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Metabolomics to Detect Response of Lettuce (*Lactuca sativa*) to $Cu(OH)_2$ Nanopesticides: Oxidative Stress Response and Detoxification Mechanisms

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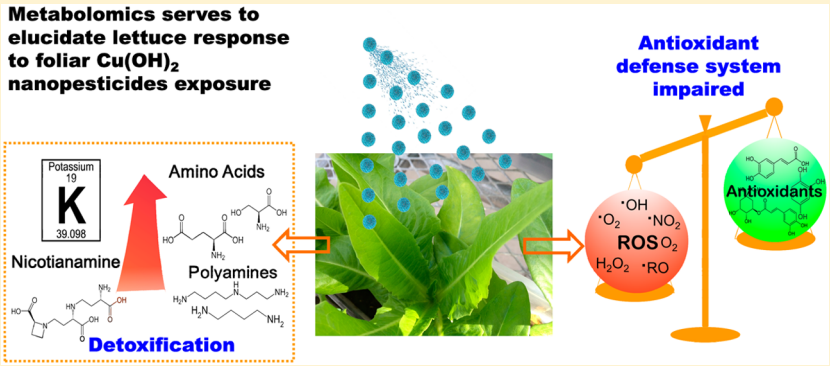
Supporting Information



ABSTRACT: There has been an increasing influx of nanopesticides into agriculture in recent years. Understanding the interaction between nanopesticides and edible plants is crucial in evaluating the potential impact of nanotechnology on the environment and agriculture. Here we exposed lettuce plants to $Cu(OH)_2$ nanopesticides (1050−2100 mg/L) through foliar spray for one month. Inductively coupled plasma-mass spectrometry (ICP-MS) results indicate that 97−99% (1353−2501 mg/kg) of copper was sequestered in the leaves and only a small percentage (1−3%) (17.5−56.9 mg/kg) was translocated to root tissues through phloem loading. Gas chromatography-time-of-flight mass spectrometry (GC-TOF-MS) based metabolomics combined with partial least squares-discriminant analysis (PLS-DA) multivariate analysis revealed that $Cu(OH)_2$ nanopesticides altered metabolite levels of lettuce leaves. Tricarboxylic (TCA) cycle and a number of amino acid-related biological pathways were disturbed. Some antioxidant levels (cis-caffeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid, dehydroascorbic acid) were significantly decreased compared to the control, indicating that oxidative stress and a defense response occurred. Nicotianamine, a copper chelator, increased by 12−27 fold compared to the control, which may represent a detoxification mechanism. The up-regulation of polyamines (spermidine and putrescine) and potassium may mitigate oxidative stress and enhance tolerance. The data presented here provide a molecular-scale perspective on the response of plants to copper nanopesticides.

INTRODUCTION

In the past decade, nanoscale fertilizers and pesticides have been increasingly proposed and used in agriculture.[1,2] In particular copper-containing nanopesticides are being introduced to the market due to their excellent antimicrobial and antifungal properties.[2,3] The increasing use of nanopesticides in agriculture has motivated researchers to consider their environmental fate, bioavailability, and toxicity to edible plants.[4] Extensive investigations of bioaccumulation and phytotoxicity of copper-based nano pesticides (CuO, Cu, $Cu(OH)_2$) on a variety of crop plants, for example, radish (*Raphanus sativus*), ryegrass (*Lolium rigidum*), cilantro (Coriandrum sativum), zucchini (*Cucurbita pepo*), bean (Phaseolus radiatus), wheat (*Triticum aestivum*), duckweeds (*Landoltia punctata*), lettuce (*Lactuca sativa*), alfalfa (*Medicago sativa*), have been conducted.[5−11] However, most studies spiked NPs in soil or via water in hydroponic systems, whereas in commercial agriculture

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most copper related pesticides are applied through foliar spray.[5−14] Compared with the soil-root transfer, the leaf-root transfer pathway(s) and toxicity mechanism(s) have rarely been investigated and are poorly understood.[12,15]

Terrestrial vascular plants have the ability to uptake metals, organic contaminants, even nanoparticles through the leaves employing different pathways.[16−18] Ions are able to penetrate the leaf cuticle (lipophilic and hydrophilic pathways) and enter the cytosol of epidermal cells or mesophyll cells.[19] For fine particles (diameter less than 2.5 μm), stomatal uptake is an important pathway.[19,20] For example, Uzu et al. reported that lettuce (*Lactuca sativa*) leaves contained 335 ± 50 mg Pb kg^{-1} (dry weight) after exposure to Pb-enriched fine particles for 43 days.[19] Hong et al. showed that CeO_2 NPs were taken up through cucumber (*Cucumis sativus*) leaves and translocated to root tissues.[21] Lettuce is a widely cultivated vegetable and usually used as a model plant in contaminant transfer studies.[19,22] Furthermore, since the leaves are the edible part of lettuces, investigating their foliar uptake of copper based nanopesticides is of high interest for risk assessment of human and ecological health.

Copper is a redox active transition metal and is involved in redox reaction in cells, generating $O_2^{\bullet-}$ and ·OH via the Haber-Weiss and Fenton reactions.[23,24] Once copper particles/ions enter into plant tissues, no matter where they are localized, they may induce oxidative stress and affect several metabolic processes. These changes should be reflected in the concentration of various metabolites after exposure. Metabolomics is a powerful approach for gaining a comprehensive understanding of biological mechanisms, including toxicity, generally by monitoring low molecular weight metabolites.[25,26] In recent years, various technologies (GC/MS/MS, LC/MS/MS, ^{1}H NMR) have been employed for metabolic investigations of organism responses to environmental stressors.[27−31] Rather than target a limited number of metabolites or physiological parameters, nontargeted metabolomics can provide information on a large number of metabolites, which results in a deeper insight into the molecular mechanisms underlying the physiological and biochemical changes. In addition, metabolomics can be used to reveal the mechanism of plant defense and detoxification of contaminants.[30,31] Pidatala et al.[30] performed metabolomics studies and revealed detoxification and tolerance mechanism of Vetiver (*Chrysopogon zizanioides*) to Pb. Our recent study on cucumber plant root exudate metabolomics revealed that exposure to nano-Cu up-regulated a number of amino acids that bind with copper NPs and ions, likely to detoxify Cu from its nearby environment.[31]

The primary aim of this work was to determine the metabolic profile changes in plants exposed to $Cu(OH)_2$ nanopesticides using GC-TOF-MS. The objective is to elucidate the toxicity and detoxification mechanisms underlying up- or down-regulated metabolites. In addition, knowledge on the uptake and translocation of $Cu(OH)_2$ nanopesticides and released Cu ions in lettuce leaves, through foliar application, is of high interest for risk assessment.

MATERIALS AND METHODS

Nanoparticles. The $Cu(OH)_2$ nanopesticide used in this study were in the form of a commercial biocide (Kocide 3000 by Dupont). Detailed physicochemical properties of Kocide 3000 have been reported before.[32,33] Specifically, the primary particle size is ∼50 to >1000 nm. The hydrodynamic diameter is 1532 ± 580 nm and the zeta potential is −47.6 ± 43 mV, measured via Dynamic Light Scattering (Malvern Zetasizer Nano ZS-90), in NanoPure water at pH 7. Although Kocide 3000 particles are mainly micron-sized, these micronized particles are made up of nanosheets of $Cu(OH)_2$ that are bound together by an organic composite and can potentially redissociate in water.[32] For this reason the pesticide is considered "nano". Copper content in Kocide 3000 is 26.5 ± 0.9%, while other elements including C, O, Na, Al, Si, S, Zn compose 73.5% of mass.[32]

Growth Condition and Foliar Application of $Cu(OH)_2$ Nanopesticide. Lettuce (*Lactuca sativa,* variety Amish Deer Tongue) seeds were purchased from Seed Savers Exchange (Iowa). The soil was collected from the Natural Reserve System of UC Santa Barbara (Sedgwick), from the top 20 cm. The soil texture is sandy loamy grassland with sand/silt/clay percentage of 54.0%, 29.0% and 17.0%. Soil pH is 5.90 ± 0.04. Loss-on-ignition organic matter is 3.11 ± 0.07%. Cation exchange capacity is 25.8 ± 0.1 mequiv 100 g^{-1}. More information regarding the soil composition was reported in a previous study.[33] Lettuce seeds were planted in pots containing 250 g of soil. Each pot contained one seed. Plants were grown in a greenhouse, which was maintained at 28 °C by day and 20 °C by night. The daily light integral (photosynthetically active radiation) was 17.3 ± 3.6 mol m^{-2} d^{-1}. When plants were 24 days old, we began to spray them with Kocide 3000 suspended in NanoPure water at 105, 155, and 210 mg Kocide/100 mL. The doses were selected generally following the manufacturer's recommendation (0.84−1.7 kg/ha). Before spraying, the suspension was sonicated (Branson 8800) for 30 min in cooled water. A hand-held spray bottle was used for spraying. The lettuces were sprayed a total of 8 times during 4 weeks; the amount sprayed each time was 8.75 (Low), 12.9 (Medium) and 17.5 (High) mg/pot. Each spray was ∼1 mL. The seven treatments were: control; low, medium, and high NPs in uncovered soil; low, medium and high NPs in covered soil. Each treatment was replicated five times. In covered samples, the soil was covered with filter paper. In covered soil, Cu detected in root should be only from leaf translocation. In uncovered soil, Cu in root not only comes from translocation, but also from soil, because the soil was also exposed to some $Cu(OH)_2$ nanopesticide during spraying. This allows us to determine whether the Cu NPs were translocated from leaf to root. At 52 days after planting, all plants were harvested.

Environmental Scanning Electron Microscopy. Environmental scanning electron microscopy (ESEM, Phillips Electron Optics, Eindhoven, Netherlands) was employed to image $Cu(OH)_2$ nanopesticides on lettuce leaf surfaces. One-month-old lettuce plants were foliar sprayed with 105 mg Kocide/100 mL. After drying for 24 h, the leaves were sampled and imaged by ESEM.

Cu and Nutrient Elements Analysis in Plant Tissues and Soil. At harvest, the lettuce plants were gently removed from the soil, thoroughly rinsed with tap water for 5 min and then rinsed with NanoPure water three times. Leaf tissue was carefully separated from vascular and mesophyll tissues (Supporting Information (SI) Figure S1). Mesophyll and root tissues were ground in liquid nitrogen and lyophilized for 5 days. Part of the freeze-dried mesophyll tissues were sent to UC Davis for metabolomics analysis, and another portion was oven-dried at 70 °C for ICP-MS analysis. Since only a small amount of vascular tissue was available, it was only oven-dried for metal analysis.

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Table 1. Copper Distribution in Different Lettuce Tissues and Soil (mg/kg dry Weight)[a]

	vascular	mesophyll	root	soil
control	9.9 ± 5	13 ± 5.9	6 ± 2.8	29.2 ± 0.9
UC-low	973 ± 145*	1695 ± 113*	34.2 ± 12.7*	50.3 ± 17.4*
UC-medium	1212 ± 139*	2157 ± 353*	56.9 ± 22.5*	44.1 ± 6.8*
UC-high	1344 ± 73*	2296 ± 302*	34.9 ± 10.4*	56.5 ± 15.6*
C-low	823 ± 110*	1353 ± 324*	19.5 ± 3.8*	37.1 ± 6.4*
C-medium	1111 ± 80*	2008 ± 438*	17.5 ± 2*	38.3 ± 7.1*
C-high	1401 ± 137*	2501 ± 295*	26.1 ± 11.7*	31 ± 1.4*

[a]Data are average of five replicate; star means statistically significant at $p < 0.05$ compared to control. UC means soil uncovered; C means soil was covered.

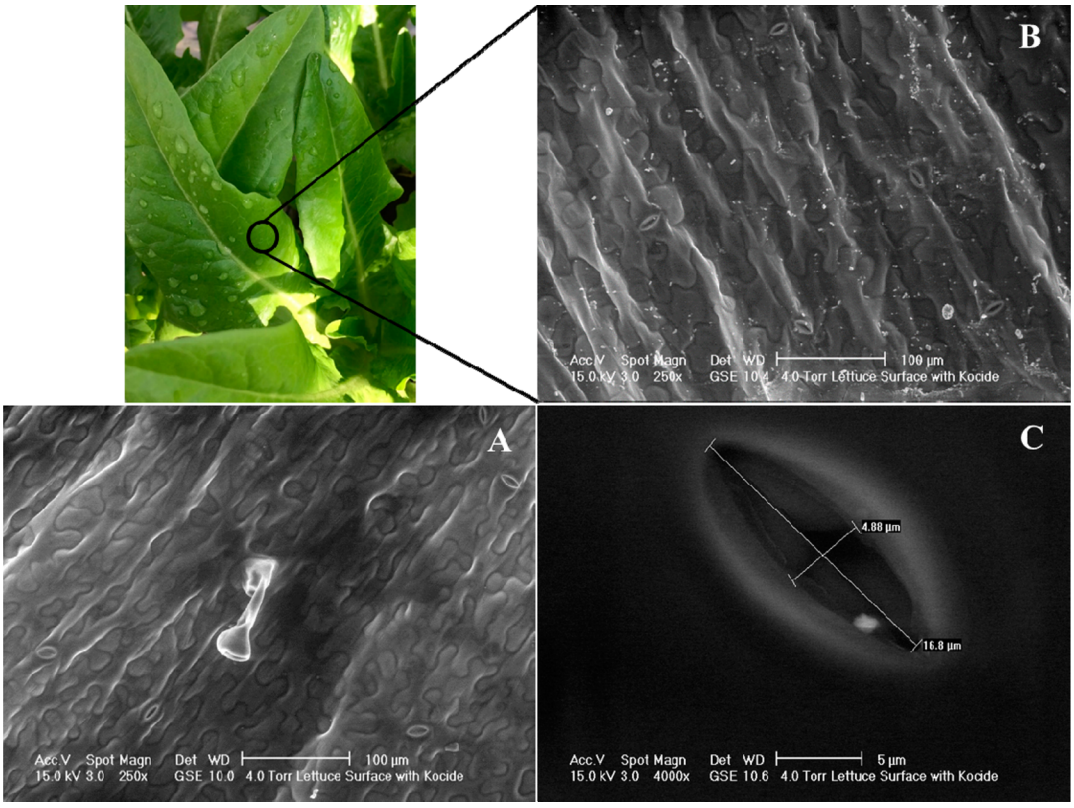

Figure 1. ESEM micrographs of lettuce surface adaxial cuticle morphology: (A) control without exposure to nanopesticides; (B) 24 h after exposure to $Cu(OH)_2$ nanopesticides, numerous NPs were distributed on the lettuce leaf surface; (C) higher magnification of B, showing an NP or aggregate trapped in a stomata.

Dried plant tissues were digested with a mixture of 4 mL of H_2O_2 and 1 mL of plasma pure HNO_3 (v/v: 4:1) using a microwave oven system (Multiwave Eco, Anton Parr) at 165 °C for 1 h. The standard reference materials NIST 1547 and 1570a were also digested and analyzed as samples. The recoveries for all elements were between 90 and 99%. Cu and other six important mineral elements (Mg, P, K, Ca, Fe, Zn) were analyzed using inductively coupled plasma mass spectrometry (ICP-MS 7900, Agilent).

Gas Chromatography-Time of Flight-Mass Spectrometry (GC-TOF-MS) Analysis of Metabolites in Root and Leaves. The freeze-dried lettuce tissues samples were subjected to GC-TOF-MS analysis at the Genome Center Core Services, University of California Davis to identify the metabolites present in lettuce tissues. A description of sample pretreatment, analytical method and instrument has been described by Fiehn et al.[34,35] Briefly, an Agilent 6890 gas chromatograph (Santa Clara, CA) containing a Rtx-5Sil MS column (30 m length × 0.25 mm internal diameter with 0.25 μm film made of 95% dimethyl/5% diphenylpolysiloxane) with an additional 10 mm integrated guard column was used to run the samples, controlled using Leco ChromaTOF software version 2.32 (http://www.leco.com). Quantification was reported as peak height using the unique ion as default. Metabolites were unambiguously assigned by the BinBase identifier numbers using retention index and mass spectrum as the two most important identification criteria. More details regarding data acquisition, data processing and data reporting are provided in the SI.

Multivariate Analysis and Biological Pathway Analysis. Partial least-squares discriminant analysis (PLS-DA) is a supervised clustering method, which uses a multiple linear regression technique to maximize the separation between groups and helps to understand which variables carry the class separating information.[36] PLS-DA was run based on GC-TOF-MS data using online resources (http://www.metaboanalyst.

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ca/).[37] Variable Importance in Projection (VIP) is the weighted sum of the squares of the PLS-DA analysis, which indicates the importance of a variable to the entire model.[36] A variable with a VIP above 1 is regarded as significant.[38] Biological pathway analysis was performed based on all detected metabolites data using MetaboAnalyst 2.0.[39] The impact value threshold calculated for pathway identification was set at 0.1.[38]

Statistical Analysis. The concentration of Cu, nutrient element and biomass was statistically analyzed using an independent two sample *t* test to determine whether concentration levels were significantly different between control and nanopesticides treatments. P-values were calculated with a two-tailed distribution.

RESULTS AND DISCUSSION

Foliar Cu Particles Uptake in Leaves. Cu in leaves (vascular and mesophyll tissues) increased in a dose-dependent manner in both covered and uncovered soil (Table 1). Cu increased 82−140 times in vascular and 115−184 times in mesophyll tissues relative to the control, which indicates a high bioaccumulation of copper/nanoparticles in leaf tissues. Even though the leaves were thoroughly washed, copper ions and NPs remained on the surface or were incorporated into leaf tissues; it is likely that washing was not 100% efficient in removing them. Leaf exudates can form weak acids in the presence of water,[40] which can accelerate dissolution of $Cu(OH)_2$ nanopesticide, releasing cupric ions as long as the water remains on the leaf. This may result in a pathway for cupric ions to penetrate the epidermis cells and translocate to other tissues.

In addition to ionized Cu, nanoparticles smaller than the stomatal diameter may enter past the guard cells. Stomatal diameters range from 8 to 12 μm for several species.[41] Even though trichomes (hair) are not abundant on lettuce leaf surfaces, ESEM images (Figure 1) taken after 24 h exposure to $Cu(OH)_2$ nanopesticides show that many small particles were deposited on the lettuce leaf surface and stomatal cavities. The typical diameter observed for lettuce stomata is 13.1 μm (Figure 1), which is large enough to permit entry to $Cu(OH)_2$ nanopesticides aggregates with an observed hydrodynamic diameter of 1530 ± 580 nm. Eichert demonstrated that 43 nm (diameter) NPs entered stomata and migrated along the surface of stomatal pores.[42] After passing the stomatal guard cells, the NPs may either attach to cell walls or move between cell walls. For example, Stamenkovic and Gustin showed the majority of foliar Hg was located in epidermal and stomatal cell walls and was rarely found in mesophyll or vascular tissue.[43] However, Hong showed that Ce was present in cucumber root phloem (vascular tissue) after foliar application of CeO_2 NPs.[12]

Translocation from Leaves to Root. As seen in Table 1, the average [Cu] in control roots is 6.0 mg/kg, while in treated plants, [Cu] in root is 17.5−26.1 mg/kg in covered soil and 34.2−56.9 mg/kg in uncovered soil. Statistical analysis showed all the NP treated plants have significantly higher [Cu] in roots compared to controls, even though application was only foliar. In covered soil, where no direct root uptake could occur, Cu in the roots was translocated from the leaves via phloem loading. Liao et al. showed that some xylem-transported Cu was recirculated to roots via the phloem in chicory (*Cichorium intybus L.* cv Grasslands Puna) and tomato (*Lycopersicon esculentum Mill.* cv Rondy) plants.[44] Even though we observed evidence of Cu translocation to the roots, 97−99% of Cu mass was retained in the leaves. In addition, the translocation rate (Cu in root/Cu in leaves) in NP-treated plants was 0.009 to 0.014, which is far lower than that in the control (0.461). This indicates plants sequestered most of the Cu in leaves.

Impact on Lettuce Growth. The threshold level for Cu to induce toxicity in plants is 20−30 mg/kg.[45] However, high concentrations (823−2501 mg/kg) of Cu in lettuce leaves did not cause any visible toxic symptoms throughout the entire exposure period (SI Figure S2). On the contrary, the leaf biomass significantly increased at low and medium levels for uncovered treatment and medium level for covered treatment (SI Figure S3). Since a high amount of Cu was retained in leaf tissues but did not induce any toxic symptoms, lettuces likely employ a detoxification mechanism to build tolerance to excess Cu.

Metabolic Response in Leaves after Foliar Application. Using untargeted GC-TOF-MS, a total of 352 compounds were detected in lettuce leaves, and 159 metabolites were identified. To visualize the overall changes in metabolites between control and groups treated at different levels of $Cu(OH)_2$ nanopesticides, PLS-DA analyses of all detected compounds were performed. The score plot (Figure 2A) shows that all treated groups were clearly separated from the control along the first principal axis (PC1), which explained 27% of the total variability. This indicates foliar application of $Cu(OH)_2$ nanopesticides significantly altered the metabolite profiles of lettuce leaves. Since there is not much separation in metabolite concentrations between different exposure levels, it is likely that the lowest exposure level already reached a threshold value for a metabolic response. In order to further identify the metabolites responsible for this separation, we performed PLS-DA based on the 159 identified compounds and determined their VIPs (SI Figure S4A showing the score plot). As seen in SI Figure S5, there were 42 metabolites with VIP > 1, which are the ones that play an important role in group separation.[38] Those metabolites include carboxylic acids (fumaric, malic, maleic, oxalic, malonic, aconitic acids), amino acids (GABA, beta-alanine, glycine, tryptophan, proline, histidine, citrulline, alanine, aspartic acid, asparagine, oxoproline, serine, glutamic acid), amines (hydroxylamine, nicotianamine, ethanolamine), sugars (lyxose, 1-kestose), fatty acid (behenic acid) and other metabolites. An independent two sample *t* test was also performed to screen metabolites which were significantly different from the controls in all treatment groups. *t* test results (SI Table S1) showed that 50 metabolites were significantly different from controls ($p < 0.05$). Twenty one of the metabolites listed in SI Table S1 overlapped with high VIP score metabolites, shown in SI Figure S3. A number of metabolites, that were not responsible for group separation but were significantly modified as determined by a *t* test, are of special interest. Four metabolites including cis-caffeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid, dehydroascorbic acid were significantly decreased in leaves exposed to $Cu(OH)_2$ nanopesticides compared to the control. In addition, oxalic and threonic acids were also down-regulated. Jansson et al. reported that the dehydroascorbic acid formed was decomposed to oxalic acid and threonic acid by hydrogen peroxide generated from Cu(I) auto oxidation in the presence of oxygen.[46] Therefore, the down-regulation of oxalic and threonic acids is due to decreased dehydroascorbic acid.

Pathway analysis indicated that six biological pathways were significantly perturbed (Figure 3A): (1) glycine, serine and threonine metabolism; (2) alanine, aspartate and glutamate metabolism; (3) tricarboxylic (TCA) cycle; (4) pantothenate

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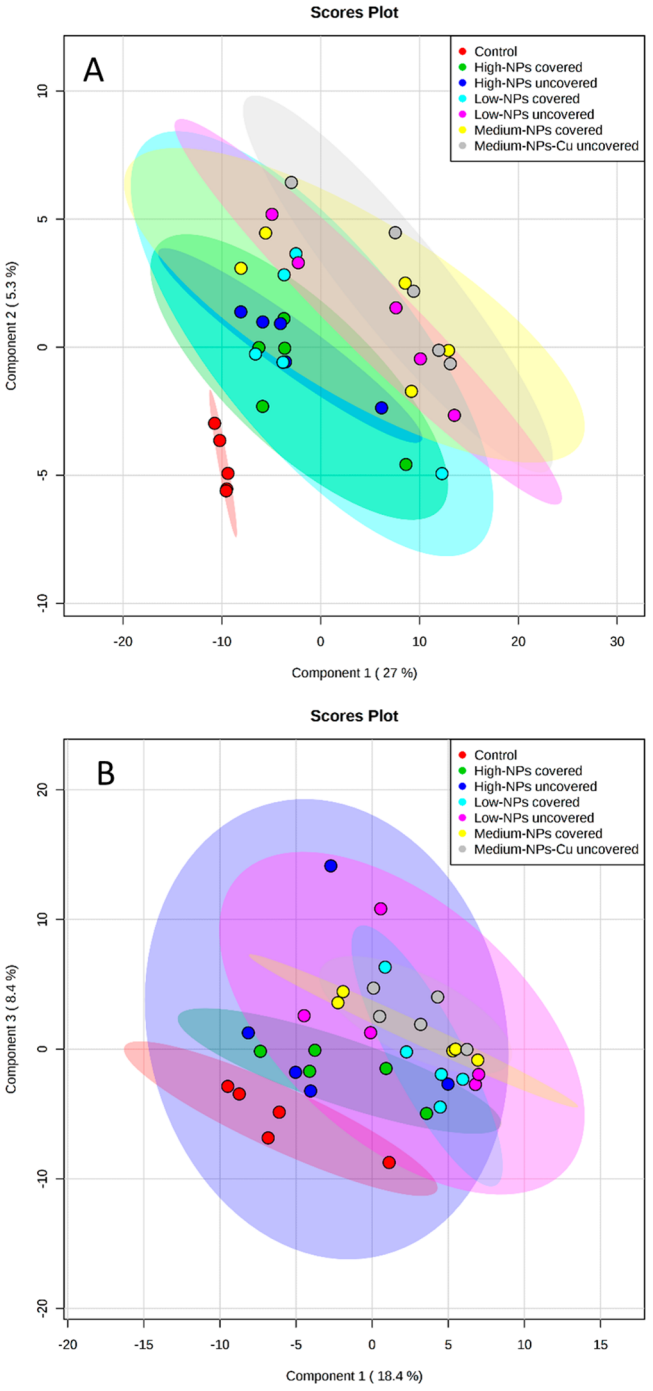

Figure 2. Partial least-squares (PLS) analysis of metabolites in lettuce (A) leaves and (B) roots as affected by different doses of $Cu(OH)_2$ nanopesticides. The data represent 352 metabolites analyzed by GC-TOF-MS.

and coenzyme-A (CoA) biosynthesis; (5) glycolysis or gluconeogenesis; and (6) pyruvate metabolism. $Cu(OH)_2$ NP treated plant leaves exhibited lower levels of TCA cycle intermediates, such as citric, isocitric, and fumaric acids; downregulation of TCA cycle appears to be a clear response. Pidatala et al.,[30] also observed that Pb induced TCA cycle disturbance in Vetiver plants. Exposure to $Cu(OH)_2$ nanopesticides increased the levels of pyruvic acid 2−5 times compared to the control, indicating three biological pathways (4, 5, and 6 in Figure 3A) in which pyruvate participates were likely perturbed. In previous studies, soluble sugars were highly sensitive to environmental stress; these sugars play an important role in signaling and stress defense.[47] In this study, sucrose, glucose, fructose and hexose concentrations did not change after exposure to $Cu(OH)_2$ nanopesticides.

Although treated and control plants can be clearly separated in the PLS-DA analysis, it was not possible to distinguish the different dose levels. It is possible that the lowest dose already reached a threshold that induced changes in the metabolite levels. The same plants that exhibited increased leaf biomass (i.e., Low uncovered, Medium uncovered and covered, from SI Figure S3) also had the most separation from the control group.

Metabolic Response in Roots after Foliar Application. As mentioned before, only 1−3% of Cu mass was translocated from leaves to roots, and root biomass was not impacted by foliar exposure to $Cu(OH)_2$ nanopesticides. As hypothesized, foliar exposure resulted in minor metabolite profile changes in roots compared to changes in leaves. *t* test statistical analysis (SI Table S1) showed that 20 metabolites were significantly different ($p < 0.05$) from the control, which is much lower than in leaves (50). There was noticeable separation between control and treated groups, based on PLS-DA analysis of all detected compounds in roots, but not as clear separation as in leaves (Figure 2B). Treated groups were separated from the control along the third principal axis (PC3), which explained 8.4% of the total variability (Figure 2B). Screened by VIP score, metabolites which were responsible for the separation include several amino acids: proline, glycine, leucine, phenylalanine, methionine, aspartic acid, oxoproline, isoleucine, glutaminc acid, citrulline, threonine, GABA, and serine (SI Figure S6). Biological pathway analysis (Figure 3B) indicates that three pathways related to amino acids were disturbed: (1) phenylalanine metabolism; (2) arginine and proline metabolism; and (3) alanine, aspartate and glutamate metabolism. Thus, root metabolism was perturbed less than leaves, and the perturbed pathways were different from those perturbed in leaves. Among the 20 significantly changed root metabolites, 14 overlapped with changed leaf metabolites. This may indicate metabolites produced in the leaves exposed to copper were transported to the roots.

Impact of Foliar Exposure on Inorganic Ions. Inorganic ions (e.g., Mg, P, K, Ca, Fe, Zn) play an important role in many metabolic processes. Previous metabolomics studies considered only organic compounds, which may not provide a comprehensive view of the changes. Compared to the control, only K^+ levels in leaves were significantly increased in all $Cu(OH)_2$ nanopesticide-treated plants (SI Table S2), with no statistically significant changes in root levels after exposure. Lahoavist et al.[48] showed that hydroxyl radical activated the permeable conductance of K^+ in *Arabidopsis*. Demidchik et al.[49] showed that free oxygen radicals regulate plasma membrane Ca^{2+} and K^+ permeable channels in plant root cells. Therefore, it is possible that exposure to $Cu(OH)_2$ nanopesticides and released Cu^{2+} generated hydroxyl radicals that induced K^+ imbalance. A number of studies have shown that copper nanoparticles are able to trigger the generation of free radicals in cell membrane.[50−53] To test this, one-month-old lettuce plants were exposed to 105 mg/100 mL of $Cu(OH)_2$ nanopesticides. At day 3, leaf samples were collected and incubated with 2′,7′-dichlorofluorescin diacetate (DCF-DA) and observed by confocal laser scanning microscopy to localize Reactive Oxygen Species (ROS). Representative leaf surfaces of

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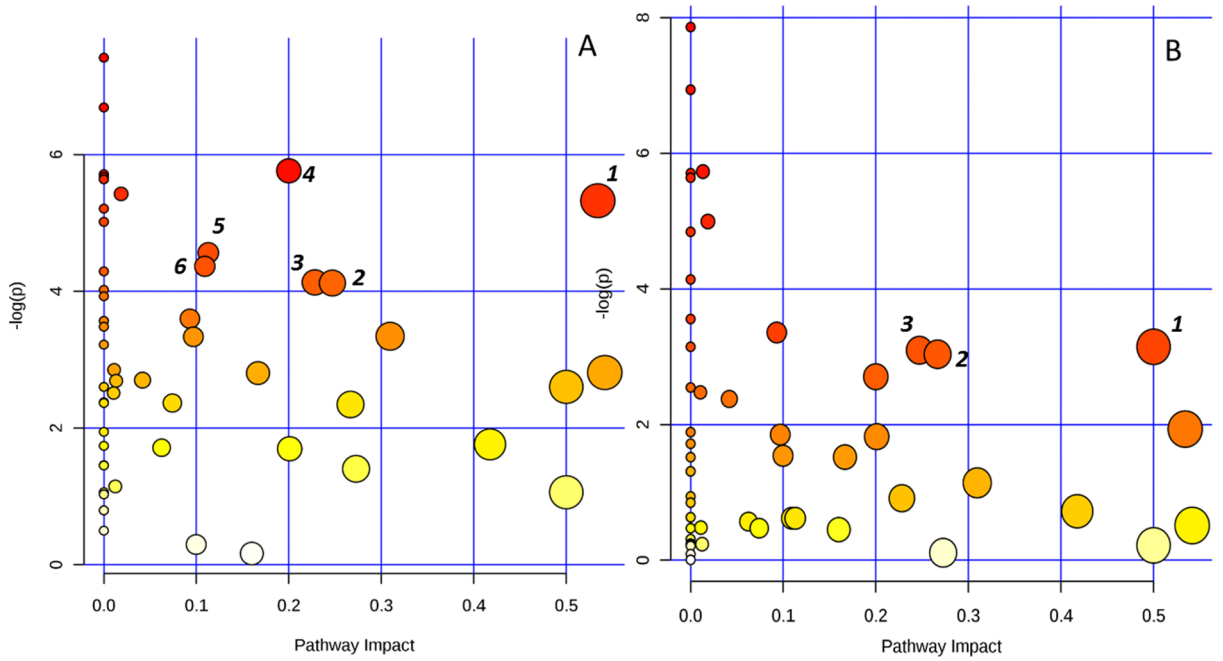

Figure 3. Summary of pathway analysis with MetaboAnalyst 2.0 in (A) leaves and (B) roots. All detected metabolites were considered in the pathway analysis. Altered pathways in leaves: (1) glycine, serine and threonine metabolism; (2) alanine, aspartate and glutamate metabolism; (3) tricarboxylic (TCA) cycle; (4) pantothenate and coenzyme-A (CoA) biosynthesis; (5) glycolysis or gluconeogenesis; (6) pyruvate metabolism. Altered pathways in roots: (1) phenylalanine metabolism; (2) arginine and proline metabolism; (3) alanine, aspartate and glutamate metabolism.

Scheme 1. Toxicity and Detoxification mechanism of lettuce to $Cu(OH)_2$ Nanopesticides

control and $Cu(OH)_2$ nanopesticide treated were imaged by confocal laser scanning microscopy. The green fluorescence signals, which represent the presence of ROS, were found to be much higher in leaves exposed to $Cu(OH)_2$ nanopesticide (SI Figure S7D), compared to the control (SI Figure S7B).

Exploring Possible Toxicity and Detoxification Mechanism. We hypothesize that lettuces must have antioxidant defenses and copper detoxification mechanisms, since there were no visual symptoms of damage on the leaves. Based on the metabolomics we aimed to identify the underlying mechanisms (Scheme 1).

Cu Chelation. Previous studies[54−57] have demonstrated that important mechanisms for plant tolerance of copper are chelation and sequestration, from the upregulated production

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of organic acids, amino acids, peptides, and polyamines. We found nicotianamine (NA) levels were significantly increased in all NPs treated plants, 12−27 times higher than that in controls (Figure 4). NA is a nonproteinaceous amino acid and is synthesized from S-adenosyl methionine.[58] NA has been shown to be an important divalent metal chelator[59] and is involved in metal transport and homeostasis in plants.[60] Previous evidence also showed overexpression of genes involved in NA synthesis increased nickel tolerance in tobacco[61] and *Arabidopsis thaliana*.[62] Liao et al. revealed that NA and histidine have the highest binding constants for Cu^{2+} in chicory and tomato.[44] Thus, the observed up-regulation of NA and histidine in leaves is a possible Cu^{2+} detoxification mechanism for lettuce plants.

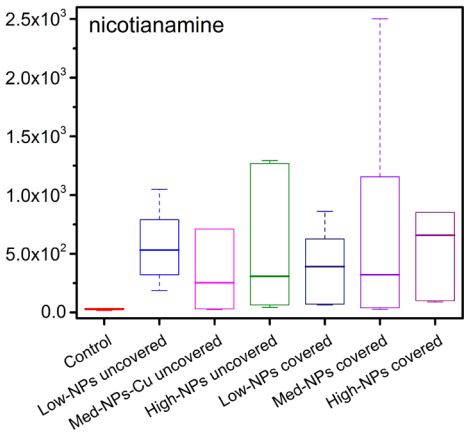

Figure 4. Box plot of relative abundance of nicotianamine (NA) in 54-day-old lettuce leaves exposure to different concentrations of $Cu(OH)_2$ nanopesticides for one month (n = 5).

Phytochelatins (PCs) and metallothioneins (MTs) have been shown to play an important role detoxifying excess Cu^{2+}.[63,64] PCs are synthesized from reduced glutathione (GSH) in a transpeptidation reaction.[65] In addition, GSH is involved in a plethora of cellular processes, including defense against ROS,[66,67] and sequestration of heavy metals.[68,69] Glycine and glutamate are the main constituents of GSH. The observed elevated levels of glycine may indicate GSH and PCs are up-regulated to detoxify excess Cu^{2+}. Pidatala et al.[30] also observed that glycine and glutamate increased in response to Pb.

Increasing Tolerance. In addition to chelating copper, lettuce plants must employ other strategies to increase their tolerance to higher copper levels. The level of spermidine and putrescine, which are important polyamines, were elevated in all treated plants (SI Table S1 and Figure S6). Previous studies showed that putrescine and spermidine play an important role in plant stress response to diverse environmental stresses by acting as antioxidants to scavenging free radicals.[70−74] Therefore, elevated polyamines may contribute to enhanced tolerance of lettuce to copper. In this study, ethanolamine (EA) levels were increased (SI Figure S5) in all NP treated plants. Rajaeian et al. suggested that EA increased salt tolerance of tobacco plants by stimulation of antioxidative responses.[75] Kogan et al. showed pretreatment with ethanolamine enhanced the tolerance of *Helianthus annuus L.* to salt stress.[76]

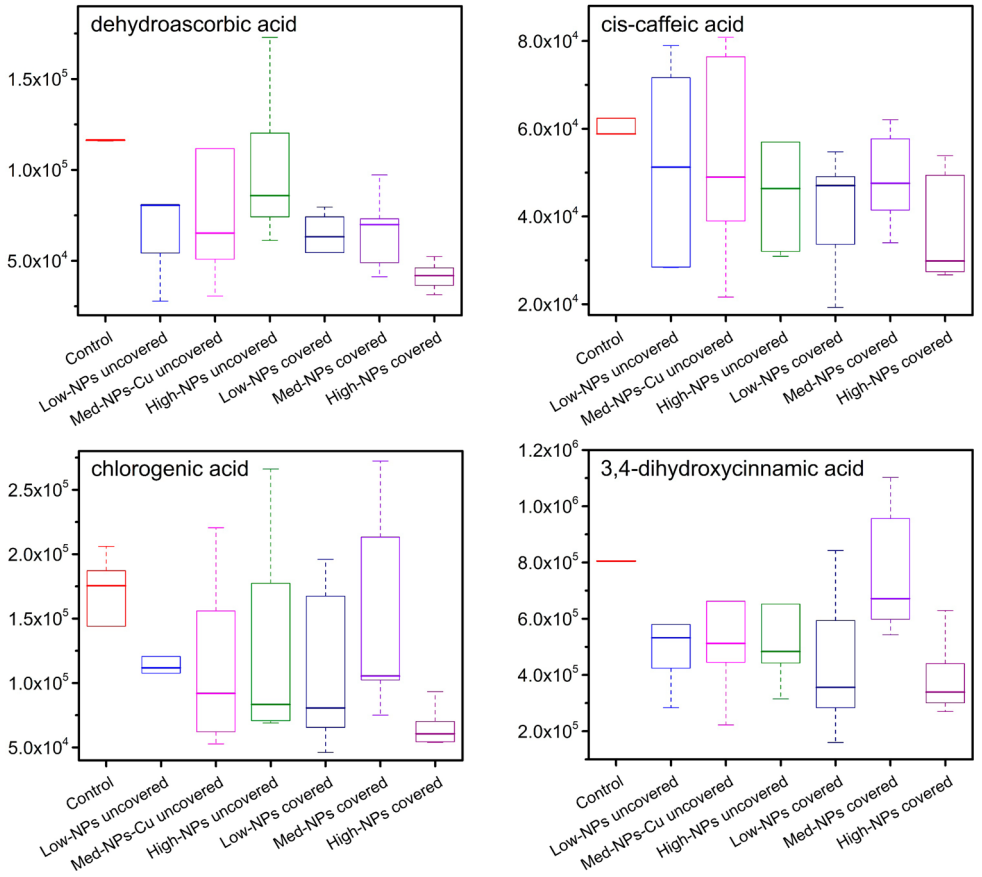

Figure 5. Box plot of relative abundance of four antioxidants (cis-caffeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid, dehydroascorbic acid) in 54-day-old lettuce leaves exposed to different concentrations of $Cu(OH)_2$ nanopesticides (n = 5).

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Elevated K^+ is another possible important tolerance mechanism. Increased K^+ in plants can lower ROS production by reducing activity of NAD(P) oxidases and maintaining photosynthetic electron transport.[77] K^+ has been implicated in regulating plant stress responses.[39] Guard cells take up mainly K^+.[78] It has been demonstrated that K^+ triggers the stomata to open. In guard cells of open stomata, K^+ was 2−4 times higher, malic acid 6 times higher, and citric acid 3 times higher, compared to closed stomata.[79] It has been repeatedly hypothesized that organic acid synthesis would accompany stomatal opening.[79] The accumulated K^+ in guard cells may promote stomata opening. Increased stomatal opening/transpiration is expected to promote photosynthesis and thereby increase plant growth.[80] Borowski and Michalek[81] showed that foliar application of potassium salts to spinach leaves resulted in more intensive gas exchange in leaves (stomatal conductance, photosynthesis, transpiration) and, as a consequence of that, increased leaf yield.

Antioxidant Defense. As reported before, Cu generates ROS in cells through the Fenton reaction. Our study also showed ROS was triggered by $Cu(OH)_2$ NPs (SI Figure S7). ROS scavenging enzymes and antioxidant molecules are a common plant response to ROS stress.[82−84] Phenolic acids and ascorbic acid are important low molecular antioxidants.[85] Previous studies indicate ROS stress increases accumulation of antioxidant molecules.[86] Up-regulated low molecular weight antioxidants can serve as scavengers of free radicals to protect plants from oxidative damage.[87,88] Interestingly, our results showed the levels of three phenolic compounds (cis-caffeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid) and dehydroascorbic acid, which are important antioxidant molecules, were significantly decreased in all nanopesticide-treated lettuce leaves (Figure 5). GABA levels also decreased. It is possibly that biosynthesis of these metabolites was activated in response to ROS stress induced by Cu at an early stage of defense. However, since the stress was sustained for one month, this induced the imbalance between ROS and the antioxidant defense system. Therefore, the antioxidant system was impaired due to the continuously generated ROS and limited ability to regulate them.

Environmental Implications. Exposure to copper-based nanopesticides is likely to increase. For lettuce, exposure via foliar application, as intended, did not result in visible leaf damage. In fact, in several cases leaf biomass increased significantly. $Cu(OH)_2$ nanopesticides can clearly enter stomata, even when aggregated. We demonstrated that Cu was translocated to the roots, although almost all the Cu mass was accumulated in leaves. Despite no visible damage, metabolomics revealed some significant changes in levels of amino acids, organic acids, carbohydrates and other important metabolites, particularly in leaves. The effect in roots was much smaller. The plants may be up-regulating some of these metabolites to increase the tolerance of plant to $Cu(OH)_2$ nanopesticide. Metabolomics can be used as a sensitive and powerful tool to understand the response of plants to nanoparticles at a molecular level. However, it is not clear if the observed metabolic changes were entirely induced by Cu ions or if NPs also contributed. Future work should address how best to use Cu ions as a control at the same level of bioavailability, to better distinguish the contribution of nano-Cu from that of the Cu ions to the observed metabolomics changes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b02763.

Separation of vascular and mesophyll tissues (Figure S1); Photograph of lettuces (Figure S2); Biomass accumulation (Figure S3); PLS-DA analysis of identified metabolites in lettuce leaves and roots (Figure S4); VIP scores from leaves and root metabolites PLS-DA analysis (Figure S5 and S6); ROS fluorescence image of leaves surface (Figure S7); Details regarding GC-TOF-MS data acquisition, data processing and data reporting (PDF)
(XLSX)
(PDF)

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Notes

The authors declare no competing financial interest.

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