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Modulation Of The Physiological And Biochemical Effects Of Copper Nanoparticles In Kidney Beans (Phaseouls Vulgaris) Treated By Kinetin

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MODULATION OF THE PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF COPPER NANOPARTICLES IN KIDNEY BEANS (*PHASEOLUS VULGARIS*) BY KINETIN

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Dedication

My beloved parents, for their unconditional love and support. One of the most important lessons you have taught me is to take pride in the things that I do – something that I always remember to live by.

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by

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Abstract

It is imperative to not only understand the impact of engineered nanomaterials (ENMs) in edible plants, but also the interactions they have with various additives used in agriculture. In this study, kidney bean plants were grown in potting soil treated with nano copper (*n*Cu), bulk copper (*b*Cu), and copper chloride ($CuCl_2$) at concentrations of 0, 50, and 100 mg/kg for 55 and 90 days. At 15 days of growth, 0, 10, and 100 µm of kinetin (KN) were applied to plants. Plant tissue samples were harvested at 55 days and seeds were reaped at 90 days. Physiological and biochemical parameters were investigated. Cu uptake was found to be highest in the roots, while a concentration-dependent increase in Cu concentration by *n*Cu x KN and *b*Cu x KN was found in leaves. *b*Cu stimulated chlorophyll production up to 28% and a hormesis dose-response was imparted by $CuCl_2$. *b*Cu and $CuCl_2$ treatments also increased Mn (up to 41%) and decreased Mg content (up to 78%) in seeds and stems, respectively. 100 mg/kg $CuCl_2$ + 100 µM KN reduced accumulation of Ca and Mg in seeds and leaves by 56% and 75%. 100 µM KN increased K and Mg accumulation in roots up to 59%, while it decreased P shoots up to 78%. *b*Cu and $CuCl_2$ increased stem length, fresh/dry weight, and water content. Seed yield was largely unaffected. Protein synthesis was stimulated by *b*Cu (11% to 12%), while it was dampened by $CuCl_2$ x KN. Our results demonstrate that *b*Cu and $CuCl_2$ were most influential in modifying the overall physiology and biochemistry of kidney bean plants and that *n*Cu + KN did not have a significant negative effect on nutritional quality or plant integrity.

Keywords: Engineered nanomaterials; copper; growth hormones; cytokinins; plant life cycle; kidney beans; elemental analysis; nutritional quality

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Nanotechnology is a rapidly growing industry that has become ubiquitous throughout modern society. The worldwide market value of products incorporating nanotechnology is expected to reach $64.2 billion by the year 2019 (Dolez, 2015). Nanomaterials (NMs) are characterized as having a width, length and/or height under 100 nm. A particle is considered to be a nanoparticle (NP) when at least two dimensions fall within the range of 1 and 100 nm (ASTM, 2014). Thus, anything greater than 100 nm is considered to be *bulk* material. While microsized particles retain constant physical properties, size-dependent characteristics are observed at the nanoscale. NPs possess enhanced and unique functional features from that of counterpart bulk material due to their small size[PJR2], which imparts a high surface-area-to-volume ratio. Among these qualities is the propensity to be highly reactive and catalytic (Bernhardt et al., 2010). Consequently, NPs are synthesized for numerous applications, such as: manufacturing, electronics, medicine, energy, environmental remediation, personal care products and consumer goods (Peralta-Videa et al., 2011).

Sitting at the base of any food chain, plants play an important role in terrestrial ecosystems. They provide an assortment of services that are essential to ecological sustainability[PJR3]. Plants are accustomed to NPs formed by chemical elements existing in nature. However, the potential applications of engineered nanomaterials (ENMs) in agriculture has generated concern. In addition to unintentional pollution, such as exposure of environmental media to contaminated wastewater or biosolids, products containing ENMs are also deliberately applied to soil (Hong et al, 2013)[PJR4]. Figure 1.1 illustrates how

nanotechnology is utilized in agriculture and how this can lead to contamination of environmental media. Studies have indicated that ENMs procure different effects in plants. For instance, changes in agronomical and physiological traits, detoxification and antioxidant defense systems, accumulation and biotransformation, yield, and nutritional quality have all been reported in a variety of plant species (Zuverza-Mena et al., 2016). Most existing studies on the effects of ENMs in plants are short-term and performed under hydroponic settings. This presents a challenge when translating results to realistic environmental conditions (Gardea-Torresdey et al., 2014)[PJR5]. Since plants present an opportunity for bioaccumulation and trophic transfer, and soil is a major sink for ENMs, it is imperative to develop a firm understanding of their interactions with one another.

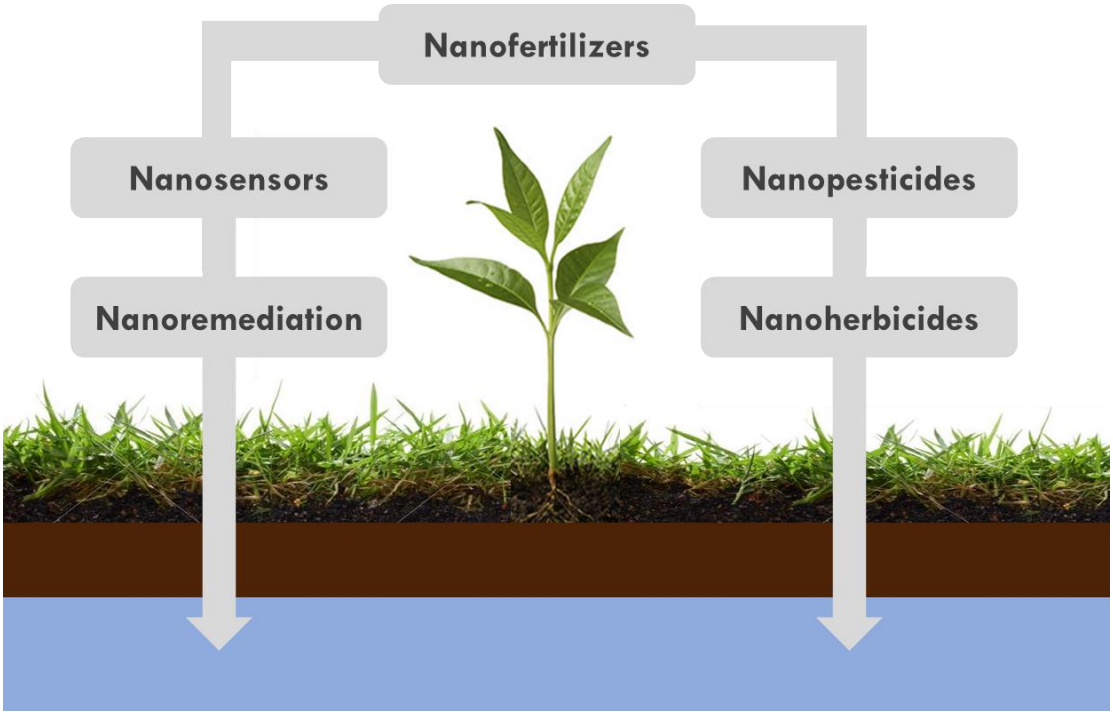

Figure 1.1 Diagram highlighting the applications and pathways of exposure of ENMs in agriculture

Copper (Cu) is a naturally occurring element that is categorized as a transition metal. Known for its high thermal and electrical conductivity, alloying, and ductility, Cu has widespread use throughout a variety of industries. It is an essential nutrient for plants and animals. An imbalance of Cu can lead to toxicity or deficiency, both of which have negative impacts on healthy metabolic functioning (Yruela, 2005). Cu fertilizers are used to correct deficiencies, especially in vulnerable soil and sensitive plant species (Gonzalez et al, 2015). Cu also possesses unique antimicrobial properties. As a result, Cu is a key ingredient in many fungicides and bactericides (Kurnik et al., 2012). The solicitation of Cu-based products in agriculture has gained popularity, as well as concern. Frequent applications of new Cu fungicide formulations were found to accumulate in the tissue of apple fruits, ultimately exceeding the 5 mg kg^{-1} maximum residue level set by the European Commission (Kurnik et al., 2012).

Nanoscale Cu (*n*Cu) possesses different malleability, ductile, and flammability properties from bulk Cu (Zuverza-Mena et al., 2015). Some of its major applications are: lithium ion batteries, lubricant oils, polymers/plastics, inks/ceramic pigments, gas sensor, catalysts, and electronics (Anjum et al., 2015). In 2010, the worldwide production of Cu-based NPs was projected to be approximately 200 tons per year, with 150, 36, 11, and 3 tons ultimately being released into landfills, soil, water, and air, respectively (Keller et al., 2013)[PJR6]. While there is an abundance of information on insufficient or excessive amounts of Cu in plants, studies on the effects of Cu-based NPs in plants, in comparison, are limited. Figure 1.2 summarizes the potential mechanisms of toxicity that are associated with *n*Cu.

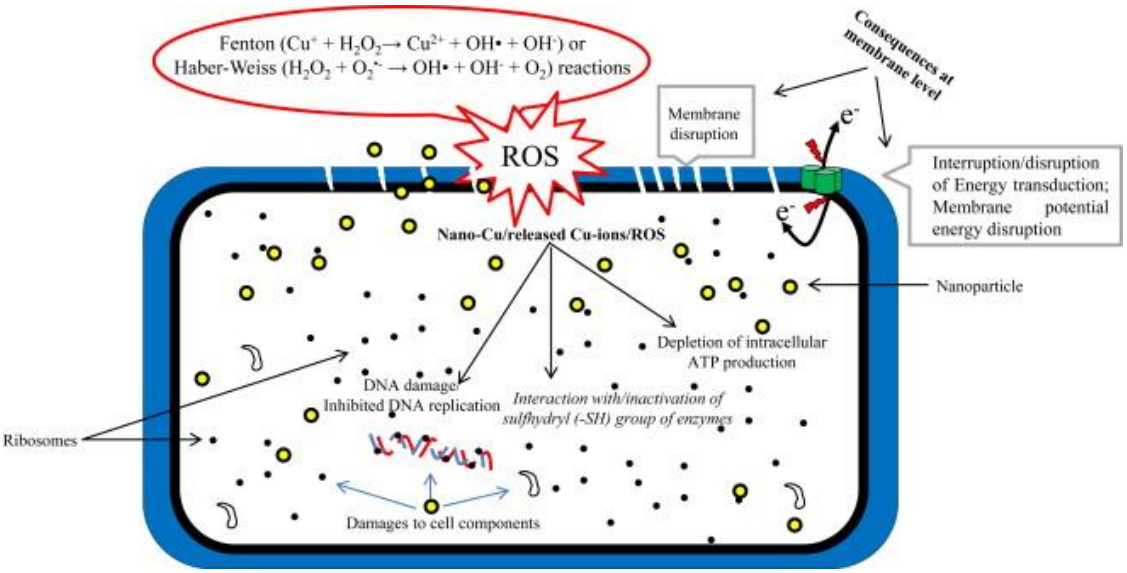

Figure 1.2 Illustration highlighting the major events associated with nCu toxicity. (Reprinted from "Nanoscale copper in the soil–plant system – toxicity and underlying potential mechanisms" by N. Anjum, 2015, Environmental Research, Volume 118, 306-325. Copyright 2015 by Elsevier[SA7])

Plant growth regulators (PGR) refer to both natural and synthetic compounds that are used to alter plants' physiological processes as a means to increase yield, improve quality, and facilitate harvesting (Nazir et al., 2015)[PJR8]. These hormones govern many growth, differentiation, and developmental processes within plants. Kinetin (KN) is a member of a group of PGR referred to as cytokinins, whose primary function is associated with cell division. Increased cellular division corresponds with amplified mitosis and, ultimately, tissue formation. The use of cytokinins has been shown to improve plant growth and stress resistance[PJR9], increase leaf expansion and chlorophyll synthesis, and delay senescence (Barbafieri et al., 2012). KN has also been touted for its phytoextraction enhancing capabilities in sites contaminated with heavy metals.

Kidney beans (*Phaseolus vulgaris*) are an important food crop. Branded as one of the top plant-based sources of protein, they are a healthy and affordable form of nourishment. Kidney beans are rich in various minerals, including: molybdenum, iron, copper, manganese, potassium, and phosphorus (USDA, 2014). In 2013, the worldwide production quantity of kidney beans was over twenty three thousand metric tons (MT)(FAOSTAT, 2013). As seen in Figure 1.3., the countries with the highest production rates were India, Brazil, China, Mexico, and the USA.

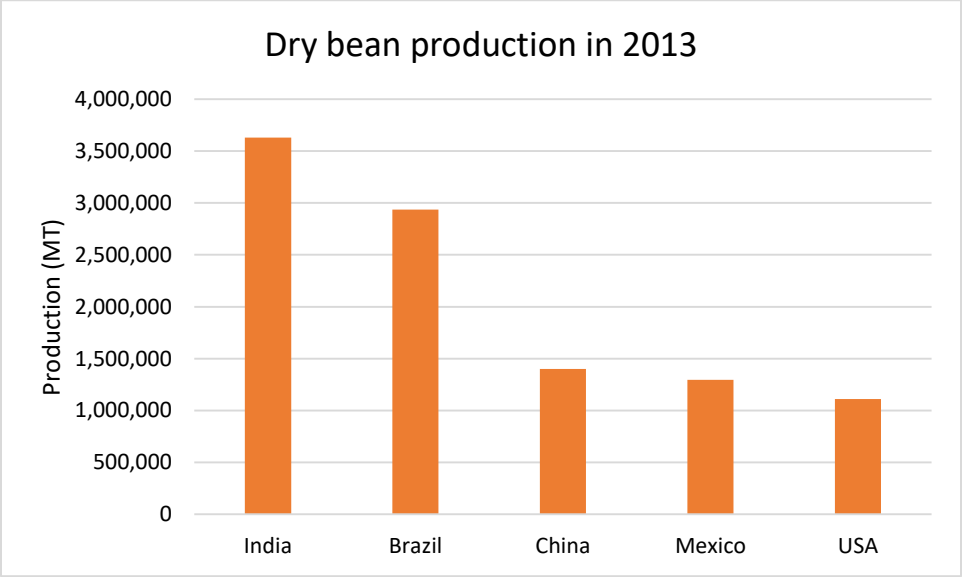

Figure 1.3. Production quantity of dry beans from the leading countries worldwide in 2013. Data retrieved from FAOSTAT, 2013.

It is crucial to not only understand the impact of ENMs in food crops, but also the interactions they have with various additives used throughout agriculture. In this study, the physiological and biochemical effects of *n*Cu+KN on kidney bean plants were assessed. The uptake, translocation, and associated phytotoxicity were investigated. The research spanned

two major phases. In *Phase 1*, plants were examined near the time of flowering. This was performed under the assumption that plants are at their peak energy level during this period in the growth cycle. Changes in plant growth and development were studied through measurement of agronomic parameters. Plant tissues (root, stem, leaf) were analyzed for variations in Cu, macro, and micronutrient content via inductively coupled plasma spectroscopy (ICP-OES). Indicators of stress (chlorophyll, catalase) were quantified using biochemical assays. *Phase 2* encompassed the investigation on harvested kidney bean seeds. Yield, protein content, and nutrient element accumulation were evaluated.

Hypothesis

Exposure of plants to KN and Cu NPs has been found to modify plant physiology and biochemistry. Hence, the combination of *n*Cu+KN can induce an alternative modulated response. This experiment was performed under the working hypothesis that KN will increase the uptake and translocation of Cu within kidney bean plants, while decreasing overall phytotoxicity through enhanced plant defense mechanisms[PJR10].

Research Objectives

The purpose of this study was to understand the impact of *n*Cu[PJR11] on the physiology and biochemistry of kidney bean plants throughout their life cycle, and how exogenous KN modulates these effects.

The specific objectives were to:

1. Examine the uptake and translocation of Cu within plant tissues

2. Assess the effects nCu + KN on the metabolic function of roots and leaves
3. Evaluate the impacts of *n*Cu + KN on plant growth and development
4. Determine changes in the elemental nutritional quality of kidney bean plants
5. Analyze the protein content of kidney seeds exposed to *n*Cu + KN

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Chapter 2: Modulation of the physiological and biochemical effects of copper nanoparticles in kidney beans treated with kinetin[UTEP12]

2.1 Introduction

Engineered nanomaterials (ENMs) comprise NPs that have been adapted for commercialization to add value and utility to various products. Since ENMs exhibit different physiochemical properties than bulk materials, this generates inquiry on the role of size in biological behavior. What remains unclear is the extent and severity of their influence – a fundamental point when considering pollution control and management. In order to fully understand the ecological impact of ENMs, it is critical to evaluate their fate, toxicity, and behavior (Klaine et al., 2008). Overall, the environmental and health implications surrounding the emerging field of nanotechnology is not well understood.

*n*Cu has been shown to influence nutritional food quality. It was reported by Zuverza-Mena et al. (2015) that Cu-based NPs depressed elemental accumulation of P in cilantro. Similarly, Hong et al. (2015) described that Cu NPs and compounds at concentrations up to 20 mg/L not only reduced the growth of lettuce and alfalfa, but also altered the nutrient content and enzyme activity. Another study found the activity of catalase (CAT) to increase, while ascorbate peroxidase (APX) decreased in roots of lettuce exposed to *n*Cu (Trujillo-Reyes et al., 2014). This reinforces the hypothesis that *n*Cu can impact the biochemistry of plants. In order to distinguish between the effects of ions and particles, a soluble Cu salt treatment is often used. Lee et al. (2008) were able to isolate mung bean and wheat seedling toxicity (length decrease) to *n*Cu, versus Cu ions. Cu agglomerates within root cellular tissues of both plant

species suggests that *n*Cu crosses the cell membrane. In Musante (2012), the growth and transpiration of squash exposed to 100 and 500 mg/L of *n*Cu was reduced by 60-70%, compared to controls. The addition of humic acid decreased the ion content of bulk Cu solution by 38-42%, also alleviating its phytotoxic effects. However, the same concentration of humic acid actually increased the ion content of *n*Cu solution by 1.4-2.9 times. This study is further indication that NP behavior differs from that of corresponding bulk material and suggests the need for studies under environmentally relevant conditions.

A study performed on Mexican Palo Verde grown in soil spiked with Cr(III) and (VI) found KN to improve Cr translocation (Zhao et al., 2011). When applied to maize seedlings, KN was found to relieve the toxic effect of zinc ions (Lukatkin et al., 2007). Nazir et al. (2015) reported that rice seedlings treated with KN and grown in nickel contaminated soil showed significantly improved quality, yield, and nutrient uptake. Further, Ni content was found to be higher in the shoots of plants treated with KN, but less in the actual grain, compared to plants grown in contaminated soil without KN.

Little is known regarding the interaction of NPs by KN. In this study, we sought to analyze the effects of *n*Cu x KN on physiological and biochemical parameters of plants, including: Cu uptake and translocation, chlorophyll content, catalase activity, agronomics, nutrient element accumulation, and protein content.

2.2 Materials and methods

2.2.1 Preparation of suspensions/solutions

Nanoparticulate Cu (*n*Cu) were acquired from The University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). *n*Cu suspensions were prepared at 50 and 100 mg/L concentrations in Millipore water (MPW) for a final volume of 100 mL. These concentrations were chosen based on the average concentration of Cu in agricultural soil (~25 mg/kg),(Schulte & Kelling, 1931). In order to avoid aggregation of particles, suspensions were sonicated (Crest Ultrasonics, Trenton, NJ) for 30 minutes at 25°C with an intensity of 180 watts. Bulk Cu (*b*Cu, Sigma Aldrich) and copper chloride ($CuCl_2$) suspensions were prepared using the same method[PJR13]. Micro sized particles were used to compare the effects of Cu at different sizes, and a Cu salt was used to differentiate between ions and particles. Characterization of *n*Cu and *b*Cu particles are shown in the supplementary data in Table S1 (Hong et al., 2014)

Powdered KN (99%) was purchased from Alfa Aesar. 2-3 mL of 0.1 N sodium hydroxide (Sigma Aldrich) was used to dissolve KN in MPW. Stock solutions were prepared at 10 and 100 µM concentrations and kept in the dark at 0°C for a maximum of seven days (Lopez-Moreno et al, 2007).

2.2.2 Seed and soil treatment

Red hawk kidney seeds (*Phaseolus vulgaris*) were supplied by Dr. James Kelly from Michigan State University. Seeds were briefly immersed in a 2% hypochlorite solution (for disinfection purposes), rinsed three times with deionized water and then submerged in MPW for 24 hours for hydration (Majumdar et al. 2014). Four hundred and twenty grams of Miracle-Gro® organic potting mix were separated into a glass container and manually mixed with Cu treatments. For control treatments, soil mixed with MPW only was used. Miracle-Gro® characteristics are included in Table S2 of the supplementary data (Barrios et al., 2015). Prepared soil treatments were placed in plastic pots (12.5 cm diameter, 14 cm height) and allowed to equilibrate for 24 hours before planting. Five seeds per replicate/treatment were equidistantly positioned in the soil at a depth of ~2.5 cm.

A factorial treatment arrangement was employed within a completely random design (Table 2.1). The factors were KN at three levels (0 µM, 10 µM, 100 µM) and Cu[PJR14] compounds (*n*Cu, *b*Cu, $CuCl_2$) at three levels (0 mg/kg, 50 mg/kg, 100 mg/kg). The interaction between Cu and KN (Cu x KN) gave a total of 21 treatments. Three replications for each treatment were used, giving a total sample size of 63[UTEP15].

Table 2.1. Factorial treatment structure of the experiment. 3=equal to the number of replicates per treatment.

KN (µM) \ Cu (mg/kg)	0	50	100
0	3	3	3
10	3	3	3
100	3	3	3

2.2.3 Plant growth

Pots were arranged in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) at 25/20°C, a 14/10 h photoperiod, 65 ± 3% relative humidity and irradiation of 340 $\mu mol^{-2} s^{-1}$. Each pot was watered with 100 mL of deionized water every other day. The seeds began to germinate after three days. On the 15th day, each pot was watered with either 100 mL of deionized water, 10 or 100 µM of KN solution. Beyond the 20th day, pots were watered with 100 mL of deionized water daily (to account for the increase in transpiration capacity in enlarged plants).

The experiment was carried out in two phases, with each phase acting as a self-containing experiment[UTEP16]. In *Phase 1*, a set of plants were treated and grown to 55 days (maximum vegetative growth), and in *Phase 2*, another set of plants were treated and grown to 90 days (full maturity).

2.2.4 Plant harvest and agronomic parameters

Phase 1

After 55 days of growth, plants were separated from soil, rinsed with tap water (to wash away excess soil) and severed into roots, stems, leaves (tri-foliate) and carpels. Tissues were rinsed three times each with 0.01 M HNO_3 (to remove *n*Cu adhered to tissues) and MPW. Stem and root length were measured using a ruler and fresh tissue weight using a balance. Samples were dried for at least 72 hours in an oven at 65°C and weighed. Water content was determined by comparing the fresh and dry tissue weights.

Phase 2

Upon reaching 90 days of growth, seeds[UTEP17] and carpals were cultivated as indicated above. Remaining plant tissues were disposed of, as they were no longer viable for analysis. Soil samples were also taken.

2.2.5 Chlorophyll contents

The method per Porra et al. (2002) was used to determine total chlorophyll, chlorophyll *a* and *b* (chl-*a* and -*b*), and pheophytin contents. Extractions were performed by grinding 0.5 g of fresh leaf tissue sample with 80% acetone. Until assays were completed, extracts were stowed in a freezer at 80°C. A UV/Vis Spectrometer (Perkin Elmer Lamda) was used to measure absorbance at 466, 663, and 645 nm.

2.2.6 Protein content

Nitrogen content was determined via the Kjeldahl method using the following procedure: acid extraction, distillation, and titration (Labconco Co., Kansas City)(AOAC2000). Samples of dry seeds (0.1 g) were placed in Kjeldahl digestion flasks, from which a tablet of K_2SO_4–catalyst mixture and 6 mL of concentrated sulfuric acid (H_2SO_4) were added. Flasks were heated at 175°C for one hour, then again at 375°C for two hours (to ensure complete digestion). Digests were left to cool. Afterwards, 20 mL of distilled water were added and digests were agitated. The distillated samples were then collected and 40 mL of 10 N sodium hydroxide (NaOH) was added and placed in a distillation chamber (Labconco Inc., Kansas City). Samples were combined with 10 mL of indicator containing boric acid (BH_3O_3) in a beaker and the 50 mL distillation was collected. Titration was performed using 0.05 N H_2SO_4 (Bremner, J. M. 1996). Nitrogen was expressed as % N (Bremner, 1996). Crude protein content was calculated as % N x 6.25 (Allen, 2002).

2.2.7 Elemental analysis

Oven dried tissues were finely ground into a powder with a coffee grinder (Hamilton Beach). Samples of 0.2 g of tissues were combined with 1 mL of plasma pure HNO_3 (SPC Science, Champlain, NY) and digested in a microwave oven ($Mars_X$, CEM, Mathews, NC). The volume of digests were adjusted to 50 mL with MPW. Inductively coupled plasma – optical emission spectrometry (ICP-OES, Perkin-Elmer Optima 4300 DV; Shelton, CT) was used to analyze digests for Cu, macroelements (Ca, Mg, K, P and S) and microelements (B, Mn, Fe, Mo,

Ni and Zn). For QA/QC, samples without plant tissues (blanks) and with spinach leaves (standard reference material),([UTEP18]1507a NIST, Gaithersburg, MD) were also digested and analyzed in the ICP-OES.

2.2.8 Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences 22.0 (SPSS, Chicago, IL). Statistical significances were evaluated using one-way analysis of variance (ANOVA). Differences among treatment means were compared using Tukey's honest significance difference (Tukey's HSD), based on a probability (*p* value) of 0.05. The interaction Cu x KN was examined using a factorial two-way ANOVA.

2.3 Results and Discussion

2.3.1 Cu uptake and translocation

The Cu concentration in seeds, leaves, stems, and roots of 55 and 90-day old plants grown in soil treated with 0 to 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0 to 100 µM KN are shown in Figure 2.1. In the seeds, Cu uptake increased by up to 10% from respective controls with the addition of all Cu treatments. However, in the highest 100 µM KN concentration this increase was subdued to approximately 5%. In contrast to those results, application of 100 µM KN resulted in the only statistically significant increases of Cu concentration in leaves (16% to 120%), compared to controls. Additionally, a concentration-dependent increase of Cu can be seen with escalating KN concentration, most notably with *n*Cu and *b*Cu treatments.[UTEP19] The interaction effect by *n*Cu x KN and *b*Cu x KN is further presented in Figure 2.2. Zhao et al., (2011) found that 250 µM KN increased Cr absorption in roots, shoots, and leaves in Mexican Palo Verde by 42%, 103%, and 72%, correspondingly. KN can increase metal uptake by stimulating biomass production and plant transpiration (Barbafieri et al., 2012). Similar patterns for Cu concentration as a function of Cu and KN treatments was observed in the stems. The greatest overall concentration of Cu was found in the due to weight of Cu, which renders it sparingly mobile in plants and causes the greatest accumulation to occur in the roots (Adrees et al., 2015). In Figure 2.3 it is shown that 100 mg/kg *b*Cu increased Cu adsorption in roots among all treatments. This could a result of particle size. Moreover, rising KN concentration resulted in a concentration dependent decrease of Cu concentration in roots. KN can bind to metal ions and increase its translocation (Olsen et al.,

1999). Further analysis is required in order to determine whether this is a result of KN saturation at the highest 100 µM KN concentration.

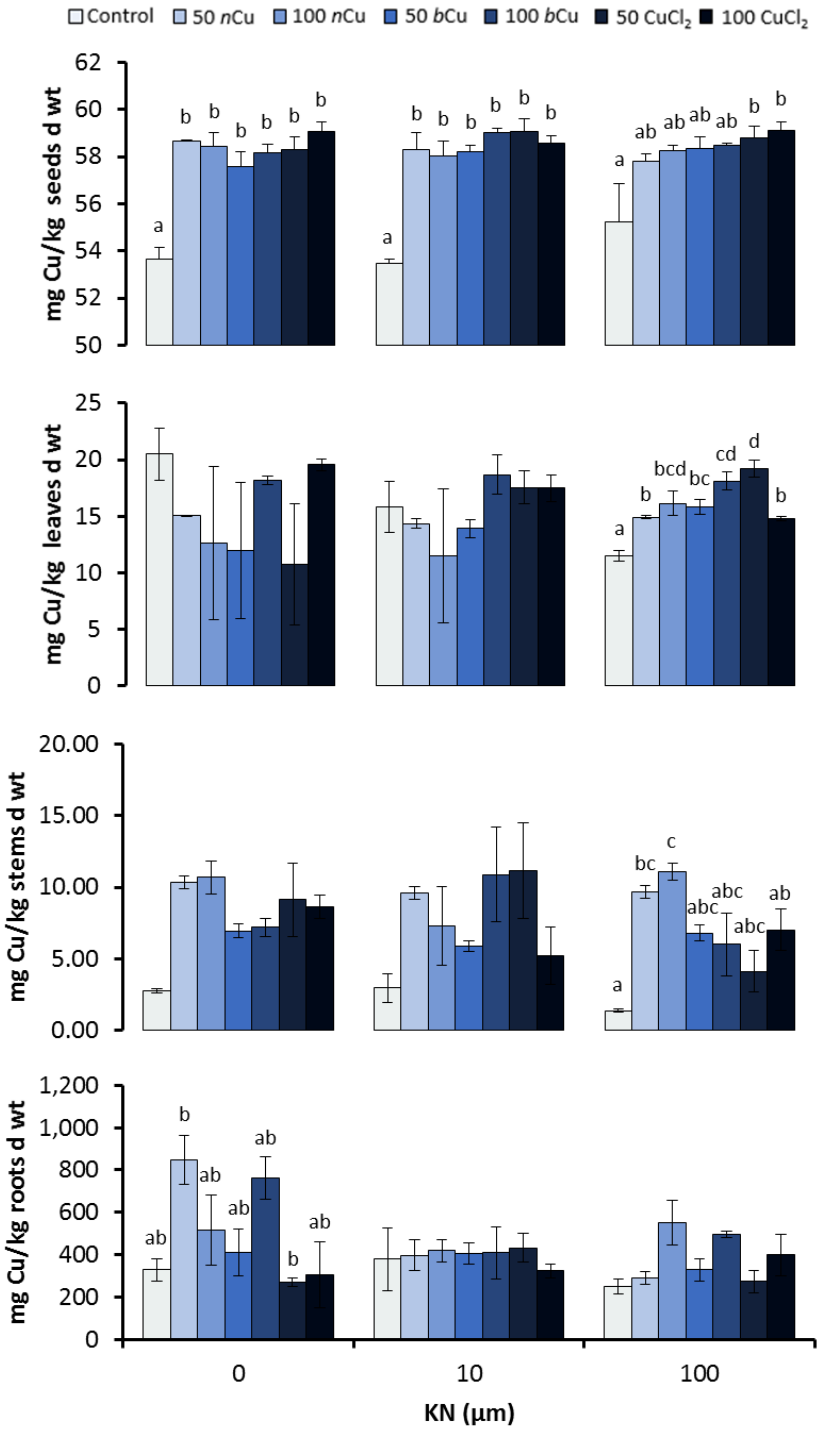

Figure 2.1 Cu content in seeds (A), leaves (B), stems (C), and roots (D) of kidney bean plants (55 and 90 days) cultivated in soil spiked with 0, 50, and 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0, 10, and 100 µM of KN. Data are means of three replicates ± SE (n = 3). Different letters represent statistically significant differences within the same KN treatment concentration at ($p \leq 0.05$).

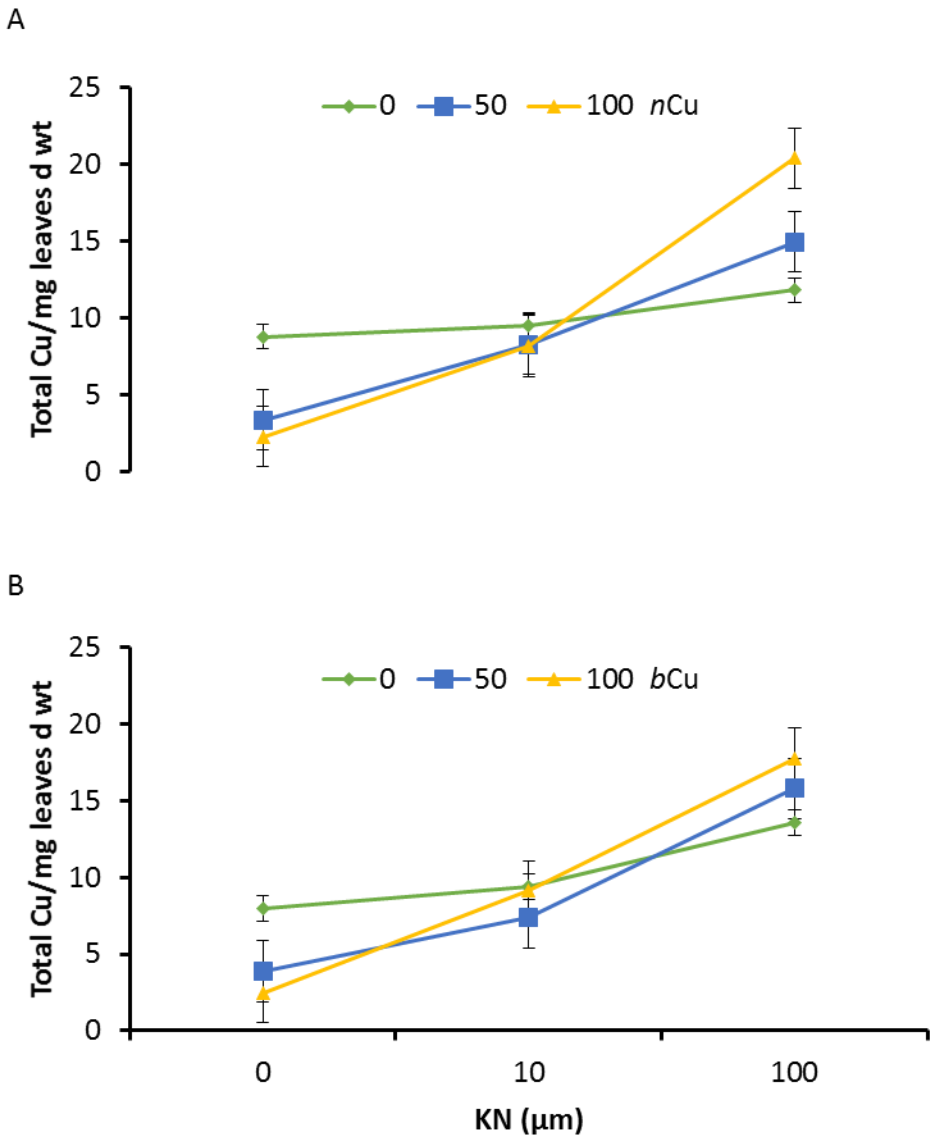

Figure 2.2 Averages of Cu content for *n*Cu x KN (A) and *b*Cu x KN (B) interactions in leaves of 55 day old kidney bean plants grown in soil treated with 0 to 100 mg/kg of nCu and bCu, and watered with 0 to 100 µM KN. Data are means of three replicates ± SE.

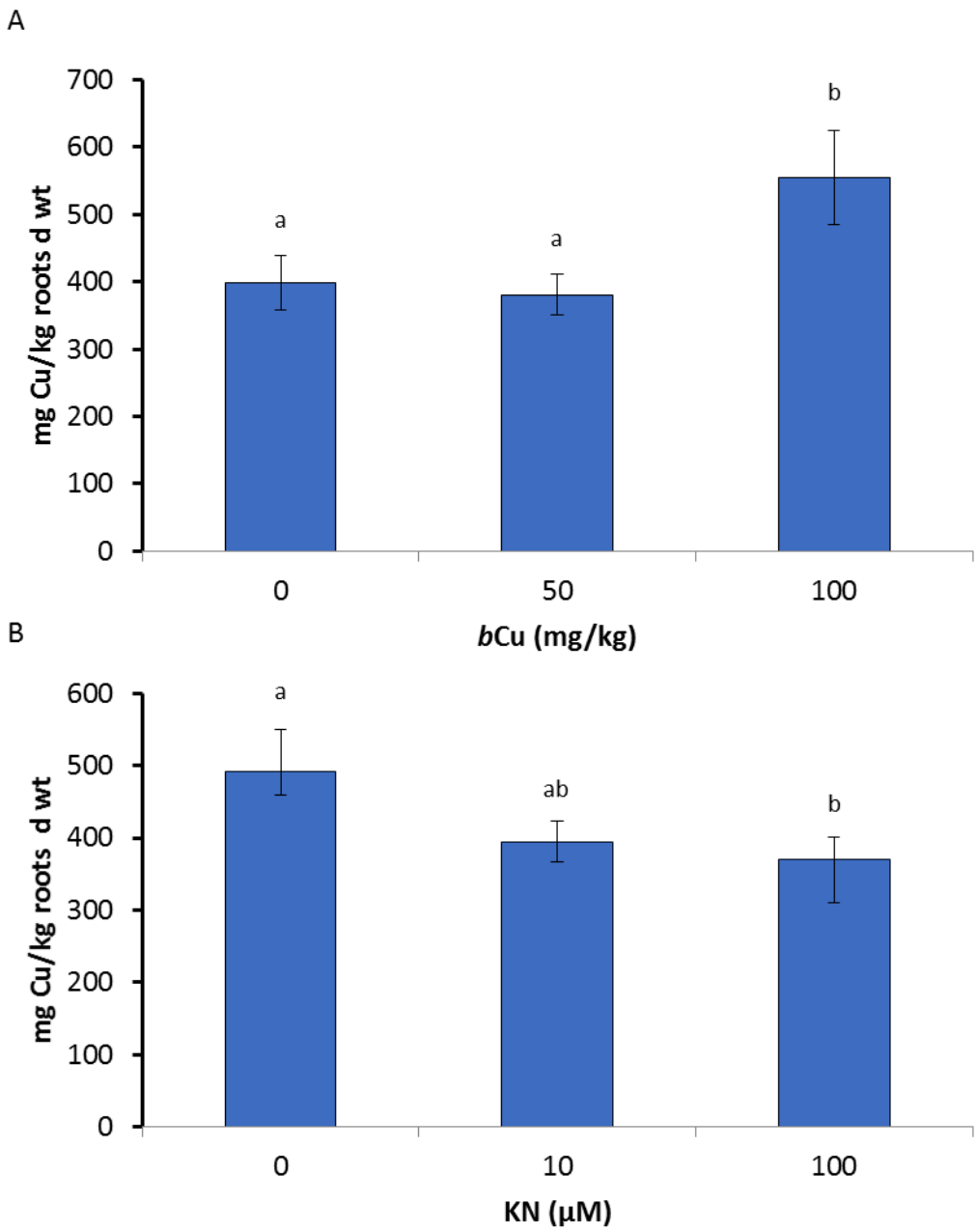

Figure 2.3 Averages of Cu content for factors *b*Cu (A) and KN (B) in roots of 55 day old kidney bean plants exposed to 0 to 100 mg/kg of *n*Cu, *b*Cu and $CuCl_2$, and 0 to 100 µM KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences ($p \leq 0.05$); n=3.

2.3.2 Chlorophyll content in leaves

Figure 2.4 displays total chlorophyll in leaves of 55-day old kidney bean plants grown in soil amended with *n*Cu, *b*Cu, $CuCl_2$, and watered with KN. Chl-*a* and chl-*b* were combined to show total chlorophyll (Table S3). Only *b*Cu and $CuCl_2$ treatments significantly impacted total chlorophyll content. While it has been widely stated that *n*CuO can induce chlorosis (Britt et al., 2012; Gopalakrishnan et al., 2014; Kumar et al., 2014; Nair et al., 2015; Prakash et al., 2014; Shaw et al., 2013; Trujillo-Reyes et al., 2014), the effects of *n*Cu on chlorophyll are varied, suggesting that environmental conditions largely determine the release of Cu ions (Trujillo-Reyes et al., 2014; Zuverza-Mena et al., 2015). Total chlorophyll production was reduced in 100 and 50 mg/kg $CuCl_2$ treatments exposed to 10 and 100 µM KN, respectively[UTEP22][UTEP23][SA24]. This U-shaped dose-response curves follows the biological phenomenon of *hormesis*, where opposite effects are seen in low doses of a toxin (inhibition) compared to high doses (stimulation) (Calabrese et al., 2011). This biphasic response is not well understood, but has received considerable interest within toxicological studies. 50 mg/kg $CuCl_2$ + 100 µM KN was found to be significantly lower compared to 100 mg/kg $CuCl_2$ + 100 µM KN. This could be due to dampened protochlorophyllide reductase activity, which contributes to chlorophyll synthesis, and enhanced activity of chlorophyllase, a chlorophyll degrading enzyme, under stress conditions caused by Cu toxicity (Eser et al., 2016; Gadallah et al., 1999). Cu^{2+} has also been found to hinder chloroplast reactions by restricting electron transport, consequently altering an essential factor of the energy-transfer mechanism (Uribe, 1982). Lowered total chlorophyll content in lupin plants exposed to Cu up to 50 mg/L was reported by Gadallah et al. (1999). However, in that same study, plants treated with 10 and 20 mg/L of KN were found to have an

overall higher total chlorophyll content, particularly those that were also exposed to metal (Gadallah et al., 1999). Our results do not demonstrate stimulation of chlorophyll production or resistance to chlorophyll loss as an effect of KN application, as found in other studies (Al, 2007; Gadallah et al., 1994, 1995; Kaul et al., 1971; Volfova, 1978).

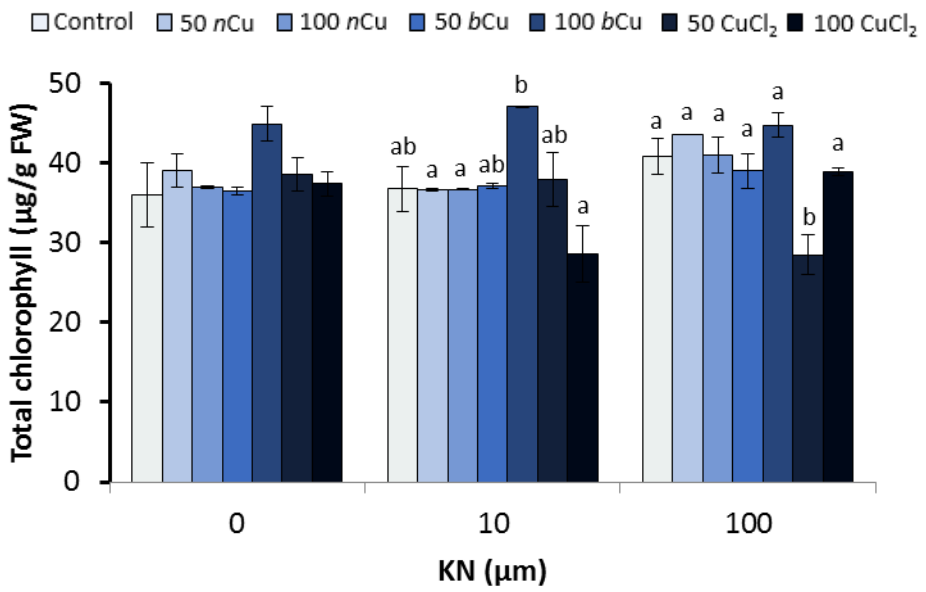

Figure 2.4 Total chlorophyll content in leaf tissue of kidney beans (55 days) grown in soil amended with 0 to 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0 to 100 µM of KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences between concentrations and treatments within the same KN treatment concentration at ($p \leq 0.05$); n=3.

2.3.3 Catalase activity in leaves and roots

The production of reactive oxygen species (ROS) is triggered by abiotic or biotic stress. Excess ROS is detoxified by various scavenger species as part of the plant antioxidant defense system (Anjum et al., 2015). Catalase (CAT) serves as a protective enzyme that mitigates ROS-mediated oxidative stress by decomposing H_2O_2 into water and oxygen (Zuverza-Mena et al., 2015). As seen in Figure 2.5 (A-B), overall CAT activity in roots was lower than in leaves. There were no statistically significant differences in CAT activity in roots among groups of hormone treatments.

In the leaves, CAT activity was reduced in 100 mg/kg *b*Cu + 10 µM KN (65%), 50 mg/kg $CuCl_2$ + 10 µM KN (82%), and 100 mg/kg $CuCl_2$ + 10 µM KN (69%) treatments, compared to respective controls[UTEP25][SA26]. The down regulation of CAT by Cu ions has been previously reported in duckweed, Brazilian waterweed, and cilantro (Hou et al., 2007; Nekrasova et al., 2011; Zuverza-Mena et al., 2015). Possible explanations are: 1) excess Cu leading to the inactivation of hemoprotein enzymes via substitution of the Fe^{2+} active center with Cu^{2+}, and 2) prompted senescence of biochemical machinery by surplus Cu resulting in antioxidant system collapse (Trujillo-Reyes et al., 2014). Exogenous KN application has been established to alleviate As and Cr heavy metal toxicity in maize seedlings and Mexican Palo Verde through improved enzyme activities (Wang et al., 2015; Zhao et al., 2011). Alternatively, CAT activity in pea seedlings was inhibited by Mn + 100 µM KN as a result of either a delay in H_2O_2 scavenging or excess H_2O_2 production (Gangwar et al., 2010). Determination of the activity level of other

antioxidant enzymes would further understanding on the metabolic response of kidney bean plants following exposure to *n*Cu, *b*Cu, $CuCl_2$, and KN.

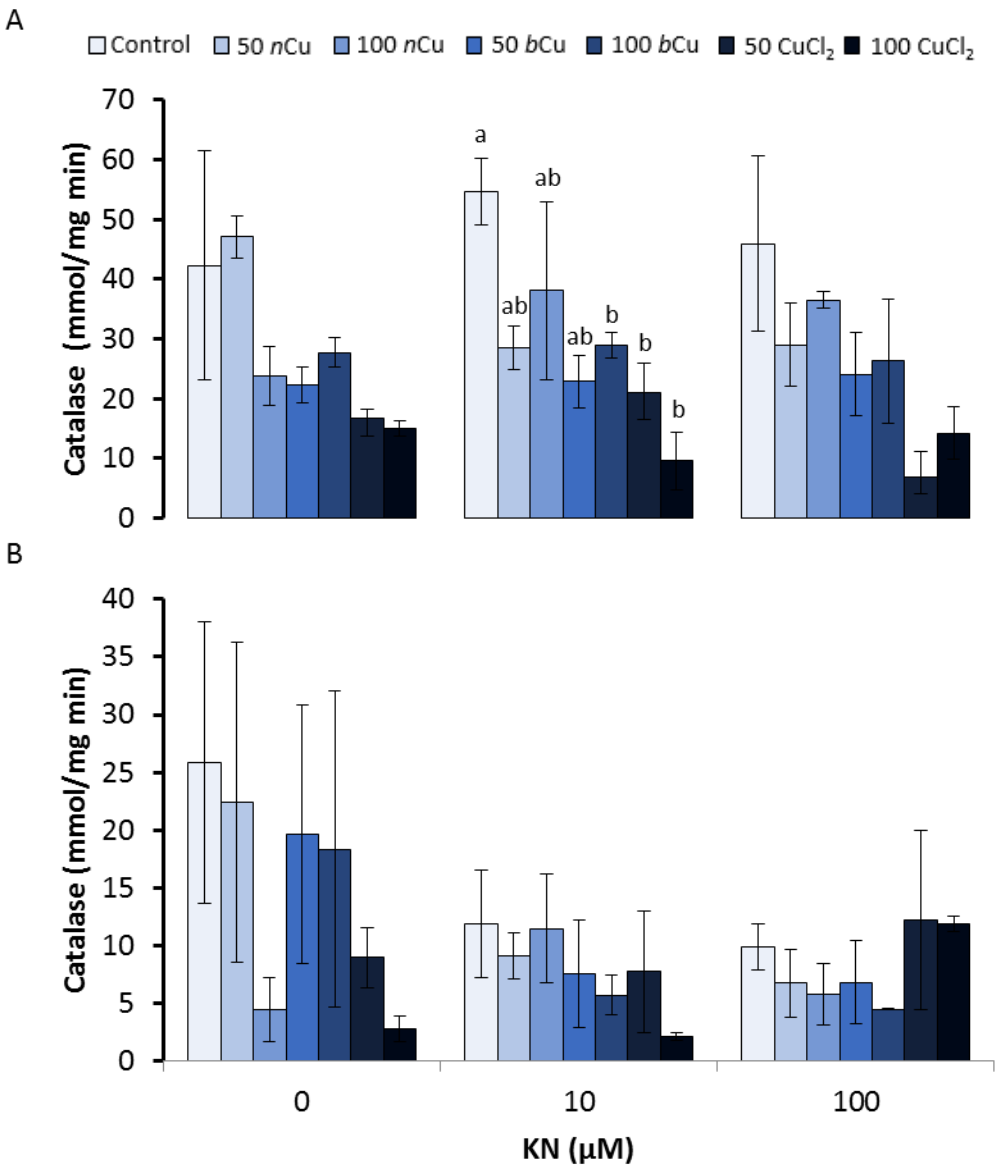

Figure 2.5 Catalase content in leaves (A) and roots (B) of 55-day old kidney bean plants cultivated in soil treated with 0, 50, and 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0, 10, and 100 µM of KN. Data are means of three replicates ± SE (n = 3). Different letters represent statistically significant differences within the same KN treatment concentration at ($p \leq 0.05$).

2.3.4 Root and stem elongation

Table 2.2 shows the stem and root length of kidney bean plants after 55 days of growth in soil amended with 0 to 100 mg/kg *n*Cu, *b*Cu, and $CuCl_2$, and treated with 0 to 100 µM KN. As indicated, root elongation was not significantly impacted by any of the treatments. These results corroborate with those found in a study by Zuverza-Mena et al., (2015), where cilantro was exposed to 20 and 80 mg/kg of nano, micro, and ionic Cu. It was concluded that the toxic effects of *n*Cu and *b*Cu are species dependent (Song et al., 2015; Trujillo-Reyes et al., 2014).

Stem length was increased by 50[UTEP27] mg/kg *b*Cu + 0 µM KN (22%), 100 mg/kg $CuCl_2$ + 0 µM KN (18%), 100 mg/kg $CuCl_2$ + 10 µM KN (16%), 100 mg/kg *b*Cu + 100 µM KN (32%), and 100 mg/kg $CuCl_2$ + 100 µM KN (9%) treatments,[UTEP28] compared to respective controls. The disagreement of these results with literature stating reduced plant growth as a consequence of Cu toxicity could be due to the growth medium (Atha et al., 2012; P M G Nair et al., 2015; Shaw et al., 2013; Trujillo-Reyes et al., 2014). Most studies on this topic have been conducted under hydroponic conditions. Binding of Cu to organic matter found in soil reduces its bioavailability to 20% or less (Adrees et al., 2015). In this study, Cu accumulation was found to be lowest in the shoots (Figure 2.1). It is possible that Cu levels were within the optimal range, thus, resulting in stem elongation. $CuSO_4$ was found to stimulate growth at concentrations less than 50 µM in mung bean (Verma et al, 2011). A 50[UTEP29]% Inhibition of stem growth was found to occur in tomato stem growth from 22 to 851 mg/kg, representing a variation of 39-fold (Ooney et al., 2006). A wider range of treatment concentrations and environmental conditions is required in order to further investigate this concept[UTEP30]. Further, KN could have interacted

with Cu to procure stem growth. Plants exposed to higher doses of KN has also been linked to stem elongation over root elongation (Hogetsu, et al., 1974; Board, 2005).

Table 2.2 Stem and root length of 55-day old kidney bean plants cultivated in soil spiked with 0, 50, and 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0, 10, and 100 µM of KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences between concentrations and treatments within the same KN treatment concentration at ($p \leq 0.05$); n=3.

KN (µM)	Treatment (mg/kg soil)	Length (cm)	
		Stem	Root
0	Control	19.4 ± 0.6 a	25.8 ± 1.2
	50 *n*Cu	19.6 ± 1.2 a	23.8 ± 1.6
	100 *n*Cu	21.6 ± 0.7 ab	24.5 ± 2.1
	50 *b*Cu	23.6 ± 1.1 ab	21.9 ± 2.0
	100 *b*Cu	24.0 ± 1.0 b	19.0 ± 0.3
	50 $CuCl_2$	24.4 ± 0.6 b	23.5 ± 1.2
	100 $CuCl_2$	22.9 ± 0.5 ab	21.5 ± 0.5
10	Control	18.1 ± 0.3 a	24.0 ± 2.4
	50 *n*Cu	21.1 ± 0.8 ab	27.9 ± 1.3
	100 *n*Cu	22.1 ± 2.0 ab	23.7 ± 2.9
	50 *b*Cu	22.3 ± 0.9 ab	22.4 ± 3.9
	100 *b*Cu	25.4 ± 0.2 abc	22.5 ± 2.7
	50 $CuCl_2$	26.8 ± 0.4 bc	27.9 ± 2.0
	100 $CuCl_2$	20.9 ± 0.8 c	30.0 ± 0.9
100	Control	20.2 ± 0.9 a	23.6 ± 2.7
	50 *n*Cu	19.7 ± 1.7 a	26.5 ± 2.6
	100 *n*Cu	21.0 ± 1.6 ab	22.1 ± 0.8
	50 *b*Cu	22.9 ± 1.4 ab	22.7 ± 2.4
	100 *b*Cu	26.6 ± 0.6 b	21.4 ± 0.8
	50 $CuCl_2$	22.1 ± 1.6 ab	26.9 ± 1.7
	100 $CuCl_2$	21.7 ± 1.1 ab	20.2 ± 1.2

2.3.5 Fresh/dry weight[UTEP31] and water content of plant tissues

The results for the weight and water content of plant tissues were varied (Table 2.3). In the leaves, 100 mg/kg bCu and 50 mg/kg $CuCl_2$ treatments with no KN increased fresh weight and water content from that of controls by 45% to 55%. While[UTEP32][SA33] the weight and water content in the stems was scattered, statistically significant differences from controls were generally due to increases by 100 mg/kg *b*Cu and 50 mg/kg $CuCl_2$ among all KN concentrations. Wang et al. (2015) found the addition of 0.1 mg/L to increase root biomass in maize seedlings, which could be associated with the role of KN in stimulating cellular division (H. Wang et al., 2015). Natural variations within plant physiological systems make it difficult to elucidate these results. Similarly, *b*Cu and $CuCl_2$ treatments accounted for significant differences from controls in the form of weight and water content increases. *b*Cu/*b*CuO has generally been found to have a negative effect on the weight of plant tissues (Atha et al., 2012; S. Lee, et al., 2013; Nair et al., 2014; Trujillo-Reyes et al., 2014; Z. Wang et al., 2012). Again, this response could be dependent on the species, environmental conditions, and growth media. Nevertheless, the increases in stem weight by 100 mg/kg *b*Cu and 50 mg/kg $CuCl_2$ + 10 µM KN correspond with the increases in stem elongation found in Table 2.2; thus, furthering the theory that optimal[UTEP34][SA35] levels of Cu were attained. An increase in plant weight shifts the lower and upper critical Cu concentration requirements for improved growth and development (Adrees et al., 2015)[UTEP36]. The size of *n*Cu has also been found to enhance its penetration potential, free location, and movement within cells (Mukherje et al., 2016). A reduction in biomass could be a manifestation of particle size induced toxicity.

Table 2.3 Fresh/dry weight and water content of kidney bean plants (55 days) grown in soil treated with 0 to 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0 to 100 µM of KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences between concentrations and treatments within the same KN treatment concentration at ($p \leq 0.05$); n=3.

Organ	KN (µM)	Treatment (mg/kg soil)	Fresh wt (g)	Dry wt (g)	Water Content (mL)
Leaves	0	Control	33.8 ± 2.7 a	3.4 ± 0.2	30.4 ± 2.5 a
		50 *n*Cu	37.9 ± 0.9 a	4.3 ± 0.2	33.6 ± 0.7 ab
		100 *n*Cu	39.9 ± 3.2 ab	4.6 ± 0.4	35.3 ± 2.8 abc
		50 *b*Cu	42.7 ± 0.9 abc	4.5 ± 0.0	38.2 ± 1.0 abc
		100 *b*Cu	50.1 ± 1.7 bc	5.7 ± 0.3	44.4 ± 1.6 bc
		50 $CuCl_2$	52.5 ± 0.7 c	5.8 ± 0.3	46.8 ± 0.7 c
		100 $CuCl_2$	44.2 ± 4.2 abc	3.6 ± 1.2	40.6 ± 5.2 abc
	10	Control	41.1 ± 1.8 a	4.5 ± 0.2 ab	36.6 ± 1.6 a
		50 *n*Cu	42.5 ± 0.9 a	4.4 ± 0.1 ab	38.1 ± 1.0 a
		100 *n*Cu	40.3 ± 4.0 a	4.5 ± 0.6 ab	35.8 ± 3.5 a
		50 *b*Cu	42.4 ± 1.8 a	5.1 ± 0.2 ab	37.3 ± 2.0 a
		100 *b*Cu	45.9 ± 7.5 a	6.4 ± 0.4 a	39.4 ± 7.5 a
		50 CuCl2	45.1 ± 2.5 a	5.3 ± 0.4 ab	39.8 ± 2.5 a
		100 $CuCl_2$	15.0 ± 5.8 b	3.5 ± 1.0 b	11.5 ± 6.2 a
	100	Control	34.8 ± 6.5	4.2 ± 0.4	30.6 ± 6.1
		50 *n*Cu	43.4 ± 1.6	4.6 ± 0.1	38.8 ± 1.5
		100 *n*Cu	41.3 ± 0.3	5.1 ± 0.2	36.2 ± 0.3
		50 *b*Cu	42.8 ± 1.2	5.7 ± 0.3	37.1 ± 1.0
		100 *b*Cu	48.8 ± 3.8	5.7 ± 0.1	43.1 ± 3.9
		50 $CuCl_2$	30.3 ± 12.4	3.9 ± 1.4	26.3 ± 11.0
		100 $CuCl_2$	41.7 ± 0.8	5.1 ± 0.3	36.6 ± 0.6
Stems	0	Control	11.5 ± 25.2 ab	1.4 ± 0.1 a	10.1 ± 0.5 ab
		50 *n*Cu	10.1 ± 50.0 a	1.6 ± 0.1 ab	8.5 ± 0.4 a
		100 *n*Cu	13.9 ± 49.7 bc	2.0 ± 0.1 bc	11.9 ± 0.3 bc
		50 *b*Cu	13.6 ± 66.0 bc	2.2 ± 0.0 c	11.4 ± 0.4 bc
		100 *b*Cu	15.1 ± 67.6 cd	2.8 ± 0.1 d	12.3 ± 0.1 bc
		50 $CuCl_2$	16.9 ± 85.2 d	2.8 ± 0.1 d	13.8 ± 0.3 c
		100 $CuCl_2$	13.9 ± 90.1 bc	3.1 ± 0.1 d	11.1 ± 1.2 abc
	10	Control	11.5 ± 26.4 ab	1.5 ± 0.0 a	10.0 ± 0.4 abc
		50 *n*Cu	11.5 ± 48.3 ab	1.7 ± 0.0 ab	9.8 ± 0.2 ab
		100 *n*Cu	11.5 ± 49.4 ab	1.8 ± 0.1 ab	9.8 ± 0.5 ab
		50 *b*Cu	13.8 ± 68.1 bc	2.3 ± 0.2 bc	11.5 ± 0.4 bcd
		100 *b*Cu	17.1 ± 65.9 c	3.2 ± 0.2 c	13.9 ± 0.8 d

		50 $CuCl_2$	16.2 ± 81.5 c	3.0 ± 0.4 c	13.2 ± 1.4 cd
		100 $CuCl_2$	9.2 ± 94.5 a	2.0 ± 0.1 ab	7.2 ± 0.4 a
	100	Control	9.8 ± 25.1	1.5 ± 0.2 a	8.3 ± 1.3
		50 *n*Cu	14.0 ± 50.3	2.0 ± 0.2 ab	12.0 ± 1.2
		100 *n*Cu	13.6 ± 50.9	2.1 ± 0.3 abc	11.5 ± 1.1
		50 *b*Cu	13.7 ± 67.4	2.2 ± 0.2 abc	11.4 ± 0.8
		100 *b*Cu	15.8 ± 64.6	3.2 ± 0.1 c	12.6 ± 0.8
		50 $CuCl_2$	12.3 ± 90.0	2.8 ± 0.4 bc	9.5 ± 2.2
		100 $CuCl_2$	12.9 ± 92.5	2.7 ± 0.3 abc	10.2 ± 0.2
	0	Control	7.4 ± 0.3 a	1.1 ± 0.1 a	6.3 ± 0.3
		50 *n*Cu	9.9 ± 1.3 ab	1.2 ± 0.2 a	8.5 ± 1.2
		100 *n*Cu	9.3 ± 0.2 ab	1.0 ± 0.0 a	8.1 ± 0.2
		50 *b*Cu	9.2 ± 0.9 ab	3.8 ± 0.0 bc	5.3 ± 0.9
		100 *b*Cu	11.8 ± 0.3 ab	4.2 ± 0.0 c	7.7 ± 0.3
		50 $CuCl_2$	13.6 ± 1.0 b	3.9 ± 0.0 bc	9.6 ± 1.0
		100 $CuCl_2$	11.5 ± 2.1 ab	3.7 ± 0.2 b	7.5 ± 1.9
	10	Control	8.2 ± 0.2	1.1 ± 0.1 a	7.0 ± 0.2
		50 *n*Cu	8.9 ± 0.3	1.2 ± 0.1 a	7.7 ± 0.3
		100 *n*Cu	8.1 ± 1.0	1.0 ± 0.2 a	7.1 ± 0.9
Roots		50 *b*Cu	10.0 ± 1.1	3.8 ± 0.0 bc	6.2 ± 1.1
		100 *b*Cu	10.4 ± 0.3	4.2 ± 0.1 c	6.2 ± 0.2
		50 $CuCl_2$	12.4 ± 2.9	3.9 ± 0.1 bc	8.6 ± 2.9
		100 $CuCl_2$	8.6 ± 1.7	3.7 ± 0.2 c	5.0 ± 1.5
	100	Control	7.1 ± 1.4 a	1.0 ± 0.1 a	6.1 ± 1.2 a
		50 *n*Cu	9.6 ± 0.5 a	1.2 ± 0.1 a	8.4 ± 0.4 ab
		100 *n*Cu	8.6 ± 0.3 a	1.2 ± 0.0 a	7.4 ± 0.3 ab
		50 *b*Cu	10.3 ± 0.8 a	4.0 ± 0.2 b	6.3 ± 0.7 a
		100 *b*Cu	11.3 ± 1.9 ab	4.2 ± 0.1 bc	7.2 ± 1.8 ab
		50 $CuCl_2$	10.4 ± 1.5 a	4.1 ± 0.23 b	6.4 ± 1.4 a
		100 $CuCl_2$	16.2 ± 0.7 b	4.3 ± 0.0 bc	11.8 ± 0.7 b

2.3.6 Seed Production

The yield and weight of seeds reaped from 90-day old plants exposed to *n*Cu, *b*Cu, $CuCl_2$, and KN are shown in Table 2.4. There were no statistically significant differences in seed yield of *n*Cu, *b*Cu, and $CuCl_2$ treatments when compared among similar hormone concentrations. Similar results were found with seed weight. The exception was the 50 mg/kg bCu +10 µM KN treatment, which was at least twice that of its respective control[UTEP37][SA38]. Since this increase was not statistically different from the seed weight among 0 µM KN, we can conclude that 50 mg/kg bCu +10 µM KN induced a reaction within the plant, causing the seeds from this treatment to weigh more. In Majumdar et al., (2015), the seed weight of kidney bean plants grown in high organic matter enriched soil treated with 62.5mg/kg to 500 mg/kg *n*CeO_2 was not significantly affected. Further, only in the highest *n*CeO_2 treatment was seed yield considerably increased (Majumdar et al., 2015). Studies on the effects of NPs on seed production of plants are limited.

Table 2.4 Yield and weight of seeds harvested from 90-day old kidney bean plants cultivated in soil amended with 0, 50, and 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0, 10, and 100 µM of KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences between concentrations and treatments within the same KN treatment concentration at ($p \leq 0.05$); n=3.

KN (µM)	Treatment (mg/kg soil)	Yield	Weight (g)
0	Control	9.7 ± 0.3	3.0 ± 0.1
	50 *n*Cu	13.0 ± 1.7	4.2 ± 0.7
	100 *n*Cu	11.3 ± 0.9	4.3 ± 0.8
	50 *b*Cu	12.0 ± 0.6	3.8 ± 0.3
	100 *b*Cu	13.0 ± 1.2	4.5 ± 0.4
	50 $CuCl_2$	11.3 ± 1.9	3.4 ± 0.6
	100 $CuCl_2$	13.0 ± 0.6	3.9 ± 0.3
10	Control	8.7 ± 0.9	2.4 ± 0.2 a
	50 *n*Cu	12.7 ± 1.7	3.8 ± 0.8 ab
	100 *n*Cu	10.7 ± 0.3	3.9 ± 0.4 ab
	50 *b*Cu	12.7 ± 1.2	4.8 ± 0.4 b
	100 *b*Cu	11.7 ± 0.3	3.9 ± 0.4 ab
	50 $CuCl_2$	11.0 ± 0.6	3.7 ± 0.3 ab
	100 $CuCl_2$	12.7 ± 1.9	3.4 ± 0.6 ab
100	Control	10.7 ± 0.9	3.4 ± 0.5
	50 *n*Cu	12.3 ± 0.3	3.7 ± 0.2
	100 *n*Cu	9.7 ± 1.2	4.2 ± 0.2
	50 *b*Cu	13.7 ± 0.9	5.0 ± 0.8
	100 *b*Cu	14.0 ± 0.0	4.4 ± 0.5
	50 $CuCl_2$	14.0 ± 1.2	4.9 ± 0.4
	100 $CuCl_2$	10.3 ± 1.2	3.5 ± 0.3

2.3.7 Nutrient element accumulation

The composition of macro- and microelements in seeds, leaves, stems, and roots of 55 and 90-day old plants grown in soil treated with 0 to 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0 to 100 µM KN are shown in Table 2.5. Mn and Fe were most significantly influenced in the seeds, with 50 and 100 mg/kg *b*Cu increasing Mn concentration by 31% to 41% and 100 *n*Cu decreasing Fe concentration by 29%. 100mg/kg $CuCl_2$ + 100 µM KN reduced Ca, Mg, and Mn content by 62% to 97%. The application of 100mg/kg $CuCl_2$ + 100 µM KN treatment reduced Ca, Mg, and Mn by at least 62% in the leaves, while it increased Fe by 183%. The greatest number and percent of alterations of nutrient elements were found in the stems. Ca, K, P, Mg, and S were all negatively influenced by at least one of the Cu treatments. Overall, *b*Cu and $CuCl_2$ has the largest impact, with most effects seen by these treatments occurring in samples without KN. Our results coincide with those found in previous literature that Cu influences the mineral uptake and accumulation of Ca, K, and Mg in the shoots of plants (Kopittke et al., 2011; Mocquot et al., 1996; Sheldon et al., 2005). In the roots, 100 µM KN stimulated K and Mg accumulation, but dampened P accumulation. It has been established that the effect of Cu varies depending on plant species, treatment concentration, growth medium, and environmental conditions (Adrees et al., 2015)[UTEP39]. Additional studies are required in order to elucidate changes in nutrient element accumulation of kidney bean plant tissues by *n*Cu, *b*Cu, $CuCl_2$, and KN.

Table 2.5 Macro and microelements altered in kidney bean plants (55 and 90 days) grown in soil amended with 0 to 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0 to 100 µM of KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences between concentrations and treatments within the same KN treatment concentration at ($p \leq 0.05$); n=3. Comparisons were made with respect to the controls and symbols + and – stand for percent of increase and decrease in nutrient concentration. Only the elements that had statistically significant differences from respective controls and were within the limit of detection by ICP-OES analysis are shown.

Organ	Element	Treatment (mg/kg soil + µM)	Concentration (mg/kg d wt tissue)	%
Seeds	**Mg**	50 *n*Cu + 10 KN	1774.5 ± 15.5	-9.3
		100 *n*Cu + 10 KN	1729.0 ± 38.3	-11.6
		50 *b*Cu + 10 KN	1762.6 ± 15.9	-9.9
		100 *b*Cu + 10 KN	1733.8 ± 35.0	-11.3
		50 $CuCl_2$ + 10 KN	1736.1 ± 45.4	-11.2
	S	50 $CuCl_2$ + 0 KN	3348.4 ± 31.3	19.0
	Fe	100 *n*Cu + 0 KN	71.2 ± 3.5	-29.1
	Mn	100 *b*Cu +0 KN	21.9 ± 1.7	31.5
		100 *b*Cu + 10 KN	22.1 ± 0.5	35.7
		50 *b*Cu + 10 KN	23.0 ± 0.9	41.4
		50 *b*Cu + 100 KN	21.4 ± 1.1	30.6
Leaves	**Ca**	100 $CuCl_2$ + 100 KN	835.3 ± 44.2	-96.7
	Mg	100 $CuCl_2$ + 100 KN	1810.2 ± 26.9	-71.6
	Fe	100 $CuCl_2$ + 100 KN	100.6 ± 5.7	183.1
	Mn	100 $CuCl_2$ + 0 KN	109.9 ± 6.2	-63.1
		100 $CuCl_2$ + 10 KN	65.1 ± 6.4	-78.0
		100 $CuCl_2$ + 100 KN	100.0 ± 4.6	-61.7
Stems	**Ca**	50 *b*Cu + 0 KN	11015.1 ± 407.5	-24.8
		100 *b*Cu + 0 KN	8543.4 ± 328.9	-41.6
		50 $CuCl_2$ + 0 KN	7611.0 ± 558.9	-48.0
		100 $CuCl_2$ + 0 KN	5379.6 ± 239.4	-63.3
		100 *b*Cu + 100 KN	4266.5 ± 2124.6	-71.6
		50 $CuCl_2$ + 100 KN	5369.6 ± 2670.0	-64.3
		100 $CuCl_2$ + 100 KN	6676.5 ± 453.1	-55.6
	K	50 *n*Cu + 0 KN	49034.0 ± 733.7	-15.2
		50 *b*Cu + 0 KN	47756.9 ± 1173.7	-17.4
		100 *b*Cu + 0 KN	39046.9 ± 2496.9	-32.4
		50 $CuCl_2$ + 0 KN	38243.0 ± 2922.5	-33.8
		100 $CuCl_2$ + 0 KN	36748.8 ± 794.7	-36.4
	P	100 *n*Cu + 0 KN	8769.0 ± 446.5	-24.8

		50 *b*Cu + 0 KN	7981.9	±	373.2	-31.5
		100 *b*Cu + 0 KN	6948.9	±	353.2	-40.4
		50 CuCl2 + 0 KN	6050.5	±	401.0	-48.1
		100 $CuCl_2$ + 0 KN	6148.0	±	204.4	-47.3
		100 $CuCl_2$ + 10 KN	2265.4	±	1115.9	-80.4
		100 *b*Cu + 100 KN	3259.9	±	1608.7	-68.8
		100 $CuCl_2$ + 100 KN	2265.4	±	1115.9	-80.4
		100 $CuCl_2$ + 100 KN	4897.4	±	627.9	-53.1
	Mg	100 $CuCl_2$ + 10 KN	671.2	±	325.0	-78.4
		50 $CuCl_2$ + 100 KN	1084.4	±	659.2	-69.1
	S	50 *n*Cu + 0 KN	3524.6	±	211.8	-21.0
		100 *n*Cu + 0 KN	3650.9	±	71.3	-18.1
		50 *b*Cu + 0 KN	3008.1	±	23.9	-32.5
		100 *b*Cu + 0 KN	2812.4	±	181.7	-36.9
		50 $CuCl_2$ + 0 KN	2779.4	±	86.6	-58.2
		100 $CuCl_2$ + 0 KN	3007.2	±	43.9	-32.6
Roots	**K**	100 *n*Cu + 100 KN	32647.8	±	2359.5	49.3
		50 CuCl2 + 100 KN	29195.1	±	2686.7	33.5
	P	100 CuCl2 + 0 KN	4676.7	±	2436.3	-66.1
		100 *b*Cu + 100 KN	10797.7	±	1365.3	-31.4
		50 $CuCl_2$ + 100 KN	5776.7	±	1638.8	-63.3
		100 $CuCl_2$ + 100 KN	4041.9	±	350.7	-74.3
	Mg	100 $CuCl_2$ + 10 KN	4056.7	±	620.9	58.5
	Al	100 $CuCl_2$ + 100 KN	124.1	±	31.7	-55.0

2.3.8 Protein content in seeds

Proteins [UTEP40][SA41]are comprised of amino acids, which are essential for plant growth and development (Khattab et al., 2009). Kidney beans also serve as a major source of protein for consumers(Torres-et al., 2006). Crude protein content is the basis on which total protein is estimated from within the food industry (Majumdar et al., 2015). There were no statistically significant differences from controls when comparisons were made among treatment groups (Figure 2.6). In Figure 2.7 (A), it is shown that 50 mg/kg and 100 mg/kg *b*Cu substantially increased protein content by 11% to 12%. Further, a concentration dependent negative trend was observed by $CuCl_2$ and $CuCl_2$ × KN treatments (Figure 2.6 (B-C)). Ionic Cu is highly reactive and could alter many physiological and biochemical processes within plant systems. The effects on macromolecule content by Cu ions are intertwined with plant metabolism, growth, and seed development mechanisms; thus, it is premature to determine whether this is a positive or negative effect[UTEP42][SA43] (Majumdar et al., 2015). This was illustrated in a study by Rico et al., (2013), where amylose content as a function of protein fractions varied in response to 500 mg/kg *n*CeO_2.

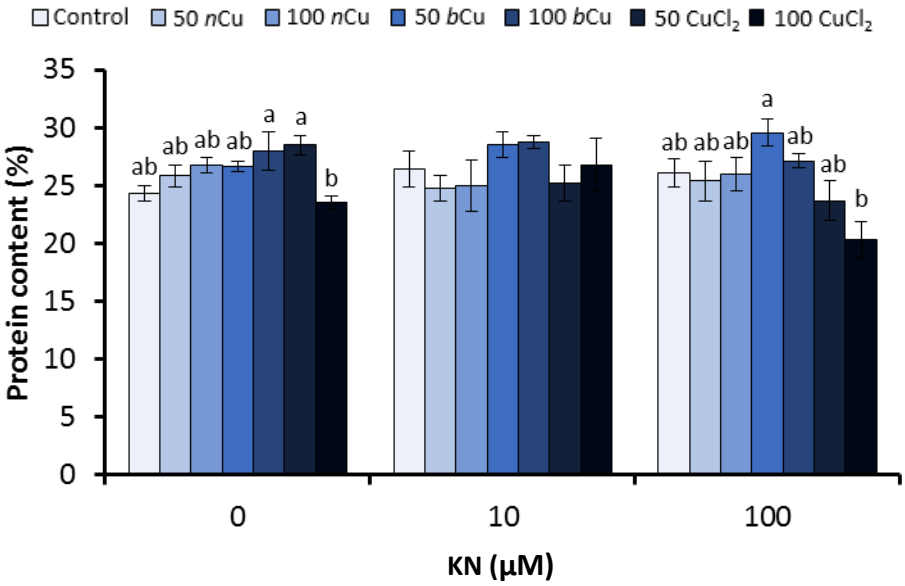

Figure 2.6 Protein content in seeds of kidney bean plants grown to full maturity (90 days) in soil rendered with 0 to 100 mg/kg of *n*Cu, *b*Cu, and $CuCl_2$, and watered with 0 to 100 µM of KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences between concentrations and treatments within the same KN treatment concentration at ($p \leq 0.05$); n=3.

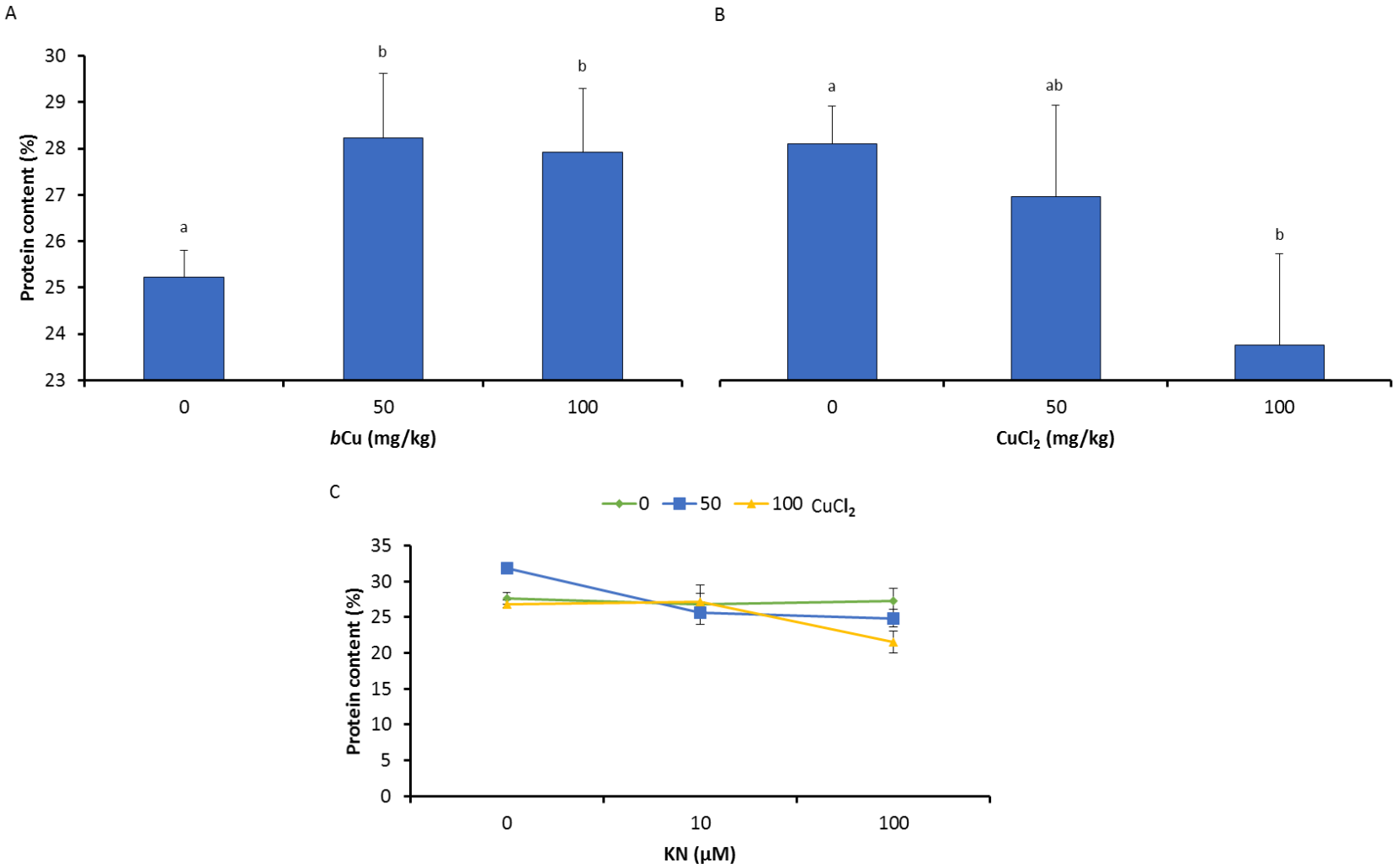

Figure 2.7 Averages of protein content for factors (A) *b*Cu, (B) $CuCl_2$, and (C) $CuCl_2$ x KN in seeds of 90-day old kidney bean plants exposed to 0, 50, and 100 mg/kg of *b*Cu and $CuCl_2$, and 0, 10, and 100 µM KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences ($p \leq 0.05$); n=3

2.4 Conclusions

In this study, we found that there was a concentration-dependent increase of Cu concentration in leaves by *n*Cu x KN and *b*Cu x KN treatments. Cu accumulation was found to be highest in the roots. Both chlorophyll and CAT were affected by *b*Cu and $CuCl_2$. These treatments also increased Mn (up to 41%) and decreased Mg content (up to 78%) in seeds and stems, correspondingly. 100 mg/kg $CuCl_2$ + 100 µM KN reduced accumulation of Ca and Mg in seeds and leaves by 56% and 75%, respectively. 100 µM KN increased K and Mg accumulation in roots up to 59%, while it decreased P shoots up to 78%. *b*Cu and $CuCl_2$ increased stem length, fresh/dry weight, and water content. Seed yield was largely unaffected. Protein synthesis was stimulated by *b*Cu (11% to 12%), while it was dampened by $CuCl_2$ x KN. Our results indicate that *b*Cu and $CuCl_2$ as main factors impacted the overall physiology and biochemistry of kidney bean plants the most. The effects of Cu were not significantly modulated by KN, despite our hypothesis that it would increase the metal uptake and accumulation through enhanced plant defense mechanisms. The interaction *n*Cu x KN is not detrimental to nutritional quality or plant integrity. Future work should entail further analysis on macromolecule composition and quality, including sugar/starch content and proteomic analysis. To our best knowledge, this is the first study examining how KN modulates the effects *n*Cu, *b*Cu, and $CuCl_2$ in kidney bean plants[UTEP44].

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Appendix

Supporting information for Chapter 2

Table S1. Physiochemical properties of *n*Cu and *b*Cu (Hong et al., 2014)

Property	***n*Cu**	***b*Cu**
Primary particle size (nm)	10^2 - 10^3	$<10^4$
Hydrodynamic diameter (nm)	2590 ± 1138	4546 ± 3940
Zeta potential (MV)	-29.4 ± 0.8	-35.4 ± 1.27
Cu content (wt. %)	83.3	98.7
Other elements present	O, C	ND
Morphology	Irregular	Dendritic, platelike, rhombus
Main Copper phase	Cu, Cu_2O	Cu
Crystal structure	Cubic	Cubic

Measurement was done at pH 7
ND = Non-detect

Table S2. Soil Composition (Barrios et al., 2015)

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

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

* A portion of the nitrogen, phosphate and potash has been coated to provide 0.15% coated slow release nitrogen (N), 0.03% coated slow release available phosphate (P_2O_5) and 0.08% coated slow release soluble potash
(K_2O)

Soil pH= 6.8-7.2

Table S3. Chl-a, chl-b, and total chlorophyll content in leaf tissue of kidney beans (55 days) grown in soil amended with 0 to 100 mg/kg of nCu, bCu, and $CuCl_2$, and watered with 0 to 100 µM of KN. Data are means of three replicates ± SE. Different letters represent statistically significant differences between concentrations and treatments within the same KN treatment concentration at ($p \leq 0.05$); n=3.

KN (µM)	Treatment (mg/kg soil)	Chl-a	Chl-b	Total chlorophyll
0	Control	30.4 ± 2.5	22.0 ± 0.9	36.0 ± 4.0
	50 *n*Cu	36.6 ± 1.6	20.7 ± 0.9	36.7 ± 2.9
	100 *n*Cu	30.6 ± 6.1	25.2 ± 0.9	40.8 ± 2.2
	50 *b*Cu	33.6 ± 0.7	24.1 ± 0.9	39.1 ± 2.1
	100 *b*Cu	38.1 ± 1.0	22.5 ± 0.9	36.6 ± 0.1
	50 $CuCl_2$	38.8 ± 1.5	26.8 ± 0.9	43.5 ± 0.0
	100 $CuCl_2$	35.3 ± 2.9	22.7 ± 0.9	37.0 ± 0.1
10	Control	35.8 ± 3.5	22.5 ± 0.9 ab	36.7 ± 0.1 ab
	50 *n*Cu	36.2 ± 0.3	25.3 ± 0.9 bc	41.0 ± 2.2 bc
	100 *n*Cu	38.2 ± 1.0	22.2 ± 0.9 bc	36.5 ± 0.5 bc
	50 *b*Cu	37.3 ± 2.0	22.9 ± 0.9 bc	37.1 ± 0.3 bc
	100 *b*Cu	37.1 ± 1.0	24.1 ± 0.9 c	39.0 ± 2.12 c
	50 $CuCl_2$	44.4 ± 1.6	27.7 ± 0.9 ab	44.9 ± 2.2 ab
	100 $CuCl_2$	39.4 ± 7.5	29.0 ± 0.9 a	47.0 ± 0.1 ab
100	Control	43.1 ± 3.9	26.3 ± 1.3 a	44.7 ± 1.5 a
	50 *n*Cu	46.8 ± 0.7	22.6 ± 0.9 a	38.6 ± 2.1 a
	100 *n*Cu	39.8 ± 2.5	19.9 ± 0.9 a	37.9 ± 3.4 a
	50 *b*Cu	26.3 ± 11.0	11.9 ± 0.9 a	28.5 ± 2.5 a
	100 *b*Cu	40.6 ± 5.2	21.5 ± 0.9 a	37.4 ± 1.5 a
	50 $CuCl_2$	11.5 ± 6.2	14.2 ± 0.9 b	28.6 ± 3.5 b
	100 $CuCl_2$	36.6 ± 0.6	23.2 ± 1.3 a	38.9 ± 0.5 a

Vita

Suzanne Annette Apodaca was born in Chesapeake, Virginia on August 3rd, 1992. She graduated from Bel Air High School Center for Health Professions under the dentistry program with her Registered Dental Assistant (RDA) license. Afterwards, she attended the University of Texas at El Paso where she majored in Environmental Science. During her time as an undergrad she interned at Miller Electric as a Safety Intern. She was accepted into the Undergraduate Research Mentoring program in Ecosystem Health in the fall of 2013 to conduct research on an independent project with Dr. Wen-Yee Lee in the chemistry department. She won "Best Poster" in Environmental Science in the school-wide Campus Office of Undergraduate Research Initiatives (COURI) symposium for her work. In December of 2013, she received her Bachelor of Science degree. She participated in the Science Undergraduate Laboratory Internship program at Lawrence Berkeley National Lab during the spring of 2014. Suzanne began her master's degree in Environmental Science under the mentorship of Dr. Jorge L. Gardea-Torresdey in the fall of 2014, of which she holds a 3.9 GPA. Her research focuses on the effects of copper nanoparticles and kinetin in kidney bean plants, emphasizing the importance in analyzing the interaction between compounds of emerging concern within the agricultural industry. In August 2016, Suzanne began her Ph.D. degree in Environmental Science and Engineering at the University of Texas at El Paso.

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