research demonstrated that plant exposure to nano-copper compounds caused visibly negative effects, such as stunted growth. In sugarcane there were no significant changes in the growth, as measured by plant height, stalk diameter, and by the number and size of secondary shoots. Similarly, zucchini plant height, root length or biomass was not altered by exposure to any of the tested compounds/materials. Although physiological measurements of the plants grown to maturity were not recorded, visibly treated plants were indistinguishable from control plants or plants from different treatments.

One important factor in determining if nano-copper exposure imposed a toxic effect was the measurement of chlorophyll. The effect of nano-copper exposure on chlorophyll content differed for sugarcane between the early life-cycle and mature studies. An important distinction between these two studies is although both were grown for the same amount of time (10–11 months), in the first study sugarcane did not mature as they did in the second study due to growing conditions. In the early life-cycle sugarcane study chlorophyll A content was at least 80% higher in plants treated with Kocide 3000, bCuO, and $CuCl_2$, while chlorophyll B content decrease with Cu NP at 40 mg kg^{-1}. When sugarcane was allowed to grow to maturity changes in chlorophyll content disappeared. The effect of nano-copper on chlorophyll production in zucchini was different to the trends seen in sugarcane. Zucchini grown for 3 weeks showed up to a 13% decrease in chlorophyll A, with treatments of nCuO and bCuO at 50 and 200 mg kg^{-1},

respectively, while chlorophyll B remained at control levels. Mature zucchini also displayed a significant decrease in chlorophyll A and B content, but changes were limited to only nCuO and bCuO at 50 and 400 mg kg^{-1}.

The second indicator of exposure toxicity utilized in these studies was the measurement of stress related enzyme activity. Enzymes such as ascorbate peroxidase (APX), catalase (CAT),or superoxide dismutase (SOD) scavenge the reactive oxygen species (ROS) H_2O_2 (APX and CAT) or O_2^- (SOD). APX activity in sugarcane treated with Cu NP at 60 mg kg^{-1} and $CuCl_2$ at 20 and 60 mg kg^{-1} increased by over 186%. CAT was both up-regulated and down-regulated, at time within the same treatment. Activity increased by more than 73% with treatments of bCuO and $CuCl_2$ at 20 mg kg^{-1} while decreasing by about 60% with treatment concentrations of 60 and 40 mg kg^{-1}, respectively. Changes in stress enzyme activity were more consistent with a general trend lowered APX and SOD activity with nano-copper treatment. Sugarcane treated with Kocide 3000, nCuO, and Cu NP at 60, 40, and 40 mg kg^{-1}, respectively, displayed a 60% decrease in APX activity. Meanwhile, all concentrations of $CuCl_2$ reduced APX activity by up to 90%. APX and CAT activity in the leaves of early life-cycle zucchini was not altered by any of the nano-copper treatments. However, APX activity in roots was diminished by 45 to 57% in plants treated with 50 and 200 mg kg^{-1} of Kocide 3000 and nCuO and 200 mg kg^{-1} of bCuO. Enzyme activity changes in mature zucchini were limited to a decrease in APX with bCuO and Cu NP at 100 mg kg^{-1} and a decrease in CAT activity with Cu NP and $CuCl_2$ at 400 mg kg^{-1}.

The uptake and translocation of Cu into plant tissues was a primary concern. Plant roots were significantly higher in nearly all treatments with both in sugarcane and zucchini, except for the mature sugarcane study where only Kocide 3000, nCuO, and Cu NP at 60 mg kg^{-1} contained elevated Cu. Immature zucchini roots contained up to 4.5 times more Cu than controls, while mature roots up to 780 mg Cu kg^{-1}. Nearly all treatments increased root Cu in immature sugarcane by at least 50%. There was significant translocation of Cu into the stems and leaves of immature zucchini, where all treatments caused elevated Cu concentrations. There was little change in Cu content of stems from mature zucchini, but high translocation to leaves and flowers

in all treatments. Zucchini flowers had the greatest change in Cu, from control levels of 9.55 mg Cu kg^{-1} to 55.14 mg Cu kg^{-1}. Significant root uptake of Cu occurred only in immature sugarcane with all treatments, while increases root Cu in mature sugarcane was limited to Kocide 3000, nCuO, and Cu NP at 60 mg kg^{-1}. Sugarcane leaf tissues display the opposite trend, with elevated Cu levels observed in the mature study and little translocation to aerial tissues occurring in the immature study. The translocation observed in the mature sugarcane study was coupled with elevated Cu concentrations in the obtained pressed cane juice. Cane juice pressed from sugarcane exposed to Kocide 3000, nCuO, and Cu NP at 40 and 60 mg kg^{-1} contained over 58% more Cu than controls. The highest Cu contamination occurred in sugarcane treated with $CuCl_2$, where Cu concentration in cane juice was 0.43 mg kg^{-1}. The elevate Cu observed in the cane juice or in other tissues did not adversely affect juice yield or sugar quality.

Based on the evidence collected both sugarcane and zucchini grown in soil treated with Kocide 3000, nCuO, bCuO, Cu NP, or $CuCl_2$ were not significantly impacted. Although uptake and translocation of Cu was significant, effects on growth did not occur, and changes to chlorophyll content and stress enzyme activity were minimal. Additionally, there was no indication that nCuO exposure was more toxic than exposure to bCuO. Although, there was significant translocation of Cu to edible tissues/products increased concentrations should not cause health concerns with normal consumption. When comparing the immature and mature studies conducted with short-term hydroponic and long-term studies in the literature it is clear that plants grown to maturity in a complex soil medium are less affected by nano-copper exposure. The experiments performed add to the growing consensus that plant exposure to nano-copper compounds, in a complex soil medium, leads to no major negative effects.

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Curriculum Vita

Carlos Tamez, Jr was born in McAllen, TX, the youngest of 5 children. He received a Bachelor's of Science in Entomology with a minor in Chemistry in 2010 from Texas A&M University in College Station. During his undergraduate studies he performed research on the interactions between blow fly (*Chrysomya rufifacies*) and the secondary screwworm (*Cochliomyia macellaria*), two forensically relevant fly species. Carlos then earned his Master's in Analytical and Environmental Chemistry at The University of Texas–Pan American (now UT–RGV). His thesis research involved the synthesis and characterization of iron oxide and manganese oxide nanoparticles and their application in the removal of copper and lead from water. This research was conducted under the mentorship of Dr. Jason Parsons.

Carlos joined the Ph.D. program in Environmental Science and Engineering at the University of Texas at El Paso in Spring 2014. He became a student researcher for the University of California Center for Environmental Implications of Nanotechnology (UC-CEIN), under the advisement of Dr. Jorge Gardea-Torresdey. During his doctoral studies he served as a Teaching Assistant for the Department of Chemistry. Mr. Tamez was the recipient of a Diana Natalicio Environmental Internship Award, allowing him to conduct research on bioavailability of lead in soil at Kansas State University. He also received a research assistantship from the Center for Education and Training in Agriculture Related Sciences. Carlos has published three peer-review journal articles and has co-authored seven more.

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