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Research paper

Application of metabolomics to assess the impact of $Cu(OH)_2$ nanopesticide on the nutritional value of lettuce (*Lactuca sativa*): Enhanced Cu intake and reduced antioxidants

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ABSTRACT

Copper-based nanopesticides are increasingly being used in the agriculture, especially in organic farming. This has triggered some concerns about their risk to environmental and human health. In this study, 24-day-old lettuce plants grown in soil were exposed via the leaves to different concentrations (0, 1050 and 1555 mg/L) of $Cu(OH)_2$ nanopesticides for one month. Results showed Cu was mainly localized in lettuce leaves (823–1111 and 1353–2008 mg/kg in vascular and photosynthetic tissues), which may potentially increase Cu intake and impact human health. In addition, foliar application of Cu(OH)2 nanopesticide significantly increased potassium concentration in lettuce leaves by 6–7% and 21–28%, in vascular and photosynthetic tissues. A Gas Chromatography-Time-of-Flight Mass Spectrometry (GC–TOF-MS) based metabolomics approach was applied to determine hundreds of organic compounds simultaneously. Using relative quantitation and Partial Least Squares-Discriminant Analysis (PLS-DA) clustering of all compounds, clear differences were observed in the metabolite profiles of control and $Cu(OH)_2$ nanopesticides treated leaves. Discriminating compounds include amino acids, organic acids, polyamines, vitamin C and polyphenols. Dehydroascorbic acid and cis-caffeic acid, which are important antioxidants, were significantly decreased (19–33% and 5–8%) due to foliar exposure to the nanopesticide. Total antioxidant capacity was significantly decreased 20–23% after exposure to $Cu(OH)_2$ nanopesticides. There was also up- and down-regulation of a number of amino acids, particularly 4-hydroxybutytic acid (GABA) which decreased 50% compared to the control, potentially affecting the overall nutritional value of lettuce leaves exposed to the $Cu(OH)_2$ nanopesticides. In future work, determining an appropriate level of nanopesticide will be important to obtain the antifungal benefits without resulting in a significant decrease in nutritional value.

1. Introduction

The field of nanotechnology has expanded rapidly in almost all industries and touched almost all aspects of human lives (White and Xing, 2016). Nanopesticide and nanofertilizers have become increasingly popular in modern agriculture for plant protection and nutritional supply (Kah, 2015). Understanding the possible effects of these nanopesticides and nanofertilizers is necessary to compare the benefits of nanotechnology to the potential risks to environmental and human health. Copper (Cu) containing pesticides have been used in agriculture for hundreds of years (Servin and White, 2016; Ma et al., 2016). In the UK the Soil Association permits the use of copper fungicides on organic land used for the production of certified organic crops (2010). According to the US Department of Agriculture, Cu-based pesticides can also be used for organic production (Zhao et al., 2016a). There are currently 209 Cu-based pesticide products registered in California (Regulation, 2009). Therefore, studying the implications of Cu-based nanopesticides which are increasingly popular for plant protection in agriculture, is of great importance.

In recent years, the fate, transport and phytotoxicity of Cu-based nanopesticides on crop plant have recently been investigated extensively (Lee et al., 2008; Stampoulis et al., 2009; Shi et al., 2011; Atha et al., 2012; Trujillo-Reyes et al., 2014; Hong et al., 2015; Zuverza-Mena et al., 2015; Wang et al., 2016). The bioaccumulation of nanopesticides in edible tissues and their impact on the nutritional supply is of great importance because this intimately relates to human health. Lettuce is an important crop widely consumed throughout the world. According to the United Nations Food and Agriculture Association, worldwide production of lettuce was over 24 million tons with a gross production

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value of over $14 billion in 2012. Thus, small nutritional alterations in lettuce could have a significant health benefits or impacts. Lettuce is rich in phytonutrients that have been acknowledged to afford protection against a range of human diseases, e.g., prevent or reduce cardiovascular diseases in rats and humans by improving cholesterol metabolism (Serafini et al., 2002; Nicolle et al., 2004b; Damerum et al., 2015). The healthy properties of lettuce are attributed to a large supply of antioxidant compounds, mainly vitamin C and polyphenols, as well as the fiber content (Serafini et al., 2002; Nicolle et al., 2004b).

Many metabolites influence taste, flavor and nutritional supply. Free amino acids and fatty acids, which are nutritional components of many fruits and vegetables, also play important roles in benefiting human health and maintaining fruit quality (Schieber et al., 2001). Sweetness depends primarily on the amounts and composition of various sugars and sugar alcohols; acidity is determined mostly by the amounts and composition of organic acids (Cuthbertson et al., 2012). Other important phytonutrients, such as carotenoids, chlorophyll, anthocyanin, also play important antioxidant roles and benefit human health (Damerum et al., 2015).

GC–TOF-MS based metabolomics enables the detection of hundreds of metabolites simultaneously. In this study, foliar application of a commercial Cu-based nanopesticide was conducted at two levels (1050 and 1555 mg/L), to 24-day-old lettuce for four weeks. At harvest, Cu and other ions on lettuce leaves were analyzed via ICP-MS and metabolites were determined via GC–TOF-MS. Multivariate analysis including principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were employed to group the treatments. In a previous study we evaluated the defense response and tolerance of lettuce plants to foliar exposure to this nanopesticide (Zhao et al., 2016b). Here we considered the impact on nutritional supply. Results revealed that the nutritional supply was altered, especially for a number of metabolites which play an important role as antioxidants.

2. Materials and methods

2.1. Copper hydroxide nanopesticides

$Cu(OH)_2$ nanoparticles used in this study were in the form of a commercial biocide, Kocide 3000 (Dupont). Detailed physicochemical properties of Kocide 3000 have been reported before (Adeleye et al., 2014; Conway et al., 2015). Specifically, the primary particle size is ~50 to 1000 nm. The hydrodynamic diameter is 1532 ± 580 nm and the zeta potential is -47.6 ± 43 mV (measured via DLS in Nanopure water at pH 7). Although Kocide 3000 particles are mainly micron-sized, these micronized particles are made up of nanosheets of $Cu(OH)_2$ that are bound together and can potentially re-dissociate in water. The nanosheets are held together by a rapidly dissolving organic composite. For this reason the pesticide is considered “nano” (Adeleye et al., 2014), although it contains a mixture of nanoparticles and micro-sized particles. Cu content in Kocide 3000 is $26.5 \pm 0.9\%$, while other elements (C, O, Na, Al, Si, S, and Zn) accounted for 73.5% of the contents.

2.2. Plant growth and exposure to $Cu(OH)_2$ nanopesticides

Lettuce (*Lactuca sativa*) seeds were purchased from Seed Savers Exchange (Iowa). Soil was collected from the Natural Reserve System of UC Santa Barbara (Sedgwick) on November 2, 2015. The soil is sandy loamy soil with pH of 5.90 ± 0.04. Cation exchange capacity is 25.8 ± 0.1 meq 100 g^{-1}. Other soil composition information was described before (Conway et al., 2015). Each lettuce seeds was planted in a Magenta box containing 250 g of soil. Each Magenta box was wrapped with aluminum foil to avoid algal growth. Three treatments (Control, 1050 and 1555 mg/L of Kocide 3000) with 5 replicates was designed. A total of 15 pots of lettuce plants were grown in a greenhouse. The temperature in the greenhouse was maintained at 28 °C at daytime and 20 °C at night. At day 24, exposure of lettuce to different concentrations of $Cu(OH)_2$ nanopesticides began: 0 (Control), 1050 mg/L (Low concentration), 1555 mg/L (High concentration). Those doses were selected generally followed the manufacturer's recommendation (0.8–1.6 kg/ha). Before spraying, the suspension was sonicated for 30 min in cooled water. A hand-held spray bottle was used for spraying. The approximate spray amount for each pot was 8.3 mL per event. The spray frequency was two times per week. A total of eight sprays were done by harvest time. At day 54, the plants were harvested.

2.3. Mineral determination

At harvest, the lettuce plants were gently removed from the soil, thoroughly rinsed with tap water for five minutes and rinsed with Nanopure water three times. We then carefully separated leaf vascular and mesophyll tissues using clean scissors. Both tissues were oven dried at 70 °C for ICP-MS analysis.

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

2.4. Gas Chromatography–Time of Flight-Mass Spectrometry (GC–TOF-MS) analysis of metabolites in leaves

At harvest, thoroughly washed lettuce leaves were dried, frozen in liquid nitrogen, and ground to powder. The frozen tissues were sent to the University of California Davis for metabolite analysis. A description of sample pretreatment, has been reported before (Badri et al., 2013; Fiehn et al., 2008). An Agilent 6890 Gas Chromatograph (Santa Clara, CA) with an additional 10 m 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. PCA and PLS-DA multivariate analysis were performed using online resources (Xia et al., 2015; Zhao et al., 2016a). The parameter of 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 (Xia et al., 2015). A variable with a VIP above 1 is regarded as important.

2.5. Determination of total antioxidant capacity

Total antioxidant capacity was determined according to (Singleton and Rossi, 1965). Ground lettuce leaves samples (0.01 g) were mixed with 5 mL of 80% methanol, and the mixture was placed in an end-over-end shaker on a Dayton-6Z412A Parallel Shaft (USA) roller mixer with a speed of 70 rpm at room temperature for 12 h to ensure full extraction. After centrifugation at 2000g for 10 min, the supernatant was used to determine the total content of phenolic compounds (Singleton and Rossi, 1965). Specifically, 50 μL of the methanolic extract was mixed with 450 μL of DI water and 250 μL of 2 M Folin − Ciocalteu reagent. The mixture was added to 1.25 mL of 20 g/L Na_2CO_3, incubated at 25 °C for 20 min and then centrifuged at 2000g for 10 min. Supernatant absorbance was measured at 735 nm using a UV–vis spectrometer (Shimadzu UV-1800, Japan). The standard curve was prepared using gallic acid (GA) with a regression $R^2 = 0.998$. The absorbance was converted to phenolic content in terms of a milligram of GA Equivalent (GAE) per gram of dried weight (DW).

2.6. Chlorophyll and carotenoids analysis

Content of chlorophyll *a* and b and total carotenoids was determined according to (SESTÁK et al., 1971) with some modification. Specifically, 0.01 g of lettuce 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 chlorophylls a and b, and at 470 nm for carotenoids. Results were expressed as milligrams of total chlorophylls (a + b) or carotenoids per gram of fresh weight or per plant.

2.7. Statistical analysis

The concentration of Cu, minerals, chlorophyll, carotenoids and total antioxidant capacity were statistically analyzed using an independent two sample *t*-test to determine whether concentration levels were significantly different between control and nanopesticide treatments. *p*-Values were calculated with a two-tailed distribution.

3. Results and discussion

3.1. Bioaccumulation of Cu in lettuce

As shown in Table 1, Cu content in control, low and high concentration treatments is 9.9, 823 and 1111 mg/kg dry weight in vascular tissues, and 13.0, 1353 and 2008 mg/kg dry weight in photosynthetic tissues of lettuce plants, respectively. This indicate lettuce leaves exposed to $Cu(OH)_2$ nanopesticides have significantly higher amount of Cu compared to the control. It is also noted that leaves have much higher Cu content in photosynthetic than in vascular tissues. Since photosynthetic tissues are the predominant biomass of lettuce leaves, the calculations below will be based on photosynthetic tissue data. The average American consumed 15 kg of lettuce in 2000 (Xia et al., 2015). That means US daily lettuce consumption was ~ 41 g/person-day (fresh weight). The average water content in lettuce leaves is 96%, so the daily consumption was 1.6 g dry weight / person-day. Thus, daily personal Cu intake from lettuce used in this study would be 21 μg for control, 2200 μg for low and 3300 μg for high levels.

According to the Food and Nutrition Board at the U.S. Institute of Medicine of the National Academies, the recommended average requirement for Cu is 700 μg/person-day, with a tolerable upper intake level of 10 mg/person-day (Trumbo et al., 2001). The third National Health and Nutrition Examination Survey (NHANES III) investigation reports showed that the average Cu intake from food alone for men and women aged 19–70 years ranged from 1540 to 1700 mg/day for men and from 1130 to 1180 μg/day for women, respectively (Trumbo et al., 2001). Therefore, Cu intake from consumption of lettuce exposed to $Cu(OH)_2$ via foliar application would be within the recommended Cu levels, even at the higher application level. However, additional sources of Cu may include other foods, drinking water, and general nutritional supplements. Therefore, total Cu intake may exceed the recommended level unless the consumer is aware of all Cu in her/his diet, and exposure via $Cu(OH)_2$ exposed lettuce could be a significant increase in daily dose. Cu, a redox active metal, is an essential element and an integral part of many important enzymes; it participates in several vital biological processes (Gaetke and Chow, 2003). However, excess Cu in the body also poses a risk to human health (Uriu-Adams and Keen, 2005). Chronic Cu toxicity can result in liver disease and severe neurological defects, as liver is the first deposition site after Cu enters the blood (Gaetke and Chow, 2003). Therefore, a higher Cu intake through eating exposed lettuce may pose a potential human health concern.

3.2. Effect of $Cu(OH)_2$ NPs on mineral content

The most abundant macro-minerals in lettuce leaves are K (50,200–64,300 mg/kg), Ca (7050–7790 mg/kg), Mg (5680–6050 mg/kg) and P (6120–6330 mg/kg) (Table 1). Generally the photosynthetic tissues contain more nutrient elements than vascular tissues. This is not surprising because vascular tissues serve as nutrient transporters. However, K is different since it accumulates significantly in vascular tissues. K content in photosynthetic tissues was significantly increased ($p < 0.05$) due to exposure to $Cu(OH)_2$, while Ca content decreased in a dose dependent way, but was not statistically significant. A similar tendency was observed in vascular tissue: K content increased after exposure to $Cu\ (OH)_2$, and Ca content was significantly decreased ($p < 0.05$). The reason for increased K after foliar exposure to $Cu(OH)_2$ nanopesticide is still unknown. Previously we hypothesized that increased K improves the tolerance of lettuce plants to Cu induced stress (Zhao et al., 2016a). Independent of the reason for the increase in K, additional K increases lettuce nutritional quality and may have beneficial effects on human health. Studies showed that increasing K intake reduces cardiovascular disease mortality (He and MacGregor, 2008). Dyer et al. evidenced that a 30- to 45-mmol increase in K intake was associated with an average reduction in population systolic blood pressure of 2–3 mm Hg (Dyer et al., 1994). In addition, reports also showed that a high-K diet may also prevent or at least slow the progression of renal disease (He and MacGregor, 2008). We also observed that exposure to $Cu(OH)_2$ nanopesticides led to significant decrease in Mg and increase in Zn, in vascular tissues. But since vascular tissues only represent a small contribution of total leaf mass, these changes may not be very important.

3.3. Univariate (t-test) and multivariate (PCA and PLS-DA) analysis and of GC-TOF-MS data

A total of 159 organic compounds were identified using GC-TOF-MS. A t-test was performed to identify metabolite concentrations that were significantly different between control and treatments. As shown in Table S1, 39 compounds were significantly different compared to the

Table 1
Effect of $Cu(OH)_2$ nanopesticide on ionic homeostasis in lettuce leaves (mg/kg dry weight).

	K	SD	Ca*	SD	Mg*	SD	P	SD	Cu*	SD	Fe	SD	Zn*	SD
Vascular tissue														
Control	88,766	8923	5651	280	3812	194	3657	265	9.9	5.0	28.3	5.2	7.3	0.4
210 mg/L	94,169	5760	5127	263	3165	303	3403	287	823	110	30.3	9.3	8.9	1.2
310 mg/L	95,837	4264	4975	201	3024	306	3276	325	1111	80	30.7	1.6	8.6	0.9
Photosynthesis tissue														
Control	50,208	1666	7787	595	5685	198	6116	402	13.0	5.9	88.5	64.6	21.8	0.2
210 mg/L	64,344	4907	7583	368	6051	348	6759	639	1353.1	324	75.7	11.8	27.9	3.1
310 mg/L	60,868	3438	7046	458	5676	329	6328	383	2007.9	438	70.7	10.2	25.5	2.4

The data are the means of five replications with Standard Deviation (SD). * means significant compared to control ($p < 0.05$).

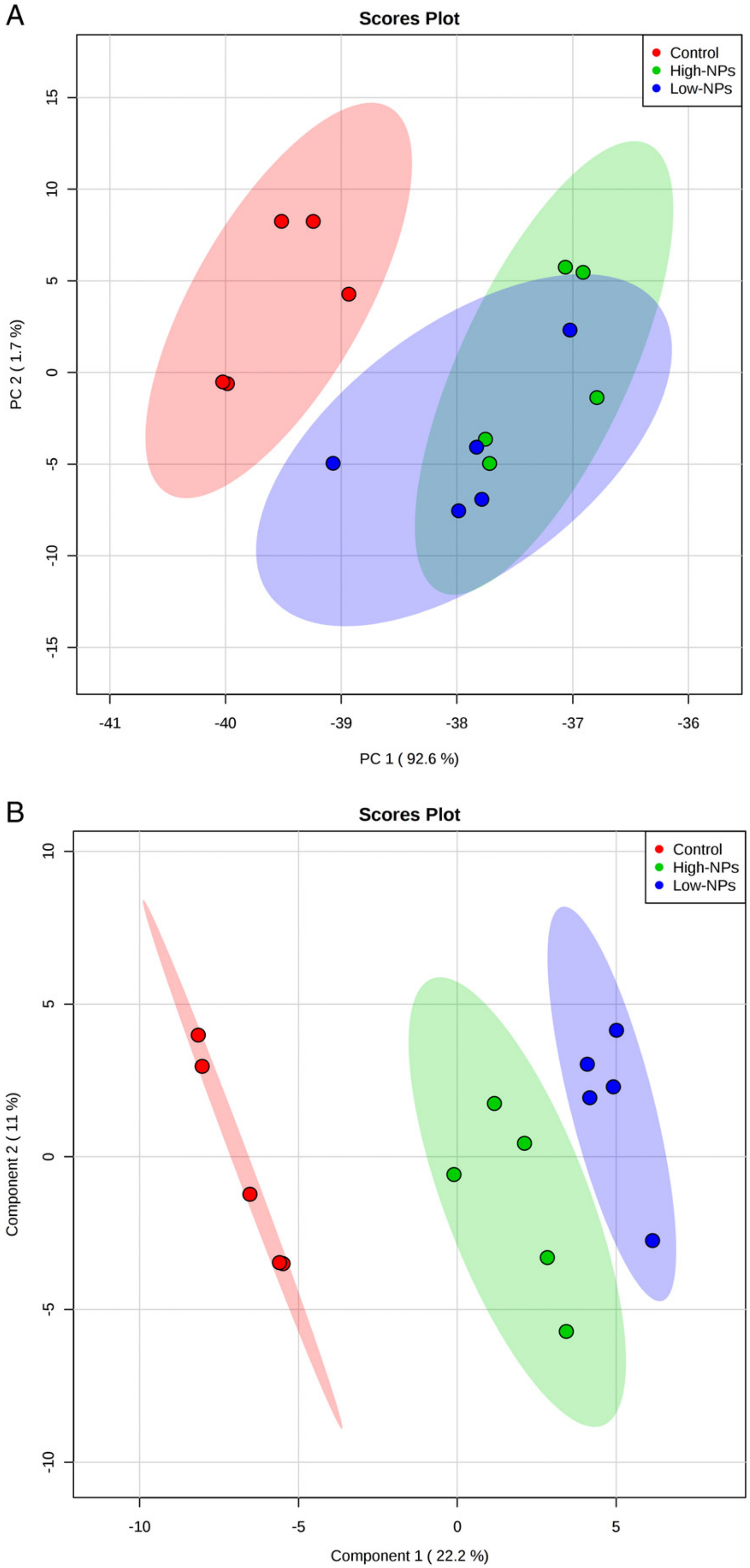

Fig. 1. Multivariate analysis of GC-TOF-MS metabolites data of lettuce phtosynthesis tissues, A: Score plot of Principle Component Analysis (PCA); B: Score plot of Partial Least Square Discriminant Analysis (PLS-DA). After 24 days lettuce plants were exposed to different concentrations (0, 1050 and 1555 mg/L) of $Cu(OH)_2$ nanopesticide for one month. At harvest, lettuce leaves were collected and analyzed by GC-TOF-MS. Each treatment had 5 replicates.

control ($p < 0.01$). Multivariate analysis methods can take into account many metabolomics features simultaneously and, consequently, they can identify relationship patterns between metabolites (Alonso et al., 2015). In order to get a general idea on grouping between control and $Cu(OH)_2$ treated lettuces, the GC-TOF-MS data were normalized by sum, followed by log transformation and analyzed by PCA. PCA is an un-supervised data analysis method, which determines the similarity patterns within the data without taking into account the sample type or class (Alonso et al., 2015). As shown in Fig. 1A, the control group and the nanopesticides treated groups were clearly separated along PC1, which explained 92% of the difference. Although the two treatment levels were separated from the control, there was not as much separation between low and high levels. PLS-DA is a supervised clustering method, which uses multiple linear regression to maximize the separation between groups and help to understand which variables carry the class-separating information (Jung et al., 2011). PLS-DA showed that three groups were completely separated along component 1 (Fig. 1 B) and the discriminating compounds were screened out by VIP value (Fig. S1). Metabolites that were significantly up- or down-regulated are presented in Table 2. Below we discuss 96 metabolites related to nutritional supply.

3.4. Primary metabolites

3.4.1. Carboxylic acid

GC-TOF-MS identified 26 carboxylic acids in lettuce leaves. As shown in Table 2, the most abundant organic acids in lettuce leaves were citric acid, glyceric acid, fumaric acid, maleic acid, and malic acid. Univariate (*t*-test) and multivariate (PLS-DA) analysis showed that the relative abundance of 8 carboxylic acids were altered in lettuce leaves due to exposure to $Cu(OH)_2$. Four of these carboxylic acids (fumaric acid, aconitic acid, threonic acid and oxalic acid) were down-regulated. Fumaric acid, a very abundant organic acid in lettuce leaves, decreased 30–54% in lettuce leaves sprayed with $Cu(OH)_2$ nanopesticides. Malic acid increased 71–96% compared to the control. In lettuce leaves without exposure to $Cu(OH)_2$, the level of fumaric acid is higher than malic acid. After exposure to $Cu(OH)_2$, the relative concentration of malic acid is higher than fumaric acid. In addition, malic acid, behenic acid, pyruvic acid and 4-hydroxybutyric acid were up-regulated after exposure to $Cu(OH)_2$, changing the profile of organic acids. Since organic acids influence flavor, these changes may alter acidity and lettuce flavor.

3.4.2. Amino acid

GC–TOF-MS identified 23 amino acids, including 9 essential or semi-essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and histidine) and 14 nonessential amino acids (proline, alanine, beta-alanine, oxoproline, asparagine, aspartic acid, glutamine, glycine, gamma-aminobutyric acid (GABA), citrulline, ornithine, glutamic acid, serine, tyrosine) (Supporting Information Excel 1). Based on the *t*-test results and VIP scores from the PLS-DA analysis, the relative concentration of 10 amino acids changed significantly, with 4 amino acids (alanine, GABA, oxoparoline and lysine,) down-regulated and 6 amino acids (aspartic acid, glutamine, tryptophan, citrulline, glycine, and asparagine) up-regulated. It is noteworthy that the relative abundance of GABA decreased 50-62% ($p < 0.05$). Although GABA is classified as a nonessential amino acid, it has been considered a bioactive plant metabolite and GABA-enriched foods have becoming popular due to the presumed health benefits (Li et al., 2013). GABA is an effective pain reliever and anxiolytic and effective in treating various nervous system disorders (Mody et al., 1994). In addition, it has been found GABA regulates blood pressure (Omori et al., 1987) and alleviates chronic alcohol-related symptoms (Oh et al., 2003). In this study, GABA content was reduced by half due to foliar application of $Cu(OH)_2$, which indicates the potential benefit from GABA due to lettuce intake will be decreased.

3.4.3. Carbohydrate

GC-TOF-MS identified 25 carbohydrates. Sucrose followed by glucose and fructose were the major sugars present in lettuce leaves. Results showed that levels of these major sugars were not significantly altered by exposure to $Cu(OH)_2$. The relative abundance of three minor carbohydrates were decreased approximately half compared to control including leucrose, levoglucosan, 1-methylgalactose, while the levels of digitoxose was increased one fold compared to control ($p < 0.05$).

3.5. Secondary metabolites

3.5.1. Polyamines

Putrescine (Put), spermidine (Spd), and spermine (Spm) form a group of aliphatic amines known as polyamines (Pinto and Ferreira, 2015). Polyamines have been shown to be involved in the differentiation of immune cells and the regulation of the inflammatory response (Ferioli et al., 2000; Moinard et al., 2005; Larqué et al., 2007). In addition, there is evidence that high polyamine intake in children is significantly correlated with preventing food allergies (Dandrifosse et al., 2000). As shown in Table 2, the level of both ethanolamine and spermidine were up-regulated in 1.7–4.2 and 1.9–2.2 fold, respectively, in lettuce leaves after foliar application of $Cu(OH)_2$ nanopesticides. In addition, putrescine increased 90–168% in lettuce leaves exposed to $Cu(OH)_2$ compared to the control. The up-regulated polyamines are possibly a plant stress response to excess Cu. Previous evidence showed exposure to salt or osmotic stress seemed to enhance polyamine biosynthesis in plants, increasing polyamine content (Friedman et al., 1989). Increased polyamine content should benefit the consumer since they are an important health factor (Soulet and Rivest, 2003; Ali et al., 2011).

3.5.2. Polyphenols

In this study 6 phenolics (benzoid acid, 3,4-dihydroxycinnamic acid, 1,2,4-benzenetriol, 4-hydroxyphenylacetic acid, chlorogenic acid) were detected in lettuce leaves. Previous studies showed that caffeic acid derivatives are the main phenolics in green lettuce varieties (Baslam et al., 2011). In our study, we found 3,4-dihydroxycinnamic acid was the most abundant polyphenol followed by chlorogenic acid and cis-caffeic acid (Table 2). The level of cis-caffeic acid significantly decreased ($p < 0.05$) in response to $Cu(OH)_2$ exposure. In addition, 3,4-dihydroxycinnamic acid and chlorogenic acid decreased 14–28%. Beneficial effects of polyphenols have been documented by several studies, including scavenging of free radicals that can cause cancer (Thavasi et al., 2006). The excellent radical scavenging properties of polyphenols are attributed to the phenolic OH groups present in the ring structures (Rice-Evans et al., 1997; Wang et al., 1997). The health potency of phenolic acid is mainly associated with its antioxidant activity. A growing body of evidence indicates phenolic acid plays a protective role against diseases associated with oxidative damage, such as coronary heart disease, stroke and cancers (Boeing et al., 2012; Cai et al., 2004; Chong et al., 2010; Liu, 2004). It has also been reported that chlorogenic acids possess strong antioxidant activity and prevent diseases caused by oxidative damage (Fujioka and Shibamoto, 2006; Fang et al., 2002; Benzie and Choi, 2014). 1,2,4-benzenetriol has also been reported to have great radical scavenging ability (Thavasi et al., 2006). The decreased levels of phenolic acids in exposed lettuce leaves may reduce their nutritional benefits.

3.5.3. Vitamins

Four vitamins were detected in lettuce samples including dehydroascorbic acid (DHA, vitamin C), gamma- and alpha-tocopherol (vitamin E), and nicotinamide (vitamin B3). DHA is an important water-soluble vitamin and is an excellent reducing agent and scavenger of free radicals, thus is an important antioxidant in food. DHA content in lettuce leaves was significantly decreased by 19–33% compared to the

Table 2
Relative abundance of compounds in lettuce leaves exposure to different concentration of $Cu(OH)_2$ nanopesticides.

		Control	Low	High	*p*-Value	VIP value	Trend
Carboxylic acid	Citric acid	1,736,398	1,140,656	1,239,902			
	Glyceric acid	1,365,428	2,108,719	1,492,753			
	Fumaric acid	**573,164**	**400,902**	**263,180**		>**1**	↓
	Maleic acid	459,140	427,778	240,720			
	Malic acid	**314,716**	**540,102**	**619,431**		>**1**	↑
	Quinic acid	143,473	159,256	129,985			
	Tartaric acid	130,275	153,754	137,909			
	Succinic acid	44,552	47,143	48,986			
	Isocitric acid	20,707	14,391	15,482			
	Shikimic acid	17,302	24,562	22,174			
	Aconitic acid	**12,032**	**6441**	**6372**		>**1**	↓
	Threonic acid	**10,365**	**5513**	**4848**	**0.018**	>**1**	↓
	Pelargonic acid	8777	9892	10,123			
	Alpha-ketoglutarate	5672	19,331	10,959			
	Behenic acid	**3061**	**264,584**	**3881**		>3	↑
	Xylonic acid	2076	2049	1216			
	Gluconic acid	1486	1683	1171			
	Benzoic acid	1410	1521	1544			
	Mucic acid	1287	2334	2250			
	Galacturonic acid	1054	1265	1423			
	Lactic acid	943	1123	1115			
	Oxalic acid	**851**	**452**	**570**		>**1**	↓
	Glycolic acid	703	662	562			
	Pentonic acid	596	795	759			
	4-Hydroxybutyric acid	**511**	**794.2**	**1602.4**	0.029		↑
	Pyruvic acid	**429**	**1784**	**1523**		>**2**	↑
	Alpha-aminoadipic acid	80	91	103			
Amino acid	Glutamic acid	140,056	116,451	100,490			
	Oxoproline	**86,649**	**35,307**	**35,380**	**0.015**	>**1**	↓
	Alanine	**54,992**	**52,973**	**49,233**	**0.042**		↓
	Aspartic acid	**34,679**	**92,582**	**95,542**		>**1**	↑
	Proline	25,447	22,480	16,559			
	Serine	23,321	39,235	41,559			
	Valine	17,342	16,974	15,552			
	Leucine	14,670	10,308	10,740			
	Glutamine	**12,721**	**27,886**	**24,203**		>**1**	↑
	Isoleucine	11,183	12,195	9484			
	Threonine	9099	14,475	11,250			
	Tyrosine	7179	6779	6376			
	Lysine	**6373**	**3453**	**4135**		>**1**	↓
	4-Aminobutyric acid (GABA)	**5881**	**2928**	**2216**	**0.029**	>**1**	↓
	Phenylalanine	5752	5508	5467			
	Tryptophan	**3951**	**12,783**	**7561**		>**2**	↑
	Ornithine	1211	1321	993			
	Citrulline	**817**	**8182**	**17,337**		>**1**	↑
	Glycine	**575**	**1879**	**1909**		>**1**	↑
	Asparagine	**570**	**1003**	**979**		>**1**	↑
	Beta-alanine	425	386	136			
	Methionine	423	636	534			
	Histidine	414	799	950			
Carbohydrate	Fructose	555,485	746,995	574,515			
	Sucrose	452,421	475,134	446,317			
	Glucose	169,937	264,784	200,649			
	N-acetylmannosamine	42,080	32,921	30,032			
	Glycerol	22,754	42,532	34,070			
	Xylose	22,143	33,443	25,574			
	Maltose	13,927	20,382	19,301			
	Fucose	12,472	15,752	15,141			
	Lactulose	11,942	8905	6384			
	Leucrose	**9574**	**5464**	**4956**	**0.051**	>**1**	↓
	Levoglucosan	**6819**	**2672**	**3462**		>**1**	↓
	Erythritol	6332	6726	6931			
	Lyxose	4604	7326	7732			
	Arabitol	3979	3747	3859			
	1-Methylgalactose	**3949**	**838**	**1999**	**0.000**	>**1**	↓
	Trehalose	3159	4245	3509			
	Sorbitol	3063	2824	2692			
	Xylulose	1873	1890	1726			
	Arabinose	1775	2339	1997			
	Ribose	1525	2174	1988			
	Malonic acid	1166	1611	1611			
	Lactitol	1124	1519	995			

(*continued on next page*)

Table 2 (*continued*)

		Control	Low	High	*p*-Value	VIP value	Trend
	Mannitol	966	1586	2771			
	Hexadecane	666	866	674			
	Digitoxose	**514**	**1159**	**1249**	**0.000**	>**1**	↑
Polyamines	Ethanolamine	16,315	69,337	27,933	0.055	>1	↑
	Putrescine	8136	15,476	21,875	0.071		↑
	Spermidine	238	531	459		>1	↑
Polyphenols	3,4-Dihydroxycinnamic acid	750,490	600,926	643,293			↑
	Chlorogenic acid	162,674	138,974	116,635			↑
	cis-Caffeic acid	**56,376**	**51,735**	**53,363**	**0.038**		↑
	4-Hydroxyphenylacetic acid	1605	1221	1375			
	Benzoic acid	1469	1521	1544			
	1,2,4-benzenetriol	258	198	524			
Vitamins	Dehydroascorbic acid (DHA)	**119,081**	**79,676**	**95,955**	**0.000**		↑
	Tocopherol gamma-	18,231	19,482	23,231			
	Tocopherol alpha-	8184	8522	9734			
Fatty acids	Stearic acid	87,071	95,791	122,239			
	Palmitic acid	15,520	19,537	24,383			
	Lauric acid	7278	12,185	11,423			
	Linolenic acid	4211	4367	4880			
	Montanic acid	3451	3797	5986			
	Myristic acid	2209	2433	2202			
	Lignoceric acid	2047	3268	4079			
	Cerotinic acid	**2038**	**2993**	**3502**	**0.006**		↑
	Arachidic acid	1873	1604	1826			
	Linoleic acid	1495	1600	2054			
Metal chelators	Nicotianamine				0.029	>**4**	↑
Others	Phytol	**30,919**	**15,251**	**18,972**	**0.009**		↓
	Uridine	27,473	37,010	38,893			
	UDP-glucuronic acid	**14,006**	**10,969**	**10,483**		>**1**	↓
	Beta-mannosylglycerate	**8780**	**24,446**	**56,593**	**0.005**	>**2**	↑
	Alpha-ketoglutarate	**5672**	**13,076**	**14,392**	**0.009**	>**1**	↑
	Hydroxylamine	**1779**	**1209**	**833**		>**1**	↓
	Adenosine	1403	1080	741			
	Mannitol	966	1586	2771			
	Fructose-6-phosphate	**549**	**757**	**1568**		>**2**	↑
	Beta-sitosterol	496	986	1541			
	Glucose-6-phosphate	**443**	**844**	**2665**		>**3**	↑
	Uracil	386	404	580			
	Arbutin	263	278	235			

The data are means of five replications. *p*-Value is from *t*-test; VIP score is from PLS-DA analysis.
Bold values means significantly changed metabolites either screened by T-test or PLS-DA analysis.
Low and High represent lettuces were exposured to 1050 and 1555 mg/L $Cu(OH)_2$ nanopesticides.

control ($p < 0.05$) (Table 2). Combining polyphenols data, it seems that most or all compounds with an antioxidant capacity were significantly decreased. This results in a reduced nutrient quality of lettuce leaves.

3.5.4. Total antioxidant capacity

Due to the chemical diversity of phenolic compounds in plant samples, it is challenging to determine each individually. The total antioxidant capacity is a better metric, particularly since their health benefits in lettuce are attributed not only to polyphenols, but also vitamins, carotenoids, fiber, and anthocyanins (Nicolle et al., 2004a). The total antioxidant capacity was significantly decreased (~20%, Fig. 2) after foliar exposure to $Cu(OH)_2$, compared to the control. Since Cu is capable of triggering the formation of ROS through Fenton reaction, nutrients/metabolites that possess antioxidant capabilities may provide protection against Cu-induced oxidative damage. Vitamins and phenolics are beneficial metabolites that may protect key biological constituents such as lipoproteins, membranes and DNA (Szeto et al., 2004). ROS generated due to exposure may be interacting with the antioxidants, decreasing total antioxidant capacity. The nutritional value of exposed lettuce leaves may decrease due to the reduction in beneficial phytonutrients.

3.5.5. Chlorophyll and carotenoids

Chlorophyll and carotenoids are also important antioxidants in lettuce. As shown in Fig. S2, there is a decrease in chlorophyll *a* and b, as well as in carotenoids due to the exposure to $Cu(OH)_2$ nanopesticides, but it was not statistically significant.

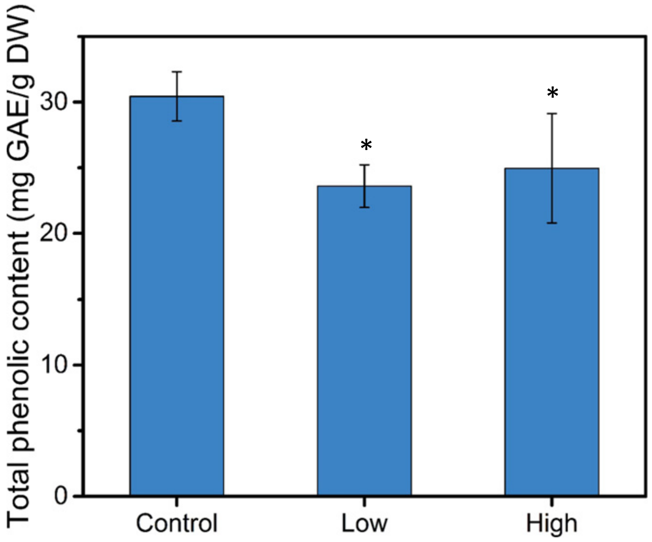

Fig. 2. Effects of different concentrations of $Cu(OH)_2$ nanopesticide on total antioxidant capacity of lettuce leaves. Error bar represents the standard deviation of five replicates. Asterisk above bar means significant difference compared to control ($p < 0.05$).

4. Conclusions

Nanopesticides may provide a number of benefits over traditional pest control methods in agriculture. However, it is important to understand their bioaccumulation in plant and impact on nutritional value. Our findings suggest that although Cu accumulated in lettuce leaves after foliar application for one month, the levels were within the recommended guidelines. However, consumers that ingest a large amount of lettuces may be exposed to levels that may be near or above the maximum recommended guidelines, particularly if they consume Cu in other foods, water and/or nutritional supplements. Foliar sprayed $Cu(OH)_2$ nanopesticides increased K concentration in lettuce leaves, which could be beneficial for human health. Cu generates ROS, the plant appears to respond by using its antioxidant capacity, in the form of phenolic compounds, vitamins (C, E and B3), and other metabolites such as cis-caffeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid and dehydroascorbic acid, which results in a decrease in the total antioxidant capacity contained in the product to be consumed. There was also up- and down-regulation of a number of amino acids, particularly GABA, which can affect the overall nutritional value of lettuce leaves exposed to the $Cu(OH)_2$ nanopesticide. Fine-tuning of the amount of nanopesticide applied will be important to obtain the antifungal benefits without resulting in a significant decrease in nutritional value.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.impact.2016.08.005.

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