NanoImpact 12 (2018) 9–17

Contents lists available at ScienceDirect

NanoImpact

journal homepage: www.elsevier.com/locate/nanoimpact

Research paper

Quantitative analysis of changes in amino acids levels for cucumber (*Cucumis sativus*) exposed to nano copper

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ARTICLE INFO

Keywords:
HILIC
LC-MS/MS
Amino acids
Cucumber
Nanoparticle
Extraction

ABSTRACT

The increasing usage of nanopesticides in agriculture poses a concern to plant crops due to unknown implications of engineered nanomaterials (ENMs). Targeted metabolomics can provide both qualitative and quantitative information at a molecular level to investigate the response of plants to emerging environmental stressors, such as nanopesticides. Here we describe a detailed protocol for the extraction and analysis of plant metabolites, specifically 23 amino acids in plants, using hydrophilic interaction liquid chromatography coupled to triple quadrupole mass spectrometry (HILIC-LC-MS/MS). Sufficient separation of 23 amino acids was achieved, without the need for derivatization, on an HILIC column with an MS/MS detector in a single run of 12 min with high sensitivity, selectivity and robustness and low LOD (0.005–15 ng/mL) and LOQ (0.02–50 ng/mL). A simple and efficient method to effectively extract amino acids from plant tissues was developed with a high recovery rate (80–120%). The protocol was then applied to determine the levels of amino acids in cucumber plants exposed to various environmentally-relevant levels of nano copper (nCu at 0, 200, 400, and 800 mg/kg soil; harvested in 60 days). Dose-dependent changes in amino acid levels were found; 13 amino acids were upregulated due to nCu stress, particularly, tyrosine increased 6.1, 8.2, and 11.0 fold after exposure to 200, 400 and 800 mg/kg nCu, respectively. The change in amino acid levels suggests an active defense response of the cucumber plant to nCu stress. We demonstrate that the HILIC-LC-MS/MS method is an effective and efficient technique to analyze underivatized amino acids in plant samples.

1. Introduction

Recently, there has been an increasing use of nanoscale fertilizers and pesticides in agriculture (Raliya et al., 2018; Dimkpa and Bindraban, 2018), and copper-containing nanopesticides (Cu NPs) are one of the most popular products on the market because of their excellent antimicrobial and antifungal properties (Bergeson, 2010; Kiaune and Singhasemanon, 2011; Keller et al., 2017). However, due to the unique physicochemical properties of engineered nanomaterials (ENMs), e.g., ultra-fine particle size, high reactivity and etc., studies indicated they can be considered as potential environmental stressors to terrestrial plants (Conway et al., 2015; Rizwan et al., 2017; Du et al., 2017). Some metallic ENMs (e.g. Cu NPs) and/or released ions (e.g. Cu^{2+}) can induce the stress to plant, e.g. the formation of reactive oxygen species (ROS) within plant cells to induce oxidative stress (Zhao et al., 2016a; Shaw et al., 2014). The levels of low molecular weight metabolites, including amino acids, represent the ultimate response of biological systems to environmental changes (Fiehn, 2002). Furthermore, by studying these metabolites, we can understand better the metabolic pathways and networks that are up- or down-regulated due to exposure to these ENM stressors (Hasler-Sheetal et al., 2016). Thus, the quantitative determination of amino acids is important in mapping the metabolomic profile and evaluating the pathway of key metabolites, as well as the nutritional supplies from plant tissues; metabolomics provides a more holistic view of plant response to these environmental stressors. In addition, since Cu^{2+} exhibits strong binding to amino acids forming complexes, increased levels of some amino acids may induce the transformation of Cu NPs within the plants and/or in soil (Huang et al., 2017). Monitoring the changes in amino acids can also serve to better understand the underlying mechanisms behind plant-ENMs interactions at a molecular level.

In our previous studies, untargeted gas chromatography-time of

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https://doi.org/10.1016/j.impact.2018.08.008
Received 5 July 2018; Received in revised form 31 July 2018; Accepted 30 August 2018
Available online 03 September 2018
2452-0748/

flight-mass spectrometry (GC-TOF-MS) and ^{1}H nuclear magnetic resonance (NMR)-based metabolomics were applied for a rapid screening of metabolite changes within crop plants as responses to the stress induced by Cu NPs (Zhao et al., 2016a, b, c, d, 2017a, b, c, d, e). Amino acid levels within plant tissues were significantly ($p < 0.05$) altered after exposure to Cu NPs (Zhao et al., 2016a, c, 2017a, c, d, e). For example, eleven amino acids (e.g., alanine, glycine, proline and etc.) were significantly up-regulated in the root exudate of cucumber, suggesting an active defense mechanism against Cu NPs stress (Zhao et al., 2016a). With untargeted analytical techniques, an overall metabolic profile can provide a molecular-scale perspective on the response of plants to stressors, such as nanopesticides. 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. Targeted metabolomics, for example liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS), can quantitatively determine the changes in secondary metabolite concentrations in plants exposed to ENMs, which provides a more-sensitive and mechanistic understanding of the biological response to a stressor (Huang et al., 2019).

Hydrophilic interaction liquid chromatography (HILIC) can separate and help quantitatively analyze a wide range of polar compounds, including amino acids (Gao et al., 2016; Dell'mour et al., 2010), peptides (Le Maux et al., 2015), carbohydrates (Schulze et al., 2017), metabolites and other biologically important compounds (Mackay et al., 2015; Buszewski and Noga, 2012). Compared to other analytical techniques (e.g., reversed-phase high-performance liquid chromatography (RP-HPLC) and/or capillary electrophoresis (CE) coupled with optical or mass spectrometry (MS) detection), no pre-treatment (derivatization with strong chromophore groups, for example, ninhydrin (Bidlingmeyer et al., 1984), *o*-phthalaldehyde (Nimura and Kinoshita, 1986), etc.) is required to analyze amino acids on HILIC. This avoids time-consuming derivatization procedures, and can minimize issues such as derivative instability, insufficient reproducibility of derivative yield, and interferences caused by the reagent (Kaspar et al., 2009). Furthermore, coupled with tandem mass spectrometry (MS/MS), HILIC-MS/MS can offer gains in selectivity and sensitivity by reaction monitoring (MRM), while avoiding issues of instrument contamination and downtime that occur with the use of derivatizing agents.

Previous LC methods exhibited long retention time for underivatized amino acids analysis (Prinsen et al., 2016; Krumpochova et al., 2015). In this study, A newly released HILIC column with small particle size (2.7 μm) was used to develop a fast and sensitive HILIC-MS/MS method for direct quantitative analysis of underivatized amino acids. A single transition was used for quantification (Prinsen et al., 2016), and a second transition was employed as qualifier for confirmation of the identity of the targeted amino acids. The HILIC-MS/MS method presented here is capable of performing quantitation at trace levels (e.g., μg/L) of these amino acids in food, biological or environmental matrices. Using the quantitative secondary metabolites (e.g., amino acids) data, targeted metabolomics were conducted to investigate the response of plants (e.g., cucumber) exposed to ENMs (e.g., Cu NPs).

2. Materials and methods

2.1. Chemicals and reagents

All analytical standards used during the study had at least > 96% purity. All amino acid standards were purchased from Sigma-Aldrich (St. Louis, MO), including: L-tyrosine (≥99.0%), L-proline (≥99.0%), L-alanine (≥98.5%), L-valine (≥98.5%), L-phenylalanine (≥98.5%), L-lysine (≥98%), L-threonine (≥99.0%), glycine (≥99.0%), L-asparagine (≥98%), L-ornithine monohydrochloride (≥99.0%), L-arginine (≥98.5%), L-glutamic acid (≥98.5%), L-tryptophan (≥99.0%), L-isoleucine (≥98.5%), L-glutamine (≥99.0%), L-leucine (≥98.5%), L-methionine (≥99.0%), L-histidine (≥99.0%), L-aspartic acid (≥99.0%), L-cysteine (≥97%), L-citrulline (≥98%), L-serine (≥99.0%) and L-homoserine (≥98%) (Table S1). Isotopically labeled internal standards (ISTD), L-isoleucine-^{15}N (98%), L-methionine-2,3,3,4,4-d_5-methyl-d_3 (98%), L-glutamic acid-^{15}N (98%), Glycine-2,2-d_2 (98%), and L-alanine-3,3,3-d_3 (99%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). DL-Lysine-3,3,4,4,5,5,6,6-d_8 dihydrochloride (99.6%) was purchased from CDN Isotopes (Pointe-Claire, Québec, Canada), used as isotopically labeled internal standard (ILIS). LC-MS grade acetonitrile (ACN) and water were purchased from Burdick and Jackson (Muskegon, MI), while LC-MS grade formic acid and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Characteristics of nCu NPs

Uncoated nCu (U.S. Research Nanomaterials) was employed here; a detailed characterization was presented in a previous study (Adeleye et al., 2014). In this study, the primary particle size is 40 nm and the hydrodynamic diameter (HDD) was measured as 2432 ± 484 nm in deionized (DI, Barnstead nanopure) water at pH 7 (0.5 mM phosphate buffer). 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 −28.8 ± 0.6 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 surface without mixing (top soil collected from Sedgwick Reserve, CA, USA; and the characteristics of the soil were provided in SI, as Table S3). The final concentration of nCu in soil (mg/kg) was 0 (Control), 200 (low), 400 (medium) and 800 (high). This total Cu concentration is within the range predicted for biosolids applied to soils (Lazareva and Keller, 2014) or due to the application of copper-based nanopesticides (Conway et al., 2015). 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 60 days in the greenhouse at a controlled temperature of 25.5 to 30.0 °C during the day and 17.7 to 18.9 °C at night.

2.4. Extraction of amino acids

Cucumber leaf tissue extracts were prepared from freshly harvested cucumber leaves, which were immediately placed in liquid nitrogen for rapid freezing. The frozen cucumber tissues were homogenized in liquid nitrogen into a fine powder using a mortar and pestle, then stored in a freezer at −85 °C. For extraction, 100 mg of frozen cucumber leaf powder was weighed into 2-mL Eppendorf microcentrifuge tubes, and 1 mL of 0.5 M aqueous HCl was added. The tubes were vortexed at 8000 rpm for 20 min, sonicated in a 25 °C water bath for 20 min, then centrifuged at 20,000 × *g* for 20 min. Finally, 250 μL of the extraction supernatant was transferred into LC vials with ISTD already added, and the mixture was diluted to 1 mL with 20% water in acetonitrile.

To determine the extraction recovery rates of amino acids, three levels of mixed amino acid standards were spiked into cucumber leaf tissue samples before and after the extraction process, to obtain pre- and post-extraction spike recovery rates, respectively. The spiked concentrations of amino acids were 20, 40 and 80 μg/g (cucumber leaf tissues).

The recovery rates were calculated using:

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

where, $C_{observed}$ is the concentration of pre- or post-extraction spiked