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Optimized extraction methodology for phenolic compounds in soil and plant tissues: Their implications in plant growth and gall formation ☆

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A R T I C L E I N F O

Method name:
Extraction of Phenolic Compounds and Total Phenolics in Soil Samples and Plant Tissues

Keywords:
Phenolic compound
LC–MS/MS
GC–MS
TTC method
Plant tissue
Seed germination
Plant-gall

A B S T R A C T

Phenolic compounds, abundant secondary metabolites in plants, profoundly influence soil ecosystems, plant growth, and interactions with herbivores. Phenolic in soil microorganisms have the potential to impact a wide range of activities in plant-soil interactions. However, the existing methods for measuring microbial activity are typically time-consuming, intricate, and expensive. In this study, we propose modifications to the method used for the extraction and quantification of various types of phenolics in soil and plant tissues. There have been substantial advancements in research aimed at extracting, identifying, and quantifying phenolic compounds in the plant and soil samples. This study discusses the use of different methodologies in the analysis of phenolic compounds. In addition, we investigated the effect of phenolics on plant growth and cues in gall-forming under environmental disturbances.

- This method is the optimum way to extract phenolic from soil and microbial activity in bulk and rhizosphere soil.
- It can be used on any soil type and plant tissue, metabolites extracted from living organisms.

Specifications table

Subject area:	Environmental Science
More specific subject area:	Metabolite analysis
Name of your method:	Extraction of Phenolic Compounds and Total Phenolics in Soil Samples and Plant Tissues
Name and reference of original method:	The methodology was adapted according to the references in Supplementary data from: N. Kanjana, Y. Li, Z. Shen, J. Mao, L. Zhang, Effect of phenolics on soil microbe distribution, plant growth, and gall formation, Sci. Total Environ. (2024) 17–1329.
Resource availability:	*N/A*

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Background

Phenolic acid is a prominent category of phenolic chemicals found in plants. It has been discovered in plants' seeds, skins, and leaves, which exist in a bound form. Phenolic acids are typically present in amides, esters, and glycosides but seldom in their free form [1]. According to the research done by Clifford (1999), hydroxybenzoic acid and hydroxycinnamic acid are the two sub-groups of phenolic acids. Hydroxycinnamic acids are often present in food as simple esters with quinic acid and glucose. Hydroxybenzoic acids are derived from benzoic acid, are found in soluble form, and are bound with cell wall fractions such as lignin [2-4].

Phenolic chemicals are not found in all plants, and their stability varies, complicating the process of extracting these compounds. Also, inhibition, observed when various plants coexist briefly, can release biologically active compounds into the environment, affecting the growth of neighboring plants [5,6]. The interaction involves releasing chemicals through plant waste decomposition, leaching, volatilization, and exudation, exerting advantageous or adverse effects on surrounding plants [7]. Chemicals in rhizospheric soil influence microbial community composition. Allelochemicals can reduce soil microbial diversity, significantly affect the genetic structure and carbon metabolism capacity of microbial communities, and lead to imbalances in soil microbial ecosystems [8]. Allelochemicals can reduce soil microbial diversity,significantly affect the genetic structure and carbon metabolismcapacity of microbial communities, and lead to imbalances insoil microbial ecosystems [8]. Phenolics constitute one of the most abundant components in soil [9] and play a vital role in the cycling of essential nutrients beneficial to plants and soil microbes [10]. Moreover, phenolic abundant secondary metabolites in plants, profoundly influence soil ecosystems, plant growth, and interactions with herbivores [11].

However, the absence of identified metabolites for additional bioactive compounds suggests possible limitations in derivatization during the study procedure. While Gas chromatography–mass spectrometry (GC–MS) offers advantages in spectral resolution and Liquid chromatography–mass spectrometry (LC-MS), its effectiveness in identifying an extensive array of metabolites might be variable [12,13]. The recovery rate of phenolic compounds from plant and soil samples may be affected by inefficient extraction methods or by attempting to extract them in a single step. Hence, carefully selecting an appropriate extraction process is crucial for successfully recovering the desired phenolic compounds. In order to gain a more comprehensive comprehension of the extraction techniques employed to collect phenolic compounds from plants and soil, this study will specifically examine the widely utilized extraction methods that offer the most effective means of extracting phenol and secondary metabolites from plant and soil samples.

Thus, our objective is to elucidate the roles of phenolic compounds in soil microorganisms and their impacts on plant development, and gall formation. Our specific aims were to (1) Develop methodologies for the extraction and quantification of phenolics in soil samples and plant tissues and (2) Investigate how phenolics influence plant growth and gall formation [11] (Fig. 1).

Method details

Materials and methods

Chemicals and reagents

The Folin–Ciocalteu (FC) reagent and sodium carbonate (Na_2CO_3) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid, triphenyl tetrazolium chloride (TTC), and phenol with a purity of 99.7 % were obtained from Adamas Reagent (Shanghai, China). Ethanol and hexane of GC grade were procured from Sinopharm Chemical Reagent (Shanghai, China).

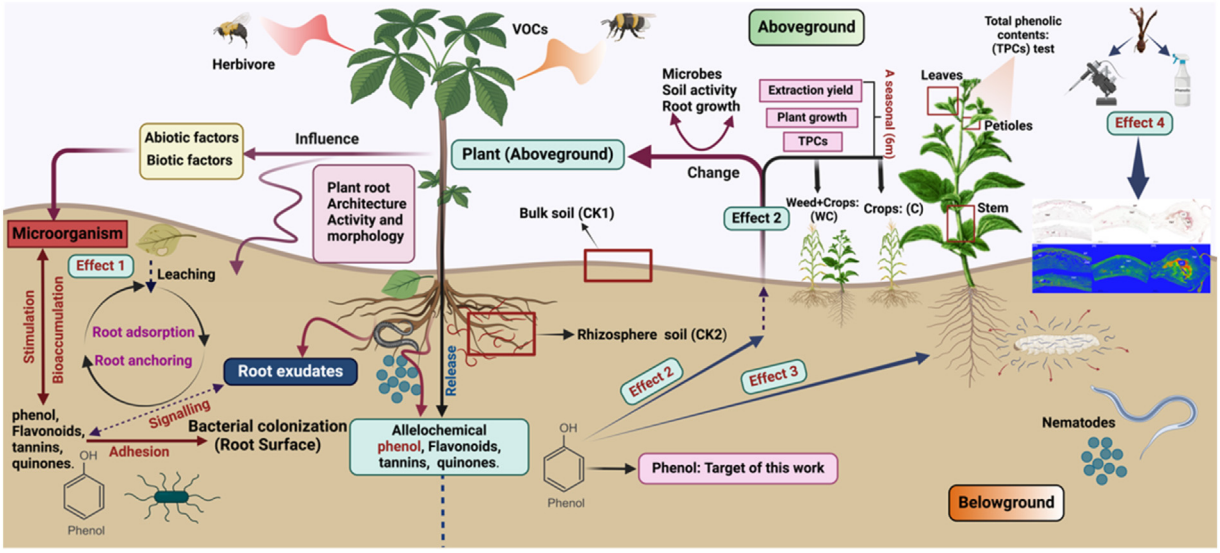

Fig. 1. Schematic of the research evolution of plant allelopathy-microbes interaction on plant growth and gall-forming [11].

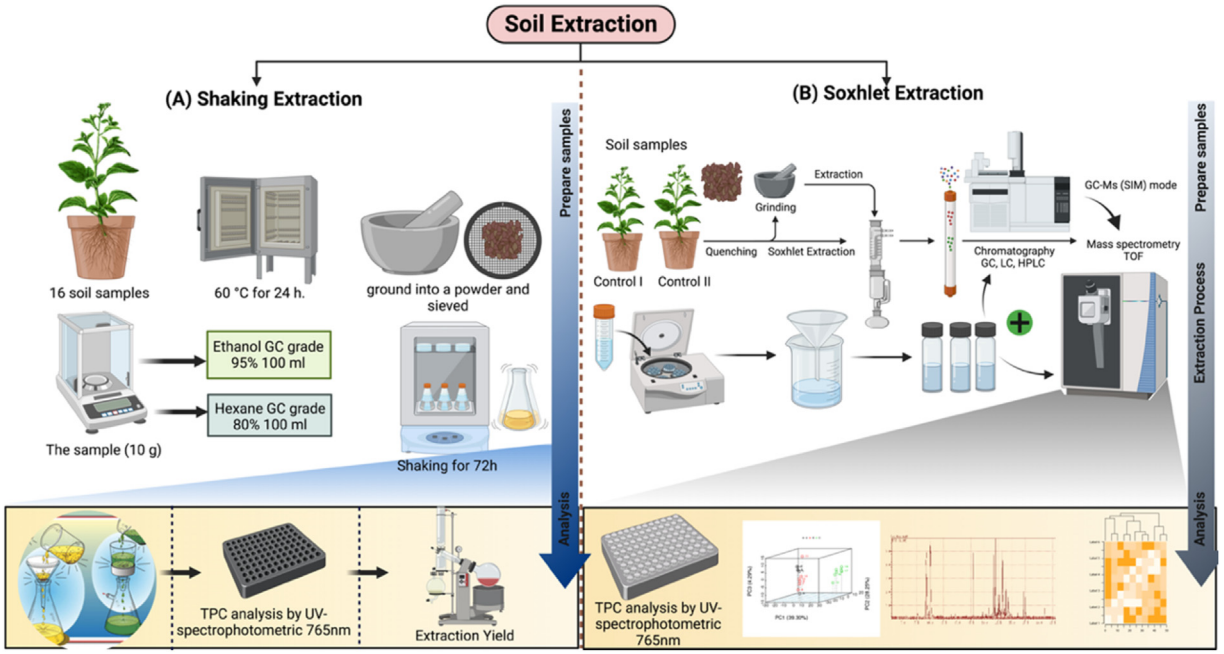

Fig. 2. Phenol extraction from soil samples using **(A)** the shaking method and **(B)** Soxhlet extraction [11].

Soil sampling and extraction

The air-dried soil sample (10 g) was macerated in a stoppered 250-mL Erlenmeyer flask containing 100 mL of 95 % ethanol and 80 % hexane. The mixture was agitated at 190 rpm for 72 h, then filtered through Whatman No. 1 filter paper (125 mm) and concentrated using a rotary evaporator (Stuart RE 400, UK). This extraction protocol was adapted from the study by Akomeng et al. [14], with minor modifications. To examine the seasonality of phenolic compounds, we weighed the dry residue of the extract and determined the extraction yield over six months. Using a Soxhlet apparatus, we dried a 10-g powdered soil sample, placed it into a thimble chamber, and then set in a thimble holder condenser attached to a 250-mL distillation flask. In addition, 10 g of soil and 200 mL of 80 % methanol were used, and the extraction was conducted at 60 ˚C for 24 h. After extraction, the residue was dried using a solvent centrifugal concentrator (Concentrator Plus, Hamburg, Germany) and filtered using a vacuum pump. The extracts were then stored in airtight containers at −20 ˚C until further analysis (Fig. 2). In addition to selecting the optimal extraction solvent, two other essential parameters affect the yield of phenolics extracted from plants and soil: time and temperature. Typically, increasing time and temperature promote analyte solubility; however, plant phenolics are generally degraded or undergo undesirable reactions, such as enzymatic oxidation by extended extraction times and high temperatures [15,16].

Plant tissue sampling and extraction

The fresh aerial parts of plant sample, including the leaves, petioles, and stems, were thoroughly washed with distilled water and then dried at 40 ˚C in a drying oven. Subsequently, samples (5 g) were subjected to ultrasonic bath extraction (KQ-600DB, 40 kHz, Kunshan Ultrasonic Instruments, China) using 220 V of power frequency and 50 mL of 70 % ethanol at 35 ˚C for 25 min, determined as the optimal frequency [17]. The extracts were then centrifuged at 5000 × *g* for 15 min (Fig. 3). The supernatant was collected and extracted again under the same conditions. The supernatant was then filtered, and the resulting extract was used immediately for property analysis.

Extraction of metabolites

Tissue samples weighing 100 mg were pulverized with liquid nitrogen, and the resultant homogenate was mixed with precooled 80 % methanol, and the mixture was incubated on ice for 5 min. Then, the samples were centrifuged at 15,000 × *g* and 4 ˚C for 20 min. A portion of the supernatant was diluted with Liquid chromatography-mass spectrometry LC–MS grade water to a final concentration of 53 % methanol. This mixture was then transferred to a new Eppendorf tube and centrifuged again at 15,000 × *g* and 4 ˚C for 20 min. Finally, the supernatant was used for LC–MS/MS analysis [18].

Individual phenolic acid content by UHPLC–MS/MS

UHPLC–MS/MS analysis was performed at Novogene (Beijing, China) by using an Orbitrap Q Exactive HF mass spectrometer and a Vanquish UHPLC system from Thermo Fisher. Samples with a particle size of 1.9 μm and dimensions of 100 × 2.1 mm^2 were injected onto a Hypersil Gold column with a flow rate of 0.2 mL/min and under a 17 min linear gradient. The eluents for the positive polarity mode were methanol and eluent A, which was 0.1 % FA in water, whereas the eluents for the negative polarity mode were

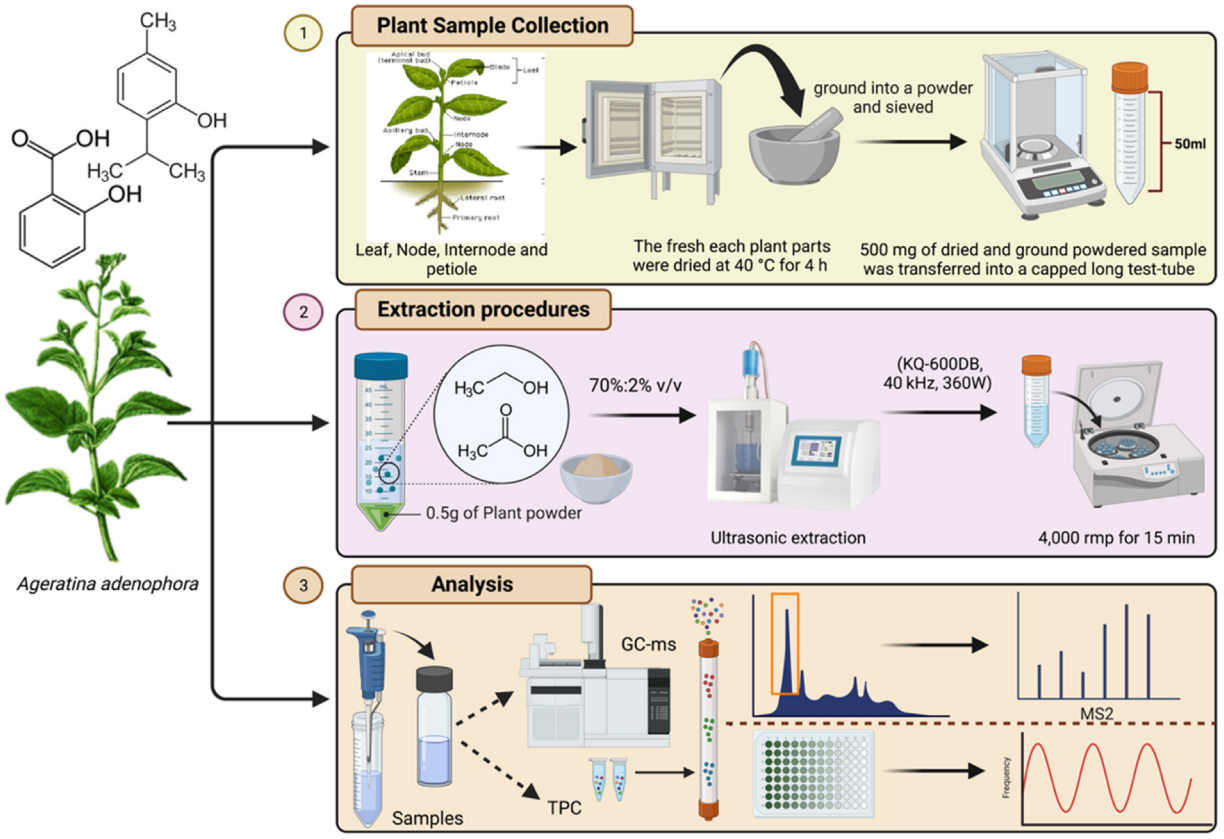

Fig. 3. Comparative analysis of phenol extraction efficiency in the stem, petiole, and leaf tissues using ultrasonic methods [11].

methanol and eluent A, which was 5 mM ammonium acetate with a pH of 9.0. The solvent gradient was set at 2 % B for 1.5 min, 2 %–85 % B for 3 min, 85 %–100 % B for 10 min, 100 %–2 % B for 10.1 min, and 2 % B for 12 min. With a spray voltage of 3.5 kV, capillary temperature of 320 °C, sheath gas flow rate of 35 psi, aux gas flow rate of 10 L/min, S-lens RF level of 60, and aux gas heater temperature of 350 °C; the Q Exactive HF mass spectrometer was used in positive/negative polarity mode. Recently, the use of high-performance TLC (HPTLC) has improved the TLC technique due to its superior resolution and reproducibility [19,20].

Microbiome assay

Before performing data analysis, a comprehensive assessment of the obtained data's reliability was conducted. Principal component analysis (PCA) was employed for contrasting the spectral data from quality control (QC) samples, which is a common practice in mass spectrometry-based metabolomics research. This comparison aimed to ensure the consistency and stability of the experimental setup. To prevent any spectral pattern overlap that could potentially influence result accuracy, a meticulous inspection of the total ion chromatograms (TICs) of the quality control samples was also carried out, drawing insights from previous studies [21,22].

Total genomic DNA was extracted from the samples by using the CTAB/SDS method, and 1 % agarose gel was employed to assess the concentration and purity of the DNA. Based on the determined concentration, DNA was diluted to 1 ng/μL by using sterile water. The 16S rDNA genes were amplified with barcode-specific primers. Each PCR reaction contained 30 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 15 μL of primers (0.2 μM for forward and reverse), and approximately 10 ng of template DNA. Thermal cycling included a 1-min initial denaturation at 98 °C, 30 cycles of 10-s denaturation at 98 °C, 30-s annealing at 50 °C, 30-s elongation at 72 °C, and a final elongation at 72 °C for 5 min. Index codes were added, and sequencing libraries were prepared using the Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) following the manufacturer's instructions. The quality of the library was assessed using the Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Sequencing was conducted on an Illumina NovaSeq platform, generating paired-end reads of 250 bp [23-25].

Determination of bioactive compounds in plant and soil samples through SPME-GC–MS

As an internal standard, 300 μL of the extraction sample was transferred into a 2-mL headspace bottle. The samples were analyzed using a gas chromatograph system connected to a mass spectrometer (GC–MS). The SPME fiber, used for detecting VOCs, was an 85-μm carboxen/polydimethyl siloxane fiber from Sigma-Aldrich, Australia (catalog number 57,334-U), with a Stableflex 24Ga manual holder and 3PK coating, selected to capture volatiles from three species and an invasive weed.

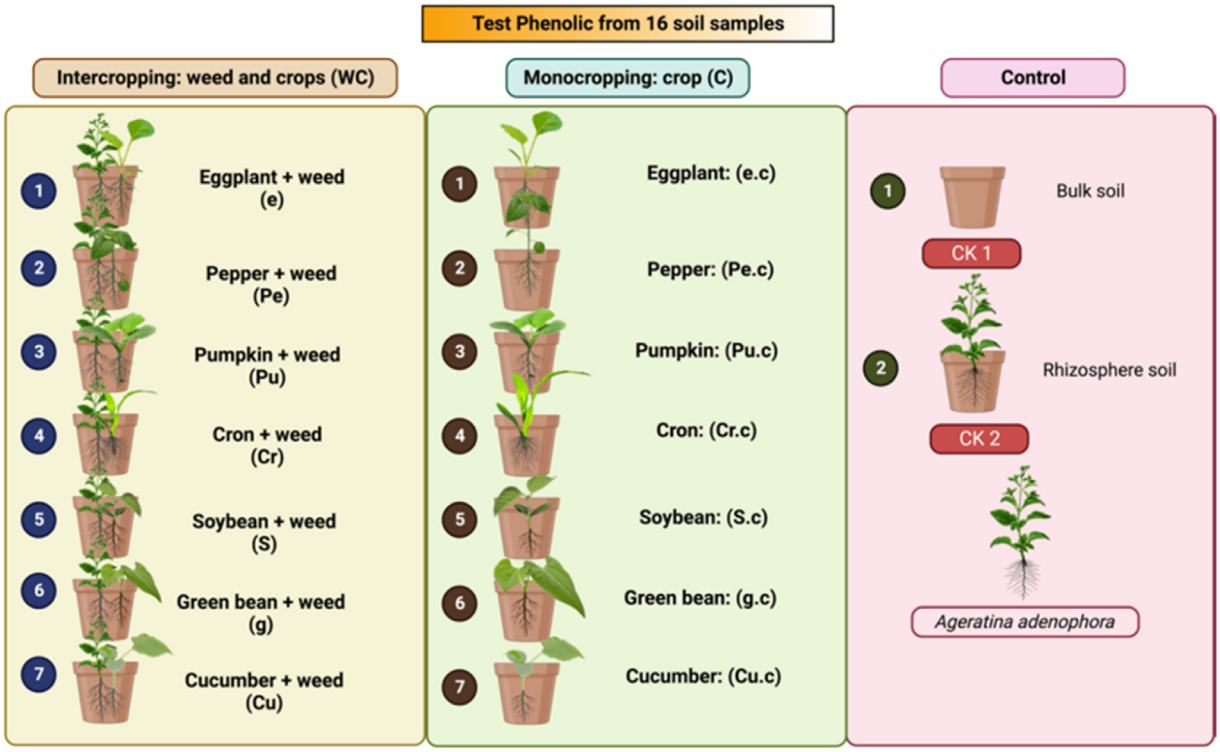

Fig. 4. Schematics of different planting patterns between crop and weed (WC) and monocropping (C) [11].

The Agilent 7890 gas chromatograph, coupled with a 5977B mass spectrometer, was used to perform Gas chromatography–mass spectrometry (GC–MS) analysis. In the PAL system's SPME cycle, desorption occurred over 4 min, with the incubator temperature set at 40 °C and a 15-min preheat time. A DB-Wax column was used in a splitless mode. The gas flow through the column was set at 1 mL/min, with a front inlet purge flow of 2.5 mL/min, and helium served as the carrier gas. The initial temperature was held at 40 °C for 3 min and then increased to 180 °C at 5 °C/min for 5 min. The temperatures for the injection, transfer line, ion source, and quadrupole were 250 °C, 250 °C, 230 °C, and 150 °C, respectively [26]. In the electron impact mode, the energy was −70 eV. Mass spectrometry data were acquired in scan mode with a solvent delay of 3 min and 20–500 *m/z* range. Compound identification was performed using GC–MS SOLUTION v. 4.20 (Shimadzu) by matching their mass spectra with those of authentic standards or reference spectra from databases (Wiley275, NIST98) [27].

Assay for total phenolic content

With a slight modification, the FC reagent was used to determine the total phenolic content (TPC) of the soil sample and the aerial portions of plant sample [28]. Then, 100 μL of the extract samples were carefully added along with 2.5 mL of FC reagent, and the mixture was left for 7 min. The sample solutions and reaction mixture were mixed with 500 μL of Na_2CO_3 (7.5 g/100 mL) and left to incubate for 45 min at room temperature in a dark environment. Finally, based on a standard gallic acid curve (0 to 400 μg/mL), referring to the method by Safdar et al. [29], the absorbance at 765 nm was determined using a UV spectrophotometric method. The TPC is expressed in milligram of gallic acid equivalent (GAE) per gram of dry weight (mg GAE/g dw). Gallic acid has been shown to specifically target the adipose tissue to suppress lipogenesis, improve insulin signaling, and concomitantly combat raised proinflammatory response and oxidative stress, as well as reduce excessive lipid storage in obese subjects. In this study, we use gallic acid to identify standard curve and identify phenol in our soil and plant samples [30].

The quantification of phenolic compounds relies on various parameters, including the compounds' chemical characteristics, the extraction process employed, the particle size, the choice of standards, and the presence of interfering substances and impurities [31].

Effect of soil phenolic seasonality on plant growth

In the previous study, we applied phenol to the invasive *Ageratina adenophora* (*A. adenophora*) and intercropping planted to investigate the phenol effect on plant growth [11]. At the Chinese Academy of Agricultural Sciences Institute of Plant Protection, a 6-month field experiment was conducted. The seeds of eggplant (*Solanum melongena*), cucumber (*Cucumis sativus*), green beans (*Phaseolus vulgaris*), pumpkin (*Cucurbita maxima*), maize (*Zea mays*), and Tabasco pepper (*Capsicum frutescens*) were used for seed germination studies. These seeds along with *A. adenophora* were used for the crop growth studies (Fig. 4). Two treatments were designed: one where the seven species were grown with *A. adenophora* and another without weeds. The experiment conducted from

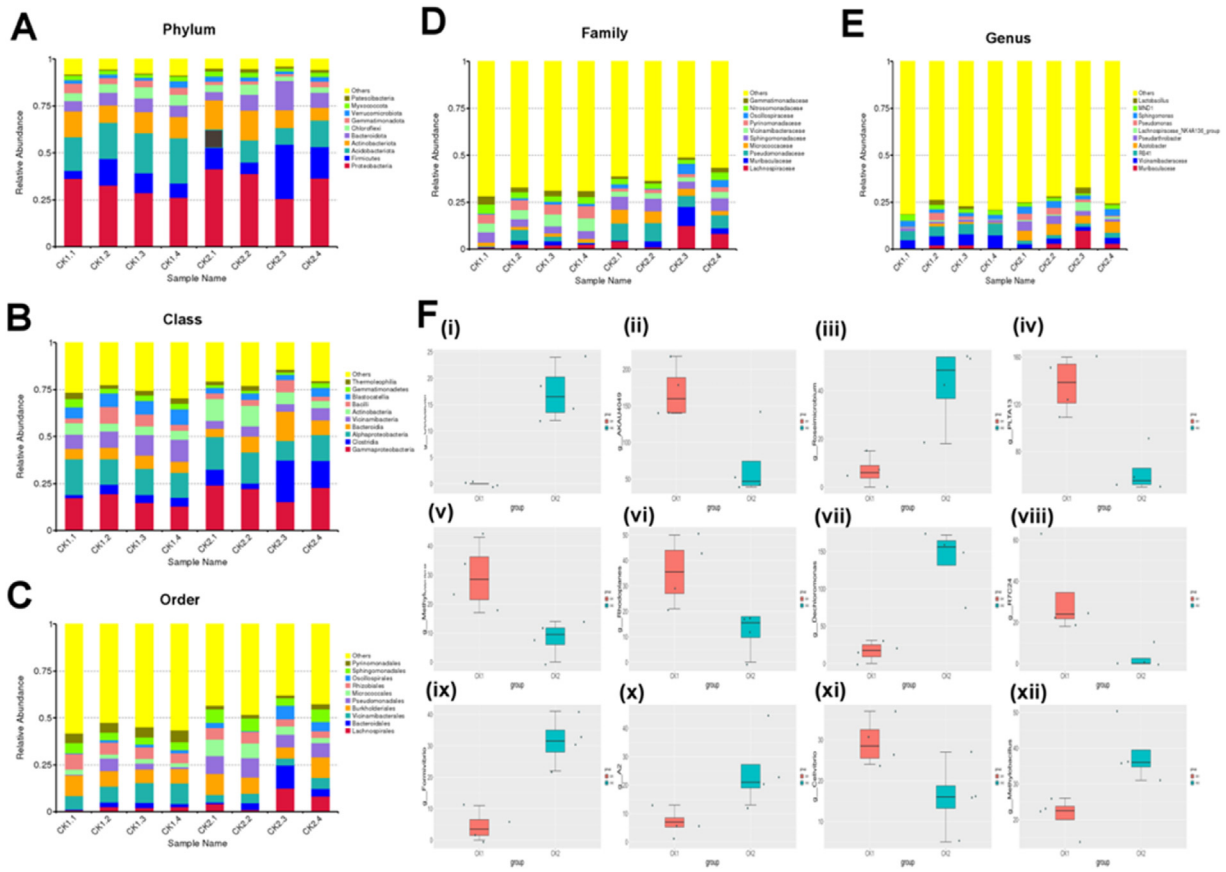

Fig. 5. Organization of soil bacterial communities: Relative abundance stack graphs at the phylum, class, order, family, and genus levels **(A–E)** and box plots of beta-diversity measures for taxa at the genus level in response to plant metabolites and time **(F)** with lower-case letters denoting multiple comparison corrections for Friedman tests (right panels) or Mann-Whitney tests (left panels) (n = 4) [11].

June to December 2022 investigated the allelopathic effects on plant root interactions, plant growth, and the relationship between crop germination and the persistence of phenolic acids in the soil rhizosphere. Weed seeds were sourced from Yunnan Agriculture University in China, whereas the crop seeds were provided by Jingyan Yinong (Beijing) Seed Sci-tech.

To prepare for the experiments that required combining plants with the seven species of grains each month, we grew weeds in 36 pots in June 2022. Twenty pots of each of the seven crop species were planted with weeds (T1) and without weeds (T2), leaving the soil in the remaining pots bare. To assess the effect of allelopathy on crop seed viability, we placed 30 ungerminated seeds of each species in 90-mm Petri dishes filled with sand and 10 mL of water. We conducted tests monthly for both T1 and T2 treatments. The Petri dishes were sealed with Parafilm, eliminating the need to replenish the water during the germination test. They were arranged in a fully randomized design with four replicates in a thermostatically controlled incubator. For 14 days, the seeds were allowed to germinate at a constant temperature of 15 ˚C ± 0.5 ˚C (optimal, control). The viability of ungerminated seeds after 14 days was determined using the TTC method at the end of the experiments [32] shown in (Fig. 6). Generally, more compounds are extracted in an ethanol solution than just phenolic compounds. Therefore, total extraction yields and TPC of the soil extracts were determined considering this factor. The presence of *A. adenophora* weed significantly affected crop germination rates [33] (Fig. 7).

Host plant response to phenolics inducing gall formation

Seeds from wild-type populations, collected from agricultural fields and stored at 15 ˚C, were provided by the Yunnan Agricultural University farm. These seeds were sown in a plastic 126 cavity seedling tray (48 mm, size: 540 × 350 mm) containing 2 kg of a soil mix (soil: sand: peat, 60:30:10; v/v) [34] in a controlled growth room. The environment was maintained at 26 ˚C ± 1 ˚C and 60 % ± 5 % humidity, with a 16/8-h light–dark cycle. After two weeks, the seedlings were transferred to plastic pots with the diameter and height of 9 cm. To examine the effects of phenolic acid on *A. adenophora*, including natural enemy release, plant growth, insect infestation, and gall formation in the gall tissue, two application methods were used: injection (10, 60, and 100 mg/μL) and spray (10, 60, and 200 mg/L). Distilled water acted as the control in this experiment.

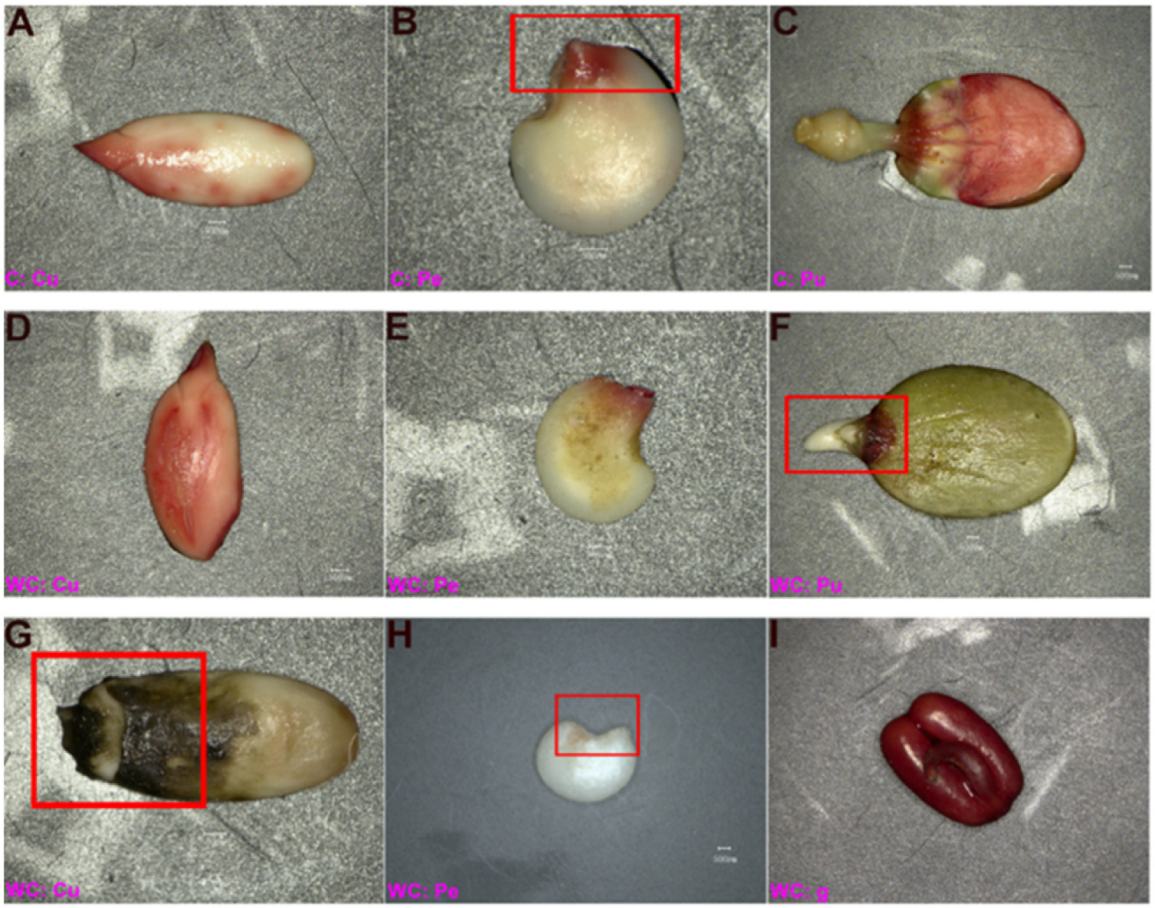

Fig. 6. Assessment of seed viability using 2,3,5-triphenyl tetrazolium chloride (TTC). **(A–F, I)**: Viable seeds (red-colored embryos); **(G and H)**: Nonviable seeds (black or colorless embryos) [11].

Statistical analysis and data visualization

The standard error of the mean (SEM) is presented with the data as the mean ± SEM. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparison test or Student's t-test. All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 19.0 (IBM Corporation, Armonk, NY, USA). A P value of <0.05 was considered statistically significant. GraphPad Prism version 8.00 (GraphPad Software, San Diego, CA, USA) was used for graphical evaluations using Pearson's correlation test. Peak alignment, peak picking, and quantitation of each metabolite were performed on UHPLC–MS/MS raw data files using Compound Discoverer 3.1 (CD3.1, Thermo Fisher) software. QIIME quality filters were initially applied to the reads, followed by OTU table creation using pick_de_novo_otus.py to cluster sequences into operational taxonomic units (OTUs) with the minimum similarity threshold of 97 %. A representative sequence was selected for each OTU for taxonomic annotation by using the RDP classifier [35]. For alpha diversity analysis, the OTU table was rarified, and three metrics were computed: Chao1 for species abundance, observed species for the number of unique OTUs per sample, and the Shannon index. These metrics were used to generate rarefaction curves.

Phylogenetic measures of beta diversity, including weighted and unweighted UniFrac, were calculated using QIIME. Principal coordinate analysis (PCoA) and clustering using the unweighted pair group method with arithmetic mean (UPGMA) were conducted with unweighted UniFrac. PCoA helps visualize complex, multidimensional data by deriving principal coordinates from a distance matrix and representing them on orthogonal axes, with each axis explaining a portion of the total variation. UPGMA, a hierarchical clustering method using average linkage, was used to interpret the distance matrix. To explore variations in microbial diversity between samples, statistical techniques such as t tests, MetaStat, LefSe, Anosim, and MRPP, were used to test the significance [36].

Soil microbe's community from between the rhizosphere and bulk soil

The soil environment, a complex ecosystem, harbors diverse microbial communities engaged in symbiotic or competitive relationships, encompassing a multitude of yet unexplored organisms [37]. Utilizing mass spectrometry (MS) enables the identification of unique metabolites in soils, shedding light on their relevance to microbial functioning pathways and ecological conditions [38]. In our study, we calculated four alpha diversity indices, including Chao and Ace for richness, and Simpson and Shannon for diversity, along with coverage to assess microbial community dynamics influenced by *A. adenophora* and seven distinct crops in vegetation-free

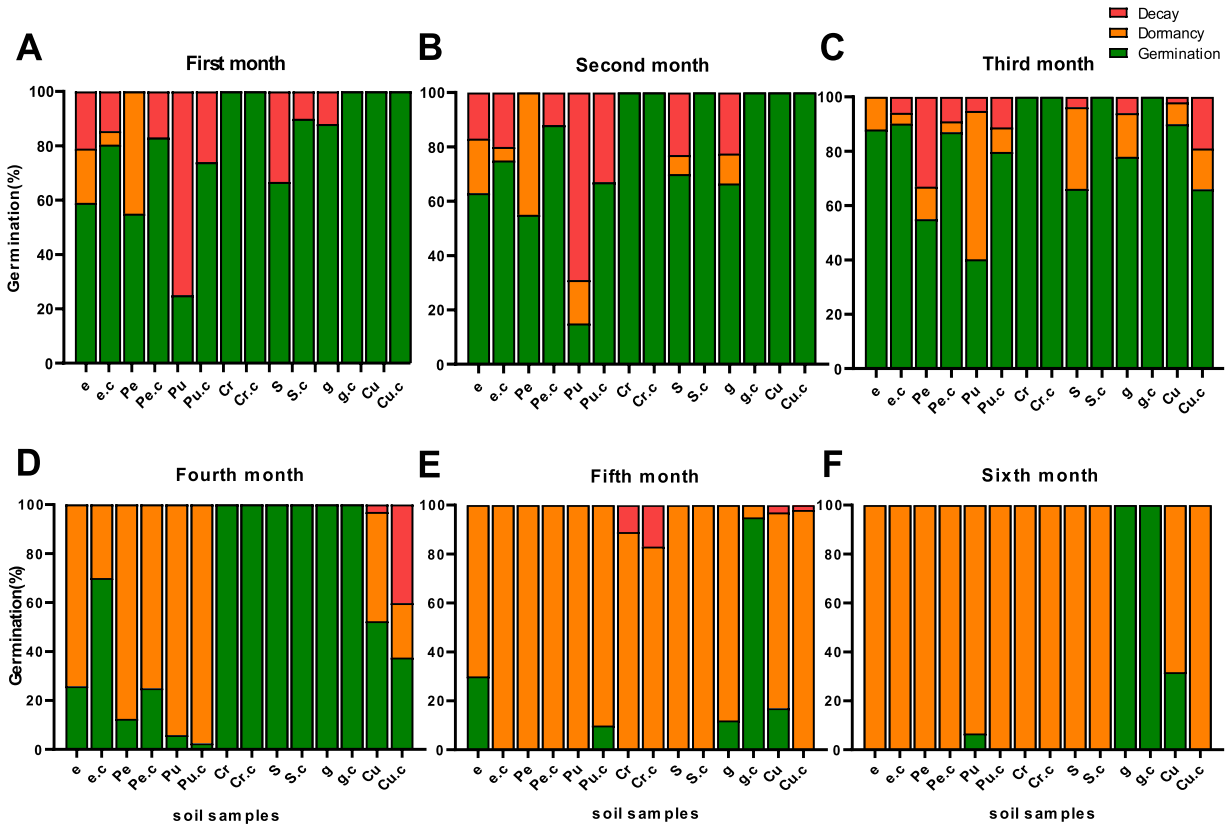

Fig. 7. Stacked graph illustrating the germination rates of longevity crops over a single season (6 months) in *Ageratina adenophora* rhizosphere soil ($n = 4$) [11].

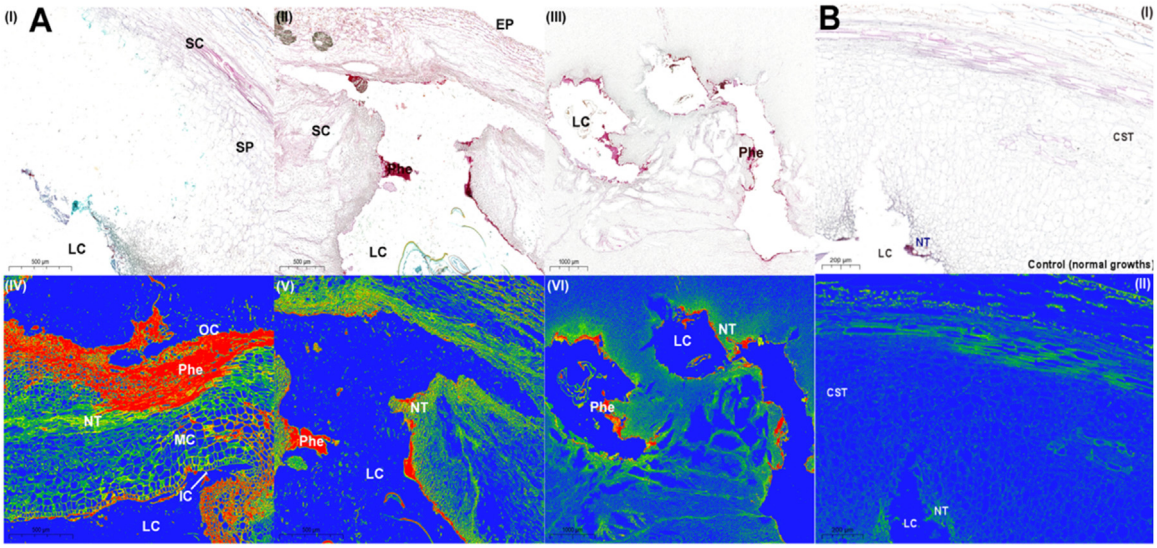

Fig. 8. Structure of the stem-galling fly's anatomy *Procecidochares utilis* injection methods with varying phenol concentrations (10, 60, and 100 mg/μL). **(A, I, IV)** Ageratina adenophora gall development in response to phenol at a concentration of 10 mg/μL. **(II, V)** Gall formation induced by phenol at a dosage of 60 mg/μL. Phenol at a concentration of 100 mg/μL **(III, VI)**. The abbreviations for the terms: LC, larval chamber; Phe, phenol (Detail of phenolic substances accumulated at the site of induction); NT, nutritive tissue; OC, outer cortex; MC, median cortex; IC, inner cortex; SC, sclerenchyma; SP spongy parenchyma. **(B)** Gall form in normal state without phenol treatment [11].

soil [39-41]. These indices were pivotal in understanding the richness and diversity of bacterial communities across different soil samples (Fig. 5).

Phenolic effect on plant growth

Phenolic effect on gall formation

As the results shown in (Fig. 8), phenols exhibited both stimulatory and inhibitory effects on plant growth, with optimal concentrations promoting emergence but higher concentrations hindering growth. Gall formation was influenced by phenolic concentrations, leading to structural alterations in stem tissue and gall morphology. Histochemical analysis revealed starch and lipid accumulation in gall tissues, indicating metabolic changes induced by phenolics [11].

In sum up, this study presence of phenolic chemicals, particularly in high concentrations, has exhibited both stimulating and inhibitory effects on gall formation, influencing the production of galls from plant cells. Additionally, phenols have been observed to cause reductions in both common storage tissue and nutritive tissue in plants. Through soil studies, phenols and certain allelochemical substances have been demonstrated to enhance plant growth, potentially affecting plant cell division.

Limitations

Not applicable.

Ethics statements

We utilized phenolic compounds in our experiments, ensuring that all adhere to our institution's safety procedures, laboratory guidelines, and environmental safety standards.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Nipapan Kanjana: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Yuyan Li:** Project administration, Writing – review & editing, Data curation, Funding acquisition. **Muhammad Afaq Ahmed:** Investigation, Software, Validation, Writing – original draft. **Zhongjian Shen:** Validation, Investigation, Writing – review & editing, Writing – original draft, Software, Data curation. **Lisheng Zhang:** Supervision, Project administration, Conceptualization, Validation, Writing – review & editing, Funding acquisition.

Data availability

Data will be made available on request.

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