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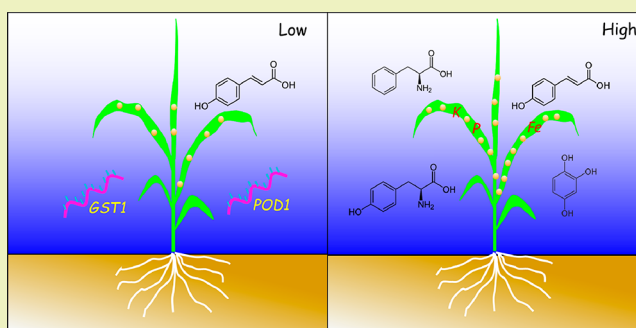
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Response at Genetic, Metabolic, and Physiological Levels of Maize (*Zea mays*) Exposed to a $\text{Cu}(\text{OH})_2$ NanopesticideLijuan Zhao,^{†,§} Qirui Hu,[‡] Yuxiong Huang,^{†,§} and Arturo A. Keller^{*,†,§}[†]Bren School of Environmental Science & Management, University of California, Santa Barbara, California 93106-5131, United States[‡]Neuroscience Research Institute and Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, California 93106, United States[§]Center for Environmental Implications of Nanotechnology, University of California, Santa Barbara, California 93106, United States

S Supporting Information

ABSTRACT: Nanopesticides are becoming more popular in modern agriculture to improve crop protection product efficacy. However, information on their implications for crop plants is needed. In this study, 3-week-old maize plants were exposed to different doses of $\text{Cu}(\text{OH})_2$ nanopesticide (0, 10, and 100 mg) for 7 days via foliar application. Gene expression of 9 antioxidant-related enzymes (*CAT1*, *POD1*, *GST1*, *SOD-1A*, *SOD-B*, *GPX*, *APX1*, *HSP1*, *PER1*) was determined using a quantitative real-time polymerase chain reaction. Using gas chromatography time-of-flight mass spectrometry, levels of 12 low-molecular-weight antioxidant compounds were measured. Results showed that a dose of 100 mg of $\text{Cu}(\text{OH})_2$ nanopesticide significantly decreased leaf chlorophyll content and biomass by 17–20%. In addition, potassium and phosphorus were up-regulated (14% and 13%, respectively) in response to this dose. Gene expression of *POD1* and *GST1* was significantly ($p < 0.05$) increased by 42.6% and 71.8%, respectively, at a dose of 10 mg, but declined at high dose (100 mg). Precursors of phenolic acids (phenylalanine and tyrosine), 4-hydroxycinnamic acid, and total phenolic content were significantly increased (24–122%) in response to 100 mg, indicating that phenolic acids may also play an important role in antioxidant defense. This study provides important information on maize plant responses to the $\text{Cu}(\text{OH})_2$ nanopesticide at genetic, metabolic, and physiological levels and may be applied to other nanoparticle/plant interaction studies.

KEYWORDS: Plant, Nanopesticide, Gene, Metabolite, Response



■ INTRODUCTION

The projected growth of the global human population will result in considerable increases in global food demand. The application of nanotechnology in modern agriculture can improve the food production efficiency of land, water, energy, fertilizers, and pesticides through the use of nanosensors, nanofertilizers, and nanopesticides.^{1,2} However, the rapid development in nanopesticide research over the last two years has increased concern over potential risks.³ Studying the bioaccumulation and toxicity of these nanopesticides to crop plants is becoming important, as they may represent the most significant intentional diffuse source of engineered nanoparticles in the environment.³

After the ban of highly toxic tributyltin, the use of copper-based pesticide increased significantly. There are currently more than 200 pesticides registered in California that use copper oxide as an active ingredient.⁴ Copper-based pesticides are regarded as less toxic than many organic chemical pesticides since copper is naturally present in most soils. However, copper ions induce the formation of reactive oxygen species (ROS) within plant cells, and their overaccumulation in plants can

result in oxidative damage of membrane lipids, proteins, and nucleic acids.⁵ A number of studies have shown that CuO nanoparticles can induce oxidative stress in various crop species such as carrot,⁶ cilantro,⁷ cucumber,⁸ and lettuce and alfalfa.⁹

Plants have efficient antioxidant systems including non-enzymatic and enzymatic antioxidants to scavenge high ROS levels. Enzymatic antioxidants include superoxide dismutase (*SOD*), peroxidase (*POD*), catalase (*CAT*), ascorbate peroxidase (*APOX*), monodehydroascorbate dehydrogenase (*MDHAR*), and dehydroascorbate reductase (*DHAR*).¹⁰ Non-enzymatic antioxidants include phenolics, flavonoids, tocopherols, ascorbic acid, and glutathione. Rico et al.¹¹ investigated the antioxidant defense system of rice in response to $n\text{CeO}_2$ nanoparticles (NPs). They reported that low $n\text{CeO}_2$ concentrations resulted in a marked decrease in the activities of *CAT*, *DHAR*, and glutathione reductase (*GR*) in rice roots, while other antioxidant-related enzymes such as *SOD*, guaiacol

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peroxidase (GPOX), and APOX were unchanged. The authors also observed that exposure to 500 mg/L $n\text{CeO}_2$ NPs triggered the up-regulation of ascorbate. Ma et al. investigated the defense mechanism of *Arabidopsis thaliana* in response to CeO_2 and In_2O_3 NPs.¹² They found that the activities of SOD, CAT, APX, and POD were significantly elevated upon exposure to 250 and 1000 mg/L CeO_2 NPs; In_2O_3 NPs triggered the activities of SOD and POD. More recently, Liu et al.¹³ observed that TiO_2 NPs alleviated the oxidative stress induced by tetracycline by down-regulating the activity of antioxidant enzymes such as SOD, CAT, APX, and POD in *A. thaliana*. These previous studies determined antioxidant enzyme activities at enzyme levels. Molecular techniques, such as the quantitative polymerase chain reaction (Q-PCR), microarray, and RNA sequencing, provide effective tools to investigate plant responses to stressors and can be supplementary to those used at the enzyme level.^{14,15} Ma et al. studied the molecular response of *Arabidopsis thaliana* (L.) to CeO_2 and In_2O_3 NPs using Q-PCR. Results showed that the expression of genes central to the stress response and detoxification, such as the sulfur assimilation and glutathione biosynthesis pathways, was altered by both types of NPs. Therefore, measuring the gene expression of antioxidant enzymes may provide a sensitive tool to evaluate plant response to NPs.

In this study, 3-week-old maize plants were exposed to different concentrations of $\text{Cu}(\text{OH})_2$ nanopesticide for a week. The impact of this nanopesticide on maize plants was evaluated at three levels: gene transcription, metabolite regulation, and physiological effects. We determined the effect on mineral homeostasis and photosynthetic pigment levels. Using Q-PCR, we examined antioxidant enzyme responses to Cu-induced oxidative stress at transcript levels. Using GC-TOF-MS, 12 metabolites which have the ability to scavenge ROS were determined.

MATERIALS AND METHODS

Experimental Design. The $\text{Cu}(\text{OH})_2$ nanopesticide (Kocide 3000) was purchased from Dupont. The primary particle size is from ~50 to >1000 nm.^{16,17} The hydrodynamic diameter of Kocide 3000 in NanoPure water (pH 7) is 1532 ± 580 nm and the ζ potential is -47.6 ± 43 mV, measured via dynamic light scattering (Malvern Zetasizer Nano ZS-90). The micronized particles in Kocide 3000 are made up of $\text{Cu}(\text{OH})_2$ nanosheets, bound together by an organic composite that disassociates in water.¹⁶ Copper content in Kocide 3000 is $26.5 \pm 0.9\%$; other elements detected by SEM-EDS are C, O, Na, Al, Si, S, and Zn.¹⁶

Corn seeds (*Zea mays*) were purchased from Seed Savers Exchange. Artificial growth media, which was used to exclude the extraneous variables introduced by soil exposure, contains sand (Quikrete washed plaster sand), Sunshine Advanced Growing Mix #4 (SunGro Horticulture), vermiculite (Therm-O-Rock), coco coir (Canna), and perlite (Therm-O-Rock) at a ratio of 1:3:1:2:2 by volume. In addition, 4-4-4 fertilizer was added at 0.4%. In total, 18 pots of corn seedlings were grown for 3 weeks in a greenhouse temperature maintained at 28 °C by day and 20 °C by night. When the corn plants were 3 weeks old, the $\text{Cu}(\text{OH})_2$ nanopesticide suspensions were applied to the corn leaves using a portable manual sprayer. $\text{Cu}(\text{OH})_2$ nanopesticide suspensions at 100 and 1000 mg/L were prepared in NanoPure water and sonicated (Branson 8800, Danbury, CT) in a temperature-control bath for 30 min prior to application. The $\text{Cu}(\text{OH})_2$ suspensions were applied to the leaves three times per day for 7 days. The total amount of $\text{Cu}(\text{OH})_2$ nanopesticide suspension applied was 2000 mL during 7 days, at the various concentrations, resulting in a total application of 0, 10, and 100 mg of $\text{Cu}(\text{OH})_2$ nanopesticide per plant.

Physiological Parameter Measurement. After 7 days of exposure to $\text{Cu}(\text{OH})_2$ nanopesticide at the various doses, the corn

plants were harvested and thoroughly washed with deionized water to remove residual nanoparticles and soil particles. Shoot and root biomass and root length were measured. All leaf tissues were ground using mortar and pestle in liquid N_2 . Half of the leaf tissues was stored at -80 °C for chlorophyll content analysis and RNA analysis. The other half was oven-dried for 3 days at 70 °C for subsequent inductively coupled plasma mass spectrometry (ICP-MS) to determine elemental content.

Photosynthetic Pigment Measurement. Chlorophyll a and b and total carotenoid content was determined on the basis of Sesták et al. with some modifications.¹⁸ Specifically, 0.01 g of corn leaves was immersed in 5 mL of 80% methanol to extract the pigments. The mixture was centrifuged for 10 min at 3000 rpm. Absorbance at 666 and 653 nm was used for chlorophyll a and b, and at 470 nm for carotenoids. Results were expressed as milligrams of total chlorophyll or carotenoids per gram of fresh weight or per plant.

Cu and Element Content Measurement. Dried leaf and root 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 Par) at 180 °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, 4 other macronutrients (Mg, P, K, and Ca), 6 micronutrients (Na, Mn, Fe, Ni, Zn, and Mo), and 13 nonessential nutrients (Al, Ti, V, Cr, Co, As, Se, Ag, Cd, Sb, Ba, Tl, and Pb) were analyzed using ICP-MS (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, and 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 .

Total Phenolics Measurement. Total antioxidant capacity was determined following Singleton and Rossi (1965).¹⁹ More details are shown in the Supporting Information.

Total RNA Extraction and Antioxidant Enzyme Gene Expression Analysis. The expression of gene transcripts related to antioxidant defense was analyzed by RT-Q-PCR in maize plants exposed to $\text{Cu}(\text{OH})_2$ nanopesticide. Details regarding total RNA extraction from maize leaves and gene expression analysis were provided in the Supporting Information.

Gas Chromatography Time-of-Flight Mass Spectrometry (GC-TOF-MS) Analysis of Low-Molecular-Mass Antioxidants. The freeze-dried maize leaf tissue samples were subjected to GC-TOF-MS analysis at Genome Center Core Services, University of California Davis, to identify the metabolites present in maize tissues. The sample pretreatment, analytical method, and instrument have been described by Fiehn et al.^{20,21} Briefly, an Agilent 6890 gas chromatograph (Agilent) containing an Rtx-5Sil MS column (30 m length $\times$ 0.25 mm internal diameter with 0.25 μm film made of 95% dimethyl-/5% diphenyl polysiloxane) 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.

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

RESULTS AND DISCUSSION

Biomass and Photosynthesis Pigments of Corn Leaves. No visible toxicity symptoms were observed in maize plants exposed to $\text{Cu}(\text{OH})_2$ nanopesticides during the entire exposure period. Leaf biomass did not change at the 10 mg dose, compared to the biomass of the control. However, the 100 mg dose significantly decreased leaf biomass ($p < 0.05$) (Figure 1). Root biomass was not impacted by either dose

(Figure 1), likely because the roots were not directly exposed to the $\text{Cu}(\text{OH})_2$ nanopesticide.

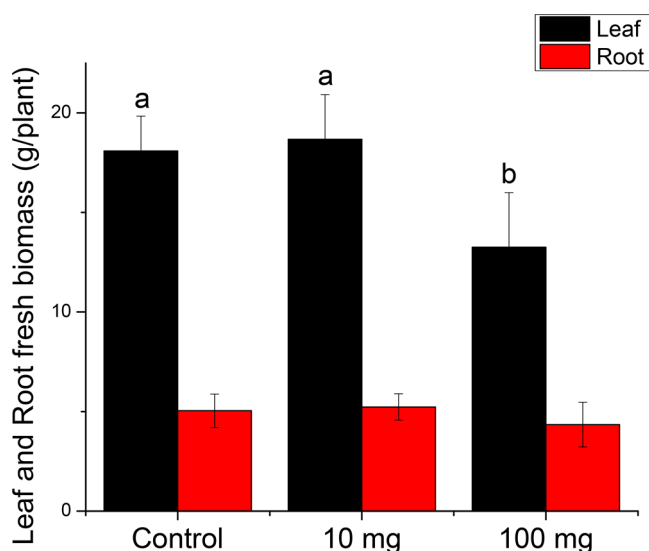


Figure 1. Effect of copper hydroxide nanopesticide on leaf and root biomass.

Chlorophyll content is considered to be one of the most important parameters in the evaluation of plant stress.²² The photosynthetic pigments (chlorophyll a and b, and carotenoids) in maize leaves exposed to 10 mg of $\text{Cu}(\text{OH})_2$ remained unchanged (Figure 2). However, the 100 mg dose significantly

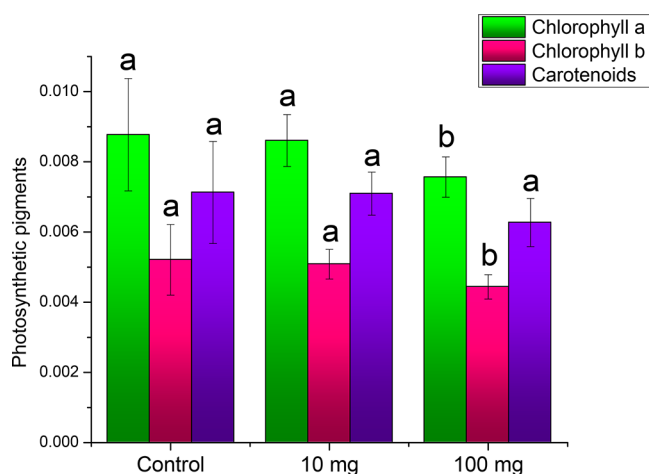


Figure 2. Photosynthetic pigment content (chlorophyll a and b and total carotenoids) of maize treated with different concentrations of $\text{Cu}(\text{OH})_2$ nanopesticide. The means are averaged from six replicates of maize leaves. The error bars correspond to the standard error of mean. Values of photosynthetic pigment followed by different letters are significantly different at $p < 0.05$.

decreased both chlorophyll a and chlorophyll b content ($p = 0.034$ and 0.033 , respectively; Figure 2). Similar to chlorophyll, carotenoid content markedly decreased at the 100 mg dose ($p = 0.088$). The decrease in chlorophyll content may explain the decrease in leaf biomass, because of reduced photosynthetic activity.

Zuverza-Mena et al.⁷ reported that CuO applied to soil at 20 mg kg^{-1} significantly reduced (26%) the relative chlorophyll

content in cilantro (*Coriandrum sativum*). The reduction of chlorophyll content induced by excess copper in chloroplasts has been observed in a variety of species such as *Ceratophyllum demersum* L.,²³ *Empetrum nigrum*,²⁴ *Brassica pekinensis* Rupr.,²⁵ and *Triticum aestivum* L.²⁶ It has been proposed that Cu at high concentrations affects plants via two main mechanisms: interference with enzymes associated with chlorophyll biosynthesis and the changes in protein composition of photosynthetic membranes.^{27,28} In addition, Cu-induced Fe deficiency²⁹ and the displacement by Cu of the Mg required for chlorophyll biosynthesis³⁰ have been proposed as possible additional damage mechanisms.

In a previous study, we exposed cucumber plants to 2.5 and 25 mg of $\text{Cu}(\text{OH})_2$ nanopesticide, and did not observe a decrease in photosynthetic pigments. On the contrary, $\text{Cu}(\text{OH})_2$ nanopesticide at 25 mg significantly increased the content of chlorophyll a and b in cucumber leaves, although the mechanism is still not known. Thus, plant response to this nanopesticide is likely species-dependent and may not always be detrimental.

Cu Bioaccumulation in Corn Leaves and Translocation to Roots. The copper content in corn leaves treated with 0, 10, and 100 mg of $\text{Cu}(\text{OH})_2$ was 12, 206, and 1404 mg kg^{-1} dry weight (DW), respectively (Table 1). This indicates that Cu was significantly ($p < 0.05$) bioaccumulated in maize leaves. However, it should be noted that significant retention of Cu on the leaf surface may exist, because water rinsing may not be effective in removing surface Cu, which may be entrapped in the cuticle or within morphological surface structures. Cu content remained unchanged in corn plant roots (Table 1), which indicates that copper was not translocated from maize leaves to root through phloem loading. Another possibility is that some Cu is just distributed on the leaf surface and does not enter the cells. Also, this indicates that dissolved Cu or $\text{Cu}(\text{OH})_2$ nanopesticide particles which may have accidentally fallen down to the soil during spraying were not significant and/or not bioavailable. Similar results were observed in cucumber plants when the $\text{Cu}(\text{OH})_2$ nanopesticide was applied via foliar spray; there was no significant increase of Cu in cucumber roots.³¹

Cu detected in corn leaves by ICP-MS represents both dissolved Cu and undissolved $\text{Cu}(\text{OH})_2$ nanoparticles. Particles deposited on the leaf surfaces are trapped in folds which makes it difficult for them to be removed even after thorough washing. While this fraction of Cu is not inside plant cells, it can undergo slow release with time.

Mineral Nutrient Homeostasis. The primary effect of abiotic stress is ion imbalance.³² Mineral nutrient concentrations in leaves, stems, and roots of maize plants exposed to different doses of $\text{Cu}(\text{OH})_2$ nanopesticide are shown in Table 1. The content of potassium and phosphorus in maize leaves exposed to 100 mg of $\text{Cu}(\text{OH})_2$ nanopesticide was significantly ($p < 0.05$) increased by 13.6% and 12.9%, respectively, compared to the content in the control. Phosphorus is a vital plant macronutrient, and a major component of essential biomolecules such as sugar phosphates, phospholipids, and phosphoproteins, and is found in energy-rich compounds like adenosine triphosphate (ATP), adenosine diphosphate, and NADPH.^{33,34} It plays crucial roles in almost all metabolic reactions including photosynthesis and respiration, energy generation, nucleic acid synthesis, membrane synthesis, enzyme activation/inactivation, and redox reactions.³⁵ Interestingly, a number of sugar phosphates such as glucose-1-phosphate,

Table 1. Effect of Cu(OH)₂ on Cu and Other Mineral Nutrient Content in Maize Leaves (mg kg⁻¹ Dry Weight)^a

	Cu	K	P	Ca	Mg	Na	Fe	Zn	Mn	Mo
control	13 ± 1.1	56623 ± 5260	6868 ± 688	4677 ± 581	3889 ± 400	91 ± 23.8	82.3 ± 4.3	55.6 ± 8.2	45.9 ± 15.2	0.25 ± 0.08
	206 ± 44*	57384 ± 3937	7236 ± 587	5073 ± 526	3934 ± 340	175 ± 73.5*	83.6 ± 3.7	58.8 ± 8.6	48.3 ± 10.9	0.21 ± 0.04
	1495 ± 283*	64340 ± 4364*	7755 ± 468*	5038 ± 805	4074 ± 603	607 ± 117*	101 ± 9.2*	61.6 ± 12	59.5 ± 11.4	0.19 ± 0.05
control	8 ± 2	137840 ± 18781	13790 ± 1298	7716 ± 1655	7659 ± 1522	79 ± 50	38.9 ± 5.7	104 ± 26	22.5 ± 8.2	0.12 ± 0.05
	63 ± 18*	152163 ± 12812	15767 ± 1573*	7596 ± 963	7705 ± 1392	217 ± 180	43.4 ± 4.7	118 ± 15	22.8 ± 5.9	0.12 ± 0.02
	523 ± 99*	138662 ± 5416	14059 ± 2317	7009 ± 1103	6747 ± 882	223 ± 21*	46.3 ± 8.5	114 ± 41	27.4 ± 5.6	0.08 ± 0.02
control	75 ± 7	28980 ± 6175	8952 ± 1238	9365 ± 1716	9611 ± 1021	11440 ± 1841	317 ± 55	142 ± 55	30.9 ± 12.2	0.31 ± 0.15
	76 ± 16	28249 ± 3306	9691 ± 968	8830 ± 1061	10317 ± 1024	11516 ± 1300	432 ± 188	138 ± 36	32.5 ± 9.8	0.27 ± 0.03
	85 ± 19	33989 ± 6527	9786 ± 1307	8849 ± 1915	8582 ± 1726	10643 ± 2395	243 ± 71	109 ± 28	34.1 ± 14.0	0.29 ± 0.06

^aData are average of 6 replicates. * indicates statistical significance ($p < 0.05$).

glucose-6-phosphate, fructose-6-phosphate, and 3-phosphoglycerate have been observed to be increased in maize leaves.³⁶ Thus, the simultaneous up-regulation of inorganic P and P-related organic compounds may indicate that the enhanced phosphate acquisition by maize plants is possibly used to up-regulate the biosynthesis of those low-molecular-weight P-related compounds to improve the energy level in plants, enhancing tolerance to excess copper. The regulation of inorganic phosphate homeostasis is possibly the adaptation of maize plants to excess bioavailable Cu.

Potassium also plays an important role in plant growth and metabolism, and it contributes greatly to the survival of plants that are under various biotic and abiotic stresses.³⁷ Potassium helps maintain cell membrane stability and is involved in detoxifying oxygen radicals.^{37,38} The up-regulation of potassium is possibly a strategy maize plants employ to defend against ROS-induced stress from excess Cu, and also a strategy to maintain cell membrane stability. Potassium has been shown to be involved in the osmotic adjustment of salt-stressed plants.³⁷ An alternative explanation for the up-regulation of potassium is to maintain osmotic adjustment induced by a higher amount of Na coming from Cu(OH)₂ nanopesticide, since Kocide 3000 contains approximately 10% sodium.

Antioxidant Defense System. It has been reported that excess Cu triggers the generation of ROS and free radicals and thus causes molecular damage to plants.³⁹ Excessive ROS can result in the irreversible oxidation of lipids, proteins, chloroplastic pigments, and nucleic acids. Plants have developed complex antioxidative defense systems comprising enzymatic and nonenzymatic components to scavenge ROS.^{40,41} In order to provide a comprehensive vision, both antioxidant enzyme RNA levels and low-molecular-weight antioxidant compounds were analyzed.

Gene Expression of Antioxidant Enzymes. Gene expression levels for genes related to the antioxidant and detoxification response in maize leaves were determined using Q-PCR (Figure 3). CATs have the potential to directly

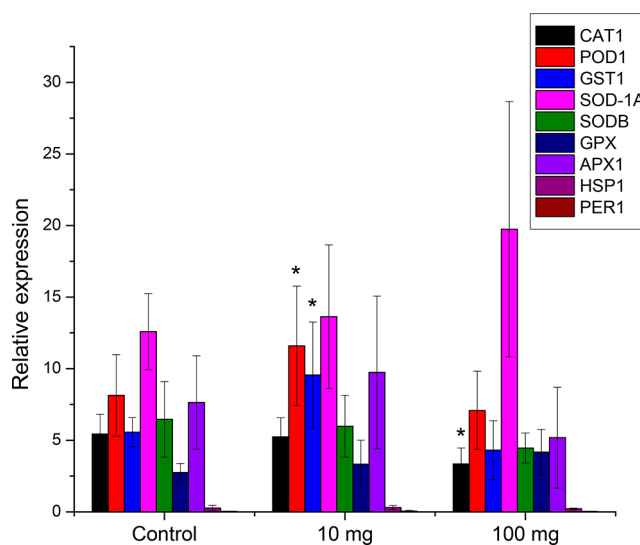


Figure 3. Relative expression of antioxidant-related genes in response to Cu(OH)₂ nanopesticide in maize leaves. The means are averaged from six replicates of maize leaves. The error bars correspond to the standard error of mean. * indicates that gene expression is significantly different from the control at $p < 0.05$.

Table 2. Effect of Cu(OH)₂ Nanopesticide on the Relative Content of Low-Molecular-Weight Antioxidants in Maize Leaves^a

	category	control	10 mg	100 mg
γ -tocopherol	vitamins	1985 $\pm$ 950	1928 $\pm$ 420	2848 $\pm$ 1683
α -tocopherol	vitamins	2285 $\pm$ 1558	2191 $\pm$ 1216	1909 $\pm$ 455
dehydroascorbic acid	vitamins	30320 $\pm$ 8587	33879 $\pm$ 8921	38072 $\pm$ 7854
phenylalanine	amino acid	8542 $\pm$ 1749	9441 $\pm$ 2526	10580 $\pm$ 1274*
tyrosine	amino acid	37692 $\pm$ 6917	43722 $\pm$ 5947	52571 $\pm$ 8634*
proline	amino acid	2893 $\pm$ 479	5252 $\pm$ 3224	7215 $\pm$ 5148
4-aminobutyric acid	amino acid	10493 $\pm$ 6414	10942 $\pm$ 6344	15330 $\pm$ 8848
benzoic acid	phenolics	2798 $\pm$ 1517	3895 $\pm$ 618	3832 $\pm$ 1307
1,2,4-benzenetriol	phenolics	408 $\pm$ 121	642 $\pm$ 273	671 $\pm$ 350
4-hydroxycinnamic acid	phenolics	1760 $\pm$ 946	3689 $\pm$ 1484*	3906 $\pm$ 1485*
3,4-dihydroxycinnamic acid	phenolics	3099 $\pm$ 945	3931 $\pm$ 1878	3401 $\pm$ 1108
ferulic acid	phenolics	2550 $\pm$ 1027	3081 $\pm$ 542	2213 $\pm$ 1203

^aThe data are means of six replications $\pm$ standard deviation. * indicates $p < 0.05$.

dismutate H₂O₂ into H₂O and O₂ and are indispensable for ROS detoxification during stressed conditions.⁴² As shown in Figure 3, the expression of CAT1 in maize leaves was unchanged at 10 mg of Cu(OH)₂ nanopesticide, but was significantly down-regulated (38.4%, $p < 0.05$) at a dose of 100 mg compared to its expression in the control, indicating that excess Cu affected CAT1 gene expression. Similarly, Srivastava et al. reported that Cu²⁺ decreased CAT activity in *A. dolium*.⁴³ We also found that POD1 and GST1 gene expressions were significantly increased, by 42.6% and 71.8%, respectively, compared to those in the control ($p < 0.05$), at a dose of 10 mg of Cu(OH)₂ nanopesticide, but expression declined at the higher dose (100 mg). POD and GST are detoxification-related enzyme genes. Peroxidase (POD) is a heme-containing glycoprotein encoded by a large multigene family in plants and is involved in various physiological processes. Kawano et al. suggested that POD plays a role in lignification, cross-linking of cell wall structure proteins, and defense against pathogens.⁴⁴ Mocquot et al. reported that POD activity significantly increased in all maize organs that exhibited higher copper content.⁴⁵ Chen et al., indicated that moso bamboo (*Phyllostachys pubescens*) can increase the activity of POD to protect it from oxidative damage induced by Cu toxicity, but high Cu stress has caused a large amount of reactive oxygen species which inhibited enzyme activity.⁴⁶ Glutathione S-transferases (GSTs) are ubiquitous enzymes that play key roles in detoxifying oxidative-stress metabolites.⁴⁷ Khatun et al.⁴⁸ reported that GST activity in *Withania somnifera* leaves was increased in the presence of Cu when compared to control plants, indicating that antioxidant enzymes played an important role in protecting the plant from Cu toxicity.

Within a cell, the superoxide dismutases (SODs) constitute the first line of defense against ROS. Surprisingly, the expressions of both SOD1-A (Cu–Zn SOD) and SOD-B (Fe SOD) were not changed in response to Cu(OH)₂ nanopesticide. Ascorbate peroxidase (APX) is the main enzyme responsible for H₂O₂ removal in the chloroplast, peroxisomes, and mitochondria. APX utilizes ascorbate as its specific electron donor to reduce H₂O₂ to water.⁴⁹ Glutathione peroxidase (GPX) is a family of isoenzymes that use glutathione to reduce H₂O₂ and organic and lipid hydroperoxides, thereby protecting cells against oxidative damage. GPX is an important H₂O₂-scavenging enzyme in mammals. Since expression of GPX and APX was not up-regulated, this indicates that these enzymes did not play a major role in maize detoxification under the test conditions.

GC-TOF-MS Determination of Nonenzymatic Antioxidants. Nonenzymatic components of the plant's antioxidant defense system include ascorbate, tocopherol, proline, glutathione, carotenoids, and phenolic compounds.⁴¹ By using GC-TOF-MS, 12 low-molecular-weight antioxidants (α -tocopherol, γ -tocopherol, dehydroascorbic acid, proline, tyrosine, phenylalanine, ferulic acid, benzoic acid, 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid, and 1,2,4-benzenetriol) were quantified (Table 2).

Vitamins. γ -Tocopherol and α -tocopherol (vitamin E), are major lipid-soluble antioxidants found in leaves, which are considered as potential scavengers of ROS and lipid radicals.⁵⁰ However, the levels of γ -tocopherol and α -tocopherol were not altered as a response to exposure of the corn leaves to the Cu(OH)₂ nanopesticide, indicating that tocopherol likely did not play a role in antioxidant defense. Ascorbic acid (vitamin C) is water-soluble and the most abundant and powerful antioxidant. It occurs in all plant tissues and is typically at higher levels in photosynthetic cells. Although dehydroascorbic acid, an oxidized form of ascorbic acid, did tend to increase at 100 mg of Cu(OH)₂ nanopesticide, this was not statistically significant.

Amino Acids. As shown in Table 2, phenylalanine and tyrosine, two aromatic amino acids (AAAs), were significantly ($p < 0.05$) increased by 23.9% and 39.5%, respectively, in response to 100 mg of Cu(OH)₂ nanopesticide. These two AAAs not only are essential components of protein synthesis, but also serve as precursors for a wide range of secondary metabolites, such as the plant hormones auxin and salicylate, as well as for several aromatic secondary metabolites that are important for plant defense.⁵¹ The up-regulation of phenylalanine and tyrosine is an indicator of the activation of the AAA biosynthesis pathway.

Apart from acting as an osmolyte for osmotic adjustment, proline is considered a potent antioxidant, scavenging free radicals and buffering cellular redox potential under stress conditions; proline also contributes to stabilizing subcellular structures (e.g., membranes and proteins),⁵² and can act as a metal chelator, thereby alleviating metal stress.⁵³ Proline was up-regulated in a dose-dependent manner when corn leaves were exposed to the nanopesticide, particularly at the higher dose ($p < 0.068$). Farago and Mullen demonstrated that proline formed a complex with Cu in Cu-tolerant *Armoria*.⁵³ 4-Aminobutyric acid (GABA) is a nonprotein amino acid. In plants, there are numerous observations of a rapid accumulation of GABA in response to biotic and abiotic stress.⁵⁴ However, in

this study, GABA was unchanged after exposure to the $\text{Cu}(\text{OH})_2$ nanopesticide at either dose.

Phenolics. Phenolic compounds are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom. The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electrons, or chelate metal cations. Phenolics can scavenge free radicals and thus are important components of the antioxidant system. Phenolic acids are divided into two main groups: benzoic and cinnamic acids.⁵⁵ As shown in Table 2, after exposure of corn leaves to the nanopesticide, benzoic acid was unchanged. 1,2,4-Benzenetriol was enhanced at both nanopesticide doses. 4-Hydroxycinnamic acid also increased by 2.2-fold compared to that in the control at both doses. That means that 4-hydroxycinnamic acid may play an important role in protecting the plant from oxidative damage.

We then analyzed the total phenolic content in maize leaves. Figure 4 shows that the total phenolic content was significantly

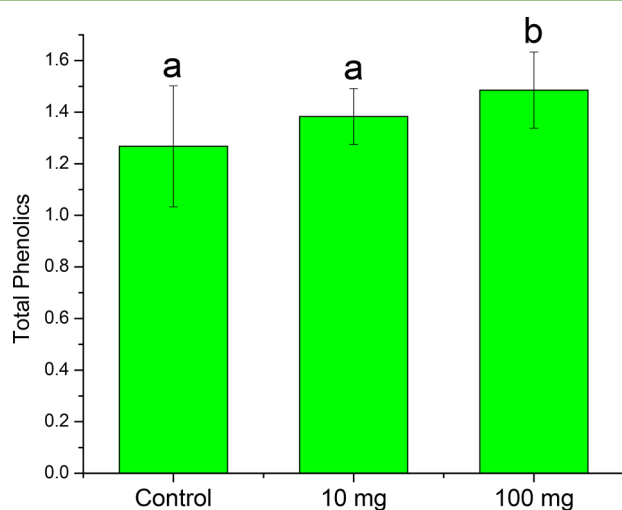


Figure 4. Effect of different concentrations of $\text{Cu}(\text{OH})_2$ nanopesticide on total phenolics in maize leaves. The means are averaged from six replicates of maize leaves. The error bars correspond to the standard error of mean. Values of total phenolics followed by different letters are significantly different at $p < 0.05$.

increased by 9.1% and 17.1% ($p = 0.077$ and 0.021) at the 10 and 100 mg doses, respectively. The biosynthesis of phenolic compounds in plants proceeds through the production of phenylalanine (Figure 5). As mentioned before, phenylalanine and tyrosine, precursors of phenolic compounds, were up-regulated. All of these metabolic changes are a strong indication that biosynthesis of phenolics is a protective mechanism for the maize plant to defend against Cu-triggered ROS. Figure 5 illustrates the biosynthesis of phenolics from shikimic acid. It has been reported that elevated levels of Cu disturbed the integrity of thylakoid membranes and changed the fatty acid composition.⁵⁶

Taken together, this study for the first time evaluates the impact of a nanopesticide on a crop plant at gene, metabolite, and physiological levels. Analysis of the antioxidant gene expression and low-molecular-weight compounds which act as an ROS scavenger provided a comprehensive understanding of the antioxidant defense system of the maize plant. Clearly, nonenzymatic antioxidants (low-molecular-mass secondary metabolites) play a much more important role compared to

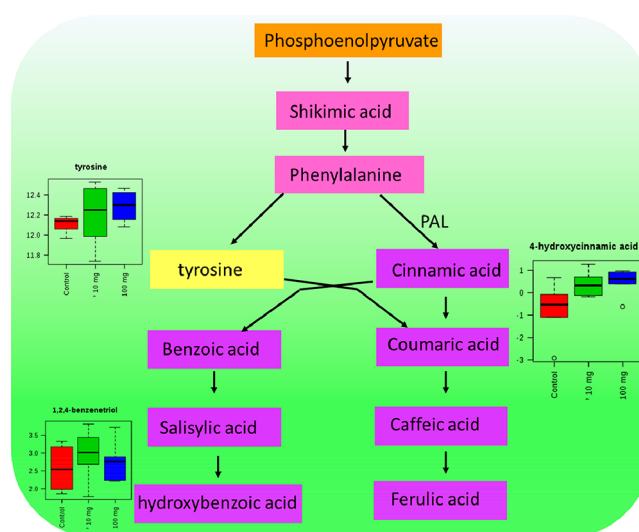


Figure 5. Schematic illustration of the biosynthesis of different phenolics from shikimic acid. PAL = phenylalanine ammonia lyase.

enzymatic antioxidants (antioxidant enzyme) in combating with ROS. Although *POD1* and *GST1* are up-regulated to alleviate the oxidative stress when maize leaves are exposed to a low concentration of $\text{Cu}(\text{OH})_2$, their biosyntheses are possibly damaged by high concentrations of $\text{Cu}(\text{OH})_2$. In contrast, nonenzymatic antioxidants were not inhibited even at high concentrations of $\text{Cu}(\text{OH})_2$. Future study on more environmentally relevant exposure throughout the whole life cycle is needed and could have significant implications for agricultural crop yield evaluation and food safety.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acssuschemeng.7b01968](https://doi.org/10.1021/acssuschemeng.7b01968).

Determination of total phenolics, total RNA extraction, antioxidant enzyme gene expression analysis, and primers used for real-time RT-PCR assays (PDF)
GC-TOF-MS method (PDF)

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Notes

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