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Research on the relationship between phenolic acids and rooting of tree peony (*Paeonia suffruticosa*) plantlets *in vitro*

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ABSTRACT

Phenolic acids, the most common plant secondary metabolites, are involved in controlling adventitious root formation and development. Plantlets of tree peony (*Paeonia suffruticosa*), a phenolic acid-rich plant, are generally difficult to root *in vitro*. In this study, 12 phenolic acids were detected in seedlings of tree peony; the most abundant was paeonol, followed by acetovanillone, paeoniflorin, benzoic acid, methyl gallate, gallic acid, shikimic acid, 4-hydroxybenzoic acid, 2,3-dihydroxy-4-methoxyacetophenone, caffeic acid, chlorogenic acid, and catechol. Paeonol, catechol, and 2, 3-dihydroxy-4-methoxyacetophenone were not detected in tree peony plantlets *in vitro*. During rooting, caffeic acid, methyl gallate, and paeoniflorin contents remained at high levels, whereas the acetovanillone content remained low. Treatment with caffeic acid (1 mg L^{-1}) had the strongest effect on rooting percentage, resulting in an increase from 10% to 64.03%. High indole-3-acetic acid levels were required for root induction in tree peony plantlets *in vitro*. We concluded that changes in the contents of endogenous phenolic acids (caffeic acid, methyl gallate, paeoniflorin, and acetovanillone) are closely related to rooting of tree peony *in vitro*, and that exogenous triphenols and especially diphenols are advantageous for rooting *in vitro*.

1. Introduction

Tree peony (*Paeonia suffruticosa*; Paeoniaceae) is a woody perennial tree that is traditionally cultivated in China (Wister, 1995). It is popular because of its large flowers with a wide range of colors, and its high medicinal value. Tissue culture of tree peony is widely used to overcome limitations of traditional breeding methods, which include the long breeding cycle, low vegetative reproduction, and poor seed development (Li et al., 1984). Several studies have reported on the micropropagation of tree peony, however, there are still problems to be overcome (Li et al., 1984; Wang and Van Staden, 2001). Rooting is one of the most important stages during tissue culture. Rooting *in vitro* is affected by various factors, including medium composition (Beruto et al., 2004; Beruto and Curir, 2007; Bouza et al., 1994; Wang et al., 2012; Wen et al., 2016) and environmental factors (Ding et al., 2010; Beruto and Curir, 2007). Other problems in establishing a tree peony propagation system include poor rhizogenesis and rooting quality and low survival rates of *in vitro* plantlets (Beruto et al., 2004; Harris and Mantell, 1991; Wang et al., 2012). Thus, the improvement of rooting quality would be a key breakthrough in tree peony tissue culture.

Various endogenous substances, including phenolic acids, can promote or inhibit adventitious rooting. Phenolic acids are the most common plant secondary metabolites, and they are involved in various metabolic processes. They accumulate in plants under biotic and abiotic stresses (Dixon and Paiva, 1995) and have been used to control adventitious root formation and development (Wu et al., 2007; Fernandez-Lorenzo et al., 2005). Phenolic acids augment auxin activity during rooting by inhibiting the indole-3-acetic acid (IAA)-oxidase system, thereby preventing the destruction of IAA (Padney and Pathak, 1981). Studies on *Eucalyptus*, *Eryngium maritimum,* and *Malus* (Fogaça and Fett-Neto, 2005; Kikowska et al., 2014; De Klerk et al., 2011) have uncovered a close association between phenolic acids and rooting. To date, most of the research on phenolic acids in tree peony, a plant rich in such compounds, has focused on the effects of major phenolic acids extracted from roots and their pharmacological effects on human illnesses (Fan et al., 2012; He et al., 2014). There have been no reports on the types of phenolic acids that accumulate during rooting, or their role in rooting of tree peony *in vitro*.

Abbreviations: IAA, indole-3-acetic acid; LC–MS, liquid chromatography-mass spectrometry; NAA, 1-naphthaleneacetic acid; 6-BA, 6-benzylaminopurine acid; PVP, Polyvinylpyrrolidone K 60; LH, L-ascorbic acid; WPM, woody plant medium; UHPLC, ultra-high performance liquid chromatography; TOF, quadrupole time-of-flight

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The aim of this study was to analyze the types of endogenous phenolic acids in tree peony, and to evaluate the effects of endogenous and exogenous phenolic acids on the rooting of tree peony plantlets *in vitro*. First, we identified endogenous phenolic acids and monitored their changes during *in vitro* rooting of tree peony plantlets. Then, we investigated the effect of various exogenous phenolic acids on this process. Identifying the correlations between phenolic acids and root initiation and development would provide vital clues to improve the micropropagation of tree peony.

2. Materials and methods

2.1. Materials

The source materials in this study were maternal plants of the tree peony cultivar 'Feng Dan Bai' cultivated in Luoyang, China. These plants were originally derived from 4-year-old *ex vitro* individuals known to be free of plant diseases and insect pests. Axillary buds were collected in February 2015, and root bark, stem bark, and leaves were collected in April 2015.

2.2. Extraction of phenolic acids from roots, stems and leaves

After collection, the root bark, stem bark, and leaves were oven-dried at 40 °C for 48 h and then ground to pass through a 40-mesh sieve. Powdered samples (1 g) were placed in 50-mL flasks. After addition of 15 mL methanol (Merck, Darmstadt, Germany) to each flask, samples were subjected to ultrasonic extraction for 30 min at 40 °C and then filtered. The above steps were repeated using the residue remaining after filtration. The combined filtrates were centrifuged at 9600 × *g* for 10 min at 40 °C. The eluant from the combined filtrates was dried and redissolved in 2 mL methanol.

2.3. Standards

The following standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): catechol, benzoic acid, 2-methoxyphenol, 4-hydroxybenzoic acid, coumaric acid, cinnamic acid, caffeic acid, 6-hydroxycoumarin, paeonol, acetovanillone, shikimic acid, gallic acid, resveratrol, chlorogenic acid, methyl gallate, and paeoniflorin. We obtained 2, 3-dihydroxy-4-methoxyacetophenone from the Shanghai Ruiqi Life Scientific Co. (Shanghai, China) and 4-hydroxybenzhydrazide from the Shanghai Source Biological Co. (Shanghai, China).

2.4. Liquid chromatography-mass spectrometry (LC–MS) analysis

The phenolic acid extracts were analyzed by LC–MS on an Agilent Technologies 1200 series liquid chromatograph coupled to an API 4400 triple quadrupole mass spectrometer. The separation was carried out on a 4.6 mm × 250 mm, 5-μm Agilent Zorbax SB-C_{18} reverse-phase column at ambient temperature and equilibrated with 50% solvent A (0.1% acetic acid in water) and 50% solvent B (methanol). The flow rate was 1 mL min^{-1}.

Electrospray ionization was performed in both positive and negative ion scanning modes (ion spray voltage = 5000 V) with nitrogen as the nebulizer (gas 1), heater (gas 2), curtain, and collision gases. The gas flow parameters (psi) were as follows: nebulizer = 50, heater = 55, and curtain = 6. The turbo ion spray temperature was set at 600 °C and the dwell time was 50 ms. Analytes and standards were monitored for quantitative analysis in multiple reaction monitoring mode. A dilution series of standards was used to construct a standard curve based on chromatographic peak areas. The injection volume was 20 μL, with samples filtered through a 0.22-μm membrane filter before injection. Peaks were identified by comparison of their retention time with those of authentic standards. Analyses were performed in triplicate. All standard and sample concentrations were determined using external standard areas vs. analyte areas.

2.5. Extraction of endogenous phenolic acids during in vitro rooting

Tree peony buds were first rinsed in running tap water for 60 min and then sterilized in 70% (v/v) ethanol for 30 s, followed by a solution of $HgCl_2$ (0.1%, w/v) for 8 min. The buds were then rinsed three times (3 min each) with sterile distilled water. The surface-sterilized buds were used as explants, and 100 explants were used for each treatment. Axillary buds (approximately 5 mm in diameter) were used as explants and were cultured for 45 days on Murashige–Skoog medium (Murashige and Skoog, 1962) supplemented with 0.5 mg L^{-1} 1-naphthaleneacetic acid (NAA; Beijing Solarbio, Beijing, China), 0.5 mg L^{-1} 6-benzylaminopurine acid (6-BA; Sigma-Aldrich), 30 g L^{-1} sucrose (Shanghai Just Scientific, Shanghai, China), and 7 g L^{-1} agar (Sigma-Aldrich). The shoots were subcultured on Murashige–Skoog medium supplemented with 0.5 mg L^{-1} NAA, 2 mg L^{-1} 6-BA, 30 g L^{-1} sucrose, 7 g L^{-1} agar powder, 1.0 g L^{-1} polyvinylpyrrolidone K 60 (PVP; Chinese Medicine Group Chemical Reagent, Beijing, China), and 50 mg L^{-1} L-ascorbic acid (LH; Wako, Osaka, Japan). After five to six rounds of subculturing, 3–5-cm-high new shoots with four to five leaves and no roots were obtained for use in subsequent experiments (Fig. 1A).

The culture conditions were as follows: 25 ± 1 °C, 12-h light/12-h dark photoperiod, illumination of 36 μmol m^{-2} s^{-1}. All media were adjusted to pH 5.8 with 1 M NaOH before autoclaving at 121 °C for 15 min.

New shoots induced from axillary buds were subcultured in 100-mL flasks containing 40 mL woody plant medium (WPM; Lloyd and McCown, 1980) supplemented with 4 mg L^{-1} IAA (Sigma-Aldrich), 1.0 g L^{-1} PVP, 50 mg L^{-1} LH, 30 g L^{-1} sucrose, and 2 g L^{-1} gellan gum (Sigma-Aldrich) (Fig. 1B). Medium without IAA was used as the control (CK). The IAA was dissolved in 100% ethanol, filter-sterilized using a 0.22-μm filter, and added to the medium after autoclaving.

Given that induction and formation of root primordia occur on the third and fifth days, respectively, and root development is completed by 20 days (He et al., 2011), we analyzed the types and contents of endogenous phenolic acids on days 0, 1, 3, 5, 7, 9, 12, 15, and 20. Each treatment consisted of three replicates of 10 shoots each. Entire *in vitro* plantlets (1-g portions) were collected at indicated times, immediately ground on ice, and extracted as described in section 2.2.

2.6. Treatment of plantlets with different phenolic acids during rooting in vitro

Rooting of plantlets was performed using shoots (3–5-cm-high new shoots with four to five leaves and no roots) after six rounds of subculturing. The shoots were cultured on WPM containing 4 mg L^{-1} IAA, 1 g L^{-1} PVP, 30 g L^{-1} sucrose, 2 g L^{-1} gellan gum, and various phenolic acids at different concentrations (0.1, 0.5, 1, 3, or 6 mg L^{-1}). Medium with no phenolic acids was used as the control. Rooting percentage, root number, and root length were recorded after 40 days of culture.

2.7. Extraction, purification and analysis of IAA

Endogenous IAA levels were determined after culturing in optimal rooting medium (4 mg L^{-1} IAA + 1 mg L^{-1} caffeic acid) for 0, 3, 5, 10, 15, and 20 days. Medium without caffeic acid (4 mg L^{-1} IAA) was used as the control. Each treatment was repeated three times. Stem bases (0.5 g fresh weight) of plantlets collected at indicated times were finely ground in liquid nitrogen, and then extracted with 10 mL 80% methanol containing 1 mM 2, 6-di-*tert*-butyl-4-methylphenol (Sigma-Aldrich) by ultrasonic disruption for 60 min at 4 °C. After 4 °C at 12 h, the extract supernatant was centrifuged at 10000*g* for 15 min at 4 °C. After concentrating the supernatant under vacuum at 35 °C to remove