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Metabolomics Reveals $Cu(OH)_2$ Nanopesticide-Activated Anti-oxidative Pathways and Decreased Beneficial Antioxidants in Spinach Leaves

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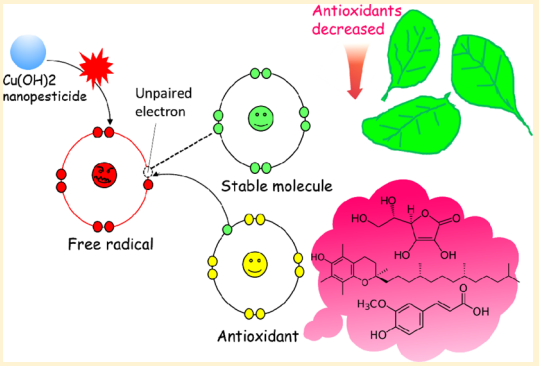
Supporting Information

ABSTRACT: While the use of nanopesticides in modern agriculture continues to increase, their effects on crop plants are still poorly understood. Here, 4 week old spinach plants grown in an artificial medium were exposed via foliar spray to $Cu(OH)_2$ nanopesticide (0.18 and 18 mg/plant) or Cu ions (0.15 and 15 mg/plant) for 7 days. A gas chromatography-time-of-flight-mass spectrometry metabolomics approach was applied to assess metabolic alterations induced by $Cu(OH)_2$ nanopesticide in spinach leaves. Exposure to $Cu(OH)_2$ nanopesticide and copper ions induced alterations in the metabolite profiles of spinach leaves. Compared to the control, exposure to 18 mg of $Cu(OH)_2$ nanopesticide induced significant reduction (29−85%) in antioxidant or defense-associated metabolites including ascorbic acid, α-tocopherol, threonic acid, β-sitosterol, 4-hydroxybutyric acid, ferulic acid, and total phenolics. The metabolic pathway for ascorbate and aldarate was disturbed in all exposed spinach plants (nanopesticide and Cu^{2+}). Cu^{2+} is responsible for the reduction in antioxidants and perturbation of the ascorbate and aldarate metabolism. However, nitrogen metabolism perturbation was nanopesticide-specific. Spinach biomass and photosynthetic pigments were not altered, indicating that metabolomics can be a rapid and sensitive tool for the detection og earlier nanopesticide effects. Consumption of antioxidants during the antioxidant defense process resulted in reduction of the nutritional value of exposed spinach.



INTRODUCTION

The increasing use of nanomaterials (NM) in agriculture has the potential to expose crop plants to these novel materials, either from direct application of nanopesticides or through the use of land applied biosolids.[1] While nanopesticides may result in lower doses with equal or better performance, there is a need to understand their effect on the crop plants and the surrounding environment. Although nanopesticides are targeted to deal with fungi, insects, or other pests, which is beneficial for the plant, plant metabolism may be perturbed.[2] Consequently, the plant metabolic network may be reprogrammed to cope with the exposure to the stressor (i.e., nanopesticide or released ions) and thus maintain essential metabolic pathways.[3] Low-molecular-weight metabolites are the end products of cellular regulatory processes, and their levels reflect the ultimate response of biological systems to environmental changes.[4] Monitoring the changes in low-molecular-mass metabolites can provide a more-holistic overview on how the plants respond to and tolerate environmental stressors. Environmental metabolomics provides a real-time representation of the interactions of organisms with their environment.[5] Recent studies[6,7] demonstrate that mass spectrometry (MS)-based environmental metabolomics is a powerful tool to detect and evaluate metabolic changes that are hard to detect via traditional monitoring of physiological parameters.

Using liquid chromatography−tandem mass spectrometry (LC−MS/MS)-based metabolic profiling, Pidatala et al. demonstrated tolerance and detoxification mechanisms of *Chrysopogon zizanioides* L. Nash (vetiver) to lead. In a recent study,[8] lettuce leaves did not exhibit obvious toxicity symptoms when exposed to $Cu(OH)_2$ nanopesticide for 4 weeks, and biomass and the levels of photosynthetic pigments were unchanged. However, gas chromatography−time-of-flight mass spectrometry (GC−TOF-MS)-based metabolomics demonstrated that lettuce leaf metabolite profiles were altered after exposure to the $Cu(OH)_2$ nanopesticide. Some metabolites that play protective roles in abiotic or biotic stress responses were found more abundant in lettuce leaves exposed to $Cu(OH)_2$ nanopesticide. More recently, using gas chromatography−qualitative time-of-flight mass spectrometry (GC−qTOF-MS)-based metabolomics, Hasler-Sheetal et al.[3] revealed cryptic metabolic changes of seagrass (*Zostera marina*) and bivalve interactions under reduced-light conditions, which could not be detected by traditional approaches. Thus, metabolomics can

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provide a more-sensitive and mechanistic understanding of biological response to a particular stressor.[9]

Spinach is commonly used in salads and are often consumed raw. Studies have shown that spinach possesses antioxidant compounds that are very beneficial to human health. Spinach is among the top 26 highest antioxidants-rich foods.[10] $Cu(OH)_2$ nanopesticides are commercial pest-control products with increasing applications in agriculture, especially organic farming. However, copper is a redox active metal known to accelerate the generation of reactive oxygen species (ROS) through the Fenton and Harweiss reaction.[11] Our previous study demonstrated that $Cu(OH)_2$ nanopesticide-induced oxidative stress in lettuce plants.[8] In higher plants, ROS may attack cell membranes and damage proteins, lipids, and genetic material. To quench excess ROS and avoid damage, plant antioxidant defense system is activated by the up-regulation of the internal synthesis of a number of low-molecular-weight antioxidants or antioxidant enzymes. Many low-molecular-weight antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), and phenolics are important components of the plant defense system. In addition, they are beneficial nutrients for human health due to their robust antioxidant capacity.

In this study, we aimed to assess and understand the metabolic response of spinach to the $Cu(OH)_2$ nanopesticide. Using multivariate analysis of metabolomics data from GC−TOF-MS, we sought to elucidate the underlying toxic or detoxification mechanisms by identifying novel biomarker profiles that may be characteristic of toxicity specific to the $Cu(OH)_2$ nanopesticide rather than just Cu ion. We also evaluated the changes in antioxidants levels in spinach leaves, which can affect their beneficial nutritional value.

MATERIALS AND METHODS

Nanopesticide and Copper Ions. The $Cu(OH)_2$ nanopesticide used in this study is a commercial biocide (Kocide 3000 by Dupont). Detailed physicochemical properties of Kocide 3000 have been reported before.[12,13] Specifically, the primary particle size is ∼50 to >1000 nm. The hydrodynamic diameter is 1532 ± 580 nm, and the ζ potential is −47.6 ± 43 mV, measured via dynamic light scattering (Malvern Zetasizer Nano ZS-90), in NanoPure water at pH 7. Kocide 3000 is not marketed as a nanopesticide by the manufacturer, however, Kocide 3000 is composed of micronized particles that contain nanosheets of $Cu(OH)_2$ that are bound together by an organic composite and can potentially redissociate in water.[12] Copper content in Kocide 3000 is 26.5 ± 0.9%, and other elements including C, O, Na, Al, Si, S, Zn compose 73.5% of mass.[12] Copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) was purchased from Sigma-Aldrich.

Exposure Experiments. Spinach (*Spinacia oleracea*) seeds were purchased from Seed Savers Exchange. The artificial growth medium contained sand (Quikrete Washed Plaster Sand), Sunshine Advanced Growing Mix no. 4 (SunGro Horticulture), vermiculite (Therm-O-Rock), coco coir (Canna), perlite (Therm-O-Rock), at a ratio of 1:3:1:2:2 by volume. In addition, organic granular 4−4−4 fertilizer was applied at 3.4 g/kg growth medium. Spinach seeds were sown 1 cm deep in 0.5 L Poly-Tainer containers (7.5 × 7.5 cm^2). In total, 15 pots of spinach were planted and kept in a greenhouse 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} day^{-1}. We applied two different materials: dissolved $CuSO_4$ and the suspended $Cu(OH)_2$ nanopesticide. Foliar application of different concentrations of Cu ions (10 and 100 mg/L, corresponding to 0.15 and 1.5 mg/plant) and $Cu(OH)_2$ nanopesticide (100 and 1000 mg/L, corresponding to 1.8 and 18 mg/plant) began after 4 weeks of initial unexposed growth. Before spraying, the nanopesticide suspension was sonicated (Branson 8800) for 30 min in cooled water until a stable dispersion was achieved. A hand-held spray bottle was used for spraying. The spinach plants were sprayed 3 times per day for 7 days; the amount sprayed each time was ∼2.9 mL per pot (7 days of exposure was chosen because preliminary experiments showed that metabolic responses occurred within 7 days). The total amount of nanopesticide suspension applied was 180 mL per treatment (3 plants) during 7 days, at various concentrations, resulting in a total application of 0, 1.8, and 18 mg Cu as $Cu(OH)_2$ nanopesticide per plant, which is in the range recommended by the manufacturer. In the case of Cu ions the dose was 0.15 and 1.5 mg of Cu per plant. The applied copper ion concentration was designed based on the previous copper ion release dynamic from $Cu(OH)_2$ nanopesticide (around 10%). It should be pointed out that the calculated Cu dose (concentration times spray volume) may overestimate the amount received by the plant because only approximately 60−70% of the spray reaches the spinach leaves, while the balance ends in the surrounding soil.

Photosynthetic Pigment and Total Phenolics Analysis. Photosynthetic pigment analysis was performed to determine chlorophyll a and b and total carotenoids content. The pigments were extracted following the protocol of Sesták et al. (1971).[14] Generally, 0.01 g of spinach leaves were mixed with 5 mL of 80% methanol for 12 h, and then the mixture was centrifuged for 10 min at 3000 rpm. A Shimazu UV−vis spectrometer was to measure the absorbance of chlorophyll a and b and carotenoids in the methanolic extract, with wavelengths at 666, 653, and 470 nm, respectively. The total content of phenolic compounds were determined with the method of Singleton and Rossi.[15] Specifically, 50 μL of the methanolic extract was diluted with 450 μL of DI water, and then 250 μL of 2 M Folin−Ciocalteu reagent and 1.25 mL of 20 g/L Na_2CO_3 were added to the mixture. After centrifugation, the absorbance of the supernatant was determined at 735 nm with UV spectra. Quality assurance and quality control include duplicated samples at 10%. The spectrophotometer was properly calibrated according to the manufacture's operating manual. The instrument's zero adjustment was checked with the solvent blank between readings.

Copper and Other Mineral Analysis. After oven drying for 3 days at 70 °C, dried leaf 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 Paar) at 180 °C for 1 h. The standard reference materials NIST 1547 and 1570a were also digested and analyzed. The recoveries for all elements were between 90 and 99%. Na, Mg, P, K, Ca, Zn, Mn, Fe, Al, Cu, Mo, and Ag were analyzed using inductively coupled plasma mass spectrometry (ICP-MS 7900, Agilent). The standard solution was diluted from an ICP-MS environmental calibration standard (Agilent), which contains 1000 mg/L each of Fe, K, Ca, Na, Mg, and 10 mg/L each of Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Tl, V, Zn, Th, and U in 10% HNO_3.

Scanning Electron Microscopy Analyses. A Nova NanoSEM 650 scanning electron microscope (SEM, FEI, Hillsboro, OR) was used to image $Cu(OH)_2$ particles on the surface of the nanopesticide-treated spinach leaves. The SEM was operated in low-vacuum mode, which allowed for the direct analysis of fresh leaves without the need for chemical fixation. The SEM's beam voltage was set at 7 kV, and a chamber pressure

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Table 1. Effect of $Cu(OH)_2$ Nanopesticide and Cu Ions on Spinach Mineral Nutrients Content (mg/kg Dry Weight)[a]

	Na	Mg	P	K	Ca	Zn
control	10710 ± 2440	16199 ± 2964	14649 ± 3750	111706 ± 20979	19386 ± 3578	207 ± 21
$Cu(OH)_2$ NP[a] 1.8 mg	10768 ± 5451	15208 ± 6391	15109 ± 6383	115513 ± 62225	18963 ± 5901	177 ± 32
$Cu(OH)_2$ NP 18 mg	10633 ± 2964	12822 ± 1588	13216 ± 2491	106070 ± 9358	17726 ± 2679	220 ± 69
Cu ion 0.15 mg	8577 ± 2385	15127 ± 1692	11312 ± 3805	123141 ± 7324	15218 ± 3306	232 ± 59
Cu ion 1.5 mg	9280 ± 2771	14407 ± 1270	11499 ± 5479	124017 ± 11915	15977 ± 1735	196 ± 13
	Mn	Fe	Al	Cu	Mo	Ag
control	45 ± 4.9	70 ± 14.1	17 ± 11.8	21 ± 7.0	0.56 ± 0.091	0.010 ± 0.0016
$Cu(OH)_2$ NP 1.8 mg	45 ± 16.9	71 ± 33.9	31 ± 10.9	377 ± 90.5[c]	0.45 ± 0.258	0.013 ± 0.0041
$Cu(OH)_2$ NP 18 mg	41 ± 5.4	103 ± 3.2[b]	130 ± 9.2[c]	3021 ± 211.3[c]	0.50 ± 0.043	0.034 ± 0.0032[c]
Cu ion 0.15 mg	42 ± 14.0	79 ± 17.4	8 ± 2.3	56 ± 12.5[b]	0.67 ± 0.127	0.007 ± 0.0014
Cu ion 1.5 mg	39 ± 1.9	70 ± 3.0	8 ± 1.9	318 ± 69.3[c]	0.67 ± 0.116	0.010 ± 0.0012

[a]Data are means of three replicates. [b]$P < 0.05$. [c]$P < 0.01$. NP indicates "nanopesticide".

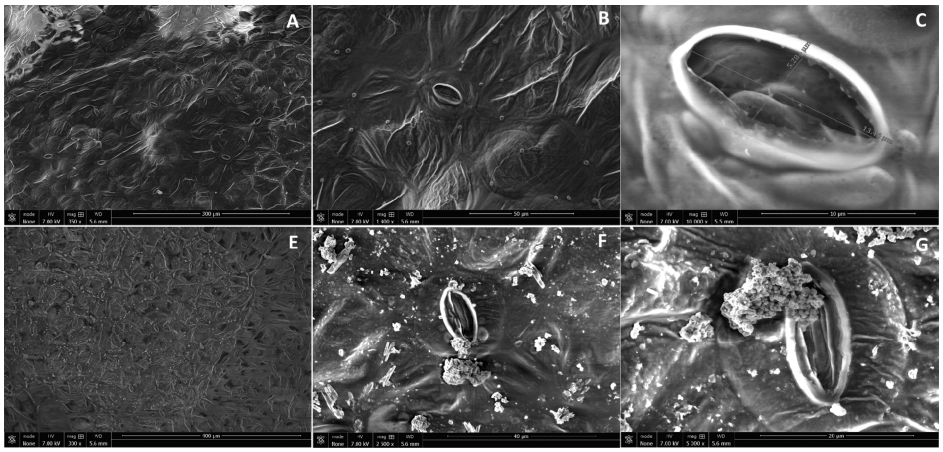

Figure 1. SEM micrographs of 4 week old spinach leaves foliar sprayed with 0 (control) (panels A–C) and 1000 mg/L $Cu(OH)_2$ nanopesticide (panels D–F).

of 0.68 Torr was used. Data was collected using a low-vacuum detector (LVD) at a working distance of ~5 mm. A paper punch was used to carefully cut out sphere-shaped leaf fractions. The paper punch allowed us to cut around the perimeter of the circular leaf fractions without disturbing the leaf surface around the core, from which SEM data was collected. The spherical leaf fractions were attached to aluminum SEM stubs using a thin layer of fast-drying colloidal silver paint (Ted Pella, Redding, CA) and viewed under the microscope without sputtering. Sample preparation for scanning electron microscopy of spinach leaves foliar-sprayed with 1000 mg/L of $Cu(OH)_2$ nanopesticide.

Gas Chromatography–Time-of-Flight Mass Spectrometry Analysis of Metabolites in Leaves and Root. The freeze-dried spinach leaves samples were subjected to GC–TOF-MS analysis at the Genome Center Core Services, University of California Davis, to identify metabolites present in spinach tissues. Sample pretreatment, analytical method and instrument have been described by Fiehn et al.[16,17] Briefly, an Agilent 6890 gas chromatograph (Santa Clara, CA) containing an 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 the data acquisition, data processing, and data reporting are provided in the Supporting Information.

Univariate and 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.[18] PLS-DA and univariate one-way analysis of variance (ANOVA) were run based on GC–TOF-MS data using online resources (http://www.metaboanalyst.ca/).[19] 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.[18] A variable with a VIP above 1 is regarded as significant.[20] Biological pathway analysis was performed based on all detected metabolites data using MetaboAnalyst 2.0.[21] The impact value threshold calculated for pathway identification was set at 0.1.[20]

Statistical Analysis. The results are mean ± SE ($n = 3$). For the concentration of elements and biomass, photosynthetic pigments and phenolics content, a one-way ANOVA followed by Tukey postdoc testing was used to determine the statistical significance between control and nanopesticides and $CuSO_4$ treatments.

RESULTS AND DISCUSSION

Cu Bioaccumulation and Uptake Pathway. In leaves sprayed with 0, 1.8, and 18 mg of $Cu(OH)_2$, Cu content was 21 ± 7, 377 ± 90 and 3021 ± 211 mg/kg dry weight (DW),

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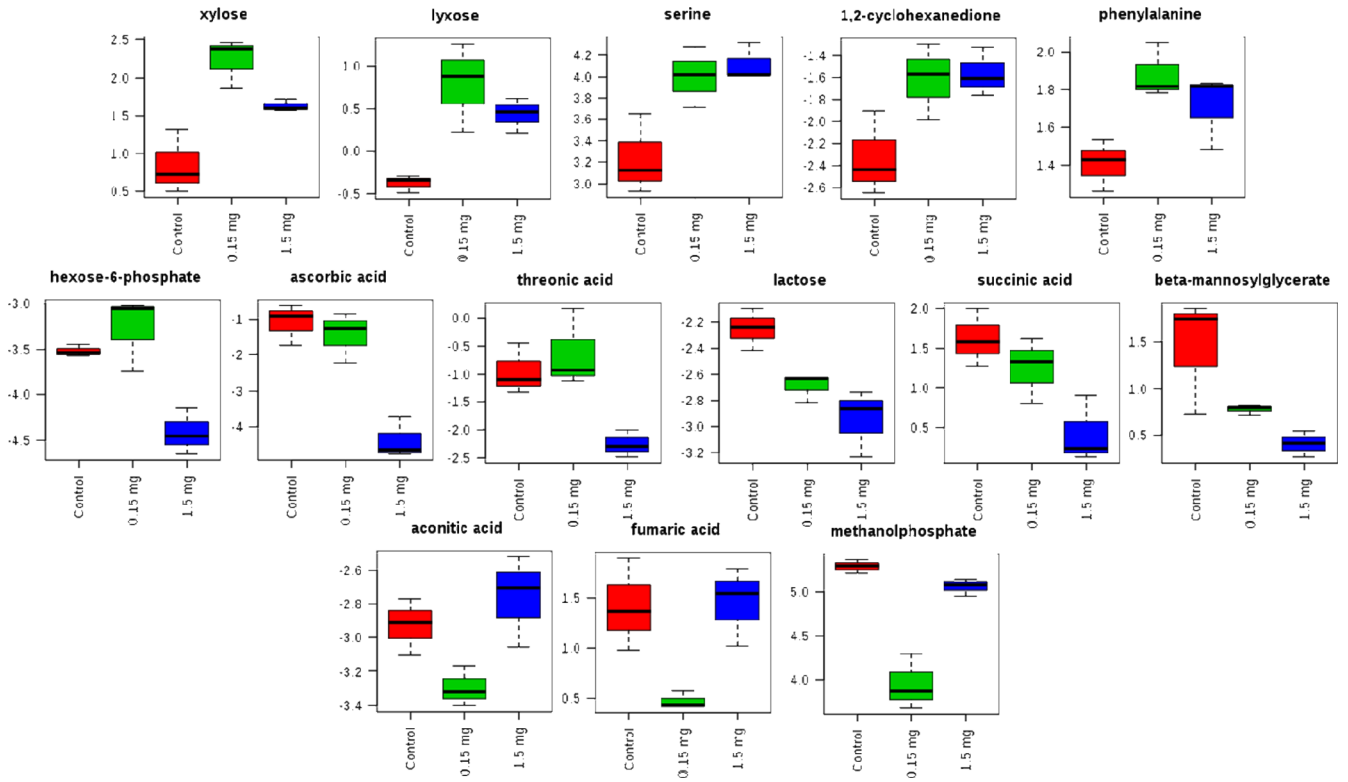

Figure 2. Box−whisker plots of GC−TOF-MS data showing relative abundance of significantly changed metabolites in 4 week old spinach plants exposed to 0, 0.15, and 1.5 mg of Cu ions for 1 week. The *y*-axis indicates the absolute signal from GC−TOF-MS.

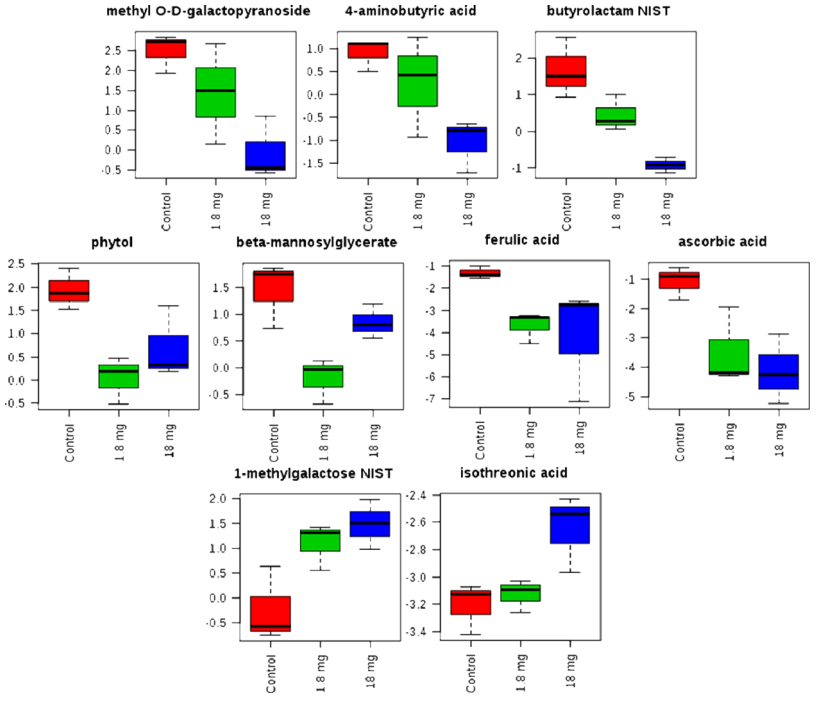

Figure 3. Box−whisker plots of GC−TOF-MS data showing the relative abundance of significantly changed metabolites in 4 week old spinach plants exposed to 0, 1.8, and 18 mg of $Cu(OH)_2$ nanopesticide for 1 week. The *y*-axis indicates the absolute signal from GC−TOF-MS.

respectively (Table 1). After foliar application of 0.15 and 1.5 mg of Cu^{2+}, leaf Cu content was 56 ± 12 and 318 ± 69 mg/kg DW, respectively. Copper content in $Cu(OH)_2$ nanopesticide-treated leaves are generally seven to nine times higher than when $CuSO_4$ is applied. On the basis of SEM observations and previous knowledge on foliar uptake of other elements,[22] four pathways are possible for foliar uptake of $Cu(OH)_2$ nanopesticide or Cu ions. (1) Particles can deposit on rough leaf surfaces (Figure 1A). (2) Particles may penetrate via stomata into inner tissues. A typical diameter of a spinach stomata is between 10 to 15 μm (Figure 1B). After entering the stomata, the particles may transport into the apoplasm, but further studies are needed to

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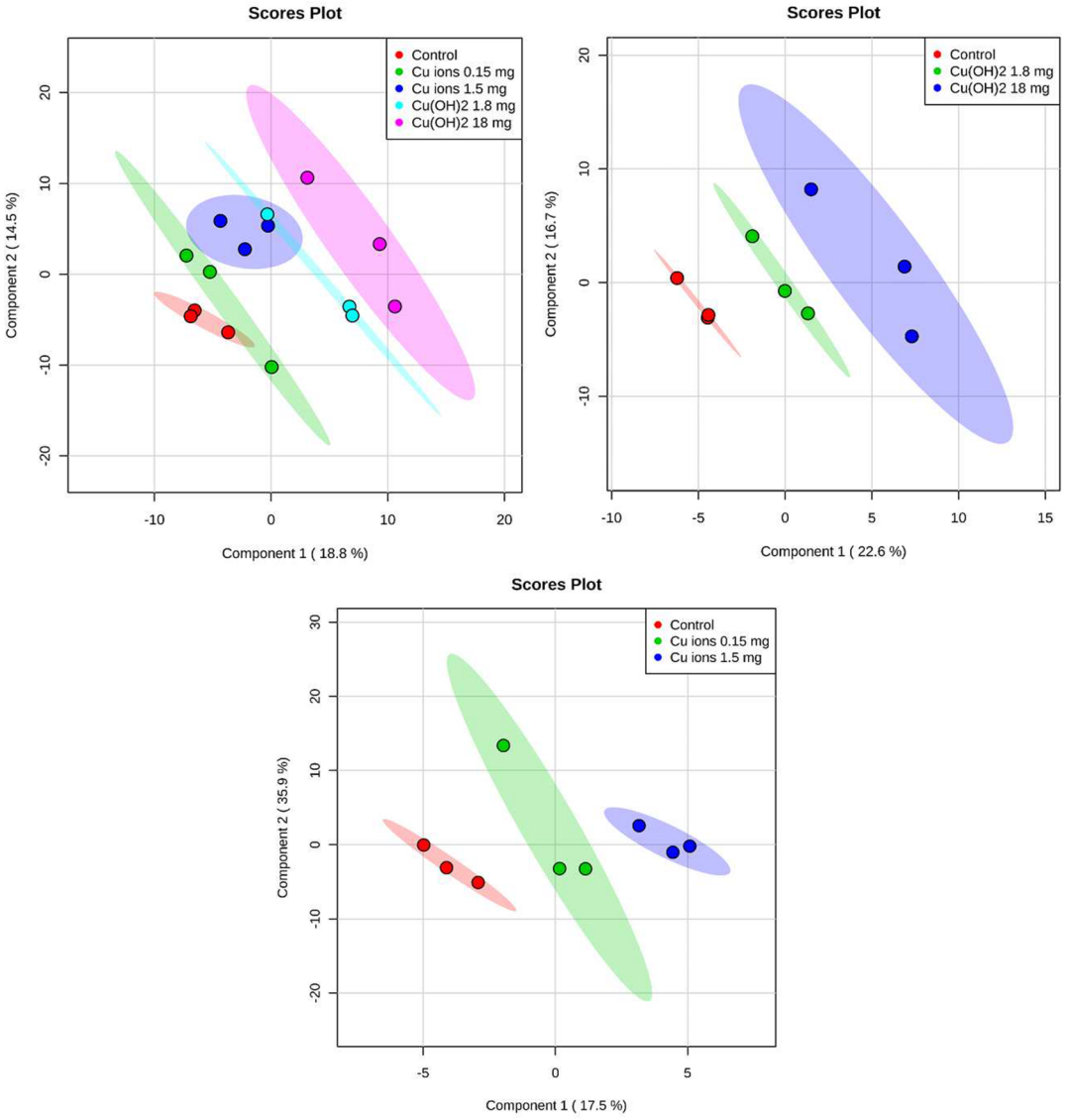

Figure 4. Partial least-squares discriminate analysis (PLS-DA) score plots of metabolic profiles in spinach leaves foliar sprayed with 0, 0.15, and 1.5 mg of Cu ions; (a) 1.8 and 18 mg of $Cu(OH)_2$ nanopesticide control vs nanopesticide and copper ions, (b) control vs nanopesticide, and (c) control vs copper ions.

verify this transport. (3) Cu ions released from $Cu(OH)_2$ may diffuse through aqueous pores of the cuticle and of the stomata, following the hydrophilic pathway.[22] (4) Cu ions may react with leaf exudates such as oxalic acid and citric acid to form insoluble salts and deposit on the leaf surface. Further work is needed to elucidate which pathways are more significant.

Physiological and Biochemical Reaction. After 7 days of exposure, no significant difference in leaf biomass was noticed between control and $Cu(OH)_2$ nanopesticide or Cu ion exposed plants (data not shown). In addition, there were no statistically significant effects on photosynthetic pigment (chlorophyll a, b and carotenoids) levels in spinach leaves (Figure S2) from either the $Cu(OH)_2$ nanopesticide or Cu ions.

Metabolic Response of Spinach Leaves to $Cu(OH)_2$ Nanopesticide and Copper Ions. A total of 357 unique metabolites were detected via GC−TOF-MS in spinach leaves, with 149 identified on the basis of their mass spectral fingerprints and retention-index matches. First, univariate one-way ANOVA testing on the GC−TOF-MS data was conducted to determine the metabolites with significant changes. Results showed (Figure S3) that the levels of 30 metabolites (14 identified and 16 unknown) out of the 357 compounds (8.4%) were markedly ($p < 0.05$) altered in response to Cu ion exposure (Figure 2). In contrast, exposure to nanopesticide showed fewer statistically significant differences; there were 24 metabolites (6.7%) (9 identified and 16 unknown) significantly changes metabolites ($p < 0.05$) (Figure 3). The 14 identified metabolites significantly ($p < 0.05$) altered by Cu ion include xylose, lyxose, serine, 1,2-cyclohexanedione, phenylalanine, hexose-6-phosphate, ascorbic acid, threonic acid, lactose, succinic acid, β-mannosylglycerate, aconitic acid, fumaric acid, methanolphosphate. Exposure to $Cu(OH)_2$ nanopesticide significantly ($p < 0.05$) altered the levels of methy *O*-D-galactopyranoside, 4-aminobutyric acid (GABA), butyrolactam NIST, phytol, β-mannosylglycerate, ferulic acid, ascorbic acid, 1-methylgalactose NIST, and isothreonic acid. This indicates that both the nanopesticide and the copper ions induced metabolic alterations (up- or down-regulation) in spinach leaves. Although Cu ions and nanopesticide exhibited some overlapping metabolic patterns (e.g., down-regulation of ascorbic acid and β-mannosylglycerate), some differences were

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also observed. Most of the significant changes in metabolite levels after exposure to copper ions were not observed in plants exposed to the nanopesticide; also, some metabolic changes induced by the nanopesticide are nanospecific. (Figure 2 and 3). In addition, it is interesting to note that some of these metabolites (GABA, β-mannosylglycerate, and 1-methylgalactose NIST) were also altered in lettuce leaves in response to the $Cu(OH)_2$ nanopesticide.[8]

Univariate methods help to select important features from the metabolomics data sets. However, they ignore the correlations that are known to be present among variables.[23] Multivariate methods take all variables simultaneously into consideration and make use of co-variances or correlations that reflect the extent of the relationships among the variables.[24] Therefore, supervised PLS-DA multivariate analysis was conducted based on the database of 357 detected metabolites. It is interesting to note that the $Cu(OH)_2$ nanopesticide, Cu ions and control groups were separated generally in a dose-dependent manner along principal component 1 (PC1) as shown in the PLS-DA score plot (Figure 4A). This indicates all nanopesticide and copper ions groups were separated from the control. In addition, there was separation between the nanopesticide group and the copper ions group. The metabolites that serve to discriminate (VIP > 1) among the different groups reveal important information (Figure S4). Among the discriminating metabolites, ascorbic acid, adenine, phytol, ferulic acid, and GABA have a negative correlation with Cu content in leaves. In contrast, sucrose, glucose, raffinose, and 1-methylgalactose NIST have positive relationships with Cu content.

We then separately ran PLS-DA on the control and $Cu(OH)_2$ nanopesticide groups and the control and Cu ion groups. The PLS-DA score plots (Figure 4B) shows a clear separation between control and $Cu(OH)_2$ nanopesticide treatments along PC1, which accounted for 23.8% of the total variance. Similarly, all Cu ion groups were separated from the control along PC1, which explained 17.5% of the total variance (Figure 4C).

The top 43 and 33 discriminating (VIP > 1) metabolites are depicted in Figure S5 for Cu ion and Figure S6 for $Cu(OH)_2$ nanopesticide. Interestingly, a number of metabolites that act as ROS scavengers were markedly decreased after exposure to either $Cu(OH)_2$ nanopesticide or Cu ions, such as ascorbic acid, threonic acid, dehydroascorbic acid, β-sitosterol, ferulic acid, 4-aminobutyric acid (GABA), and α-tocopherol. The decrease of antioxidants suggests that the antioxidant defense system was activated, and these low-molecular-weight antioxidants were partially consumed to quench overproduced ROS induced by the increased Cu levels. The reduction of low-molecular-weight antioxidants, such as dehydroascorbic acid, cis-caffeic acid, chlorogenic acid, and 3,4-dihydroxycinnamic acid, in lettuce leaves foliar -sprayed with $Cu(OH)_2$ nanopesticide were observed in a previous study.[8] In this study, both 3,4-dihydroxycinnamic acid and 4-hydroxycinnamic acid were not changed by either nanopesticide or copper ions.

Ascorbic acid was the top significantly altered metabolite level after exposure to both $Cu(OH)_2$ nanopesticide and Cu ions (Figures S5 and S6); it was reduced almost 10-fold at a dose of 1.5 mg Cu ion and 7-fold at a dose of 18 mg of $Cu(OH)_2$ nanopesticide. Note that the dissolved Cu elicited a stronger response than the nanopesticide, likely due to the slower release of Cu^{2+} from $Cu(OH)_2$ in this formulation. Moreover, threonic acid, an oxidation product of ascorbic acid, was found to have a similar trend as ascorbic acid: decreased by both 18 mg of nanopesticide and 1.5 mg of copper ions. Ascorbic acid is one of the most-abundant water-soluble antioxidants in plants, present in the cellular fluids such as cytosol, or the cytoplasmic matrix.[25,26] The mechanism for ascorbic acid to scavenge free radicals is that ascorbate transforms to an ascorbate radical by donating an electron to the lipid radical to terminate the lipid peroxidation chain reaction. Pairs of ascorbate radicals react rapidly to produce one molecule of ascorbate and one molecule of dehydroascorbate. The dehydroascorbate does not have any antioxidant capacity.[11] The level of dehydroascorbic acid was decreased by exposure to 1.5 mg of copper ions but remain unchanged when plants were exposed to the nanopesticide. The significantly reduced levels of ascorbic acid and oxidized products are a strong indicator that ROS was over-generated in spinach cells when exposed to Cu ions and $Cu(OH)_2$ nanopesticide. As mentioned above, Cu ions can catalyze the generation of ROS through the Fenton reaction.

Exposure to both $Cu(OH)_2$ nanopesticide and Cu ions decreased α-tocopherol (vitamin E) levels in spinach leaves (Figures S5 and S6). While the decrease in ascorbic acid levels was only seen at a dose of 1.5 mg of Cu ions, vitamin E levels were decreased even at a lower Cu ion dose (0.15 mg). Both levels of $Cu(OH)_2$ induced a significant decrease of α-tocopherol in spinach leaves. Vitamin E is a lipid-soluble antioxidant and is predominantly located in cell membranes.[11] Therefore, we presume the ROS was generated not only in the cytosol but also in the cell membranes. Vitamin E is an efficient lipid soluble antioxidant that functions as a chain breaker during lipid peroxidation in cell membranes. It helps to intercept lipid peroxyl radicals ($LOO^{\bullet}$) and to terminate the lipid peroxidation chain reactions ($LOO^{\bullet}$ + α-tocopherol−OH → LOOH + α-tocopherol-$O^{\bullet}$).[11]

Ferulic acid (3-methoxy-4-hydroxycinnamic acid), an antioxidant found naturally in plant cell walls, was significantly decreased by both Cu ions and $Cu(OH)_2$ nanopesticide (Figures S5 and S6).[27] Ferulic acid has been reported as one of the most effective photoprotectors, providing protection to photosynthesis during drought stress; it also participates in the pathway of phenylpropanoid biosynthesis.[28] Ferulic acid has also been considered as a key metabolite for rice drought-tolerance.[29] Ferulic acid is the main phenolic acid occurring in cell walls of monocotyledons.[30] We thus measured total phenolics content in spinach leaves and found both $Cu(OH)_2$ nanopesticide and Cu ions significantly reduced the total phenolic content in spinach leaves (Figure S7). Generally we found a negative relationship between leaf Cu content and total phenolics content. Phenolic hydroxyl groups are good hydrogen donors: hydrogen-donating antioxidants can react with reactive oxygen and reactive nitrogen species in a termination reaction, which breaks the cycle of generation of new radicals.[31]

In addition to ascorbic acid, α-tocopherol, and ferulic acid, β-sitosterol has also been reported to have an antioxidant function.[32] β-sitosterol levels also decreased in response to Cu ions (Figure S5). Low-molecular-weight antioxidants help to prevent oxidative damage in chloroplasts. The simultaneous decrease of ascorbic acid, α-tocopherol, β-sitosterol, ferulic acid, and total phenolics are indicators of oxidative stress and activation of the plant anti-oxidative defense response, induced by Cu ions. Similarly, these compounds (ascorbic acid, α-tocopherol, isothreonic acid, and ferulic acid) were markedly changed in spinach leaves treated with $Cu(OH)_2$ nanopesticide. This suggests the activation of the antioxidant pathway induced by $Cu(OH)_2$ nanopesticides is driven by the released Cu ions.

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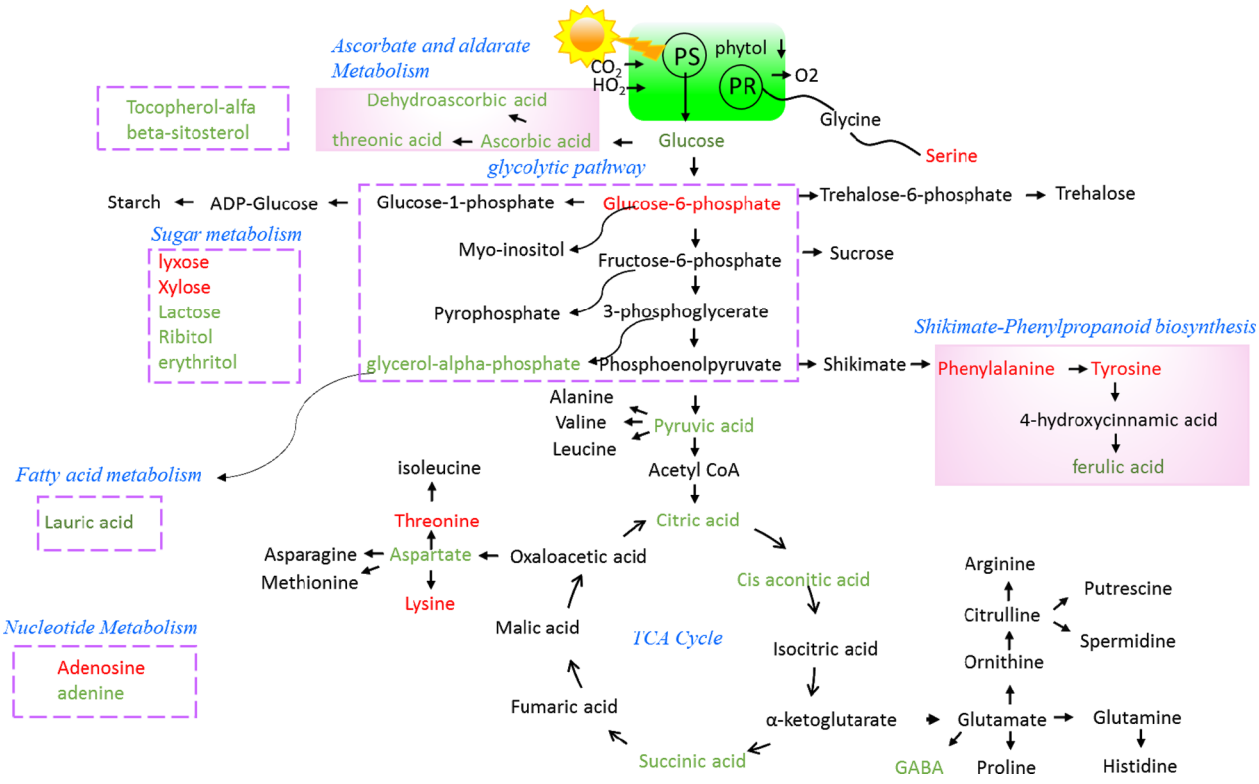

Figure 5. Schematic diagram of proposed metabolic pathways of spinach. Central metabolic pathways (glycolytic pathway, TCA cycle, shikimate–phenylpropanoid biosynthesis) and other metabolite biosynthetic pathways (sugar, amino acid, lipids, and fatty acid) are shown. Red and green circles indicate that the metabolites increased or decreased in response to the Cu ions.

Phytol and β-β-mannosylglycerate. In addition to the previously mentioned ROS scavengers, phytol and β-mannosylglycerate are metabolites that were significantly down-regulated by both Cu ions and nanopesticide (Figures S5 and S6). We found phytol levels and copper content showed strong negative correlation (Pearson's $r = -0.77$) (see the Supporting Information). Meanwhile, strong positive correlations also exist between levels of phytol and several antioxidants (0.60–0.80). Phytol is a chlorophyll degradation product that accumulates during plant senescence.[33] The up-regulation of phytol initiates tissue degradation and propagates cell death.[3] Phytol may also have the capacity to quench ROS and act as an antioxidant. Santos et al.[34] demonstrated that phytol was able to reduce the production of free radicals. They hypothesized that this activity can be attributed to phytol's structural features because phytol is a branched-chain unsaturated alcohol, and its antioxidant properties may be related to the hydroxyl group (OH). It is likely that phytol, by reacting with a free radical, donates hydrogen atoms with an unpaired electron (H·), converting free radicals into less-reactive species.[35] In a recent study, phytol also decreased in cucumber (*Cucumis sativus*) and corn (*Zea mays*) exposed to the same $Cu(OH)_2$ nanopesticide.[36] Therefore, phytol may be an important biomarker in several plant species as a response to copper-based pesticides. The role of phytol in plant defense may be underestimated. Future studies on the function and role of phytol in stress response are needed. In addition, the levels of β-mannosylglycerate were significantly down-regulated (53%~69%) by exposure to Cu ions and nanopesticide (Figures 2 and 3). β-mannosylglycerate is the major organic solute accumulated in response to increased salinity.[37] The reason for the induced down-regulation of β-mannosylglycerate by Cu ions and nanopesticide is still unknown.

Phenylpropanoid Biosynthesis and TCA Cycle. In addition to the similarities in metabolic changes, some clear differences were observed between plants exposed to Cu ions and nanopesticide. The abundance of two aromatic amino acids, phenylalanine and tyrosine, were found increased 1.2- and 1.8-fold, respectively, in response to 1.5 mg of Cu ions (Figures 2 and S5). Phenylalanine and tyrosine are the precursors of the general phenylpropanoid pathway. Phenylpropanoid metabolism generates a large array of secondary metabolites based on the few intermediates of the shikimate pathway as the core unit.[38] The higher levels of phenylalanine and tyrosine in plants treated with Cu ion may serve as an increased supply for phenylpropanoid synthesis, which could partially mitigate the toxicity of Cu ions.[39] Around 20% of the carbon fixed by photosynthesis is channeled into the phenylpropanoid pathway.[40] Reprogramming of the metabolism to divert more photosynthetic fixed carbon toward the aromatic amino acid precursors of the phenylpropanoid pathway may lead to decreased carbon input to energy pathways.[41] This may explain the observed decrease in TCA cycle intermediate (e.g., succinic acid, citric acid, fumaric acid, malic acid, and aconitic acid) levels by 1.3–2.3-fold after exposure to Cu ions. In contrast, phenylalanine and tyrosine were unchanged in spinach leaves treated by $Cu(OH)_2$ nanopesticide, indicating no up-regulation of the phenylpropanoid pathway. Similarly, levels of TCA cycle intermediates were unchanged after exposure to $Cu(OH)_2$ nanopesticide. Moreover, pyruvic acid, the precursor of TCA cycle, was down-regulated by Cu ions yet up-regulated by nanopesticide. This indicates that Cu ions cannot fully explain the metabolic changes in spinach leaves induced by nanopesticide. Some metabolic responses are nanopesticide-specific. The differential response may reflect the relatively slow Cu^{2+}

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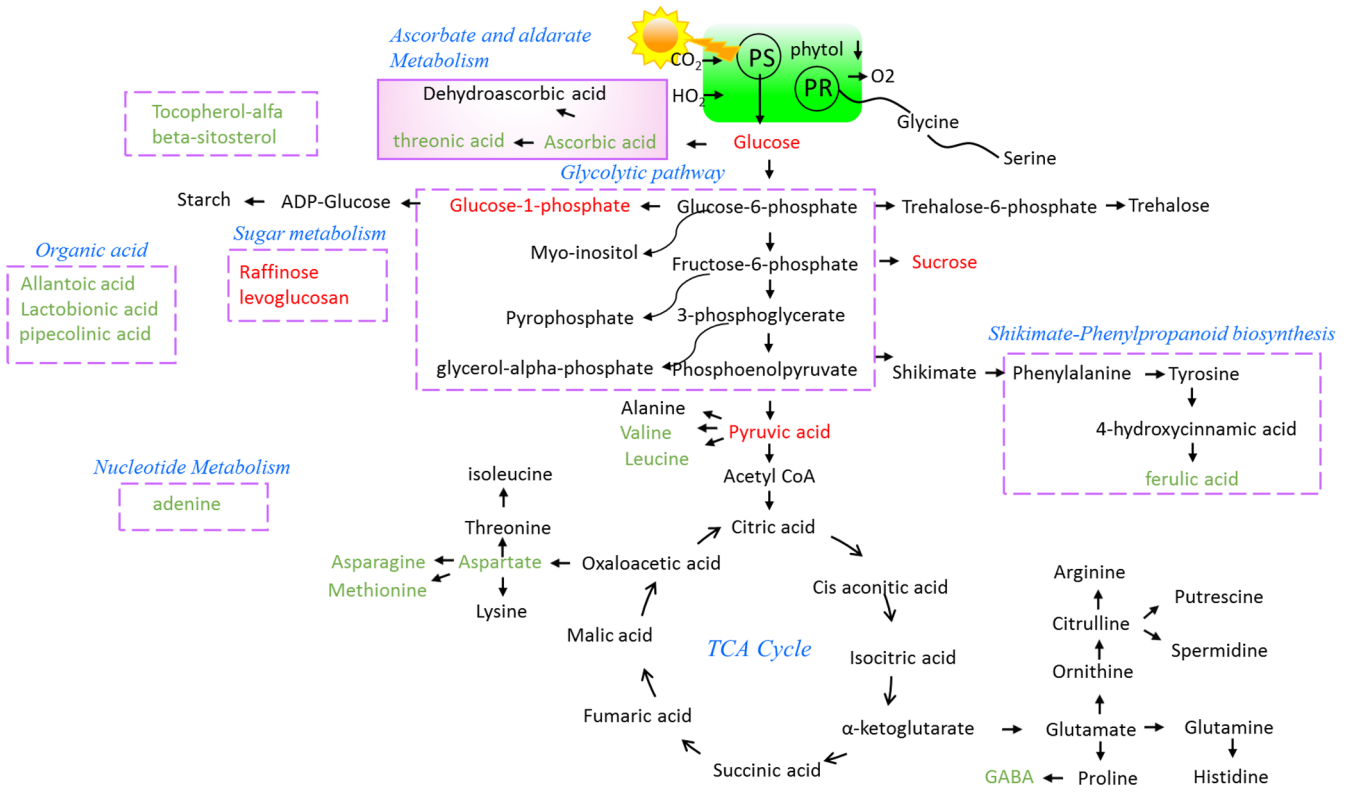

Figure 6. Schematic diagram of proposed metabolic pathways of spinach. Central metabolic pathways (glycolytic pathway, TCA cycle, shikimate–phenylpropanoid biosynthesis) and other metabolite biosynthetic pathways (sugar, amino acid, lipids, and fatty acid) are shown. Red and green circles indicate that the metabolites increased or decreased in response to the $Cu(OH)_2$ nanopesticide.

release process from $Cu(OH)_2$ nanopesticide,[13] but further study is needed.

Biological pathway Analysis in Spinach Leaves. Pathway-enrichment analysis showed that the ascorbate and aldarate pathway and the tyrosine pathway were significantly altered by Cu ions (Figure S8A). Both of the perturbed pathways are related to plant antioxidation activities. Similar to Cu ions, $Cu(OH)_2$ nanopesticide also perturbed the ascorbate and aldarate pathway (Figure S8B). However, exposure to $Cu(OH)_2$ nanopesticide did not activate the tyrosine pathway. In contrast the $Cu(OH)_2$ nanopesticide did alter alanine, asparate, and glutamate metabolism. Most metabolites (aspartic acid, alanine, asparagine, and GABA) involved in this metabolism were down-regulated by $Cu(OH)_2$ nanopesticide. This indicates that nitrogen metabolism was impacted by $Cu(OH)_2$ nanopesticide and that this reaction is not Cu-ion-specific.

Global Visualization of Pathway Changes in Spinach Leaves Exposed to Cu Ions. To obtain a global perspective of the metabolic changes, central C (glycolytic pathway, TCA cycle, and shikimate–phenylpropanoid biosynthesis) and N metabolism pathways were mapped into a network in which the altered metabolites were labeled. In addition to the changes in the ascorbate and aldarate metabolism and shikimate–phenylpropanoid biosynthesis mentioned above, exposure to Cu ions induced decreased levels of a TCA cycle precursor (pyruvic acid) and intermediates (citric acid and succinic acid), indicating that energy production was altered due to exposure to Cu ions (Figure 5). α-aminoadipic acid increased almost 2-fold, compared to the control, when plants were exposed to 1.5 mg of Cu ion. α-Aminoadipic acid is an intermediate in the α-aminoadipic acid pathway for the metabolism of lysine and saccharopine. Lysine catabolism in plants is a super-regulated metabolic pathway,[42] which efficiently converts lysine to glutamate and then to other metabolites in response to stress and in certain developmental programs.[42] The up-regulation of α-aminoadipic acid may indicate that stress-defense-related pathways were activated in response to Cu ion. In addition to the TCA cycle, sugar metabolism was also affected by Cu ions. Xylose is a monomer of hemicellulose, which is an important structural polysaccharide in plant cell walls. Previous studies showed that the cell wall plays an important role in the defense response of plants to metals.[43] The composition of the cell wall may be actively modified under heavy metal stress. The modifications of the cell wall can increase the capacity of the cell wall to bind metals and decrease its permeability to trace metal migration into protoplast.[43] Protein, amino acids, and phenolics also take part in the metal-binding process. The up-regulation of xylose and phenolics, which are a component of cell walls, are a strong indicator that spinach plants may employ cell wall composition changes as a strategy to increase the tolerance to Cu by either binding Cu ions on the cell wall or decreasing the permeability of cell wall. However, two sugar alcohols (ribitol and erythritol) decreased in a dose-dependent way after exposure to Cu ions. The reason for their reduction in response to Cu ion exposure is unknown.

Global Visualization of Pathway Changes in Spinach Leaves Exposed to $Cu(OH)_2$ Nanopesticide. As shown in Figure 6, exposure of spinach leaves to $Cu(OH)_2$ nanopesticide resulted in an increase in primary products of photosynthesis, such as soluble sugars (sucrose and glucose), indicating that spinach leaves attempt to fix more carbon in response to the $Cu(OH)_2$ nanopesticide. In addition to sucrose and glucose, a trisaccharide (raffinose) was up-regulated by the nanopesticide. Raffinose can quench ROS produced by plants in response to

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abiotic stress.[44] Increased intracellular levels of raffinose might be a protective mechanism of plants for coping with oxidative stress.[45]

In addition to sugar metabolism, exposure to the $Cu(OH)_2$ nanopesticide-perturbed N metabolism. Branch-chain amino acids (valine and leucine) were decreased (36−39%) by the nanopesticide compared to the control (Figure 6). In addition, some amino acids synthesized from TCA cycle intermediates, such as aspartate, asparagine, and methionine, were decreased by 24−56%, compared to the control. This indicates that N metabolism is partially inhibited by exposure to $Cu(OH)_2$ nanopesticide. This response is specific to the $Cu(OH)_2$ nanopesticide because it was not observed when spinach plants were exposed to Cu ions.

Beneficial Nutritional Supply Alteration. Average daily spinach consumption in the United States is 2.5 g/day-capita (fresh weight).[46] The average water content in lettuce leaves is 91.4%, so the average daily consumption is 0.215 g/day-capita (DW). Thus, the estimated average daily dietary exposure to Cu through the consumption of spinach exposed to $Cu(OH)_2$ nanopesticide would be 4.5, 81, and 650 μg for control, with 1.8 and 18 mg doses, respectively. According to the Food and Nutrition Board at the U.S. Institute of Medicine of the National Academies, the tolerable upper safe intake level of copper is 10 mg/person-day.[47] Therefore, Cu intake from consumption of spinach exposed to $Cu(OH)_2$ via foliar application would be well within the safe Cu levels, even at the higher application level. However, it has to be mentioned that the hazard associated with NPs forms in tissues exposure in humans is still fundamentally unknown. According to the Soil Association, the total copper that can be applied to organic-designated land is 6 kg/ha-year. Typical planting density for spinach is 150 000 plants per hectare. Therefore, up to 40 mg of Cu may be applied to each plant. This dose is a higher than the highest dose (25 mg of Cu per plant) in the current study but still much lower than the recommended upper safe limit.

In addition, we found that mineral nutrient profiles were altered in plants exposed to the nanopesticide. For example, 18 mg of $Cu(OH)_2$ nanopesticide significantly ($p < 0.05$) increased the content of Al, Fe, and Ag in spinach leaves, while macronutrients such as P, K, Mg, Zn, Mn, Ca, Na, and Mo were unchanged (Table 1). In contrast, exposure to Cu ions did not change any mineral nutrient levels. This indicates the enhanced Al, Fe, and Ag is specific to the $Cu(OH)_2$ nanopesticide.

Antioxidant and Amino Acids. In addition to sugars, amino acids, and proteins, spinach is a good source of natural antioxidants such as vitamin C, carotenoids, and phenolic compounds. Dietary antioxidants can act as scavengers of free radicals to protect from excess free radicals.[48] Previous studies have shown a strong and consistent protective effect of vegetable consumption against the risk of free radical-related diseases such as aging, cancer, and cardiovascular disease.[49] β-Sitosterol is an important phytosterol found in plants. It has been shown to have antiproliferative and antiatherosclerosis effects on cancers of the colon, breast, stomach, and prostate.[50] The decrease in ascorbic acid, α-tocopherol, β-sitosterol, ferulic acid, and GABA in spinach leaves exposed to $Cu(OH)_2$ nanopesticide suggest potentially compromising nutritional benefit. Meanwhile, the reduction of a number of amino acids (alanine, valine, leucine, asparagine, methionine, and aspartic acid) in leaves exposed to $Cu(OH)_2$ nanopesticide also reduced the nutritional content.

Environmental Implications. Using metabolomics we demonstrated that the $Cu(OH)_2$ nanopesticide applied at agriculturally relevant doses affected anti-oxidative pathways and significantly decreased a number of low-molecular-mass antioxidants, which are beneficial nutrients for humans. Some of the responses, such as the reduction of antioxidants, are induced by the released Cu ions from either the nanopesticide or the Cu salt, which means that other copper-based nanopesticide may induce similar affects. The observed metabolic reprogramming could not be explained entirely by Cu ions, although some metabolic patterns did overlap. For example, some of the reactions are nanopesticide-specific, such as changes in N metabolism. Moreover, perturbation on the TCA cycle and activation of phenylpropanoid biosynthesis were observed only in Cu-ion exposure. This is the first study that reports that a copper-based nanopesticide can induce significant decrease in antioxidants in spinach leaves and that there is a differential metabolic response between exposure to Cu ions and exposure to this nanopesticide.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b02163.

Figures showing sample preparation, the effect of copper ions and $Cu(OH)_2$ nanopesticide, one-way ANOVA analysis, discriminating metabolites, VIP scores, the effect of Cu ion and Cu(OH)2 nanopesticide, and pathway enrichment analysis. (PDF)

Data dictionary of primary metabolism by automated liner exchange cold injection system (ALEX-CIS), gas chromatography time of flight mass spectrometry (GC−TOF-MS). (PDF)

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Notes

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