

Water Response Characteristics of Phenolic Acid Allelochemicals in Reed Decomposition Soil (Postprint)

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Abstract

Phragmites australis and *Glyceria maxima* both possess strong pollutant removal capabilities and are frequently configured as wetland plants in the same constructed wetland for wastewater treatment. As a strongly allelopathic plant, *Phragmites australis* exhibits pronounced allelopathic effects on *Glyceria maxima*, leading to the replacement of *Glyceria maxima* by *Phragmites australis* in both natural and constructed wetlands. This phenomenon is associated with soil water content. Furthermore, research has demonstrated that the allelopathic inhibitory effect of *Phragmites australis* decomposition soil on *Glyceria maxima* is closely correlated with the total phenolic acid content in the decomposition soil. Therefore, this study employed high-performance liquid chromatography (HPLC) to separate and identify phenolic acids in *Phragmites australis* decomposition soil under different moisture conditions, investigated the moisture response characteristics of major phenolic acids in the decomposition soil, and screened for phenolic acid species exhibiting pronounced moisture responses. Through regulation of wetland soil moisture, the objective was to achieve competitive balance between *Glyceria maxima* and *Phragmites australis*, thereby maintaining long-term stable coexistence of both species in constructed wetlands. The results revealed that eight phenolic acids could be separated from *Phragmites australis* decomposition soil: gallic acid, coumaric acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, salicylic acid, and benzoic acid. Among these, coumaric acid, benzoic acid, and ferulic acid were present at relatively high concentrations. The contents of all eight phenolic acids showed significant linear negative correlations with the relative water content of the decomposition soil; specifically, as the relative water content of the decomposition soil increased, the phenolic acid contents decreased. The moisture response trends of each phenolic acid could be well fitted by linear equations. Among them, coumaric acid, gallic acid, and ferulic acid exhibited the most pronounced moisture responses in *Phragmites australis* decomposition soil. Therefore, coumaric

acid, gallic acid, and ferulic acid can be designated as primary regulation targets. By controlling soil moisture content in wetlands, the allelopathic inhibitory effect of *Phragmites australis* on *Glyceria maxima* can be attenuated, thereby maintaining stability of the *Glyceria maxima*-*Phragmites australis* community.

Full Text

Preamble

Characteristics of Response of Phenolic Acid Allelochemicals in *Phragmites australis* Decomposing Soil to Soil Moisture

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Abstract

Both *Phragmites australis* and *Phalaris arundinacea* exhibit strong pollutant removal capacity and are frequently deployed together as wetland plants in constructed wetlands for sewage treatment. As a potent allelopathic plant, *P. australis* exerts strong allelopathic effects on *P. arundinacea*, leading to the gradual replacement of *P. arundinacea* by *P. australis* in both natural and constructed wetlands. This phenomenon is associated with soil moisture content, and previous studies have demonstrated that the allelopathic inhibitory effect of *P. australis* decomposing soil on *P. arundinacea* is closely related to the total phenolic acid content in the decomposing soil. Therefore, this study employed high-performance liquid chromatography (HPLC) to separate and identify phenolic acid substances in *P. australis* decomposing soil under different moisture conditions, investigating the moisture response characteristics of major phenolic acids and screening for those showing pronounced moisture responses. Through regulation of wetland soil moisture, the competitive balance between *P. australis* and *P. arundinacea* can be achieved, thereby maintaining their long-term stable coexistence in constructed wetlands.

The results showed that eight phenolic acid substances were separated and identified: gallic acid, coumaric acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, salicylic acid, and benzoic acid. Among these, coumaric acid, benzoic acid, and ferulic acid were present at relatively higher concentrations. The content of each identified phenolic acid exhibited a significant linear negative correlation with the relative moisture content of the decomposing soil; that is, as the relative moisture content of the decomposing soil increased, the content of each phenolic acid decreased. The moisture response trends of all phenolic acids could be well fitted using linear equations, with coumaric acid, gallic acid, and ferulic acid showing the most pronounced responses to soil moisture in *P.*

australis decomposing soil. Consequently, these three phenolic acids can serve as primary regulatory targets. By controlling wetland soil moisture content, the allelopathic inhibitory effect of *P. australis* on *P. arundinacea* can be weakened, thus maintaining the stability of the *P. australis*-*P. arundinacea* community.

Keywords: *Phragmites australis*, phenolic acids, decomposition, allelopathy, moisture response

Introduction

Phragmites australis is a perennial rhizomatous grass with strong vegetative propagation capability. Natural populations primarily reproduce and regenerate through rhizomes, often forming monodominant communities in habitats, and are widely distributed along rivers, lakes, coastal areas, and floodplains (Zhuang et al., 2010). Numerous domestic and international studies have confirmed that *P. australis* is a potent allelopathic plant that exhibits strong allelopathic inhibition against algae such as cyanobacteria (Nakai S, 2006) and various higher plants, including invasive species like *Spartina alterniflora* (Zheng, 2009) and *Solidago canadensis* (Liu et al., 2014) (Fu et al., 2013).

Despite this strong allelopathic inhibition of *P. australis* on *P. arundinacea*, the phenomenon of *P. australis* populations gradually replacing *P. arundinacea* populations occurs in both natural and constructed wetlands as the wetland substrate accumulates and elevates (Březinová et al., 2014; Wei-Guo Fu, 2013). Given that *P. australis* is a potent allelopathic plant and that allelopathic effects exhibit strong water-environment response characteristics—specifically, “allelopathic inhibition effects increase as soil moisture decreases” (Wang et al., 2011)—we hypothesize that the gradual replacement process represents an enhancement of allelopathic inhibition by *P. australis* on *P. arundinacea* as substrate elevation leads to reduced soil moisture content.

Allelochemicals are released through pathways including rainfall leaching, volatilization, plant residue degradation, and root exudation (Lin et al., 2007). However, only root exudates and chemical substances from decomposing plant residues that enter the soil show high correlation with soil moisture content. Yin (2015) investigated the allelopathic inhibitory effects of *P. australis* root exudates on *P. arundinacea* and their water-environment response characteristics, identifying and separating seven phenolic acids in roots and rhizosphere soil: coumaric acid, vanillic acid, gallic acid, benzoic acid, syringic acid, ferulic acid, and salicylic acid. Japanese scholar Nakai S isolated phenolic allelochemicals including coumaric acid, ferulic acid, gallic acid, and vanillic acid from *P. australis* decomposing solution. Regarding plant residues and litter, another study used phosphomolybdic-phosphotungstic acid colorimetry to measure total phenolic acid content in decomposing *P. australis* litter and its allelopathic inhibitory effect on *P. arundinacea* (Fu et al., 2015), but did not further identify, separate, or quantify the specific phenolic acids present in the decomposing material, nor investigate their moisture response characteristics.

Therefore, this study will employ HPLC to prepare decomposing soil from *P. australis* litter, identify, separate, and quantify various phenolic acids under different moisture conditions, and select those showing pronounced moisture responses as primary regulatory targets. By regulating wetland soil moisture content, we aim to maintain long-term competitive coexistence of *P. australis* and *P. arundinacea* populations, thereby providing theoretical support and technical guidance for community stability management in *P. australis*-*P. arundinacea* constructed wetlands.

1.1 Study Area Overview

The Zhenjiang riverside wetland in the lower reaches of the Yangtze River (32°15 N, 119°28 E) experiences a transitional monsoon climate from warm temperate to north subtropical zones. Influenced by monsoons, the area has distinct seasons with warm, humid conditions. The mean annual temperature is 15.4°C, annual precipitation is 1074.0 mm, and annual evaporation ranges from 847 to 1755.9 mm. Formed through long-term sediment deposition carried by river water, the wetland has complex hydrological conditions, including daily variations in tidal river sections and seasonal changes during flood, normal, and dry periods. Vegetation grows luxuriantly, developing through primary succession on bare river beaches. As the wetland substrate elevates, *P. australis* communities gradually replace *P. arundinacea* communities to become the climax community.

1.2 Experimental Materials and Design

Collection and processing of *P. australis* litter: In early November 2016, dead *P. australis* plants were collected from the Zhenjiang riverside wetland, sun-dried, and ground into powder. Concurrently, newly deposited soil was collected from bare beaches in the same wetland, sun-dried, crushed, cleaned, and sieved. The soil and *P. australis* powder were then mixed uniformly at a 3:1 volume ratio (based on the unit area ratio of soil to *P. australis* residues in the riverside wetland). The mixed samples were placed in 12 plastic buckets (specifications: 24 cm upper diameter, 26 cm lower diameter, 22 cm height) for decomposition. Four moisture treatments were established during decomposition: T1, T2, T3, and T4 (Table 1), with three replicates per treatment (each bucket representing one replicate). The gravimetric method was used to maintain soil moisture content within the designated ranges throughout the decomposition period. On November 1, 2017, phenolic acids were separated, identified, and measured in each treatment, with mean values used for analysis.

1.3.1 Instruments and Reagents

The following instruments were used: Thermo UltiMate 3000 HPLC system (quaternary pump, UV detector, Chromeleon 7 workstation, Thermo Fisher Scientific); R-201 rotary evaporator (Shanghai Yarong Biochemical Instrument Factory); SHZ-D(III) vacuum pump (Henan Yuhua Instrument Co., Ltd.); KH-100 ultrasonic cleaner (Kunshan Hechuang Ultrasonic Instrument Co., Ltd.);

Neofuge 18R high-speed centrifuge (Heal Force Bio-Meditech); CHA-S air-bath thermostatic oscillator (Jintan Ronghua Instrument Manufacturing Co., Ltd.); and BSA224S electronic analytical balance (0.1 mg precision, Sartorius, Germany). Standard compounds including gallic acid, ferulic acid, benzoic acid, vanillic acid, coumaric acid, syringic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, salicylic acid, caffeic acid, sinapic acid, and chlorogenic acid were purchased from Shanghai Chenyi Biotechnology Co., Ltd.

1.3.2 Chromatographic Conditions

The chromatographic column was a Thermo Accucore XL C18 (250 mm × 4.6 mm, 4 μm). Mobile phase A was HPLC-grade methanol, and mobile phase B was 1% acetic acid aqueous solution. UV detection wavelength was set at 280 nm, column temperature at 30°C, and gradient elution conditions were: 0–8 min, methanol 5%–40%; 8–19 min, methanol 40%–35%; 19–30 min, methanol 10%. Flow rate was 1 mL/min.

1.3.3 Standard Solution Preparation

Accurately weighed 10 mg of each of the 10 phenolic acid standards was placed in a 10 mL volumetric flask, dissolved and diluted to volume with 50% methanol aqueous solution to obtain a 1 mg · mL⁻¹ mixed stock solution of the 10 phenolic acids. This stock solution was serially diluted 2, 10, 50, and 100 times with 50% methanol aqueous solution to prepare a series of standard mixed solutions. Additionally, 1 mg of each of the 10 phenolic acid standards was accurately weighed and placed in separate 10 mL volumetric flasks, dissolved and diluted to volume with 50% methanol aqueous solution to obtain 1 mg · mL⁻¹ individual phenolic acid solutions for peak identification and qualification in HPLC.

1.3.4 Preparation of *P. australis* Decomposing Soil Sample Solutions

Ten grams of *P. australis* decomposing soil was placed in a conical flask, extracted with 100 mL of 50% methanol aqueous solution by shaking at room temperature for 6 hours, then centrifuged to collect the supernatant. The supernatant was concentrated to 1 mL using a rotary evaporator (vacuum, 30°C) and filtered through a 0.22 μm organic membrane.

1.4 Statistical Analysis

Linear regression analysis was performed to examine the response relationship between phenolic acid content in *P. australis* decomposing soil and relative soil moisture content. Data processing and significance analysis were conducted using Excel 2007 and SPSS 11.5 statistical software.

2.11 Selection of Detection Wavelength

The maximum absorption spectra of the 10 phenolic acid standard samples were scanned across a wavelength range of 190–400 nm. Analysis of the maximum absorption spectra revealed that all 10 phenolic acids had significant absorption peaks around 280 nm. Therefore, 280 nm was selected as the quantitative detection wavelength for phenolic acids.

2.12 Selection and Optimization of Mobile Phase

Because phenolic hydroxyl and carboxyl groups in phenolic acids readily ionize in aqueous solution, enhancing polarity and causing dual retention on the stationary phase, severe peak tailing occurs. Adding an appropriate acidic modifier can suppress ionization of polyphenols to some extent, reduce polarity, enhance retention on the stationary phase, and improve separation and peak shape (Liu et al., 2002). Acetic acid was used as the acidic modifier for the mobile phase, with its concentration affecting both retention time and peak shape of phenolic acids. Different concentrations of acetic acid aqueous solution (0.5%–2%) were tested for separation efficiency of the 10 phenolic acids, with 1% acetic acid aqueous solution ultimately selected. Isocratic elution provided unsatisfactory peak separation, whereas gradient elution achieved good separation. After multiple optimization trials, the optimal gradient conditions were: 0–8 min, methanol 5%–40%; 8–19 min, methanol 40%–35%; 19–30 min, methanol 10%; flow rate 1 mL · min⁻¹. Under these gradient conditions, all 10 phenolic acids were well separated with good peak shape and stable retention times.

2.2 Identification of Phenolic Acid Types in *P. australis* Decomposing Soil

Appropriate amounts of individual phenolic acid standard solutions were added to the mixed phenolic acid standard for analysis. By comparing the chromatograms with and without the added individual standard, peaks showing significantly increased area were identified as corresponding to that phenolic acid. This method was used to confirm the retention positions and times of all 10 phenolic acids. By comparing retention times, the chromatogram of the *P. australis* decomposing soil sample solution was matched against the mixed standard chromatogram, revealing the presence of eight phenolic compounds: gallic acid, coumaric acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, salicylic acid, and benzoic acid. The chromatograms of the mixed phenolic acid standards and the *P. australis* decomposing soil sample solution are shown in Figure 1 [Figure 1: see original paper].

2.2 Moisture Response Relationships of Phenolic Acids in *P. australis* Decomposing Soil

The serially diluted mixed solutions of the 10 phenolic acids were injected (10 L each), and standard curves were plotted with phenolic acid concentration

x (g/mL) as the abscissa and peak area S (mAU) as the ordinate. Linear regression equations, correlation coefficients, and linear ranges were calculated, with detection limits determined based on a signal-to-noise ratio ($S/N = 3$) (Table 2). The determination coefficients of the linear regression equations for all 10 phenolic acids exceeded 0.999, showing extremely significant correlations at $P < 0.01$, making them suitable for quantitative HPLC analysis of phenolic acids in *P. australis* decomposing soil.

Based on these standard curve equations, the contents of various phenolic acids in *P. australis* decomposing soil samples under different treatments were calculated. Linear regression was then performed between each phenolic acid content and the corresponding relative moisture content of the decomposing soil, yielding fitted equations and plots (Figure 2 [Figure 2: see original paper]). The determination coefficients indicated that fitting the moisture response of each phenolic acid using the linear equation $Y = ax + b$ produced excellent results. Significance analysis revealed that the contents of all eight identified phenolic acids showed extremely significant negative correlations with the relative moisture content of the decomposing soil ($P < 0.01$). Based on the slope of the fitted equations, coumaric acid showed the most pronounced response to relative moisture content in decomposing soil, followed by gallic acid and ferulic acid, then vanillic acid, *p*-coumaric acid, salicylic acid, and benzoic acid, with syringic acid showing the weakest response.

3. Conclusions and Discussion

Soil microorganisms can utilize phenolic acids as carbon and energy sources. Under limiting conditions, phenolic acids can provide carbon sources for nitrogen-fixing bacteria, which is a determining factor for whether allelopathic activity is expressed. In other words, microorganisms can either reduce or amplify phenolic acid toxicity. Soil moisture is the primary controlling factor for the availability and mobility of soluble organic matter in soil (Casals et al., 2000), and soluble organic matter is the main respiratory substrate and energy source for soil microorganisms (Chen, 2003). Therefore, changes in soil moisture conditions profoundly affect microbial respiration in soil, thereby influencing the allelopathic activity of phenolic acids. Meanwhile, increased soil moisture reduces soil aeration and oxygen content, which also affects nitrification and nitrogen fixation in soil to some extent. Soil microbial nitrogen fixation partially determines the transformation and allelopathic activity of phenolic acids (Kefeli et al., 2003). Consequently, the moisture response of phenolic acid allelochemicals in *P. australis* decomposing soil may be achieved through the combined effects of multiple factors including soil aeration, soil nitrogen cycling, and microbial activity.

Plant litter can release phenolic acids with significant allelopathic effects on itself or other plants through decomposition. Wu (2014) detected six phenolic acids in the rhizosphere soil of *Panax notoginseng*: *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and benzoic acid, and confirmed

that their concentrations had reached levels that significantly inhibited *P. notoginseng* growth. Li et al. (2011) found that benzoic acid, phenylpropionic acid, and salicylic acid from grape root decomposition products had significant allelopathic inhibitory effects on tissue culture seedlings of *Vitis amurensis* × *Vitis riparia*. Using 10 common allelopathic phenolic acids as standards, this study identified eight phenolic acids in *P. australis* decomposing soil using HPLC: gallic acid, coumaric acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, salicylic acid, and benzoic acid. Compared with phenolic acids isolated from *P. australis* roots by Yin (2015), *p*-coumaric acid was additionally detected in this study. Compared with phenolic acids isolated from *P. australis* decomposing solution by Nakai S (2006), syringic acid, *p*-coumaric acid, salicylic acid, and benzoic acid were additionally detected. These differences suggest that soil microorganisms may influence the allelopathic activity of phenolic acids to some extent. The contents of all eight phenolic acids in *P. australis* decomposing soil showed significant linear negative correlations with relative soil moisture content; that is, as the relative moisture content of *P. australis* decomposing soil increased, the contents of all phenolic acids decreased, and the relationship between each phenolic acid content and decomposing soil moisture could be fitted with linear equations. Different phenolic acids showed varying response intensities to moisture in decomposing soil, with coumaric acid showing the most pronounced response, followed by gallic acid and ferulic acid.

Through investigating the moisture response relationships of phenolic acid contents in *P. australis* decomposing soil, we identified coumaric acid, gallic acid, and ferulic acid as three phenolic acids with strong moisture response characteristics—showing more significant decreases in content as soil moisture increased. Therefore, in maintaining the stability of *P. arundinacea*-*P. australis* constructed wetland plant communities, these three phenolic acids can serve as primary regulatory targets. By controlling wetland soil moisture content, the allelopathic inhibitory effect of *P. australis* on *P. arundinacea* can be weakened, thus maintaining community stability. This conclusion is based on moisture response relationships of phenolic acid contents. However, among the various phenolic acids, some may show only minor content decreases with increasing soil moisture (i.e., weak moisture response) but exhibit significant reductions in allelopathic inhibitory effect. Such allelochemicals could also serve as primary regulatory targets. Therefore, dose-effect studies on allelopathic inhibition of different phenolic acids are needed to more precisely and comprehensively identify appropriate regulatory targets for achieving competitive balance between *P. arundinacea* and *P. australis* through wetland soil moisture regulation, thereby maintaining long-term stable coexistence of both species in constructed wetlands.

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