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Antioxidant response of cucumber (*Cucumis sativus*) exposed to nano copper pesticide: Quantitative determination via LC-MS/MS

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ABSTRACT

Targeted metabolomics aims to provide a new approach to investigate metabolites and gather both qualitative and quantitative information. We describe a protocol for extraction and analysis of plant metabolites, specifically 13 secondary metabolites (antioxidants) using liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS), with high linearity (R2 > 0.99) and reproducibility (0.23–6.23 R%) with low limits of detection (> 0.001 ng/mL) and quantification (> 0.2 ng/mL). The protocol was applied to study the antioxidant response of cucumber plants exposed to nanocopper pesticide. Dose-dependent changes in antioxidant concentrations were found, and 10 antioxidants were significantly consumed to scavenge reactive oxygen species, protecting plants from damage. Levels of three antioxidants were up-regulated, as a response to the depletion of the other antioxidants, signaling activation of the defense system. We demonstrated that the reported LC-MS/MS method provides a quantitative analysis of antioxidants in plant tissues, for example to investigate interactions between plants and nanomaterials.

1. Introduction

Antioxidants in plant cells and tissue play an important role in the response to environmental stressors that induce reactive oxygen species (ROS); these compounds are generally present in relatively low concentrations but contribute to inhibiting or delaying the oxidation of substrates (Matkowski, 2008). In plants, phenolic acids (e.g. flavonoids such as ferulic, caffeic, p-coumaric, and vanillic acids) and vitamins (e.g. α-tocopherol) are important low molecular weight antioxidants, which play an important role in the defense to hierarchical oxidative stress (Blokhina, Virolainen, & Fagerstedt, 2003). Some antioxidants (e.g. α-tocopherol) act as chain-breaking inhibitors of lipid peroxidation when ROS are generated *in vivo*, and interfere with free radical propagation cascades (Salah et al., 1995). Previous studies have semi-quantitatively demonstrated that ROS stress influences the levels of antioxidant in various plants (Soria, Montes, Bisson, Atilla-Gokcumen, & Aga, 2017; Zhao, Huang, Adeleye, & Keller, 2017; Zhao, Ortiz, et al., 2016).

The rapid development of nanotechnology in the past decade has resulted in increased consideration and use of nanoscale fertilizers and pesticides in agriculture (Khot, Sankaran, Maja, Ehsani, & Schuster, 2012). In particular copper-containing nanopesticides (Cu NPs) are being introduced to the market due to their excellent antimicrobial and antifungal properties (Keller et al., 2017). However, there is an increasing concern on the environmental fate, bioavailability and toxicity of engineered nanomaterials (ENMs) to terrestrial plants (Kah & Hofmann, 2014). Some metallic ENMs (e.g. Cu NPs) and/or released ions (e.g. Cu^{2+}) can induce the formation of ROS within plant cells, and their over-accumulation in plants can result in oxidative damage of membrane lipids, proteins, and nucleic acids (Gill & Tuteja, 2010). A number of studies have shown, via indirect methods such as antioxidant assays, that Cu NPs can induce oxidative stress in various crop species such as alfalfa (Hong et al., 2015), barley (Shaw et al., 2014), cilantro (Zuverza-Mena et al., 2015), chickpeas and soybeans (Adhikari, Kundu, Biswas, Tarafdar, & Rao, 2012), mung beans and wheat (W. M. Lee, An, Yoon, & Kweon, 2008), radish (Atha et al., 2012), carrot (Ebbs et al., 2016) and corn (Zhao, Hu, Huang, & Keller, 2017). As a result, the metabolic pathways may be up- or down-regulated due to exposure to these ENM stressors (Hasler-Sheetal, Castorani, Glud, Canfield, & Holmer, 2016). Low molecular weight metabolites are the end products

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of cellular regulatory processes, and their levels reflect the ultimate response of biological systems to environmental changes (Fiehn, 2002). Monitoring the changes in concentrations of low molecular mass metabolites can provide a more holistic view of plant response to these environmental stressors.

Untargeted metabolomics can be used to perform a rapid screening of the metabolites that are over- or under-expressed due to a change in conditions. It generates a large list of metabolites that are significantly affected by a stressor. For example, recently we applied untargeted gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) and ^{1}H nuclear magnetic resonance (NMR)-based metabolomics to detect and evaluate the responses induced by various Cu NPs (i.e. nano-Cu, nano-CuO and nano-$Cu(OH)_2$) on various crop plants (i.e. cucumber, lettuce, spinach and corn) (Zhao, Hu, Huang, Fulton, et al., 2017; Zhao, Huang, et al., 2017; Zhao, Huang, Hannah-Bick, Fulton, & Keller, 2016; Zhao, Ortiz, et al., 2016). In some instances, up to 357 unique metabolites were detected via GC-TOF-MS, and around 150 metabolites were identified on the basis of their mass spectral fingerprints and retention-index matches. Of these, levels of around 30 were significantly ($p < 0.05$) altered (Zhao, Huang, et al., 2017), including several anti-oxidants. Other non-targeted metabolomics studies also indicated that anti-oxidants are almost always affected by exposure to these ENMs (Zhao, Hu, Huang, Fulton, et al., 2017; Zhao, Huang, et al., 2017; Zhao, Huang, Hannah-Bick, et al., 2016; Zhao, Ortiz, et al., 2016). However, the untargeted metabolomics analysis provides semi-quantitative information on the changes in metabolite levels, since there is no rigorous assessment of the recovery of metabolites during extraction, or calibration of the GC-TOF-MS responses. Thus, there is a need for a quantitative determination of the changes using rigorous quantitation methods.

Based on the previous untargeted metabolomics studies, levels of several antioxidants (e.g. ferulic acid, α-tocopherol) showed significant changes in cucumber plants exposed to Cu NPs (Gill & Tuteja, 2010; Zhao, Huang, et al., 2017; Zhao, Huang, Hannah-Bick, et al., 2016; Zhao, Ortiz, et al., 2016). Thus, 13 compounds that are highly related to the anti-oxidative defense of these crop plants were chosen as analytes (Table S1) to be evaluated in this study. Since most of the analytes possess relatively high polarities, liquid chromatography was considered as a separation method. LC–MS/MS can provide high sensitivity to analyze many metabolites and generally requires less sample pretreatment (Junot, Fenaille, Colsch, & Becher, 2014). Thus, LC-MS/MS based mass spectrometric technique may be used as a quantitative environmental metabolomics platform.

2. Materials and methods

2.1. Chemicals and reagents

All analytical standards used during the study were at least > 96% purity and every effort was made to use standards of the highest purity commercially available. Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO), including: p-coumaric acid (purity ≥ 98%), caffeic acid (≥98%), benzoic acid (99.5%), 2-hydroxycinnamic acid, predominantly trans (97%), l-glutathione reduced (≥98.0%); curcumin (≥98%), α-d-glucose 1-phosphate disodium salt hydrate (≥98%), and (±)-α-tocopherol (≥96%)). Vanillic acid (98%) was obtained from Alfa Aesar (Ward Hill, MA), gallic acid hydrate (> 98%) from TCI Chemicals (Japan), ferulic acid (99.6%) from MP Biomedicals (Solon, OH) and 4-(trifluoromethyl)cinnamic acid (98%) from Matrix scientific (Columbia, SC) (Table S1). Isotopically-labeled internal standards (benzoic acid-^{13}C, 99%) and ferulic acid-$^{13}C_3$, 99%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol, acetonitrile (ACN), isopropyl alcohol (IPA) and LC-MS grade water were purchased from Burdick and Jackson (Muskegon, MI), while formic acid (LC-MS grade), and ammonium acetate (LC-MS grade) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Characteristics of nCu

Uncoated nCu (U.S. Research Nanomaterials) was employed here; a detailed characterization was presented in previous studies (Adeleye, Conway, Perez, Rutten, & Keller, 2014). Briefly, the primary particle size is 40 nm and the hydrodynamic diameter (HDD) is 2590 ± 1138 nm in deionized (DI, Barnstead nanopure) water at pH 7 (0.5 mM phosphate buffer), at similar concentrations as in this study. Scanning electron microscope (SEM) and transmission electron microscopy (TEM) images of nCu are presented in the Supporting Information (Fig. S1). The surface charge, expressed as zeta potential in 0.5 mM phosphate buffer solution, is −29.4 ± 0.8 mV at pH 7.

2.3. Plant exposure and growth conditions

Cucumber (*Cucumis sativus*) seeds were purchased from Seed Savers Exchange (Iowa, USA). nCu was suspended in DI water and sonicated for 30 min before being applied to the soil (Sedgwick soil). The final concentration of nCu in soil (mg/kg) was 0 (Control), 400 (low) and 800 (high). This total Cu concentration is within the range predicted for biosolids applied to soils or due to the application of copper-based nanopesticides. Each treatment had four replicates. In each replicate, pairs of cucumber seedlings were grown in 3.0 L Poly-Tainer containers. The cucumber plants were grown for 60 days in the greenhouse at a controlled temperature of 25.5–30.0 °C during the day and 17.7–18.9 °C at night.

2.4. Extraction of antioxidants

At harvest, the fresh cucumber leaves were immediately placed in liquid nitrogen for rapid freezing. The frozen tissues were homogenized in liquid nitrogen into a fine powder using a pestle and mortar, and then stored at −85 °C in a freezer (VIP Series Ultra-Low Temperature Freezer, Sanyo Scientific, Bensenville, IL). For the extractions, 100 mg of frozen cucumber leaf powder was weighted into 2 mL Eppendorf microcentrifuge tubes, and then a 1 mL extraction solution (80:20 methanol and water with 2% formic acid) was added. The microcentrifuge tubes were vortexed at 3000 rpm for 30 min, and then sonicated in a 25 °C water bath for 30 min. A final centrifugation at 20,000*g* was done for 20 min. The supernatant (0.5 mL) was used for LC-MS/MS analysis.

To determine the recovery of antioxidants during the extraction, two levels of mixed antioxidants standards (50 and 100 ng/mL) were spiked into cucumber leaves tissues samples before and after the extraction process, to obtain pre- and post-extraction spike recovery, respectively. The recovery was calculated using:

$$Recovery(\%) = \frac{C_{observed} - C_{neat}}{C_{expected}} \times 100$$

where $C_{observed}$ is the concentration of pre- or post-extraction spiked sample with mix antioxidants standards; C_{neat} is the concentration of non-spiked (control) sample; $C_{expected}$ is the concentration that was spiked into samples.

2.5. Liquid chromatography

An Agilent 1260 UHPLC binary pump was used to perform liquid chromatography for all analyses. An Agilent ZORBAX StableBond 80 Å C18 (4.6 mm × 50 mm, 3.5 μm) column was used for chromatographic separation of all analytes. The column was maintained at 30 °C throughout the run. A dual eluent mobile phase comprised of water with 0.1% formic acid and 5 mM ammonia formate (A) and methanol (B) at 500 μL/min was used for separation. The mobile phase was held at 2% solvent B for 2 min. At 2 min, solvent B was linearly increased to 100% for 4 min. This gradient was held for 3 min, before returning to the initial condition after a total of 10 min. A 4.5 min post-run column

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