ABSTRACT

Interactive Effects of Copper Oxide Nanoparticles and Arsenic on Rice (*O.sativa japonica* 'Koshihikari') Plant Growth and Development

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The emerging applications of nanomaterials (NMs) encompass a wide range of industries, including agriculture (e.g., as fertilizers, pesticides, and biosensors). NMs applied in the crop-field may influence plant growth and uptake of heavy metal(loid)s including arsenic (As). Being ubiquitous in the environment and readily bioavailable in aquatic system, As is taken up by rice plants, causes phytotoxicity and accumulates in rice grains. Classified as a Class I carcinogen and causing many diseases, As in rice grains potentially causes human health effects, particularly for infants who are eating rice to transition from breast milk to solid food, and Asian populations with regular daily rice consumption. Although As in water can be removed dramatically by copper oxide nanoparticles (nCuO) due to their high adsorption capacity for As, the interaction of nCuO and As has not been well elucidated on rice plant growth and As accumulation.

This project was the first to investigate the interaction of nCuO and As on rice (*Oryza sativa japonica*'Koshihikari') plant growth and development during a life cycle (from seed germination to seed maturation). The effects of nCuO and As were

determined, individually and interactively, on rice seed germination and early seedling growth in sand and an artificial soil mixture of clay and topsoil. A greenhouse study on the life cycle growth of rice plants in the artificial soil mixture approximated the real agriculture scenario and identified the nCuO dependent acceleration of heading process of rice plants. Particularly, As accumulation in dehusked rice grains was decreased by nCuO to 128 ng/g, 36% lower than the WHO maximum safe concentration of As in white rice (200 ng/g) for humans. Arsenic distribution and speciation inside the plant growth were also affected by nCuO. Transgenerational effects of nCuO and As were also verified on seed germination and early seedling growth of rice plants in this project.

These results contribute to the fundamental database of endpoint effects on rice plants, direct future research about wide-scale application of NMs in crop field as fertilizers or amendments, and provide insightful information to investigate the mechanism of the interaction between nCuO and As on plant growth.

Interactive Effects of Copper Oxide Nanoparticles and Arsenic on Rice (*O.sativa japonica* 'Koshihikari') Plant Growth and Development

by

Jing Liu, Bachelor's, Master's

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Jing Liu

At Baylor University, Waco Texas, USA

DEDICATION

To my late uncle, Tianfeng Liu,
and my beloved parents, Tiangan Liu and Fengxian Bi.

ATTRIBUTIONS

Five chapters of this dissertation have been published or submitted to peer-reviewed journals. Attributions of all the authors were list as below.

Jing Liu, the first author of all the articles, designed the project under the supervision of Dr.George P Cobb, and composed all the manuscripts.

Dr. Birendra Dhungana, the coauthor of Chapter One and Chapter Two, reviewed the manuscripts and helped with sample measurement.

Madie Simms made great contribution to Chapter Three by helping conduct the experiment in the greenhouse, and doing sample measurement and analysis in the lab.

Dr.Shui Song, who is also an engineer, coauthored Chapter Three. He helped with the layout and experiment conduction in the greenhouse.

Dr.Ryan S King contributed to the statistical analysis and interpretation of the results in Chapter Three.

Kyle Wolf was the coauthor of Chapter Four and Five. He helped with the sample analysis and experiment conduction.

Dr.Phillip M Potter contributed to the analysis of copper and arsenic spectra of X-ray Absorption Near Edge Structure collected from Argonne National Lab and interpretation of the results in Chapter Four.

CHAPTER ONE

Environmental Behavior, Potential Phytotoxicity, and Accumulation of Copper Oxide Nanoparticles and Arsenic to Rice Plants [1]

Abstract

Copper oxide nanoparticles (nCuO) are widely used in many industries. The increasing release of nCuO from both intentional and unintentional sources into the environment may pose risks to rice plants, thereby reducing the quality or quantity of this staple grain in the human diet. Arsenic (As) contamination has not only decreased rice yield, As accumulation in rice has also been a great human health concern for a few decades. New technologies have succeeded in removing As from water by nanomaterials. By all accounts, few studies address nCuO phytotoxicity to rice, and the interactions of nCuO with As are poorly described. The present article a) reviews studies about the environmental behavior and phytotoxicity of nCuO and As, and research about the interaction of nCuO with As in the environment, b) discusses critically the potential mechanisms of nCuO and As toxicity in plants and their interaction, c) proposes future research directions for solving the As problem in rice.

Keywords: copper oxide nanoparticles, arsenic, phytotoxicity, bioaccumulation, speciation, rice

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[1] This chapter was published as a critical review article in *Environmental Toxicology and Chemistry,* and also serves as an overall introduction chapter for the whole dissertation.

Introduction

Rice has served as an important human food source for more than 8000 years and played an essential role in the process of Asian civilization and urbanization [1]. Historically, rice has been and continues to be the main food source for half of the world's population due to its easy transportation and storage, and relatively short cooking time [2, 3]. Globally, 42% of the 2868 Kcal energy consumed daily by the average person comes from cereal crops in which rice is the dominant grain [4]. Rice also serves as a mainstay or supplementary food for people on restricted diets. For example, rice replaces simple carbohydrates, meat, and dairy products for lactose intolerant people or for those on a macrobiotic diet, due to its low percentage of gluten and a slow and continuous release of glucose into the blood [5]. However, the production of rice is not keeping pace with increasing demand as the global population increases [6]. By 2050, the production of rice may be insufficient to feed the rapidly increasing human population [1].

Several pressures currently limit rice production: diminishing clean water availability due to weather system shifts; increasing salinity from sea-level rise; conversion of agricultural land to other uses [6]. In addition, contamination and pollution from various anthropogenic activities disrupt or severely damage wetland functionality [7]. These activities include domestic, agricultural, and industrial activities such as surface runoff from concentrated animal feeding areas, effluent disposal of wastewater treatment plant, mine and factory discharges, fertilizer and pesticide application. These activities can transport excessive nutrients, dissolved and suspended metals, and organic pollutants to wetland areas, including rice paddies. Particularly, As contamination has

reduced rice yield and has become a great concern for causing a variety of adverse chronic human health effects [8].

To solve the problem of decreasing rice yield and poor quality caused by As and other contaminants, significant research is underway [9-17]. For example, the 3000 Rice Genomes Project is trying to identify the most important genes for rice production [18]. In addition, genetic engineering may discover or create new rice varieties - to increase yields and nutritional value, and to make cultivars more resistant to diseases and pests and more tolerant to severe weather such as droughts and floods. For example, the genetically modified (GM) Golden Rice has the potential to solve the vitamin A deficiency (VAD) problem, which has caused much of the death and disease in developing countries such as Africa and Southeast Asia [19]. However, the impact of GM food has yet to be directly or widely tested, and whether it will be a life saver or not is as yet undetermined [18, 20-22]. While scientific and regulatory agencies deem that biotech foods are safe, some environmental organizations strongly opposed GM crops including Golden Rice [22]. Resolving these differences will require input and agreement by a wide array of stakeholders such as scientists, rice producers and consumers, and regulatory agencies. In addition, nanotechnology application in agriculture may provide another promising approach to increase rice production and improve rice quality caused by As contaminants. However, some nanoparticles (NPs) are phytotoxic [23-34], and the effects from As combined with nano metal oxides are unknown (Figure 1.1).

This present article reviews literature in the year range of 1900 to 2017. Key topic words (including nanotechnology, nanomaterials, nanoparticles, copper oxide, arsenic, rice, agriculture, phytotoxicity, toxicity, dissolution) were searched in multiple databases

including "Web of Science", "Scopus", and "PubMed". Additionally, new publication alerts were created with the same key topic words to keep the information current. To the date of completion, 138 references closely related to the following topics were selected and reviewed:

a) The phytotoxicity of As and NPs, particularly copper oxide nanoparticles (nCuO), b) the environmental behavior and interaction of nCuO with As in the context of environment and toxicity to plants, c) potential mechanisms of nCuO and As toxicity in plants and their interaction.
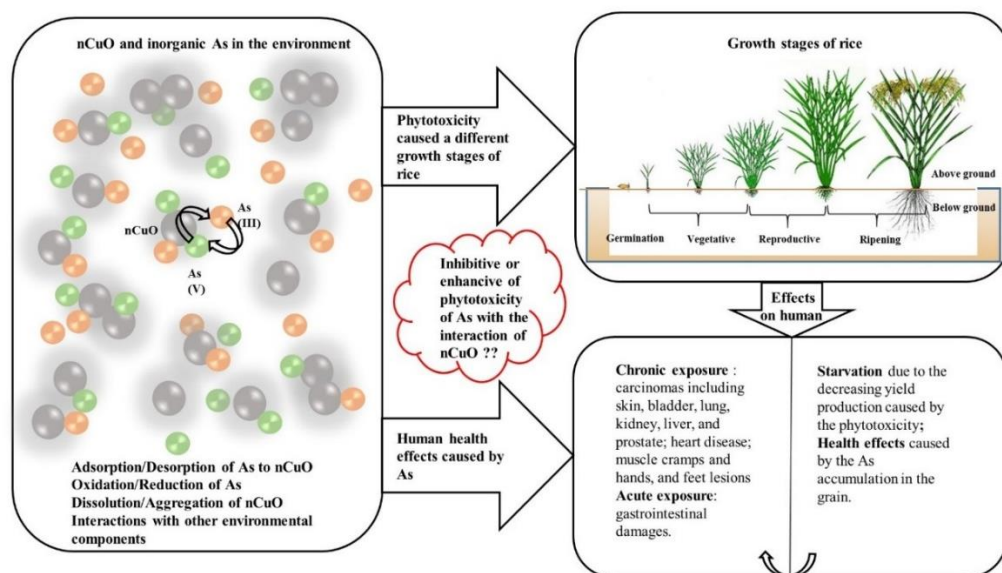
Figure 1.1. A simplified conceptual model showing the interaction of nCuO and As (mainly inorganic As species) in the environment, the phytotoxicity caused to rice plants at different growth stages and the possible human health effects. Graphics of growth stages of rice are reproduced with permission of the International Rice Research Institute (IRRI).

Environmental Behavior of Copper Oxide Nanoparticles and Arsenic

Interaction of copper oxide nanoparticles and arsenic in the environment.

Nanotechnology has been used in many industries including agriculture. Nanomaterials (NMs) exhibit significantly different properties from their corresponding

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bulk materials and may interact differently with other chemicals relative to their bulk size [35, 36]. Adsorption of As by NMs has been proposed as alternatives to conventional adsorbents for remediation [37]. This is probably because NMs have enhanced properties and improved effectiveness compared to their bulk counterparts when interacting with As. Various metal oxide NPs, such as iron (hydr)oxides, alumina, titanium dioxide, zinc oxides and copper oxide, have been used as nano adsorbent for As removal [37]. In particular, the maximum adsorption capacity of nCuO was much higher than its bulk counterpart. The maximum adsorption capacity of nCuO was reported to be 26.9 mg/g nCuO for As (III) and 22.6 mg/g nCuO for As (V), while the maximum removal of As from water by bulk CuO was about 369 µg As/g CuO due to its relative lower affinity of bulk CuO to As [38, 39]. Moreover, As (III) adsorption to nCuO showed greater dependence on pH (6 to11) and ranged from 62% to 83%, whereas, As (V) was relatively independent of pH in the range and consistent from 90% to 97%. In addition, As (III) can be oxidized into As (V) when sorbed to the surface of nCuO [40]. Nevertheless, compared with conventional adsorbents, the sorption capacity of nCuO allows it to be used effectively without adjusting the pH or oxidizing As (III) into As (V). Moreover, the presence of competing anions (sulfate, silicate and phosphate) did not have a significant effect on As adsorption even at exceptionally high concentrations [39]. Desorption, regeneration, and reuse of nCuO also solve the problem of waste sludge or spent media disposal. In addition, As collected during the regeneration process of nCuO can be reused in the industry. The water chemistry such as pH, major elements, and trace elements including Cu of the treated water was seldom affected by the regeneration process of nCuO [40, 41].

However, no additional studies have been conducted to evaluate the interaction of As and nCuO in environmental systems including soils, uptake by plants, and phytotoxicity (Figure 1.1). Hypothetically, nCuO may decrease the bioavailability of As via adsorption process mentioned above in this section, thus potentially reducing As toxicity to plants; however, this is yet to be studied.

Production, application, and disposal of copper oxide nanoparticles.

Copper and its compounds are naturally present, and they have been widely used for about 10,000 years [42]. Copper is an essential element for all known living creatures and is ranked as the 3rd most important metal for human service due to widespread use in everyday life and in almost every industry [43].

Copper was first used as a fungicide in 1882 in Bordeaux, France, for protecting grape plants from *Plasmopara viticola* fungi. Known as Bordeaux mixture, the Cu-containing mixture is still in use in several crops for preventing damage by various fungi [44-46]. However, due to their low water solubility and concomitant low bioavailability to plants, relatively large amount of these traditional Cu-containing agrochemicals are applied to the crops, which may cause phytotoxicity to the plant while protecting it from the phytopathogenic fungi [44]. Copper phytotoxicity manifests as seedling growth inhibition, ROS generation, gene alteration, DNA damages, etc. [25-34, 47, 48]. In addition, the widespread use of conventional Cu-containing agrochemicals accounted for a noticeable proportion in causing serious environmental and human health problems reviewed in the copper toxicological profile [49]. Moreover, Cu has been identified at 921 of 1630 sites on the EPA National Priorities List in 2015 [50]. Therefore, it is essential to develop new products which have higher biological activity and contain less

Cu in the formulation. Fortunately, the emergence and development of nanotechnology

can make it a reality. Given that nanotechnology allows the precise control of nano-scale

manufacturing of the agrochemicals and the delivery vehicles, the stability of the active

ingredients can be improved against transformation in the environment. Thereby, the

excess amount of Cu-containing mixture can be reduced by using nano-sized materials

(e.g., nCuO) with high surface activity, which increases the effectiveness of the

agrochemical. For example, three different Cu-based NPs including nCuO were tested to

be more effective against phytophthora infestans than the four registered Cu-based

agrochemicals including Bordeaux mixture [44]. In addition, nCuO improved the pest

resistance of transgenic insecticidal crops by significantly enhancing the Bt toxin protein

expression in the leaves and roots of Bt-transgenic cotton at the low concentration (10

mg/L) [34]. Furthermore, pollution caused by agrochemical run-off can also be reduced

when using their nano-forms, thus diminishing adverse environmental consequences [51].

Overall, the development of nanotechnology greatly promotes the application of

Cu and its compounds in many industries. Cu-based engineered nanomaterials (ENMs)

are unique among the most widely used ENMs because Cu contains three oxidation states

(Cu0, Cu1+, and Cu2+) and exhibits many unique and useful physicochemical properties

[52, 53]. In particular, nCuO is being widely used in many applications including high

efficient catalysts [54, 55], energy-saving materials [56, 57], high-temperature

superconductors [58], gas sensors [59], antimicrobial agents [54, 60], environmental

remediation [41, 61], and friction-reduction and anti-wear additives [62].

Research on new applications (e.g., biomarkers and biomedical use) is

undertaken. A conservative and an optimistic estimate of the annual global market for

nCuO from 2010 to 2025 were 200 to 830 tons/year and 330 to 1600 tons/year, respectively; and the 16 yr totals were 7075 and 14320 tons, respectively [63]. On the whole, the application of nCuO will keep increasing. However, the application of nCuO and other NMs in agriculture is relatively new and has not yet become a common practice, although there are nano-fertilizers freely available in the market [64, 65]. Thus environmental toxicologists and chemists have the opportunity to assess potential beneficial and adverse effects from using nCuO and other NMs before they are widely adopted in agriculture. An article reviewed potential applications of NMs in agriculture for several aspects: help seed germination and plant growth, protect plants and promote food production, detect pesticide residue using nano-sensor, and detect plant pathogen with the nano-diagnostic tool [64].

Like any other material, nCuO may be released to the environment during any stage of a product's life cycle including manufacturing, delivery, application and disposal [66-70]. Therefore, nCuO may also enter the environment from sources other than direct use in agriculture. NPs are introduced into the environment by two general pathways: the intentional and the unintentional release. The intentional release includes remediation of contaminated soils and groundwater with NPs, such as nCuO being used as antibacterial agent [54], and as an adsorbent to remove As from drinking water [39]. Atmospheric emissions, solid waste and liquid sewage from production industry facilities are unintentional release pathways of nCuO into the environment [54, 71]. Wastewater effluents, direct discharges, or accidental spillages to the aquatic systems are all possible ways for nCuO traveling a long distance from their sources. In addition, the wind or rainwater runoff can also transport nCuO and redistribute it in the environment. Keller,

et.al estimated the fate of the global ENMs with a time-integrated, mass balance approach [72, 73]. In 2010, of the 260,000 to 309,000 metric tons of total global ENM production, including about 200 metric tons of nCu and nCuO, 63% to 91% ended up in landfills, 8% to 28% in soils, 0.4% to 7% in water bodies, and 0.1% to 1.5% in the atmosphere. Eventually, most of those in the atmosphere will deposit on land and water surfaces.

Environmental behavior of copper oxide nanoparticles.

The high demand and application of nCuO will likely increase their release into the environment, but the environmental behavior of nCuO has not yet been well characterized. For example, most previous studies determined the dissolved Cu percentage after a specific time, however, the NP concentration, supersaturated with CuO varied by several orders of magnitude (0.025% to 60%) [74]. And only a few studies reported the dissolution rates of nCuO [74-78].

The behavior of nCuO depends on its intrinsic physiochemical properties and the chemistry of its surrounding environment [79]. In aqueous systems, aggregation, sedimentation, and dissolution control the stability of nCuO, and stability is a key factor determining the transformation, transport, fate, and toxicity of nCuO in different environment media. Generally, water properties like ionic strength (IS), pH, salinity, TOC, NOM, redox potential and other chemical components influence the short and long term behavior of nCuO [79-81]. Surface charge, controlling zeta potential and being altered with the pH change, influences aggregation and disaggregation of nCuO. The maximum hydrodynamic diameter of nCuO aggregates occurs near pH 10, which was defined as the isoelectric point [79]. In addition, aggregation of nCuO was shown to have a strong positive correlation with the IS of natural waters. This relationship was most

pronounced within the concentration range (0.03 to 0.15 M) of nCuO and was independent of pH [79]. The dissolution of aqueous nCuO and the ionic copper fraction were significantly influenced by complex-forming ions and the presence of NOM [80]. These constituents also influence aggregation via electrostatic stabilization mechanisms and electrosteric repulsion, because the adsorption of NOM to nCuO surface may reduce the positive charges of the particle [79]. Sedimentation of nCuO was enhanced by high salinity in the water column. Particularly, nCuO sedimentation appeared to be controlled by phosphate, because phosphate has the strong ability to covalently bond to metal oxides, making it a precursor in nCuO sedimentation [80]. In addition, fate and toxicology of nCuO were also influenced when the surface charge of nCuO shifted from positive to negative, thereby altering their interactions with other ions and substances. Sulfidation of nCuO with soluble sulfide was also studied. A rapid sulfidation was observed, and the dissolution-precipitation mechanism was involved [82].

Bioavailability of metal-based NPs is controlled by their environmental behaviors, which depends upon many factors mentioned earlier in this section, especially the dissolution in the aqueous phase [83, 84]. Quantifying the bioavailability of NMs and the released constituents is key to explaining the toxicity [77]. An intelligent non-experimental modeling method, nano-QSARs (quantitative structure-activity relationship), was developed to predict the toxicity of metal-based NPs. This method was based on 26 physicochemical properties of the metal and their cytotoxic effects in Escherichia coli. This model can be useful in evaluating the bioavailability and toxicity of metal-based NPs in the future [85]

Environmental behavior of arsenic.

Arsenic occurs naturally in the environment and is generally combined with other elements, especially in minerals and ores. Arsenic minerals are often associated with base metals such as copper, lead, tin and zinc, and precious ores such as gold and silver. Primary natural sources of As include weathering, bioturbation and dissolution of As minerals. Mining and processing (e.g., extraction and refining) of such ores have produced a wide scale of industrial pollution by inorganic As-containing waste and remain a primary anthropogenic source of As in the water bodies [41]. Other anthropogenic sources of As include solid by-product disposal and water discharge from various industrial processes (e.g., coal combustion, wood preservation, glass production, and in situ extraction processes of oil and natural gas), and arsenical pesticides application in agriculture [49, 86, 87]. Moreover, As has been identified at 1143 of 1630 sites on the EPA NPL in 2015, ranking 1st among the 848 substances found at hazardous waste sites [50].

Arsenic has a relatively high mobility over a wide range of redox conditions in aquatic systems [88]. The two common inorganic As forms [As (III) and As (V)] usually co-exist together, while As (V) predominates in an oxidizing environment and As (III) predominates in reducing conditions [49]. Redox potential, together with pH, are the most important factors influencing the As speciation [88, 89]. In oxidizing conditions, $H_2AsO_4^-$ is the dominant species at low pH (< 6.9), while $HAsO_4^{2-}$ becomes dominant at higher pH. In extremely acidic and alkaline conditions, H_3AsO_4 and AsO_4^{3-} may be present, respectively. In addition, the uncharged As acid (H_3AsO_3) dominates in reducing conditions at most environmentally relevant pH (< 9.2) [88, 90, 91]. Speciation,

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temperature, salinity, redox potential, pH, and ionic strength are important factors in determining As bioavailability as are many minerals (e.g., mackinawite - FeS), which bind or sequester metals thereby controlling As distribution among biotic and abiotic compartments [49, 92-95].

As (V) and As (III) have different affinities for minerals, which influence the mobility of As in the environment and bioavailability to plants. As (V) has a high affinity for iron oxyhyroxides, manganese oxides, aluminum (hydr)oxides and clay minerals [11, 14, 96]. Under oxidizing conditions and when pH is below 8.5, As (V) tends to adsorb tightly onto those mineral constituents which makes it relatively less mobile in the soil. As (III) has a lower affinity for these solid phases, which makes it more mobile. Under strongly reducing conditions, As can precipitate as sulfide minerals [88]. As (V) and As (III) formed surface complexes on FeS at pH 5.5 to 6.5 with high As loadings [97]. However, with the co-existence of phosphate which competes with As for sorption sites on FeS, the mobility of As can be increased [95]. Therefore, FeS minerals greatly influence the speciation, mobility and partition of As in sulfidic environment [95]. Moreover, As (III) has a greater tendency to partition into the solution phase in the presence of ferric (hydr)oxide [9, 11, 98]. Although the adsorption capacity of ferric (hydr)oxide is greater for As (III) than for As (V), the desorption rate of As (III) is much greater than that of As (V) [99]. This is because As (III) has an outer-sphere and multiple inner-sphere complexes whereas As (V) has only one inner-sphere complex [100, 101]. It was demonstrated that As concentration in the flooded paddies was increased under anaerobic conditions by reductive dissolution of As (e.g., due to the activity of Fe-reducing bacteria) [9]. Although not so common, reduction of As (III) to As (II) was also

observed under a slightly acidic environment with high As loadings [102, 103]. All the behaviors mentioned in this section influence the As bioavailability and accumulation by rice plants.

Bioaccumulation and Phytotoxicity of Copper Oxide Nanoparticles and Arsenic

Plants provide an important pathway for NP bioaccumulation into the food chain. The pore diameter of plant cell wall ranges from 5 to 20 nm [104], functioning as a sieving process, which allows NPs with sizes less than the pore diameter to traverse the cell wall easily. In addition, NP interaction with cell walls may enlarge the pore size of the plant cell wall and facilitate the entrance of NPs [30]. Once through the cell wall, NPs may traverse the plasma membrane assisted by the embedded protein transport carrier, or they may enter the interior of the cell directly through ion channels [30]. When applied to leaves, NPs can also enter the plants through stomatal openings and trichomes [30]. The ability of NPs to enter leaf cells depends on the plant species. For example, nCuO aggregated on the epidermis of conventional cotton leaves, while it entered transgenic cotton leaf cells by endocytosis [34].

Nanomaterials (e.g., ZnO, Al2O3, and CuO NPs) can cause phytotoxicity after entering the plant [23, 24]. The wide array of ENMs may affect different plants through various routes, thereby causing a range of physiological effects. Additionally, a single NM may have differential effects on several plant species [25-34]. The bioaccumulation of Cu -based ENMs was found to cause toxicity in many types of organisms, such as fungi [105, 106], mussels [107], amphipods [108], Daphnia magna [84, 109], worms [110], and plants [25-34]. In particular, nCuO was reported to cause phytotoxicity to various plants. Exposure of Indian mustard (Brassica juncea L.) to nCuO (0, 20, 50, 100,

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200, and 500 mg/L) inhibited both root and shoot growth in a dose-dependent manner [32]. nCuO at 500 mg/L inhibited the growth (height, fresh weight, leaf surface area) of maize (Zea mays L.) [47]. nCuO significantly inhibited the growth (height, root length, root number, and biomass production) of transgenic and conventional cotton plants at concentrations greater than 10 mg/L [34]. nCuO (10 to 100 mg/L) also induced DNA (e.g., 7,8-dihydro-8-oxoguanine) damage in agricultural and grassland plants (e.g., radish, perennial ryegrass, and annual ryegrass) [26]. Notably, in rice, nCuO caused a variety of phytotoxicity at the target concentrations at different life stages of rice growth. nCuO at 5 mg/L severely inhibited the root growth of rice seedlings by generating ROS and influencing the expression level of two genes, OsCDC2 and OsCYCD, which are associated with the root growth [27]. nCuO at concentrations of 0.5 mM, 1.0 mM and 1.5 mM decreased seed germination, shoot and root growth, and also caused oxidative damage to rice seedlings [48]. High concentrations (500 and 1000 mg/L) of nCuO caused adverse effects on rice growth during the whole lifecycle [111]. Plant length and biomass were decreased since the tillering stage. Fatal effects occurred after plants were treated with the high concentration (1000 mg/L) of nCuO at the heading stage. The two higher concentrations of nCuO also decreased the grain yield. Based on the previous studies, potential mechanisms of NP toxicity are summarized: 1) DNA damage or gene alteration [26, 27], 2) ROS generation and oxidative stress [82], 3) NPs penetration into the cell interfering with intracellular metabolism [112], 4) Metal ions released from the NPs hindering enzyme function, and 5) adsorption of NPs on the surface of organism (e.g., seed) generating locally concentrated ions [113].

In contrast to nCuO, of which the toxicity mainly depends on the small size and concentration of the particle, the toxicity of As is speciation and concentration dependent [114, 115]. Inorganic As species are generally more toxic than organic species, and As (III) is much more toxic than ionized As (V) [116]. As described in the previous section (Environmental behavior of arsenic), As speciation can be quite complicated [117]. In the process of plant uptake and transport, deprotonated As species [As (III), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)] can behave as silicic acid analogs and the arsenate as phosphate analogs [17, 118]. Thus, As is absorbed by competing for the same carriers in the root plasmalemma [14, 119]. A study on Spartina alterniflora indicated the potential mechanism of the As phytotoxicity [120]. Chemical forms and concentrations of As affected the macro-and micro-nutrient uptake by the plant. Due to sharing the same uptake system of the root with phosphate (P), As (V) uptake was increased under insufficient P condition, while organic As species decreased the P uptake by damaging the root cell metabolism. In addition, organic arsenical depressed the potassium (K) uptake while increased the sodium (Na) concentration in the plant root which was coordinated with the antagonism between K and Na. The significant concentration reduction of several essential macro nutrients P, K, Ca and Mg, and micronutrients B, Cu and Fe may contribute to the phytotoxicity of MMA, which was the most phytotoxic species to this marsh grass. Notably, for rice, phosphate is an essential and usually limiting macronutrient, thus making it efficient for rice to assimilate As analogs of the phosphate moieties [121]. However, the presence of iron plaque showed more complex impacts on the As (III) and (V) uptake by rice plant root [14]. Once within the plant, As species undergo metabolism, complexation, symplastic transport,

subcellular localization, and xylem transport during different life stages. Thereafter, As can be remobilized from shoots to grains via phloem during grain filling process and accumulate in the grains [119]. Within the plant, As species exert toxicological action via inhibiting ATP formation and other phosphorylation processes, causing oxidative stress and binding to protein sulfhydryl groups amongst others [122]. As a result, growth inhibition and the grain yield reduction of the plants occur because of the toxicological action [13]. In addition, the bioaccumulation of As in rice grains was increased because of the elevated concentration of As in rice paddies and the high mobility of As under the anaerobic conditions (e.g., flooded paddy) [11, 121, 123]. For example, rice from mine impacted regions had a higher total As concentration (a high percentage of inorganic As) than that from non-mine impacted regions [124]. Moreover, the seasonal rainfall influenced the As concentration in irrigated ground water and paddy field soil, thus the As concentration in rice grains also varied in a seasonal pattern [10].

Studies have shown that NMs (e.g., graphene oxide, silicon NPs, MnO2 NPs, nano-Fe2O3 and nano-Al2O3) amplified or alleviated the phytotoxicity of As [125-128]. However, it is as yet unknown for plants whether As and nCuO interaction increases or decreases the toxicity relative to each individual toxicant.

Human Health Effects Caused by Arsenic Accumulation in Rice

The As species of toxicological concern may be inorganic [arsenate including all As (V) species, and arsenite including all As (III)] or organometallic (MMA, DMA, trimethylarsineoxide, tetramethylarsonium, arsenochloline, arsenobetaine, and arsenosugars). Generally, inorganic species cause more acute toxicity to animals than do organic As species [129]. Although inorganic As was widely used as a pesticide in the

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past, inorganic As has no longer be used in agriculture since 1993 [130]. Whereas, some arsenicals are still in use, especially on non-food crops such as cotton and turf [49]. Presently, about 90% of As overall is used as wood preservatives, for which the production was phased out in the US by 2004, while the stock piles could still be sold and used [49]. Arsenic may enter the air, water and soil, and then be accumulated by organisms including plants. Humans can be exposed to As by eating food, drinking water, and breathing air. Rice is considered to be a major source of inorganic As in the human diet, which poses great risks to human health because rice plants intakes As about ten times more than other grain crops [15]. Moreover, the major As species (up to 90% of the total content) in rice grain are inorganic (arsenate and arsenite) [131]. This is primarily caused by the anoxic condition in which rice plants are submerged in water, and due to the unique physiology of the rice plant which allows it to scavenge As from the environment and accumulate it mostly in the inorganic form [8, 12]. Overall, As in rice grain can be elevated by three major anthropogenic scenarios: irrigation using surface water with elevated As to rice paddies, industrial activities contaminating paddy soils, and conversion of soils previously treated with arsenical pesticides to rice paddies [121]. Among the three scenarios, irrigation related contamination of As is becoming worse because of the shortage of clean surface water supplies [132], which may also force more people to drink groundwater. Higher As concentrations are common in deeper geologic formations [132, 133]. Arsenic contaminated rice will add to exposures experienced by people who drink As laden groundwater.

Inorganic As compounds are considered as class 1 carcinogen, causing various carcinomas including skin, bladder, lung, kidney, liver, and prostate [121]. Chronic As

exposure has also been linked with heart disease, muscle cramps and skin lesions, while

acute As exposure can cause gastrointestinal damages [49]. The Joint Food and

Agriculture Organization of United Nations/World Health Organization (FAO/WHO)

Expert Committee on Food Additives derived the lowest benchmark dose (BMDL0.5) of

inorganic arsenic, which was 3.0 µg/kg body weight/day [134]. This value could range 2

to 7 µg/kg body weight/day based on dietary exposure, resulting in a 0.5% risk increase

in lung cancer. Currently, together with As problem in drinking water, As contamination

in rice also presents a serious global concern in respect of human health [121]. The WHO

proposed maximum safe concentrations (ML) of As in rice at 0.2 mg/kg for white rice in

July 2014 [135], and 0.35 mg/kg for brown rice in July 2016 [136]. In the future, the

WHO may target a lower concentration for brown rice when more data from all regions

are available. Due to the dominant role of rice in the total diet, China introduced a more

restrictive regulatory threshold value of As as 0.15 mg/kg rice [137]. Human uptake of

As from rice grain consumption not only depends on the cooking process, but also the

nature of As contamination (e.g., speciation, concentration, and distribution) in the

original production area. Nevertheless, to decrease rice in the diet is an efficient way to

avoid extra exposure overall, especially in developing countries where rice permeates the

culture [138]. However, this poses nutritional challenges for large populations.

Research Needs

Arsenic is known to adversely impact rice plant by causing phytotoxicity,

reducing the grain yield and accumulating in the grain thereby causing human health

effect through consumption of rice. However, few studies focused on the impact of nCuO

on rice plants, and no studies have evaluated the toxicity of nCuO in combination with As. Overall, more research needs to be done:

a) To characterize the mechanistic bioavailability and uptake of nCuO to rice plants and eventually to the rice grain. In order to do this, properties of media need to be characterized, and rice plant physiology must be well understood regarding the uptake, transport and accumulation of nCuO in the plant.

b) To determine the toxicity of nCuO in combination with As to the rice plants. Environmental behaviors (such as chemical speciation, fate and transport) of nCuO should be characterized. Proteomics and relative enzyme (e.g., superoxide dismutase and catalase) activities in the plant need to be evaluated. The interaction (additive, synergistic or antagonistic) between nCuO and As to cause positive or negative effects at the cellular and whole organism level also needs to be determined.

c) To develop feasible farming methods to minimize As uptake by rice and other food crops. Although nCuO can be used as adsorbents to remove As in water, it is not determined whether it can be used to decrease As bioavailability to plants. In addition, there have been studies which determined ways to decrease As uptake in rice; however, these methods are not feasible for farming applications. Altogether, it is necessary to develop methods that will minimize As uptake by rice and reduce risks from consumption of rice.

Acknowledgments

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CHAPTER TWO

Copper Oxide Nanoparticles and Arsenic Interact to Alter Seedling Growth of Rice
(*Oryza sativa japonica*)

Abstract

Arsenic (As) causes phytotoxicity to rice plants, decreases rice production and

causes serious human health concerns due to rice consumption. Additional stresses may

be posed to rice plants due to the increasing release into the environment by the

expanding production and application of copper oxide nanoparticles (nCuO). The

influence of nCuO on As uptake in and effects on rice (*Oryza sativa japonica)* are

explored here for the first time. An 18-d factorial experiment was conducted to determine

main effects of nCuO (0, 0.1, 1.0, 10, 50, and 100 mg/L) and As (0 and 10 mg/kg), and

the interaction effect between nCuO and As on rice seed germination and seedling

growth. Arsenic alone decreased the germination percentage. Both As and nCuO reduced

seedling shoot and root length, and exhibited interactive effects. nCuO and As also

produced an interaction effect on the number of root branches (NRB) of rice seedlings.

Notably, high nCuO concentrations (50 and 100 mg/L) mitigated the negative effect of

As on the NRB. Copper uptake in shoots and roots was linearly correlated with Cu

concentration in the sand without As addition ($R^2 > 0.756$). Whereas, As addition to the

sand produced non-monotonic changes in Cu concentrations in shoots and roots versus

Cu concentration in the sand ($R^2 > 0.890$). Arsenic concentration in shoots had a slightly negative linear correlation with Cu concentration in the sand ($R^2 = 0.275$).

Keywords: copper oxide nanoparticle, arsenic, interaction, germination, rice, phytotoxicity

Introduction

Arsenic (As) contamination lowers rice yields, and As accumulation in rice grains has emerged as a global health concern, since rice (*Oryza sativa*) is a staple food source for about half of the population worldwide (Panaullah et al., 2008; Meharg and Zhao, 2012; Sohn, 2014; Hojsak et al., 2015). Naturally occurring As is ubiquitous in the environment, and As concentrations in soil and water bodies can be elevated by anthropogenic activities, which consequently increase As concentrations in rice grains (Meharg and Zhao, 2012).

Human health effects of As have been widely investigated and extensively reviewed (Saha et al., 1999; Ratnaike, 2003; ATSDR, 2007; Meharg and Zhao, 2012). In particular, inorganic As species [As (V) and As (III)] are Class 1 Carcinogens due to their causal relationships to various carcinomas (Meharg and Zhao, 2012). Adverse effects, including heart disease, muscle cramps, and skin lesions on hands and feet, are often caused by chronic exposures (e.g., drinking water with elevated As concentrations) (Kapaj et al., 2006). Acute As exposure can cause gastrointestinal distress including nausea, vomiting, abdominal pain, and severe diarrhea (Saha et al., 1999; Ratnaike, 2003; ATSDR, 2007).

Rice is a major source of inorganic As in human diet due to rice plants' unique ability to uptake about 10 times more As than other food crops (Williams et al., 2007;

Hojsak et al., 2015). This trait is likely related to the evolutionary pressure to thrive with

root systems that are completely submerged in anoxic media, where available As may be

increased by reductive dissolution of As (e.g., due to the activity of Fe-reducing bacteria)

(Takahashi et al., 2004). In rice grains, up to 90% of As is inorganic (Meharg and Zhao,

2012). As(III) represents about 64% and 70% of total As in Korean and US rice samples,

respectively (Kim et al., 2013). Arsenic-contaminated rice is of greater concern for

infants and toddlers, as rice is the common transition from breast milk to solid food

(Meharg et al., 2008). Maximum safe concentrations of As in rice were proposed by the

World Health Organization (WHO): 0.20 mg/kg for white rice, and 0.35 mg/kg for brown

rice. A lower As concentration for brown rice will be targeted by the WHO when more

data are available (Commission, 2014, 2016). Rice plays a dominant role in the total diet

in China, where a more restrictive regulatory threshold of As was adopted as 0.15 mg/kg

regardless of rice type (Gundert-Remy et al., 2015). It is suggested that a balanced diet

can minimize overall exposure of As via rice consumption, especially in developing

countries where rice permeates the culture (Zeigler, 2014).

The phytotoxicity of As has been extensively studied (Carbonell et al., 1998;

Meharg and Hartley-Whitaker, 2002; Liu et al., 2005; Li et al., 2007; Panaullah et al.,

2008). Germination percentages of wheat decreased with exposure to increasing As

concentrations (0–16 mg/L). The shoot and root growth were also inhibited in a

dose-dependent fashion by As exposure (Liu et al., 2005). However, another study

showed that low As concentrations (0–1 mg/kg) stimulated wheat (*Triticum aestivum L*)

seed germination and root and shoot growth, while all these parameters were gradually

decreased at high As concentrations (5–20 mg/kg) (Li et al., 2007). Rice (*Oryza sativa L.*)

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seed germination percentage, shoot length, root length, and fresh biomass decreased with

As treatments [As(III) (50 and 100 mM) and As(V) (100 and 500 mM)]. Arsenic also

generated oxidative stress in rice seedlings and up-regulated some antioxidant enzyme

activities, particularly in As(III) treatments (Shri et al., 2009).

Efforts have been devoted to As removal from the water or remediation of soil

with As contamination. Copper oxide nanoparticles (nCuO) sorb As from water

(Martinson and Reddy, 2009), and may reduce As impacts on rice plants. Compared with

other conventional adsorbents (including oxides of aluminum, iron, titanium, zirconium,

and manganese, ferric phosphate, zeolites, and coal combustion by-products), nCuO does

not have limitations such as pH adjustment requirement, oxidation of water, and

removing of competing ions (Reddy et al., 2013). In addition, nanotechnologies were

envisioned to revolutionize agriculture systems. Several research areas were highlighted

in the 2003 United States Department of Agriculture (USDA) report, including the

detection of pathogens and contaminants, smart treatment delivery systems, and smart

agricultural integration systems (USDA, 2003). The unique anti-microbial activity of Cu

and the high surface to volume ratio of nanoparticles (NPs) make Cu-based NMs more

effective than conventional Cu-containing agrochemicals such as algaecides, fungicides,

and in some cases as herbicides (Stoimenov et al., 2002; Ren et al., 2009; Wang et al.,

2013). Agricultural applications involving NPs are relatively new and have not been

widely used. However, copper-based nanomaterials (NMs) have been widely applied in

many industries and released to the environment (Stoimenov et al., 2002; Chowdhuri et

al., 2004; Zhou et al., 2006; Namburu et al., 2007; Dar et al., 2008; Ren et al., 2009; Jatti

and Singh, 2015; An et al., 2016). Although Cu is an essential nutrient element for plants,

little is known about the overall effects of Cu-based NPs (e.g., nCuO) on crops. To date, it is unknown whether As and nCuO interaction increases or decreases the toxicity to plants relative to each individual "toxicant" (Liu et al., 2018).

In our study, rice (*Oryza sativa japonica)* seed germination and early seedling growth were investigated in the presence of nCuO and As, separately and in combination. Effects on seed germination percentages, shoot length (SL), root length (RL), dry weight (DW), and the number of root branches (NRB) under 12 treatments were evaluated. These experiments were designed to test the hypotheses that 1) As and nCuO have individual main effects on rice seed germination and seedling growth, 2) As and nCuO interactions alter the rice seed germination or seedlings growth, and 3) nCuO affects As uptake and accumulation in rice seedlings.

Methods

Seed germination and seedling growth tests

The effects of nCuO and As on rice seed (*Oryza sativa japonica,* Kitazawa Seed Company, CA, USA) germination and seedling growth were evaluated. Two As treatments, 0 and 10 As mg/kg sand (~ average soil concentration in the TX, US (Scanlon et al., 2005)), were prepared by homogenizing powdered $Na_2HAsO_4 \cdot 7H_2O$ (98%, S9663, Sigma-Aldrich) in sand medium. Aqueous nCuO treatments (0, 0.1, 1.0, 10, 50 and 100 mg/L) were prepared to encompass concentrations that elicit most observed phytotoxic effects resulting from nCuO exposure (Atha et al., 2012; Wang et al., 2015; Le Van et al., 2016). Solutions were prepared with the nCuO powder (NanoArc®, 97.5%, 23-37 nm, APS powder, Alfa Aesar, MA, USA) in 1/5th Hoagland's solution (Appendix A, Table A.7). Therefore, there were 12 treatments (2×6 combinations of the nCuO and As), and

the treatment with 0 mg/kg of As and 0 mg/L of nCuO was used as the control. The

treatment with 10 mg/kg As and 0 mg/L of nCuO served as an As control treatment.

nCuO solutions were only applied on the first day. The Hoagland's solution was used as

the nutrient source and to maintain the water level afterwards. Each treatment had 20

replicate growth cells (88.7 mL in volume, without holes at the bottom, each received 55

g sand) with 2 seeds sowed in each cell. Seed germination and seedling growth tests

proceeded in an incubator (VWR diurnal growth incubator, model 2015, Oregon, USA)

for 18 d. The temperature was $25 \pm 1\,^\circ\text{C}$ during the day (16 h), and $20 \pm 1\,^\circ\text{C}$ at night (8

h). The humidity ranged from 40–90%. Luminance in the photosynthetically active

spectral region was directly measured to be $100 \pm 20\,\mu\text{mol/m}^2/\text{sec}$. Data were acquired on

Day 0 with a Quantum Meter.

Seed germination and seedling growth determination

Seed germination percentage in each growth cell was monitored daily. Seedlings

were removed after 18-d growth, rinsed with DI water to remove the external materials,

and blotted with tissue to remove excess water. The SL (from the root-shoot junction to

the tip of the longest leaf) and the RL (from the root-shoot junction to the tip of the

primary root) were recorded. The NRB was also counted on roots originating from the

planted seed. The shoot and root of individual seedlings were separated and dried at

$60\,^\circ\text{C}$ for 72 h to measure the DW. Dried samples were stored in plastic bags at room

temperature until analysis.

Total copper and arsenic concentrations in samples

Five growth cells from each treatment were randomly chosen, from which dry sand samples (0.5 g) were analyzed before sowing seeds and after removing seedlings. Five water samples (2 mL) were obtained every 6 d for each treatment (Supplemental Materials in Appendix A). Dry seedling root/shoot samples (n = 2–6) from a given treatment were grouped into 5 replicates with approximate dry mass. Sand and dry seedling samples were digested using a method adapted from USEPA method 3050B (USEPA, 1996). Solution samples were digested using a method adapted from USEPA method 200.8 (USEPA, 1994). Concentrations of Cu and As were determined in digestates with an Agilent 7900 ICP-MS in helium collision mode.

Statistical analyses

Generalized linear models (GLMs) were used to analyze rice seed germination percentages, seedling growth parameters (SL, RL, NRB, DWs), and concentrations of Cu and As in seedlings and growth media in response to nCuO and As treatment. A binomial distribution was specified for the germination model, because response variables were proportions but not over dispersed (Crawley, 2013). A gamma distribution was specified for other models, because response variables were continuous and right skewed (Crawley, 2013). Models were assessed by examining the residual plots, and were accepted with low heterogeneity. Analysis of variance (ANOVA) was conducted for the models to study the main effects of nCuO and As, and the interaction effect of nCuO and As. Means were considered significantly different when $p < 0.05$. Regression models were developed to analyze the relationships between parameters. All statistical analyses were performed in R (version 3.3.1).

Total Copper and Arsenic Concentrations in Test Media

Total Cu and As concentrations in sand media were measured before seeds were sowed and after seedlings were removed (Table A.1). Prior to receiving nCuO treatments, no difference was observed in total Cu concentration or in As concentration in respective groups with or without As addition. Therefore, the sand condition was well controlled before seedling growth. After seedling removal, total Cu concentration in sand increased in treatments that received higher concentrations (10, 50 and 100 mg/L) of nCuO ($p < 0.05$), and no difference was observed in treatments that received lower nCuO concentrations. Arsenic concentrations in the sand (with or without As addition) showed no difference than those prior to nCuO treatments. Thus, the approximate amount of nCuO was properly added to the corresponding treatment, and seedling growth did not significantly decrease As concentration in the sand.

Effects on Seed Germination

Arsenic had a main effect on rice seed germination ($p < 0.05$). Germination decreased in the As only treatment (82.5%) compared with control (97.5%) ($p < 0.05$) (Figure 2.1). Although germination percentages were the same in As+nCuO 1.0 and As alone treatments, data distributions in these two treatments were not the same. As had a significant main effect, which was the primary reason why values for As+nCuO1.0 and As were similar. An extra one-way ANOVA was conducted to compare the Control vs As treatment and Control vs As+nCuO1.0 treatment (which is usually not necessary when no significant difference was shown). The *p*-value for Control vs As treatment is 0.038, which is below the significance level 0.05, but the *p*-value for Control vs

As+nCuO1.0 is 0.074, which is quite close to the significance level 0.05, but we have to

say no significant difference is shown.

Moreover, no significant effect of nCuO was observed on seed germination ($p =$

0.571). And there was no interaction effect between nCuO and As ($p = 0.070$).
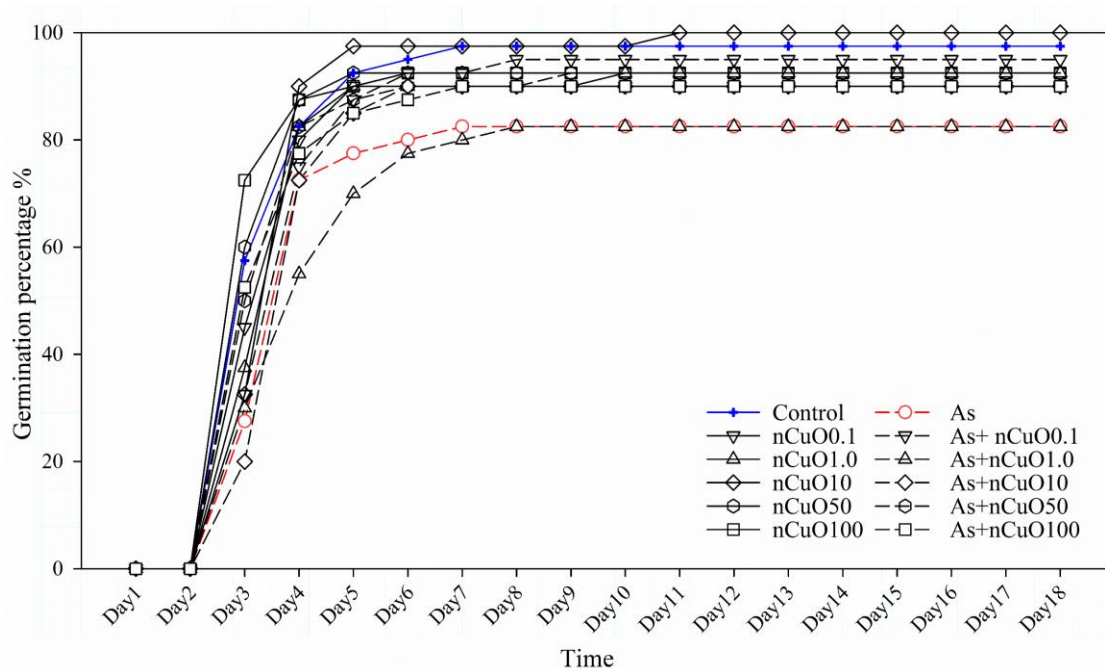
Figure 2.1. Rice (*Oryza sativa japonica*) seed germination curves in the 18-d laboratory
experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution (n =
20)

Effects on Seedling Growth Parameters

Both nCuO and As affected the SL and the RL ($p < 0.05$), and they had

significant interaction effects on the two parameters ($p < 0.001$) (Figure 2.2, and Table

A.4). Compared with control, As alone caused a prominent reduction in the SL and the

RL by 56% and 81% ($p < 0.001$), respectively. The higher concentrations (10, 50, and

100 mg/L) of nCuO without As addition decreased the SL by 38%–42% ($p < 0.05$).

Lower concentrations (0.1, 1.0 and 10 mg/L) of nCuO without As addition decreased the

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RL by 43%–59% ($p < 0.05$). In contrast, As addition decreased the SL by 28%–50% in treatments with lower nCuO concentrations (0.1, 1.0, and 10 mg/L) ($p < 0.05$), while As did not decrease the SL in treatments with higher concentrations of nCuO (50 and 100 mg/L). The RL was decreased 63%–76% by As addition in all nCuO treatments ($p < 0.001$). Arsenic and nCuO also had a significant effect on the NRB ($p < 0.001$), and they had an interaction effect on the parameter ($p < 0.05$) (Figure 2.2, and Table A.5). However, neither As alone nor any nCuO treatment without As influenced the NRB, compared with the control. In contrast, As addition reduced the NRB in treatments containing lower nCuO concentrations (0.1 and 1.0 mg/L) by 39.0% and 28.6% ($p < 0.05$), respectively. However, As did not alter the NRB in treatments containing 50 or 100 mg/L of nCuO, compared to the corresponding treatment without As. Furthermore, nCuO had no effect on shoot DW, nor had an interaction effect with As on the parameter (Figure 2.2, and Table A.5). Arsenic alone decreased shoot DW by 64% ($p < 0.001$). Whereas, both nCuO and As affected root DW ($p < 0.001$), but no interaction effect was observed between the two factors. Arsenic alone decreased root DW by 76% ($p < 0.001$). Arsenic addition to nCuO treatments decreased root DW by 63%–78% ($p < 0.05$). Without As addition, the SL decreased ($p < 0.05$) as the Cu concentration in sand increased (Figure 2.3). In contrast, when As was added to sand, the SL increased ($p < 0.05$) with increased Cu concentration in the sand (Figure 2.3), which indicates a mitigation of As adverse effect by nCuO. Overall, the DWs of shoot and root were closely correlated with their corresponding lengths ($R^2 = 0.88$ for shoots, $R^2 = 0.92$ for roots).
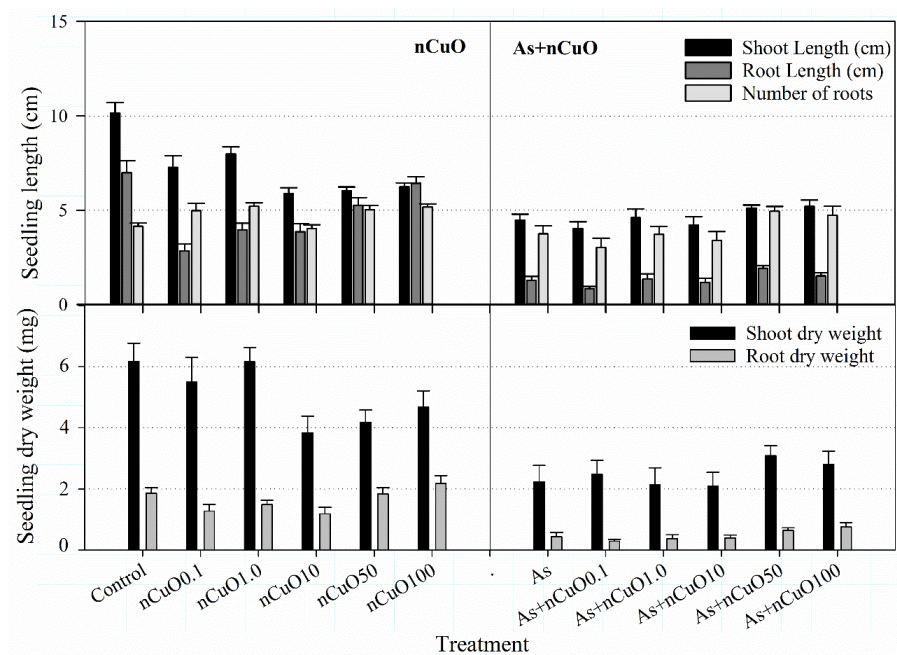
Figure 2.2. Rice (*Oryza sativa japonica*) seedling growth parameters after the 18-d laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution (Replicate numbers are shown in Appendix A Table A.4-5 for each treatment)
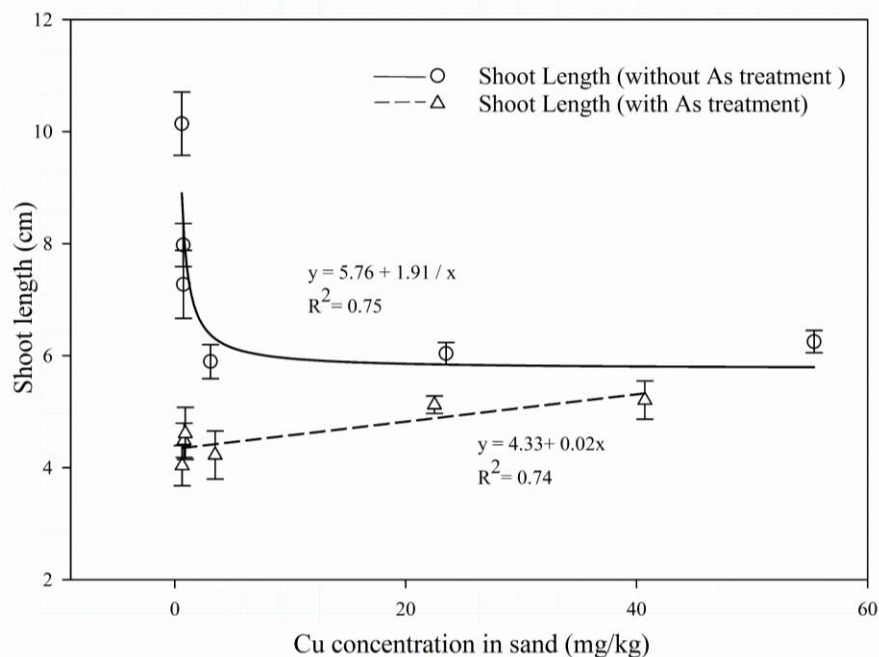
Figure 2.3. Relationships between seedling length and the concentration of copper in the sand after seedling growth in the 18-d laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution (Replicate numbers are shown in Appendix A Table A.4 for each treatment)

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Total Copper and Arsenic Accumulation in Seedlings

Total Cu concentration in shoots and roots were influenced by nCuO and As ($p <$ 0.05), and there were interaction effects between the two factors on total Cu in shoots and roots ($p < 0.001$, Figure 2.4, and Table A.6). Compared with control, the As alone treatment decreased Cu concentration in shoots by 47% ($p < 0.05$), but increased Cu concentration in roots by 99% ($p < 0.001$). However, compared to control, higher concentrations (10, 50 and 100 mg/L) of nCuO increased the Cu concentration in shoots and roots with or without As addition. Whereas, the addition of As decreased Cu uptake into shoots and roots as compared to corresponding nCuO exposure groups without As addition ($p < 0.05$).

Furthermore, both nCuO and As significantly affected the As concentration in shoots and roots ($p < 0.001$, Figure 2.4, and Table A.6). There was an interaction effect of the two factors on the As concentration in shoots ($p < 0.001$), while no interaction effect existed in the As concentration in roots ($p = 0.395$). Arsenic addition to sand caused higher As accumulation in shoots, compared to the accumulation in the corresponding nCuO treatment without As addition ($p < 0.001$). Additionally, the As bioaccumulation factor (BAF) in shoots from nCuO treatment groups ranged from 6.7–15.8, while the BAF was 10.5 in the absence of nCuO.

Moreover, As had no significant main effect on the Cu concentration ratio in shoot: root (Cu ratio), which was influenced by nCuO addition ($p < 0.001$) and the interaction of nCuO with As ($p < 0.001$). Meanwhile, nCuO had no significant main effect on the As concentration ratio in shoot: root (As ratio), which was influenced by As addition ($p < 0.001$) and the interaction of As with nCuO ($p < 0.05$) (Figure 2.5, and

Table A.6). The Cu ratio generally followed a decreasing trend with respect to nCuO

treatment with or without As addition ($p < 0.001$, Figure 2.5, and Table A.6), whereas

increasing trends of the Cu concentration in shoots and roots with corresponding Cu

concentration in the sand were observed ($p < 0.001$) (Figure 2.6). The As ratio had no

significant difference compared to control in nCuO only treatment group; however, in the

presence of As and nCuO simultaneously, the As ratio decreased in most treatments

compared to the corresponding treatment containing nCuO alone (except 1.0 mg/L had

no change) (Figure 2.5, and Table A.6).

The Cu concentration in shoots and roots were linearly correlated with the Cu

concentration in the sand in nCuO treatment group without As addition ($R^2 = 0.756$ and

0.948, respectively, $p < 0.001$) (Figure 2.6). However, with As addition, the Cu

concentrations in shoots and roots showed a non-monotonic relationship with the Cu

concentration in the sand ($R^2 = 0.890$ and 0.921, respectively, $p < 0.001$) (Figure 2.6).

There was a peak of Cu concentration in shoots, while a plateau of Cu concentration was

reached in roots. Moreover, in As+nCuO treatment group, a slightly negative correlation

was demonstrated between As concentration in shoots with the Cu concentration in the

sand ($R^2 = 0.275$, $p < 0.05$) (Figure 2.7).
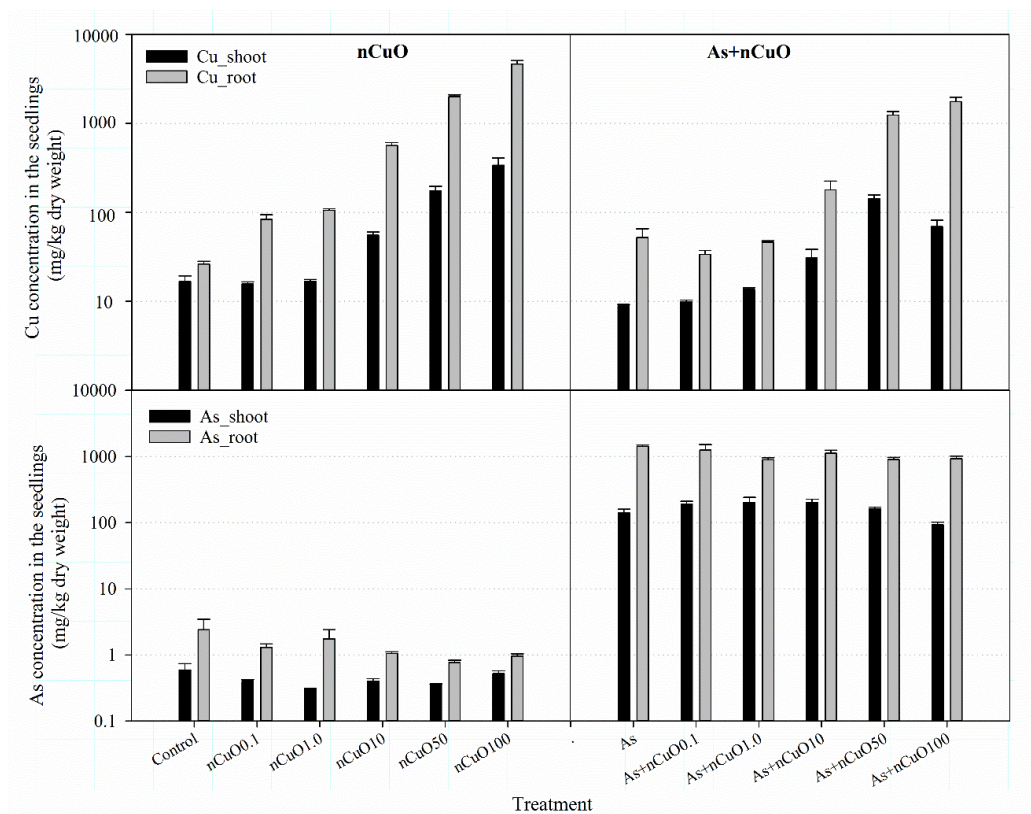
Figure 2.4. Total copper and arsenic concentrations in rice seedlings after the 18-d laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution (n = 5)
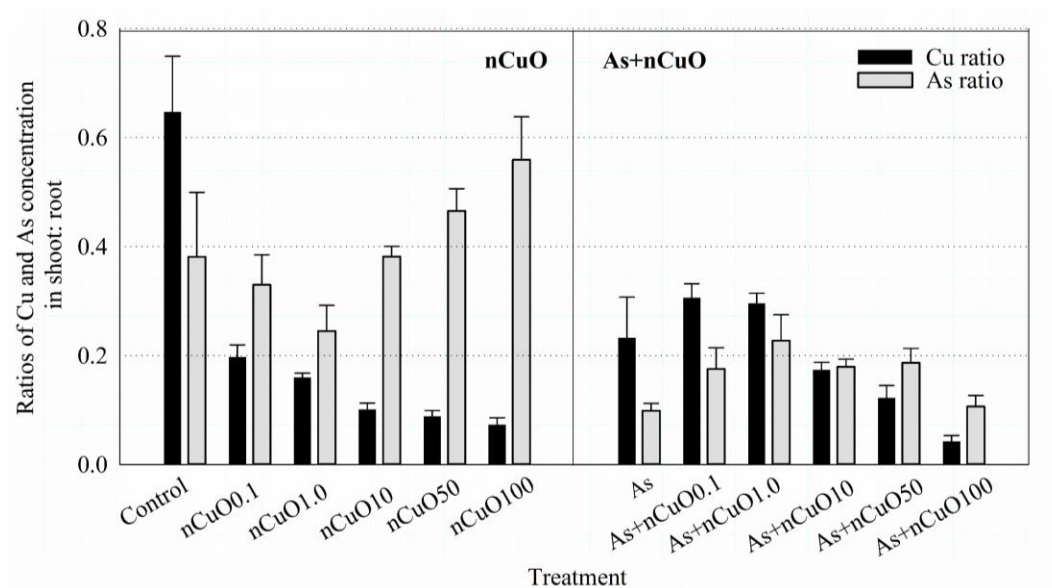
Figure 2.5. Concentration ratios in shoot: root of copper and arsenic after the 18-d laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution

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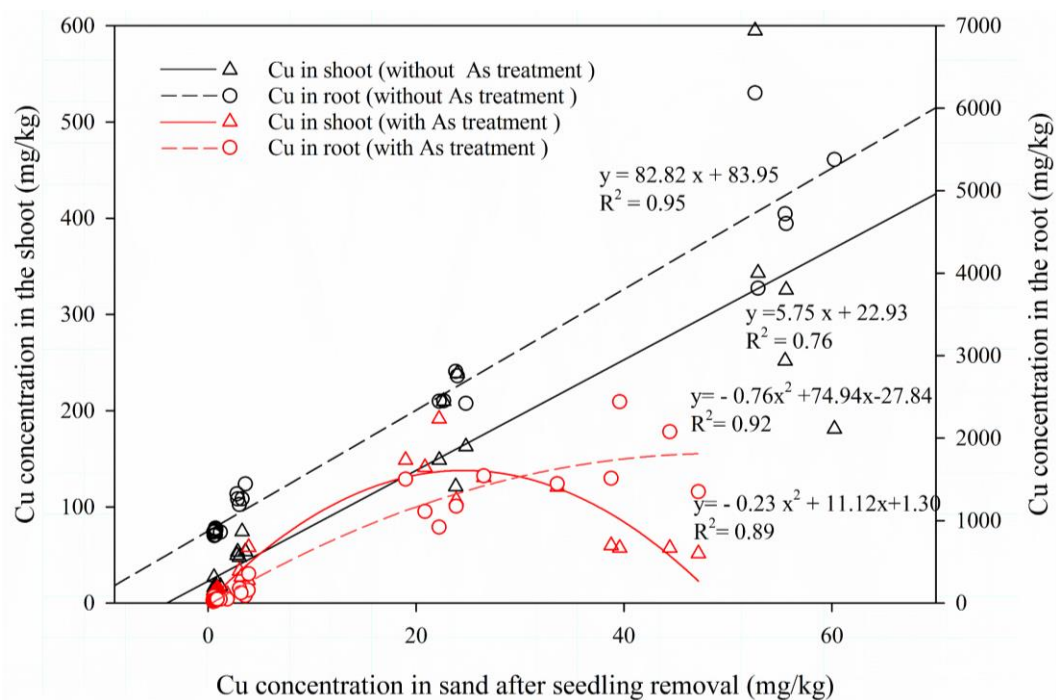
Figure 2.6. Total copper concentration (mg/kg) in rice seedlings after the 18-d laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution (n = 5)
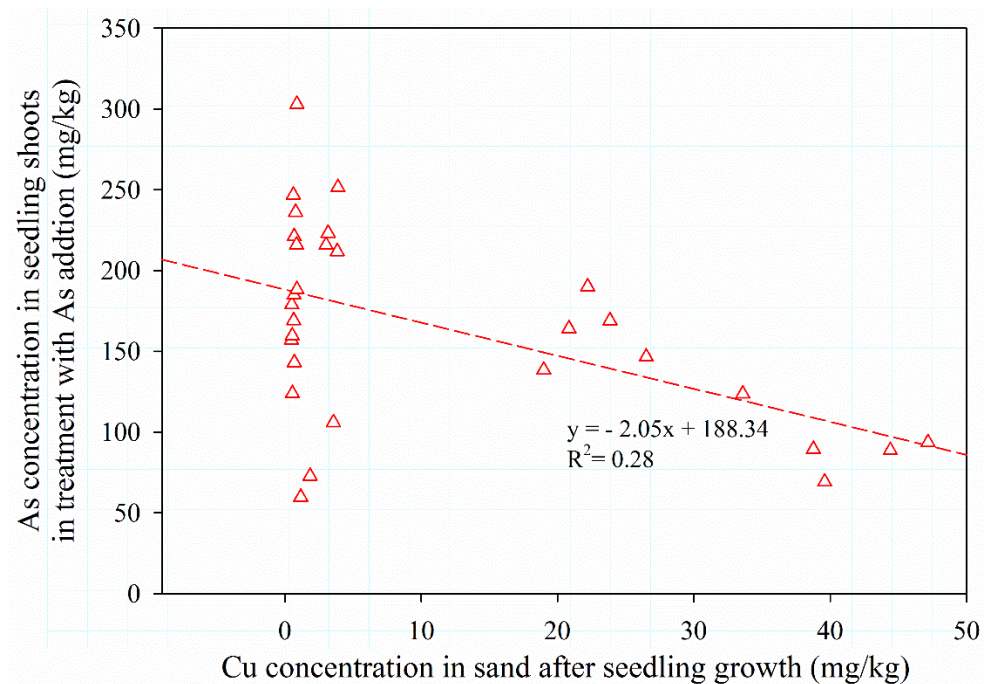
Figure 2.7. Arsenic concentration (mg/kg) in rice seedlings after the 18-d laboratory experiment exposed to arsenic in the sand and copper oxide nanoparticles in nutrient solution (n = 5)

Discussion

The sensitivity of vegetative response endpoints to metal and As impacts has been postulated to follow the order: the RL > root mass > the SL > total mass > shoot mass > germination (Kapustka et al., 1995). In our study, germination, length, dry biomass, and the NRB, were used to evaluate the effects of nCuO and As on rice, and confirmed the postulated order except that the NRB is also a sensitive endpoint. These data are important for our understanding of rice cultivation given the known presence of As in soils where rice is grown. Furthermore, nCuO is proposed to be used as agrochemicals, and the release of nCuO into the environment is supposed to be increased. Therefore, it is significant to study the interaction effect of As and nCuO on rice plant growth.

Arsenic Behavior and Effects

The addition of 10 mg/kg As considerably inhibited rice seed germination and reduced seedling length and biomass. Therefore, in practical field conditions, high As concentrations in soil (up to18 mg/kg reported in Texas, US) or irrigation with As contaminated water could have adverse effects on rice seed germination and young seedling establishment. These observations support previous studies addressing rice and other crop seedlings (Abedin and Meharg, 2002; Liu et al., 2005; Imran et al., 2015). In particular, a significant reduction in the RL of rice seedling by As is reasonable, since roots are the first point of contact of the toxicant in growth media (Abedin and Meharg, 2002). Soil texture is one of the most influential factors for inorganic As phytotoxicity. Inorganic As toxicity to a broad range of crops was five times more in sand than in clay soils (Sheppard, 1992).

Soil properties (e.g., pH, redox potential, organic matter, and microbial activity) play fundamental roles in controlling available As in the porewater (Onken and Hossner, 1995). Basically, As toxicity is determined by its speciation and concentration (Cobb et al., 2000; Akter et al., 2005). In our study, As(V) was added to sand media and was mobilized with the solution addition. Therefore, the total As concentration in the solution media within individual treatments increased over time ($p < 0.05$) (Table A.2 and Figure A.1). Arsenic re-distribution and possible As(V) reduction to As(III) may increase As bioavailability to seedlings, thus increasing the As uptake by the seedlings and causing phytotoxicity. In addition, the solid growth media in our study was 100% sand cleaned with 2% HNO_3. The total organic carbon was 0.076 ± 0.006 % before sowing the seeds, 0.101 ± 0.005% after seedling removal. Total nitrogen was 0.029 ± 0.003% before sowing the seeds, 0.031 ± 0.001 % after seedling removal (Table A.2). The small percentage of organic matter would be expected to have negligible influence on the behavior of As and nCuO compared to organic matter in natural soils.

As previously reported, As(V) was most likely assimilated by phosphate transporters in plant roots, and prevented the formation of high energy phosphoryl bonds (Carbonell et al., 1998; Summers, 2009). Moreover, As(V) can be reduced to As(III) by the reductase enzyme or further reduced via methylation (Farooq et al., 2016). Toxic effects may be induced during As(V) reduction to As(III), which can bind sulfhydryl groups influencing catalytic functions (Summers, 2009; Farooq et al., 2016). Reactive oxygen species could also be produced by plants when exposed to As. The resulting oxidative stress is considered an underlying cause of As phytotoxicity (Shri et al., 2009).

nCuO and As Interactions

In the present study, most As toxic effects were altered by the addition of nCuO (0–100 mg/L), which was probably due to the variation in As chemical behavior and uptake by seedlings resulting from the interaction of nCuO and As in the growth media. With the pH lower than the point of zero charge of nCuO (pH < 9.4) (Martinson and Reddy, 2009), the positive surface of nCuO with relatively high particle concentration facilitates sorption of the negatively charged As anions, thus reducing the bioavailability of As to the seedlings. Similarly, As addition may have changed the chemical behavior of nCuO or disrupted the uptake of nCuO by the plants, which caused the decrease of Cu in shoots and roots compared to corresponding nCuO treatment without As addition. Although As concentrations in the solution within individual treatments slowly increased over time, nCuO addition altered the final As concentrations, and the minimum occurred in treatments with the highest nCuO concentration (Table A.2 and Figure A.1). Additionally, the accumulation ratios of Cu and As in the shoot: root implied different mechanisms for the uptake by roots and/or transport within seedlings when nCuO and As were present simultaneously. Moreover, without As addition, most nCuO effects on rice seedling growth were dose-dependent in our study. Therefore, rice had different metabolic responses to low, medium and high nCuO concentrations in seedling growth. Notably, the fact that nCuO exposure decreased SL but did not alter shoot DW suggests a change in the carbon partitioning process may have decreased SL but increased the thickness or density.

nCuO and As Behavior in Solutions

In our study, the temporal decrease of nCuO and total Cu concentrations in solutions suggested particle aggregation and concomitant precipitation occurred, particularly for treatments with higher nCuO concentrations. This was also verified by a 72-h dissolution and aggregation experiment (72-h experiment) (Supplemental Materials in Appendix A). Meanwhile, Cu concentration in the solution increased again on day 18 compared to day 12, which was probably due to the increasing dissolution when particle aggregation reached the maximum (Table A.3 and Figure A.1). Moreover, all As concentrations decreased over time in the six solutions during the 72-h experiment ($p <$ 0.05, Figure A.2). Arsenic interaction with constituents in the solution would cause As removal from all solutions. In addition, the As concentration decreased the most in the solution with the highest nCuO concentration (100 mg/L), which was probably due to the largest active surface area to adsorb As and decrease As solubility. Moreover, lower nCuO concentration more fully dissolved in the presence of As. There are complex chemistries at play in the test systems containing Hoagland's solution. One possible reason for the increased dissolution of the nCuO in As containing solutions may be attributable to a small increase in pH following the addition of $HAsO_4^{-2}$ to test solutions, thus increasing the deprotonation of EDTA. This would increase the fraction of Cu complexed by EDTA in various states of deprotonation and increased dissolution rates. The smaller particles would have been more likely to dissolve or reach sizes that were not retained by the 3 kDa filters. Also, the Hoagland's solution contained 11 components, six of which were anions or possessed amine functional groups, including EDTA and the PIPES buffer, which interacted with Cu. There are also 9 cationic species within these

solutions and these may well interact with As. Also, the solution pH influences speciation.

Unlike 72-h experiment solutions, plant growth chambers contained sand and plants that could alter As concentrations in solution. Arsenic dissolution from the sand appears to have exceeded plant uptake from solutions in the 18-d seedling growth experiment, which allowed As to increase during the course of the experiment. Several studies also demonstrated that nCuO can sorb As (Martinson and Reddy, 2009; Reddy et al., 2013; McDonald et al., 2015; Schilz et al., 2015). Therefore, nCuO potentially reduced As bioavailability to the seeds and seedlings in the growth media, which may explain the mitigation of As toxic effect by nCuO on the seedling growth in our study. We will pursue this question in upcoming studies by evaluating nCuO and As speciation, size, and distribution in rice seedlings using X-ray Absorption Near Edge Spectroscopy (XANES). Under normal field conditions, As mobility and distribution are controlled by an array of factors including As speciation, redox potential, pH, ionic strength, and mineral substrates (USEPA, 1979; Wakao et al., 1988; ATSDR, 2007; Bataillard et al., 2014; Niazi and Burton, 2016). Further studies are needed to gain insight into mechanisms of the interaction between the two chemicals in the uptake by roots and transportation within seedlings. There is a minimal possibility that organic carbon in the treatments influenced the experiment outcome. However, since the growth media was sand, any difference in organic carbon would most likely have resulted from root exudates or rhizosphere organisms. Each of those evaluations could be a stand-alone study.

Regulatory Mechanism of Plant Growth

Seed germination and seedling growth are regulated by genes which can be influenced by environmental conditions. In root development, the cell-division cycle (G1, S, G2 stages, and mitotic phase) is controlled by plant D-type cyclins (CYCD) and cell-division cycle genes (CYC) (Wang et al., 2015). A 24-h root exposure study to nCuO at 5 mg/L suppressed the expression of two genes of *Oryza sativa* (CYCD by 35.6%, and CDC2 by17.8%) compared with control, thus inhibiting root growth by disturbing the normal cell-division cycle (Wang et al., 2015). Moreover, other influencing mechanisms have also been reported: NPs entering the cell and interfering intracellular metabolisms (Limbach et al., 2007), metal ions released from NPs hindering enzyme function, and NPs sorbed on the seed surface generating locally concentrated ions which further affect the normal metabolism of seeds (Tang et al., 2013).

In our study, high nCuO concentrations (50 and 100 mg/L) mitigated As adverse effects on the NRB ($p < 0.05$). Root branching, characterizing the adaptability of a plant to its environment, is fundamental to secure anchorage, assure water, minerals and nutrient supplies, and store photoassimilates by increasing the surface area (Atkinson et al., 2014; Bellini et al., 2014). Root branching is regulated (induced or inhibited) by endogenous regulatory factors (e.g., genetic program and hormonal signals) determining cell fate, cell division, and root primordia initiation, emergence, and elongation. Root branching is also influenced by environmental conditions (e.g., nutrient, light, drought, and biotic and abiotic stresses) (Bellini et al., 2014). In our study, nCuO, as an abiotic stressor, may be sensed by relative genes and stimulate the hormonal formulation to

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up-regulate root branching as a response, thus alleviating As adverse effects (Bellini et al., 2014).

Conclusion

We investigated the effects of nCuO (0.1–100 mg/L) and As (10 mg/kg) on rice seed germination and seedling growth. The As treatment caused severe inhibition of seed germination and reduction in seedling growth, especially root growth. This suggests significant reduction in rice productivity where As concentration is high. In As treatments, nCuO addition altered the seedling growth, and mitigated the As adverse effect observed in the SL and the NRB. The uptake of As in shoots was negatively correlated to the Cu concentration in the growth media. These data imply that nCuO may reduce As uptake into rice grains, thereby lowering human exposure to As. This study is the first to examine the influence of nCuO in combination with As. Broader concentration ranges of the two chemicals, different As species, and the practical medium/field conditions would be factors to evaluate in order to achieve more extensive understanding. Studies are to underway to evaluate the effect of As in combination with nCuO addition on the whole life cycle growth of rice plants and the As accumulation in ripe grains, especially in agriculture environment.

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Declaration of Interest

The authors report no conflicts of interest, and are responsible for the content and writing of the publication.

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Supplemental Materials (Appendix A)

Supplemental Materials related to this article are provided in a separate file.

CHAPTER THREE

Physiological Effects of Copper Oxide Nanoparticles and Arsenic on the Growth and Life
Cycle of Rice (*O.sativa japonica* 'Koshihikari')

Abstract

A factorial study was conducted to evaluate the phytotoxicity of copper oxide
nanoparticles (nCuO, 0.1–100 mg/L), arsenic (As, 0 and 10 mg/kg) and their interaction
to rice plants (*O.sativa japonica* 'Koshihikari') during the life cycle. No significant effect
was observed on seed germination. Main effects of nCuO and As were observed on
lengths and biomasses of seedling shoots and roots, and on root branching. The
interaction between nCuO and As also significantly influenced these parameters. nCuO
addition increased Cu uptake in seedlings and generally improved seedling growth. With
As addition, As was highly concentrated in roots and increased in shoots, and seedling
growth was also inhibited. Additionally, nCuO and As had significant main and
interaction effects on mature plant dry biomass, panicle number, total grain weight,
average grain weight, and several other panicle parameters. Moreover, nCuO and As
interacted to affect panicle emergence. nCuO also decreased As accumulation in
dehusked-grains. The accelerated heading stage by nCuO may help shorten the life cycle
of rice plants, thereby reducing As accumulation in grains. This study is the first to
examine the influence of nCuO in combination with As on the life cycle of rice plants.

Introduction

High geogenic content of arsenic (As) or anthropogenic activities (e.g., mining, smelting, agrochemical application) elevate As concentrations in biota and abiotic media, which poses direct or indirect ecological and human health risks. Arsenic causes phytotoxicity in various plants, including rice (*Oryza sativa*) [1-5]. Soil/water-plant systems also limit the transfer of excessive metals to the food chain. Even so, food from terrestrial and aquatic plants can be a primary source of metals for humans. Rice is the main food source of inorganic As for humans, relative to other dietary staples [6], because the unique physiology of rice plants for living in flooded conditions facilitates As accumulation from water [7]. Rice-dominated diets present a chronic exposure route that may cause global health concerns, since rice nourishes about half of the world's population [8]. The relationship between As concentrations in human hair and those in rice and agricultural soils supports the soil-plant exposure pathway of humans to As and the inextricable linkage between ecosystems and humans [9].

Nanotechnology serves various industrial and domestic purposes including removal of heavy metals (e.g., As, Cr, Cd, Pb, etc.) from water systems using nano-absorbents [10, 11]. Particularly, nano-copper oxide (nCuO), one of the most widely used nanoparticles (NPs), has advantages over other nanomaterials as an As sorbent [12], which may in turn alleviate As phytotoxicity. Moreover, Cu-based chemicals have been used in agriculture over several centuries [13]. nCuO has the potential to enhance the antimicrobial and fungicidal efficiencies of Cu due to the large surface area to volume ratio. Soils in

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certain areas of the world are copper deficient, which affects plant growth and crop food quality [14]. nCuO exposure can increase Cu concentrations in growing plants [15, 16]. Rapid nanotechnology development may pose environmental and human health risks while benefiting society. nCuO can enter the environment from manufacturing byproducts, wastewater discharge in nano-industry, and nano-product disposal after application. Adverse effects of nanomaterials, including nCuO, have been observed in plants including rice [17, 18]. Understanding of potential nCuO effects on crops is needed to minimize phytotoxicity to plants before widespread agricultural application, and to evaluate any differentiating effects as compared to bulk CuO. Furthermore, the interaction of nCuO and As in the environment is poorly understood, as are the mechanisms of resultant uptake by rice plants. This research investigated the effects of nCuO and As co-exposure during the entire life cycle of rice plants. The hypotheses are: 1) nCuO and As have individual main effects on rice seed germination and developmental parameters during the life cycle, 2) nCuO and As interactions alter rice plant growth during the life cycle, and 3) nCuO can alleviate some adverse effects of As on rice plant growth and reduce As accumulation in dehusked rice grains.

Materials and Methods

Life Cycle Test in the Greenhouse

Two types of commercial soils (60% Grainger, Catalog # 2258, 40% Lowe's, # 235384) were homogenized as growth media for rice (*O.sativa japonica* 'Koshihikari', Kitazawa Seed Company, CA, USA). Arsenic (0 and 10 mg/kg) in the form of $Na_2HAsO_4 \cdot 7H_2O$ (Sigma-Aldrich, lot # BCBM0939V) was added to the soil mixture and equilibrated for one week. nCuO (Nano-Arc®, 97.5%, 23–37 nm, APS powder, Alfa

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Aesar, MA, USA) was prepared at six concentrations (0, 0.1, 1.0, 10, 50,100 mg/L) in 20% Hoagland's solution [Appendix B, Table B.1]. There were 12 treatments (2×6 combinations of nCuO and As), including one control with neither As nor nCuO, and one treatment that received only As. Twenty replicate growth containers (Berry Plastics ID: T60785CP, 2.5 L) were prepared for each treatment. Ten seeds were wet-seeded in each container, and seedlings were thinned to 2 well-established ones on day 18. The study was conducted in a greenhouse for 131 d. Procedural details are included in SI.

Characterization of Nanoparticles and Soils

Hydrodynamic diameter and zeta potential of nCuO in Hoagland's solution were determined with a Malvern Zetasizer Nano ZS before addition to growth containers (Table B.2). nCuO dry powder was imaged with a scanning electron microscope (SEM, FEI Company). NPs in solutions were sampled on day 14 and characterized using a transmission electron microscope (TEM, JEM-1010, JEOL Inc.) with a dried aliquot on a formvar-carbon coated 200 mesh Cu-TEM grid (Figure B.4). Soil particles were characterized with Mastersizer 2000 for size distribution. Standard soil properties were also characterized using standard methods (Table B.3).

Seed Germination and Seedling Growth Measurements

Seed germination was monitored daily until seedling collection on day 18. Shoot length (SL, from the root-shoot junction to the tip of the longest leaf) and root length (RL, from the root-shoot junction to the tip of the primary root) were measured along with the number of root branches (NRB) (primary root and adventitious roots) and dry weights (DWs) of shoots and roots.

Heading Process, Panicle and Plant Biomass Measurements

Panicles numbers were recorded daily once the heading process began, when the tip of a panicle emerged from the sheath of a flag leaf. The following parameters were recorded for each replicate container after harvesting panicles and plants: total number of ripe panicles (TNRP), each panicle axis length (PAL), numbers of primary branches and secondary branches per panicle (PBN and SBN), spikelet numbers per panicle (SN), total dry weight of panicles (TPW, including the axes of panicles and branches, and all spikelets), total grain dry weight (TGW), dry weights (DWs) of mature plant straw and roots. Average grain dry weight (AGW) was determined for each treatment. Grain to straw and root to straw dry weight ratios (GSR and RSR) were also determined.

Copper and Arsenic Concentrations in Growth Media, Seedlings, and Grains

Total Cu and As concentrations in the growth media (soil and solution), 18-d seedling shoots and roots, and dehusked-grains were determined by ICP-MS (Agilent 7900) after digestion (adapted method from USEPA 3050B for solid samples, adapted method from USEPA 200.8 for solution samples) [19, 20]. Total Cu and As concentrations were measured in the test solution and in the soil mixture before adding solutions and after harvesting plants (Table B.4-6).

Statistical Analysis

Statistical analyses were performed with individual growth containers as the statistical unit. Generalized linear models (GLMs) were used to analyze the effects of nCuO and As on the growth parameters of rice plants and the interaction of nCuO and As on these parameters. Generalized linear mixed modeling (GLMM) was used to analyze

the effect of nCuO and As on the heading process with repeated measurement throughout the experiment. Analysis of variance (ANOVA) was conducted to determine the main effects of nCuO and As, and their interaction effects. Means were considered significantly different when $p < 0.05$. Regression models were developed between parameters to analyze their relationships. All statistical analyses were performed in R (version 3.3.2) with details provided in SI.

Results

The soil particle characterized by size with Mastersizer 2000 was 4.2% ± 0.2%, 56% ± 1% and 40% ± 1.2% of clay, silt and sand, respectively. The soil texture was classified as silt loam [21]. Organic matter content ranged from 3.5% to 3.9% in soils before growing plants, which explained the high exchange capacity (18.8–29.0 meq /100 g) of soil colloids (Table B.3). Exchangeable cations associated with soil colloids were Ca^{2+}, Mg^{2+}, K^+, and Na^+. Their base saturation percentages were 70.2%–76.1%, 12.3%–15.4%, 9.0%–11.6%, 2.7%–4.1%, respectively. These exchangeable cations are generally available to plants by replacing hydrogen ions from root hairs [21]. The measured high phosphorus in basic soils after rice harvest indicated a proper nutrient application [22].

Seed Germination and 18-d Seedling Growth

Mean rice seed germination success ranged from 87%–94% and was independent of nCuO or As addition ($p > 0.31$, Figure B.5).

Significant individual main effects of As and nCuO were observed on the SL, although no difference was found in nCuO treatments compared with control. The interaction between As and nCuO was also significant ($p < 0.05$) (Figure 3.1, Table B.7).

nCuO decreased SL at 100 mg/L compared with 1.0 mg/L, and As alone increased SL by 12% compared with control. Arsenic addition to the high nCuO treatment (100 mg/L) increased the SL by 18% relative to the corresponding nCuO treatment alone. More prominent main effects and interaction of As and nCuO were observed for shoot DW compared to the SL. nCuO treatments at or above 1.0 mg/L increased shoot DW by 20%–30% ($p < 0.05$). Whereas, As addition to higher nCuO concentrations (50 and 100 mg/L) decreased shoot DW by 13% and 15%, respectively, corresponding to the same nCuO concentration alone ($p < 0.05$).

Seedling RL was influenced by both As and nCuO ($p < 0.05$), and there was a significant interaction between the two substances. Specifically, nCuO alone increased RL by 34%–49% compared with control (in treatments at or above 1.0 mg/L), while RL was decreased by 16%–18% by As addition compared with the same concentration (1.0, 10 and 100 mg/L) of nCuO ($p < 0.05$). Addition of 10–100 mg/L nCuO to As treatments, increased RL by 27–30%, compared with treatments receiving As alone. Main effects of As and nCuO were also observed in root DW along with differing interactions between As and nCuO ($p < 0.001$). Addition of nCuO increased root DW by 32%–105%, although root DW decreased at 0.1 mg/L. Arsenic addition decreased root DW by 14%–53% compared with the same nCuO concentration ($p < 0.05$). Decreased root DW was also observed in As+nCuO 0.1 mg/L treatment as compared to As alone ($p < 0.05$). The NRB was also influenced by As and nCuO individually and interactively ($p < 0.001$). Specifically, high concentrations of nCuO at 50 and 100 mg/L increased the NRB by 21% and 18%, respectively, compared with control, while As addition decreased NRB by 15%–22% relative to nCuO treatments alone (0.1, 50 and 100 mg/L) ($p < 0.05$).
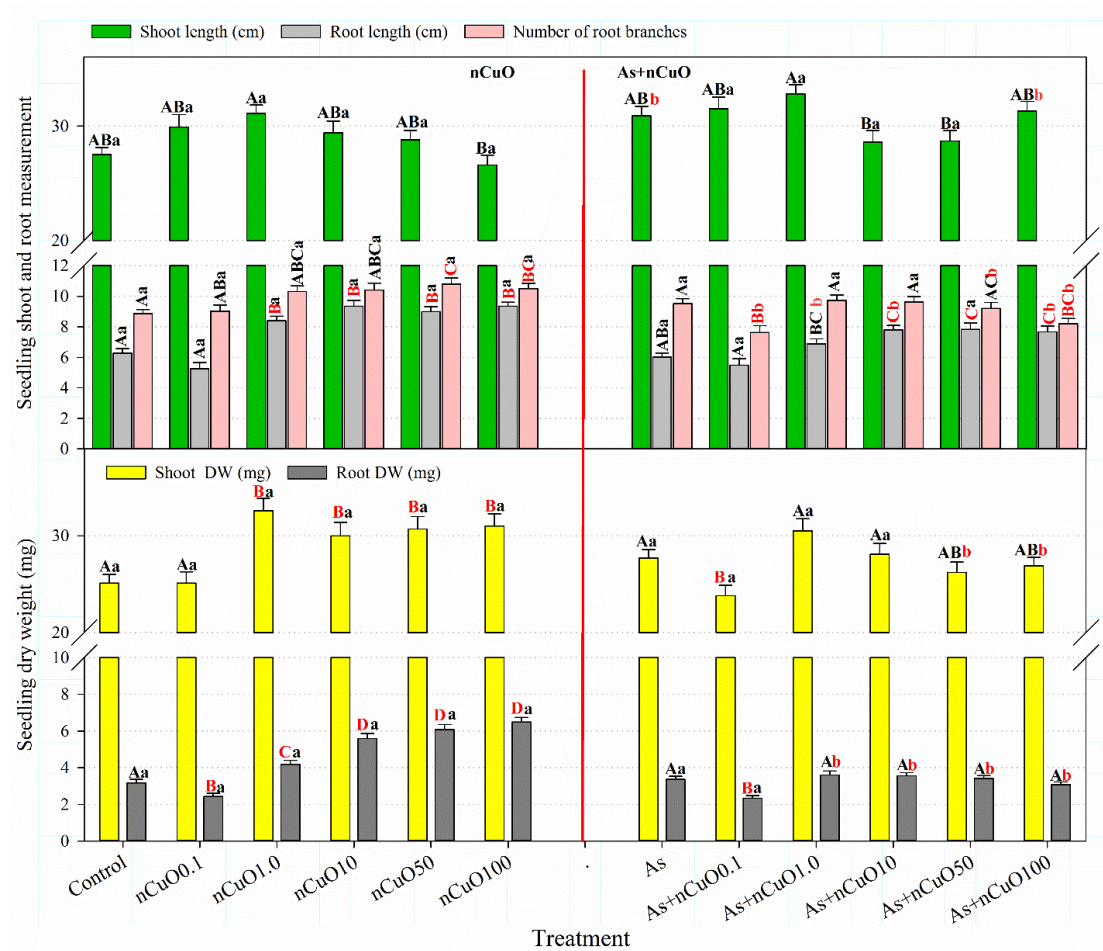
Figure 3.1. 18-d seedling growth parameters during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (number of replicates are shown in Appendix B Table B.7). Means of treatments at the same As level with a common superscript letter (A-D) are similar ($p < 0.05$). Means of treatments at the same nCuO level with a common superscript letter (a and b) are similar ($p < 0.05$)

Heading Process

Rice plants started heading on day 62 (Figure 3.2). New tillers kept emerging while water remained in the growth container, but not all tillers were effective at bearing panicles and becoming fertilized. Only panicles emerging before day 100 had matured by harvest day, since the ripening of rice panicles normally needs over 30 days [23]. The number of panicles exserting from the boots increased with time, and was influenced by nCuO, As, and their interaction ($p < 0.001$). nCuO (without As addition) significantly

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increased rice panicle numbers with increasing concentration ($p < 0.001$). Particularly, high concentrations (50 and 100 mg/L) of nCuO increased panicle numbers by 17%– 192% compared with control throughout the heading process ($p < 0.05$). Arsenic addition significantly decreased the panicle number by 2–100% in most treatments (except As+nCuO 0.1 mg/L) relative to corresponding nCuO treatments, particularly in the latter five weeks (days 96-131) of the study ($p < 0.05$). However, As treatment alone did not significantly decrease the panicle number compared with control.
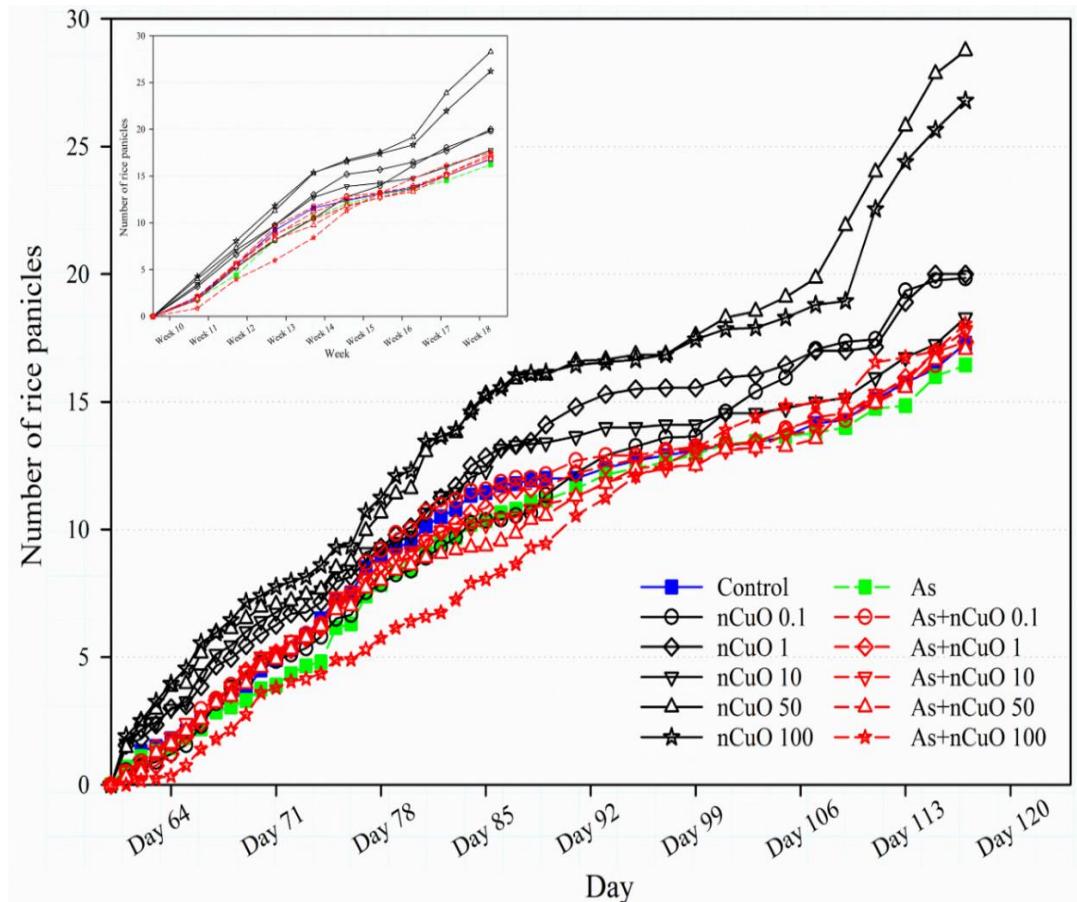
Figure 3.2. Heading process of rice panicles during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 20). The figure inset at the left-up corner depicts weekly average data.

Panicle and Grain Measurements

The intrinsic physiology of rice panicles implies that many of the measurements covary (Figure 3.3, Table B.9). For example, the SN and the SBN depended on the PBN regardless of treatment (Figure B.6). The slope difference in groups with and without As addition suggests As had positive effects on the SBN and SN ($p < 0.05$). Specifically, these physiological parameters were influenced by As and nCuO individually and interactively ($p < 0.05$) (Table B.9). Generally, high concentrations (50 and 100 mg/L) of nCuO increased TNRP ($p < 0.05$), while As decreased TNRP relative to nCuO treatments alone ($p < 0.05$). The PAL, however, was increased by As in combination with nCuO (1.0–100 mg/L) compared with the same nCuO concentration without As ($p < 0.05$). Similar increases by As and nCuO interaction was observed in the PBN, SBN, SN and TPW.

Filled grains were manually threshed from panicles and the TGW were measured. Arsenic and nCuO exerted main effects on the TGW, and the interaction of As and nCuO was also significant ($p < 0.01$) (Figure 3.4 and Table B.10). Specifically, compared with control, nCuO (10–100 mg/L) increased TGW by 17%–25%, while As alone increased TGW by 13% ($p < 0.05$). With As addition, low nCuO concentrations (0.1 and 1 mg/L) increased TGW by 58% and 19%, respectively, in comparison with the same concentration of nCuO without As addition ($p < 0.05$).

The AGW was influenced by As and nCuO individually and interactively ($p < 0.05$, Figure 3.4 and Table B.10). nCuO at 10 mg/L increased AGW by 4%, whereas As addition to 10 mg/L nCuO treatments decreased the parameter by 8% ($p < 0.05$). Treatments containing As+nCuO 10 mg/L also decreased AGW compared to As alone

treatment ($p < 0.05$). However, in treatments receiving As alone and treatments receiving both As and nCuO (except at 10 mg/L), AGW was increased by 5%−9% compared to the similar nCuO treatments ($p < 0.05$).
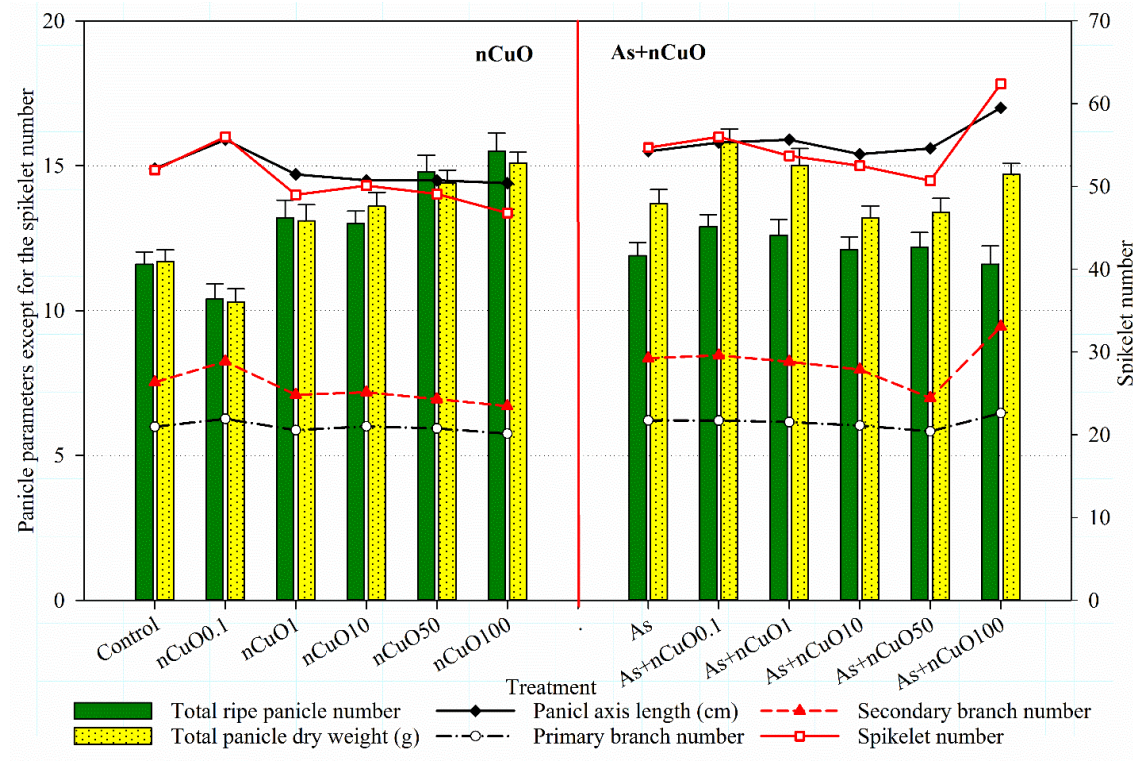
Figure 3.3. Panicle parameters in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 20)

Rice Plant Biomass

Straw DW was increased ($p < 0.05$) by 23%, 24% and 20% compared with the control in the low and two high nCuO treatments (0.1, 50, and 100 mg/L), respectively (Figure 3.5 and Table B.11). Arsenic addition to the 0.1 and 50 mg/L nCuO treatments decreased straw DW by 13% and 27%, respectively ($p < 0.05$). The addition of high nCuO concentration (100 mg/L) to As increased straw DW compared to treatments receiving As alone ($p < 0.05$). Main and interactive effects of As and nCuO were also

observed in mature rice plant root DW ($p < 0.001$). Two relative high concentrations of

nCuO (50 and 100 mg/L) increased root DW by 60% and 43% ($p < 0.05$), respectively,

compared with control. Arsenic decreased root DW compared with corresponding nCuO

concentrations (50 and 100 mg/L) without As by 50% and 19% ($p < 0.05$), respectively.

Grain to Straw Ratio and Relative Growth of Rice Plants

Straw DW is an indicator of rice plant photosynthetic performance [24]. We

employed a ratio of grain to straw dry weights (GSR), similar to the commonly used

harvest index [24]. The GSR of rice crops ranges from 0.5 for traditional tall varieties to 1.0

for improved short varieties. The rice cultivar in our study, *O.sativa japonica*

'Koshihikari', belongs to the short variety. The GSR was affected by As, nCuO and their

interaction ($p < 0.001$) (Figure 3.6 and Table B.11). nCuO decreased the GSR at the low

concentration (0.1 mg/L) and increased the GSR at the mid-concentration (10 mg/L) ($p <$

0.05). Arsenic treatment alone did not influence the GSR. Three As+nCuO treatments

(0.1, 1.0 and 50 mg/L) increased the GSR compared with corresponding nCuO treatments

alone ($p < 0.05$).

The relative growth of root to shoot is also an important indicator of plant growth.

Root and shoot growth normally covary. The RSR of dry weights was used to

characterize the relative growth of rice plants in our current study (Figure 3.6, and Table

B.11). Arsenic and nCuO did not show main effects on the RSR; however, the interaction

of the two substances was significant ($p = 0.004$). Additions of As to 1.0 and 50 mg/L

nCuO treatments decreased the RSR by 20.3% and 31.8%, respectively ($p < 0.05$).
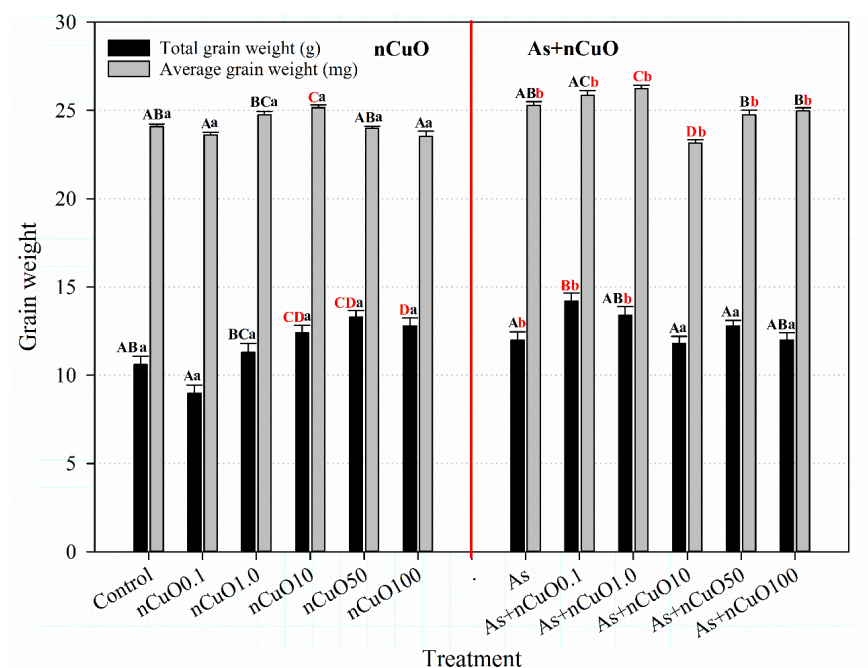
Figure 3.4. Total grain dry weight (g) and average grain weight (mg) in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 20 for total grain dry weight, n = 5 for average grain dry weight). Means of treatments at the same As level with a common superscript letter (A-D) are similar ($p < 0.05$). Means of treatments at the same nCuO level with a common superscript letter (a and b) are similar ($p < 0.05$)
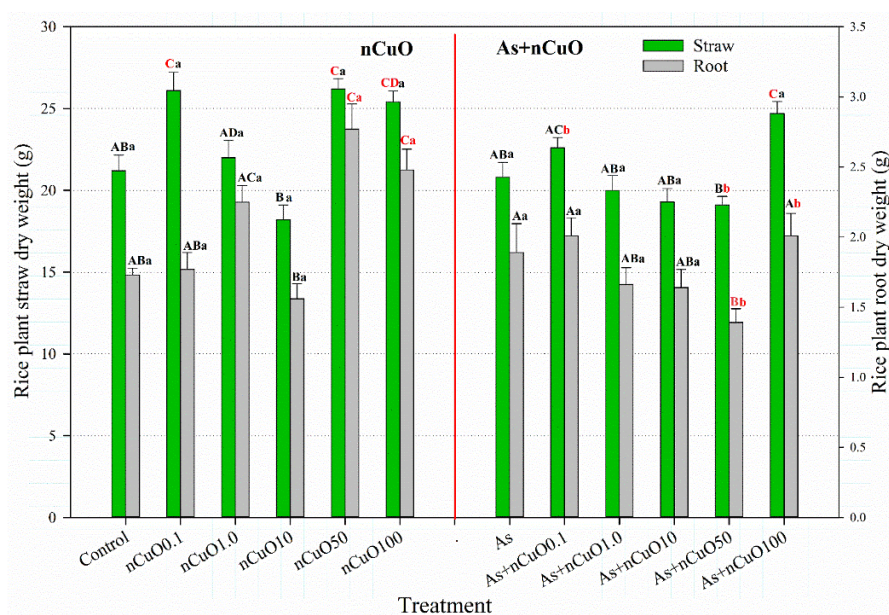
Figure 3.5. Rice straw biomass (g) after panicle harvest in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 20). Means of treatments at the same As level with a common superscript letter (A-D) are similar ($p < 0.05$). Means of treatments at the same nCuO level with a common superscript letter (a and b) are similar ($p < 0.05$)

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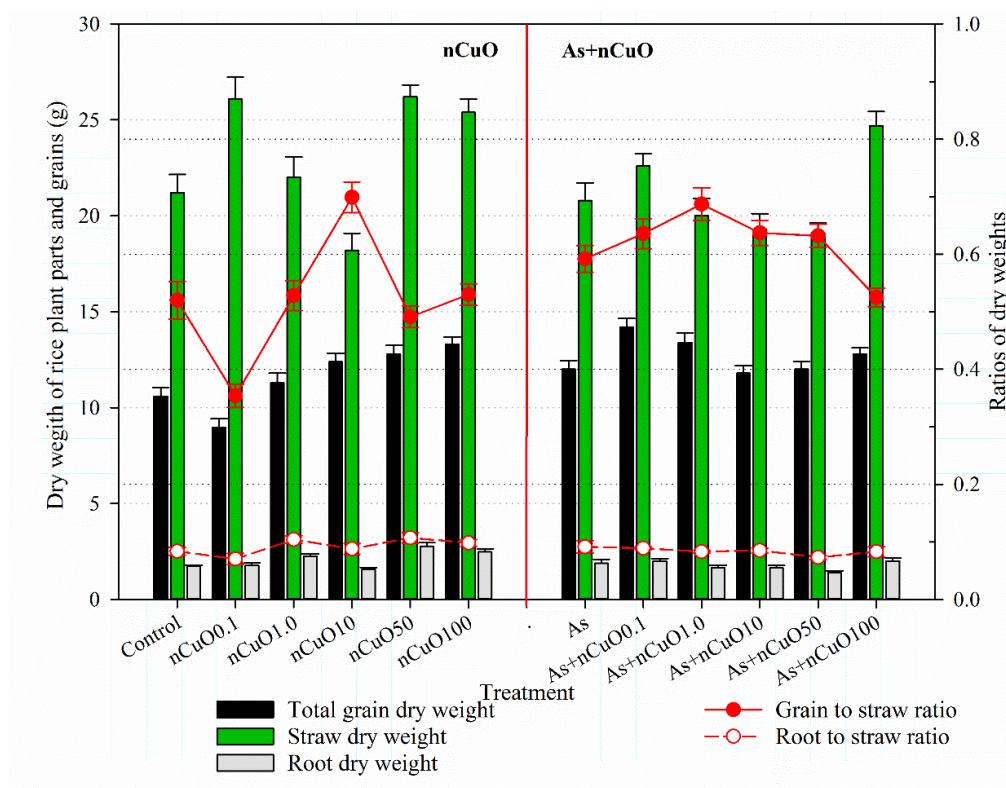
Figure 3.6. Rice plant biomass and grain yield in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n =20)

Accumulation of Copper and Arsenic in Dehusked-grains

Copper accumulation in dehusked-grains was influenced by nCuO and As individually and interactively ($p < 0.05$, Figure 3.7, and Table B.12). With As addition, Cu in dehusked-grains generally increased with nCuO concentration. Compared to the same nCuO concentration without As, Cu in dehusked-grains was decreased by As addition at nCuO 0.1 mg/L, and increased at nCuO 50 mg/L ($p < 0.05$). Arsenic accumulation in dehusked-grains was significantly increased by As and influenced by nCuO and As interaction ($p < 0.05$). nCuO at 50 mg/L decreased As accumulation in dehusked-grains by 35% compared with As alone treatment ($p < 0.05$).
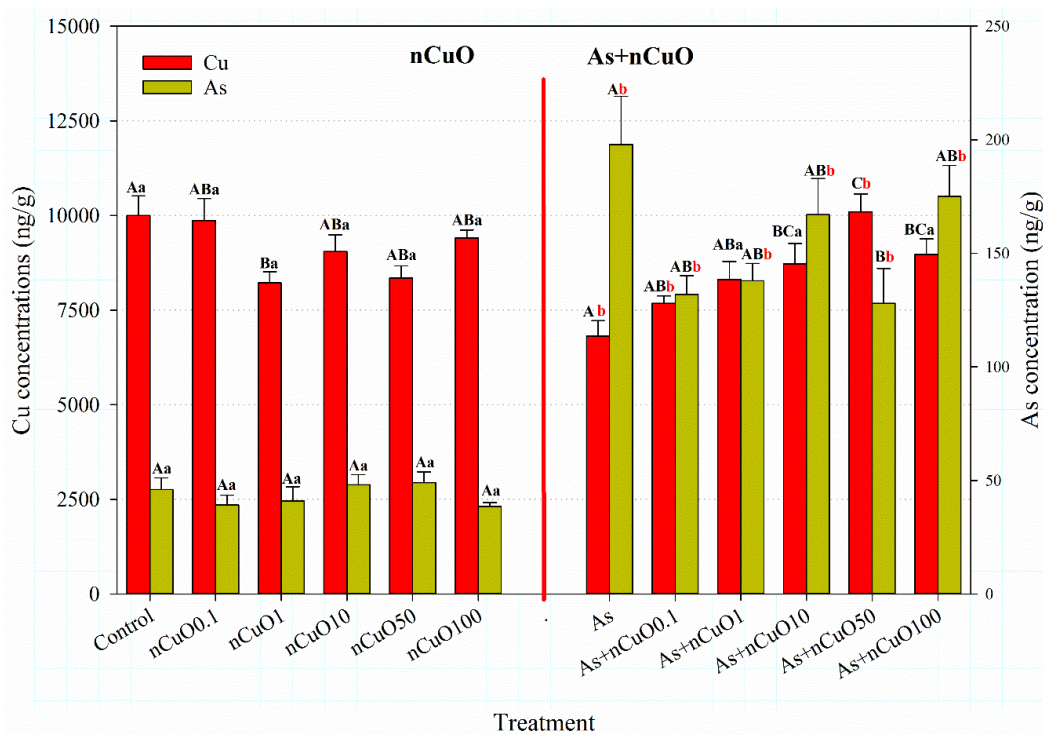
Figure 3.7. Accumulation of copper and arsenic in dehusked rice grains in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 20). Means of treatments at the same As level with a common superscript letter (A-C) are similar ($p < 0.05$). Means of treatments at the same nCuO level with a common superscript letter (a and b) are similar ($p < 0.05$).

Discussion

Effects on Rice Plant Growth

Agronomically, rice plants undergo two distinct growth phases in a regulated and coordinated manner: vegetative (seed germination, early seedling growth and tillering) and reproductive (booting, heading, and ripening) phases [25, 26]. Plant growth is controlled by chemical reactions which are regulated by structural and functional proteins encoded by genes [25], and also influenced by external environmental stresses (e.g., temperature, light, water, and contaminants). Grain yield and straw biomass are normally determined by plant health at all growth stages. Arsenic or nCuO can cause toxicity to rice plants by

several mechanisms. Thus, many parameters may serve as effective endpoints for different growth stages [27, 28].

The reproductive stage (especially booting, heading and flowering) has been postulated as especially sensitive to biotic and abiotic stressors, followed by grain filling and vegetative growth stages [29], which was verified by our study. The heading process was significantly promoted by nCuO treatment ($p < 0.001$), which indicates nCuO may shorten the time-to-harvest of rice plants, thereby decreasing As accumulation in grains. Panicle axis, primary branches, secondary branches and florets are important elements of a panicle [24]. Rice grain production is determined by fertilized grain number, which depends on the PAL, the PBN, the SBN and SN. Although the TNRP was decreased by As in our study, PAL, PBN, SBN, SN per panicle, and the AGW were generally increased in the presence of As. Therefore, rice grain yield or the TGW was not adversely influenced by As. In addition, nCuO had a positive interaction effect with As on most of the panicle parameters. nCuO alone increased the TNRP and TGW in 10–100 mg/L treatments.

As for rice growth in the vegetative stage, early seedling growth conditions play a fundamental role in later growth stages of rice plants. For example, the increased NRB and root DW facilitate plant growth improvement in the later vegetative stage since roots anchor the plant and provide water and nutrients. Whereas, the influence of As and nCuO on mature plants was not as prominent as on early seedlings regarding shoot/straw and root biomass. This was probably due to plant acclimation in the growth media during the life cycle, and the reduced bioavailability of As and Cu resulting from the long-term

interaction (after week 1 and week 9 for As and Cu, respectively) with other components in the media (Table B.5−6).

In addition, straw is usually treated as a by-product of grain production. However, increasing attention has been afforded to straw biomass because of its importance for feeding animals, improving soils, fuel generation, masonry, and paper making [30, 31]. Being genetically stable and easily measured, the GSR is often used to select high yield varieties of rice [32]. The variation of GSR in different treatments in our study may enhance cultivation and selection of high yield rice varieties when grain production is desired. The results may also provide useful information when straw biomass is preferable. Furthermore, in field work, it is unrealistic to acquire an entire root biomass. The RSR in our study provides data that can improve estimates of root biomass and carbon input into the soil based on the above-ground biomass and carbon input models [33].

Behaviors of Arsenic and Copper Oxide Nanoparticles

Arsenic equilibration time with soil before addition of water or seeds allowed soil constituents to sorb or react with As, thereby decreasing the bioavailability and mobility of As [34, 35]. Soil properties (e.g., pH, redox potential, mineral content and microbial activity) also play a fundamental role in controlling long-term As availability to rice plants [36]. The resultant As concentration in water overlying sediments was increased in the first week and decreased thereafter (Table B.6). It was measured up to 0.2 mg/L, much lower than that in hydroponic studies by other research groups, wherein germination was adversely affected by As at 0.5–8.0 mg/L [37]. Moreover, in our previous work, the same As concentration was added to a sand growth media and produced aqueous As concentration of 1.4–7.0 mg/ L as well as decreased rice seed germination [38].

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Aggregation and dissolution of nCuO were also influenced by test media properties and by nCuO concentration. For example, at 50 mg/L, nCuO aggregation played a dominant role in solutions in the first week after addition to the growth container. The concomitant precipitation of nCuO decreased Cu concentration in solution (Table B.5). Dissolution dominated the behavior of nCuO and increased Cu in the solution by the end of week 3. In contrast, at low nCuO concentration (0.1–1.0 mg/L), dissolution dominated nCuO behavior in solutions in the first week, and nCuO aggregation was more prominent and decreased Cu concentration in solutions by the end of week 3. nCuO dissolution is influenced by interaction with ligands (e.g., EDTA) in the growth media. However, high organic matter content in the grow media may have facilitated nCuO sorption and decreased nCuO dissolution and Cu bioavailability [39, 40]. Moreover, research by other groups showed that high ionic strength influenced size distribution and dissolution of nCuO by regulating surface charge of NPs [39]. Particularly, the presence of phosphate may enhance nCuO sedimentation, which reduced nCuO concentration in solutions [39]. As a result, no effect was observed in seed germination by nCuO. However, once roots emerged from the seed, growth medium conditions influenced plant growth. Arsenate was taken up by plants as a phosphate analog and exerted adverse effects such as preventing the formation of high energy phosphoryl bonds or influencing catalytic functions by binding sulfhydryl groups after being reduced to As(III) [3, 4, 41]. Research by other groups showed that As (> 10 mg/kg) decreased the photosynthetic performance of rice leaves by decreasing the chlorophyll content [28], thus decreasing the biomass. This supports our findings of decreased seedling biomass in As containing treatments, where As was more accumulated in seedling roots than in soil and solution (Table B.4, 6, and

13). Research by other groups also showed that nCuO, once entering the plant, can cause phytotoxicity by generating reactive oxidative species (ROS), damaging DNA, or inhibiting gene expressions [e.g., rice plant D-type cyclins (OsCYCD2) and cell-division cycle (OsCDC) genes] [42-44].

Moreover, As (III and V) was effectively adsorbed onto nCuO between pH 6 and 10 [12], which covered the post-treatment pH of soils in our study (7.4–7.9). However, high concentrations of phosphate (> 6.2 mg/L phosphorus), which were observed in our study, may also have enhanced As desorption from nCuO or other sorption surfaces due to the competition for adsorption sites[12]. In addition, organic and inorganic root exudates (e.g., low molecular organic acids such as citrate and malate, and CO_2) also changed the environment (e.g., pH, Eh, organic matter content, CEC) in the rhizosphere [39, 45]. Thus, the interaction of As with nCuO was more complex in our study. The increase in shoot biomass that we observed at high nCuO concentrations (50 and 100 mg/L) was possibly due to the adequate uptake of Cu in the shoot (17.3 and 18.3 mg/kg), which is within the range of 15–26 mg/kg found by other research groups [46]. For example, organic matter decreases the Cu bioavailability, while polyfunctional acids in plant exudates increase Cu bioavailability [40, 45]. According to our previous 72-h aggregation and dissolution experiment [38], decreased seedling biomass caused by 0.1 mg/L nCuO, was probably because nCuO equilibrated at a smaller size after the aggregation dominated phase (48 h) and these smaller nCuO particles interacted with plants.

Intrinsic Regulatory Mechanism

Overall, roots are more sensitive than shoots to environmental stressors because roots are the dominant location for hormone (e.g., cytokinins) generation [47]. Roots not

only anchor the plant, assure supplies of water, minerals and nutrients, but also influence hormonal messages in shoots. Roots can change the output of root hormones or precursors (e.g. cytokinins or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid) to shoots or act as sinks for shoot produced phloem-mobile hormones [e.g. abscisic acid (ABA)] [48].

Effects on root growth can be explained by inter-regulated intrinsic and extrinsic response pathways. External stress (e.g., As) can influence hormone messages in roots and shoots by altering gene expressions that regulate phytohormone biosynthesis, inactivation, and signaling [49]. In our study, RBN was increased by nCuO and decreased by As, indicating that both chemicals may have differentially influenced processes that regulate root cell division and branching process, which needs further study.

Human Health Implication of Copper and Arsenic Accumulation in Dehusked-grains

Dehusked rice grains, the edible part for humans, present an exposure pathway for As to potentially affect human health. In our study, As accumulation in dehusked-grains decreased at all test nCuO+As treatments compared with As alone treatment (Table B.12). The lowest As accumulation in the dehusked-grains among all As treatments was 128 ± 15.4 ng/g, which was observed in the As+nCuO 50 mg/L treatment and was 36% lower than the WHO maximum safe concentration of As in white rice (200 ng/g) for humans [50]. Whereas, the confidence interval of As in dehusked-grains for As alone treatment included 200 ng/g (Table B.12). A 60-kg person with a daily ingestion of 200 g of rice containing 200 ng/g As would experience an As exposure rate at 0.67 µg/kg/day. This is lower than the no-observed-adverse-effect level (NOAEL, 0.80 µg/kg/day) for human cardiovascular effects (ischemic heart disease, mortality), hepatic effects

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(increased serum alkaline phosphatase and bilirubin), dermal effects (hyperkeratosis and hyperpigmentation), and intelligence performance [51]. In contrast, rice containing 128 ng/g As translates to an exposure of 0.43 µg/kg/day, which approximates the NOAEL (0.40 µg/kg/day) for gastrointestinal effects (gastrointestinal irritation, diarrhea, nausea), dermal effects (pigmentation changes with hyperkeratosis). The Minimal Risk Level for effects (other than cancer) is also set at 0.4 µg/kg/day [51]. Therefore, 128 µg/kg/day can lower As exposure and avoid known human effects as reported by US ATSDR for humans who consume at least 200 g of rice per day [51].

Furthermore, although As inhibited nCuO induced increases in panicle emergence, the negative correlation between Cu and As accumulation in dehusked-grains indicated an antiport process of the two elements by rice grains, of which the mechanism is yet unclear.

Our study is the first study to evaluate the interaction of As and nCuO on the effects during the life cycle of rice plants [52]. By combining nCuO and As into one study and working in silt loam growth media containing As at concentrations near the average As concentration in soil from Texas, USA and lower than previously reported experiments, we have extended the knowledge of rice sensitivity to commonly encountered elements in the environments. nCuO not only contains the essential element Cu which can serve as an antimicrobial agent for rice plant, nCuO also has the potential to decrease As bioavailability These are promising characteristics for application in agriculture. More studies are underway to better understand nCuO and As accumulation and speciation in rice plants and grains, which may facilitate existing work to address possible influences of nCuO and As on food safety and the nutritional quality of rice.

Declaration of Interest Statement

The authors report no competing financial interest, and are responsible for the publication.

Supporting Information Available (Appendix B)

Additional text describing fertilizer application, soil property characterization and statistical analysis; 6 figures showing the conceptual model of treatment preparation and exposure process, record of light intensity, temperature and humidity, images with SEM and TEM of grain husk and nanoparticles, seed germination percentages, and relationships between rice panicle parameters; 13 tables showing Hoagland's solution composition, nCuO characterization, Cu and As concentrations in the growth media, and physiological parameters of 18-d rice seedlings and mature rice plants, uptake of Cu and As in seedling shoots and roots, and accumulation of Cu and As in dehusked-grains. This information is available free of charge via the Internet at http://pubs.acs.org.

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CHAPTER FOUR

Distribution and Speciation of Copper and Arsenic in Rice Plants (*O.sativa japonica* 'Koshihikari') Treated with Copper Oxide Nanoparticles and Arsenic during a Life Cycle

Abstract

A 6×2 factorial study was conducted to investigate the effects of copper oxide nanoparticles (nCuO, 0.1–100 mg/L), arsenic (As, 0–10 mg/kg) and their interaction on uptake, distribution and speciation of Cu and As in rice plants (*Oryza sativa japonica* 'Koshihikari'). Arsenic (in As-addition treatments) and Cu in seedling roots (SRs) were 1.45 and 1.58 times of those in soil, respectively. Arsenic and Cu concentrations further increased in mature plant roots (MRs), which were 2.06 and 2.35times of those in soil, respectively. Arsenic and Cu concentrations in seedling shoots (SSs) were 79% and 54% lower than in SRs, respectively. The mature stems, however, contained only 3% and 44% of As and Cu in SSs. Copper in flag leaves did not vary much compared with stems, while As was 14.5 times of that in stems. Species transformations of Cu and As were observed in rice including reductions of Cu (II) to Cu (I) and As (V) to As (III). Arsenic in dehusked-grains was negatively correlated with Cu and was lowered by nCuO below the WHO (World Health Organization) maximum safe concentration for white rice (200 ng/g). This may alleviate As adverse effects on humans from rice consumption.

Introduction

High arsenic (As) concentrations in the environment pose direct and indirect adverse effects on humans. Rice (*Oryza sativa*) plant growth throughout the life cycle has been adversely affected by As [1-4]. Seed germination and biomass of rice plants has been shown to be decreased by As, and the nutritional quality of rice grains have been lowered due to As accumulation [1-4]. Rice grains containing high As concentrations provide a chronic As exposure pathway for humans. Therefore, regulations or guidelines have been developed for acceptable As concentrations in rice. The WHO (World Health Organization) proposed maximum safe concentrations of inorganic As at 0.2 mg/kg for white rice and 0.35 mg/kg for brown rice [5, 6]. The US Food and Drug Administration (FDA) proposed an action level (or limit) for inorganic As in infant rice cereals at 0.1 mg/kg [7]. Studies have been conducted to reduce As accumulation in rice grains by adding soil amendments, water management (e.g., irrigation and drainage), and genetic manipulation [8-12].

Nanotechnology has revolutionized agriculture in the last decade to increase crop yield and improve food quality [13]. Nanomaterials (NMs) can be introduced in plant growth systems as nanofertilizers, nanopesticides, and nanobiosensors [13]. Copper oxide nanoparticles (nCuO) may serve as nanofertilizers by providing important micronutrients for plants, as well as nanopesticides due to antimicrobial properties [14]. Moreover, nCuO has been studied to remove As from water systems [15, 16]. All of these functions allow nCuO to improve rice plant growth, reduce As uptake into rice plants, and diminish As

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accumulation in the grains. In addition to the intentional applications, the unintentional discharge of nCuO is likely to increase due to emerging applications of its unique properties and high sustainability (wide-range industrial suitability, 100% recyclability, abundant availability, and effective antimicrobial properties) [14]. Although some studies have evaluated effects of NMs or As on the life cycle of rice plants, no studies have been done that describe the interaction of nCuO and As during the life cycle growth of rice plants [17,18-20]. The uptake, transport, and speciation of NMs in plants are also dependent on species and growth medium conditions.

We conducted a factorial experiment to study effects of As (0 and 10 mg/kg soil) and nCuO (0–100 mg/L) individually and in combination on rice plants during the life cycle (from seed germination to seed maturation). Several hypotheses were investigated: nCuO and As interact to influence the uptake of As and Cu by rice plants during the life cycle; nCuO and As interact to affect the distribution and speciation of As and Cu in different parts of rice plants during the life cycle; and the accumulation of Cu and As in rice grains are influenced by nCuO and As interaction in the growth media.

Methods

Life Cycle Exposure in the Greenhouse

Arsenic in the form of $Na_2HAsO_4 \cdot 7H_2O$ [21, 22] (Sigma-Aldrich, lot # BCBM0939V) was added at 0 and 10 mg/kg (near average As concentration in Texas, US [23]) to an artificial soil mixture (60% Grainger clay soil, Catalog # 2258, 40% Lowe's topsoil, # 235384). nCuO (Nano-Arc®, 97.5%, 23–37 nm, APS powder, Alfa Aesar, MA, USA) was prepared at six concentrations (0, 0.1, 1.0, 10, 50, 100 mg/L) in 20% Hoagland's solution. Twelve treatments (2×6 combinations of the nCuO and As) were

studied including one control (without either As or nCuO), one As-only treatment, and 20

replicate growth containers (Berry Plastics ID: T60785CP, 2.5 L) were prepared for each

treatment. Ten seeds (*Oryza sativa japonica* 'Koshihikari', Kitazawa Seed Company,

CA, USA) were wet-sown in each container after adding nCuO-containing solutions.

Seedlings were thinned on day 18 and two well-established seedlings were kept for a

131-d life cycle in the greenhouse. Materials and procedural details are extensively

presented in the Supporting Information (Appendix C), and were published in our

previous article [24].

Uptake, Distribution and Speciation of Arsenic and Copper Oxide Nanoparticles

Early seedlings shoots (SSs) and roots (SRs), mature plant roots (MRs), stems,

flag leaves and their paired grains (husk and dehusked-grains) (Figure C.4) were

collected and digested before being analyzed for total As and Cu with inductively

coupled plasma mass spectrometry (ICP-MS, Agilent 7900) (Appendix C).

Micro-XRF maps and X-Ray Absorption Near Edge Structure (XANES) with

spectral acquisition near the Cu K-edge (8979 eV) and the As K-edge (11867 eV) were

collected for samples (seedlings and grains) and reference standards at Beamline 13-ID-E

in Argonne National Laboratory. Copper reference materials were: nCuO, CuO,

$CuSO_4 \cdot 5H_2O$, $Cu_3(PO_4)_2$, cupric acetate, cuprous acetate, cupric oxalate, and synthesized

cupric arsenate. Arsenic reference materials were: As_2O_3, As_2S_3, AsS, As_2O_5,

schneiderhonite ($Fe_4As_5O_{13}$), scorodite ($FeAsO_4 \cdot 2H_2O$), dimethylarsinic acid (DMA),

methylarsinic acid (MAA), and synthesized cupric arsenate. Details about sample

preparation and data acquisition are included in the Appendix C [25].

XRF mapping data were processed with Larch software [26]. XANES data were

processed with Athena (Demeter 0.9.26) [27] and analyzed with linear combination fitting

method. R factor and reduced χ^2 were criteria for determining the best fit.

Statistical Analyses

Generalized linear models (GLMs) were used to analyze the main and interactive

effects of nCuO and As in growth media on their uptake into rice. Data distributions were

defined in the model. Models were validated and accepted with low residual

heterogeneity, followed by the analysis of variance (ANOVA) to study the individual

main effects of nCuO and As and their interaction. Data were expressed as means ±

S.E.M (standard error of measurement). Means were considered different when $p < 0.05$.

All p values were adjusted with Tukey method. With the exception of spectral

deconvolution, all statistical analyses were performed in R (version 3.3.2) [28].

Results and Discussion

Primary effects of nCuO and As and their interaction on major morphological and

physiological parameters of rice plants during a life cycle growth has been discussed

previously [24]. Arsenic mainly inhibited seedling growth, and nCuO generally mitigated

As adverse effects. Arsenic decelerated panicle heading process, while nCuO accelerated

this process. Both As and nCuO increased grain yield in the studied concentration range.

Total Cu and As uptake, transport and speciation inside plants were also shown to be

dependent on treatments

Uptake and Distribution of Copper and Arsenic in Rice Plants

18-d Seedlings. Copper was highly concentrated in rice SRs and was influenced by nCuO concentration, As addition, and the interaction of As and nCuO ($p < 0.05$) (Figure 4.1, Figure C.7, Table C.5). Copper concentration in SRs exponentially increased with that in soil in nCuO alone treatment and tended to reach a plateau (Figure 4.1), while Cu in SRs linearly increased with that in soil in As+nCuO treatments. In contrast, Cu in SSs increased in an exponential manner with Cu concentration in soil and plateaued in both treatment groups. Compared with control, Cu concentrations in SRs decreased at nCuO 10 mg/L but increased at nCuO 50 and 100 mg/L (Figure C.7, Table C.5). Arsenic-only treatment decreased Cu in SRs compared with control. Arsenic addition to nCuO at 50 mg/L also decreased Cu in SRs compared with nCuO alone treatment at 50 mg/L ($p < 0.05$). Arsenic addition increased Cu in SRs at a medium and a high nCuO concentration (10 and 100 mg/L) ($p < 0.05$). Copper in SSs was much lower than in SRs and soils ($p < 0.001$), and was influenced by nCuO, As and the interaction of As and nCuO ($p < 0.001$) (Figure 4.1, Figure C.7, Table C.2, Table C.5). Arsenic alone or addition to nCuO at low concentrations (0.1 and 1 mg/L) decreased Cu concentration in SSs, while As addition to nCuO at 100 mg/L increased Cu in SSs ($p < 0.05$).

Arsenic concentrations in SRs and SSs in treatments without As addition were lower than those in soil, but As in SRs in As-addition treatments was accumulated to concentrations higher than those in soil ($p < 0.001$) (Figure C.7, Table C.2, Table C.7). Arsenic in SRs was influenced by As addition, nCuO concentration, and the interaction of As and nCuO ($p < 0.001$). Arsenic addition significantly increased As uptake by SRs, but As interacting with nCuO at 50 mg/L decreased As concentration in SRs compared to

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the As-only treatment ($p < 0.05$). Arsenic in SSs was lower than that in SRs and soils ($p < 0.001$) and was influenced by As and the interaction between As and nCuO ($p < 0.001$), but not influenced by nCuO ($p = 0.894$).
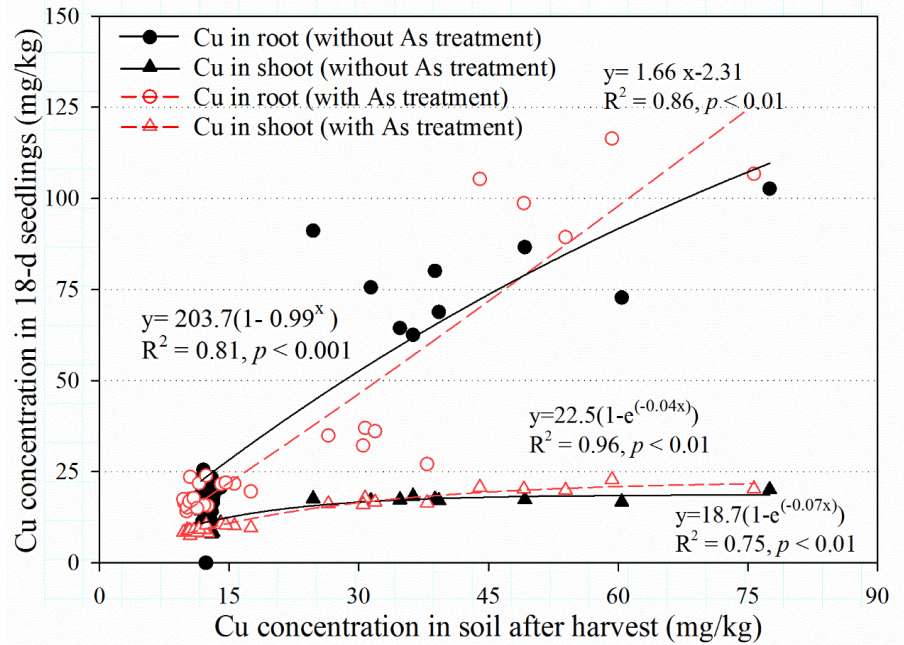
Figure 4.1. Copper concentrations in the seedlings collected on day 18 from a greenhouse study of rice (O.sativa japonica 'Koshihikari') with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5)

Mature Plant Roots and Stems. Copper concentrations in MRs were higher than in soil, generally increased with nCuO concentration, and influenced by CuO and As interaction ($p < 0.05$) (Figure 4.2, Table C.2, Table C.6). Compared with treatments receiving nCuO alone, As addition to nCuO 10 mg/L treatment decreased Cu accumulation in MRs, while As addition to nCuO 50 mg/L treatment increased Cu accumulation. Overall, Cu in MRs was linearly correlated with Cu in soil from nCuO alone treatments, while in As+nCuO treatment, Cu in MRs was quadratically correlated with Cu in soil (Figure 4.3). Arsenic was highly concentrated in MRs compared to that in

soil, and was mainly increased by As addition ($p < 0.001$). Overall, Cu and As concentrations increased in MRs compared with SRs.

Copper and As concentrations in stems were only 44% and 3% of those in SSs, respectively, and they were only 15% and less than 1% of those in MRs, respectively ($p < 0.001$) (Figure 4.2, Table C.6, Figure C.8). However, Cu concentration in stems was not influenced by As, nCuO, and their interaction. Arsenic concentration in stems was increased by As addition in nCuO 10 and 100 mg/L treatments, relative to control ($p < 0.001$).

Flag leaves and grains. Grains were paired with the flag leaf from the same tiller of a given rice plant and were separated as husks and dehusked-grains. Flag leaves and grains were collected at two time points (Day 120, and Day 131) and were analyzed for Cu and As concentrations (Figure 4.4, Table C.7-8). Flag leaves contained elevated Cu and As concentrations at both time points compared with those in stems ($p < 0.001$). Copper and As concentrations in flag leaves and dehusked-grains were generally higher on Day 131 than on Day 120 ($p < 0.001$), so was As in husks. Conversely, Cu in husks decreased in most treatments on Day 131 compared to Day 120. Cu and As concentrations on Day 131 represented the final measurements in plants (Figure 4.4, Table C.8). Final Cu concentration in flag leaves was influenced by nCuO and As interaction ($p = 0.047$), and was lowest in the As+nCuO100 mg/L treatment. Final Cu in dehusked-grains was generally higher than that in flag leaves, while Cu in flag leaves was higher than that in husks. In addition, in nCuO treatment alone, final Cu concentration in husks was decreased at 10, 50 and 100 mg/L nCuO concentrations, compared with

control ($p < 0.05$). In treatments of As+nCuO, Cu in husks increased with nCuO compared with As-only treatment ($p < 0.05$). Particularly, Cu in husks in As+nCuO 50 mg/L was higher than nCuO 50 mg/L alone treatment ($p < 0.05$). Moreover, the final Cu concentration in dehusked-grains was influenced by As and the interaction between As and nCuO ($p < 0.05$). Compared to the same nCuO concentration without As, Cu in dehusked-grains was decreased by As addition at nCuO 0.1 mg/L, and increased at nCuO 50 mg/L (Figure 4.4, Table C.8). Generally, Cu in dehusked-grains increased in a linear function of Cu concentration in husks (Figure C.9).

Unlike Cu distribution, final As in dehusked-grains was lower than that in husks, which was lower than that in flag leaves (Figure 4.4, Table C.8). Arsenic in dehusked-grains was linearly correlated with those in flag leaves and in husks (Figure C.10). Final As concentrations in flag leaves were influenced by As, nCuO and their interaction ($p < 0.05$). Arsenic addition increased As accumulation in flag leaves, while nCuO addition at 0.1 mg/L decrease As accumulation compared to As-only treatment ($p < 0.05$). Final As concentration in husks was also influenced by nCuO, As, and their interaction ($p < 0.05$). Arsenic in husks was significantly increased by As. nCuO addition at 0.1 and 1.0 mg/L decreased As accumulation in husks compared with the As-only treatment ($p < 0.05$). Final As concentrations in dehusked-grains were increased by As and influenced by the interaction of nCuO and As ($p < 0.05$). nCuO at 50 mg/L decreased As accumulation in dehusked-grains by 35% compared with As-only treatment ($p < 0.05$). Particularly, As concentration in husks was negatively correlated with Cu concentration in husks, and As concentration in dehusked-grains followed a similar negative correlation dependency on Cu concentration in dehusked-grains (Figure 4.5).

Total Cu and As distributions in different parts of mature rice plants were depicted in all treatments (Figure C.11-12). Although accumulating in flag leaves or grains, Cu and As concentrations generally decreased in the above-ground parts of rice plants as compared to below-ground parts.

Copper concentrations generally followed the order:

a) nCuO treatment: root > dehusked-grain > flag leaf > husk ~ stem; b) As+nCuO treatment: root > dehusked-grain > flag leaf > stem ~ husk.

Arsenic concentrations generally followed the order:

a) nCuO treatment: root > flag leaf > stem > husk > dehusked-grain; b) As+nCuO treatment: root > flag leaf > husk > dehusked-grain > stem.

Uptake Mechanisms of Copper and Arsenic by Rice Plants. Being an essential micronutrient, Cu was taken into the plant from roots via specific Cu transporters (e.g., COPT) [29]. Adequate Cu uptake in rice plants was within the range of 15–26 mg/kg found by other researchers [30]. This explains the rapid Cu uptake (16–21.2 mg/kg) in SRs at nCuO < 10 mg/L in our study (Table C.5). When excess Cu is provided, plants employ complex homeostatic regulations to address the dual nature of Cu as being essential and toxic by assuring Cu delivery to cuproproteins and inhibiting excess Cu uptake (e.g., by sequestering excess metal with polypeptides) [29, 31]. Research by other groups showed that the TCP16 from TCP (teosinte branched 1, cycloidea and proliferating cell factor 1) family of transcription factors significantly down-regulated the expression of Cu intracellular transporter (COPT3) in A. thaliana with hydroponic exposure to Cu^{2+} (635 µg/L) [32]. This concentration is lower than the highest Cu concentration in solutions from

in our study (Table C.3). Therefore, total Cu uptake in SRs in treatments without As addition tended to reach relatively high maximum concentrations (Figure 4.1). Meanwhile, As addition to the growth media disrupted normal homeostatic Cu regulation [22]. Therefore, in treatments with As addition, Cu in SRs increased linearly with Cu in soil. However, Cu uptake in MRs followed different patterns compared with 18-d SRs in our study. This was probably because Cu and As were transported to other parts when the plant grew and the biomass increased [33, 34].

Unlike Cu, As is not required for plant health, but As species can enter plants as analogues of other essential elements. As(V) enters plant roots through phosphate transporters (e.g., OsPT4) [35]. As(III) is taken up by rice roots through silicic acid transporters (Nodulin 26-like intrinsic membrane proteins, such as Lsi1) [20]. Arsenic is more available for rice plants compared to other crop plants, because As bioavailability is relatively high in water than in other environmental compartments [36]. Overall, As uptake was differentiated at various growth stages (Table C.5-8). High phosphate concentrations (> 6.2 mg/L phosphorus), which were observed in our study, may also have enhanced As desorption from soil mineral particles due to the competition for adsorption sites [16]. However, a study by other groups demonstrated that As (V) and As(III) were both effectively adsorbed onto nCuO between pH 6–10, with little competition from other anions (e.g., phosphate, silica and sulfate) [15, 16]. The post-treatment pH of soils in our study was covered in this range (7.4–7.9) [24]. Our previous study also demonstrated that As concentration in the Hoagland solution decreased over time in the presence of nCuO [37]. In addition, microbes in the growth media and root exudates (e,g., organic acid) to the rhizosphere also interacted with As and nCuO, thus influencing their bioavailability and

uptake to rice plants [38]. Soil type also significantly influences Cu and As availability [39, 40].

Our previous study with sand as the growth media showed that Cu concentrations in 18-d

SRs and SSs were about 12 and 3 times greater, respectively, than those in our current

study [37]. While As concentrations in 18-d SRs and SSs grown in sand were 70 and 54

times greater, respectively, than those in our current study.
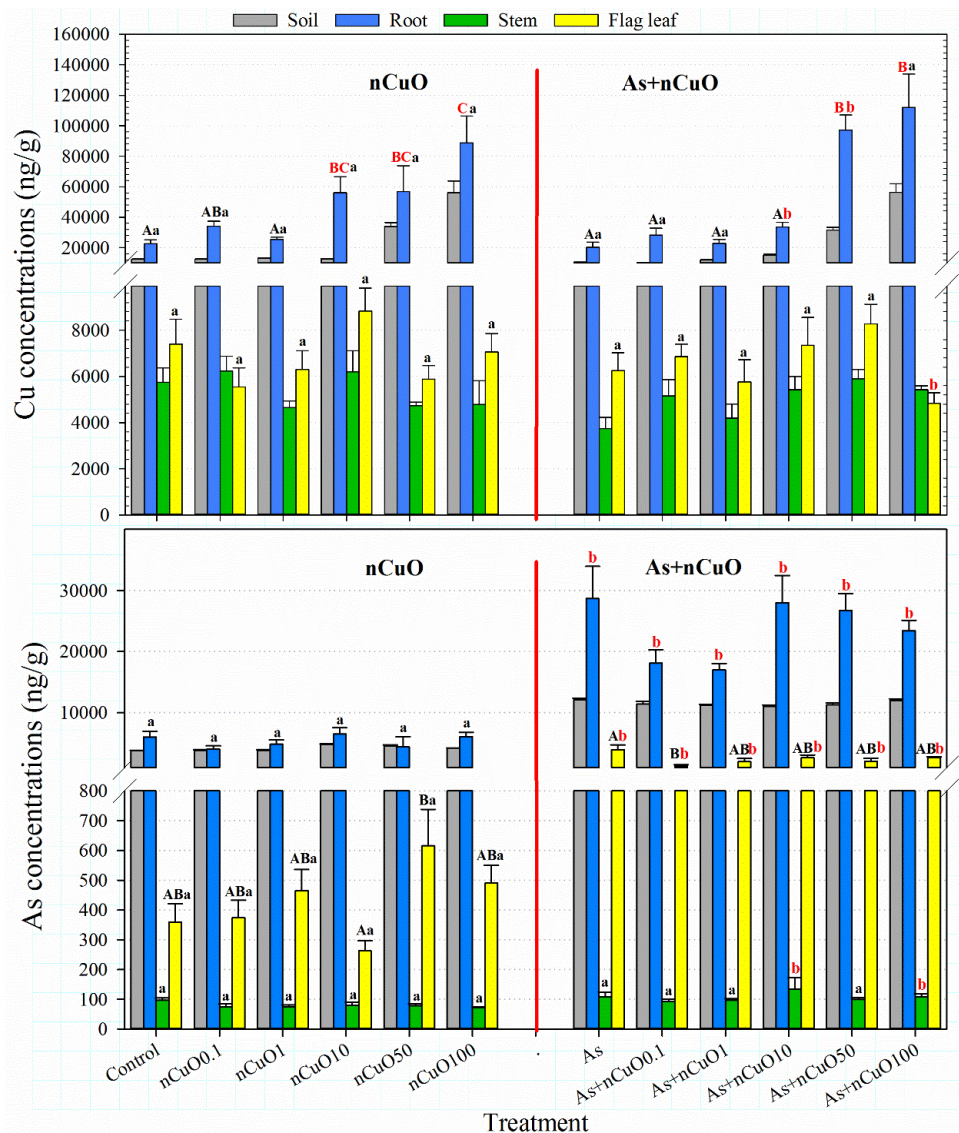
Figure 4.2. Copper and arsenic concentrations in the mature rice plants from a greenhouse study of rice (O.sativa japonica 'Koshihikari') with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5)
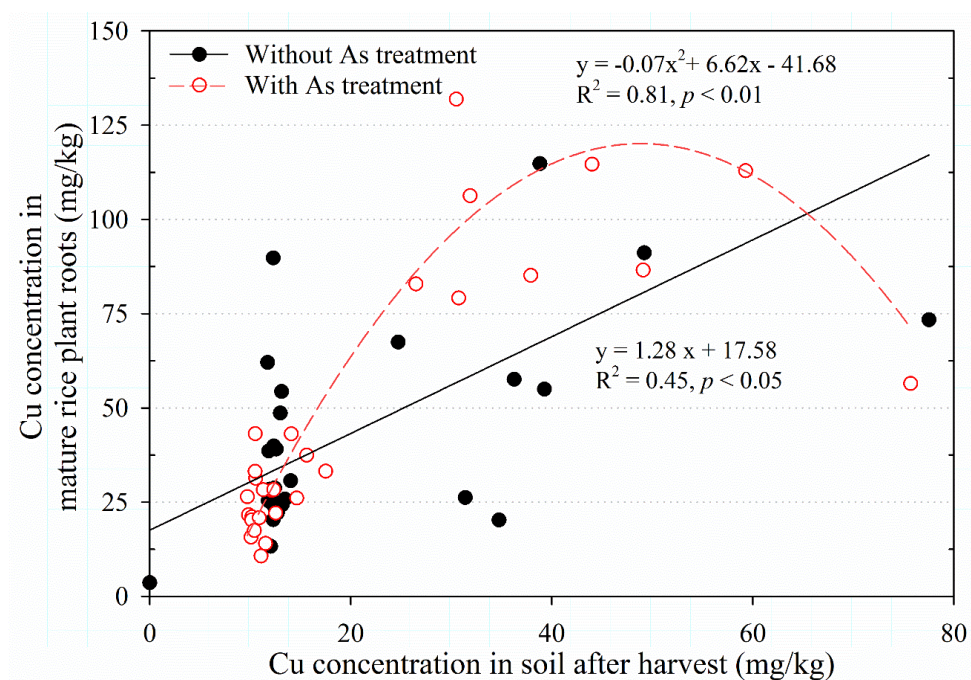
Figure 4.3. Copper concentration in the mature rice plant roots from a greenhouse study of rice (O.sativa japonica 'Koshihikari') with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5)
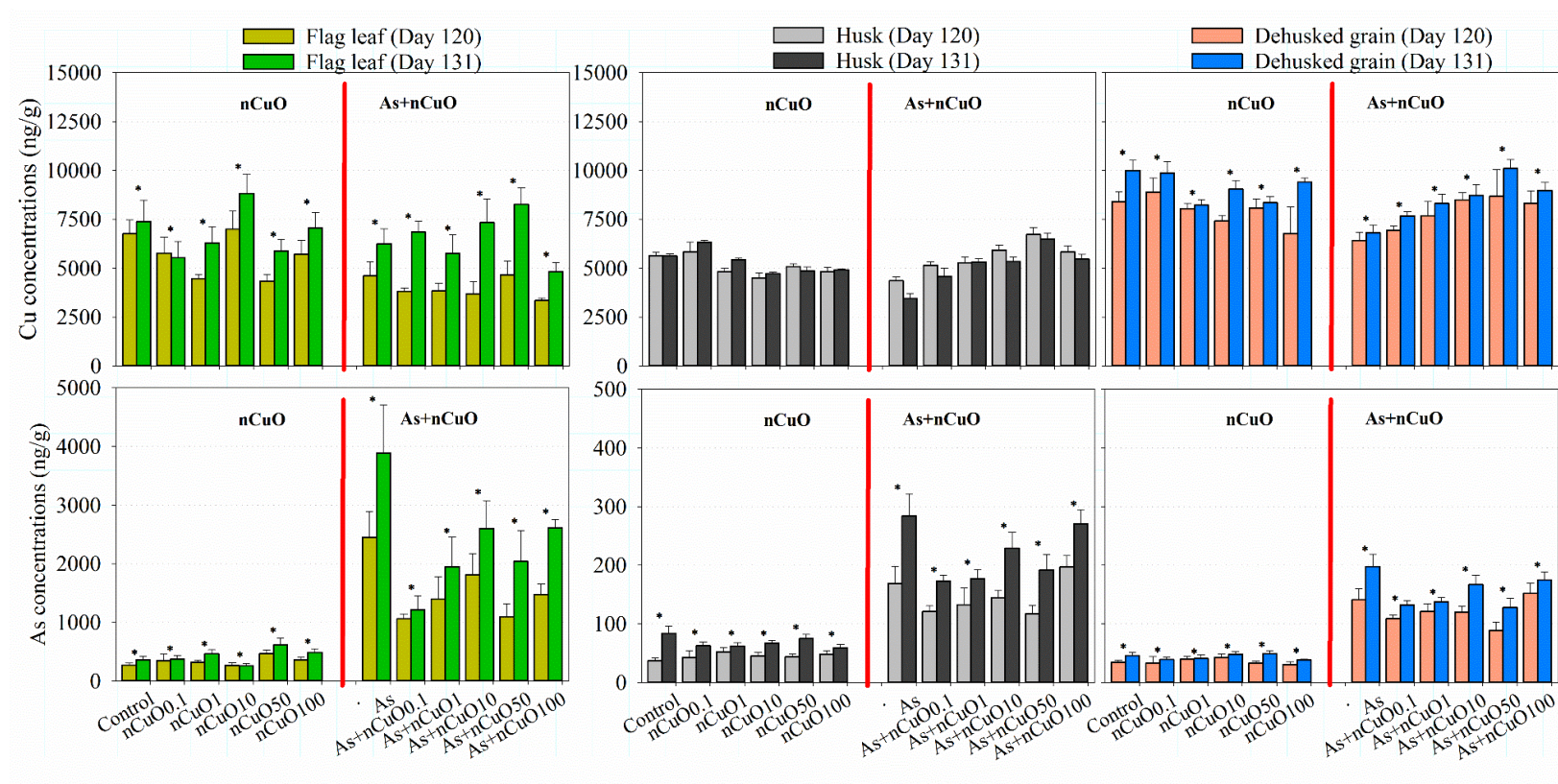
Figure 4.4. Comparison of copper and arsenic concentrations in different parts of rice plants at two time points (Day 120 and Day 131, 2017) from a greenhouse study of rice (O.sativa japonica 'Koshihikari') with 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5). * means of the same treatment at the two time points are different ($p < 0.05$)
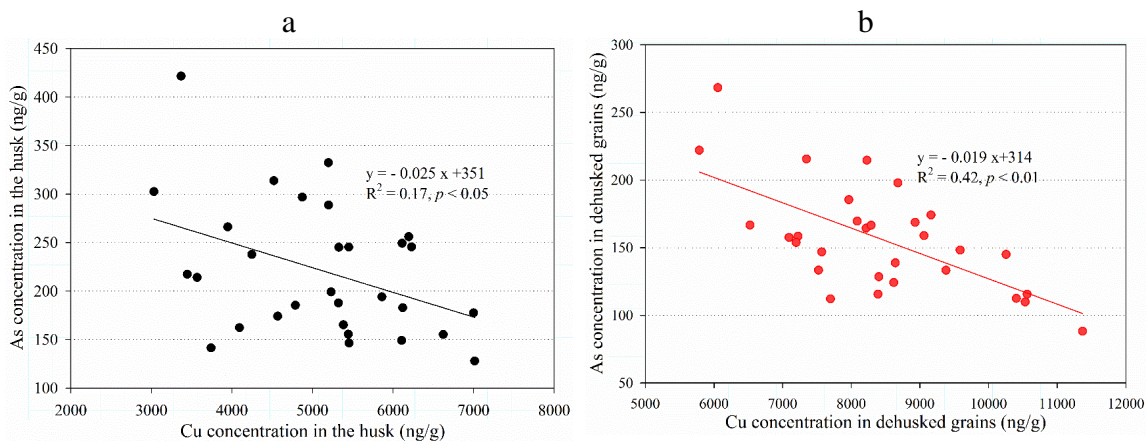
Figure 4.5. Relationships between copper and arsenic concentrations in the rice grain husk (a) and dehusked grains (b) in treatments from a greenhouse study of rice (*O.sativa japonica* 'Koshihikari') with 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5)

Transport and Speciation of Copper and Arsenic in Rice Plants

The Cu XANES spectra of bulk CuO and nCuO were indistinguishable as the concentration we measured in plants [41]. Thus we used "CuO" for the speciation in our samples. Spectral deconvolution of Cu XANES showed that Cu species in seedlings mainly included cupric acetate, cupric oxalate, CuO, and cuprous acetate (Figure 4.6, Table 4.1). Arsenic species in seedlings mainly included As_2S_3, As_2O_5, scorodite, dimethylarsinic acid, and methylarsonic acid (Figure 4.6, Table 4.2). Copper oxide was observed in all samples, and the dominant species in SSs from nCuO 100 mg/L treatment. Cupric oxalate was observed in control and As+nCuO100 mg/L treatment, and the percentage in SSs increased than in SRs from the same treatment. Cupric acetate was the dominant species observed in SRs from control and in SSs from As+nCuO100 mg/L treatment. Cuprous acetate was the main species observed in SRs from treatments receiving nCuO 100 mg/L.

Copper oxide nanoparticles undergoes a series of transformations (e.g., aggregation, sedimentation, dissolution and speciation) in the environment. Ultimately, ionic Cu is the dominant species taken up by rice plants via Cu transporters, but nCuO could also enter plant roots via several pathways, and several barriers (e.g., mucilage, cuticle and cell wall, blockage of the pores of cell walls due to NP aggregation) hinder the process [42]. Possible subcellular NP uptake pathways across the barriers were summarized in a review article, including passive diffusion through permeable region of cuticle and cell wall pores and facilitated transport by natural organic matter (e.g., humic acids and root exudates) and microorganisms (e.g., algae, bacteria, and fungi) [42]. Once across the barriers, NPs penetrated cell membranes by binding to the receptor and subsequent fluid-phase endocytosis (FPE), by passive diffusion or by physically damaging the membrane [42]. After entering the root, NPs could travel from root to xylem via apoplastic and symplastic routes, and then be translocated to other locations of the plants by xylem and phloem. This was verified by other research groups that nCuO was transported from roots to shoots via xylem and back translocated from shoots to roots via phloem in Maize (*Zea mays L.*) [43]. Based on this research, the observed CuO in SRs and SSs in our study (Table 4.1) could be NPs [43]. This previous research also observed that Cu(II) was reduced to Cu(I), which was consistent with our result that cuprous acetate content increased in SRs from nCuO treatment compared with control (Table 4.1). nCuO itself cannot be utilized by plants but may affect plant growth by interacting with other components (e.g., As). Although dissociated Cu ions from nCuO can be used as micronutrient, nCuO dissolution inside the plants has not been well understood. Moreover, during plant growth and development, different root exudates were secreted

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and influenced the behavior of nCuO in the rhizosphere and further uptake of Cu ion and nCuO [38]. Concurrently, As and other components in the growth media also exerted dynamic influence on Cu speciation.

Dominant As species in SRs and SSs varied among treatments (Figure 4.6, Table 4.2). Arsenic concentrations in SRs and SSs were relatively low, therefore there were more non-detectable species. DMA was dominant in SRs from control and SSs from As-only treatment. Scorodite was dominant in SRs from As and As+nCuO treatments, and SSs from As+nCuO treatment. As_2S_3 was observed in all samples except in SSs from As-only treatment, while As_2O_5 were observed in both SSs and SRs from As-only treatment, and in SSs from As+nCuO treatment. Methylarsonic acid (MAA) was only observed in SSs from As-only treatment. for the literature suggests that the higher energy peak (11872 eV) in the spectra of SSs samples from As-only treatment is very similar to synthetic As(III)-(GLU)$_3$ standard [44]. This confirms to our understanding of the physiological mechanism of plants interacting with As [31].

In our study, As(V) was the original test species added in the soil. However, As(V) could easily be reduced to As(III) under submerged rice cultivation in anaerobic or microaerobic soil and desorbed from the soil particles due to the lower sorption capacity of As(III) than As(V) [45]. As(III) could also be oxidized to As(V) on the surface of nCuO [16]. Once assimilated by the plant, As(V) could be relocated to the xylem and phloem by phosphate transporters such as OsPT1 and OsPT4 [35, 46], while As(III) is transported from root towards stele through efflux transporter (Lis2) for silicic acid [20]. Given the shared transport pathway in the xylem and phloem, As and nCuO could interact with each other

inside the plants. Arsenic speciation inside the plants was dependent on the locations in

the plant, which was also affected by nCuO in our study.
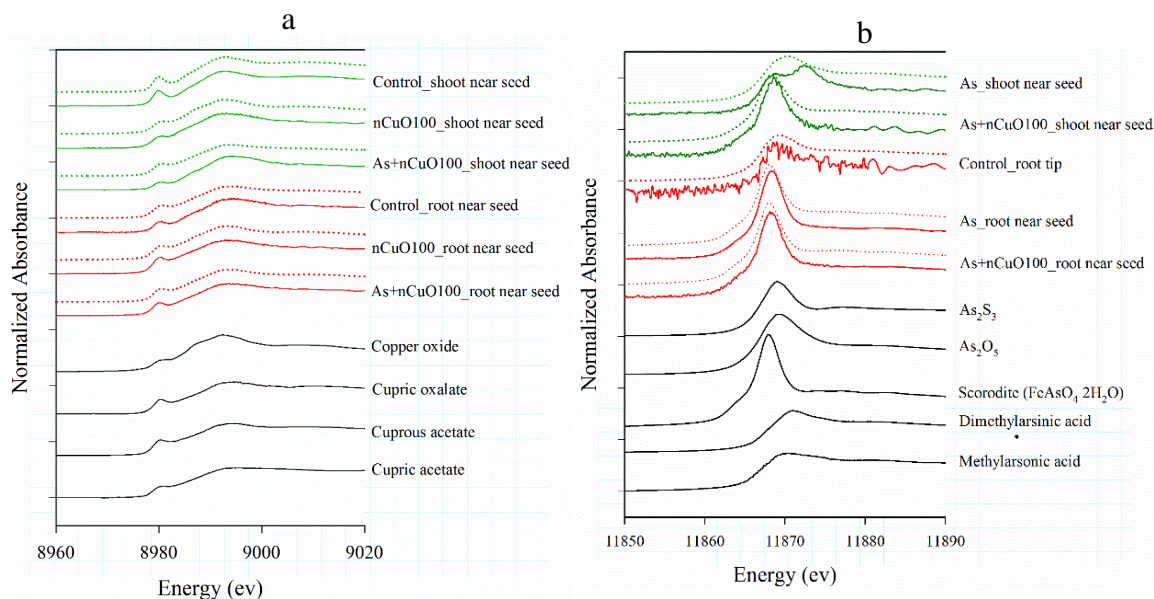
Figure 4.6. Copper (a) and arsenic (b) K-edge XANES spectra of reference standards and 18-d rice seedling root near seed and shoot near seed samples in control and treatments of nCuO100, and As+nCuO100 from a greenhouse study of rice (*O.sativa japonica* 'Koshihikari') with 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution . Solid lines are spectra for the samples, and dashed lines are linear combination fitting results for the sample.

Table 4.1. Copper speciation based on copper K edge XANES spectra of 18-d seedling root and shoot samples in control and treatments of nCuO and As+nCuO100 from a greenhouse study of rice (*O.sativa japonica* 'Koshihikari') with 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution. R factor, the residual sum of squares; ND=not detectable; Reduced $\chi 2$, a weighted sum of squared deviations. All standards were evaluated but some were omitted from the table for not present.

Samples	Fitting Criteria		CuO	Cupric oxalate	Cupric acetate	Cuprous acetate	Fraction Sum
	R factor	Reduced $\chi 2$	Fraction %	Fraction %	Fraction %	Fraction %	
Control_root near seed	0.011	0.002	16.6	41.2	42.2	ND	100
nCuO100_root near seed	0.005	0.001	39.7	ND	ND	60.3	100
As+nCuO100_root near seed	0.003	0.001	22.25	23.1	26.05	28.6	100
Control_shoot near seed	0.007	0.002	28.3	71.7	ND	ND	100
nCuO100_shoot near seed	0.007	0.002	58.05	ND	10.25	31.7	100
As+nCuO100_shoot near seed	0.027	0.006	15.73	25.57	58.7	ND	100

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Table 4.2. Arsenic speciation based on arsenic K edge XANES spectra of 18-d seedling root and shoot samples in control and treatments of nCuO and As+nCuO100 from a greenhouse study of rice (*O.sativa japonica* 'Koshihikari') with 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution. R factor, the residual sum of squares; ND=not detectable; Reduced $\chi 2$, a weighted sum of squared deviations. All standards were evaluated but some were omitted from the table for not present.

Samples	Fitting Criteria		As_2S_3	As_2O_5	Scorodite ($FeAsO_4$ •$2H_2O$)	Dimethyl-arsinic Acid [DMA, As(V)]	Methyl-arsonic acid [MAA, As(V)]	Fraction Sum
	R-factor	Reduced $\chi 2$	Fraction %	Fraction %	Fraction %	Fraction %	Fraction %	
Control_root tip	0.05	0.02	45.10	ND	8.90	46.00	ND	100
As_root near seed	0.02	0.02	18.10	10.77	71.13	ND	ND	100
As+nCuO100_ root near seed	0.01	0.01	23.80	ND	76.20	ND	ND	100
As_shoot near seed	0.05	0.02	ND	26.13	5.20	51.53	17.13	100
As+nCuO100_ shoot near seed	0.03	0.02	36.20	20.25	43.55	ND	ND	100

Agriculture and Human Health Implication

Rice straw is an alternative of fertilizers used on-site in rice paddies, because nutrients in rice straw can be released to the field after straw decomposition for future rice cultivation [47]. Meanwhile, As can also be remobilized from the straw residual and be available for rice growing in the next generation. In a previous study by other groups [47], the released As from incubated mixture of rice straw (5 g, 1.1 ± 0.34 mg As/kg dry weight) and soil (800 g, 9.49 mg As /kg dry weight) in 500 mL H_2O was up to 1.3 % (~ 200 µg/L in pore water). Based on this percentage, As concentration from a mixture with the same relative ratio of straw, soil and water would be estimated up to 240 µg/L in our study, which is much lower than previously reported As concentration (500–8000 µg /L) in hydroponic studies that adversely affected rice seed germination and early seedling growth [21]. Therefore, rice straw in Texas can be applied directly in the field as fertilizers.

Copper and As concentrations in grains were related with those in flag leaves (Figure C11–12). The flag leaf of a tiller emerges as the last leaf, and it contributes most to the grain filling process by transporting photosynthetic products into the spikelet [48]. Relatively high concentrations of Cu and As in flag leaves as compared to stems also implied that flag leaves were an important pathway to transport As and Cu from the lower parts of plants to grains. Studies by other research groups showed that a phosphate transporter probably facilitated As mobilization from flag leaves to panicles, and a C-type ATP-binding cassette transporter (OsABCC1) was also shown to limit As transport to grains [35, 49]. In our study, As in the dehusked-grain was lower than in husks, while Cu in the dehusked-grain was higher than in husks. Moreover, there was a negative correlation between Cu and As in dehusked-grains. These relationships implied an antiport process of Cu and As in the grain. However, it is unclear about the role of flag leaves during the process. Foliar application of NP suspension may be more efficient if Cu ion and nCuO can be absorbed and transported through flag leaves to grains and antiport As from grains simultaneously by interacting with the related transporters, which needs further study. In our study, As in dehusked-grains decreased at all nCuO+As treatments compared with As-only treatment (Table C8). The lowest As accumulation in dehusked-grains among all As-addition treatments (128 ± 15.4 ng/g) was observed in As+nCuO 50 mg/L treatment. It was 36% lower than the WHO maximum safe concentration of As in white rice (200 ng/g) for humans [50]. Rice containing 128 ng/g As converts to an exposure (for a 60-kg person consuming 200 g rice daily) of 0.43 µg/kg/day, approximating the Minimal Risk Level for effects (other than cancer), which was set at 0.4 µg/kg/day [51]. Therefore, 128 µg/kg/day can reduce As exposure and avoid the reported human effects by US Agency

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for Toxic Substances and Disease Registry (ATSDR) for humans who consume at least 200 g rice per day [51].

Arsenic contamination is a big issue globally and causes rice yield reduction and food safety concerns. Thus, As remediation has become essential for healthy human life of the increasing population worldwide. Although gene technology is efficient to lower As transport to the grains [52, 53], rice yield may be decreased due to limited uptake of other nutrients. The nutrient value may also be influenced consequently. Moreover, the safety of genetically modified food is still a big controversy. Among a wide variety of NMs, Cu-based NMs exhibit great potential to remediate As-contaminated environment, and simultaneously release the micronutrient and antimicrobial agent Cu to improve plant growth. Therefore, nCuO provides an alternative to alleviate As phytotoxicity in rice plants and reduce As accumulation in rice grains.

Declaration of Interest Statement

The authors report no conflicts of interest, and are responsible for the publication.

Supporting Information (Appendix C)

Additional text providing the materials and procedural details of the study; 12 figures showing the environment condition in the greenhouse, the characterization of nCuO, a flag leaf paired with a panicle, portions of seedlings for XANES analysis, the elemental components of synthesized cupric arsenate, Cu and As concentrations in 18-d seedlings, depicted Cu and As distribution in rice seedlings, and relationships of Cu and As concentrations in different parts of the plants; 7 tables demonstrating soil characteristics, concentrations of Cu and As in the growth media and plants, and Cu speciation in dehusked rice grains.

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CHAPTER FIVE

Exposure to Copper Oxide Nanoparticles and Arsenic Causes Intergenerational Effects
on Rice (*Oryza sativa japonica* 'Koshihikari') Seed Germination and Seedling Growth

Abstract

An 18-d F1 rice (*Oryza sativa japonica* 'Koshihikari') seed germination and

seedling growth test was conducted to investigate intergenerational effects of arsenic (As)

and copper oxide nanoparticles (nCuO), with seeds harvested from a life cycle study

exposed to As (0, 10 mg/kg) and nCuO (0, 0.1, 1.0, 10, 50, 100 mg/L). F1 seed

germination and seedling growth were influenced by treatments experienced by F0 plants

($p < 0.05$). Seeds produced from plants in F0 treatment with nCuO 50 mg/L had the

lowest germination percentage and shortest seedling shoot length (SL) and root length

(RL), in F1control and F1As alone treatment ($p < 0.05$). The SL and RL were decreased

while the number of root branches (NRB) was increased in F1As treatment compared

with F1control ($p < 0.001$). Interaction of As and nCuO also caused differential seed

germination and seedling growth at various nCuO concentrations in quasi-F0 treatment

(seeds receiving the same exposure as F0 plants) ($p < 0.05$). Copper and As uptake in

F1control seedlings was not affected by seeds' F0 exposure, which indicated the

transgenerational effects on rice seedling growth were not dependent on total Cu or As

uptake in seedlings. The enhanced effects on seedlings from quasi-F0 treatment were

influenced by additional exposure to nCuO and As, which also interacted to affect Cu and As uptake in seedlings.

Keywords: intergenerational effect, copper oxide nanoparticles, arsenic, interaction, rice

1.Introduction

Arsenic (As) uptake in rice plants impairs plant growth and reduces grain production (Rahman et al., 2007; Panaullah et al., 2008; Shri et al., 2009). Arsenic accumulation in rice grains also potentially affects human health, especially infants (Carey et al., 2010; Sohn, 2014; Signes-Pastor et al., 2016). Researchers have sought to treat As in waste, soil, water, and rice paddies for decades (Angency, 2002; Suriyagoda et al., 2018). Different technologies have been used in soil remediation (e.g., solidification / stabilization, soil washing / acid extraction) and water treatment (e.g., precipitation, membrane filtration, adsorption, ion exchange). Particularly in rice cultivation, methods have been developed to reduce As bioavailability and accumulation for rice plants, and on-going studies seek to further decrease As availability (Deng et al., 2018; Suriyagoda et al., 2018). These treatment approaches include irrigation water, fertilizer amendment (biochar, sulfur, silicon and phosphorus), smart farming strategies (intermittent flooding, intercropping with other As-hyperaccumulating plants), and developing new rice varieties with genetic modification. However, most approaches cannot achieve both high production and good quality of rice. Other unwanted environmental side-effects (e.g., eutrophication of nearby water systems) may also occur when excess fertilizers are added.

Nanomaterials (NMs) are promising alternatives to conventional environmental remediation methods (e.g., water purification by removal of heavy metals and other pollutants, removal of pathogenic microorganisms and detoxification of toxic contamination, etc.) (Adeleye et al., 2016; Guerra et al., 2018; Singh, 2018). Compared with traditional methods, NMs are more cost-effective for removing toxicants and pathogens. Applications of NMs in agriculture have been proposed for many purposes: increasing production rate and crop yield, enhancing food quality and safety, increasing resource utilization efficiency, reducing agricultural inputs, and decreasing waste production (Kah and Hofmann, 2014; Prasad et al., 2017; Guerra et al., 2018; Li et al., 2019). Inorganic nanopesticides, following polymer-based formulations, have received increasing attention for crop protection (Kah and Hofmann, 2014). Addition of NMs in soils to remove, stabilize or degrade pollutants from soils has proven feasible in agriculture. Studies have evaluated the application of iron-, manganese-, silicon-containing nanoparticles (NPs) and other nanocomposites to remediate As in soil or to alleviate As phytotoxicity and accumulation in rice plants (Wang et al., 2017; Yu et al., 2017; Li et al., 2019). Copper oxide nanoparticles (nCuO) have been widely used in many industries, including human health sanitation and environmental remediation (Grigore et al., 2016; Singh, 2018). nCuO also has the potential to remediate As contamination in rice paddies (Liu et al., 2018c). However, scarce research has been conducted to characterize the interaction of nCuO and As on rice plants (Liu et al., 2018b). Our previous studies demonstrated that nCuO interacted with As to alter seed germination and seedling growth in different growth media, and also to influence plant growth at different stages and the final As accumulation in rice grains (Liu et al., 2018a;

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Liu et al., 2018c). Specifically, final As accumulation in rice grains was reduced by nCuO application at 50 mg/L (Liu et al., 2018c).

Moreover, external environmental stressors can cause transmissible effects to subsequent generations (Ou et al., 2012; Burton and Metcalfe, 2014). Nevertheless, few studies have investigated the potential effects of nCuO or As on seed germination and plant growth of future generations (Ou et al., 2012). Our current study conducted an F1 rice (*Oryza sativa japonica* 'Koshihikari') seed germination and seedling growth test to investigate the intergenerational (used interchangeably with transgenerational, multigenerational, and cross-generational) effects of nCuO and As from F0 exposure. The hypotheses of the current study were: 1) F1 rice seed germination and seedling growth are influenced by seed source (F0 treatment); 2) F1 rice seed germination and seedling growth are influenced by F1 treatment; 3) the uptake of Cu and As in F1 seedlings is also determined by seed source and F1 treatment.

2.Methods and Materials

2.1.F1 Seed Germination and Seedling Growth Tests

The present study involved two generations of rice plants, which were referred to as F0 generation (parent) and F1 generation (offspring). F1 seeds were harvested from F0 rice plants (*Oryza sativa japonica* 'Koshihikari') that were grown in one of 12 treatments containing As (0, 10 mg/kg) in soil and nCuO (0, 0.1, 1.0, 10, 50, 100 mg/L) that was added on day 1 (Liu et al., 2018c). The F0 study was performed in a greenhouse for 131 days (Liu et al., 2018c).

F1 treatments included: control (F1C), As at 10 mg/kg (F1As), and quasi-F0 treatments (defined as F1 treatments with the same exposures as those of F0 plants

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producing the seeds) combining As and nCuO (F1Cux and F1AsCux, where x refers to 0.1, 1.0, 10, 50 and 100 mg/L). The notation "F1treatment[F0treatment]" refers to the F1treatment of seeds from plants experiencing F0 treatment. For example, the term F1As[F0Cu50] denotes As treatment of F1seeds that had been collected from F0 plants exposed to nCuO at 50 mg/L. Here, we used seed source as a concept referring to different F0 treatments producing seeds. Acid-cleaned sand (Quikrete pool filter sand #1153) was rinsed with DI water, dried in the oven and used as the growth media. Arsenic (in $Na_2HAsO_4 \cdot 7H_2O$) at 10 mg/kg was prepared in sand. Hoagland's solution (20% strength) provided nutrients, and nCuO concentrations in nutrient solution mirrored F0 treatments (0.1, 1.0, 10, 50, 100 mg/L). All 12 types of F1 seeds were germinated in F1C and F1As. Each type of seeds was also germinated in the corresponding Quasi-F0 treatment (F1Cux, and F1AsCux) with the same exposure to As and nCuO as the parent plant producing the seeds. Therefore, 34 treatments were prepared in total (Table D.1).

Each F1 treatment contained ten replicate growth cells, with individual volumes of 88.7 mL and no drainage holes. Each growth cell received 55 g sand, and 4 seeds were sown in each cell. nCuO solutions were only applied on the first day. The Hoagland's solution provided nutrients and maintained the water level afterwards. F1 seed germination and seedling growth tests were conducted in an incubator (VWR diurnal growth incubator, model 2015, Oregon, USA) for 18 d. The photoperiod was set as 16 h light ($25 \pm 1°C$), and 8 h dark (20 ± 1 °C). The humidity in the incubator ranged from 40–90%. Luminance in the incubator was 100 ± 20 µmol/m^2/sec photosynthetically-active radiation, which was measured with a Quantum Meter (BQM, Apogee Instrument Inc., Logan, UT, USA).

2.2.F1 Seed Germination and Seedling Growth Determination

Seed germination was monitored daily in each growth cell. After 18-d growth, entire seedlings were collected for measurement of shoot length (SL), root length (RL), the number of root branches (NRB), and the dry weights (DWs) of shoots and roots of individual seedlings. Method details of these measurements can be found in our previous study (Liu et al., 2018c).

2.3.Total Copper and Arsenic Concentrations in F1 Samples

Dry sand samples (0.5 g) from two randomly chosen growth cells in each treatment were analyzed before sowing seeds and after removing seedlings. Two solution samples (2 mL) for each treatment were obtained every 6 d (Supplemental Data). Dry shoot/root samples (n = 2–6) were grouped into 3 replicates (with approximate dry mass) for each treatment. Sand, dry seedlings and solutions were digested separately, and concentrations of Cu and As in digestates were determined with an Agilent 7900 ICP-MS, operated in helium collision mode (Liu et al., 2018a).

2.4.Statistical Analyses

Generalized linear models (GLMs) and mix effect models (GLMM) were used to analyze the effect of seed sources, nCuO and As on seed germination percentages, 18-d seedling parameters (SL, RL, NRB, DWs), and Cu and As concentrations in seedlings and growth media. Data distributions for the parameters listed above were defined in the corresponding models. Models with low heterogeneity of the residuals were accepted. Analysis of variance (ANOVA) was conducted for the models to study the main effects of type of seeds, nCuO and As, and the interaction effect of seed source, nCuO and As.

All *p* values for comparisons were adjusted with Tukey's method. Comparisons of the

same parameter between two treatments or two generations were analyzed with Student's

t-test. Means were considered significantly different when $p < 0.05$. Relationships

between parameters were analyzed with regression models. We performed all statistical

analyses in R (version 3.3.1).

3.Results

Copper concentration in grains did not differ by nCuO concentrations in the F0Cu

treatment (Table D.2). Whereas, Cu in grains increased at relatively high concentrations

of nCuO (10, 50, and100 mg/L) in F0AsCu treatment. Arsenic concentration in grains

increased with As addition in F0As and F0AsCu treatments, while As in grains from

F0AsCu50 treatment was decreased compared with F0As treatment. The small

percentage of organic matter in sand for F1 treatment would be expected to have

negligible influence on the behavior of As and nCuO relative to organic matter in natural

soils (Table D.3). Copper and As concentrations in sand before sowing seeds and adding

solutions and after seedling removal demonstrate appropriate application of nCuO and As

(Table D.4–5). Concentrations of Cu and As in solutions during the experiment show the

changing bioavailability of Cu and As to rice seedlings over time (Table D.6–8).

3.1.F1 Seed Germination and Seedling Growth

Intergenerational effects were observed in F1 seed germination. F1C[F0Cu50]

and F1As[F0Cu50] had significant lower germination percentages in F1C and F1As

(67.5% and 52.5%, respectively) compared with other F1 seeds (85–100%) ($p < 0.05$)

(Figure 5.1 and Table D.9). However, no significant difference was observed between

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F1C and F1As from the same seed source. In quasi-F0 treatments, F1Cu50[F0Cu50] had lower germination (70%) than others (87.5–100%) ($p < 0.05$). The germination percentage increased by 25% in F1AsCu50[F0AsCu50] compared with F1Cu50[F0Cu50] ($p < 0.05$).

Among F1C, decreases were observed in the SL, RL and NRB in the F1C[F0Cu50] ($p < 0.05$), which were 33.7%, 23.8 and 18.6%, respectively, compared with F1C[F0C] (Figure 5.2 and Table D.10). A similar decrease was observed in SL, RL and NRB in the F1As[F0Cu50] compared with F1As[F0C] ($p < 0.05$) (Figure 5.2 and Table D.11), and percentage decreases in the SL, RL, and NRB were 36.5%, 49.3% and 22.0%, respectively. The SL and RL were decreased by 5%–19.3%, and 51.3–71.0%, respectively, in F1As as compared to F1C with the same seed source ($p < 0.05$).

In quasi-F0 treatments, SL was influenced by nCuO and the interaction of nCuO and As ($p < 0.05$) (Figure 5.2 and Table D.12). High concentrations of nCuO (10–100 mg/L) decreased SL at high concentrations of nCuO (10–100 mg/L) in the F1Cux[F0Cux] treatment. With As addition (i.e., in F1AsCux[F0AsCux]), we saw a decrease in the SL in relatively low nCuO exposures (0.1–10 mg/L), but an increase in the SL at relatively high nCuO exposures (50 and 100 mg/L) in presence of As. A decreased RL was also observed with high concentrations of nCuO (50 and 100 mg/L) in quasi-F0 treatments ($p < 0.05$). The presence of As in F1AsCux[F0AsCux] decreased the RL at all nCuO treatments compared with F1Cux[F0Cux] receiving the same nCuO concentration ($p < 0.05$). The NRB was increased in F1AsCu50[F0AsCu50] compared with F1Cu50[F0Cu50] ($p < 0.05$).

Similar but more prominent effects in DWs were observed in F1C and F1As compared with results of seedling length (Figure 5.3 and Table D.10–11). F1 seedlings in F1C[F0Cu50] and F1As[F0Cu50] showed a significant decrease in shoot and root DWs, respectively, in F1C and F1As ($p < 0.05$). In F1C, decreases in shoot and root DWs in the F1C[F0Cu50] were 40.7% and 42.1% respectively, compared with F1C[F0C] (Figure 5.3 and Table D.10). While in F1As, decreases were 41.5% and 50.3% respectively in shoot and root DWs in F1As[F0Cu50], compared with F1As[F0C] (Figure 5.3 and Table D.11). Compared with F1C, F1As with same seed sources decreased the DWs of both shoots and roots by 0–23.2% and 41.1–63.3%, respectively ($p < 0.05$) (Figure 5.3 and Table D.10–11).

In quasi-F0 treatments, nCuO, As, and their interaction also affected DWs of shoots and roots ($p < 0.05$) (Figure 5.3 and Table D.10). Both shoot and root DWs were decreased at high concentrations of nCuO exposures (50 and 100 mg/L) in F1Cux[F0Cux] ($p < 0.05$). With As presence in F1AsCux[F0AsCux], shoot DW was decreased in relatively low nCuO concentration (0.1 mg/L), and shoot DW was increased at nCuO 50 mg/L ($p < 0.05$). The root DW in all F1AsCux[F0AsCux] was decreased compared to the F1Cux[F0Cux] at the same nCuO concentration alone ($p < 0.05$).
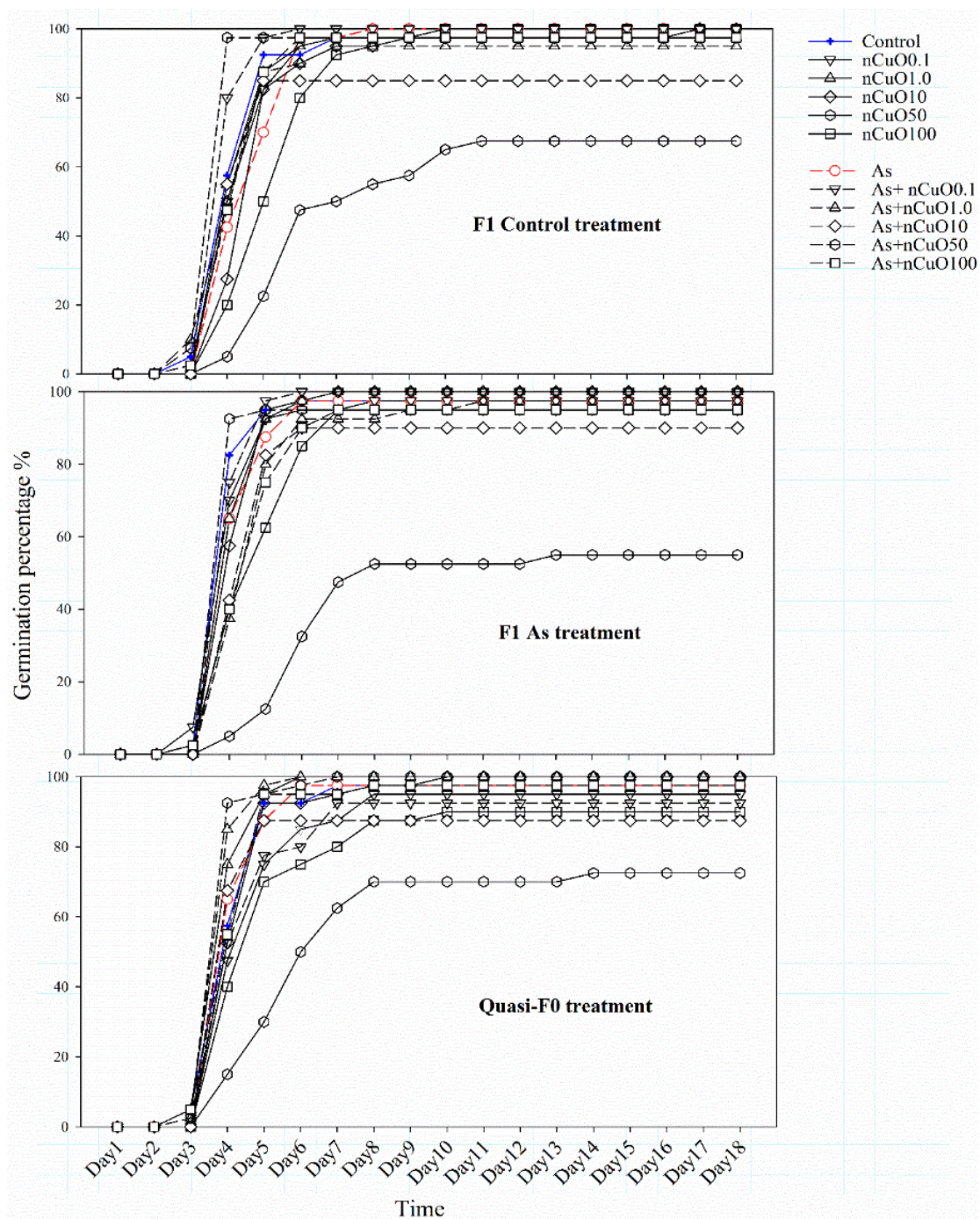
Figure 5.1. F1 seed germination curve in an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in the nutrient solutions (n = 10).
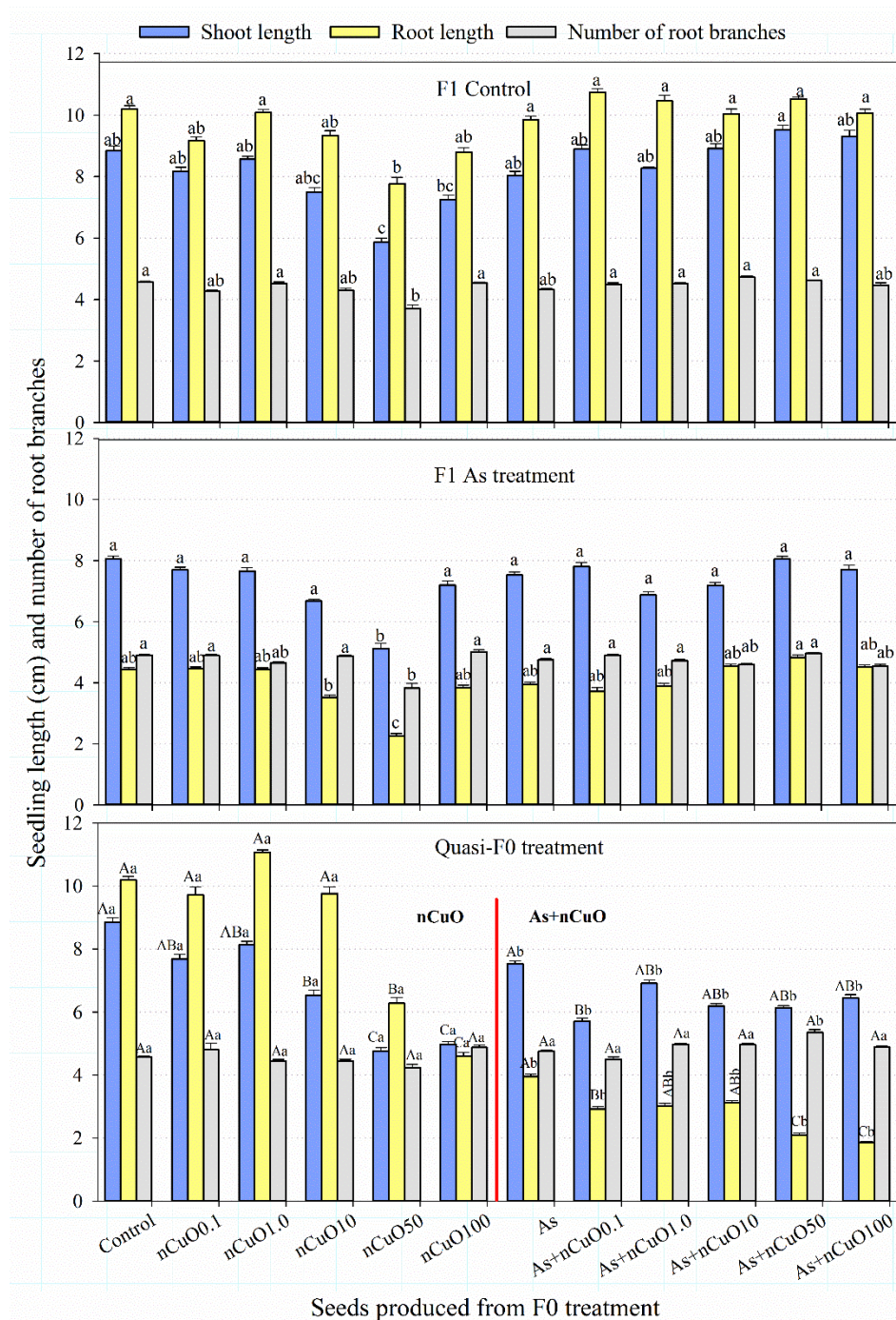
Figure 5.2. 18-d F1 seedling growth parameters (lengths of shoots and roots, and number of root branches) in an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in nutrient solutions (n = 10). In F1 Control and F1 As treatment, means of treatments with a common superscript letter (a-c) are similar ($p < 0.05$). In Quasi-F0 treatment, means of treatments at the same As level with a common superscript letter (A-C) are similar ($p < 0.05$). Means of treatments at the same nCuO level with a common superscript letter (a-b) are similar ($p < 0.05$).
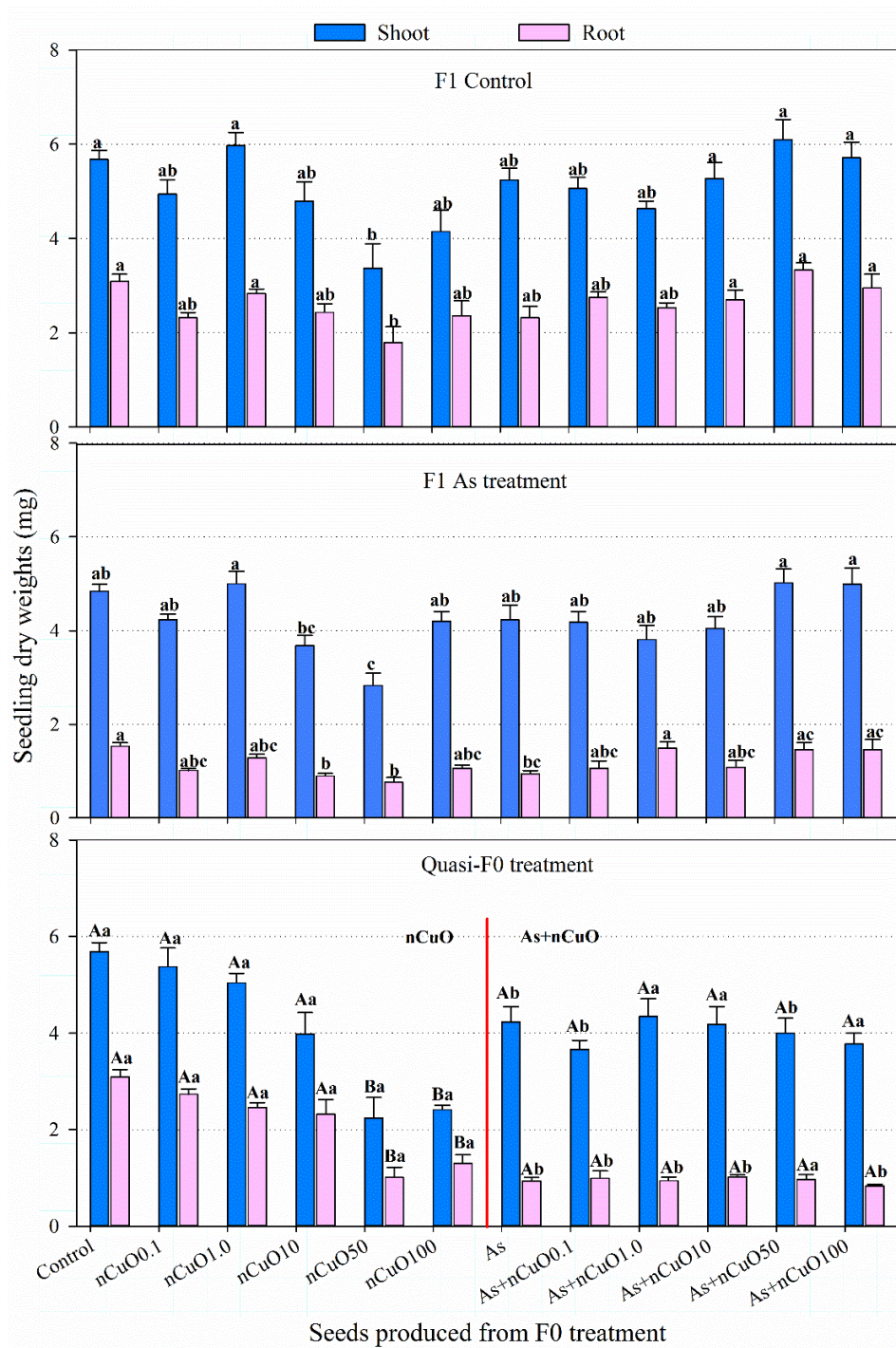
Figure 5.3. 18-d F1 seedling growth parameters (dry weights of shoots and roots) in an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in the nutrient solutions (n = 10). In F1 Control and F1 As treatment, means of treatments with a common superscript letter (a-c) are similar ($p < 0.05$). In Quasi-F0 treatment, means of treatments at the same As level with a common superscript letter (A-B) are similar ($p < 0.05$). Means of treatments at the same nCuO level with a common superscript letter (a-b) are similar ($p < 0.05$).

3.2.Total Copper and Arsenic Uptake in F1 Seedlings

Seed source did not affect Cu uptake in F1 SRs or SSs from F1C ($p = 0.28$ and 0.89, respectively) (Figure D.1 and Table D.13). Arsenic uptake in F1 SRs was not influenced by seed source in F1C ($p = 0.06$). Although seed source significantly influenced As uptake in F1 SSs ($p = 0.02$), no significant comparison was found.

In F1As, Cu uptake in SRs was affected by seed source ($p < 0.001$) (Figure D.2 and Table D.14). Copper uptake in SRs in F1As[F0Cu50] was 10% higher than F1As[F0C] ($p < 0.05$), while Cu uptake in F1 SSs was similar in F1As regardless of the seed source ($p = 0.66$). Arsenic uptakes in F1 SRs and SSs were both dependent on seed source ($p < 0.001$) in F1As. However, no difference in As uptake by SRs was observed in F1As from other seed sources compared with F1As[F0C]. Arsenic in SSs in F1As[F0AsCu50] was 28% lower than F1As[F0C] ($p < 0.05$). Copper uptake in F1 SRs was affected by nCuO concentration and the interaction between nCuO and As in quasi-F0 treatments ($p < 0.001$) (Figure 5.4, Table D.15), while Cu uptake in SSs was affected by nCuO concentration, As addition, and the interaction between nCuO and As in quasi-F0 treatments ($p < 0.01$). Copper uptake in SRs and SSs was increased with nCuO concentration in F1Cux[F0Cux] ($p < 0.05$) (Figure 5.4, Table D.15), and closely correlated with Cu concentrations in sand after harvesting the F1 seedlings (Figure 5.5). In addition, F1 seedling SL and RL were negatively correlated with Cu concentration in sand after F1 seedling removal ($p < 0.01$) (Figure 5.6). Arsenic presence in F1AsCux[F0AsCux] at the lowest and relative high concentrations (0.1 and 50 mg/L) decreased Cu concentration in F1 SRs. F1AsCux[F0AsCux] at high nCuO concentrations (50 and 100 mg/L) decreased Cu concentration in F1 SSs.

Relatively low concentrations of As were observed in F1Cu*x*[F0Cu*x*] at low nCuO concentrations (0.1 and 1.0 mg/L) compared with F1C[F0C] in F1C, while As presence in F1AsCu*x*[F0AsCu*x*] increased As uptake in both SRs and SSs ($p < 0.05$).
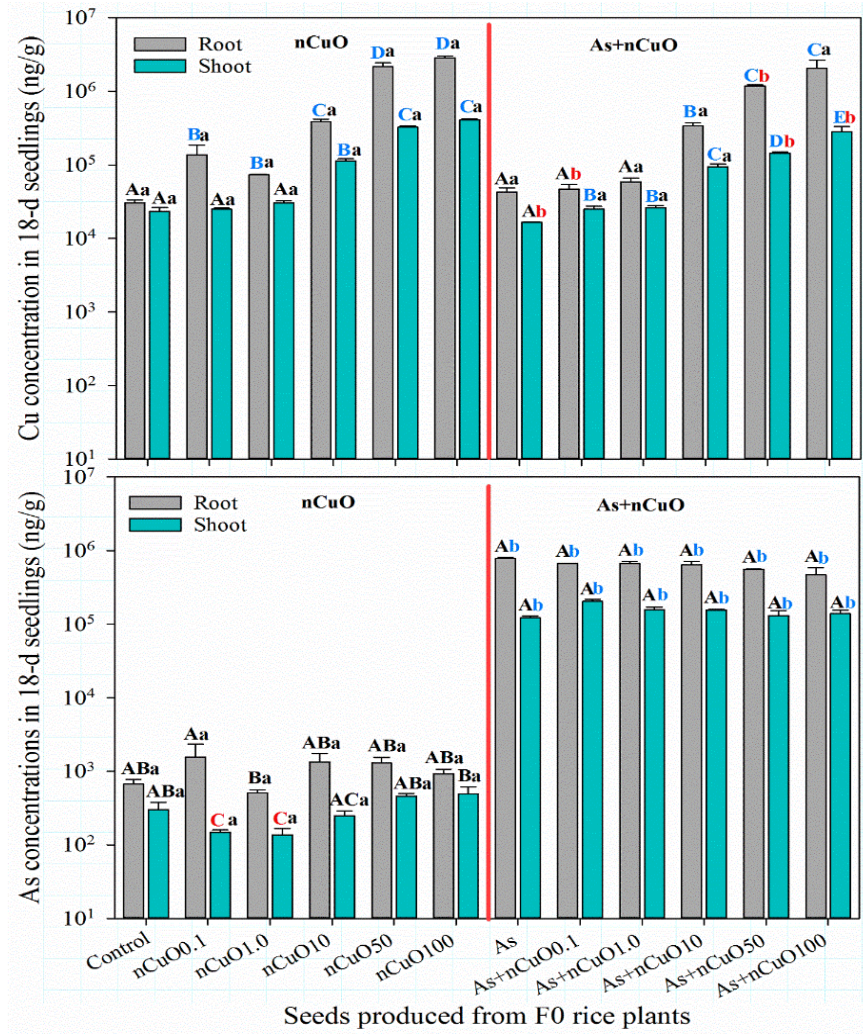
Figure 5.4. Copper and arsenic concentrations in F1 seedlings in quasi-F0 treatment from an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in the nutrient solutions (n = 10). Means of treatments at the same As level with a common superscript letter (A-D) are similar ($p < 0.05$). Means of treatments at the same nCuO level with a common superscript letter (a-b) are similar ($p < 0.05$).
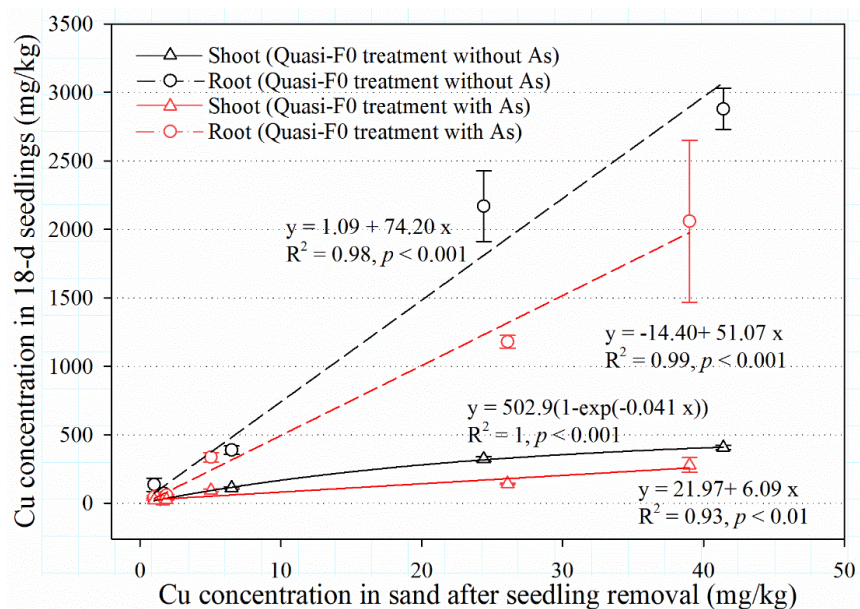
Figure 5.5. Relationship between copper concentrations in 18-d seedlings and that in sand in quasi-F0 treatment from an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in the nutrient solutions (n = 10).
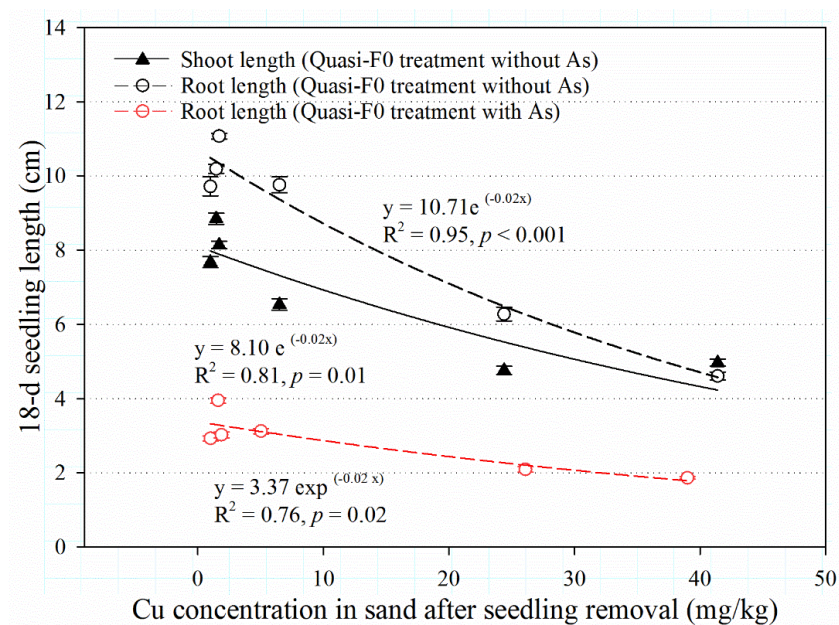
Figure 5.6. Relationship between 18-d seedling measurement and Cu concentration in sand in quasi-F0 treatment from an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in the nutrient solutions (n = 10).

4.Discussion

4.1. Intergenerational Effects from Comparison of F0 and F1 Generations

Copper and arsenic accumulation in F1 seeds. Copper, being an essential element for plants, is taken up by plant roots and transported to other parts of the plants via specific protein carriers (e.g., COPT) (Peñarrubia et al., 2015). Whereas, being a potentially toxic element, As enters plants by mimicking other essential elements (e.g., phosphorus, silicon) (Ma et al., 2008; Ye et al., 2017). In our previous study on the life cycle growth of F0 rice plants, Cu and As accumulation in rice grains (F1 seeds) was influenced by exposure to nCuO and As, both individually and interactively (Figure 7 and Table D.12 in the previous study (Liu et al., 2018c)). Therefore, the 12 types of F1 seeds contained different concentrations of Cu and As. Together with other essential nutrients, Cu and As were carried over from F0 treatment to the F1 seeds and would influence the seed germination and seedling growth in the F1 generation.

F1 seed germination and seedling growth. F1 seeds produced from F0Cu50 had the lowest seed germination as well as the shortest SL, RL and NRB in both F1C and F1As. However, the Cu and As concentrations in the F1 seeds from F0Cu50 were not different from other F0 treatments (Liu et al., 2018c). These results indicated that total concentrations of Cu or As in F1 seeds were not key factor influencing F1 seed germination and seedling growth. Speciation of Cu and As in seeds, other chemical constituents in seeds (e.g., nutrients and hormones), and external environmental conditions, also matter. For example, As speciation in seeds interferes the biochemical process (e.g., preventing the formation of high energy phosphoryl bonds, and inhibiting

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catalytic functions) during seed germination and seedling growth process (Carbonell et al., 1998; Summers, 2009; Farooq et al., 2016).

Our previous study with F0 seeds (from the same source for the 12 F0 treatment), performed under the same growth condition as this current F1 study, demonstrated the effects of nCuO and As on F0 seed germination and seedling growth (Liu et al., 2018a). Comparison of the previous study on F0 generation with this current F1 study showed clear intergeneration effects of nCuO and As on seed germination and seedling growth. For example, the seed germination was reduced by 27.0%, 40.5% and 21.6% in F1C[F0Cu50], F1As[F0Cu50], and F1Cu50[F0Cu50], respectively, compared to F0Cu50 treatment in F0 generation ($p < 0.001$). The SL also showed difference in the two generations ($p < 0.05$). The SL in F0 treatment was 3.67–10.1 cm (F1C was 10.1 cm), while the F1 SLs were 7.25–9.52 cm in F1C, 5.12–8.06 cm in F1As, and 4.76–8.84 cm in quasi-F0 treatments, respectively (Table D.10–12). More prominent difference between the two generation seedlings was observed in RL. The RL in F0 treatments was 0.76– 6.99 cm, while the RLs in F1 treatments were 7.77–10.7 cm in F1C, which was significantly increased compared to F0 generations ($p < 0.001$). Similar comparisons were observed in DWs of shoots and roots between F0 and F1 seedlings as in lengths of shoots and roots, respectively.

Although no significant difference in Cu or As uptake in seedlings was observed between F0 and F1 generations, the relationships between seedling measurements and Cu concentrations in sand were different. In quasi-F0 treatments, the SL and RL were decreased with Cu concentration in sand with As addition (Figure 5.6); whereas, the SL

was increased with Cu concentration in sand with As addition in F0 treatment (Fig.3 in the previous study (Liu et al., 2018a)).

4.2. Mechanisms of Intergenerational Effects

As mentioned in *4.1.2,* total Cu or As concentrations were not key factors controlling F1 seed germination and seedling growth. nCuO and As influences on other seed components (e.g., micronutrients, hormones, and proteins) and F1 treatment chemistry may reveal dominant mechanisms of the intergenerational effects.

Epigenetic alteration causing gene modification. The up/down regulation of gene expression due to epigenetic alterations could explain the cross-generational effects of environmental factors in the present study (Riggs et al., 1996; Burton and Metcalfe, 2014). A profile of gene expression is demonstrated when the organism develops. Epigenetic modification can occur in "early life" stages and be inherited by successive generations (Ou et al., 2012; Burton and Metcalfe, 2014). The "early life" stages are particularly sensitive to environmental stressors, because epigenetic alterations involve a relatively large portion of cells in the fully-grown plant, which is regulated by relatively high level hormonal expression. A study from other research groups demonstrated heavy metal(loid) (e.g., Cr, Cu, Hg) induced modification of DNA methylation in parental generation was carried to the next two generations of rice plants (Ou et al., 2012). In our previous F0 life cycle study of rice plants, the exposure to nCuO and As started before seed germination, which covered the "early life" stages. Therefore, epigenetic alteration could have occurred in "early life" of F0 plants and induced the transgenerational effects to F1 seed germination and seedling growth.

Nutritional and hormonal changes in seeds and seedlings. In addition to the ongoing early expression of genes, the seed / early seedling is more vulnerable because of its small size and incompletely developed self-protective mechanism and self-sufficiency. The average weight of seeds was also influenced by F0 treatment based on our previous research (Liu et al., 2018c), thus influencing the nutrient and hormone storage, which regulates the germination and future growth of the F1 seedlings (Shu et al., 2016). Under environmental stress, plants may terminate their vegetative growth prematurely and transition more rapidly to produce seeds, resulting in smaller plants with fewer leaves that provide photosynthate (Taiz et al., 2018). Thus, smaller seeds may be produced under suboptimal growth conditions. Small and big seeds are also qualitatively different. For example, they are different in their abscisic acid: gibberellins ratio (primary determinant of seed dormancy), food storage, ability to germinate from depth, and relative growth of roots and shoots (Shu et al., 2016; Taiz et al., 2018). This was supported by previous research from other groups that the relative growth rate of seedlings in the first 3 weeks was inversely proportional to the embryo weight, which was proportional to the seed weight (Fenner, 1983).

Food reserves (carbohydrates, lipids, proteins, and minerals) in rice grains are not only important for human health but also for future rice seedling growth. The stored reserves are massively mobilized from the endosperm to provide nutrients to growing seedlings after seed germination (Taiz et al., 2018). However, nutrient reserves including mineral concentrations in the seeds (grains) could be changed by the exterior environment during the plant growth process (Chen et al., 2012). Our previous life cycle study of F0 rice plants showed that zinc (Zn), magnesium (Mg), and molybdenum (Mo)

in the F0 grains (F1 seeds) were influenced by treatment with exposure to nCuO and As (Table D.2). For example, Mg in grains was decreased in treatments with high concentrations (50 and 100 mg/L) of nCuO ($p < 0.05$) regardless of As addition. Zinc in grains was decreased by one middle and two high concentrations (1.0, 50 and 100 mg/L) of nCuO in treatments without As addition ($p < 0.05$). Molybdenum was mainly decreased by As addition ($p < 0.05$). These elements play important roles in protein synthesis, membrane construction, gene expression and tolerance of oxidative stress during seed germination and seedling growth processes (Cakmak, 2000; Lu et al., 2013).

Plants are sessile and constantly adjust their responses to the environment via sophisticated signal transduction pathways either to make good use of favorable conditions or to survive unfavorable circumstances (Taiz et al., 2018). Hormones serve as secondary signals (messengers) and play a quite important role in initiating the responses of plants to the environmental input (the primary signals). Hormones regulate the uptake of water and nutrients (e.g., Cu) into the plants. Hormone synthesis is also influenced by exterior environment conditions. Copper is known for its structural role in many proteins (e.g., ethylene receptors, Mo cofactor), which are required for biosynthesizing hormones (e.g., ethylene, abscisic acid, and auxin indol-3-acetic (IAA) (Peñarrubia et al., 2015). Nevertheless, under excess Cu stress, complex cross-regulated signaling pathways are involved to produce physiological alterations (e.g. ROS generation).

Chemistry in spermosphere and rhizosphere. Soil texture, components of solutions, and exudates from seed or seeding roots all influence the chemistry in the spermosphere around the seed (before seed germination) or rhizosphere around the

seedling roots (after seed germination). The two previous studies from our research group used sand and artificial soil as growth media with the same exposure combination of nCuO and As. These studies showed different effects in seed germination and 18-d seedling growth (Liu et al., 2018a; Liu et al., 2018c). nCuO and As in sand exhibited more toxic effect than in artificial soils.

Various compound exudates are excreted in the spermosphere from germinating seeds and in the rhizosphere from seedling roots (Bacilio-Jiménez et al., 2003; Schiltz et al., 2015). These exudates include carbohydrate, organic acids, alcohols fatty acids, amino acids, proteins, secondary metabolites and inorganic ions. These organic and inorganic components can further influence the behaviors of nCuO and As (e.g., dissolution of nCuO, As adsorption to and desorption from nCuO) in the microenvironments (Martinson and Reddy, 2009; McManus, 2016).

In F1C, the average As concentrations in solutions increased 28% with seeds from F0As and F0AsCu treatments (with As addition) compared with treatments with seeds from F0C and F0Cu treatments (without As addition) ($p < 0.01$) (Table D.6). This indicated that F1 seeds or seedling roots also excreted As from inside the seeds or seedlings containing relative high As. However, with high concentrations of As in the growth media, As can also enter the seedlings, displacing phosphate and silicates in biological reactions, inhibiting metabolic processes, and mediating gene expression (Carbonell et al., 1998; Summers, 2009; Pathare et al., 2016).

5.Conclusion

F0Cu50 in the absence of additional As exposure in the growth media showed transgenerational effects by decreasing F1 seed germination and seedling growth,

although no adverse physiological effects on F0 plants were observed in F0Cu50 treatment in our previous study (Liu et al., 2018c). Moreover, no overt effects on rice seed production were observed from plants in F0Cu50. In contrast, nCuO and As co-presence in the growth media not only alleviated the toxicity of As in F0 plants, but also mitigated the transgenerational effects on F1 seed germination and seedling growth. These results imply that nCuO alone may cause adverse effects, but it can be used in the environment where high As concentrations occur. Although nanotechnology has the potential to bring promising improvement in agriculture, arising concerns about the fate of NMs in the field and the long-term effects (e.g., multigenerational effects) to plants have not been fully addressed. Moreover, increasing attention has focused on epigenetic changes, which provide potentially long-term adaptation to abiotic stress (Taiz et al., 2018). The stress-induced chromatin modification in epigenetic changes might have evolutionary implications to rice plants. Our study depicts basic intergenerational effects of nCuO and co-exposure of nCuO with As on rice seedling growth. These data in combination with our greenhouse results may help to develop environmental quality criteria of nCuO or/and As for protecting aquatic plants including rice. Future studies can investigate the intergenerational effects of nCuO and As on life cycle growth of rice plants in a more realistic scenario, wherein a different growth profile may be observed.

from the Center for Reservoir & Aquatic Systems Research (CRASR) for analysis of total organic carbon and nitrogen in sand samples.

Declaration of Interest Statement

The authors report no conflicts of interest, and are responsible for the content and writing of the publication.

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Supplemental Data (Appendix D.)

The Supplemental Data are available on the Wiley Online Library at DOI:

10.1002/etc.xxxx.

CHAPTER SIX

Conclusion

Arsenic (As) reduces the production and lowers the quality of rice. Chronic As exposure via rice consumption has the potential to cause adverse effects on human health. It is possible to efficiently reduce As health effects in rice by decreasing or even stopping As transport to rice grains with genetic technology. However, the controversial safety concerns of genetically modified food crops make it unacceptable overall to the general public. The emerging applications of nanoparticles (NPs) in agriculture, such as fertilizers, pesticides, or biosensors, also hold the potential to alleviate As phytotoxicity, which has not been well understood.

Cu-containing compounds have been used as pesticides for over a century. One of the concerns for using Cu-containing pesticides is the buildup of application over extended time period may cause phytotoxicity. This concern can be addressed by transforming Cu-containing compounds into their nano forms. On the nano scale, antimicrobial properties of Cu are enhanced due to a tremendous increase in the surface area to volume ratio. The release of Cu is also slower from the Cu-containing nanoparticles into the water. Thus, the amount of Cu-containing agrochemical application could be reduced to avoid or alleviate possible phytotoxicity. In addition, the high demand and application of nCuO in numerous industries will increase their unintentional release into the environment, especially to aquatic system. Thus, it is necessary to understand the environmental behavior and effects of nCuO to rice plants. The beneficial

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and toxic properties of Cu/nCuO depends on interactions with other environmental components (e.g., As concentrations) and plant species. Diverse tolerance mechanisms for As toxicity have also been developed by different plant species. Due to the space limit, we only studied one variety of rice species, Oryaza sativa japonica 'Koshihikari', which is a very prevalent rice variety in Asian countries due to its aroma, sweet flavor, and sticky texture. This variety is widely adaptable and has also been introduced to other areas worldwide, including the USA. However, it is necessary to compare As tolerance and uptake by different rice species in the future as well as from the perspective of grain production and human health effects.

The two-way factorial design enabled this project to assess the individual main effects of As (0 and 10 mg/kg, near the average As concentration in soil from Texas, US) and nCuO (0–100 mg/L), and their interaction on rice plant growth. Studies were conducted at an earlier vegetative stage (from seed germination to 18-d seedling growth) in an incubator and during a life cycle (from seed germination to offspring seed maturation) in a greenhouse.

Results from the F0 incubator study revealed that As generally inhibitory effects rice plant growth (15.5% reduction in germination, 56–81% reduction in SL and RL, and 64%–76% reduction in DWs of shoots and roots) at the same test concentration (10 mg/kg) in sand compared to control. With an artificial soil as the growth media, As effects on the above parameters of seedlings were not as prominent as seedlings grown in sand. For example, the reduction in root DWs was 14%–53% by As addition compared to the nCuO alone treatment. Except the inhibition at early growth stage, As at 10 mg/kg increased final grain yield by 13.2% compared to control. nCuO at relatively high

concentration also caused some reduction in rice plant growth (RL, SL, and DWs of shoots and roots) in the study conducted in sand media, but the interaction of nCuO with As overall alleviated As adverse effects compared with As alone treatment. In most cases, nCuO brought benefit to rice plant growth especially in the greenhouse study, where rice plants were grown in an artificial soil approximating real agriculture scenarios. The benefit from nCuO particularly presented at later growing stages. The final grain production was also increased 17.0– 25.5% by nCuO (10–100 mg/L) relative to control. Particularly, the heading process of rice panicles was accelerated by nCuO especially at higher concentration (50 and 100 mg/L), and the deceleration of the heading process by As was mitigated by nCuO addition. The acceleration of heading process by nCuO may shorten the harvest time of rice plant, which will bring many benefits in agriculture. For example, the likelihood for plants to contract diseases will be lowered. Additionally, less physical labor will be required to maintain the paddy field. Moreover, less water will be needed to keep the paddy field flooded, and the farmers will have more time to prepare for the next growing season. Finally, the growing season can be more flexible and would accommodate alterations in cultivation due to inclement weather. In fact, a shortened growing season would allow replanting of a crop damaged by bad weather and the plants can still reach full maturation on time. These results suggest that growth media play an important role in the effects of nCuO and As on plant growth. Amendment to the soil is mandatory in some rice paddies with higher sand content to mitigate As toxicity. However, the influence from other components of the soil and the overlying water need to be addressed in specific conditions. For example, aggregation of nCuO is inevitable without either engineered or incidental coatings (Lowry et al., 2012),

and decreases the surface area (i.e., reactivity) of the NPs. In addition to the homoaggregation of nCuO, heteroaggregation among nCuO and other soil particles also affect the behavior of nCuO and its interaction with As. Hereoaggregation occurs in most cases where environmental particles have a greater concentration than NPs. Coating agents (e.g., synthetic and organic macromolecules, natural organic matters) may be useful to stabilize nCuO, control its aggregation and dissolution, and increase its efficiency for As adsorption. Although it is not feasible to determine the interaction between nCuO and all types of surfaces in the environment, it is easier to estimate the behavior of nCuO when coherent coatings are used. All surfaces would be expected to behave similarly, thus a simpler homoaggreagation process can be used to approximate a complex heteroaggreation. However, further studies need to be conducted to evaluate appropriate coating agents to optimize the stability or dissolution rate for a given growth medium. Moreover, organic matter in the natural soil is also a key factor influencing the aggregation, sorption, dissolution and biological effects of nCuO by contributing to the corona formed on the surface of NPs. Evaluation the influence of organic matter on nCuO could be done by mimicking the real environments with changing the organic matter content.

This project also suggests that nCuO at 50 mg/L decreased As accumulation in the dehusked grains to 128 ng/g, which was 36% lower than the proposed maximum safe concentration of inorganic As in white rice by WHO. Therefore, more adverse human health effects (e.g., ischemic heart disease, hepatic effects, dermal effects, gastrointestinal irritation, diarrhea, nausea, and intelligence performance) can be avoided from rice consumption with this alternative containing less As. This finding will benefit a large

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sub-population including people who have regular daily rice consumption especially Asian people, those who have dietary restriction, and infants who use rice to transition from breast milk to solid food. In addition, this project also demonstrated that As kept accumulating in the grains throughout the maturation stage. Therefore, the time to harvest also plays an important role in the final As accumulation in grains. By shortening the harvest time, nCuO addition can further reduce As accumulation in rice grains.

Although Cu and As uptake by the root, distribution in other parts of the plant, and accumulation in the grains were observed in this study, the mechanisms for these processes are still not clear. In particular, the antiport mechanisms of Cu and As in rice grains need to be elucidated. The specific transport channels and paired ions or molecules involved with Cu/nCuO and As antiport process should be studied inside the plants. Copper is an important co-factor of many proteins, which may be involved in As efflux from grains. The inhibition of As methylation in the presence of nCuO in the treatment, as observed in rice plants in this study, also indicated a potential interaction between nCuO with microbes in the growth media, since plants are not able to methylate As. Arsenic methylation in the growth media could also contribute to the reduced uptake of As into the plants, because methylated As cannot be taken up by plants as efficiently as inorganic As.

Being the final leaf to transport most of the photosynthetic products into the grains, the flag leaf may play an important role in transporting Cu and As into the rice panicle as well as be involved in the antiport process between Cu and As. Therefore, compared to application in the growth media, foliar application could be more efficient if nCuO can be taken up by flag leaves. Moreover, Cu concentration in the water will be

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decreased when nCuO is applied on leaves, and the possible adverse effects of Cu would be reduced when water in rice paddies is drained into the aquatic systems near the end of the life cycle growth of rice plants.

With the seeds produced from the greenhouse study exposed to nCuO and As, this project expands the knowledge in the effects of nCuO and As to the next generation. Results demonstrated that a transgenerational inhibition of seed germination and seedling growth was from seeds produced from parent plants treated with nCuO at 50 mg/L. This result will provide some implication in seed selection and preservation for sustaining future generations. However, the mechanism needs to be clarified for the transgenerational effects occurred only at 50 mg/L nCuO.

Furthermore, we are not clear whether nCuO entered the plants, how it interacted mechanistically with As inside the plants, and how it influenced the plant regulation system on the molecular level (e.g., ROS, enzyme activity, hormone synthesis). Although we observed nCuO with TEM in the solution after 14 d of exposure, we were not able to distinguish nCuO from bulk CuO in the plant tissues, since the XANES spectra of nCuO and bulk CuO were too similar. Due to the limited time schedule and the unexpected shutdown of the beamline at Advanced Photon Source in Argonne National Laboratory, we were not able to investigate the species of Cu and As in the soil and more mature plant tissues.

Overall, as the first to study the interaction of nCuO and As on rice plant growth, results from this project add to the body of knowledge in nanophytotoxicity of nCuO, phytotoxicity of As, and the interaction of nCuO and As, regarding rice plant growth and As uptake. These results also provide implications in sustainable agriculture development

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by combining the applications of nCuO as pesticides, fertilizers and amendments counteracting As toxicity. The threefold benefits may also give advantages to nCuO over other mitigation agents of As phytotoxicity.

APPENDICES

APPENDIX A

Supplemental materials for Chapter Two: Copper Oxide Nanoparticles and Arsenic Interact to Alter Seed Germination and Seedling Growth of Rice (*Oryza Sativa Japonica*)

Data Handling of Seed Germination and Seedling Growth

Two seeds were planted in each growth cell. Thus, the germination percentage for each replicate cell could have been 0%, 50%, and 100%. There 20 statistical units for each parameter (germination percentage, seedling length and dry weight) in each of the 12 treatments. Germination and growth data were analyzed with generalized linear models (GLM) depending on the data distributions.

Solution Sampling

Water samples (0.50 mL out of 50 mL) were collected using the pipette every 6 d from each growth cell. Five composite water samples were collected within each treatment. Each composite contained four distinct water samples with volumes of 2.0 mL. The effect of water removed (1%) on the As or Cu concentrations is negligible.

Detection Limits

Sand samples

For ICP-MS analysis, the digested mass of soil was 0.50 g, and digestates were diluted to 50 mL. The linear calibration range of Cu and As was 5–1000 µg/L.

Quality control recoveries at 125 µg/L were: Cu 105 ± 1.08%, As 100 ± 1.20%. No quantifiable concentrations were measured in reagent blanks (same matrix as the

sample solution, but no added analyte), which were estimated to be below the calibration range. Three out of 60 concentration values of the samples were below the lowest calibration standard (5 µg/L) and considered no detectable (ND) [1]. For statistical analysis, values reported by the instrument were used for the 3 samples.

Seedling samples

For ICP-MS analysis, the average mass of seedlings was 8.43 mg, and digestates were diluted to 10 mL. The linear calibration range of Cu and As was 1–50 µg/L.

Quality control recoveries for 10 µg/L: Cu 98.8 ± 0.69%, As 97.4 ± 0.87%. No quantifiable concentrations were measured in reagent blanks (same matrix as the sample solution, but no added analyte), which were estimated to be below the calibration range. Three out of 60 concentration values of the samples were below the lowest calibration standard (1 µg/L) and considered no detectable (ND) (Pan, Ochoa et al. 2013). For statistical analysis, values reported by the instrument were used for the 3 samples.

Total Organic Carbon and Nitrogen

Sand samples (45-60 mg) from 3 treatments before sowing seeds and after seedling removal were taken for analyzing total organic carbon and nitrogen with the Flash 1112 NC Soil Analyzer. Samples were fumigated with hydrochloride acid (acid fumigation) and dried in the oven to remove carbonates prior to total organic carbon analysis.

Overall, total organic carbon in the samples was 0.076 ± 0.006 % before sowing the seeds, 0.101 ± 0.005% after seedling removal (Table A.2). Total nitrogen was 0.029 ± 0.003% before sowing the seeds, 0.031 ± 0.001 % after seedling removal (Table A.2).

Table A.1. Total copper and arsenic concentrations in sand media (mg/kg sand) before and after 18-d laboratory experiment of rice (*Oryza sativa japonica*) seed germination and seedling growth test exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution

Variable		Cu		As	
As (mg/kg)	nCuO (mg/L)	Before	After	Before	After
0	0	$0.633 \pm 0.065^{\text{Aa}}$	$0.609 \pm 0.027^{\text{Aa}}$	$0.915 \pm 0.069^{\text{Aa*}}$	$0.95 \pm 0.039^{\text{ABa}}$
	0.1	$0.627 \pm 0.027^{\text{Aa}}$	$0.745 \pm 0.115^{\text{Aa}}$	$0.841 \pm 0.047^{\text{Aa}}$	$1.08 \pm 0.199^{\text{Aa}}$
	1.0	$0.846 \pm 0.226^{\text{Aa}}$	$0.741 \pm 0.005^{\text{Aa}}$	$0.885 \pm 0.044^{\text{Aa}}$	$0.873 \pm 0.024^{\text{ABa}}$
	10	$0.54 \pm 0.025^{\text{Aa}}$	$3.1 \pm 0.149^{\text{Ba*}}$	$0.766 \pm 0.034^{\text{Aa}}$	$0.837 \pm 0.052^{\text{Ba}}$
	50	$0.648 \pm 0.065^{\text{Aa}}$	$23.5 \pm 0.464^{\text{Ca*}}$	$0.842 \pm 0.069^{\text{Aa}}$	$0.924 \pm 0.054^{\text{ABa}}$
	100	$0.643 \pm 0.045^{\text{Aa}}$	$55.4 \pm 1.37^{\text{Da*}}$	$0.839 \pm 0.069^{\text{Aa}}$	$0.703 \pm 0.021^{\text{ABa}}$
10	0	$0.697 \pm 0.041^{\text{Aa}}$	$0.807 \pm 0.262^{\text{Aa}}$	$14.7 \pm 3.26^{\text{Ab}}$	$12 \pm 1.48^{\text{Ab}}$
	0.1	$0.655 \pm 0.062^{\text{Aa}}$	$0.64 \pm 0.027^{\text{Aa}}$	$11.2 \pm 1.49^{\text{Ab}}$	$15 \pm 0.566^{\text{Ab}}$
	1.0	$0.603 \pm 0.015^{\text{Aa}}$	$0.915 \pm 0.064^{\text{Aa}}$	$10.6 \pm 1.34^{\text{Ab}}$	$14.9 \pm 1.85^{\text{Ab}}$
	10	$0.613 \pm 0.024^{\text{Aa}}$	$3.5 \pm 0.175^{\text{Ba*}}$	$12.5 \pm 2.24^{\text{Ab}}$	$14.7 \pm 0.471^{\text{Ab}}$
	50	$0.648 \pm 0.038^{\text{Aa}}$	$22.5 \pm 1.29^{\text{Ca*}}$	$17.8 \pm 5.13^{\text{Ab}}$	$15.1 \pm 0.519^{\text{Ab}}$
	100	$0.641 \pm 0.047^{\text{Aa}}$	$40.7 \pm 2.36^{\text{Da*}}$	$14.5 \pm 3.52^{\text{Ab}}$	$13.2 \pm 0.276^{\text{Ab}}$
p-value	As	0.951	0.211	< 0.001	< 0.001
	nCuO	0.407	< 0.001	0.977	0.007
	A×N[1]	0.233	0.119	0.153	0.283

Values are means ± SEM, n = 5 per treatment group.
[A-D] Means in a column at the same As level without a common superscript letter differ ($p < 0.05$)
[ab] Means in a column at the same nCuO level without a common superscript letter differ ($p < 0.05$).
[*] Means in a row for Cu or As differ between before and after ($p < 0.05$).
[1] A × N = As × nCuO interaction effect.

Table A.2. Total organic carbon and nitrogen in the sand media before and after seedling growth in the 18-day laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution

Variable		Mass (mg)	% Carbon	% Nitrogen
As	Before	51.0 ± 2.7	0.062 ± 0.004	0.032 ± 0.002
	After	53.6 ± 0.922	0.107 ± 0.009	0.032 ± 0.002
Control	Before	51.1 ± 2.520	0.065 ± 0.008	0.024 ± 0.007
	After	51.9 ± 2.000	0.100 ± 0.010	0.030 ± 0.002
nCuO100	Before	53.2 ± 1.610	0.102 ± 0.005	0.032 ± 0.002
	After	54.4 ± 1.830	0.095 ± 0.009	0.028 ± 0.003

Values are means $\pm$ SEM, n = 5 per treatment group.
The recovery for standards of carbon and nitrogen are in the range from 97.3%–109.0% and 96.1%–103.6%.

Table A.3. Total copper and arsenic concentration (mg/L) in solution media in the 18-day laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution (n = 5 for each time points)

Variable			Cu	Variable			Cu	As
As (mg/kg)	nCuO (mg/L)	Day	(mg/L)	As (mg/kg)	nCuO (mg/L)	Day	(mg/L)	(mg/L)
0	0	1	0.021 ± 0.000^{Aa}	10	0	1	0.020 ± 0.001^{Aa}	1.44 ± 0.067^{Aabc}
		6	0.034 ± 0.001^{Ba}			6	0.054 ± 0.003^{Ba}	4.62 ± 0.204^{Ba}
		12	0.044 ± 0.002^{Ca}			12	0.081 ± 0.004^{Ca}	4.52 ± 0.258^{Ba}
		18	0.060 ± 0.002^{Da}			18	0.117 ± 0.003^{Da}	7 ± 0.272^{Cab}
	0.1	1	0.068 ± 0.001^{ABb}		0.1	1	0.068 ± 0.005^{ABb}	1.68 ± 0.083^{Aab}
		6	0.049 ± 0.002^{Cb}			6	0.064 ± 0.002^{Bab}	5.46 ± 0.208^{Ba}
		12	0.058 ± 0.002^{ACb}			12	0.080 ± 0.001^{Aa}	4.58 ± 0.163^{Ba}
		18	0.081 ± 0.002^{Bb}			18	0.135 ± 0.009^{Ca}	7.26 ± 0.555^{Ca}
	1.0	1	0.254 ± 0.009^{Ac}		1.0	1	0.224 ± 0.041^{Ac}	1.37 ± 0.113^{Aac}
		6	0.084 ± 0.004^{Bc}			6	0.079 ± 0.004^{Bb}	4.64 ± 0.187^{Ba}
		12	0.128 ± 0.002^{Cc}			12	0.115 ± 0.004^{Cb}	4.88 ± 0.168^{Ba}
		18	0.191 ± 0.009^{Dc}			18	0.21 ± 0.008^{Ab}	7.54 ± 0.214^{Ca}
	10	1	3.69 ± 0.746^{Ad}		10	1	4.11 ± 0.141^{Ad}	1.75 ± 0.155^{Aab}
		6	0.58 ± 0.023^{Bd}			6	0.297 ± 0.013^{Bd}	5.15 ± 0.173^{Ba}
		12	0.981 ± 0.052^{Cd}			12	0.367 ± 0.022^{Bc}	4.76 ± 0.098^{Ba}
		18	1.7 ± 0.046^{Dd}			18	1.11 ± 0.050^{Cc}	8.28 ± 0.377^{Ca}
	50	1	39.3 ± 1.01^{Ae}		50	1	41 ± 0.299^{Ae}	1.1 ± 0.108^{Ac}
		6	3.03 ± 0.092^{Be}			6	2.36 ± 0.055^{Bc}	4.8 ± 0.172^{Ba}
		12	2.53 ± 0.061^{Be}			12	2.39 ± 0.044^{Bd}	4.85 ± 0.159^{Ba}
		18	5.51 ± 0.136^{Ce}			18	5.3 ± 0.666^{Cd}	6.95 ± 0.248^{Cab}
	100	1	86.3 ± 2.29^{Af}		100	1	84.8 ± 1.07^{Af}	1.87 ± 0.411^{Ab}
		6	2.87 ± 0.024^{Be}			6	2.92 ± 0.186^{Bc}	5.45 ± 0.248^{Ba}
		12	2.87 ± 0.057^{Be}			12	2.65 ± 0.086^{Bd}	4.32 ± 0.132^{Ba}
		18	6.05 ± 0.260^{Ce}			18	4.27 ± 0.103^{Cd}	5.48 ± 0.197^{Bb}
p-value	nCuO		< 0.001	p-value	nCuO		< 0.001	< 0.001
	Time		< 0.001		Time		< 0.001	< 0.001
	N×T1		< 0.001		N×T1		< 0.001	< 0.05

Values are means ± SEM, n = 5 per treatment group.
[A-D] Means in a column at the same nCuO level without a common superscript letter differ ($p < 0.05$).
[a-f] Means in a column within the same day without a common superscript letter differ ($p < 0.05$).
1N × T = nCuO × Time interaction effect.

Table A.4. Statistical description of rice (*Oryza sativa japonica*) seedling growth parameters after the 18-day laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	Sample size (n)	SL(cm)	RL(cm)	NRB
0	0	20	10.1 ± 0.758^{Aa}	6.99 ± 0.636^{Aa}	4.15 ± 0.189^{Aa}
	0.1	19	7.27 ± 0.789^{ABa}	2.84 ± 0.376^{Ba}	4.97 ± 0.396^{Aa}
	1.0	19	7.98 ± 0.529^{ABa}	3.96 ± 0.350^{BCDa}	5.21 ± 0.185^{Aa}
	10	20	5.89 ± 0.430^{Ba}	$3.86 \pm 0.437^{BC}a$	4.03 ± 0.207^{Aa}
	50	20	6.04 ± 0.273^{Ba}	5.27 ± 0.406^{ACDa}	5.03 ± 0.228^{Aa}
	100	20	6.25 ± 0.263^{Ba}	6.43 ± 0.343^{ADa}	5.18 ± 0.171^{Aa}
10	0	18	4.49 ± 0.408^{Ab}	1.29 ± 0.195^{ABb}	3.75 ± 0.426^{ABa}
	0.1	18	3.67 ± 0.398^{Ab}	0.756 ± 0.114^{Ab}	3.03 ± 0.489^{Ab}
	1.0	18	4.61 ± 0.627^{Ab}	1.36 ± 0.269^{ABb}	3.72 ± 0.421^{ABb}
	10	19	4.22 ± 0.564^{Ab}	1.18 ± 0.20^{ABb}	3.39 ± 0.475^{ABa}
	50	18	5.12 ± 0.217^{Aa}	1.93 ± 0.15^{Bb}	4.94 ± 0.252^{Ba}
	100	19	5.21 ± 0.463^{Aa}	1.52 ± 0.158^{Bb}	4.74 ± 0.470^{Ba}
p-value	As		< 0.001	< 0.001	< 0.001
	nCuO		0.042	< 0.001	< 0.001
	A×N1		< 0.001	< 0.001	< 0.05

Values are means ± SEM.
[A-D] Means in a column at the same As level without a common superscript letter differ ($p < 0.05$).
[ab] Means in a column at the same nCuO level without a common superscript letter differ ($p < 0.05$).
[1] A × N = As × nCuO interaction effect.

Table A.5. Statistical description of rice (*Oryza sativa japonica*) seedling growth parameters after the 18-day laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution

Variable			Shoot_DW (mg)	Root_DW (mg)
As (mg/kg)	nCuO (mg/L)	Sample size(n)		
0	0	17	6.18 ± 0.593Aa	1.86 ± 0.190Aa
	0.1	18	5.5 ± 0.803Aa	1.28 ± 0.211Aa
	1.0	17	6.16 ± 0.470Aa	1.49 ± 0.135Aa
	10	19	3.84 ± 0.542Aa	1.19 ± 0.216Aa
	50	18	4.19 ± 0.396Aa	1.84 ± 0.206Aa
	100	19	4.68 ± 0.535Aa	2.19 ± 0.251Aa
10	0	11	2.24 ± 0.533Ab	0.441 ± 0.136ABb
	0.1	14	2.48 ± 0.459Ab	0.282 ± 0.0646Ab
	1.0	15	2.2 ± 0.549Ab	0.381 ± 0.123ABb
	10	15	2.09 ± 0.451Ab	0.396 ± 0.099ABb
	50	16	3.09 ± 0.321Aa	0.647 ± 0.081ABb
	100	14	3.01 ± 0.412Aa	0.811 ± 0.142Bb
p-value	As		< 0.001	< 0.001
	nCuO		0.496	< 0.001
	A×N[1]		0.075	0.669

Values are means ± SEM.
[A-B] Means in a column at the same As level without a common superscript letter differ ($p < 0.05$).
[ab] Means in a column at the same nCuO level without a common superscript letter differ ($p < 0.05$).
[1] A × N = As × nCuO interaction effect.

Table A.6. Total copper and arsenic concentrations in seedlings (mg/kg dry sample) after the 18-day laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution (n = 5)

Variable		Cu		As		Cu ratio	As ratio
As (mg/kg)	nCuO (mg/L)	Shoot	Root	Shoot	Root	Shoot: root	Shoot: root
0	0	16.8 ± 2.56^{Aa}	26.4 ± 1.87^{Aa}	0.590 ± 0.145^{Aa}	2.40 ± 1.04^{Aa}	0.647 ± 0.102^{Aa}	0.381 ± 0.119^{ABa}
	0.1	15.8 ± 0.823^{Aa}	83.9 ± 10.3^{Ba}	0.394 ± 0.025^{ABCa}	1.30 ± 0.169^{ABa}	0.197 ± 0.022^{Ba}	0.329 ± 0.056^{ABa}
	1.0	16.9 ± 0.713^{Aa}	106 ± 3.36^{Ba}	0.304 ± 0.012^{Ba}	1.74 ± 0.673^{ABa}	0.16 ± 0.008^{BCa}	0.246 ± 0.047^{Aa}
	10	55.7 ± 4.77^{Ba}	565 ± 47.3^{Ca}	0.400 ± 0.034^{ABCa}	1.05 ± 0.061^{ABa}	0.101 ± 0.012^{BCDa}	0.380 ± 0.019^{ABa}
	50	176 ± 21.2^{Ca}	2010 ± 95.1^{Da}	0.35 ± 0.013^{BCa}	0.772 ± 0.057^{Ba}	0.089 ± 0.011^{CDa}	0.464 ± 0.039^{ABa}
	100	339 ± 70.1^{Ca}	4670 ± 449^{Ea}	0.516 ± 0.051^{ACa}	0.958 ± 0.077^{Ba}	0.073 ± 0.012^{Da}	0.556 ± 0.079^{Ba}
10	0	8.88 ± 0.380^{Ab}	52.5 ± 13.1^{Ab}	140 ± 19.3^{ABb}	1430 ± 67.3^{Ab}	0.233 ± 0.074^{ABb}	0.099 ± 0.014^{Ab}
	0.1	9.99 ± 0.429^{Ab}	33.8 ± 3.53^{Ab}	191 ± 19.1^{Ab}	1250 ± 267^{Ab}	0.306 ± 0.026^{Aa}	0.175 ± 0.039^{ABb}
	1.0	13.6 ± 0.640^{Aa}	46.4 ± 1.78^{Ab}	201 ± 40^{Ab}	896 ± 62.0^{Ab}	0.295 ± 0.019^{Ab}	0.227 ± 0.048^{Ba}
	10	31 ± 7.57^{Bb}	180 ± 46.1^{Bb}	202 ± 24.9^{Ab}	1120 ± 128^{Ab}	0.174 ± 0.014^{ABb}	0.179 ± 0.014^{ABb}
	50	144 ± 13.8^{Ca}	1250 ± 119^{Cb}	162 ± 9.01^{Ab}	903 ± 69.0^{Ab}	0.122 ± 0.023^{Ba}	0.186 ± 0.027^{ABb}
	100	69.4 ± 13.0^{Db}	1770 ± 211^{Cb}	92.9 ± 8.78^{Bb}	925 ± 84.5^{Ab}	0.043 ± 0.011^{Cb}	0.106 ± 0.020^{ABb}
p-value	As	< 0.001	< 0.05	< 0.01	< 0.001	0.977	< 0.001
	nCuO	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.060
	A×N[1]	< 0.001	< 0.001	< 0.001	0.395	< 0.001	< 0.01

Values are means ± SEM, n = 5 per treatment group.
[A-D] Means in a column at the same As level without a common superscript letter differ ($p < 0.05$).
[ab] Means in a column at the same nCuO level without a common superscript letter differ ($p < 0.05$).
[1] A × N = As × nCuO interaction effect.

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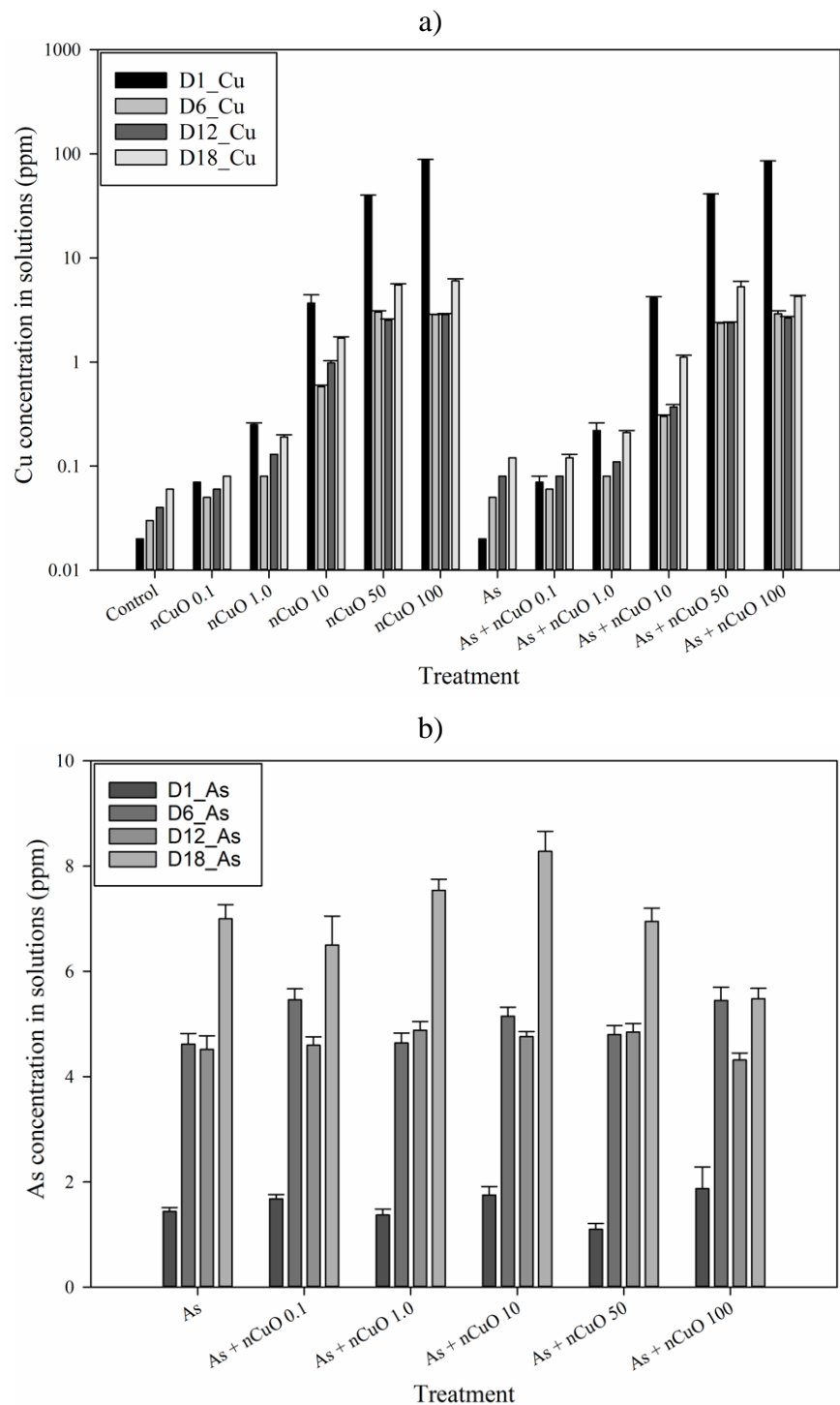
Figure A.1. a). Copper and b). arsenic concentration change in the solution sample during the 18–day laboratory experiment exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution

The 72–hour dissolution and aggregation experiment.

The dissolution kinetics of copper oxide nanoparticles (nCuO) with and without

arsenic (As) in the 1/5th Hoagland's nutrient solution (Table A.7) was monitored over 72

h [2] in duplicate experiments. Stock solution (500 mg/L) of nCuO was made with the

nutrient solution and dispersed using a sonication probe (Sonic Dismembrator Model

500, Fisher Scientific) for 30 min. The stock solution was then used to obtain eight

different solutions with desired concentrations (0, 0.1, 10, and 100 mg/L) of nCuO and

As (0 and 4.5 mg/L, and the concentration of As 4.5 mg/L was the average As

concentration as measured in the solution samples of As treatment in the seedling growth

test). Eight solutions were sonicated (Fisherbrand, FB11201) for 30 min.

Dissolution experiment:

Duplicate samples of 4 mL from each solution were transferred to the Amicon

Ultra filter tube (Fisher Scientific, 3 kDa cutoff, Catalog Number UFC800396), and

spinned in a Beckman Avanti JXN-26 Floor Centrifuge with a swinging bucket rotor at

4,000 $\times$ g at room temperature (20℃) [3-5]. Different samples for each solution were

centrifuged at multiple time points (0, 6h, 12h, 24h, 48h, 60h and 72h). The filtrates were

transferred and diluted from 1 mL to 50 ml with 2% HNO3 to be acidified. Then the

solution was analyzed with ICP–MS for measuring total dissolved Cu and As

concentrations.

Aggregation experiment:

After sonication, 1.5 ml of each of the eight solutions were taken and the effective

diameter of the particles in the solution were determined via Dynamic Light Scattering

(DLS) (Malvern Zetasizer Nano ZS) at different time point. The experiment was run in

triplicates, and the results presented were the mean value of each run.

Table A.7. Composition of 1/5th Hoagland's solution used as nutrient in the 18-day laboratory experiment of rice (*Oryza sativa japonica*) seed germination and seedling growth exposed to arsenic in sand and copper oxide nanoparticles in nutrient solution

	Compound	Concentration (µM)
Hoagland's Solution	$MgCl_2$	400
	$CaCl_2 \cdot 2H_2O$	2000
	KCl	1000
	H_3BO_3	22.5
	$MnCl_2 \cdot 4H_2O$	4.5
	$ZnSO_4 \cdot 7H_2O$	0.5
	$CuSO_4 \cdot 5H_2O$	0.15
	MoO_3	0.07
	Fe-EDTA	45
	$Na_2SiO_3 \cdot 5H_2O$	1500
	PIPES buffer	5000

Note: PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)) was used to maintain the pH (6.8–7.2) of the solution.
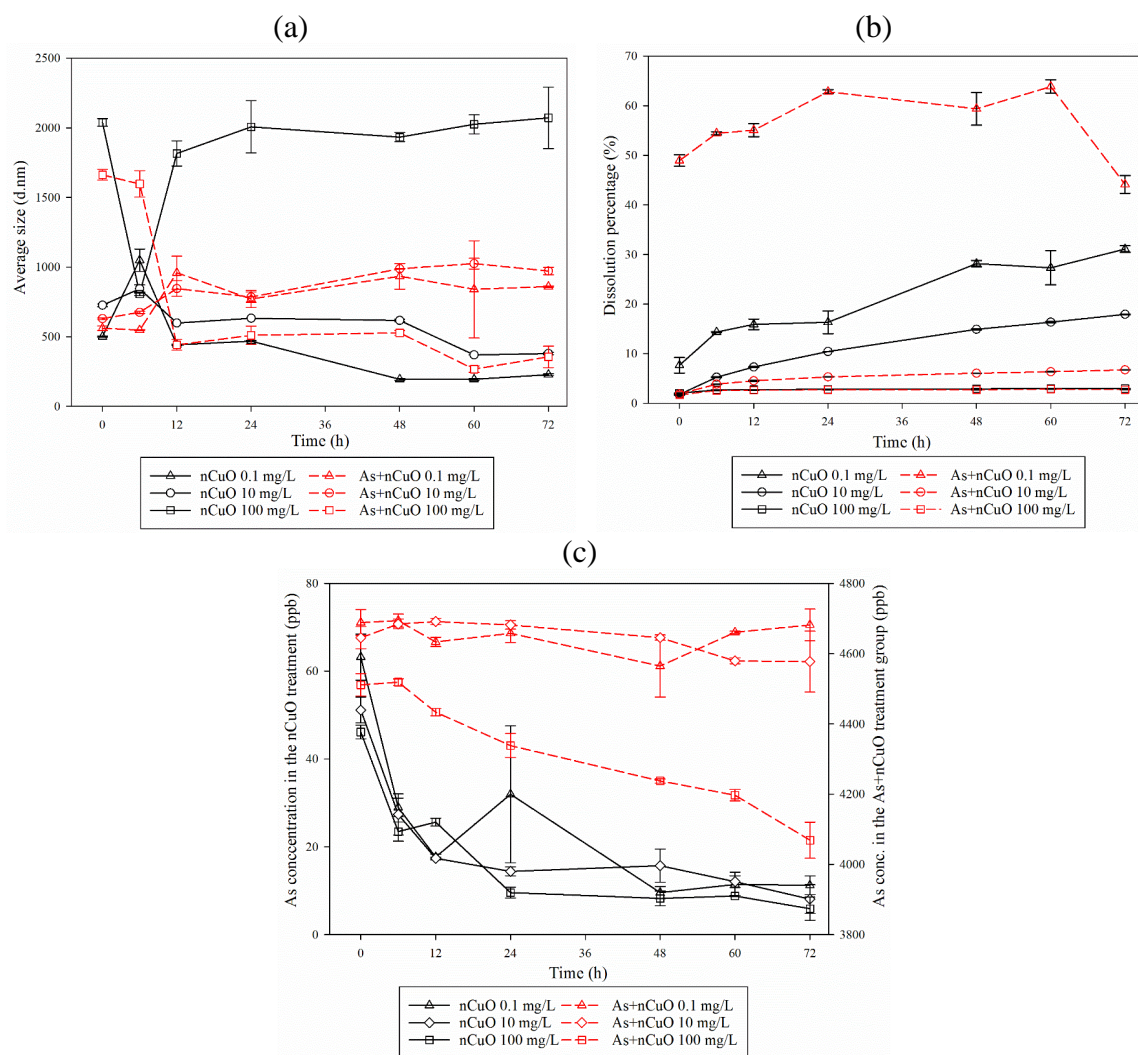
Figure A.2. Particle size change (a), the dissolution percentage of copper oxide nanoparticles (b), and arsenic concentration (c) in the 1/5th Hoagland's solution with / without As addition over 72-h (Duplicate samples for each time point)

References

[1]   Pan, X., et al., Absorption, distribution, and biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine in B6C3F1 mice (Mus musculus): RDX transformation and distribution in mice. Environmental Toxicology and Chemistry, 2013. 32:1295-1303

[2]   Jiang C, Castellon BT, Matson CW, Aiken GR, Hsu-Kim H. 2017. Relative contributions of copper oxide nanoparticles and dissolved copper to cu uptake kinetics of gulf killifish (Fundulus grandis) Embryos.

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[4]   Miller RJ, Lenihan HS, Muller EB, Tseng N, Hanna SK, Keller AA. 2010. Impacts of metal oxide nanoparticles on marine phytoplankton. *Environmental science & technology* 44:7329-7334.

[5]   Wang Z, Von Dem Bussche A, Kabadi PK, Kane AB, Hurt RH. 2013. Biological and environmental transformations of copper-based nanomaterials. *ACS nano* 7:8715-8727.

APPENDIX B

Supporting information for Chapter Three: Physiological Effects of Copper Oxide Nanoparticles and Arsenic on the Growth and Life Cycle of Rice (*O.sativa japonica* 'Koshihikari')

Number of pages: 25

Number of figures: 6

Number of tables: 13

Additional text is provided about fertilizer application, soil characterization and detailed statistical analysis. Tabular data in this document support the depicted figures within the main text of the manuscript, and may also be beneficial for those who are conducting research for direct comparisons of means and ranges, or those engaged in meta-analysis of ongoing research to address plant sensitivity to metals.

Methods

Whole life growth test in the greenhouse

Commercial substances (60% Grainger clay soil, Catalog # 2258, 40% Lowe's topsoil, # 235384) were purchased to prepare test soils. The clay soil was ground with an automatic continuous hammer mill grinder (DF-15, ECO-WORTHY). The topsoil was sifted through a 2-mm sieve. Then the two types of soils were homogenized. The As treatment (10 mg/kg soil) were prepared by spraying the $Na_2HAsO_4 \cdot 7H_2O$ (Sigma-Aldrich, lot # BCBM0939V) containing solution onto the spread soil surface and homogenizing (Figure B.1). nCuO (Nano-Arc®, 97.5%, 23–37 nm, APS powder, Alfa Aesar, MA, USA) treatments were prepared at 6 levels (0.1, 1.0, 10, 50, and 100 mg/L) in 20% Hoagland's solution (Table B1). Hydrodynamic diameter and zeta potential of nCuO in Hoagland's solution were determined with Malvern Zetasizer Nano ZS (Table B.2). Twelve treatments (6×2 combinations of the nCuO and As) were prepared in total. Each treatment had 20 replicate growth containers (Berry Plastics ID: T60785CP, 2.5 L) with 1.5 kg of soil mixture in each. The soil in each container was saturated with deionized (DI) water. Nutrient solution with appropriate nCuO concentrations were added on the first day to cover the soil with 4 cm (150 mL) of liquid. nCuO solutions

were replenished on day 35. Ten seeds (*O.sativa japonica* 'Koshihikari', Kitazawa Seed Company, CA, USA, pre-soaked for 12 hours in DI water) were water-sowed in each container. Water depth was maintained with DI water. Hoagland's solution was used as nutrient every other day for the first two weeks of seedling growth. Thereafter, additional fertilizers (see section of Fertilizer Application) were applied to optimize plant growth.

One day 18, seedlings were thinned to 2 about 4" apart. Seedling samples were used for morphological measurement and chemical analysis. Solutions were sampled weekly during the growth period. Flag leaves and grains from the same tiller (day 118 and day 131) were taken for analysis at two time points. Watering was stopped when the majority of panicles started to mature. Plants were harvested after one week without watering. The experiment was conducted in the greenhouse for about 131 days with monitoring of the light intensity (measured with Odyssey Photosynthetic Active Radiation Logger and calibrated to the PAR), humidity and temperature (with the monitor of humidity and temperature) (Figure B.2-B.3).

In addition, As was mixed into the soil because: 1) As was naturally occurring in the soil, and As soil contamination was worsened by all kind of human activities, especially previous arsenic-containing agrochemical application. 2) the target concentration of As exposure in this study approximated the concentration in soil from Texas, US. nCuO was added into the solution because: 1) nCuO is not naturally occurring in appreciable amounts, but is released from industrial discharge into the water system. 2) Copper-containing pesticides are usually applied as suspension.

Fertilizer application

Two types of fertilizer stock solutions were made and diluted **1:100** just before use. Solution A was made with 4.5 kg of Peters Water Soluble Fertilizer N.P.K-15-5-15 in 19 L water. Solution B was made with 4.5 kg of Peters Water Soluble Fertilizer N.P.K-15-5-15 and 0.9 kg of Sprint 330 in 19 L of water. Dilutions (1:100) of Solution A and B were alternatively applied into the growth container on the water surface from day 49 until heading (flowering). Solution B was stopped when plants flowered.

Because Cu is an essential element for rice plant growth, minimum Cu is needed to maintain plant health. Copper concentration in Hoagland's solution is shown in Table B.1, which is equivalent to 0.95 µg/kg soil when added to the system. The estimated Cu contribution from Hoagland's solution is below 48 µg/kg from one-time application of fertilizers. The Cu additions from both Hoagland's solution and additional fertilizers were all below the background soil Cu concentration ("Cu before" in Table B.1). Therefore, the influence is negligible compared to both background Cu in soil and the added nCuO concentrations.

Soil property characterization

There were two types of soils before adding solutions and seeds: control soils and As containing soils. Post-treatment soils were obtained after 131-d exposure of As and nCuO. Before adding solutions and seeds, and after harvesting rice plants, dry soils were characterized for organic matter content, phosphorus content, concentrations of exchangeable cations, cation exchange capacity, pH and percent base saturation by Midwest Laboratory, Nebraska, US (Table B.6).

Statistical analysis

Generalized linear models (GLMs) were used to analyze the effects of nCuO and As on the growth parameters of rice plants and the interaction effect of nCuO and As on these parameters. A binomial distribution was specified for the germination model, because response variables were proportions but were not over dispersed [1]. A gaussian distribution was used for TGW, AGW. A gamma distribution was specified for other models, because response variables were continuous and the distributions were right-skewed. Generalized liner mixed modeling (GLMM) was used to analyze the effect of nCuO and As on the heading process with days of the experiment, because GLMM accommodates specification of appropriate covariance structure to account for repeated measures from the same containers over time (nested within time) and a random effect function to handle the heterogeneous variances among different growth containers. Heading data were averaged weekly due to the functional limit of the software. Models were assessed and accepted with low heterogeneity of the residual. Analysis of variance (ANOVA) was conducted to determine the main effects of nCuO and As, and their interaction effects. The p-values were adjusted with Tukey method to decrease the family-wise error rate. Data were reported as the mean value $\pm$ standard error of measurement (S.E.M) for each treatment group. Means were considered significantly different when $p < 0.05$. Regression models were developed between parameters to analyze the relationships. All statistical analyses were performed in R (version 3.3.2).
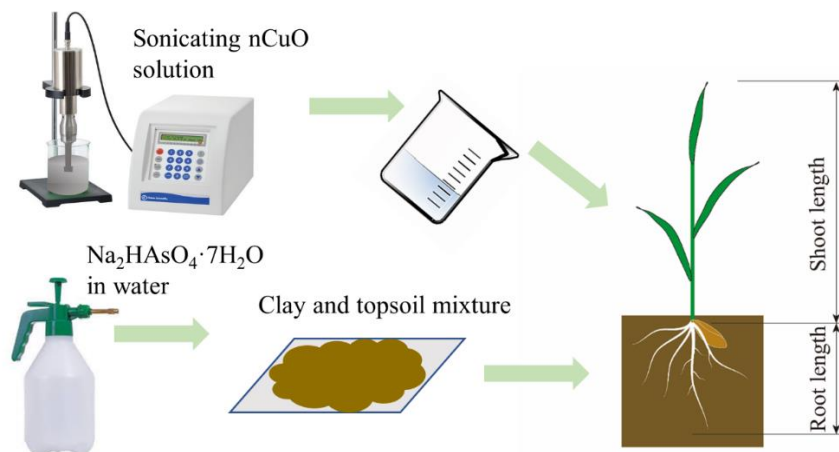
Figure B.1. Conceptual model of treatment preparation and exposure process in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution.
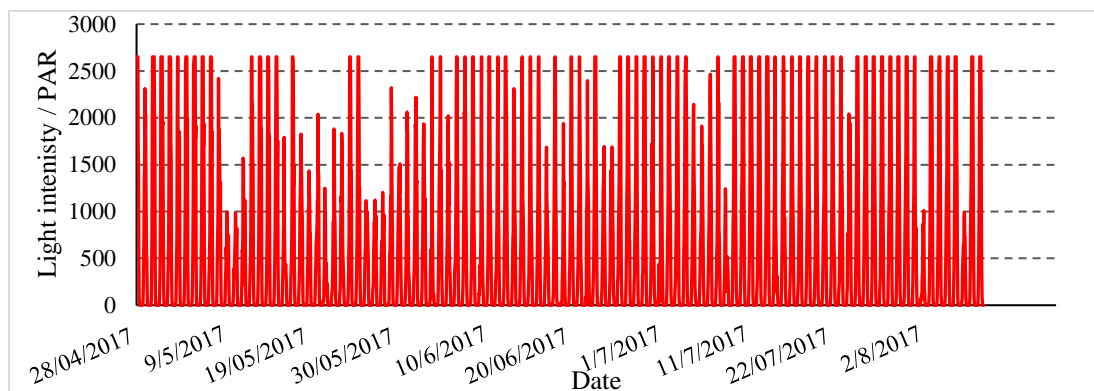
Figure B.2. Light intensity record in the greenhouse (Photosynthetically Active Radiation, PAR) during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution.
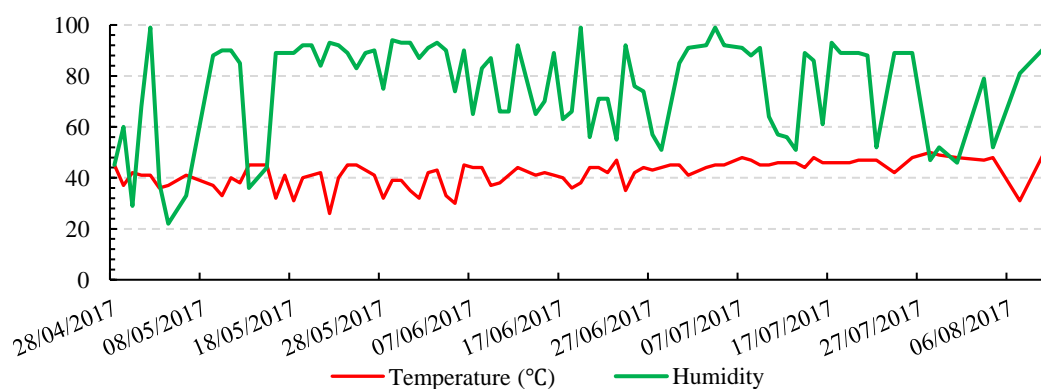
Figure B.3. Temperature and humidity records in the greenhouse during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution.
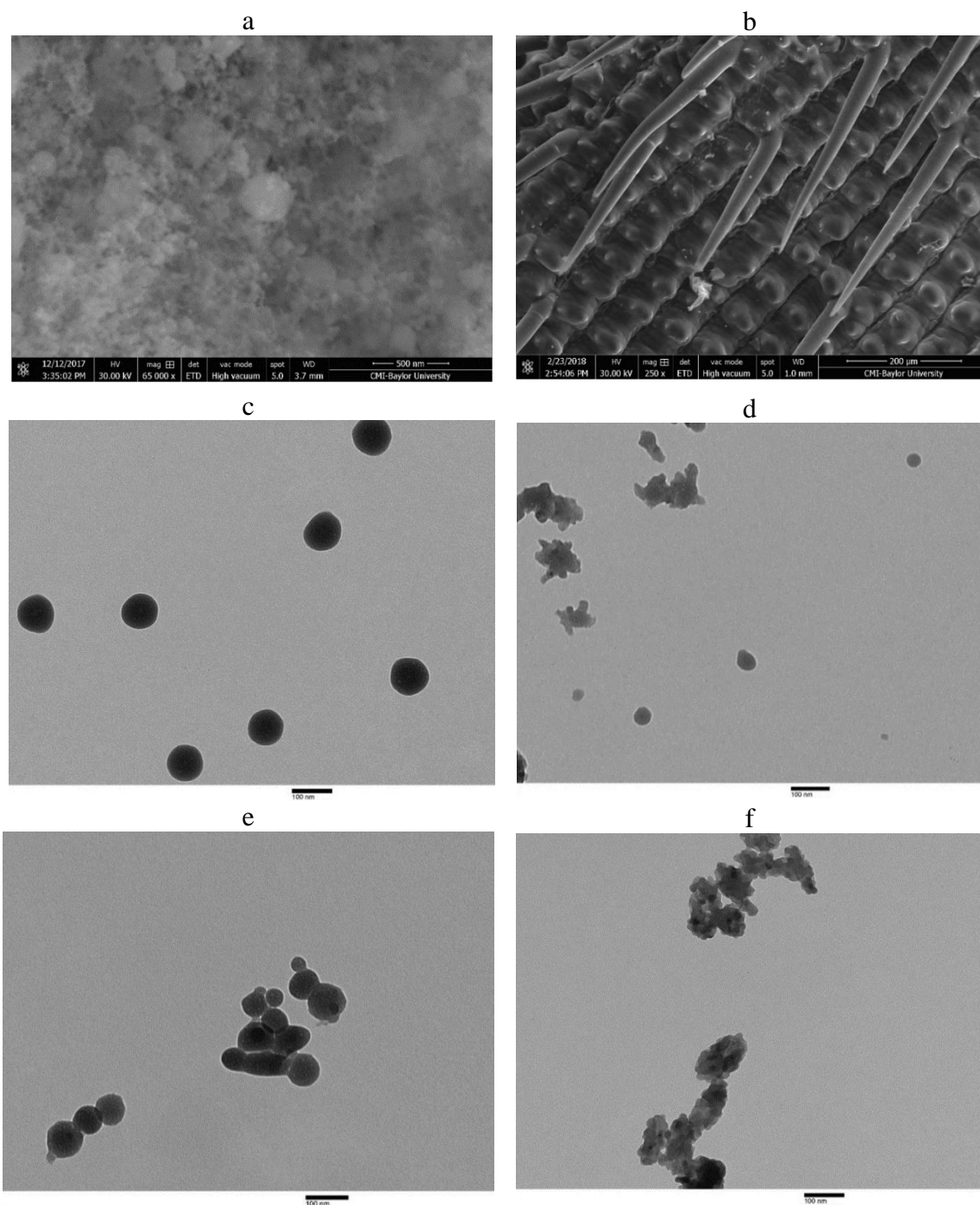
Figure B.4. SEM and TEM images from samples collected during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution. a. SEM image of copper oxide nanoparticle powder; b. SEM image of rice husk outer surface of grain samples collected on day 131; c–f. TEM images of nanoparticles and their aggregation in the solution samples collected on day 14(c. As+nCuO 100 mg/L, d. nCuO100 mg/L, e. As+nCuO 0.1mg/L and f. nCuO0.1 mg/L).
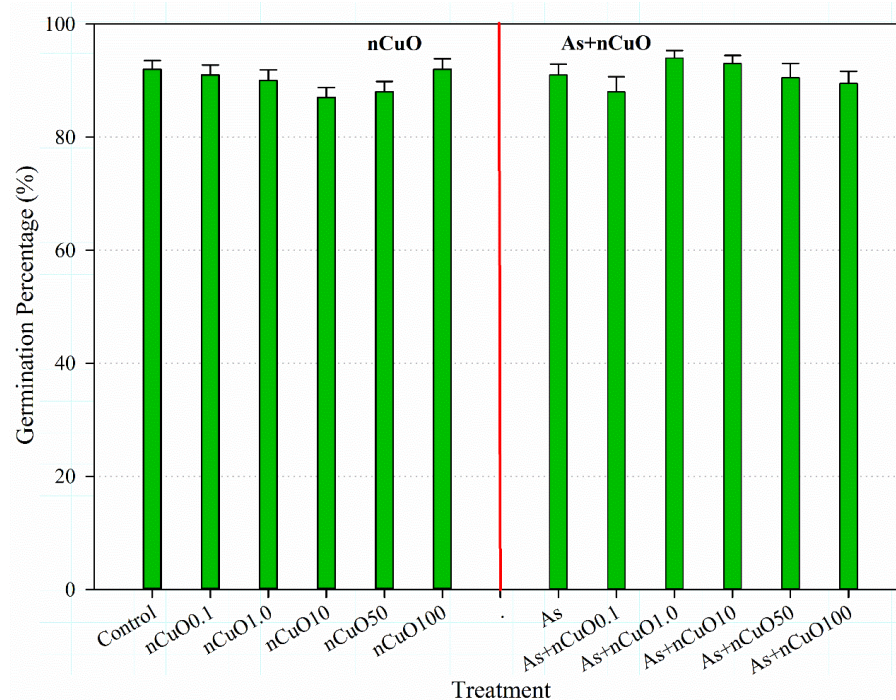
Figure B.5. Rice (*O.sativa japonica* 'Koshihikari') seed germination percentages during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 20).
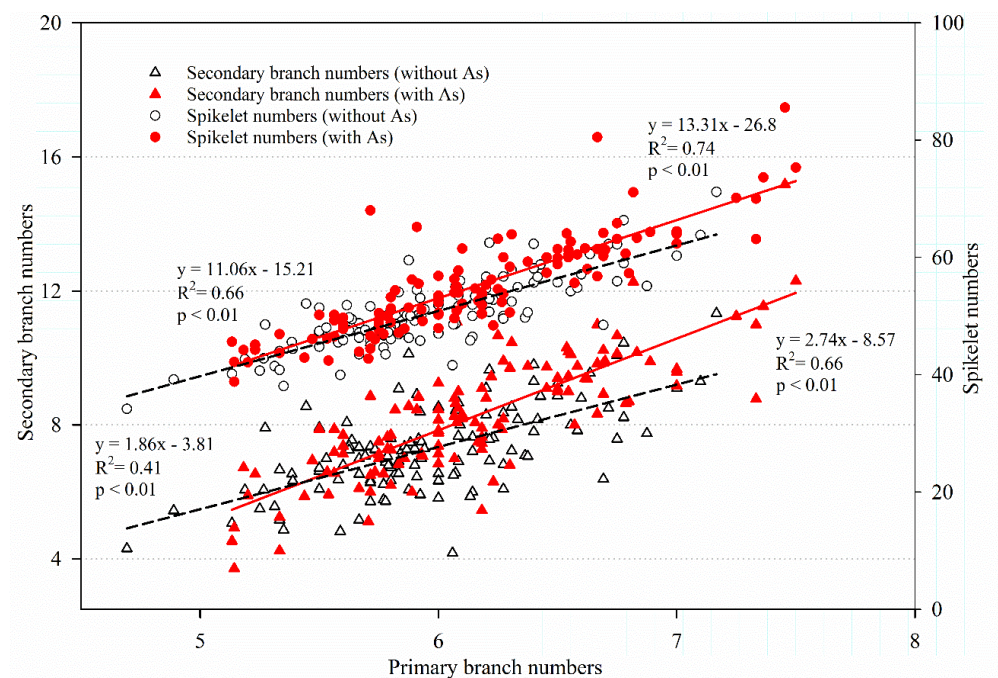
Figure B.6. Relationship between rice panicle parameters and grain numbers in treatment group with or without As addition in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 20).

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Table B.1. Composition of 20% Hoagland's solution used as nutrient for the early growth stage of rice seedlings during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

	Compound	Concentration (μM)
Hoagland's Solution	$MgCl_2$	400
	$CaCl_2 \cdot 2H_2O$	2000
	KCl	1000
	H_3BO_3	22.5
	$MnCl_2 \cdot 4H_2O$	4.5
	$ZnSO_4 \cdot 7H_2O$	0.5
	$CuSO_4 \cdot 5H_2O$	0.15
	MoO_3	0.07
	Fe-EDTA	45
	$Na_2SiO_3 \cdot 5H_2O$	1500
	PIPES buffer	5000

PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)) was used to maintain the pH (6.8–7.2) of the solution.

Table B.2. Characterization of copper oxide nanoparticles as exposure to the rice plants in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

Nominal concentration of nCuO (mg/L)	Hydrodynamic diameter (nm) [a]	Zeta potential (mV) [a]	PDI [a]
0.1	295 ± 3.4	-13.37 ± 0.69	0.33 ± 0.026
1.0	198 ± 3.4	-19.23 ± 0.58	0.27 ± 0.027
10	181 ± 1.4	-20.97 ± 0.93	0.15 ± 0.005
50	622 ± 16.5	-20.17 ± 0.46	0.23 ± 0.003
100	1246 ± 14.0	-16.50 ± 0.50	0.50 ± 0.038

The primary particle size of nCuO was 23-37 nm. The measurement was done near pH 7 at 25 ℃ with a Malvern Zetasizer Nano when the solution was made fresh and before being dosed in the growth pot. Each measurement was run in triplicate.

Table B.3. Soil characteristics before and after harvesting rice plants in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

Before adding nCuO containing solution

Soil type	Organic matter %	Phosphorus			Neutral ammonium acetate (exchangeable)				pH	CEC[2] (meq/100g)	Percent base saturation (computed)			
		P 1 (weak bray) (ppm)	P2 (strong bray) (ppm)	OB.P[1] (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)			%K	Mg%	Ca%	Na%
Control soil	3.9 ± 0.4	104 ± 8	131 ± 11	87 ± 39	843 ± 15	335 ± 0	2754 ± 55	NA	6.8 ± 0.1	18.8 ± 0.3	11.6 ± 0.4	14.9 ± 0.2	73.6 ± 0.6	NA
As containing soil	3.5 ± 0.2	100 ± 4	119 ± 25	94 ± 4	1033 ± 44	446 ± 58	3397 ± 515	190±47	7.0 ± 0.2	24.2 ± 3.4	11.1 ± 1.1	15.4 ± 0.1	70.2 ± 0.9	3.4± 0.4

After rice harvest

Soil type variables		Organic matter %	Phosphorus			Neutral ammonium acetate (exchangeable)				pH	CEC[2] (meq/100g)	Percent base saturation (computed)			
As (mg/kg)	nCuO (mg/L)		P 1 (weak bray) (ppm)	P2 (strong bray) (ppm)	OB.P[1] (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)			%K	Mg%	Ca%	Na%
0	0	4.5 ± 0.2	77 ± 8	129 ±10	96 ± 15	743 ± 40	318 ± 15	3161 ± 92	NA	7.9 ± 0.1	20.4 ± 0.7	9.4 ± 0.2	13.0 ± 0.2	77.7 ± 0.4	NA
0	0.1	4.3 ± 0.2	74 ± 4	142 ± 8	94 ± 5	864 ± 23	406 ± 3	3610 ± 27	184 ± 7	7.6 ± 0.1	24.4 ± 0.1	9.1± 0.3	13.8 ± 0.1	73.8 ± 0.4	3.3 ± 0.2
	1	4.3 ± 0.3	77 ± 1	142 ± 2	82 ± 5	928 ± 43	426 ± 23	3750 ± 192	212 ± 15	7.5 ± 0.1	25.6 ± 1.3	9.3 ± 0	13.8 ± 0.1	73.2 ± 0.2	3.6 ± 0.1
	10	3.7 ± 0.1	65 ± 4	122 ± 3	75 ± 2	1070 ± 65	452 ± 29	4320 ± 113	204 ± 11	7.5 ± 0.1	29.0 ± 1.0	9.5 ± 0.3	13.0 ± 0.4	74.6 ± 0.7	3.1 ± 0.1
	50	3.7 ± 0.5	75 ± 1	126 ± 8	78 ± 3	996 ± 19	438 ± 3	4260 ± 196	188 ± 4	7.5 ± 0.1	28.3 ± 1.1	9.0 ± 0.2	12.9 ± 0.4	75.2 ± 0.7	2.9 ± 0.1
	100	3.8 ± 0.1	58 ± 1	126 ± 14	69 ± 1	916 ± 0	386 ± 2	3980 ± 35	163 ± 2	7.5 ± 0	26.2 ± 0.2	9.0 ± 0.1	12.3 ± 0.1	76.1 ± 0.2	2.7 ± 0.0
	0.1	4.4 ± 0.5	83 ± 2	145 ± 1	96 ± 6	907 ± 80	428 ± 51	3550 ± 220	206 ± 20	7.6 ± 0.1	24.5 ± 1.9	9.5 ± 0.2	14.4 ± 0.7	72.4 ± 0.9	3.7 ± 0.1
	1	4.4 ± 0.4	92 ± 3	145 ± 4	102 ± 1	907 ± 119	420 ± 62	3390 ± 400	224 ± 39	7.5 ± 0.2	23.8 ± 3.0	9.8 ± 0.1	14.7 ± 0.3	71.4 ± 0.6	4.1 ± 0.2
10	10	5. 5 ± 0.5	91 ± 5	141 ± 7	112 ± 3	968 ± 34	450 ± 8	3550 ± 16	238 ± 7	7. 5 ± 0.1	25 ± 0.3	10.0 ± 0.3	15.0 ± 0.1	71.0 ± 0.5	4.1 ± 0.1
	50	5.2 ± 0.6	95 ± 3	152 ± 5	101 ± 11	889 ± 21	420 ± 28	3360 ± 137	212 ± 9	7.4 ± 0.2	23.5 ± 1	9.7 ± 0.2	14.8 ± 0.4	71.6 ± 0.2	3.9 ± 0.0
	100	4.7 ± 0.1	86 ± 6	142 ± 7	104 ± 0	866 ± 65	408 ± 32	3320 ± 238	198 ± 15	7.5 ± 0.1	23.1 ± 1.7	9.6 ± 0.0	14.7 ± 0.1	72.0 ± 0.1	3.7 ± 0.0

Values are means ± S.E.M, n = 2. [1]OB.P represents Olsen Bicarbonate P. [2]CEC represents Cation exchange capacity. NA: not available

Table B.4. Copper and arsenic concentration in the soil mixture before and after plant growth in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

Variable		Cu (µg/kg)		As (µg/kg)	
As (mg/kg)	CuO (mg/L)	Before	After	Before	After
0	0	11400 ± 211^{Aa}	12300 ± 199^{Aa}	4060 ± 48.3^{Aa}	3740 ± 101^{Aa}
	0.1	11600 ± 175^{Aa}	12300 ± 200^{Aa}	3950 ± 42.7^{Aa}	3800 ± 119^{Aa}
	1	11400 ± 112^{Aa}	13100 ± 290^{Aa}	4080 ± 48.2^{Aa}	3840 ± 80.7^{Aa}
	10	9020 ± 119^{Ba}	12600 ± 277^{Aa}	4950 ± 163^{Ba}	4790 ± 79.1^{Ba}
	50	8590 ± 98.5^{Ba}	33800 ± 2680^{Ba}	5050 ± 219^{Ba}	4500 ± 203^{BCa}
	100	8940 ± 263^{Ba}	55900 ± 7830^{Ca}	4810 ± 242^{Ba}	4170 ± 45.4^{ACa}
10	0	9300 ± 134^{Ab}	10300 ± 218^{Ab}	13500 ± 414^{Ab}	12100 ± 236^{Ab}
	0.1	10700 ± 489^{Bb}	10200 ± 147^{Ab}	13200 ± 343^{Ab}	11400 ± 423^{Ab}
	1	10300 ± 92.5^{Bb}	11700 ± 291^{Aa}	13300 ± 334^{Ab}	11200 ± 157^{Ab}
	10	10800 ± 436^{Bb}	14800 ± 854^{Ba}	12900 ± 184^{Ab}	11000 ± 230^{Ab}
	50	10600 ± 135^{Bb}	31500 ± 1840^{Ca}	13400 ± 479^{Ab}	11300 ± 290^{Ab}
	100	10100 ± 362^{ABb}	56400 ± 5450^{Da}	13800 ± 205^{Ab}	12000 ± 256^{Ab}
p-value	As	< 0.05	< 0.05	< 0.001	< 0.001
	nCuO	< 0.001	< 0.001	< 0.001	< 0.001
	A×N[1]	< 0.001	0.056	0.062	< 0.001

Values are means ± S.E.M, $n = 5$ per treatment group.
[1]A × N = As × nCuO interaction effect.
[A-C]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A×N = As × nCuO interaction effect.

Table B.5. Total copper concentration in solution media in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	Cu (µg/L)							
		Day1	Day7	Day21	Day35	Day49	Day63	Day84	Day112
0	0	66.6 ± 20.4	84.6 ± 13.1	80.9 ± 6.4	87.5 ± 6.8	53.8 ± 1.0	103 ± 13.4	40.2 ± 4.3	27.4 ± 1.3
	0.1	40.6 ± 7.8	78.5 ± 4.1	74.5 ± 5.8	63.6 ± 2.9	58.5 ± 3.3	129 ± 3.2	30.3 ± 2.7	26.2 ± 1.7
	1.0	92.4 ± 19.4	152 ± 42.9	81.6 ± 14.3	58.7 ± 2.4	68.3 ± 3.8	101 ± 4.77	37.2 ± 4.2	26.9 ± 1.4
	10	61.2 ± 23.7	76.6 ± 2.0	80.8 ± 8.8	69.5 ± 7.6	50.6 ± 3.2	208 ± 27.5	51.8 ± 9.9	47.3 ± 8.5
	50	394 ± 11.5	56.8 ± 4.7	149 ± 5.8	84.0 ± 8.6	82.2 ± 7.3	751 ± 145	87.5 ± 8.9	40.5 ± 3.7
	100	543 ± 14.2	97.6 ± 6.5	119 ± 5.5	87.2 ± 7.6	121 ± 26.1	589 ± 149	102.0 ± 9.9	41.1 ± 2.9
10	0	27.6 ± 3.47	79.9 ± 5.0	40.1 ± 9.8	78.4 ± 4.7	67.7 ± 18.5	100 ± 3.8	43.9 ± 2.6	32.4 ± 5.3
	0.1	60.5 ± 13.0	74.9 ± 3.8	67.6 ± 4.3	77.9 ± 5.3	54.0 ± 2.0	103 ± 2.3	30.4 ± 1.2	34.5 ± 5.2
	1.0	38.4 ± 10.6	74.9 ± 4.5	54.5 ± 2.2	65.4 ± 2.6	53.3 ± 3.5	110 ± 7.4	30.6 ± 1.2	28.9 ± 1.4
	10	152 ± 19.9	112 ± 8.7	75.1 ± 5.5	58.5 ± 2.7	44.7 ± 3.1	219 ± 12.1	31.6 ± 1.9	26.2 ± 0.6
	50	733 ± 150	231 ± 13.7	418 ± 44.4	88.4 ± 5.0	69.4 ± 4.1	927 ± 80.2	68.6 ± 10.7	54.4 ± 5.9
	100	727 ± 193	653 ± 199	228 ± 39.6	94.7 ± 3.5	76.6 ± 5.5	1530 ± 114	78.1 ± 6.6	71.7 ± 16.6
p-value	nCuO	< 0.001							
	As	< 0.001							
	Day	< 0.001							
	N×A[1]	< 0.001							
	N×D[2]	< 0.001							
	A×D[3]	< 0.001							
	N×A×D[4]	< 0.001							

Values are means ± S.E.M, $n = 5$ per treatment group.
[1]N×A1= nCuO × As interaction effect.
[2]N×D2 = nCuO × Day interaction effect.
[3]A×D3= As × Day interaction effect.
[4]N×A×D4= nCuO × As × Day interaction effect

Table B.6. Total arsenic concentration in solution media in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	As (µg/L)							
		Day1	Day7	Day21	Day35	Day49	Day63	Day84	Day112
0	0	24.5 ± 5.9	28.6 ± 3.8	19.4 ± 2.1	14.4 ± 0.9	14.3 ± 0.7	15.4 ± 0.4	7.7 ± 0.8	7.0 ± 1.2
	0.1	17.7 ± 2.7	26.6 ± 0.8	16.9 ± 2.0	10.1 ± 0.4	13.5 ± 0.9	14.4 ± 1.0	6.6 ± 1.3	3.5 ± 0.8
	1.0	21.7 ± 4.7	36.1 ± 2.3	20 ± 3.3	11.4 ± 0.9	13.5 ± 1.5	20.4 ± 1.1	8.1 ± 1.3	4.9 ± 1.2
	10	9.7 ± 1.2	15.6 ± 0.5	11.9 ± 0.8	8.5 ± 0.5	10.1 ± 0.7	13.4 ± 0.5	9.1 ± 1.1	8.1 ± 1.2
	50	8.7 ± 0.4	12.7 ± 0.3	11.9 ± 0.2	8.1 ± 0.5	10.4 ± 1.4	14.6 ± 0.8	9.3 ± 0.9	4.2 ± 0.1
	100	7.9 ± 0.6	13.4 ± 1.4	12.6 ± 0.8	7.4 ± 0.2	11.5 ± 1.3	13.0 ± 1.3	7.1 ± 0.5	4.4 ± 0.4
10	0	79.6 ± 8.9	168 ± 2.4	158 ± 15.1	61.5 ± 7.41	55.9 ± 5.8	73.4 ± 6.8	54.1 ± 4.09	37.5 ± 4.8
	0.1	73.1 ± 7.8	167 ± 5.0	112 ± 5.0	98.2 ± 2.79	79.3 ± 2.1	69.2 ± 5.1	40.3 ± 3.05	20.0 ± 2.2
	1.0	100 ± 12.6	186 ± 6.7	102 ± 3.0	77.3 ± 8.08	73.2 ± 7.9	71.7 ± 2.2	40.8 ± 2.42	27.4 ± 2.6
	10	91.9 ± 3.8	194 ± 9.0	116 ± 5.1	57.7 ± 7.36	73.6 ± 3.1	72.4 ± 3.5	45.3 ± 3.57	32.6 ± 4.8
	50	90.4 ± 4.9	179 ± 2.9	133 ± 12.8	82.0 ± 4.51	92.9 ± 3.3	77.8 ± 5.9	42.9 ± 4.69	25.8 ± 4.0
	100	91.1 ± 7.8	201 ± 16.4	130 ± 8.7	71.0 ± 5.48	92.1 ± 7.2	73.7 ± 5.5	37.5 ± 3.95	16.4 ± 2.0
P-value	nCuO	0.708							
	As	< 0.001							
	Day	< 0.001							
	N×A[1]	< 0.001							
	N×D[2]	< 0.001							
	A×D[3]	< 0.001							
	N×A×D[4]	< 0.001							

Values are means ± S.E.M, $n = 5$ per treatment group.
[1]N×A1= nCuO × As interaction effect.
[2]N×D2 = nCuO × Day interaction effect.
[3]A×D3= As × Day interaction effect.
[4]N×A×D4= nCuO × As × Day interaction effect.

Table B.7. 18-d seedling growth parameters in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	Sample numbers	Shoot length (cm)	Root length (cm)	Number of Root Branches
0	0	(n = 49)	27.5 ± 0.62 ^{ABa}	6.3 ± 0.29 ^{Aa}	8.9 ± 0.27 ^{Aa}
	0.1	(n = 48)	29.9 ± 1.09 ^{ABa}	5.3 ± 0.41 ^{Aa}	9.0 ± 0.40 ^{ABa}
	1	(n = 47)	31.1 ± 0.73 ^{Aa}	8.4 ± 0.29 ^{Ba}	10.3 ± 0.38 ^{ABCa}
	10	(n = 43)	29.4 ± 1.04 ^{ABa}	9.4 ± 0.37 ^{Ba}	10.4 ± 0.45 ^{ABCa}
	50	(n = 42)	28.8 ± 0.81 ^{ABa}	9.0 ± 0.32 ^{Ba}	10.8 ± 0.40 ^{Ca}
	100	(n = 54)	26.6 ± 0.86 ^{Ba}	9.4 ± 0.26 ^{Ba}	10.5 ± 0.35 ^{BCa}
10	0	(n = 69)	30.9 ± 0.81 ^{ABb}	6.0 ± 0.27 ^{ABa}	9.5 ± 0.32 ^{Aa}
	0.1	(n = 35)	31.5 ± 1.00 ^{ABa}	5.5 ± 0.42 ^{Aa}	7.6 ± 0.45 ^{Bb}
	1	(n = 51)	32.8 ± 0.80 ^{Aa}	6.9 ± 0.33 ^{BCb}	9.7 ± 0.35 ^{Aa}
	10	(n = 55)	28.6 ± 1.00 ^{Ba}	7.8 ± 0.28 ^{Cb}	9.6 ± 0.36 ^{Aa}
	50	(n = 50)	28.7 ± 0.91 ^{Ba}	7.8 ± 0.42 ^{Ca}	9.2 ± 0.40 ^{ACb}
	100	(n = 49)	31.3 ± 0.84 ^{ABb}	7.7 ± 0.39 ^{Cb}	8.2 ± 0.34 ^{BCb}
p-value	As		< 0.001	< 0.001	< 0.001
	nCuO		0.001	< 0.001	< 0.001
	A×N[1]		0.012	0.012	< 0.001

Values are means ± S.E.M.
$^{A-C}$Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
$^{a-b}$Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A×N = As × nCuO interaction effect.

Table B.8. 18-d seedling growth parameters in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	Sample numbers	Shoot dry weight (mg)	Root dry weight (mg)
0	0	(n = 53)	25.1 ± 0.91^{Aa}	3.2 ± 0.19^{Aa}
	0.1	(n = 48)	25.1 ± 1.18^{Aa}	2.4 ± 0.19^{Ba}
	1	(n = 47)	32.6 ± 1.29^{Ba}	4.2 ± 0.22^{Ca}
	10	(n = 43)	30.0 ± 1.39^{Ba}	5.6 ± 0.27^{Da}
	50	(n = 45)	30.7 ± 1.29^{Ba}	6.1 ± 0.28^{Da}
	100	(n = 47)	31.0 ± 1.29^{Ba}	6.5 ± 0.25^{Da}
10	0	(n = 80)	27.7 ± 0.87^{Aa}	3.4 ± 0.17^{Aa}
	0.1	(n = 45)	23.8 ± 1.07^{Ba}	2.3 ± 0.15^{Ba}
	1	(n = 50)	30.5 ± 1.26^{Aa}	3.6 ± 0.23^{Ab}
	10	(n = 50)	28.1 ± 1.14^{Aa}	3.6 ± 0.17^{Ab}
	50	(n = 47)	26.2 ± 1.07^{ABb}	3.4 ± 0.16^{Ab}
	100	(n = 45)	26.9 ± 0.85^{ABb}	3.1 ± 0.15^{Ab}
p-value	As		< 0.001	<0.001
	nCuO		0.018	<0.001
	A×N[1]		0.009	<0.001

Values are means ± S.E.M.
[A-D]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A×N = As × nCuO interaction effect.

Table B.9. Parameters of the mature rice panicles harvested from each replicate growth container in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	CuO (mg/L)	TNRP	PAL	PBN	SBN	SN	PW	TPW
0	0	11.6 ± 0.43 [ABa]	14.9 ± 0.12 [ABa]	6.0 ± 0.09 [ABa]	7.5 ± 0.16 [ABa]	52.0 ± 0.87 [ABa]	1.0 ± 0.04 [Aa]	11.7 ± 0.40 [ABa]
	0.1	10.4 ± 0.52 [Aa]	15.9 ± 0.25 [Aa]	6.3 ± 0.12 [Aa]	8.2 ± 0.36 [Aa]	56.0 ± 1.6 [Aa]	1.0 ± 0.03 [Aa]	10.3 ± 0.45 [Aa]
	1	13.2 ± 0.62 [BCDa]	14.7 ± 0.23 [Ba]	5.9 ± 0.12 [ABa]	7.1 ± 0.34 [ABa]	49.0 ± 1.52 [Ba]	1.0 ± 0.03 [Aa]	13.1 ± 0.55 [BCa]
	10	13.0 ± 0.44 [BCa]	14.5 ± 0.16 [Ba]	6.0 ± 0.08 [ABa]	7.2 ± 0.26 [ABa]	50.1 ± 1.13 [Ba]	1.1 ± 0.02 [Aa]	13.6 ± 0.48 [Ca]
	50	14.8 ± 0.56 [CDa]	14.5 ± 0.14 [Ba]	5.9 ± 0.08 [ABa]	6.9 ± 0.22 [Ba]	49.1 ± 0.94 [Ba]	1.0 ± 0.02 [Aa]	14.4 ± 0.45 [Ca]
	100	15.5 ± 0.63 [Da]	14.4 ± 0.37 [Ba]	5.8 ± 0.10 [Ba]	6.7 ± 0.27 [Ba]	46.8 ± 1.18 [Ba]	1.0 ± 0.03 [Aa]	15.1 ± 0.37 [Ca]
10	0	11.9 ± 0.45 [Aa]	15.5 ± 0.28 [Aa]	6.2 ± 0.10 [ABa]	8.4 ± 0.35 [ABa]	54.7 ± 1.64 [Aa]	1.2 ± 0.04 [Ab]	13.7 ± 0.48 [Ab]
	0.1	12.9 ± 0.42 [Ab]	15.8 ± 0.19 [Aa]	6.2 ± 0.09 [ABa]	8.5 ± 0.37 [ABa]	56.0 ± 1.77 [ABa]	1.3 ± 0.05 [ABb]	15.8 ± 0.47 [Bb]
	1	12.6 ± 0.56 [Aa]	15.9 ± 0.39 [Ab]	6.2 ± 0.13 [ABa]	8.2 ± 0.33 [ABCb]	53.7 ± 1.53 [Ab]	1.2 ± 0.04 [ABb]	15.0 ± 0.60 [ABb]
	10	12.1 ± 0.44 [Aa]	15.4 ± 0.19 [Ab]	6.0 ± 0.09 [ABa]	8.0 ± 0.26 [ACa]	52.5 ± 1.21 [Aa]	1.1 ± 0.03 [Aa]	13.2 ± 0.41 [Aa]
	50	12.2 ± 0.49 [Ab]	15.6 ± 0.21 [Ab]	5.8 ± 0.11 [Aa]	7.0 ± 0.41 [Ca]	50.7 ± 1.40 [Aa]	1.1 ± 0.03 [Ab]	13.4 ± 0.48 [Aa]
	100	11.6 ± 0.63 [Ab]	17.0 ± 0.36 [Bb]	6.5 ± 0.15 [Bb]	9.5 ± 0.49 [Bb]	62.4 ± 2.37 [Bb]	1.3 ± 0.05 [Bb]	14.7 ± 0.38 [ABa]
p-value	As	< 0.05	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001
	nCuO	< 0.001	< 0.001	< 0.05	< 0.001	< 0.001	0.102	< 0.001
	A×N[1]	< 0.001	< 0.001	< 0.01	< 0.001	< 0.001	< 0.01	< 0.001

TNRP: Total number of ripe panicles. PAL: panicle axis length (cm). PBN: primary branch number. SBN: secondary branch number per panicle.
SN: total spikelet numbers per panicle. PW: average panicle weight (g). TPW: total panicle weight in each replicate container (g).
Values are means ± S.E.M, $n = 20$ per treatment group.
[A-D] Means in a column at the same As level with a common superscript letter are similar ($p < 0.05$).
[a-b] Means in a column at the same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1] A × N = As × nCuO interaction effect.

Table B.10. Average grain weight and total grain weight in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	Total grain weight (g)	Average grain weight (mg)
0	0	10.6 ± 0.46 [ABa]	24.1 ± 0.15[ABa]
	0.1	9.0 ± 0.45 [Aa]	23.6 ± 0.15[Aa]
	1	11.3 ± 0.50 [BCa]	24.7 ± 0.19 [BCa]
	10	12.4 ± 0.44 [CDa]	25.2 ± 0.16 [Ca]
	50	12.8 ± 0.44 [CDa]	24.0 ± 0.11 [ABa]
	100	13.3 ± 0.38 [Da]	23.5 ± 0.29 [Aa]
10	0	12.0 ± 0.46 [Ab]	25.3 ± 0.22 [ABb]
	0.1	14.2 ± 0.45 [Bb]	25.9 ± 0.27 [ACb]
	1	13.4 ± 0.48 [ABb]	26.2 ± 0.19 [Cb]
	10	11.8 ± 0.41 [Aa]	23.2 ± 0.19 [Db]
	50	12.0 ± 0.41 [Aa]	24.7 ± 0.28 [Bb]
	100	12.8 ± 0.31 [ABa]	25.0 ± 0.19 [Bb]
p-value	As	< 0.001	<0.001
	nCuO	0.001	<0.001
	A×N[1]	< 0.001	<0.001

Values are means ± S.E.M.
$n = 20$ per treatment for total grain weight.
$n = 5$ per treatment for average grain weight.
[A-D]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
1A×N = As × nCuO interaction effect.

Table B.11. Mature plants straw and root biomass and ratios of straw-grain and root-straw in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	Straw DW (g)	Grain to straw ratio	Rice root DW (g)	Root to straw ratio
0	0	21.2 ± 1.56 [ABa]	0.52 ± 0.030 [Aa]	1.7 ± 0.05 [ABa]	0.08 ± 0.007 [ABa]
	0.1	26.1 ± 1.78 [Ca]	0.35 ± 0.0203 [Ba]	1.8 ± 0.12 [ABa]	0.07 ± 0.010 [Aa]
	1.0	22.0 ± 1.60 [ADa]	0.53 ± 0.026 [Aa]	2.3 ± 0.12 [ACa]	0.10 ± 0.007 [Ba]
	10	18.2 ± 1.28 [Ba]	0.70 ± 0.027 [Ca]	1.6 ± 0.11 [Ba]	0.09 ± 0.009 [ABa]
	50	26.2 ± 0.64 [Ca]	0.49 ± 0.019 [Aa]	2.8 ± 0.18 [Ca]	0.11 ± 0.009 [Ba]
	100	25.4 ± 0.57 [CDa]	0.53 ± 0.019 [Aa]	2.5 ± 0.15 [Ca]	0.10 ± 0.006 [ABa]
10	0	20.8 ± 1.08 [ABa]	0.59 ± 0.023 [ABa]	1.9 ± 0.21 [Aa]	0.09 ± 0.011 [Aa]
	0.1	22.6 ± 0.74 [ACb]	0.64 ± 0.026 [ABb]	2.0 ± 0.13 [Aa]	0.09 ± 0.003 [Aa]
	1.0	20.0 ± 0.83 [ABa]	0.69 ± 0.028 [Ab]	1.7 ± 0.12 [ABb]	0.08 ± 0.003 [Ab]
	10	19.3 ± 1.38 [ABa]	0.64 ± 0.022 [ABa]	1.6 ± 0.13 [ABa]	0.09 ± 0.005 [Aa]
	50	19.1 ± 0.73 [Bb]	0.63 ± 0.020 [ABb]	1.4 ± 0.10 [Bb]	0.07 ± 0.004 [Ab]
	100	24.7 ± 0.81 [Ca]	0.53 ± 0.016 [Ba]	2.0 ± 0.16 [Ab]	0.08 ± 0.009 [Aa]
p-value	As	<0.001	<0.001	<0.001	0.113
	nCuO	<0.001	<0.001	<0.001	0.709
	A×N[1]	<0.001	<0.001	<0.001	0.004

Values are means ± S.E.M, $n = 20$ per treatment group for straw DW, $n = 5$ per treatment group for rice root DW.
[A-C]Means in a column at the same As concentration with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at the same nCuO concentration with a common superscript letter are similar ($p < 0.05$).
[1]A × N = As × nCuO interaction effect.

Table B.12. Accumulation of copper and arsenic in dehusked rice grains in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

Variable		Cu (ng/g)	As (ng/g)
As (mg/kg)	CuO (mg/L)	Dehusked grain	Dehusked grain
0	0	9890 ± 471 [Aa]	44.4 ± 4.70 [Aa]
	0.1	9870 ± 577 [ABa]	39.2 ± 4.33 [Aa]
	1	8220 ± 292 [Ba]	40.9 ± 6.32 [Aa]
	10	9050 ± 438 [ABa]	48.1 ± 4.41 [Aa]
	50	8350 ± 319 [ABa]	49.0 ± 4.75 [Aa]
	100	9410 ± 211 [ABa]	38.5 ± 1.75 [Aa]
10	0	6820 ± 400 [Ab]	198 ± 21.1 [Ab]
	0.1	7680 ± 197 [ABb]	132 ± 8.25 [ABb]
	1	8310 ± 477 [ABa]	138 ± 7.5 [ABb]
	10	8720 ± 544 [BCa]	167 ± 15.9 [ABb]
	50	10100 ± 466 [Cb]	128 ± 15.4 [Bb]
	100	8970 ± 415 [BCa]	175 ± 13.7 [ABb]
p-value	As	0.005	< 0.001
	nCuO	0.090	0.205
	A×N[1]	< 0.001	0.01

Values are means ± S.E.M, $n = 5$ per treatment group.
[A-C]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A×N = As × nCuO interaction effect

Table B.13. Uptake of copper and arsenic in 18-d seedlings during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

Variable		Cu (ng/g)		As (ng/g)	
As (mg/kg)	nCuO (mg L)	Root	Shoot	Root	Shoot
0	0	21200 ± 2590 [Aa]	12800 ± 1150 [Aa]	1920 ± 362 [Aa]	612 ± 70.3 [ABa]
	0.1	19000 ± 1990 [Aa]	11400 ± 486 [Aa]	4430 ± 652 [Ba]	714 ± 41.2 [Aa]
	1	20100 ± 517 [ABa]	11000 ± 150 [Aa]	3100 ± 599 [ABa]	511 ± 21.3 [Ba]
	10	16000 ± 1140 [Ba]	9620 ± 674 [Aa]	2790 ± 278 [ABa]	585 ± 49.7 [ABa]
	50	76000 ± 4640 [Ca]	17300 ± 90.7 [Ba]	2590 ± 176 [ABa]	532 ± 17.6 [Ba]
	100	81300 ± 6750 [Ca]	18300 ± 580 [Ba]	2190 ± 147 [Aa]	543 ± 17.4 [Ba]
10	0	16000 ± 535 [Ab]	8680 ± 133 [Ab]	21200 ± 2630 [Ab]	2850 ± 63.3 [Ab]
	0.1	18100 ± 1420 [Ab]	8430 ± 244 [ABa]	17800 ± 2730 [Ab]	2980 ± 81.3 [Ab]
	1	17200 ± 1240 [ABb]	8780 ± 203 [ABa]	16000 ± 1750 [Ab]	3240 ± 175 [Ab]
	10	21700 ± 691 [Ba]	10200 ± 159 [Bb]	16400 ± 2250 [Ab]	3090 ± 187 [Ab]
	50	33400 ± 1790 [Ca]	16600 ± 304 [Cb]	8110 ± 643 [Bb]	3170 ± 131 [Ab]
	100	103000 ± 4490 [Db]	20800 ± 525 [Db]	20700 ± 2510 [Ab]	3060 ± 130 [Ab]
p-value	As	0.028	< 0.001	< 0.001	< 0.001
	nCuO	< 0.001	< 0.001	< 0.001	0.894
	A×N[1]	< 0.001	< 0.001	< 0.001	< 0.001

Values are means ± S.E.M, $n = 5$ per treatment group.
[A-D]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A×N = As × nCuO interaction effect.

References

1. Crawley, M. J., *The R book*. 2nd ed.; Wiley: Chichester, West Sussex, United Kingdom, 2013.

APPENDIX C

Supporting Information for Chapter Four: Distribution and Speciation of Copper and Arsenic in Rice Plants (*O.sativa japonica* 'Koshihikari') Treated with Copper Oxide Nanoparticles and Arsenic during a Life Cycle

Number of pages: 28

Number of tables: 9

Number of figures: 12

Additional text is provided about details of sample preparation for XANES analysis, and synthesis and characterization of cupric arsenate. Tabular data in this document support the depicted figures in the main text of the manuscript, and may also be useful for those who are conducting research to compare means and ranges, or those engaged in meta-analysis of ongoing research addressing plant sensitivity to metals.

Materials and Methods

Materials and procedural details of rice (*O.sativa japonica* 'Koshihikari') life cycle exposure to nCuO and As were included in our previous publication [1]. The soil mixture was composed of two commercial substances (60% Grainger clay soil, Catalog # 2258, 40% Lowe's topsoil, # 235384). The clay soil was ground with an automatic continuous hammer mill grinder (DF-15, ECO-WORTHY). The topsoil was sifted through a 2-mm sieve. The two types of soils were homogenized by spreading on the tarp, turning and spreading the soil with a trowel, and folding the tarp. These steps were repeated multiple times (> 20). The As treatment (10 mg/kg soil) were prepared by

spraying water solution containing $Na_2HAsO_4 \cdot 7H_2O$ (Sigma-Aldrich, lot #

BCBM0939V) onto the spread soil mixture surface and homogenizing by mixing as

described above. nCuO (Nano-Arc®, 97.5%, 23–37 nm, APS powder, Alfa Aesar, MA,

USA) treatments were prepared at 6 nominal concentrations (0.1, 1.0, 10, 50, and 100

mg/L) in 20% Hoagland's solution. Twelve treatments (6×2 combinations of the nCuO

and As) were prepared. Each treatment had 20 replicates, with 1.5 kg of soil mixture in

each replicate container (Berry Plastics ID: T60785CP, 2.5 L). The soil was saturated

with deionized (DI) water in each container. The next day, nutrient solutions with

appropriate nCuO concentrations were added to cover the soil with 4 cm (150 mL) of

liquid. nCuO solutions were replenished on day 35. Ten rice seeds (*Oryza sativa*

japonica, Kitazawa Seed Company, CA, USA, pre-soaked for 12 hours in DI water) were

water-sowed in each container. Hoagland's solution was added to the container as

nutrient every other day for the first two weeks of seedling growth. Additional fertilizers

were applied to optimize plant growth in later stages. Two types of fertilizer stock

solutions were made and diluted 1:100 just prior to use. Solution A was made with 4.5 kg

of Peters Water Soluble Fertilizer N.P.K-15-5-15 in 19 L water. Solution B was made

with 4.5 kg of Peters Water Soluble Fertilizer N.P.K-15-5-15 and 0.9 kg of Sprint 330 in

19 L of water. Dilutions (1:100) of Solution A and B were alternatively applied into the

growth container on the water surface from day 49 until heading. Solution B was stopped

when plants flowered. Water depth was maintained with DI water for the rest of the time.

On day 18, only 2 well-established seedlings were kept in the container with 4"

separating them for the life cycle growth. Water addition was stopped when the majority

of panicles started to mature. Plants were harvested after one week without watering on

day 131. The experiment was conducted in a greenhouse. The light intensity (measured with Odyssey Photosynthetic Active Radiation Logger and calibrated to the PAR), temperature and humidity (monitored with an Acurite Digital monitor of humidity and temperature) in the greenhouse was recorded during the entire study (Figure C1-C2) [1].

Sampling and Analysis

Growth Media

Before adding solutions and sowing the seeds, and after harvesting rice plants, dry soils were characterized for organic matter content, phosphorus content, concentrations of exchangeable cations, cation exchange capacity, pH and percent base saturation by Midwest Laboratory, Nebraska, US (Table C1). Total Cu and As concentrations were also determined in the soil samples with ICP-MS after digestion with HNO_3 and H_2O_2 (adapted method from USEPA 3050B for solid samples [2]) (Table C2).

Solutions (0.5 mL) were sampled from each of the 20 growth containers and grouped to 5 replicates (2 mL) for each treatment weekly during the growth period. Cu and As concentrations were measured in the solution with ICP-MS after being digested with HNO_3 and HCl (adapted method from USEPA 200.8[3]).

Nanoparticle characterization of nCuO in solutions was conducted before adding to the growth container and also during the process of rice plant growth (Figure C3).

Early Seedlings and Mature Rice Plant Samples

Early seedlings samples were collected on day 18. Flag leaves paired with husks and dehusked grains (Figure C4) were samples on day 120 and day 131. Mature plants roots and stems were sampled after harvest the rice panicles on day 131. Total

concentrations of Cu and As were measured in early seedling shoots and roots, mature plant roots, stems, flag leaves, husks and dehusked grains with ICP-MS after digestion (adapted method from USEPA 3050B [2]).

<center>*X-Ray Absorption Near Edge Structure (XANES) Analysis*</center>

Sample and Reference Preparation

Seedling samples were dried in the oven at 40 ℃. Grain samples were sliced into 70 nm in thickness along the longest direction with microtome and attached onto the Kapton tape. All samples were then taped onto the stage with double-sided tapes under the beam. Different portions of seedlings were chosen for collecting the XANES information (Figure C5).

All standard reference materials were ground in a mortar, spread onto a strip of one-sided tape and fold to cover the compound, and loaded onto the stage with double-sided tape.

Synthesis and Characterization of Cupric Arsenate

Solution A was made with dissolving 0.05g NaOH in 25 ml genpure water, and adding 0.78 g $Na_2HAsO_4 \cdot 7H2O$ afterwards. Solution B was made with dissolving 0.188 g $CuSO4 \cdot 5H_2O$ in 25 ml genpure water. Solution A and B were mixed together and interacted with a magnetic bar stirring in the beaker. After 1 h of reaction, the mixture was filter with the final product leaving on the surface of the filter paper. The synthesized compound was dried in the oven and ground into fine powder for elemental analysis with scanning electron microscopy-energy dispersive x-ray spectroscopy (SEM-EDX, FEI

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Company). The atomic percentages for the synthesized mixture were Cu 8.65%, As 2.46% and O 32.69% (Figure C6), which confirms the presence of cupric arsenate.

Data Acquisition at the Beamline

Micro-XRF maps and Cu and As K-edge XANES were measured at GSECARS X-ray microprobe beamline 13-ID-E of the Advanced Photon Source and Argonne National Lab. This beamline uses a 36 mm period undulator, a cryogenically-cooled Si(111) monochromator, and Kirkpatrick-Baez focusing mirrors to make a focused monochromatic X-ray beam of 2 x 2 microns with about 10^{11} photons per second. The incident beam intensity was measured with an ion chamber, and the X-ray fluorescence was measured with a 4-element Si-drift detector with fast electronics allowing count rates above 1 MHz per detector element with modest deadtime. X-ray fluorescence maps were measured by continuously moving the sample in the beam, typically binning the data into 5 microns pixels, with dwell times of 30 to 50 milliseconds per pixel. XRF Mapping data was processed with the Larch software [4]. XANES spectra for reference standards were measured in transmission mode, while XANES for samples were measured in fluorescence mode. For micro-XANES, energies selected by the Si(111) monochromator were scanned from 8900 to 9215 eV for Cu K-edge XAFS using 0.1 eV steps between 8970 and 9005 eV and larger steps outside this region. For As K-edge XAFS, the energy was scanned from 11765 to 12110 eV, with 0.15 eV steps between 11852 and 11882 eV. XAFS was collected by continuously scanning the monochomator energy and undulator energy, with typical dwell times of 2 seconds per energy point. For most XAFS scans measured from points selected from the XRF maps, only 1 scan was collected per point.

The Athena software was applied to process and analyze XANES data [5]. The spectra were energy-calibrated and normalized. Linear combination fitting (LCF) was used to calculate the percentage of Cu and As species at a range of -20 –50 and -20–30 with respect to the Cu and As K-edge, respectively, and no more than four standards were included in one fit. R factor and reduced χ^2 were chosen as parameters to determine the best fit.

Table C.1. Soil characteristics before and after harvesting rice (*O.sativa japonica* 'Koshihikari') plants in a greenhouse study with131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

Before adding nCuO containing solution

Soil type	Organic matter %	P 1 (weak bray) (ppm)	P2 (strong bray) (ppm)	OB.P[1] (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	pH	CEC[2] (meq/100g)	%K	Mg%	Ca%	Na%
Control soil	3.9 ± 0.4	104 ± 8	131 ± 11	87 ± 39	843 ± 15	335 ± 0	2754 ± 55	NA	6.8 ± 0.1	18.8 ± 0.3	11.6 ± 0.4	14.9 ± 0.2	73.6 ± 0.6	NA
As containing soil	3.5 ± 0.2	100 ± 4	119 ± 25	94 ± 4	1033 ± 44	446 ± 58	3397 ± 515	190±47	7.0 ± 0.2	24.2 ± 3.4	11.1 ± 1.1	15.4 ± 0.1	70.2 ± 0.9	3.4± 0.4

After rice harvest

As (mg/kg)	nCuO (mg/L)	Organic matter %	P 1 (weak bray) (ppm)	P2 (strong bray) (ppm)	OB.P[1] (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	pH	CEC[2] (meq/100g)	%K	Mg%	Ca%	Na%
0	0	4.5 ± 0.2	77 ± 8	129 ±10	96 ± 15	743 ± 40	318 ± 15	3161 ± 92	NA	7.9 ± 0.1	20.4 ± 0.7	9.4 ± 0.2	13.0 ± 0.2	77.7 ± 0.4	NA
0	0.1	4.3 ± 0.2	74 ± 4	142 ± 8	94 ± 5	864 ± 23	406 ± 3	3610 ± 27	184 ± 7	7.6 ± 0.1	24.4 ± 0.1	9.1± 0.3	13.8 ± 0.1	73.8 ± 0.4	3.3 ± 0.2
	1	4.3 ± 0.3	77 ± 1	142 ± 2	82 ± 5	928 ± 43	426 ± 23	3750 ± 192	212 ± 15	7.5 ± 0.1	25.6 ± 1.3	9.3 ± 0	13.8 ± 0.1	73.2 ± 0.2	3.6 ± 0.1
	10	3.7 ± 0.1	65 ± 4	122 ± 3	75 ± 2	1070 ± 65	452 ± 29	4320 ± 113	204 ± 11	7.5 ± 0.1	29.0 ± 1.0	9.5 ± 0.3	13.0 ± 0.4	74.6 ± 0.7	3.1 ± 0.1
	50	3.7 ± 0.5	75 ± 1	126 ± 8	78 ± 3	996 ± 19	438 ± 3	4260 ± 196	188 ± 4	7.5 ± 0.1	28.3 ± 1.1	9.0 ± 0.2	12.9 ± 0.4	75.2 ± 0.7	2.9 ± 0.1
	100	3.8 ± 0.1	58 ± 1	126 ± 14	69 ± 1	916 ± 0	386 ± 2	3980 ± 35	163 ± 2	7.5 ± 0	26.2 ± 0.2	9.0 ± 0.1	12.3 ± 0.1	76.1 ± 0.2	2.7 ± 0.0
10	0.1	4.4 ± 0.5	83 ± 2	145 ± 1	96 ± 6	907 ± 80	428 ± 51	3550 ± 220	206 ± 20	7.6 ± 0.1	24.5 ± 1.9	9.5 ± 0.2	14.4 ± 0.7	72.4 ± 0.9	3.7 ± 0.1
	1	4.4 ± 0.4	92 ± 3	145 ± 4	102 ± 1	907 ± 119	420 ± 62	3390 ± 400	224 ± 39	7.5 ± 0.2	23.8 ± 3.0	9.8 ± 0.1	14.7 ± 0.3	71.4 ± 0.6	4.1 ± 0.2
	10	5. 5 ± 0.5	91 ± 5	141 ± 7	112 ± 3	968 ± 34	450 ± 8	3550 ± 16	238 ± 7	7. 5 ± 0.1	25 ± 0.3	10.0 ± 0.3	15.0 ± 0.1	71.0 ± 0.5	4.1 ± 0.1
	50	5.2 ± 0.6	95 ± 3	152 ± 5	101 ± 11	889 ± 21	420 ± 28	3360 ± 137	212 ± 9	7.4 ± 0.2	23.5 ± 1	9.7 ± 0.2	14.8 ± 0.4	71.6 ± 0.2	3.9 ± 0.0
	100	4.7 ± 0.1	86 ± 6	142 ± 7	104 ± 0	866 ± 65	408 ± 32	3320 ± 238	198 ± 15	7.5 ± 0.1	23.1 ± 1.7	9.6 ± 0.0	14.7 ± 0.1	72.0 ± 0.1	3.7 ± 0.0

Values are means ± S.E.M, n = 2. [1]OB.P represents Olsen Bicarbonate P. [2]CEC represents Cation exchange capacity. NA: not available

Table C.2. Copper and arsenic concentration in the soil mixture before and after rice (*O.sativa japonica* 'Koshihikari') plant growth in a greenhouse study 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

Variable		Cu (µg/kg)		As (µg/kg)	
As (mg/kg)	CuO (mg/L)	Before	After	Before	After
0	0	11400 ± 211Aa	12300 ± 199Aa	4060 ± 48.3 Aa	3740 ± 101Aa
	0.1	11600 ± 175 Aa	12300 ± 200Aa	3950 ± 42.7 Aa	3800 ± 119Aa
	1	11400 ± 112 Aa	13100 ± 290Aa	4080 ± 48.2 Aa	3840 ± 80.7Aa
	10	9020 ± 119 Ba	12600 ± 277Aa	4950 ± 163 Ba	4790 ± 79.1Ba
	50	8590 ± 98.5 Ba	33800 ± 2680Ba	5050 ± 219 Ba	4500 ± 203BCa
	100	8940 ± 263 Ba	55900 ± 7830Ca	4810 ± 242 Ba	4170 ± 45.4ACa
10	0	9300 ± 134 Ab	10300 ± 218Ab	13500 ± 414 Ab	12100 ± 236Ab
	0.1	10700 ± 489 Bb	10200 ± 147Ab	13200 ± 343 Ab	11400 ± 423Ab
	1	10300 ± 92.5 Bb	11700 ± 291Aa	13300 ± 334 Ab	11200 ± 157Ab
	10	10800 ± 436 Bb	14800 ± 854Ba	12900 ± 184 Ab	11000 ± 230Ab
	50	10600 ± 135 Bb	31500 ± 1840Ca	13400 ± 479 Ab	11300 ± 290Ab
	100	10100 ± 362 ABb	56400 ± 5450Da	13800 ± 205 Ab	12000 ± 256Ab
p-value	As	< 0.05	< 0.05	< 0.001	< 0.001
	nCuO	< 0.001	< 0.001	< 0.001	< 0.001
	A×N1	< 0.001	0.056	0.062	< 0.001

Values are means $\pm$ S.E.M, $n = 5$ per treatment group.
[1]A $\times$ N = As $\times$ nCuO interaction effect.
[A-C]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A×N = As $\times$ nCuO interaction effect.

Table C.3. Total copper concentration in solution media in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	Cu (µg/L)							
		Day1	Day7	Day21	Day35	Day49	Day63	Day84	Day112
0	0	66.6 ± 20.4	84.6 ± 13.1	80.9 ± 6.4	87.5 ± 6.8	53.8 ± 1.0	103 ± 13.4	40.2 ± 4.3	27.4 ± 1.3
	0.1	40.6 ± 7.8	78.5 ± 4.1	74.5 ± 5.8	63.6 ± 2.9	58.5 ± 3.3	129 ± 3.2	30.3 ± 2.7	26.2 ± 1.7
	1.0	92.4 ± 19.4	152 ± 42.9	81.6 ± 14.3	58.7 ± 2.4	68.3 ± 3.8	101 ± 4.77	37.2 ± 4.2	26.9 ± 1.4
	10	61.2 ± 23.7	76.6 ± 2.0	80.8 ± 8.8	69.5 ± 7.6	50.6 ± 3.2	208 ± 27.5	51.8 ± 9.9	47.3 ± 8.5
	50	394 ± 11.5	56.8 ± 4.7	149 ± 5.8	84.0 ± 8.6	82.2 ± 7.3	751 ± 145	87.5 ± 8.9	40.5 ± 3.7
	100	543 ± 14.2	97.6 ± 6.5	119 ± 5.5	87.2 ± 7.6	121 ± 26.1	589 ± 149	102.0 ± 9.9	41.1 ± 2.9
10	0	27.6 ± 3.47	79.9 ± 5.0	40.1 ± 9.8	78.4 ± 4.7	67.7 ± 18.5	100 ± 3.8	43.9 ± 2.6	32.4 ± 5.3
	0.1	60.5 ± 13.0	74.9 ± 3.8	67.6 ± 4.3	77.9 ± 5.3	54.0 ± 2.0	103 ± 2.3	30.4 ± 1.2	34.5 ± 5.2
	1.0	38.4 ± 10.6	74.9 ± 4.5	54.5 ± 2.2	65.4 ± 2.6	53.3 ± 3.5	110 ± 7.4	30.6 ± 1.2	28.9 ± 1.4
	10	152 ± 19.9	112 ± 8.7	75.1 ± 5.5	58.5 ± 2.7	44.7 ± 3.1	219 ± 12.1	31.6 ± 1.9	26.2 ± 0.6
	50	733 ± 150	231 ± 13.7	418 ± 44.4	88.4 ± 5.0	69.4 ± 4.1	927 ± 80.2	68.6 ± 10.7	54.4 ± 5.9
	100	727 ± 193	653 ± 199	228 ± 39.6	94.7 ± 3.5	76.6 ± 5.5	1530 ± 114	78.1 ± 6.6	71.7 ± 16.6
p-value	nCuO	< 0.001							
	As	< 0.001							
	Day	< 0.001							
	N×A[1]	< 0.001							
	N×D[2]	< 0.001							
	A×D[3]	< 0.001							
	N×A×D[4]	< 0.001							

Values are means ± S.E.M, $n = 5$ per treatment group.
[1]N×A1= nCuO × As interaction effect.
[2]N×D2 = nCuO × Day interaction effect.
[3]A×D3= As × Day interaction effect.
[4]N×A×D4= nCuO × As × Day interaction effect

Table C.4. Total arsenic concentration in solution media in a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution

As (mg/kg)	nCuO (mg/L)	As (µg/L)							
		Day1	Day7	Day21	Day35	Day49	Day63	Day84	Day112
0	0	24.5 ± 5.9	28.6 ± 3.8	19.4 ± 2.1	14.4 ± 0.9	14.3 ± 0.7	15.4 ± 0.4	7.7 ± 0.8	7.0 ± 1.2
	0.1	17.7 ± 2.7	26.6 ± 0.8	16.9 ± 2.0	10.1 ± 0.4	13.5 ± 0.9	14.4 ± 1.0	6.6 ± 1.3	3.5 ± 0.8
	1.0	21.7 ± 4.7	36.1 ± 2.3	20 ± 3.3	11.4 ± 0.9	13.5 ± 1.5	20.4 ± 1.1	8.1 ± 1.3	4.9 ± 1.2
	10	9.7 ± 1.2	15.6 ± 0.5	11.9 ± 0.8	8.5 ± 0.5	10.1 ± 0.7	13.4 ± 0.5	9.1 ± 1.1	8.1 ± 1.2
	50	8.7 ± 0.4	12.7 ± 0.3	11.9 ± 0.2	8.1 ± 0.5	10.4 ± 1.4	14.6 ± 0.8	9.3 ± 0.9	4.2 ± 0.1
	100	7.9 ± 0.6	13.4 ± 1.4	12.6 ± 0.8	7.4 ± 0.2	11.5 ± 1.3	13.0 ± 1.3	7.1 ± 0.5	4.4 ± 0.4
10	0	79.6 ± 8.9	168 ± 2.4	158 ± 15.1	61.5 ± 7.41	55.9 ± 5.8	73.4 ± 6.8	54.1 ± 4.09	37.5 ± 4.8
	0.1	73.1 ± 7.8	167 ± 5.0	112 ± 5.0	98.2 ± 2.79	79.3 ± 2.1	69.2 ± 5.1	40.3 ± 3.05	20.0 ± 2.2
	1.0	100 ± 12.6	186 ± 6.7	102 ± 3.0	77.3 ± 8.08	73.2 ± 7.9	71.7 ± 2.2	40.8 ± 2.42	27.4 ± 2.6
	10	91.9 ± 3.8	194 ± 9.0	116 ± 5.1	57.7 ± 7.36	73.6 ± 3.1	72.4 ± 3.5	45.3 ± 3.57	32.6 ± 4.8
	50	90.4 ± 4.9	179 ± 2.9	133 ± 12.8	82.0 ± 4.51	92.9 ± 3.3	77.8 ± 5.9	42.9 ± 4.69	25.8 ± 4.0
	100	91.1 ± 7.8	201 ± 16.4	130 ± 8.7	71.0 ± 5.48	92.1 ± 7.2	73.7 ± 5.5	37.5 ± 3.95	16.4 ± 2.0
p-value	nCuO	0.708							
	As	< 0.001							
	Day	< 0.001							
	N×A[1]	< 0.001							
	N×D[2]	< 0.001							
	A×D[3]	< 0.001							
	N×A×D[4]	< 0.001							

Values are means ± S.E.M, $n = 5$ per treatment group.
[1]N×A1= nCuO × As interaction effect.
[2]N×D2 = nCuO × Day interaction effect.
[3]A×D3= As × Day interaction effect.
[4]N×A×D4= nCuO × As × Day interaction effect.

Table C.5. Copper and arsenic concentration in the 18 d rice seedling (*O.sativa japonica* 'Koshihikari') shoots and roots from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5).

Variable		Cu (ng/g)		As (ng/g)	
As (mg/kg)	nCuO (mg L)	Root	Shoot	Root	Shoot
0	0	21200 ± 2590 [Aa]	12800 ± 1150 [Aa]	1920 ± 362 [Aa]	612 ± 70.3 [ABa]
	0.1	19000 ± 1990 [Aa]	11400 ± 486 [Aa]	4430 ± 652 [Ba]	714 ± 41.2 [Aa]
	1	20100 ± 517 [Aa]	11000 ± 150 [ABa]	3100 ± 599 [ABa]	511 ± 21.3 [Ba]
	10	16000 ± 1140 [Aa]	9620 ± 674 [Ba]	2790 ± 278 [ABa]	585 ± 49.7 [ABa]
	50	76000 ± 4640 [Ba]	17300 ± 90.7 [Ca]	2590 ± 176 [ABa]	532 ± 17.6 [Ba]
	100	81300 ± 6750 [Ba]	18300 ± 580 [Ca]	2190 ± 147 [Aa]	543 ± 17.4 [Ba]
10	0	16000 ± 535 [Ab]	8680 ± 133 [Ab]	21200 ± 2630 [Ab]	2850 ± 63.3 [Ab]
	0.1	18100 ± 1420 [ABa]	8430 ± 244 [Ab]	17800 ± 2730 [Ab]	2980 ± 81.3 [Ab]
	1	17200 ± 1240 [ABa]	8780 ± 203 [ABb]	16000 ± 1750 [Ab]	3240 ± 175 [Ab]
	10	21700 ± 691 [Bb]	10200 ± 159 [Ba]	16400 ± 2250 [Ab]	3090 ± 187 [Ab]
	50	33400 ± 1790 [Cb]	16600 ± 304 [Ca]	8110 ± 643 [Bb]	3170 ± 131 [Ab]
	100	103000 ± 4490 [Db]	20800 ± 525 [Db]	20700 ± 2510 [Ab]	3060 ± 130 [Ab]
p-value	As	0.028	< 0.001	< 0.001	< 0.001
	nCuO	< 0.001	< 0.001	< 0.001	0.894
	A×N[1]	< 0.001	< 0.001	< 0.001	< 0.001

Values are means ± S.E.M, n = 5 per treatment group.
[A-D]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A×N = As × nCuO interaction effect.

Table C.6. Copper and arsenic concentration in the mature rice (*O.sativa japonica* 'Koshihikari') plant roots and stems from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5).

Variables		Cu (ng/g)		As (ng/g)	
As (mg/kg)	nCuO (mg/L)	Root	Stem	Root	Stem
0	0	22500 ± 2680 [Aa]	5750 ± 624	6000 ± 947[Aa]	96.1 ± 8.97[a]
	0.1	34100 ± 3200 [ABa]	6230 ± 640	4030 ± 522 [Aa]	73.9 ± 10.7[a]
	1	25400 ± 1440 [Aa]	4650 ± 292	4820 ± 702 [Aa]	74.9 ± 5.34[a]
	10	55900 ± 10500 [BCa]	6200 ± 910	6500 ± 1000 [Aa]	79.2 ± 10.4[a]
	50	56700 ± 17000 [BCa]	4740 ± 152	4380 ± 1650 [Aa]	77.9 ± 6.36[a]
	100	88700 ± 17600 [Ca]	4780 ± 1040	6050 ± 716 [Aa]	71.4 ± 3.4[a]
10	0	20100 ± 3440 [Aa]	3740 ± 497	28700 ± 5260 [Ab]	109 ± 15.2[a]
	0.1	28100 ± 4640 [Aa]	5150 ± 712	18100 ± 2150 [Ab]	93 ± 7.48[a]
	1	22700 ± 2640 [Aa]	4190 ± 614	17000 ± 1020 [Ab]	95.9 ± 6.18[a]
	10	33600 ± 3080 [Ab]	5420 ± 584	28000 ± 4460 [Ab]	134 ± 37.9[b]
	50	97100 ± 9910 [Bb]	5890 ± 415	26700 ± 2820 [Ab]	98.9 ± 6.97[a]
	100	112000 ± 21800 [Ba]	5420 ± 177	23400 ± 1710 [Ab]	108 ± 9.26[b]
p-value	As	0.285	0.237	< 0.001	< 0.001
	nCuO	< 0.001	0.173	0.115	0.253
	A×N[1]	0.046	0.092	0.231	0.674

Values are means ± S.E.M, n = 5 per treatment group.
[A-C]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A × N = As × nCuO interaction effect.

Table C.7. Copper and arsenic concentration in the rice (*O.sativa japonica* 'Koshihikari') plant flag leaves, rice grain husks and dehusked grains on day 120 from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5).

Variable		Cu (ng/g)			As (ng/g)		
As (mg/kg)	CuO (mg/L)	Flag leaf	Husk	Dehusked grain	Flag leaf	Husk	Dehusked grain
0	0	6760 ± 708 [Aa]	5650 ± 192[Aa]	8410 ± 513 [Aa]	271 ± 38.3 [Aa]	37.2 ± 4.39 [Aa]	34.9 ± 3.46 [Aa]
	0.1	5780 ± 810 [Aa]	5840 ± 495 [Aa]	8890 ± 731 [Aa]	348 ± 110 [Aa]	42.7 ± 11.4 [Aa]	32.8 ± 11.4 [Aa]
	1	4470 ± 213 [Aa]	4830 ± 164 [ABa]	8060 ± 261 [Aa]	319 ± 29.9 [Aa]	52.4 ± 7.19 [Aa]	39.6 ± 5.53 [Aa]
	10	7000 ± 918 [Aa]	4520 ± 239 [Ba]	7420 ± 294 [Aa]	268 ± 45.3 [Aa]	45.5 ± 6.06 [Aa]	42.8 ± 5.88 [Aa]
	50	4350 ± 319 [Aa]	5090 ± 138 [ABa]	8070 ± 476 [Aa]	469 ± 65.7 [Aa]	43.8 ± 4.81 [Aa]	32.8 ± 4.06 [Aa]
	100	5720 ± 710 [Aa]	4840 ± 216 [ABa]	6770 ± 1360 [Aa]	364 ± 39 [Aa]	48.7 ± 5.62 [Aa]	30.6 ± 5.22 [Aa]
10	0	4610 ± 721 [Ab]	4370 ± 197 [Ab]	6420 ± 428 [Ab]	2450 ± 447 [Ab]	169 ± 28.7 [Ab]	141 ± 18.8 [Ab]
	0.1	3830 ± 160 [Ab]	5150 ± 184 [ABa]	6930 ± 240 [Aa]	1060 ± 83.5 [Bb]	121 ± 9.59 [Ab]	109 ± 6.29 [Ab]
	1	3840 ± 399 [Aa]	5290 ± 287 [ABa]	7690 ± 722 [Aa]	1400 ± 380 [ABb]	133 ± 29 [Ab]	121 ± 13.4 [Ab]
	10	3700 ± 632 [Ab]	5930 ± 252 [BCb]	8500 ± 368 [Aa]	1810 ± 360 [ABb]	145 ± 11.6 [Ab]	120 ± 10.3[Ab]
	50	4660 ± 707 [Aa]	6730 ± 335 [Cb]	8670 ± 1380 [Aa]	1110 ± 222 [ABb]	117 ± 14.7 [Ab]	88.5 ± 14.7 [Ab]
	100	3350 ± 124 [Ab]	5840 ± 301 [BCb]	8320 ± 620 [Aa]	1480 ± 179 [ABb]	197 ± 19.4 [Ab]	152 ± 17.2 [Ab]
p-value	As	< 0.001	0.006	0.636	< 0.001	< 0.001	< 0.001
	nCuO	0.078	0.008	0.856	0.034	0.103	0.283
	A×N[1]	0.051	< 0.001	0.036	0.064	0.595	0.583

Values are means ± S.E.M, n = 5 per treatment group.
[A-D]Means in a column at same As level with a common superscript letter are similar ($p < 0.05$).
[a-b]Means in a column at same nCuO level with a common superscript letter are similar ($p < 0.05$).
1A × N = As × nCuO interaction effect.

Table C.8. Copper and arsenic concentration in the rice (*O.sativa japonica* 'Koshihikari') plant flag leaves, rice grain husks and dehusked grains on day 131 from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5).

Variable		Cu (ng/g)			As (ng/g)		
As (mg/kg)	CuO (mg/L)	Flag leaf	Husk	Dehusked grain	Flag leaf	Husk	Dehusked grain
0	0	7390 ± 1090 [a]	5520 ± 313 [ABa]	9890 ± 471 [Aa]	359 ± 62.1 [ABa]	74.2 ± 14.1 [Aa]	44.4 ± 4.70 [Aa]
	0.1	5540 ± 831 [a]	6330 ± 379 [Aa]	9870 ± 577 [ABa]	373 ± 59.7 [ABa]	62.9 ± 6.13 [Aa]	39.2 ± 4.33 [Aa]
	1	6300 ± 814 [a]	5440 ± 335 [ABa]	8220 ± 292 [Ba]	464 ± 71.5 [ABa]	62.1 ± 6.15 [Aa]	40.9 ± 6.32 [Aa]
	10	8830 ± 993 [a]	4730 ± 241 [Ba]	9050 ± 438 [ABa]	262 ± 34.1 [Aa]	67.0 ± 4.70 [Aa]	48.1 ± 4.41 [Aa]
	50	5880 ± 591 [a]	4870 ± 301 [Ba]	8350 ± 319 [ABa]	615 ± 123 [Ba]	74.9 ± 6.89 [Aa]	49.0 ± 4.75 [Aa]
	100	7050 ± 810 [a]	4920 ± 199 [Ba]	9410 ± 211 [ABa]	490 ± 60.1 [ABa]	59.4 ± 5.68 [Aa]	38.5 ± 1.75 [Aa]
10	0	6250 ± 773 [a]	3470 ± 148 [Ab]	6820 ± 400 [Ab]	3890 ± 816 [Ab]	284 ± 38.0 [Ab]	198 ± 21.1 [Ab]
	0.1	6850 ± 546 [a]	4590 ± 310 [ABb]	7680 ± 197 [ABb]	1220 ± 233 [Bb]	173 ± 10.0 [Bb]	132 ± 8.25 [ABb]
	1	5760 ± 959 [a]	5330 ± 303 [BCa]	8310 ± 477 [ABa]	1950 ± 503 [ABb]	177 ± 16.2 [BCb]	138 ± 7.5 [ABb]
	10	7340 ± 1220 [a]	5340 ± 363 [BCa]	8720 ± 544 [BCa]	2600 ± 477 [ABb]	229 ± 27.4 [ABCb]	167 ± 15.9 [ABb]
	50	8270 ± 854 [a]	6490 ± 213 [Cb]	10100 ± 466 [Cb]	2040 ± 521 [ABb]	192 ± 26.0 [ABCb]	128 ± 15.4 [Bb]
	100	4830 ± 455 [b]	5470 ± 249 [BCa]	8970 ± 415 [BCa]	2610 ± 148 [ABb]	271 ± 23.8 [ACb]	175 ± 13.7 [ABb]
p-value	As	0.568	0.323	0.005	< 0.001	< 0.001	< 0.001
	nCuO	0.114	< 0.001	0.090	0.001	0.044	0.205
	A×N[1]	0.047	< 0.001	< 0.001	0.016	0.003	0.01

Values are means ± S.E.M, n = 5 per treatment group.
[A-C] Means in a column at the same As level with a common superscript letter are similar ($p < 0.05$).
[a-b] Means in a column at the same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1]A × N = As × nCuO interaction effect.

Table C.9. Copper speciation based on copper K edge XANES spectra of rice (*O.sativa japonica* 'Koshihikari') dehusked grains on day 131 from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution.

| Treatment | Fitting Criteria | | CuO | $Cu_3(PO4)_2$ | $CuSO_4 \cdot 5H_2O$ | Cupric acetate | CuC_2O_4 | Cuprous acetate | Fraction Sum |
	R factor	Reduced $\chi2$	Fraction %	Fraction %	Fraction %	Fraction %	Fraction %	Fraction %	Fraction %
Control	0.005	0.001	12.3	ND	ND	ND	20.5	67.2	100
nCuO100	0.006	0.001	ND	18.1	63.6	ND	18.3	ND	100
As+nCuO100	0.025	0.004	ND	ND	ND	68.6	31.4	ND	100

Note:
R factor, the residual sum of squares; ND=not detectable; Reduced $\chi2$, a weighted sum of squared deviations.
All standards were evaluated but some were omitted from the table for not present.
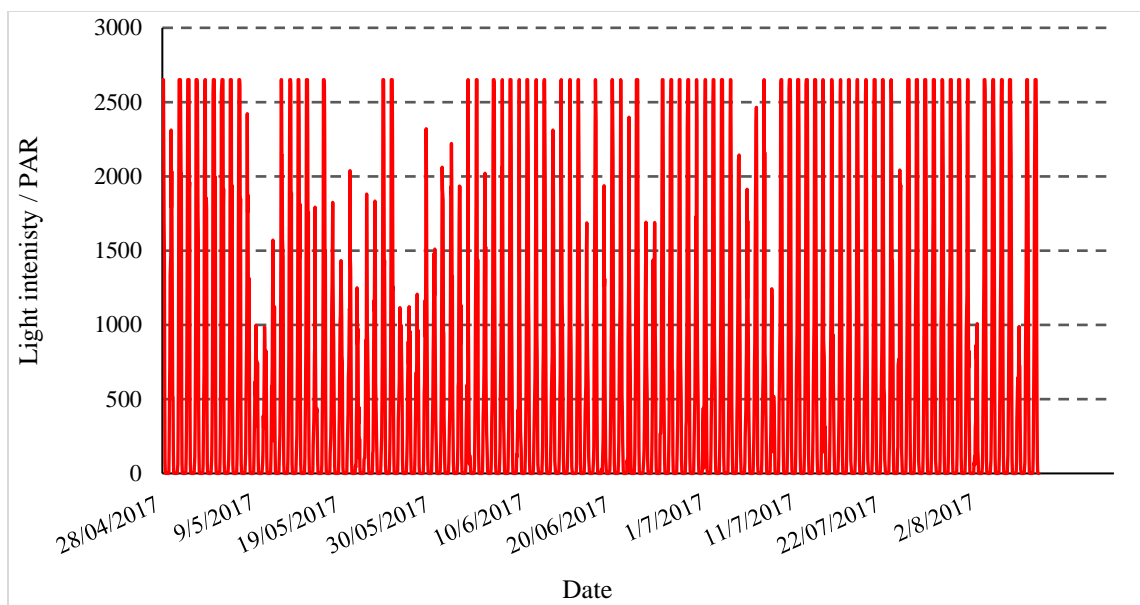
Figure C.1. Light intensity record in the greenhouse (Photosynthetically Active Radiation, PAR) during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution
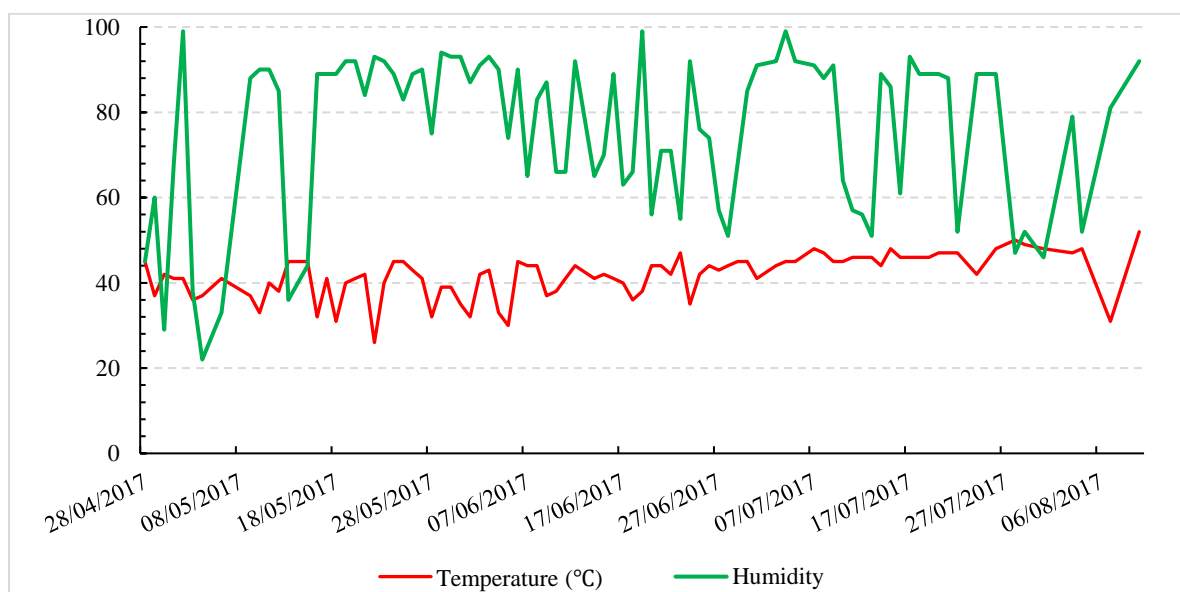
Figure C.2. Temperature and humidity records in the greenhouse during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution
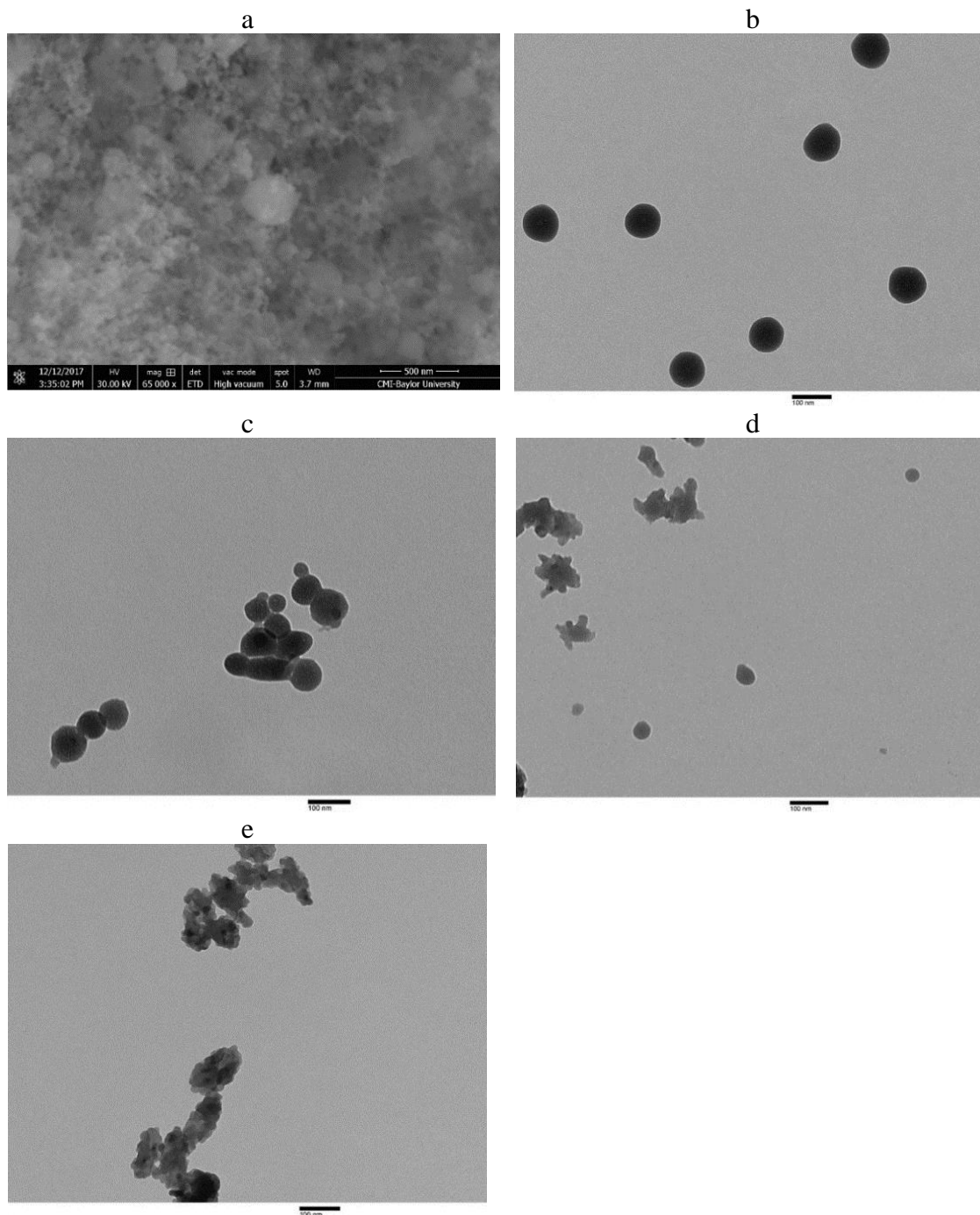
Figure C.3. SEM and TEM images from samples collected during a 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution. a. SEM image of copper oxide nanoparticle powder; b–e. TEM images of nanoparticles and their aggregation in the solution samples collected on day 14(c. As+nCuO 100 mg/L, d. nCuO100 mg/L, e. As+nCuO 0.1mg/L and f. nCuO0.1 mg/L)

Flag leaf   Panicle   Husks and dehusked grains
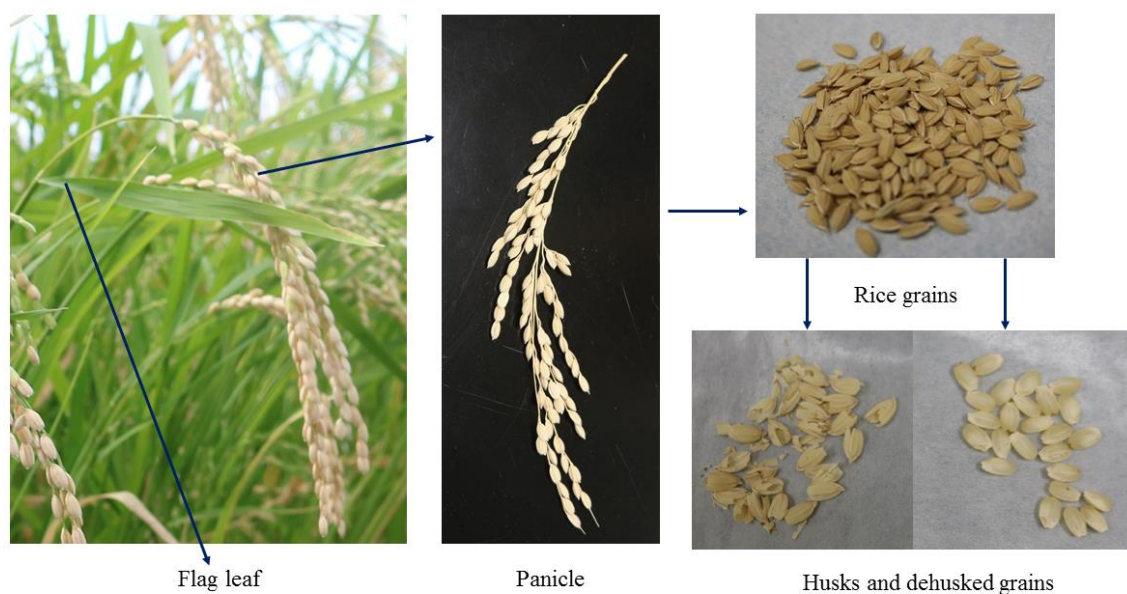
Figure C.4. A Pair of a rice (*O.sativa japonica* 'Koshihikari') plant flag leaf and a panicle with an exserted panicle and separated husk and dehusked grains from selected grain samples from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution.
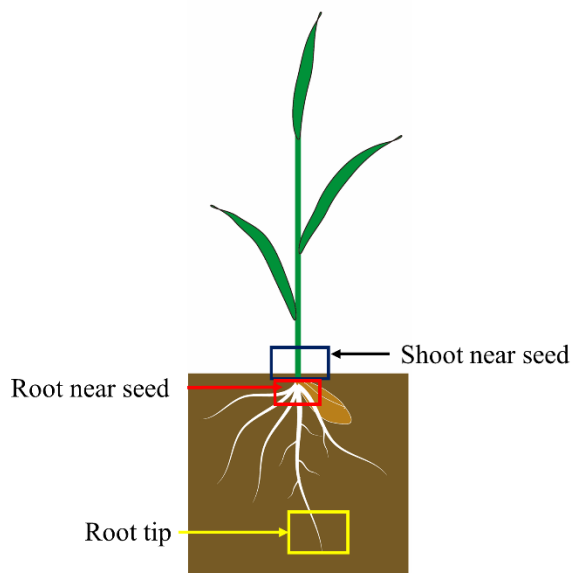
Figure C.5. Portions of seedlings analyzed with X-Ray Absorption Near Edge Structure (XANES) for identifying speciation of copper and arsenic
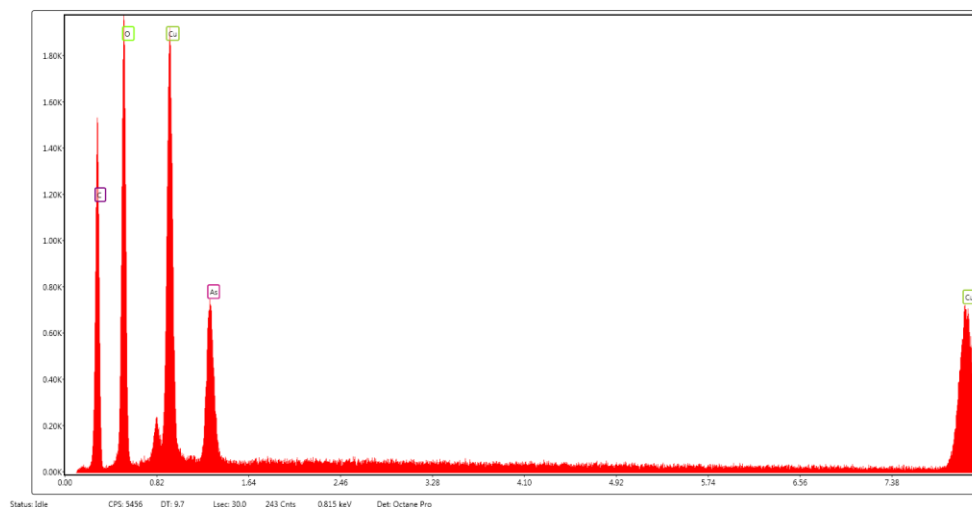
Figure C.6. Elemental analysis of synthesized cupric arsenate by SEM-EDX
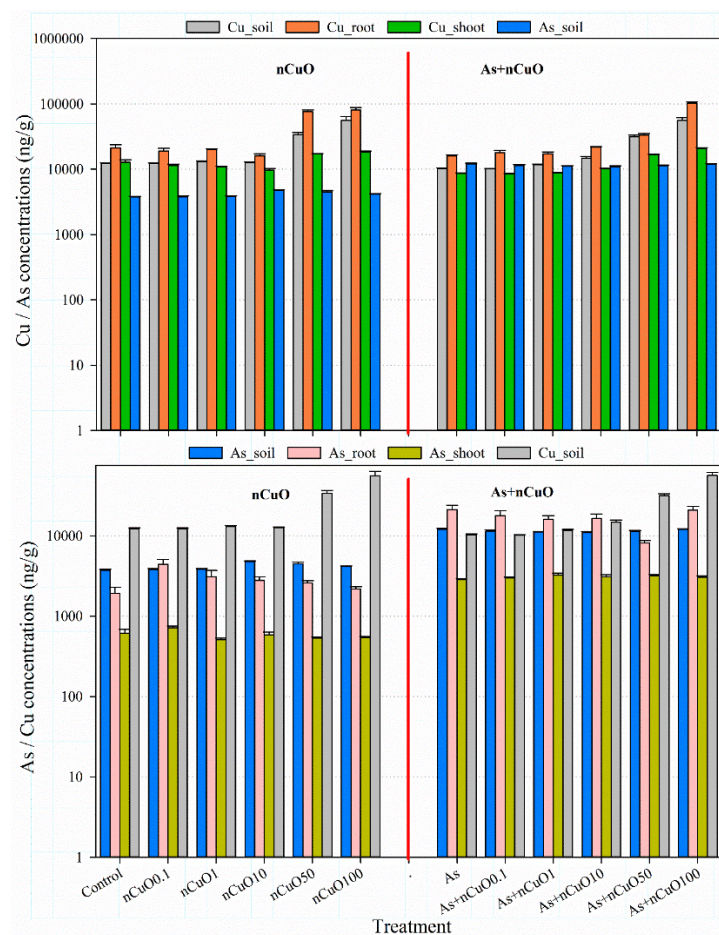
Figure C.7. Copper and arsenic concentration in the 18-d rice (*O.sativa japonica* 'Koshihikari') seedlings from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5).
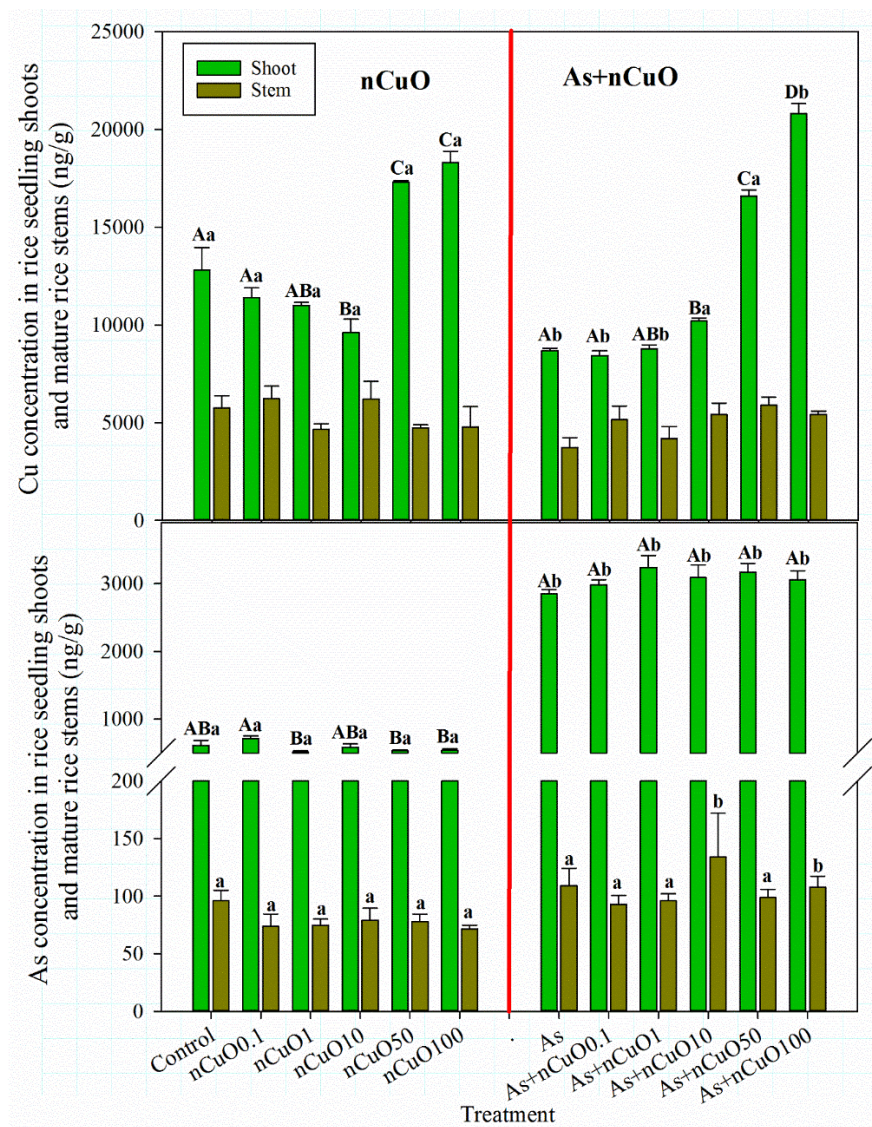
Figure C.8. Copper and arsenic concentration in 18-d rice seedling shoots and mature rice plant stems from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution.
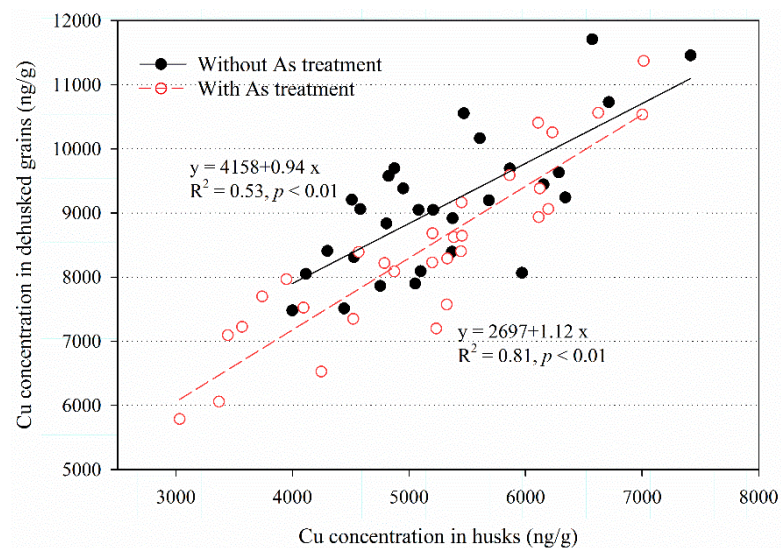
Figure C.9. Relationship of copper concentrations between rice (*O.sativa japonica* 'Koshihikari') dehusked grains and husk from a greenhouse study with 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5).
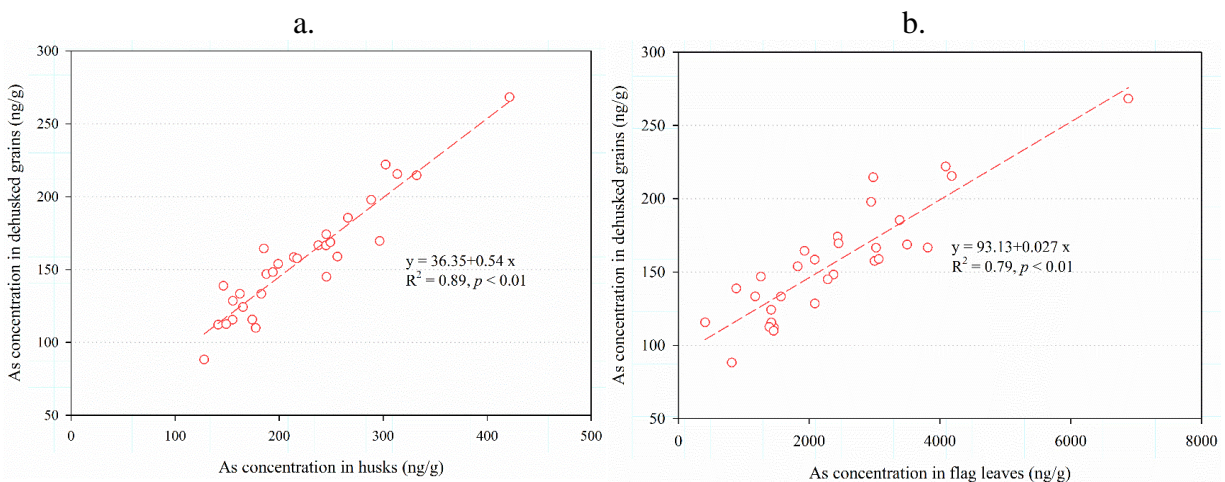
Figure C.10. Relationship of arsenic concentrations between rice (*O.sativa japonica* 'Koshihikari') dehusked grains and husks (a) and relationship of arsenic concentrations between dehusked grains and flag leaves (b) from a greenhouse study with 131-d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5)

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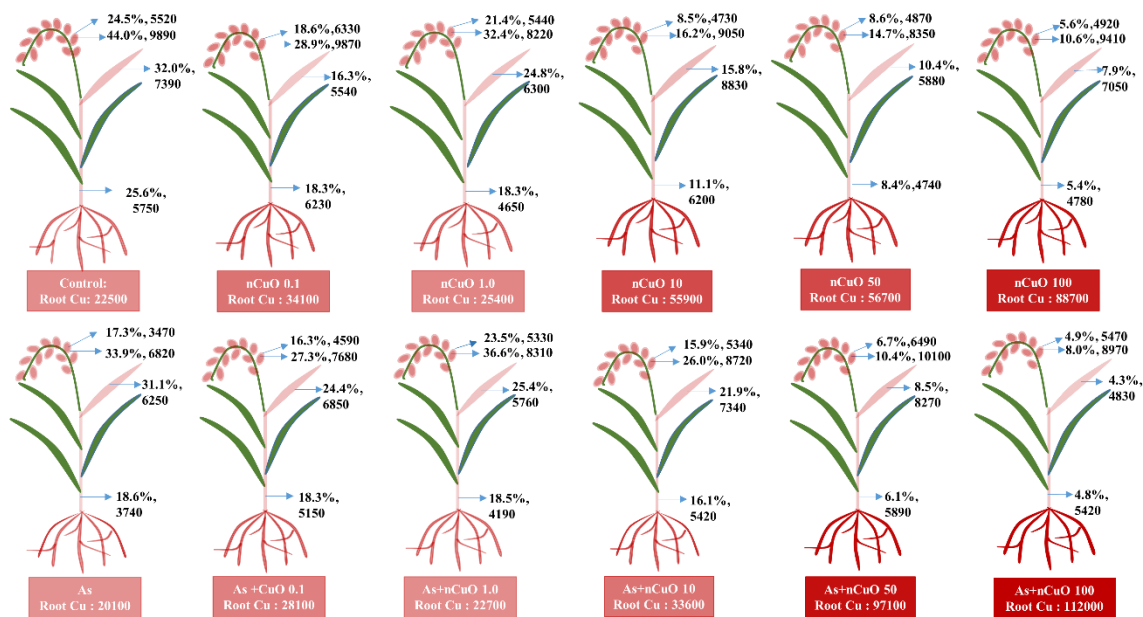
Figure C.11. Copper distribution (ng/g in roots) in rice (*O.sativa japonica* 'Koshihikari') plants from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5). Percentages (%) are relative to concentrations in the roots.
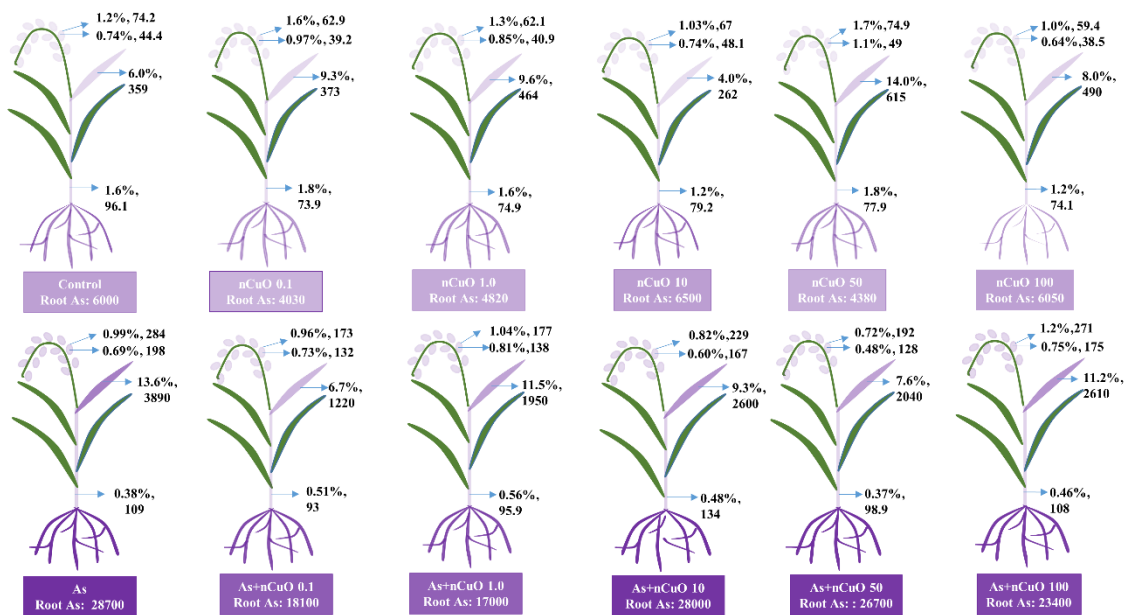
Figure C.12. Arsenic distribution (ng/g in roots) in rice (*O.sativa japonica* 'Koshihikari') plants from a greenhouse study with 131 d exposure to arsenic in soil and copper oxide nanoparticles in nutrient solution (n = 5). Percentages (%) are relative to concentrations in the roots.

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APPENDIX D

Supplemental Data for Chapter Five: Exposure to Copper Oxide Nanoparticles and
Arsenic Causes Intergenerational Effects on Rice (*Oryza sativa japonica* 'Koshihikari')
Seed Germination and Seedling Growth

Number of pages: 21

Number of tables: 15

Number of figures: 2

Additional text is provided about data handling with growth cells as statistical unit and solution sampling details. Tabular data in this document support the depicted figures within the main text of the manuscript, and may also be beneficial for those who are conducting research for direct comparisons of means and ranges, or those engaged in meta-analysis of ongoing research to address plant sensitivity to metals.

Data Handling of Seed Germination and Seedling Growth

The statistical unit was individual growth cell. There were 10 statistical units for each parameter (germination percentage, seedling length and dry weight) in each of the 34 treatments. Germination and growth data were analyzed with generalized linear models (GLM) depending on the data distributions.

Solution Sampling

Water samples (0.40 mL out of 50 mL) were collected using the pipette every 6 d from each growth cell. Two composite water samples were collected within each treatment. Each composite contained four distinct water samples with volumes of 2.0 mL. The effect of water removed (0.8%) on the As or Cu concentrations is negligible.

Table D.1. Overall treatment design for F1 seed germination and seedling growth study with seeds harvested from F0 plants in a life cycle greenhouse study with exposure to nCuO and As

Seeds from 12 F0 treatments	F1 treatment											
	Control	As	nCuO0.1	nCuO1.0	nCuO10	nCuO50	nCuO100	As+ nCuO0.1	As+ nCuO1.0	As+ nCuO10	As+ nCuO50	As+ nCuO100
Control	F1C [F0C]	F1As [F0C]										
As	F1C [F0As]	F1As [F0As]										
nCuO0.1	F1C [F0Cu0.1]	F1As [F0Cu0.1]	F1Cu0.1 [F0Cu0.1]									
nCuO1.0	F1C [F0Cu1.0]	F1As [F0Cu1.0]		F1Cu1.0 [F0Cu1.0]								
nCuO10	F1C [F0Cu10]	F1As [F0Cu10]			F1Cu10 [F0Cu10]							
nCuO50	F1C [F0Cu50]	F1As [F0Cu50]				F1Cu50 [F0Cu50]						
nCuO100	F1C [F0Cu100]	F1As [F0Cu100]					F1Cu100 [F0Cu100]					
As+ nCuO0.1	F1C [F0AsCu0.1]	F1As [F0AsCu0.1]						F1AsCu0.1 [F0AsCu0.1]				
As+ nCuO1.0	F1C [F0AsCu1.0]	F1As [F0AsCu1.0]							F1AsCu1.0 [F0AsCu1.0			
As+nCuO10	F1C [F0AsCu10]	F1As [F0AsCu10]								F1AsCu10 [F0AsCu10]		
As+nCuO50	F1C [F0AsCu50]	F1As [F0AsCu50]									F1AsCu50 [F0AsCu50]	
As+nCuO100	F1C [F0AsCu100]	F1As [F0AsCu100]										F1AsCu100 [F0AsCu100

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Table D.2. Concentrations of copper, arsenic and several other essential metals in F0 dehusked grains (F1 seeds) from a life cycle greenhouse study of rice plants with exposure to nCuO and As

F1 Seed type	As (mg/kg)	nCuO (mg/L)	Cu (ng/g)	As (ng/g)	Mg (μg/g)	Mn (μg/g)	Zn (μg/g)	Mo (μg/g)
Control		0	9890 ± 471 [Aa]	44.4 ± 4.70 [Aa]	1580 ± 116 [Aa]	22.4 ± 1.59	37.6 ± 1.98 [Aa]	11.3 ± 0.43 [a]
As		0.1	9870 ± 577 [ABa]	39.2 ± 4.33 [Aa]	1460 ± 30.4 [ABa]	22.3 ± 0.98	35.8 ± 1.41 [ABa]	12.6 ± 0.73 [a]
nCuO0.1	0	1	8220 ± 292 [Ba]	40.9 ± 6.32 [Aa]	1390 ± 15.8 [BCa]	22.2 ± 0.60	32.6 ± 0.62 [BCa]	11.2 ± 0.39 [a]
nCuO1.0		10	9050 ± 438 [ABa]	48.1 ± 4.41 [Aa]	1530 ± 23.9 [Aa]	23.1 ± 0.88	35.8 ± 1.02 [ABa]	11.5 ± 0.28 [a]
nCuO10		50	8350 ± 319 [ABa]	49.0 ± 4.75 [Aa]	1310 ± 19.5 [Ca]	23.7 ± 0.73	31 ± 0.61 [Ca]	11.8 ± 0.52 [a]
nCuO50		100	9410 ± 211 [ABa]	38.5 ± 1.75 [Aa]	1330 ± 12.7 [Ca]	22.8 ± 0.36	32.3 ± 0.56 [BCa]	10.7 ± 0.33 [a]
nCuO100		0	6820 ± 400 [Ab]	198 ± 21.1 [Ab]	1450 ± 32.8 [Ab]	22.3 ± 0.20	33.2 ± 0.93 [Ab]	10.7 ± 0.78 [a]
As+nCuO0.1		0.1	7680 ± 197 [ABb]	132 ± 8.25 [ABb]	1410 ± 20.4 [ABa]	22.1 ± 0.38	32.5 ± 0.96 [Ab]	10.3 ± 0.29 [b]
As+nCuO1.0	10	1	8310 ± 477 [ABa]	138 ± 7.5 [ABb]	1420 ± 16.9 [ABa]	23.4 ± 0.49	34.8 ± 1.09 [Aa]	10.7 ± 0.42 [a]
As+nCuO10		10	8720 ± 544 [BCa]	167 ± 15.9 [ABb]	1390 ± 20.1 [ABCb]	23.1 ± 0.59	34.1 ± 0.90 [Aa]	10.7 ± 0.58 [a]
As+nCuO50		50	10100 ± 466 [Cb]	128 ± 15.4 [Bb]	1300 ± 17.9 [BCa]	23.5 ± 0.70	32.1 ± 0.88 [Aa]	10.6 ± 0.46 [a]
As+nCuO100		100	8970 ± 415 [BCa]	175 ± 13.7 [ABb]	1270 ± 17.1 [Ca]	25 ± 0.31	31.6 ± 0.38 [Aa]	10.4 ± 0.38 [a]
	As		0.005	< 0.001	0.002	0.224	0.061	< 0.001
P-value	nCuO		0.090	0.205	<0.001	0.085	< 0.001	0.600
	A×N[1]		< 0.001	0.01	0.065	0.415	0.007	0.370

Values are means ± SEM, n = 5 per treatment group.
[A-D] Means in a column at the same As level with a common superscript letter are similar ($p < 0.05$).
[ab] Means in a column at the same nCuO level with a common superscript letter are similar ($p < 0.05$).
[1] A × N = As × nCuO interaction effect.

Table D.3.Total organic carbon and nitrogen in the sand media before and after seedling growth in an18-day laboratory experiment of F1 rice seed germination and seedling growth

Variable		Mass (mg)	% Carbon	% Nitrogen
As	Before	51.0 ± 2.7	0.062 ± 0.004	0.032 ± 0.002
	After	53.6 ± 0.92	0.107 ± 0.009	0.032 ± 0.002
Control	Before	51.1 ± 2.52	0.065 ± 0.008	0.024 ± 0.007
	After	51.9 ± 2.00	0.100 ± 0.010	0.030± 0.002
nCuO100	Before	53.2 ± 1.61	0.102 ± 0.005	0.032 ± 0.002
	After	54.4 ± 1.83	0.095 ± 0.009	0.028 ± 0.003

Values are means ± SEM, n = 5 per treatment group.
The recovery for standards of carbon and nitrogen are in the range from 97.3%–109.0% and 96.1%–103.6%.

Table D.4. Copper and arsenic concentrations in sand before seedling growth from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Control treatment		As treatment		Quai-F0 treatment	
	Cu (ng/g)	As(ng/g)	Cu (ng/g)	As (ng/g)	Cu (ng/g)	As (ng/g)
control	1040 ± 25.4	975 ± 46.1	991 ± 62.1	8470 ± 155	1040 ± 25.4	975 ± 46.1
nCuO0.1	992 ± 59	1230 ± 340	952 ± 117	8140 ± 344	1400 ± 49.8	1120 ± 76.7
nCuO1.0	1030 ± 114	1170 ± 218	1380 ± 65.7	8350 ± 271	1390 ± 69.6	1150 ± 125
nCuO10	1110 ± 112	1220 ± 188	1790 ± 216	9610 ± 163	1190 ± 30.7	827 ± 35.1
nCuO50	1140 ± 75.1	1150 ± 55.4	988 ± 73.2	8280 ± 261	1210 ± 66.5	782 ± 131
nCuO100	1190 ± 78.7	1350 ± 122	999 ± 70.1	8920 ± 171	1320 ± 103	949 ± 169
As	966 ± 51.1	956 ± 71	1000 ± 16.7	8790 ± 389	1000 ± 16.7	8790 ± 389
As+nCuO0.1	1100 ± 75.9	1160 ± 118	929 ± 41.7	8460 ± 268	1190 ± 29	8460 ± 284
As+nCuO1.0	1150 ± 121	1050 ± 222	936 ± 26.4	8190 ± 408	1160 ± 77.2	8240 ± 338
As+nCuO10	1470 ± 154	902 ± 86	911 ± 45.3	8280 ± 383	1280 ± 64.9	8820 ± 308
As+nCuO50	1430 ± 227	1030 ± 169	1270 ± 126	9810 ± 594	1220 ± 16.6	8590 ± 758
As+nCuO100	1680 ± 164	1020 ± 63.6	960 ± 50.3	7590 ± 332	1200 ± 44.2	8430 ± 341

Values are means $\pm$ SEM, n = 5 per treatment group.

Table D.5. Copper and arsenic concentrations in sand after seedling removal from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Control		As treatment		Quasi-F0 treatment	
	Cu (ng/g)	As (ng/g)	Cu (ng/g)	As (ng/g)	Cu (ng/g)	As (ng/g)
Control	1460 ± 147	1360 ± 261	1850 ± 300	11100 ± 670	1460 ± 147	1360 ± 261
nCuO0.1	1310 ± 202	1120 ± 120	1970 ± 319	12500 ± 1500	1000 ± 74.8	1130 ± 56.4
nCuO1.0	1510 ± 5.61	1660 ± 6.95	1530 ± 180	10500 ± 799	1700 ± 194	1090 ± 145
nCuO10	1090 ± 139	982 ± 63.2	1620 ± 41.2	11900 ± 727	6500 ± 655	934 ± 140
nCuO50	1300 ± 119	1150 ± 240	2910 ± 1470	10700 ± 1020	24400 ± 507	1070 ± 142
nCuO100	1390 ± 158	1360 ± 457	1940 ± 45.2	11900 ± 473	41400 ± 1850	879 ± 46.3
As	1420 ± 70	1800 ± 704	1610 ± 9.58	12300 ± 219	1610 ± 9.58	12300 ± 219
As+nCuO0.1	1320 ± 117	1140 ± 212	1910 ± 139	12100 ± 1280	1010 ± 78.9	8920 ± 760
As+nCuO1.0	1400 ± 89.6	1080 ± 112	1610 ± 96.8	11900 ± 1860	1880 ± 302	9740 ± 1010
As+nCuO10	1710 ± 121	1390 ± 138	1750 ± 128	11300 ± 961	5040 ± 171	8110 ± 345
As+nCuO50	1550 ± 24.7	1290 ± 129	1770 ± 97.4	12600 ± 1210	26100 ± 2010	8500 ± 578
As+nCuO100	1760 ± 177	1490 ± 144	1550 ± 36.2	10200 ± 857	39000 ± 4030	7810 ± 501

Table D.6. Copper and arsenic concentrations in solution in control after seedling removal from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Cu (ng/ml)			As (ng/ml)		
	day6	day12	day18	day6	day12	day18
C	173 ± 5.18	99.1 ± 10	122 ± 4.52	4.79 ± 0.36	4.18 ± 0.44	2.97 ± 0.05
nCuO0.1	174 ± 19.4	118 ± 8.93	120 ± 7.32	3.55 ± 0.41	3.22 ± 0.13	2.58 ± 0.20
nCuO1.0	156 ± 26.8	114 ± 1.6	112 ± 7.23	3.23 ± 0.03	3.59 ± 0.23	2.82 ± 0.01
nCuO10	174 ± 15.7	115 ± 6.24	123 ± 0.43	2.44 ± 0.04	3.57 ± 0.04	2.78 ± 0.09
nCuO50	351 ± 129	103 ± 0.05	110 ± 1.36	2.82 ± 0.00	3.91 ± 0.19	2.49 ± 0.22
nCuO100	182 ± 22.7	108 ± 2.03	119 ± 18.3	2.79 ± 0.04	3.39 ± 0.30	2.18 ± 0.25
As	146 ± 12.1	104 ± 7.13	118 ± 0.94	3 ± 0.42	3.72 ± 0.40	3.16 ± 0.32
As+nCuO0.1	240 ± 73.3	150 ± 31	131 ± 2.65	5.93 ± 0.26	4.02 ± 0.55	4.32 ± 0.07
As+nCuO1.0	193 ± 11.7	163 ± 1.99	144 ± 3.37	5.81 ± 1.12	3.31 ± 0.36	3.82 ± 0.14
As+nCuO10	177 ± 47.2	178 ± 6.31	135 ± 0.45	3.91 ± 0.12	3.89 ± 0.24	2.97 ± 0.09
As+nCuO50	130 ± 7.35	170 ± 2.57	138 ± 0.99	4.63 ± 0.05	4.22 ± 0.16	3.35 ± 0.12
As+nCuO100	132 ± 3.56	177 ± 13.5	125 ± 5.01	4.98 ± 0.15	4.42 ± 0.11	3.87 ± 0.16

Table D.7. Copper and arsenic concentrations in solution in As treatment after seedling removal from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Cu (ng/ml)			As (ng/ml)		
	day6	day12	day18	day6	day12	day18
Control	101 ± 11.3	196 ± 14.3	126 ± 11	1550 ± 593	2920 ± 196	2770 ± 129
nCuO0.1	110 ± 0.58	190 ± 1.46	113 ± 4.84	1880 ± 95.7	2890 ± 66.9	2920 ± 117
nCuO1.0	146 ± 19.1	202 ± 12.9	108 ± 1.42	2320 ± 521	2820 ± 154	2650 ± 15.2
nCuO10	141 ± 5.1	183 ± 4.13	118 ± 14.2	2040 ± 24.4	2850 ± 126	2880 ± 308
nCuO50	113 ± 10.1	212 ± 23.9	119 ± 2.04	1880 ± 43.9	2390 ± 772	3170 ± 28.7
nCuO100	114 ± 3.2	188 ± 9.23	120 ± 3.55	1870 ± 187	2840 ± 26	2830 ± 25.4
As	101 ± 16	188 ± 9.26	106 ± 6.51	1810 ± 94.7	3010 ± 52.2	2910 ± 121
As+nCuO0.1	107 ± 1.65	195 ± 8.92	117 ± 16.8	2060 ± 17.4	2940 ± 39.3	3010 ± 391
As+nCuO1.0	99.2 ± 2.33	178 ± 9.64	88 ± 2.08	1890 ± 207	2820 ± 63.7	2500 ± 70.7
As+nCuO10	112 ± 9.24	364 ± 175	115 ± 7.99	1810 ± 141	2880 ± 177	2570 ± 178
As+nCuO50	113 ± 1.99	176 ± 6.52	95.6 ± 3.79	1880 ± 125	2480 ± 4.12	2230 ± 27.3
As+nCuO100	96.1 ± 4.94	214 ± 19.3	90.7 ± 0.20	1910 ± 64.8	2910 ± 447	2360 ± 217

Table D.8. Copper and arsenic concentrations in solution in quai-F0 treatment after seedling removal from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Quasi-F0 treatment		Seed type	Cu (ng/ml)			As (ng/ml)		
As (mg/kg)	nCuO (mg/L)		day6	day12	day18	day6	day12	day18
0	0	Control	173 ± 5.18	99.1 ± 10	122 ± 4.52	4.79 ± 0.36	4.18 ± 0.44	2.97 ± 0.05
	0.1	nCuO0.1	140 ± 3.69	165 ± 32.1	106 ± 7.48	2.99 ± 0.01	4.76 ± 0.12	ND
	1	nCuO1.0	732 ± 13.2	941 ± 40.4	796 ± 26.4	3.47 ± 0.55	5.88 ± 0.63	ND
	10	nCuO10	2730 ± 62.9	2760 ± 58.1	2430 ± 22	1.66 ± 0.01	5.76 ± 1.46	ND
	50	nCuO50	2270 ± 99.4	3050 ± 31.4	$3180 \pm 68.9b$	1.39 ± 0.02	3.41 ± 0.08	ND
	100	nCuO100	2230 ± 39.8	3530 ± 42	3560 ± 67.2	1.27 ± 0.19	3.28 ± 0.44	ND
10	0	As	101 ± 16	188 ± 9.26	106 ± 6.51	1810 ± 94.7	3010 ± 52.2	2910 ± 121
	0.1	As+nCuO0.1	129 ± 20.3	144 ± 4.13	105 ± 9.93	1790 ± 252	2600 ± 62.8	2510 ± 361
	1	As+nCuO1.0	644 ± 28.1	783 ± 76.6	674 ± 10.1	2330 ± 191	3420 ± 298	2870 ± 17.4
	10	As+nCuO10	1920 ± 208	2430 ± 11.5	2360 ± 55.7	1860 ± 140	2870 ± 26	2770 ± 151
	50	As+nCuO50	3120 ± 303	3520 ± 26.3	3350 ± 110	2610 ± 382	2610 ± 160	2360 ± 166
	100	As+nCuO100	2650 ± 191	3960 ± 56.9	3450 ± 42.6	2080 ± 191	2570 ± 1.81	2160 ± 3.73

Table D.9. Seed germination in an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Control	As treatment	Quai-F0 treatment
Control	97.5 ± 2.5% [A]	97.5 ± 2.5% [A]	97.5 ± 2.5% [Aa]
nCuO0.1	100 ± 0.0% [A]	97.5 ± 2.5% [A]	95 ± 3.3% [Aa]
nCuO1.0	97.5 ± 3.0% [A]	100 ± 0.0% [A]	100 ± 0.0% [Aa]
nCuO10	95 ± 5.0% [A]	100 ± 0.0% [A]	100 ± 0.0% [Aa]
nCuO50	67.5 ± 7.5% [B]	52.5 ± 10.8% [B]	70 ± 8.2% [Ba]
nCuO100	100 ± 0.0% [A]	88.2 ± 9.3% [AB]	90 ± 4.1% [Aa]
As	100 ± 0.0% [A]	97.5 ± 2.5% [A]	97.5 ± 2.5% [Aa]
As+nCuO0.1	100 ± 0.0% [A]	100 ± 0.0% [A]	92.5 ± 3.8% [Aa]
As+nCuO1.0	95 ± 3.3% [A]	97.5 ± 2.5% [AB]	100 ± 0.0% [Aa]
As+nCuO10	85 ± 7.6% [A]	90 ± 5.5% [A]	87.5 ± 4.2% [Aa]
As+nCuO50	97.5 ± 2.5% [A]	95 ± 3.3% [A]	100 ± 0.0% [Ab]
As+nCuO100	97.5 ± 2.5% [A]	95 ± 3.3% [AB]	97.5 ± 2.5% [Aa]
p	Seed: < 0.001	Seed: < 0.001	As: 0.25 nCuO: 0.12 As×nCuO: 0.02

Values are means ± SEM, n = 10 per treatment group.
[A-B] Means in a Control and As treatment column with a common superscript letter are similar ($p < 0.05$).
[A-B] Means in Quai-F0 treatment column at the same As level with a common superscript letter are similar ($p < 0.05$).
[ab] Means in Quai-F0 treatment column at the same nCuO level with a common superscript letter are similar ($p < 0.05$).
As × nCuO: interaction effect.

Table D.10. Seedling growth parameters in control from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Shoot length (cm)	Root length (cm)	Number of root branches	Shoot dry weight (mg)	Root dry weight (mg)
Control	8.84 ± 0.48^{ab}	10.2 ± 0.369^{a}	4.57 ± 0.0903^{a}	5.68 ± 0.191^{ab}	3.09 ± 0.151^{a}
nCuO0.1	8.16 ± 0.457^{ab}	9.17 ± 0.389^{ab}	4.28 ± 0.087^{ab}	4.94 ± 0.307^{ac}	2.32 ± 0.111^{ab}
nCuO1.0	8.56 ± 0.309^{ab}	10.1 ± 0.301^{a}	4.53 ± 0.142^{a}	5.97 ± 0.282^{a}	2.83 ± 0.101^{a}
nCuO10	7.49 ± 0.427^{ac}	9.32 ± 0.475^{ab}	4.31 ± 0.167^{ab}	4.79 ± 0.413^{ac}	2.43 ± 0.187^{ab}
nCuO50	5.86 ± 0.446^{c}	7.77 ± 0.642^{b}	3.72 ± 0.358^{b}	3.37 ± 0.518^{c}	1.79 ± 0.339^{b}
nCuO100	7.25 ± 0.445^{bc}	8.78 ± 0.517^{ab}	4.53 ± 0.106^{a}	4.15 ± 0.449^{bc}	2.36 ± 0.317^{ab}
As	8.03 ± 0.446^{ab}	9.86 ± 0.349^{a}	4.32 ± 0.131^{ab}	5.24 ± 0.253^{ab}	2.32 ± 0.237^{ab}
As+nCuO0.1	8.9 ± 0.403^{ab}	10.7 ± 0.364^{a}	4.5 ± 0.149^{a}	5.06 ± 0.237^{ab}	2.75 ± 0.118^{ab}
As+nCuO1.0	8.27 ± 0.133^{ab}	10.5 ± 0.539^{a}	4.52 ± 0.106^{a}	4.64 ± 0.155^{ac}	2.53 ± 0.106^{ab}
As+nCuO10	8.91 ± 0.474^{ab}	10 ± 0.515^{a}	4.72 ± 0.146^{a}	5.27 ± 0.345^{ab}	2.7 ± 0.204^{ab}
As+nCuO50	9.52 ± 0.477^{a}	10.5 ± 0.185^{a}	4.62 ± 0.0844^{a}	6.1 ± 0.425^{a}	3.33 ± 0.156^{a}
As+nCuO100	9.31 ± 0.565^{a}	10.1 ± 0.359^{a}	4.47 ± 0.211^{ab}	5.71 ± 0.334^{ab}	2.95 ± 0.297^{a}

Values are means $\pm$ SEM, n = 10 per treatment group.
[a-c] Means in a column with a common superscript letter are similar ($p < 0.05$).

Table D.11. Seedling growth parameters in arsenic alone treatment from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Shoot length (cm)	Root length (cm)	Number of root branches	Shoot dry weight (mg)	Root dry weight (mg)
Control	8.06 ± 0.284^a	4.44 ± 0.206^{ab}	4.9 ± 0.093^a	$4.84 \pm 0.148ab$	$1.53 \pm 0.075a$
nCuO0.1	7.7 ± 0.288^a	4.47 ± 0.169^{ab}	4.89 ± 0.14^a	$4.23 \pm 0.12ab$	$1.01 \pm 0.046ad$
nCuO1.0	7.66 ± 0.38^a	4.43 ± 0.199^{ab}	4.65 ± 0.14^{ab}	$5 \pm 0.267a$	$1.28 \pm 0.079ad$
nCuO10	6.68 ± 0.175^{ab}	3.53 ± 0.227^b	4.88 ± 0.13^a	$3.68 \pm 0.22bc$	$0.892 \pm 0.053cd$
nCuO50	5.12 ± 0.533^b	2.25 ± 0.254^c	3.82 ± 0.44^b	$2.83 \pm 0.258c$	$0.76 \pm 0.098d$
nCuO100	7.2 ± 0.449^a	3.84 ± 0.251^{ab}	5.01 ± 0.25^a	$4.2 \pm 0.206ab$	$1.06 \pm 0.068ad$
As	7.53 ± 0.295^a	3.95 ± 0.228^{ab}	4.75 ± 0.129^a	$4.23 \pm 0.312ab$	$0.936 \pm 0.073bd$
As+nCuO0.1	7.8 ± 0.453^a	3.72 ± 0.402^{ab}	4.9 ± 0.107^a	$4.18 \pm 0.229ab$	$1.06 \pm 0.155ad$
As+nCuO1.0	6.88 ± 0.34^a	3.9 ± 0.273^{ab}	4.72 ± 0.126^a	$3.81 \pm 0.298ac$	$1.49 \pm 0.132ab$
As+nCuO10	7.19 ± 0.333^a	4.55 ± 0.204^{ab}	4.6 ± 0.096^{ab}	$4.05 \pm 0.246ac$	$1.08 \pm 0.147ad$
As+nCuO50	8.06 ± 0.284^a	4.83 ± 0.279^a	4.97 ± 0.090^a	$5.02 \pm 0.295a$	$1.46 \pm 0.146abc$
As+nCuO100	7.71 ± 0.416^a	4.52 ± 0.2^{ab}	4.55 ± 0.178^{ab}	$4.99 \pm 0.345a$	$1.46 \pm 0.215ab$

Values are means $\pm$ SEM, n = 10 per treatment group.
[a-c] Means in a column with a common superscript letter are similar ($p < 0.05$).

Table D.12. Seedling growth parameters in F1 quai-F0 treatment from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Quasi-F0 treatment		Seed type	Shoot length (cm)	Root length (cm)	Number of root branches	Shoot dry weight (mg)	Root dry weight (mg)
As (mg/kg)	nCuO (mg/L)						
0	0	Control	8.84 ± 0.48ab	10.2 ± 0.37a	4.57 ± 0.09a	5.68 ± 0.19^a	3.09 ± 0.15^a
	0.1	nCuO0.1	7.68 ± 0.47^{ab}	9.71 ± 0.81^a	4.80 ± 0.67	5.37 ± 0.39^{ab}	2.73 ± 0.11^{ab}
	1.0	nCuO1.0	8.14 ± 0.32^a	11.10 ± 0.25^a	4.45 ± 0.13	5.04 ± 0.20^{ac}	2.46 ± 0.10^{ab}
	10	nCuO10	6.54 ± 0.50^{bd}	9.76 ± 0.67^a	4.45 ± 0.15	3.98 ± 0.44^{bc}	2.32 ± 0.30^b
	50	nCuO50	4.76 ± 0.36^e	6.28 ± 0.59^b	4.24 ± 0.29	2.24 ± 0.43^e	1.01 ± 0.21^c
	100	nCuO100	4.97 ± 0.29^{de}	4.61 ± 0.33^{bc}	4.88 ± 0.23	2.41 ± 0.09^{de}	1.30 ± 0.18^c
10	0	As	7.53 ± 0.30^a	3.95 ± 0.23^{ab}	4.75 ± 0.13^a	4.23 ± 0.31^{ac}	0.94 ± 0.07^c
	0.1	As+nCuO0.1	5.71 ± 0.31^{cde}	2.93 ± 0.21^{cd}	4.50 ± 0.24	3.66 ± 0.19^{ce}	0.99 ± 0.15^c
	1.0	As+nCuO1.0	6.91 ± 0.32^{abc}	3.02 ± 0.25^{cd}	4.97 ± 0.10	4.34 ± 0.37^{ac}	0.94 ± 0.08^c
	10	As+nCuO10	6.19 ± 0.26^{be}	3.12 ± 0.22^{cd}	4.96 ± 0.18	4.18 ± 0.37^{ac}	1.02 ± 0.04^c
	50	As+nCuO50	6.14 ± 0.21^{be}	2.09 ± 0.23^d	5.35 ± 0.31	4.00 ± 0.31^{bc}	0.97 ± 0.10^c
	100	As+nCuO100	6.45 ± 0.33^{bd}	1.86 ± 0.10^d	4.88 ± 0.14	3.77 ± 0.23^{cd}	0.83 ± 0.04^c
p	**As**		0.95	< 0.001	0.03	0.02	< 0.001
	nCuO		< 0.001	< 0.001	0.96	< 0.001	0.004
	A×N		< 0.001	< 0.001	0.14	< 0.001	< 0.001

Values are means ± SEM, n = 10 per treatment group.
[A-B] Means in a at the same As level with a common superscript letter are similar ($p < 0.05$).
[ab] Means in a column at the same nCuO level with a common superscript letter are similar ($p < 0.05$).
As × nCuO: interaction effect.

Table D.13. Copper and arsenic concentrations in F1 seedlings in control from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Root		Shoot	
	Cu (μg/g)	As (ng/g)	Cu (μg/g)	As (ng/g)
Control	30.60 ± 2.90	671 ± 105	23.30 ± 3.09	302 ± 75.4
nCuO0.1	30.50 ± 2.15	692 ± 250	22.00 ± 0.69	270 ± 23.5
nCuO1.0	30.20 ± 2.57	490 ± 114	23.00 ± 2.73	231 ± 21
nCuO10	32.50 ± 2.44	466 ± 9.35	22.00 ± 2.14	266 ± 26
nCuO50	33.70 ± 2.23	487 ± 52.2	25.10 ± 1.56	364 ± 76.5
nCuO100	33.20 ± 2.10	494 ± 85.7	21.20 ± 1.61	328 ± 15.3
As	32.40 ± 2.37	427 ± 52.7	21.10 ± 0.93	255 ± 37.9
As+nCuO0.1	29.10 ± 2.00	509 ± 54.9	21.10 ± 2.09	207 ± 10.9
As+nCuO1.0	32.60 ± 2.34	432 ± 51.3	20.80 ± 1.30	259 ± 39.9
As+nCuO10	26.20 ± 0.87	542 ± 92.8	21.00 ± 0.29	210 ± 8.95
As+nCuO50	37.10 ± 5.92	452 ± 40.6	21.60 ± 0.50	236 ± 8.99
As+nCuO100	35.50 ± 2.72	291 ± 16.5	20.80 ± 2.35	218 ± 5.52

Values are means ± SEM, n = 3 per treatment group.
[a-c] Means in a column with a common superscript letter are similar ($p < 0.05$).

Table D.14. Copper and arsenic concentrations in F1 seedlings in As alone treatment from an18-day laboratory experiment of F1 rice seed germination and seedling growth

Seed type	Root		Shoot	
	Cu (µg/g)	As (µg/g)	Cu (µg /g)	As (µg /g)
Control	36.4 ± 3.86 a	668.0 ± 17.5 ab	19.3 ± 1.13	150.0 ± 5.07 ab
nCuO0.1	42.5 ± 6.67 ab	631.0 ± 62.2 ab	20.6 ± 0.86	140.0 ± 7.73 abc
nCuO1.0	33.9 ± 2.13 a	582.0 ± 12.6 ab	18.2 ± 1.68	126.0 ± 2.89 abc
nCuO10	43.3 ± 6.56 ab	647.0 ± 44.6 ab	19.4 ± 2.41	135.0 ± 6.03 abc
nCuO50	77.2 ± 20.40 b	486.0 ± 112.0 a	22.0 ± 4.40	113.0 ± 14.80 ac
nCuO100	38.4 ± 4.89 a	677.0 ± 19.7 ab	18.7 ± 1.45	117.0 ± 3.43 abc
As	42.9 ± 6.10 ab	778.0 ± 35.8 b	16.6 ± 0.31	122.0 ± 7.63 abc
As+nCuO0.1	37.9 ± 2.24 a	750.0 ± 7.02 ab	18.2 ± 1.13	147.0 ± 5.58 ab
As+nCuO1.0	30.9 ± 3.80 a	638.0 ± 39.4 ab	16.6 ± 1.54	143.0 ± 11.00 abc
As+nCuO10	44.1 ± 5.48 ab	675.0 ± 16.0 ab	20.6 ± 1.20	158.0 ± 13.00 b
As+nCuO50	31.8 ± 2.94 a	665.0 ± 13.6 ab	18.8 ± 0.92	108.0 ± 5.31 c
As+nCuO100	33.2 ± 1.29 a	610.0 ± 48.1 ab	19.3 ± 0.53	133.0 ± 8.13 abc

Values are means ± SEM, n = 3 per treatment group.
[a-c] Means in a column with a common superscript letter are similar ($p < 0.05$).

Table D.15. Copper and arsenic concentrations in F1 seedlings in F1 quai-F0 treatment from an18-day laboratory experiment of F1 rice seed germination and seedling growth

As (mg/kg)	nCuO (mg/L)	Seed type	Root		Shoot	
			Cu (µg /g)	As (µg /g)	Cu (µg /g)	As (µg /g)
0	0	Control	30.60 ± 2.90 [Aa]	0.67 ± 0.11 [ABa]	23.3 ± 3.09 [Aa]	0.30 ± 0.08 [ABa]
	0.1	nCuO0.1	137.0 ± 48.6 [Ba]	1.57 ± 0.77 [Aa]	25.0 ± 0.84 [Aa]	0.15 ± 0.01 [Ca]
	1.0	nCuO1.0	73.20 ± 1.30 [Ba]	0.51 ± 0.05 [Ba]	30.4 ± 2.22 [Aa]	0.14 ± 0.03 [Ca]
	10	nCuO10	389.0 ± 30.0 [Ca]	1.34 ± 0.40 [ABa]	113.0 ± 7.73 [Ba]	0.25 ± 0.04 [ACa]
	50	nCuO50	2170.0 ± 258.0 [Da]	1.29 ± 0.26 [ABa]	324.0 ± 16.80 [Ca]	0.46 ± 0.04 [ABa]
	100	nCuO100	2880.0 ± 150.0 [Da]	0.93 ± 0.14 [ABa]	408.0 ± 17.20 [Ca]	0.49 ± 0.12 [Ba]
10	0	As	42.900 ± 6.10 [Aa]	778.0 ± 35.80 [Ab]	16.60 ± 0.31 [Ab]	122.0 ± 7.63 [Ab]
	0.1	As+nCuO0.1	46.700 ± 7.47 [Ab]	672.0 ± 0.59 [Ab]	24.80 ± 2.48 [Ba]	204.0 ± 14.70 [Ab]
	1.0	As+nCuO1.0	59.2 ± 7.05 [Aa]	667.0 ± 47.70 [Ab]	26.10 ± 1.93 [Ba]	158.0 ± 13.10 [Ab]
	10	As+nCuO10	337.0 ± 34.3 [Ba]	643.0 ± 70.80 [Ab]	95.20 ± 8.13 [Ca]	156.0 ± 4.31 [Ab]
	50	As+nCuO50	1180.0 ± 45.8 [Cb]	559.0 ± 2.91 [Ab]	144.0 ± 6.23 [Db]	131.0 ± 22.50 [Ab]
	100	As+nCuO100	2060.0 ± 591.0 [Ca]	469.0 ± 120.0 [Ab]	280.0 ± 53.3 [Eb]	139.0 ± 15.40 [Ab]
p		As	0.38	< 0.001	< 0.01	< 0.001
		nCuO	< 0.001	< 0.001	< 0.001	< 0.001
		A×N	< 0.001	0.63	< 0.001	0.16

Values are means ± SEM, n = 3 per treatment group.
[A-E] Means in a at the same As level with a common superscript letter are similar ($p < 0.05$).
[ab] Means in a column at the same nCuO level with a common superscript letter are similar ($p < 0.05$).
A×N: As × nCuO interaction effect.
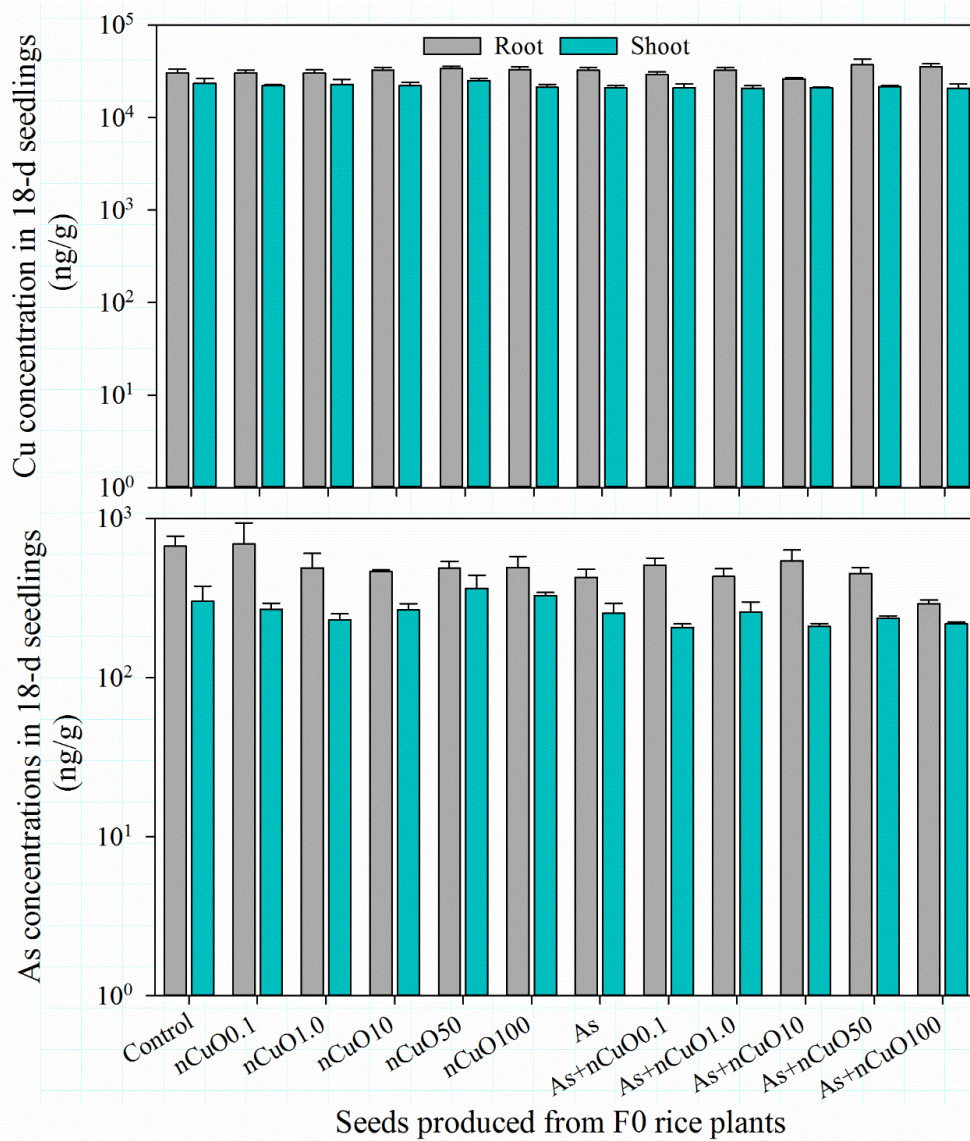
Figure D.1. Copper and arsenic concentrations in F1 seedlings in control treatment from an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in the nutrient solutions (n = 10).
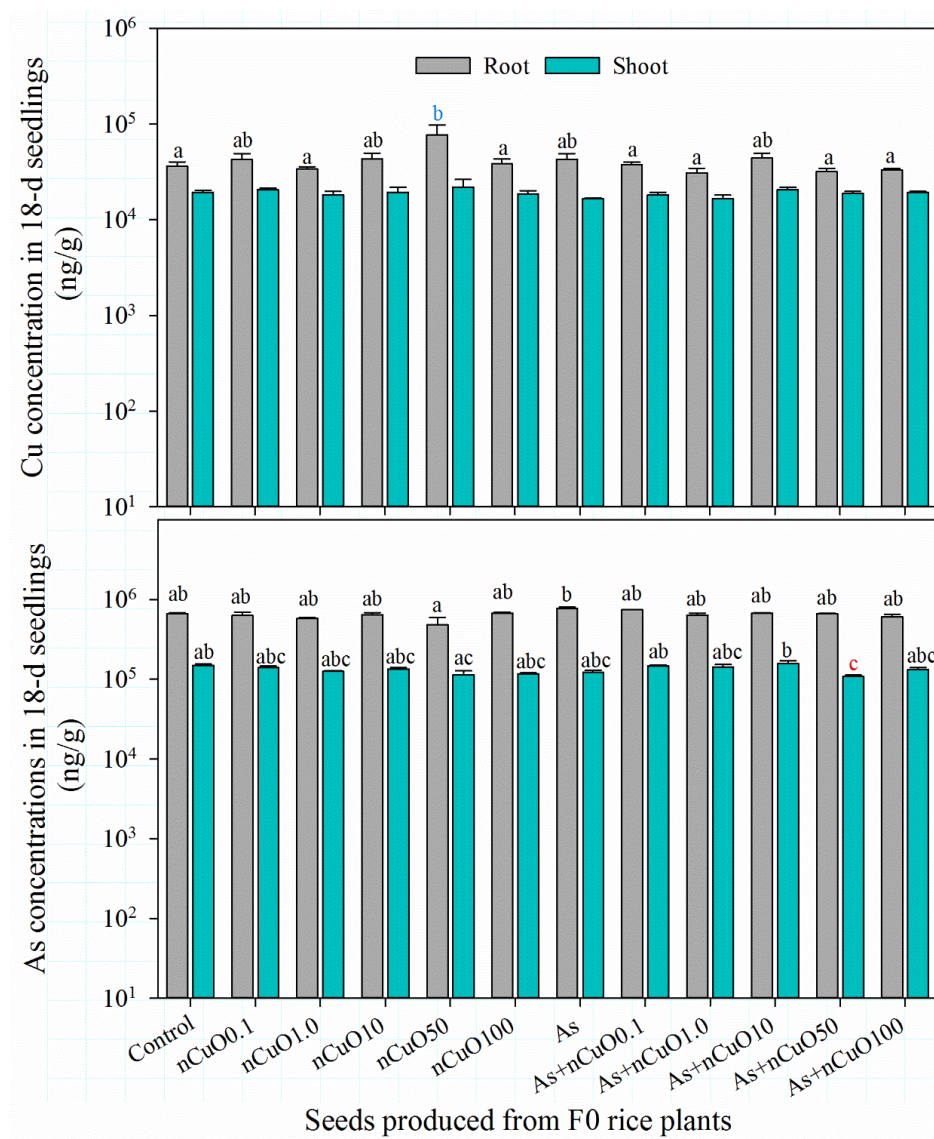
Figure D.2. Copper and arsenic concentrations in F1 seedlings in As alone treatment from an 18-d seed germination and seedling growth test with exposure to arsenic in the sand and copper oxide nanoparticles in the nutrient solutions (n = 10). Means of treatments with a common superscript letter (a-c) are similar ($p < 0.05$).

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