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Analysis of phytohormones in vermicompost using a novel combinative sample preparation strategy of ultrasound-assisted extraction and solid-phase extraction coupled with liquid chromatography–tandem mass spectrometry

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ABSTRACT

Vermicompost (VC), a widely used premium organic fertilizer, is the by-product of symbiotic interactions between earthworms and microorganisms living within them. It has been postulated that phytohormones are plausible "magic compounds" in VC that are responsible for making them such good fertilizers. Thus, a novel approach involving ultrasound-assisted extraction (UAE) and solid-phase extraction (SPE) was developed as a fast and efficient sample preparation method to screen for different classes of phytohormones in VC by liquid chromatography–tandem mass spectrometric (LC–MS/MS) analysis. Nine phytohormones from three different classes, including *trans*-zeatin (*tZ*), kinetin (K), N^6-[2-isopentyl]adenine (iP), N^6-benzyladenine (BA), N^6-isopentenyladenosine (iPR), indole-3-acetic acid (IAA), 4-[3-indolyl]butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and (+)-abscisic acid (ABA), were simultaneously screened. The extraction parameters influencing UAE efficiency were optimized to provide comparable recovery to the conventional mix-stirring (MSt) method. The optimized UAE method was subsequently applied on the analysis of phytohormones in VC, i.e. phytohormone extract was further pre-concentrated and purified using C18 and MCX SPE cartridges prior to LC–MS/MS analysis. The following phytohormones, namely iP, iPR and IAA, were detected and quantified to be 0.49, 0.53, 79.78 ng g^{-1}, respectively; *tZ* was found to be below the limit of quantitation. Recoveries of 10.2%, 9.1%, 18.9% and 0.3% for *tZ*, iP, iPR and IAA were obtained. This is one of the few reported works for the successful detection and quantitation of cytokinins and auxins in VC, that provided the key empirical evidence to explain the growth efficacy of applying VC in promoting plant growth. Additionally, this pioneering work could potentially be applicable for the analysis of other types of organic fertilizers such as composts and activated composted materials awaiting phytohormone analyzes for quality assessment and control.

1. Introduction

Vermicompost (VC) is a finely divided peat-like material with high porosity, good aeration and water-holding capacities, and low C:N ratios. It is the by-product of symbiotic interactions between earthworms and microorganisms [1]. VC, as a premium biofertilizer, has attracted worldwide attention in agricultural and horticultural industries due to its environmental friendliness, growth efficacy and organic nutrient richness. It is known that prolonged application of chemical fertilizers would cause soil degradation by altering soil structure and decreasing the availability of microorganisms, especially the beneficial ones [1]. Uncontrolled and excessive use of chemical fertilizers, coupled with poor land use management, could result in eutrophication of adjacent waterbodies. Similar to chemical

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fertilizers, VC contains useful minerals such as nitrates, exchangeable phosphorus, soluble potassium, calcium and magnesium, which are essential for plant growth [2]. VC also contains organic nutrients like fulvic and humic acids, and putative trace amounts of phytohormones, such as auxins, gibberellins (GAs) and cytokinins (CKs) [2]. Similar growth patterns of plants were observed for addition of aqueous extracts from VC as with addition of exogenous auxins, GAs and CKs through the soil [3]. The presence of indole-3-acetic acid (IAA, an auxin) in humic acid extracted from VC was identified using gas chromatography–mass spectrometry (GC–MS) [4]. Recently, we discovered the presence of CKs in vermicompost tea (the liquid "wash" of VC) using mass spectrometry [5]. To our knowledge, however, the rapid extraction and simultaneous screening of the different classes of phytohormones in VC have not been studied. The quantification of phytohormones in VC will be crucial globally as a form of quality assessment for the rapidly emerging area of biofertilizer usage, production and quality assurance.

Phytohormones play pivotal roles in regulating plant growth, development, and in responding to biotic and abiotic stresses [6–8]. Thus, the simultaneous quantitative profiling of different classes of phytohormones will provide a useful basis for defining additive, synergistic or antagonistic hormonal activities in any biofertilizer or natural compounds of interest [9,10]. Phytohormones are typically present in (ultra)trace levels in complex biological matrix, which make their analysis rather challenging. With the development of newer analytical instrumentation and techniques, more accurate and sensitive identification and quantitation methods for phytohormones are becoming readily available, and several reviews have been published recently [9,11–14]. Due to the highly complex nature of biological matrix and (ultra)trace levels of phytohormones in samples, the lack of proper sample preparation methods can lead to major instrumentation and downstream analytical challenges. Traditional sample preparation techniques, including solvent extraction of the sample (i.e., solid plant material) followed by liquid–liquid extraction (LLE) or solid-phase extraction (SPE), are well-known procedures applied to isolate and purify phytohormones, prior to analysis. The first solvent extraction step is commonly achieved by mix-stirring (MSt) of sample with different extraction solvents [11]. However, this process is relatively time consuming compared to the recently established techniques, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). UAE and MAE have been successfully employed to extract bioactive phytochemicals from various sources, such as extracting isoflavones from peanuts [15] and soybeans [16,17], and taxanes from the taxus plant [18]. When compared to MAE, UAE is a more attractive option when lower cost of equipment and safety is taken into consideration. However, research in the extraction of phytohormones by UAE is still lacking.

Several sensitive and specific detection methods have been established for analyzing phytohormones, such as GC–MS [19], enzyme- or radioimmunoassay (ELISA, RIA) [20], capillary electrophoresis (CE) [21], high-performance liquid chromatography (HPLC) [22] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [23]. GC–MS is generally used to analyze volatile compounds, and thus most phytohormones have to undergo derivatization step prior to analysis. Additionally, the high temperature in GC can lead to degradation of the thermally labile compounds. Immunoassay-related techniques are useful approaches in achieving specificity due to their unique ligand–antibody binding. However, due to their precise recognition process for specific solute(s), the use of those techniques for simultaneous determination of two or more classes of phytohormones, with different molecular structures, in a given sample is challenging. HPLC is potentially a suitable technique for these polar compounds that are thermally labile. At present, the identification and determination of phytohormones by LC–MS(/MS) is the most widely used technique in phytohormone research [11–14]. In addition to the unequivocal identification of different phytohormones, these LC–MS and related MS methods also enable the quantification of a broad spectrum of analytes in a single analysis.

The aim of this study is to develop an efficient method for the extraction and simultaneous detection of different classes of phytohormones in VC based on UAE. The efficiency of this technique was compared with the classical MSt method. Nine phytohormone standards from three different classes *trans*-zeatin (*t*Z), kinetin (K), N^6-[2-isopentyl]adenine (iP), N^6-isopentenyladenosine (iPR), N^6-benzyladenine (BA), (+)-abscisic acid (ABA), IAA, 4-[3-indolyl]butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) were used as test analytes. The chemical structures of the target phytohormones are shown in Fig. 1. The parameters influencing the extraction were investigated, including extraction solvent type, solvent composition, extraction time, amount of acid, and solvent to sample ratio. Under optimized UAE conditions, by combining with an efficient solid-phase purification method, the developed method was later evaluated using a real VC sample prior to LC–MS/MS analysis. The presence of putative phytohormones was identified by LC–MS/MS based on their characteristic fragmentation pattern and retention time. Our literature review suggested that this is the first report on the UAE extraction and full screening of major phytohormones in VC.

2. Material and methods

2.1. Chemicals and reagents

Pure phytohormone standards *t*Z, IAA and IBA were obtained from Sigma-Aldrich (St. Louis, Missouri, USA); K, iP, BA, ABA and NAA were purchased from PhytoTechnology Laboratories (Shawnee Mission, USA); iPR were bought from OlChemIm Ltd, Czech Republic. Stock solutions (1 mg mL^{-1} of each analyte) were prepared separately in methanol and stored at 4 °C. HPLC grade methanol (MeOH) and isopropyl alcohol (IPA) were obtained from Fisher (Trinidad, USA) and Tedia (Fairfield Ohio, USA), respectively; triethylamine (TEA) (HPLC grade) was supplied by BDH (England, UK). Absolute ethanol (EtOH) was purchased from VWR International (Radnor, PA, USA). The formic acid used in phytohormone extraction (Scharlau, Spain), acetic acid (Riedel-de Haën®, Germany), acetone (Univar, USA) and ammonium hydroxide (Fisons, Ipswich, UK) were of the reagent grade. The formic acid used in HPLC analysis was bought from Fluka (Steinheim, Switzerland). Ultrapure water was obtained from PURELAB Ultra (ELGA, Saint Maurice, France) water purification system throughout the study. The pH value was measured with a pH meter (Corning 440, Corning Glass Works, NY, USA).

2.2. Vermicompost preparation

VC was produced by two earthworm species (*Perionyx excavatus* and *Eisenia foetida* at 65:35 ratio) feeding on organic wastes such as vegetables, fruit peels and water hyacinth at a frequency of 2–3 days per feed [5]. After three months, VC was collected and freeze-dried using a freeze dryer (ModulyoD Freeze Dryer,Thermo Electron Corporator, UK) for a few days till dryness, before it was ground to fine powder size of 0.5 mm (MF10 Basic, IKA Werke, Staufen, Germany). The powdered VC was stored in a dry cabinet prior to analysis. The organic matter composition of the VC is presented in Table 1. The vermicompost had a C:N ratio of 11:1, with pH 7.4.

To prepare spiked VC samples, VC was first mixed with acetone until the VC was completely submerged. Appropriate volumes of the phytohormone stock solutions were spiked into the slurry to obtain