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Research article

Phenolic compounds responding to zinc and/or cadmium treatments in *Gynura pseudochina* (L.) DC. extracts and biomass

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ABSTRACT

Gynura pseudochina (L.) DC. is a Zn/Cd hyperaccumulative plant. In an *in vivo* system under controlled plant age, this research reveals that phenolic compounds and lignification play beneficial roles in protecting *G. pseudochina* from exposure to an excess of Zn and/or Cd, and Zn reduces Cd toxicity under the dual treatments. The total phenolic content (TPC), total flavonoid content (TFC), and half-maximal inhibitory concentration (IC_{50}) values correspond to the metal dose-response curves. Liquid chromatography-electrospray ionization-quadrupole time of flight-tandem mass spectrometry (LC-ESI-QTOF-MS/MS) is used to characterize phenolic compounds and their glycosides, which could play roles in antioxidant activities and in the esterification of the cell wall, especially derivatives of *p*-coumaric and caffeic acid. Confocal laser scanning microscopy (CLSM) and micro X-ray fluorescence (μ-XRF) imaging revealed that the accumulation of Zn and Cd in the cell wall involves flavonoid compounds. Low extractable pools of Cd and Zn in the leaf extracts indicate that these elements are tightly bound to the plant biomass structures. The bulk X-ray absorption near edge structure (XANES) spectra indicate that Zn^{2+} and Cd^{2+} dominate with O and S ligands, which could be provided by cell walls, phenolic compounds, and sulphur protein. Consequently, the benefit of these results is to support the growth of *G. pseudochina* for phytoremediation in a Zn- and/or Cd-contaminated site.

1. Introduction

Cadmium (Cd) appears mostly as a guest metal in zinc (Zn) mineralisation (Prasad et al., 2015). *Gynura pseudochina* (L.) DC., a perennial plant in the Asteraceae family (Vanijajiva, 2009), is a Zn and Cd hyperaccumulative plant that grows in a Zn rich area of the province of Tak, Thailand (Phaenark et al., 2009). In addition, *G. pseudochina* is a medicinal herb and a source of phenolic compounds (Siriwatanametanon et al., 2010; Siriwatanametanon and Heinrich, 2011; Moektiwardoyo et al., 2014). Heavy metals, which behave as oxidizing agents due to the presence of a vacant *d*-orbital, possess the ability to produce reactive oxygen species (ROS) (Flora, 2009), and heavy metal-tolerant species produce high levels of ROS scavenging antioxidant enzymes and non-enzymatic, especially phenolic compounds that protect the plants from oxidative damage (Gill and Tuteja, 2010; Sytar et al., 2013). Zn and Cd are periodic table group II metals, and therefore have many physical and chemical similarities (Chang, 2010). Cd is not an essential element, whereas Zn is an essential micronutrient for normal plant growth at a low concentration (Kabata-Pendias, 2011). Although Zn and Cd are not directly involved in the Haber-Weiss and Fenton reactions, a supra-optimal Zn concentration and Cd toxicity induce oxidative stress (Flora, 2009; Lin and Aarts, 2012; Gallego et al., 2012). Protein chelators and sequestration in trichromes are detoxification methods for Zn and Cd in *G. pseudochina* (Nakbanpote et al., 2010; Panitlertumpai et al., 2013). However, there is still uncertainty concerning the functions of phenolic compounds when responding to Zn and/or Cd.

Plant phenolic compounds scavenge harmful active oxygen species and chelate heavy metals (Michalak, 2006; Malešev and Kuntic, 2007; Sytar et al., 2013). The expression of phenolic compounds in dicots under Zn and/or Cd stresses has been reported in *Camellia sinensis* (Zagoskina et al., 2007), *Vaccinium myrtillus*

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(Białońska et al., 2007), *Matricaria chamomilla* (Kováčik et al., 2009), and *Erica andevalensis* (Márquez-García et al., 2012). The antioxidant and/or prooxidant activity of phenolics depends on many factors, such as metal-reducing potential, chelating behaviour, pH, and solubility characteristics (Sakihama et al., 2002). Phenolic compounds, particularly flavonoids, have a high tendency to chelate heavy metals, and thus, they inhibit the generation of ROS as well as reduce the ROS once formed (Agati et al., 2012; Michalak, 2006; Wei and Guo, 2014; Bai et al., 2004). Confocal laser scanning microscopy (CLSM) and fluorescent staining provide opportunities to study tissue localization of phenolic compounds (Hutzler et al., 1998; Gavin and Durako, 2011). Synchrotron radiation-based micro-X-ray fluorescence analysis (SR-μ-XRF) imaging provides spatially resolved maps of elements at the cellular scale (Zhao et al., 2014; Isaure et al., 2006; Harada et al., 2010; Mongkhonsin et al., 2011).

This research aims to study the responses of phenolic compounds to Zn, Cd, and Zn plus Cd treatments on *G. pseudochina*. Rhizobacteria and endophytes possess properties that promote plant growth and reduce oxidative stress under abiotic stress, such as exposure to heavy metals (Ma et al., 2011), and plants usually secrete phenolic compounds to combat pathogen attacks (Wojtaszek, 1997). Therefore, an *in vitro* tissue culture system was used to control the environment and the effects of microorganisms. The effects of the metals on plant growth, total phenolic content (TPC), total flavonoid content (TFC), free radical scavenging activity (FRSA), as well as the main components in leaf extracts were investigated. The quantities of Zn and Cd in extracts and digested biomass were analysed, and the distribution of flavonoids and metals were studied in plant tissues and bulk biomass. These investigations discovered the biochemical processes of phenolic compounds that connect with the Zn/Cd hyperaccumulation ability, which could support the growth of *G. pseudochina* for phytoremediation.

2. Materials and methods

2.1. Plant material and Zn and/or Cd treatments

A voucher specimen of *G. pseudochina* (L.) DC. (KKU No. 28875) was deposited at the Herbarium of Khon Kaen University (KKU), Thailand. The *in vitro* propagation of *G. pseudochina* followed the method of Panitlertumpai et al. (2013). The sterilised explants were cultured aseptically with 20 mL of full strength Murashige and Skoog nutrient agar (Murashige and Skoog, 1962) at 25 °C under a 1500 lux light intensity for a 12 h photoperiod, in plant tissue culture glass bottles (diameter 4.5 cm, height 8.5 cm). Four-week-old healthy plants with similar leaf numbers and heights were selected for treatment. Zn and Cd solutions were prepared using $ZnSO_4 \cdot 7H_2O$ and $CdSO_4 \cdot 8H_2O$, respectively, and the pHs were adjusted to 5.5. The metals solutions were sterilised before being applied to the Murashige and Skoog media agar of the *in vitro* system. The plant samples were separately treated with 2 mL of the Zn solution (100, 250, 500, 750, and 1000 mg L^{-1}) or the Cd solution (5, 20, 50, 100, and 150 mg L^{-1}). The Zn/Cd dual treatments were conducted with 1000 mg L^{-1} of Zn plus various Cd concentrations (50, 100, and 150 mg L^{-1}). Accordingly, the final concentrations of Zn and/or Cd in the 20 mL of the Murashige and Skoog agar were 10–100 mg L^{-1} of Zn, 0.5–15 mg L^{-1} of Cd, and 100 mg L^{-1} of Zn plus 5–15 mg L^{-1} of Cd. For the control system, plants were treated with 2 mL of sterile deionised water, pH 5.5. Therefore, the control had 0.04 mg L^{-1} of Zn from the Murashige and Skoog components. After two weeks of treatment, the plants were washed with an excess of deionised water. Whole plants were weighed to obtain the wet weight and then dried at 80 °C for 24 h to record the dry weight.

2.2. Plant extraction

The leaves of the plants from each treatment were collected, freeze dried, and then ground into a fine powder. A percolation and solvent partition method (Liu, 2011) was conducted in a 3 mL disposable syringe (1.0 cm inner diameter NIPRO, Japan), with a series of solvents in order of increasing polarity (99.5% (v/v) hexane, 99.9% (v/v) ethanol, 99.9% (v/v) methanol, and 50% (v/v) methanol). A set was 0.450 g of leaf powder packed into the syringe to obtain a 1.5 mL bed volume. An effluent of 45 mL per loading was collected as a fraction, and three fractions were collected for each solvent. The flow rate was controlled at 0.1–0.2 mL min^{-1} by a vacuum manifold (12-Port Teknokrama, Spain). All extracts were kept in separate amber vials with tight stoppers at 4 °C until analysis. The extracts were assayed for TPC, TFC, FRSA, and concentrations of Zn and Cd.

2.3. TPC, TFC, and FRSA

The TPC was determined using a modified Folin-Ciocalteau method (Cicco et al., 2009). A 100 μL leaf extract was pipetted into 1.0 mL Eppendorf tubes and 500 μL of 10% (v/v) Folin-Ciocalteau reagent was applied. The mixture was left to stand in the dark for 3 min and then 400 μL of 7.5% (w/v) Na_2CO_3 was added. After 30 min in the dark, the absorbance was determined at 731 nm using a UV/visible spectrometer (Beckman Coulter DU 730 Life Science, USA). A standard curve was prepared from 10, 20, 40, 80, and 100 mg L^{-1} of caffeic acid. The TPC was expressed in terms of a caffeic acid equivalent (μmol CAE g^{-1} dry wt.).

The TFC was analysed using a colorimetric method (Pękal and Pyrzynska, 2014). Briefly, 1.25 mL of deionised water and 250 μL of leaf extract were added to 15 mL brown glass vials. Then, 75 μL of 5% (w/v) $NaNO_2$ was added. The mixtures were left in the dark for 5 min before 150 μL of 10% (w/v) $AlCl_3$ was added. After standing for 6 min, 500 μL of 1 M NaOH and 275 μL of deionised water were added. After 5 min in the dark, the absorbance was measured at 510 nm. A standard curve was prepared from 10, 20, 40, 80, and 100 mg L^{-1} of epicatechin. The TFC was expressed in terms of an epicatechin equivalent (μmol EPE g^{-1} dry wt.).

The FRSA was evaluated based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical method (Brand-Williams et al., 1995). An amount of 100 μL of leaf extract or blank was pipetted into separate 1.0 mL Eppendrof tubes, and 900 μL of 80 μM DPPH solution was added to each. The mixture was left in the dark for 30 min, and the absorbance was measured at 515 nm. The ability of the extract to scavenge DPPH free radicals was calculated by equation (1).

$$FRSA(\%) = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the test sample. The IC_{50} value was the concentration of the extract (mg extract mL^{-1}) required to scavenge 50% of the DPPH. Therefore, an IC_{50} was calculated from the plot of the serial dilutions vs the % of the *FRSA*.

2.4. Determination of total Zn and Cd in plant

The total amounts of Zn and Cd in the dried leaf biomass after the extraction were determined using a modified nitric-perchloric (HNO_3-$HClO_4$) acid digestion method (Miller, 1998) where 0.15 g of dried biomass was digested with 3 mL of 65% (v/v) HNO_3 at 150 °C for 1 h in a digestion tube. Then, 1 mL of 70% (v/v) $HClO_4$ was