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Metabolomics Reveals the Molecular Mechanisms of Copper Induced Cucumber Leaf (*Cucumis sativus*) Senescence

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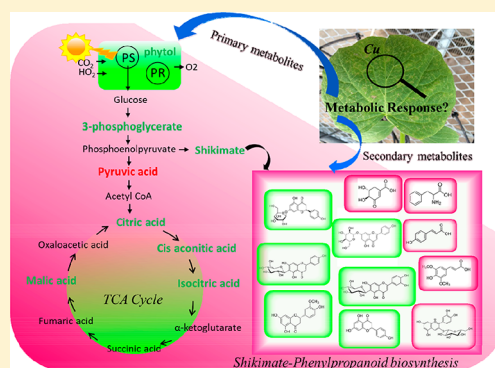
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Supporting Information

ABSTRACT: Excess copper may disturb plant photosynthesis and induce leaf senescence. The underlying toxicity mechanism is not well understood. Here, 3-week-old cucumber plants were foliar exposed to different copper concentrations (10, 100, and 500 mg/L) for a final dose of 0.21, 2.1, and 10 mg/plant, using CuSO₄ as the Cu ion source for 7 days, three times per day. Metabolomics quantified 149 primary and 79 secondary metabolites. A number of intermediates of the tricarboxylic acid (TCA) cycle were significantly down-regulated 1.4–2.4 fold, indicating a perturbed carbohydrate metabolism. Ascorbate and aldarate metabolism and shikimate-phenylpropanoid biosynthesis (antioxidant and defense related pathways) were perturbed by excess copper. These metabolic responses occur even at the lowest copper dose considered although no phenotype changes were observed at this dose. High copper dose resulted in a 2-fold increase in phytol, a degradation product of chlorophyll. Polyphenol metabolomics revealed that some flavonoids were down-regulated, while the nonflavonoid 4-hydroxycinnamic acid and *trans*-2-hydroxycinnamic acid were significantly up-regulated 4- and 26-fold compared to the control. This study enhances current understanding of copper toxicity to plants and demonstrates that metabolomics profiling provides a more comprehensive view of plant responses to stressors, which can be applied to other plant species and contaminants.



INTRODUCTION

Due to their antifungal characteristics, copper-based pesticides are widely used in agriculture to control fungal diseases in a number of crops around the world.¹ Repeated applications of copper-containing fungicides can lead to elevated copper concentrations in surface soils, as high as 1280 mg/kg.² Recently, copper nanoparticles have been proposed as bionanosensors and electrical-nanosensors to detect potential pathogens in plants.³ In addition to these intentional inputs of copper in agriculture, mining activities, and sewage sludge application also contribute to copper accumulation in agricultural soils. Therefore, exposure to copper is a common environmental stress encountered by crop plants.

Copper is also an essential element for plants, participating in different metabolic processes such as photosynthesis and is a component of important antioxidant enzymes such as superoxide dismutase and ascorbate oxidase.⁴ Previous reports demonstrated that 0.05 mg/L copper sulfate promotes *Vigna radiata* growth and increases carbon and nitrogen assimilation.⁵ However, excess copper in plants triggers overproduction of

free radicals that can induce lipid peroxidation and DNA damage.⁶ Pradhan et al. showed that copper above 0.5 mg/L induced severe tissue injuries.⁷ Our previous studies showed that a Cu(OH)₂ nanopesticide triggered oxidative stress and metabolic adaptation in a variety of crop plants including cucumber,⁸ corn,⁹ lettuce,¹⁰ and spinach.¹¹ The released Cu ions are regarded as the active ingredient of many copper-containing pesticides. Thus, elucidating the effects of Cu ions to plants is crucial. Cu may induce a number of physiological and biochemical defects including impacting photosynthesis and inducing early leaf senescence.¹² However, the underlying mechanisms of copper-induced senescence are still unknown. Moreover, metabolite level reprogramming by the plant to resist and tolerate the toxic effects of copper is poorly understood.

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Metabolomics are defined as the simultaneous measurement of many metabolites in a given biological system.¹³ Low molecular weight metabolites are the final products of gene expression, thus the changes of metabolites reflect the final response of organisms to environmental stress.¹⁴ Using a metabolomics platform, global patterns of plant metabolite changes in response to exposure to stressors can be monitored. Plants have diverse and complex metabolic pathways that generate primary and secondary metabolites.¹⁵ The primary metabolites, including sugars, amino acids, and lipids, are indispensable for the growth and development of plants by providing the necessary energy and molecular building blocks. Monitoring the primary metabolites may reflect the metabolic status during plant growth. In addition, plants produce a large number of secondary metabolites such as flavonoids, terpenoids, and glucosinolates, which are crucial for interactions with the environment, including providing resistance against biotic and abiotic stresses.¹⁵ A number of secondary metabolites protect plants from oxidative damage, such as ascorbic acid, glutathione, tocopherols, polyphenols, anthocyanins, and carotenoids by scavenging active oxygen species.¹⁶ The phenylpropanoid pathway is involved in the regulation of plant mechanisms in response to various stressful conditions.¹⁷ For example, it has been reported that copper stress leads to activation of the phenylpropanoid pathway and growth inhibition in burdock (*Arctium lappa* L.).¹⁸ Investigating the intermediates of the phenylpropanoid pathway enables a better understanding of the antioxidant mechanisms.

In this study, 4-week-old cucumber plants were foliar exposed to different doses of copper ions for 7 days. Two metabolomics platforms (GC-TOF-MS and LC-MS/MS) were employed to quantify primary and secondary metabolites. Multivariate analysis of metabolites data combined with biological pathway analysis were used to provide insight into the response and tolerance mechanisms. A previous study reported the phenotypic data from this experiment.⁸ Results showed that a dose of 10 mg/plant copper induced leaf chlorosis and the decline of photosynthetic pigment content. In this study the molecular responses were linked to the previously reported phenotypes, which helps to provide a deeper insight into the mechanisms employed by plants to cope with copper induced stress.

MATERIALS AND METHODS

Growth Condition and Foliar Application of CuSO₄

Cucumis sativus seeds were purchased from Seed Savers Exchange (Iowa, U.S.A.). CuSO₄·5H₂O was purchased from Sigma-Aldrich. Growth media was composed of 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, organic granular 4–4–4 fertilizer was applied at 3.4 g/kg artificial growth medium. The temperature in the greenhouse was maintained at 28 °C by day and 20 °C by night. The daily light integral (photosynthetically active radiation) was $17.3 \pm 3.6 \text{ mol m}^{-2} \text{ d}^{-1}$. A total of 16 pots (four replicates in each treatment) of cucumber seedlings were grown in a greenhouse for 3 weeks before foliar application of CuSO₄. After that, cucumber plants were foliar exposed to different concentrations (0, 10, 100, and 500 mg/L) of CuSO₄ for 1 week. A 7 day exposure time was chosen because preliminary experiments showed early reactive oxygen species (ROS) response started within 7 days. A hand-held spray bottle

was used for spraying. During spraying, the spray bottle was set around 20 cm above the plants to ensure the aerosols were applied directly onto the leaf surfaces. The cucumber plants were sprayed a total of 3 times per day for 7 days; the amount sprayed each time was approximately 4 mL per pot. The total amount of CuSO₄ suspension applied was 500 mL during 7 days, resulting in a total application of 0, 0.21, 2.1, and 10 mg Cu per plant at the various concentrations.

Gas Chromatography-Time of Flight-Mass Spectrometry (GC-TOF-MS) Analysis of Primary Metabolites in Cucumber Leaves. At harvest, leaf tissues were freeze-dried in liquid nitrogen and ground to a fine powder. The powders were analyzed using GC-QTOF-MS at the West Coast Metabolomics Center at UC Davis (<http://metabolomics.ucdavis.edu/>). A description of sample pretreatment, analytical method, and instrument has been provided by Fiehn et al.^{19,20} Briefly, an Agilent 6890 gas chromatograph (Santa Clara, CA) containing a Rtx-SSi1 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 Supporting Information (SI).

Liquid Chromatography-Time of Flight-Mass Spectrometry (LC-MS/MS) Analysis of Polyphenols in Cucumber Leaves. Identification and quantification of polyphenols in cucumber leaves were performed via LC-MS/MS. The detailed method was reported by Ma et al.²¹ Specifically, 10 mg of samples from the cucumber leaves were extracted with 1000 μL of cold 80:20 MeOH/H₂O. Samples were mixed for 10 s after adding the extraction solvent using a VWR Mini Vortexer (Radnor, PA, U.S.A.). Samples were then centrifuged for 5 min at 14 000 relative centrifugal force (RCF) using an Eppendorf Centrifuge 5415D (Hauppauge, NY, U.S.A.). After removing the supernatant, 2 aliquots of 450 μL each were dried down. Dried samples were resuspended in 100 μL of 10:90 ACN/H₂O with 1 μg/mL 12-(cyclohexylcarbamoylamino) dodecanoic acid (CUDA) for LC-MS/MS analysis.

LC-MS/MS data were acquired on a high resolution Thermo Q Exactive HF mass spectrometer using a Thermo Vanquish UHPLC+ in positive and negative electrospray ionization (ESI) modes. The LC method was modified from Ma et al.²¹ Analytes were separated using a Phenomenex Kinetex C18 column (100 × 2.1 mm², 1.7 μm) with a KrudKatcher Ultra HPLC in-line filter (0.5 μm Depth Filter × 0.004 in ID). The mobile phases were (A) H₂O with 0.1% acetic acid and (B) ACN with 0.1% acetic acid. Gradient elution was performed at a flow rate of 0.5 mL/min under the following program: from 0–10 min B changed linearly from 10%–90%, held at 90% B for 2.50 min, returned to 10% B over the next 2.50 min, and held at 10% B for equilibration over 5 min. The column temperature was kept at 45 °C. The operating parameters for the Q Exactive HF in positive ion mode were: sheath gas flow rate at 60, auxiliary gas flow rate at 25, sweep gas flow rate at 2, spray voltage at 3.60 kV, capillary temperature at 300 °C, S-lens RF level at 50, and auxiliary gas heater temperature at 370 °C. For negative ion

mode all parameters remained the same except the spray voltage was set to 3.00 kV. Mass spectral data was collected using full scan MS1 and data-dependent MS2. Full scan MS1 had the following parameter settings: scan range from a m/z 150–2000 with the resolution set to 120 000, AGC target set to 1×10^6 , and maximum ion injection set to 300 ms. Data-dependent MS2 had the following parameter settings: scan range from m/z 150–2000 with the resolution set to 15 000, AGC target set to 1×10^5 , maximum injection time set to 50 ms, loop count set to 3, and TopN set to trigger the top-3 most abundant ions, with an isolation window of 1.0 m/z , and higher-energy collisional dissociation (HCD) was conducted using three normalized collision energies: 35, 45, and 65. The injection volume for each sample was 5 μ L.

Multivariate and Univariate 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.²² PLS-DA was run on the GC-TOF-MS results using online resources (<http://www.metaboanalyst.ca/>).²³ 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.²² A metabolite with a VIP above 1 is regarded as significant.²⁴ In addition, univariate (one way ANOVA) analysis was performed on the LC-MS/MS results using online resources (<http://www.metaboanalyst.ca/>). Biological pathway analysis was performed based on all detected metabolites data using MetaboAnalyst 2.0.²⁵ The impact value threshold calculated for pathway identification was set at 0.1.²⁴

RESULTS AND DISCUSSION

The phenotypic analysis including biomass and chlorophyll content was reported in our previous study⁸ and is summarized in the Supporting Information (Figures S1–S3). Results showed that cucumber leaves exposed to the highest dose of copper (10 mg/plant) exhibit leaf chlorosis (Figure S1) and the content of chlorophyll a, b and carotenoids were significantly decreased (Figure S2).⁸ However, leaf biomass was unchanged at all copper doses (up to 10 mg) (Figure S3).⁸ In the following sections, the metabolic responses will be linked to the phenotype observations.

Impact of Copper ions on Primary Metabolites Profiles. The GC-QTOF-MS analysis identified and quantified 149 metabolites from the extracts of cucumber leaves exposed to different concentrations of Cu ions. The 149 identified compounds are mainly from the primary metabolism, including sugars, sugar alcohols, and sugar derivatives, amino acids, small organic acids, fatty acids, and nucleotides and their derivatives. Several secondary metabolites such as vitamins (tocopherol, ascorbic acid), polyphenols (3,4-dihydroxycinnamic acid), and phenolamides (putrescine) were also detected. PLS-DA multivariate analysis was conducted on the metabolomics data to determine whether there were significant differences in metabolic profiles across treatments and compared to the control. Figure 1 shows that the plants treated with copper ions at different doses are clearly separated from the control, generally in a dose-dependent manner. The metabolites which induced the separation between copper ion treatments and the control, named the discriminating compounds, were screened by VIP score (Figure S4). The compounds which led to the separation between control and copper-exposed groups are

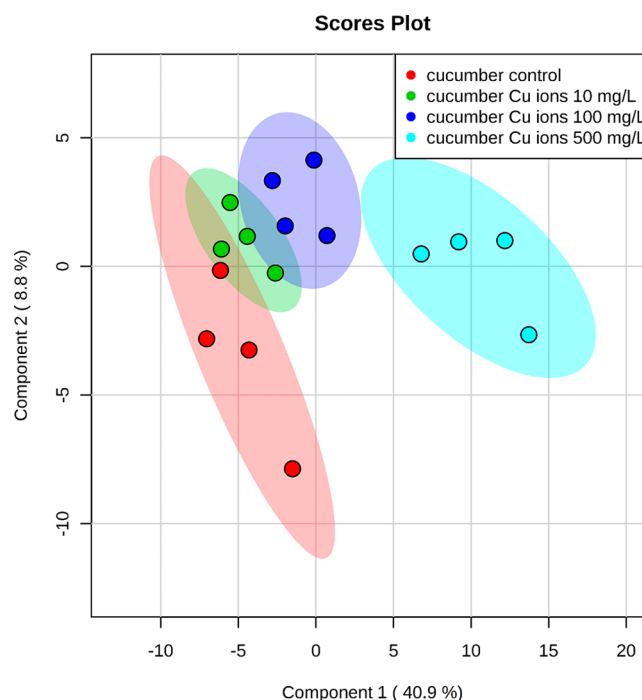


Figure 1. Partial least-squares discriminant analysis (PLS-DA) score plot of four treatments (control, 0.21, 2.1, and 10 mg Cu). Data are based on semiquantitative results of 149 metabolites from GC-TOF-MS. The quantitative variances of the relative abundances of 149 metabolites were clustered to reveal the difference and relative similarities of metabolite profiles of cucumber leaf treated with different concentrations of copper. 95% confidence regions are displayed for each treatment.

sulfuric acid, 2-ketoglucose, shikimic acid, trehalose, 3-dehydroshikimic acid, 4-hydroxycinnamic acid, 3-phosphoglycerate, gluconic acid, and ferulic acid. However, this multivariate analysis is based on the combined data from the three treatments, without considering the difference between doses. Thus, we separately conducted PLS-DA analyses for control vs low (0 and 0.21 mg Cu), control vs medium (0 and 2.1 mg Cu), and control vs high (0 and 10 mg Cu), to elucidate the metabolic response at different doses.

Metabolite Profile Changes from Low Copper Dosing. The PLS-DA score plot and VIP values for control vs low copper dose are shown in Figures S5 and S6. At the lowest dose the metabolite with the most significant change in concentration was glyceric acid, which increased 6-fold compared to the control. Glyceric acid is an intermediate of glycerolipid metabolism. The low copper dose also decreased the level of a number of organic acids such as citric acid (39.6%, $p < 0.05$), aconitic acid (32.1%, $p < 0.01$), malic acid (31.5%, $p = 0.01$), and isocitric acid (26.1%, $p = 0.12$) (Figure S6). These organic acids are key metabolic components in the tricarboxylic acid cycle (TCA cycle). Glycolysis and TCA cycle are major energy metabolism pathways, generating large amounts of energy compounds (ATP and NADH) and precursors for amino acid synthesis. The down-regulation of TCA cycle intermediates indicates that copper perturbs energy metabolism even at low doses, although visible toxicity was not observed at this dose (Figures S1–S3).⁸ Previous reports showed that visible symptoms, such as yellow dots and decreased chlorophyll content, occurred only at a high dose of copper (Figures S1 and S2).

Table 1. Perturbed Biological Pathways by Low Dose of Copper (0.21 mg Cu per Plant)

	perturbed pathway	<i>p</i>	impact	match status	involved metabolites
1	glyoxylate and dicarboxylate metabolism	7.56×10^{-3}	0.48	5/17	citric acid, malic acid, isocitric acid
2	citrate cycle (TCA cycle)	7.83×10^{-3}	0.26	6/20	citric acid, isocitric acid, malic acid, succinic acid
3	glycerolipid metabolism	2.28×10^{-2}	0.10	2/14	glyceric acid

Table 2. Perturbed Biological Pathways by Low Dose of Copper (2.1 mg Cu per Plant)

	perturbed pathway	<i>p</i>	impact	match status	involved metabolites
1	citrate cycle (TCA cycle)	8.01×10^{-4}	0.26	6/20	malic acid, alanine, pyruvic acid
2	glyoxylate and dicarboxylate metabolism	8.59×10^{-4}	0.48	5/17	citric acid, isocitric acid, malic acid
3	glycerolipid metabolism	1.54×10^{-2}	0.1	2/14	glyceric acid
4	starch and sucrose metabolism	1.75×10^{-2}	0.37	6/25	glucose, maltose, sucrose, fructose
5	carbon fixation in photosynthetic organisms	3.58×10^{-2}	0.04	5/21	malic acid, alanine

Other changes are the relative abundances of phenylalanine and tyrosine (Figure S6), which are elevated 15.6% and 38.3% compared to the control even by a low dose of copper ions. These aromatic amino acids are precursors of the shikimate-phenylpropanoid pathway, which generates numerous antioxidants (e.g., flavonoids, phenolics, and lignins) to scavenge ROS. It is known that copper is a redox reactive metal and can trigger the over generation of ROS in cells. The activation of this pathway is likely to quench the excess ROS. It is interesting to note that downstream shikimate-phenylpropanoid pathway compounds (e.g., 3,4-dihydroxycinnamic acid and ferulic acid), were significantly decreased approximately 2-fold compared to the control ($p < 0.05$). The downstream compounds of this pathway are apparently used to control ROS and their consumption activates the shikimate-phenylpropanoid pathway to compensate for the consumed antioxidants. While not evaluated here, it is likely that the regulatory gene AtMYB12 may be expressed to up-regulate the shikimate and phenylalanine biosynthetic pathways to supply more aromatic amino acids for secondary metabolism.²⁶

In addition, threonic acid, which is the intermediate of ascorbate and aldarate metabolism, increased 1-fold compared to the control ($p = 0.051$) (ANOVA) (Figure S6). Ascorbate and aldarate metabolism is also an antioxidant defense related pathway. Thus, a dose of just 0.21 mg Cu triggered the nonenzymatic antioxidant defense system via these various pathways. Our previous study had shown that a 0.21 mg Cu dose induced the up-regulation of a number of antioxidant enzymes including *POD*, *MDAR*, *GPX*, and *SOD*.⁸ This low dose activates both the enzymatic and nonenzyme antioxidant defense systems, though changes in phenotypic responses such as biomass and chlorophyll content were not observed at this dose.⁸ This confirms that metabolomics is a very sensitive tool that can be used for early detection of plant response to environmental stress.

This low Cu dose also increased levels of several fatty acids, such as arachidic acid (2-fold), linoleic acid (1.6-fold), stearic acid (1.7-fold), palmitic acid (1.5-fold), and 1-monopalmitin (1.6-fold) compared to the control (Figure S6). Fatty acids and sterols with phospholipids are primary components of the plasma membrane. The plant plasma membrane may be regarded as the first “living” structure that is the target of copper toxicity.²⁷ Abnormal function of the plasma membrane, including increased cell leakage, can be induced by excess metals, particularly copper.²⁷ Changes in the composition of the plasma membrane, such as relative levels of sterols and fatty acids, may contribute to changes in membrane fluidity and

permeability and can be used as a mechanism of stress adaptation. The cell membrane may play an important role in metal homeostasis.²⁷ The up-regulation of fatty acids may change the plasma membrane composition and therefore change metal homeostasis. Jung et al.¹⁸ also observed the increased levels of unsaturated fatty acids such as linoleic acid, linolenic acid, and palmitic acid when burdock roots were exposed to copper. They postulated that the high level of membrane lipid unsaturation helps to maintain the fluidity necessary for proper membrane function. The fluidity of biological membranes is largely influenced by the type of fatty acids, the amount of sterols and the nature of the phospholipids.²⁸

The biological pathways most significantly impacted by this low copper dose, as calculated by MetaboAnalyst 3.0, are shown in Table 1. The impact pathway value was calculated as the sum of the matched important metabolites from all the metabolites in each pathway.²⁹ Impact values greater than 0.1 and *P* values less than 0.05 were considered to determine significantly impacted pathways. As shown in Table 1, the lowest Cu dose perturbed three metabolic pathways: glyoxylate and dicarboxylate metabolism, TCA cycle, and glycerolipid metabolism, all of which indicates that carbon metabolism was negatively affected.

Metabolite Profile Changes from Medium Copper Dosing. The changes in metabolite levels between the control and the medium dose (2.1 mg Cu) are shown in Figure S8. In addition to the changes observed at the low dose (e.g., decrease in key components of the TCA cycle such as malic acid (43.5%), citric acid (58.7%), aconitic acid (48.9%), isocitric acid (43%), and fumaric acid (34.0%); significant increases in phenylalanine (37.3%), tyrosine (32.1%), and dehydroascorbic acid (47%)), the medium Cu dose altered additional metabolite levels. For example, the concentrations of a number of sugars, including maltose, lactose, fructose, trehalose, glucose and sucrose, decreased 23–66%, indicating that 2.1 mg Cu affects carbohydrate metabolism. However, changes in fatty acid composition were only observed at the low dose (0.21 mg Cu) (Figure S6) and not at the medium dose; the reason is unknown. Cu dosing at 2.1 mg perturbed two more biological pathway, namely starch and sucrose metabolism and carbon fixation in photosynthetic organisms (Table 2). These changes indicate that copper at low and medium dose also perturbed carbon metabolism.

Metabolite Profile Changes from High Copper Dosing. The highest dose (10 mg Cu) induced the most significant alteration of metabolite levels in cucumber leaves. As expected,

Table 3. Perturbed Biological Pathways by Low Dose of Copper (10 mg Cu per Plant)

	perturbed pathway	<i>p</i>	impact	match status	involved metabolites
1	glyoxylate and dicarboxylate metabolism	1.80×10^{-6}	0.48	5/17	glycolic acid, citric acid, succinic acid
2	phenylalanine, tyrosine and tryptophan biosynthesis	2.45×10^{-4}	0.11	4/22	shikimic acid, tryptophan
3	citrate cycle (TCA cycle)	4.84×10^{-4}	0.26	6/20	succinic acid, malic acid, citric acid, isocitric acid
4	tryptophan metabolism	8.60×10^{-4}	0.31	1/25	tryptophan
5	tyrosine metabolism	2.38×10^{-3}	0.27	3/18	tyrosine, succinic acid
6	isoquinoline alkaloid biosynthesis	9.85×10^{-3}	0.5	1/6	tyrosine
7	phenylalanine metabolism	1.54×10^{-2}	0.46	2/11	phenylalanine
8	ascorbate and aldarate metabolism	1.78×10^{-2}	1.0	2/14	uridine diphosphate glucuronic acid
9	linoleic acid metabolism	2.64×10^{-2}	1.0	1/5	linoleic acid
10	galactose metabolism	2.44×10^{-2}	0.22	8/26	raffinose, lactose, sucrose, glycerol, glucose
11	starch and sucrose metabolism	3.26×10^{-2}	0.37	6/25	uridine diphosphate glucuronic acid, glucose sucrose, maltose
12	phenylpropanoid biosynthesis	4.23×10^{-3}	0.08	4/31	phenylalanine tyrosine

intermediate metabolites of the TCA cycle (e.g., citric acid, isocitric acid, malic acid and aconitic acid) decreased in a dose-dependent fashion (Figure S10). In addition, a number of intermediates of the shikimate-phenylpropanoid biosynthesis pathway decreased, including shikimate, tyrosine, 4-hydroxycinnamic acid, and ferulic acid. Two aromatic amino acids, phenylalanine and tryptophan, increased 22.6% ($p < 0.05$) and 117% ($p < 0.01$), respectively, compared to the control. Two byproducts of the ascorbate and aldarate metabolic pathway, dehydroascorbic acid, and threonic acid, decreased 41% ($p < 0.05$) and 54% ($p = 0.10$), respectively, at the highest dose. These changes indicate that the high Cu dose likely generated high amounts of ROS and started to inhibit this antioxidant related pathway.

In addition, at the higher dose the levels of several additional metabolites were either significantly increased (gluconic acid, ribose, methionine, adenine, 1,2-cyclohexanedione, erythritol) or decreased (3-phosphoglycerate, 2-ketoglucose dimethylacetal NIST, glycolic acid, ribitol, phytol, β -mannosylglycerate, β -sitosterol). These changes were not observed at the low and medium doses. The decrease in phytol, a side chain of chlorophylls, may indicate that chlorophyll degradation is occurring. Luna et al.,³⁰ reported that high concentrations of Cu^{2+} caused the breakdown of chlorophyll, and caused rapid senescence of plant leaves via oxidative reactions. In this study, detection of the down-regulation of phytol explains the underlying mechanism of leaf chlorosis. Furthermore, levels of gluconic acid in cucumber leaves exposed to 10 mg copper increased 9-fold compared to the control ($p < 0.01$). Since gluconic acid is a product of glucose oxidation, a significant increase in gluconic acid levels may be an indicator of glucose oxidation.

Levels of β -mannosylglycerate significantly decreased 33.2% ($p < 0.05$) at the high Cu dose compared to control, while no change was observed at low and medium doses. The outstanding ability of β -mannosylglycerate to stabilize protein structure has been demonstrated. Mannosylglycerate (MG) is an intracellular organic solute. MG also plays an important role in osmoadaptation. The reason for its decrease is still unknown, but may affect protein structure. Similarly, the level of β -sitosterol decreased 47.5% compared to the control. Jung et al.¹⁸ also found decreased sitosterol levels following the exposure of burdock roots to copper ion. Sitosterol has been found to regulate membrane fluidity and water permeability.³¹ A decrease in β -sitosterol levels may indicate membrane damage.

In general, trehalose decreased in a dose-dependent manner, and at the highest Cu dose trehalose levels decreased 3-fold compared to the control. The disaccharide trehalose is involved in stress response in many organisms.³² However, in plants, its precise role remains unclear, although some data indicate that trehalose plays a protective role during abiotic stresses. In contrast, some trehalose metabolism mutants exhibit growth aberrations, revealing potential negative effects on plant physiology. Contradictory effects also appear under biotic stress conditions.³²

An increase in glycine at the higher Cu dose may reflect activation of the photorespiratory pathway. Methionine, which also did not increase at the lower doses, was significantly ($p < 0.01$) increased by 10 mg Cu. Methionine may act as a catalytic antioxidant, protecting proteins from ROS.³³

Twelve biological pathways were perturbed when the dose increased to 10 mg Cu, (Table 3). This higher dose of copper resulted in perturbation of defense related metabolic pathways such as ascorbate and aldarate, and phenylpropanoid. Moreover, some amino acid related pathways were also perturbed by the high dose of copper, such as phenylalanine, tyrosine, and tryptophan biosynthesis; tryptophan metabolism; and tyrosine metabolism (Table 3), which were not observed at the low and medium copper treatments (Tables 1 and 2). This indicates that a higher dose of copper can affect nitrogen metabolism in cucumber leaves. The isoquinoline alkaloid, linoleic acid, and galactose metabolic pathways were also altered ($p < 0.05$) (Table 3).

Accumulation of Secondary Metabolites (Polyphenols). The chemical interaction between plants and their environment is mediated mainly by the biosynthesis of secondary metabolites.³⁴ Polyphenols are ubiquitous secondary metabolites that are generally involved in signaling and antioxidant defense against biotic and abiotic stress. Flavonoids can undergo single electron oxidation and, thus, are capable of reducing free radicals. They act as antioxidants by direct quenching free radicals through transferring a proton from the A and/or B rings and generating less active flavonoid radicals.

By using LC-MS/MS, 79 polyphenols were identified and quantified. One way ANOVA analysis revealed that 26 compounds were significantly changed in cucumber leaves exposed to copper ions ($p < 0.05$) (Figure S11 for up-regulated and S12 for down-regulated polyphenols, respectively). As shown in Figure S11, some polyphenols showed a significant increase ($p < 0.05$) in accumulation in plants exposed to the higher dose of copper, including cyanidin-3-glucoside (8.8-fold), acetylucine (9.3-fold), *N*-acetyl-L-phenylalanine (10-

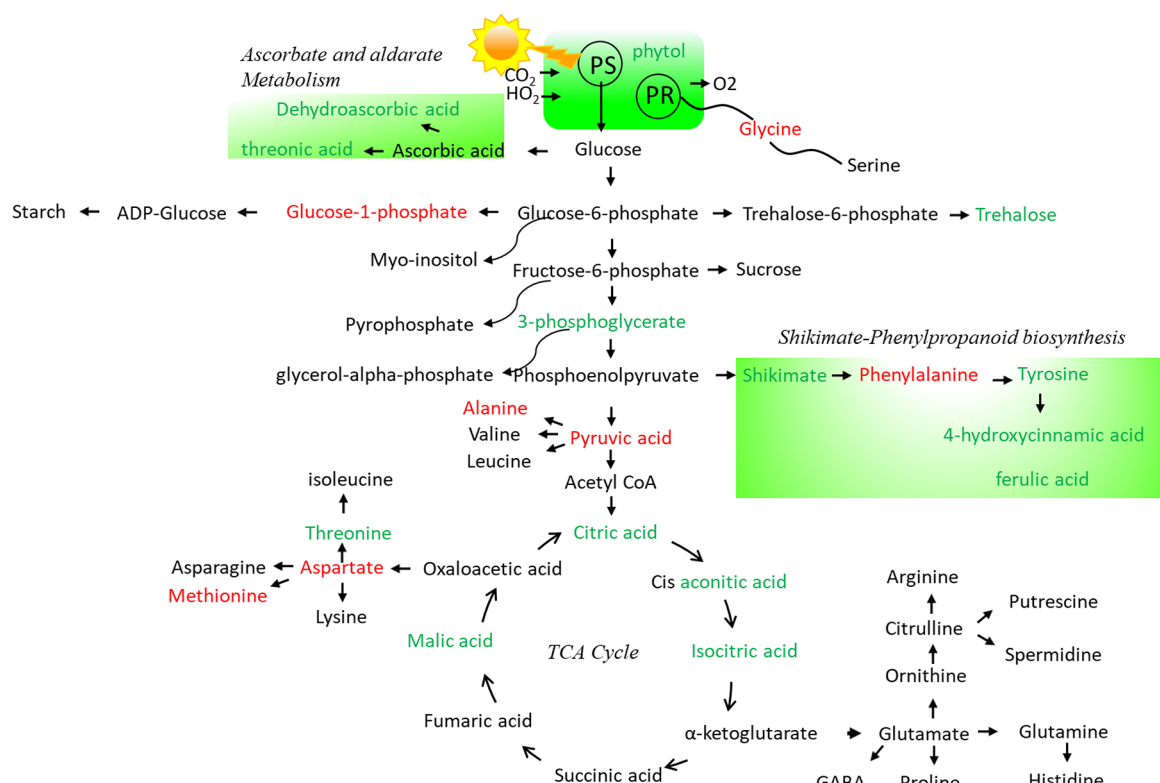


Figure 2. Schematic diagram of proposed metabolic pathways of cucumber leaf exposed to 10 mg copper/plant. Central metabolic pathways (TCA cycle, shikimate-phenylpropanoid biosynthesis, ascorbate, and aldarate metabolism) are shown. Red and green circles indicate that the metabolites increased or decreased in response to the Cu ions.

fold), *N*-acetyltryptophan (11-fold), pyroglu-pro (2.5-fold), 4-coumaric acid (3.8-fold), tryptophan (2.2-fold), *L*-methionine, *N*-acetyl- (60-fold), sinapic acid (5-fold), *cis*-ferulic acid (2.7-fold), riboflavin (9-fold), 3-dehydroshikimic acid (2.5 fold), 17-trans-prostaglandin E3 (3.1-fold), malvidin-3-*O*- β -D-galactoside (6.6-fold). In contrast, a number of secondary metabolites decreased significantly when exposure to high dose of copper, including xanthurenic acid (2.4-fold), prunin (19-fold), naringenin-7-*O*-glucoside (14-fold), naringenin (11-fold), cosmosiin (4-fold), isovitexin (19-fold), *trans*-2-hydroxycinnamic acid (27-fold), 5'-methylthiadenosine (1.8-fold), *trans*-ferulic acid (1.9-fold), isoorientin (72-fold), *trans*-EKODE-(E)-Ib (13-fold), and homoeriodictyol (18-fold).

As mentioned before, 10 mg copper induced leaf senescence (Figure S1). Previous studies demonstrated that some secondary compounds that accumulate in senescing and ripening tissues are products of phenylpropanoid metabolism. Anthocyanins are of adaptive value to plants in stress responses. The anthocyanin responsible for the red color of autumn leaves is commonly cyaniding-3-glucoside.³⁵ Taken together, the up-regulated phytol and anthocyanin are indicators that higher doses of Cu accelerate cucumber leaf senescence.

Phenylpropanoids are a large group of polyphenolic compounds synthesized in plants via the phenylpropanoid pathway, the best understood plant secondary metabolism pathway (Figure 2).³⁶ Upstream metabolites of the phenylpropanoid pathway such as 3-dehydroshikimic acid, phenylalanine, cinnamic acid (sinapic acid), and 4-coumaric acid were significantly increased by 10 mg copper, while the downstream metabolites (naringenin, prunin, cosmosiin, isovitexin, isoorientin, and homoeriodictyol) of the phenylpropanoid pathway were significantly decreased by 10 mg copper (Figure 3).

Naringenin defines a key branch point for the synthesis of several major classes of flavonoids, including flavanones, flavonols, and anthocyanins.³⁷ Thus, a decrease in naringenin may affect all of these downstream metabolites.

Hydroxycinnamic acids are phenolic compounds derived from the phenylalanine and tyrosine pathway³⁸ and belong to the nonflavonoid polyphenols (Figure 3). A very interesting finding is that several secondary metabolites which were up-regulated in response to copper ions are hydroxycinnamic acid derivatives (e.g., 4-coumaric acid, sinapic acid, *cis*-ferulic acid) (Figure 3), while metabolites which were down-regulated by copper are flavonoid derivatives (e.g., prunin, naringenin-7-*O*-glucoside, naringenin, apigenin-7-*O*-glucoside, isovitexin, isoorientin, homoeriodictyol) (Figure 3). 4-Coumaric acid is a key phenylpropanoid intermediate which is further modified to generate different phenolic acids.

Cyanidin-3-*O*- β -glucoside (C3G) was also upregulated (Figure S11). C3G belongs to a class of dietary polyphenols known as anthocyanins, which are water-soluble pigments widely distributed among fruits and vegetables, as well as present in red wine. Jung et al. evaluated the antioxidant and anti-inflammatory activities of C3G.³⁹

Isoorientin and naringenin-7-*O*-glucoside were significantly decreased. Isoorientin (ISO) is an important flavonoid-like compound, responsible for antioxidant, anti-inflammatory, and antinociceptive activities.⁴⁰ Evidence showed that naringenin-7-*O*-glucoside (NARG) could prevent cardiomyocytes from DOX-induced toxicity by stabilizing the cell membrane and reducing ROS generation.⁴¹

Our previous study found the 10 mg copper triggered the up-regulation of gene expression of antioxidant enzymes.⁸ Antioxidant enzymes have long been proposed as representing

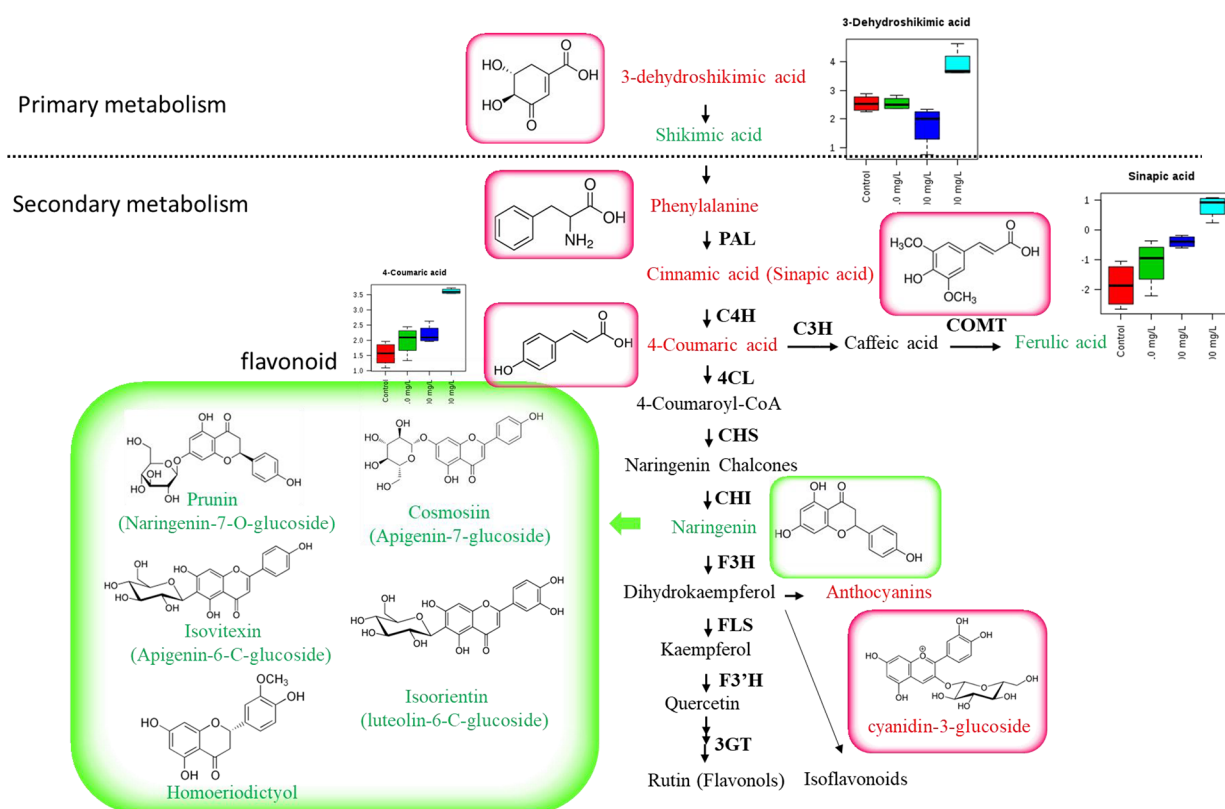


Figure 3. Schematic representation of phenylpropanoid/flavonoid pathway as affected by copper ions. PAL, phenylalanine ammonium lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; F3'H, flavonoid-3'-hydroxylase; 3GT, flavonoid 3-O-glucosyltransferase; C3H, *p*-coumarate-3-hydroxylase; and COMT, caffeate O-methyltransferase.

the first line of defense against ROS generation, but their action needs to be complemented by other ROS scavenging systems during severe stress conditions.⁴² Fini⁴² proposed that flavonoids constitute a secondary ROS-scavenging system in plants exposed to stress conditions. The polyphenol metabolomics results combined with gene expression data, provide a holistic view of the antioxidant defense system of cucumber plant under copper stress.

Additional polyphenols (e.g., *N*-acetyltryptophan, *N*-acetyl-L-methionine, and 5-methylthioadenosine) that are not intermediates of the phenylpropanoid/flavonoid pathway also play important protective functions and their levels were also significantly changed. *N*-Acetyltryptophan has been used as a protein stabilizer.⁴³ It prevents protein molecules from oxidative degradation by scavenging oxygen dissolved in protein solutions. *N*-Acetyl-L-methionine (*N*-AcMet) was found to be a superior ROS scavenger and an efficient stabilizer of albumin structure exposed to ROS.⁴⁴ 5-Methylthioadenosine (MTA) is a critical metabolic byproduct resulting from multiple essential cellular processes, such as polyamine synthesis for cell proliferation and homoserine lactone production in quorum sensing.

ENVIRONMENTAL IMPLICATIONS

In this research, we investigated the metabolic response of cucumber plants exposed to copper via foliar application. In particular, secondary metabolites that play an important role in resistance were determined. Results showed that copper altered the metabolite profile of cucumber leaves, but that the magnitude of alteration was strongly dose-dependent. Levels

of a number of intermediates of the TCA cycle were decreased even at a low copper dose, indicating that copper disturbs energy metabolism. In addition, even a low dose of copper resulted in a significant increase in the accumulation of threonine, indicating antioxidant defense related pathways were activated. The higher dose of copper also altered polyphenol levels in cucumber leaves. The present study elucidated the underlying molecular mechanism of copper induced cucumber leaf senescence. It has to be noted that some of the observed effects may be due to sulfate, but this was not studied. Overall, the present study provides a global vision of the metabolic response of plants to copper. The methodology can be applied to other species and metals or nanoparticles to study the interaction between stressor and plants. More importantly, copper is widely used in agriculture as an active ingredient in many fungicides. The low and medium doses applied in this study are comparable with realistic application doses. The metabolic changes observed in this study serve to better understand the potential toxicity at higher doses, which can serve to develop guidance for the safe use of copper containing fungicides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b00742.

Image of 4-week-old cucumber leaves treated with water and 10 mg Cu (Figure S1); effects of copper on photosynthetic pigment content (Figure S2); effects of copper on cucumber root length and biomass (Figure

S3); VIP scores from PLS-DA analysis of cucumber leaves treated with different doses of copper (Figure S4); score plot and VIP scores from PLS-DA model for low, medium, and high dose of copper (Figures S5–S10); up-regulated and down-regulated polyphenols in cucumber leaves (Figures S11 and 12); and details regarding GC-TOF-MS data acquisition, data processing, and data reporting (PDF)

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Notes

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