Leaf litter contributes to the obstacles of Cynanchum auriculatum Royle ex Wight continuous cropping

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Research Article

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Abstract

Aims

To test a hypothesis that fallen leaf litter results in the continuous cropping obstacle (CCO) in *Cynanchum auriculatum* Royle ex Wight (*CA*) via growth promotion and invasion reinforcement of soil-borne fungal pathogen.

Methods

Water extracts of leaf (LE) and root (RE) were compared for their effects on seed germination, seedling growth indices, and plant defense enzymes activities. Besides, the impacts of LE on a fungal pathogen were investigated under laboratory conditions following isolation and verification. Then, the effects of LE on soil microbial communities were determined by using high-throughput sequencing technology.

Key Results

A fungal strain D1 belonging to *Fusarium solani* causing root rot disease was isolated and confirmed for its potential contribution to CCO. Both LE and RE inhibited seed germination, seedling growth, and plant defense enzymes activities. Extracts especially LE coupled with D1 aggravated the impacts. Apart from the induction of propagation of D1 in soil, extracts could also promote hypha weight, spore number, and spore germination rate of D1 under the culture conditions. Compared with RE, LE showed more promoting-effects on the pathogenesis-related enzymes activities of D1. Moreover, caffeic acid and ferulic acid were the possible active substances contributing to the events. Besides, not bacterial but fungal community were shifted by LE especially by LE+D1.

Conclusions

These results suggested that water extract of leaf litter promoted the growth and propagation of strain D1, and enhanced its pathogenicity towards *CA*, which synthetically contributed to the CCO process.

Introduction

Plants are often subjected to the continuous planting process, resulting in inhibition of seed germination and plant growth, which is called continuous cropping obstacle (CCO). CCO happens in many plants especially in medicinal plants, such as *Panax quinquefolius* L. (Dong et al. 2016; Tan et al. 2017; Liao et al. 2018; Li et al. 2020, 2021; Zhang et al. 2020), *Pogostemon cablin* (Zeng et al. 2020), *Angelica sinensis* (Xinhui 2010), and *Amomum villosum* (Wang et al. 2022a). CCO also takes place in *Cynanchum auriculatum* Royle ex Wight (*CA*), which is commonly known as “Binhai Baishouwu” mainly distributed in Binhai county, Jiangsu Province, China (Jiang et al. 2011). Studies have indicated that *CA* has high medicinal values including anti-tumor, immunomodulatory, hepatoprotection, anti-inflammatory, and anti-depressant activities (Chen et al. 2019), and thus is widely planted in China and Korea for their high economic benefits (Kim et al. 2018). However, CCO restricts the development of these medicinal plants including *CA* to some degree (Chen et al. 2022b).

Mechanisms on CCO have also been explored from many perspectives. Alters in microbial structure and diversity as well as deteriorations of soil properties via accumulations of phenolic acids and other autotoxins were widely reported and commonly accepted (Dong et al. 2016; Tan et al. 2017; Bai et al. 2019; Li et al. 2020; Wang et al. 2020; Chen et al. 2022b; Wang et al. 2022a). Peng et al. further pointed out that different phases had distinct changes of CCO in strawberry (Chen et al. 2020). Based on these findings, methods such as addition of arbuscular mycorrhizal fungi and calcium (CUI et al. 2019), microalgae (Feng et al. 2022), novel bioorganic fertilizer (Ling et al. 2014), using stereoscopic cultivation (Liao et al. 2018) and intercropping systems (Zeng et al. 2020) were proposed and have achieved some positive outcomes.

To date, no reports were found to be relevant to CCO in *CA*. Through field surveys, we noticed that the aboveground parts of *CA* (leaf mainly) fell to the ground without harvesting and utilization for their poor palatability for animals and thus were plowed into the ploughed layers after harvesting roots. Despite that leaf litter contributes more to soil organic carbon than fine roots in some plantations (Cao et al. 2020) and plays protective roles in runoff and erosion (Li et al. 2014), this situation was not similar in medicinal plants. Lu et al. showed that leachate (water-extraction solution) from the stems and leaves of common medicinal materials displayed significant inhibitory effects on the release of carbon, nitrogen and phosphorus from litter decomposition and the activities of all seven kinds of soil enzymes (Yu-Peng et al. 2017). In consideration of the massive leaf litter of *CA* falling into the soil during each planting season, plus with the fact that kinds of allelopathic substances existed in leaves of many medicinal plants (Basotra et al. 2005; Gupta 2016; Aniya et al. 2020), we hypothesized that the falling leaf litter contributes more to the inhibitory effects on *CA* growth than root extraction. Past studies have revealed the possibility of the inhibitory effect of leaf litter on plant growth, and the main connection between leaf litter and plant growth index is the changes of soil microbial structure and diversity (Chen et al. 2022b), especially mirrored by increased pathogenic bacteria, and decreased numbers of beneficial microorganisms (Pang et al. 2021; Wei-Ye et al. 2022). However, these conclusions were concluded from the results of high-throughput sequencing, few evidences on pure cultures were exhibited before.

Thus, in the present study, we first isolated a vital fungal pathogen causing root rot disease, and then the different effects of root and leaf extracts on the indices relevant to plant growth and soil quality, as well as pathogen were compared. The findings of this study provide a theoretical basis for uncovering the mechanism of CCO and for better field management of *CA*.

Materials and Methods

Isolation and identification of fungal pathogen causing root rot disease

On 22 May 2022, soil (0–20 cm) was collected from the rhizosphere of *CA* suffering from root rot disease in a planting base (117.71071 E, 39.0032 N) with at least 15 years of cultivation history. For the soil sampling, *CA* roots were shaken vigorously to separate the soil that were not tightly adhered, and the left soil was regarded as rhizosphere soil. Then, the soil sample was transported to the laboratory at Yancheng Teachers University in a cooler box within 12 h. The properties of the sandy loam soil were: pH $_{(H20)}$ 8.30, organic matter 1.5%, and total nitrogen 1.07 g/kg. Soil pH was measured in a 1:2 soil/distilled water suspension using a pre-calibrated glass electrode (Mclean 2015). Organic matter and total N were determined using the routine methods (Houba et al. 2000; Bremner 2018). Soil sample was diluted with sterile distilled water (SDW) and vortexed for 3 min followed by spreading on a Potato Dextrose Agar (PDA) medium containing 10 mg/L rifampicin and 200 mg/L of ampicillin. The plates were kept at room temperature in the laboratory for 5 to 7 days. Fungal colonies on the plate were repeatedly streaked to a new plate until pure cultures obtained.

Afterwards, a total of seven fungal strains with different morphologies were separately tested for their pathogenicity. *CA* seedlings with 3–4 leaves growing in quartz sand were transferred to pots (2 kg soil each). The soil for the pot experiment was collected from the campus of Yancheng Teachers University. Every five pots were inoculated with one of the seven fungal spore suspensions around the root at the final concentration of 10^9 colony-forming unit (CFU) per kg soil. Meanwhile, control soil without inoculation was conducted. The disease severity index of *CA* leaves was rated on a scale of 0 to 4, where 0 = no disease symptom, 1 = 0.1 to 5%, 2 = 5.1 to 20%, 3 = 20.1 to 40%, and 4 = 40.1 to 100% (the percentage of diseased leaf area). The disease index was calculated using the following formula: Disease index (%) = [Σ (the number of diseased leaves × disease severity index)/(4 × the number of leaves rated)] ×100 (Lee et al. 2006). Based on the pathogenicity results and the symptoms, the fungal pathogen with the strongest infectivity was selected and reisolated from the plant roots. Then, the reisolated strain was testified for its same pathogenicity towards *CA*.

DNA of the selected pathogenic fungus was extracted using a Rapid Fungi Genomic DNA Isolation Kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturers' protocol. The internal transcribed spacer (ITS) region of the ribosomal RNA was amplified using the universal primer ITS1/ITS4 (forward primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3', reverse primer: ITS4 5'-TCCTCC GCTTATTGATATGC-3') (White et al. 1990). The PCR product was sent to Sangon Biotech. Co., Ltd for sequencing, then the result was deposited in NCBI database and the accession number (OP677989) was obtained. Afterwards, a phylogenetic tree was constructed using DNA sequences by software Mega X neighbor-joining method (Kumar et al. 2018). Finally, a fungal strain named D1 that was responsible for the rot disease of *CA* was identified as *Fusarium solani*.

Effects of leaf, root extracts, and fungal pathogen on seed germination and plant growth indices

Fallen yellow leaves and mature roots were collected from the planting base followed by grinding and drying to constant weight at 65℃. Thirty grams of dried leaves or roots were soaked in 1 L SDW at 24℃ for 48 h, and the liquid was shaken every 12 h. After 48 h, the liquid was centrifuged at 10000 r/min for 20 min and the supernatant was extracted. The extracts were separately freeze-dried and concentrated to the concentration of 90 g/L. Thus, the water extracts of leaves (LE) and roots (RE) were separately obtained and stored at 4℃ for later use. Then, the extracts were sterile filtered (0.22 μm pore size) and autoclaved, respectively. Besides, *F. solani* D1 was cultured with PDA media at 28℃ for 3 days. Afterwards, the spores on the plates were collected with SDW and adjusted to 10^6 CFU/mL and also stored at 4℃ less than one week.

For the germination experiment in the laboratory, at least 360 seeds of *CA* (variety: Binwu No. 1) were surface sterilized with 50% bleach solution for 15 min, washed 3 times with SDW, and placed at 4℃ overnight before use. Half the seeds were soaked in the spore suspension (10^6 CFU/mL) for 20 min at room temperature, and then air dried. Meanwhile, the left seeds were soaked in SDW at the same condition. These two parts were regarded as inoculated and uninoculated seeds, respectively. Besides, LE and RE were diluted to 30 g/L as stock solutions. Six treatments were designed, namely control (uninoculated seeds, 2 mL SDW every 2 days), D1 (inoculated seeds, 2 mL SDW every 2 days), LE (uninoculated seeds, 2 mL LE stock solution every 2 days), LE + D1 (inoculated seeds, 2 mL LE stock solution every 2 days), RE (uninoculated seeds, 2 mL RE stock solution every 2 days), and RE + D1 (inoculated seeds, 2 mL RE stock solution every 2 days). Every 20 seeds were placed on a sterile filter paper in a petri dish containing different additives shown above and germinated at 24°C. Three experimental replicates were conducted for each treatment and control. On day 10, germination rate of each petri dish was calculated. To know if the effective substances in extracts were heat resistant, autoclaved and filtered extracts were also compared via the germination experiment.

To better understand the effects of LE, RE, and D1 on seed germination and seedling growth in soil system, a pot experiment was also conducted. For each treatment, 3 seeds were sown in the pot filled with 2 kg soil collected from the campus. A total of 12 replicates for each treatment were prepared, and random three pots were sampled at every sampling time. The treatments were the same as above. For the additions of extracts, 667 mL of LE and RE (90 g/L) were separately and evenly added to 2 kg of soil, and the final concentration of extracts in soil was 30 g/kg. For those D1-contained treatments, D1 spore suspensions were added to soil around the seeds at the final concentrations of 10^9 CFU/kg. On day 15, germination rates were calculated based on the data observed. Besides, seedlings from each treatment were sampled randomly on day 15 for plant growth determination. Lengths of shoot and root were measured separately, and the dry weights of which were weighted after drying to constant weights at 70°C for 72 h.

Due to the sensitivity of some plant indicators in response to environmental stress, the contents of Chlorophyll Chl *a*, Chl *b*, carotenoid (Car), and phenylalanine ammonia lyase (PAL), as well as the activities of superoxide dismutase (SOD), and peroxidase (POD) were also determined (Datt 1999; Huang et al. 2010; Kong et al. 2014; Zameer et al. 2022). Six treatments and the methods for the pot experiment were the same as above. Differently, seedlings at the third true leaf stage were transplanted to each pot (one seedling per pot), and at least 12 pots for each treatment were prepared. On day 0, 15, and 50, leaves from three random pots for each treatment were sampled. For the assays of photosynthetic pigment contents, a updated procedure with a new simultaneous equations were adopted (Chazaux et al. 2022). The simultaneous equations for quantifying chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoid (Car) are: Chl $a = 12.18 \times A_{663.6} - 2.36 \times A_{646.6}$; Chl $b = 20.19 \times A_{646.6} - 4.59 \times A_{663.6}$; Car = (1000 × A_{470} − 2.05 × Chl *a* − 114.8 × Chl *b*) / 245, where A = absorbance in 1.00-centimeter cuvettes. Crude extracts of the antioxidant enzymes were prepared by homogenization of frozen leaves in buffer medium. Briefly, 10 g of the sample was homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM ascorbic acid and 0.5% (w/v) polyvinylpyrrolidone for 5 min at 4°C. The homogenate was filtered through three layers of gauze towel and then the filtrate was centrifuged at 5,000 × g for 15 min followed by a collection of

the supernatants. PAL activity was assayed by measuring the absorbance of trans-cinnamic acid at 290 nm modified by González-Mendoza et al. (González-Mendoza et al. 2018). One unit (U) of this enzymatic activity was defined as the amount causing an increase of 0.01 in the absorbance per h. SOD was determined by measuring the inhibition in photoreduction of nitro-blue tetrazolium (NBT) by SOD enzyme (Kumar et al. 2012). One U of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT. POD activity was determined by measuring the increase in the absorption at 420 nm spectrophotometrically caused by oxidation of 4-methylcatechol by H_2O_2. One U of the enzymatic activity was defined as 0.001 change in absorbance per min (Onsa et al. 2004).

Effects of leaf, root extracts, and fungal pathogen on soil enzymes

During the pot experiment introduced above, on day 0, 15, and 50, soils from the three random pots for determination were also sampled. Briefly, soils coring to 10 cm depth around root were sampled with a soil corer of 3 cm diameter. After removal of root residues, the soils were stored at 4°C and − 80°C for enzyme activity determination within one week and molecular analysis, respectively. Invertase activity was determined with 3,5-dinitrosalicylic acid colorimetric method and was expressed as mg of released reducing sugars per gram of soil per 24 h at 37℃ (Frankeberger and Johanson 1983). Urease activity was assayed using colorimetric determination of ammonium and was expressed as mg of NH_3-N in 1 g of soil after 24 h (Kandeler and Gerber 1988). Alkaline phosphatase activity was detected by using p-nitrophenyl phosphate (pNPP) as chromogenic substrate and was shown as mg p-nitrophenol (pNP) per gram of soil per 24 h (Zuberer 2018).

Quantitative polymerase chain reaction (qPCR) analysis of D1

The total microbial DNA was extracted from the ~ 0.5 g soil using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA) and checked using agarose gel electrophoresis. A sequence within the TEF1-alpha gene (TEF1) was selected for the qPCR determination of abundance of strain D1 with a specific primer pair F8 (5'-GCTCAGCGGCTTCCTATTG-3') and R8 (5'-CGGGGTATTCATCATTCACTTCA-3') (Peng et al. 2022).

Detection of phenolic acids in soil and extracts and their influences on the growth of mycelium growth rate of strain D1

The soils that had been planted with *CA* for 0 (0 a), 2 (2 a) and 3 years (3 a) were sampled for comparing the contents of phenolic compounds by using High-performance Liquid Chromatography (HPLC). One hundred grams of fresh sample were placed into a centrifuge tube, to which 100 mL of 1 mol/L NaOH was added, allowing for extraction to occur over 24 h. The samples were then shaken for 60 min. Following centrifugation, the solution was acidified to pH 2.5 using 12 mol/L HCl for 2 h. Then, the phenolic acids were obtained by centrifugation, following which the supernatant was passed through a filter membrane of 0.22 μm. The filtrates were then measured by HPLC, and the results were converted according to the weight of the dried soil.

A homogenized dried plant sample (0.3 g) was placed into a 50 mL graduated plastic test tube and homogenized in 7 mL methanol solution (adjusted to pH = 2 with 1 mol/L HCl). Sample extracts were ultrasonicated at 45℃ for 40 min and cooled to room temperature, and then diluted to 10 mL with SDW. After a centrifugation at 10000 r/min for 20 min, 1 mL supernatant was purified using a membrane filter with pore-size 0.22 μm for the HPLC analysis (Mattila and Kumpulainen 2002). The results were converted according to the weight of the dried samples.

For the HPLC analysis, a SunFire™ C18 column (4.6 mm ×250 mm, 5 μm) was loaded to the Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA). Injection volume of 10 μL, flow rate of 0.6 mL/min, column oven temperature of 30°C, and UV detection wavelength of 280 nm were adopted in the process. The elution mobile phases A and B were methanol and 1% aqueous acetic acid solution (pH 2.5), respectively, and the retention time were from 0 to 30 min. The proportion of the mobile phase A and B was 1:3. A 10 min delay at the end of each cycle was set up to remove the effects of interfering components and ensure the stability and repeatability of the results. According to the previous study, 7 phenolic acids were selected as standard samples for determination. The standard samples (Sigma) included coumaric acid, p-coumaric acid, caffeic acid, p-hydroxybenzoic acid, vanillin, ferulic acid, and syringic acid. All the phenolic acids were quantified using the external standard method. The samples were identified based on the retention time and peak area of the analytical standards (Bai et al. 2019).

Besides, to testify the possibilities of the detected phenolic acids (p-hydroxybenzoic acid, caffeic acid, p-coumaric acid and ferulic acid) that might be responsible for CCO, the influence of the four phenolic acids on the mycelium dimeters of *F. solani* D1 were measured and compared. Briefly, the mycelia with the same growth from the colony edge were taken with a hole punch (5 mm dimeter) and inserted into PDA media containing different concentrations (0, 2, 6 mg/L) of the four phenolic acids. The colony diameters were then measured after 2, 3 and 4 days of culture at 28℃.

Effects of leaf and root extracts on the growth of D1 and activities of pathogenesis-related enzymes

LE or RE were separately added to 50 mL PDA liquid media with four final concentrations, namely 0, 10, 20, and 30 g/L, and were inoculated with strain D1 at the final concentration of 10^6 CFU/mL. The triangular flasks were incubated at 28°C and 180 rpm followed by a time-course measurement of the dry weights of hypha. Two milliliters of culture solutions from each treatment were taken every 12 h and centrifuged at 8000 r/min for 15 min. Then, the precipitations were washed with SDW and then dried to constant weight, and the dry weights of hyphae were weighed. Besides, different concentrations (see above) of LE or RE were added to the PDA media, then a patch of D1 was transplanted in the center of each plate using a hole puncher from a precultured plate. Colony diameter was measured for each plate at 84 h. Spore numbers on each plate were counted in a Sysmex-5000i automated hematology counter following spore washing with SDW. Moreover, spore germination rates were detected with hemocytometer after 24 h of culture in PDB media containing filtrated LE or RE at the final concentrations of 10, 20, and 30 g/L following inoculation with the same numbers of spores of strain D1.

For understanding the effects of LE and RE on enzymes involving in pathogenesis, four invasion-related enzymes including pectinase, cellulase, β-glucosidase, and α-amylase activities were determined and compared among treatments (Zhang et al. 2006; Hasan et al. 2013; Bethke et al. 2016; Huang et al. 2021). Briefly, pectinase was assayed by determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by 3,5-dinitro-salicylic acid reagent

(DNS) method (Miller 1959). Pectinase activity was determined using pectin as substrate. The reaction mixture containing equal amount of substrate (2%) prepared in citrate buffer (0.05 mol/L, pH 4.4) and suitably diluted enzyme was incubated at 50℃ for 30 min in water bath. The release of reducing sugars was measured by DNS method using galacturonic acid as standard. The pectinase enzymatic activity was defined as the amount of enzyme required to release 1 μmol equivalent of galacturonic acid/min under assay conditions. The cellulase activity was carried out in citrate buffer (0.05 mol/L, pH 4.8) and suitably diluted enzyme was incubated at 50℃ for 60 min in water bath and measured by DNS method using 50 mg Whatman No. 1 filter paper strip (1.0 x 6.0 cm) as a substrate. The cellulase enzymatic activity was defined as the amount of enzyme required to reduce sugars equivalent to 1 μg of glucose per mL per min (Ghose 1987). β-Glucosidase activity was carried out in acetic acid buffer (50 mmol/L, pH 5.0) and suitably diluted enzyme was incubated at 60℃ for 10 min in water bath assayed by measuring the hydrolysis of p-Nitrophenyl β-D-glucopyranoside. β-Glucosidase activity was defined as the amount of enzyme releasing 1 μmol p-nitrophenol per minute as 1 unit of enzyme activity (Cai et al. 1999). α-Amylase activity was determined by measuring the hydrolysis of starch into glucose using DNS method, and one U was defined as the amount of 1 mg of reducing sugar (calculated as maltose) released from starch per mL per min under the specified conditions (Lorentz 1979).

Scanning Electron Microscopy (SEM) observation of the morphologies of strain D1 following different treatments

Seed cells of strain D1 were equally inoculated on the PDA plates with different concentrations of LE and RE (0 and 30 mg/L) and cultured at 28℃ for 84 h. Hypha from each plate were picked and put on a coverslip, and then fixed in 2.5% glutaraldehyde in 0.01 mol/L PBS for 1 h at room temperature. The coverslip was put into 30%, 50%, 70%, 80% and 90% ethanol for 10 min, then dehydrated with anhydrous ethanol for 10 min for 2–3 times. Then, samples were put onto the objective table for observations after spraying of gold, and the working distance and voltage were 6.6 mm and 3.0 kV, respectively.

Microbial community analysis

Microbial DNA were extracted from four pot soil samples (control, D1, LE and LE + D1) at different sampling points (0, 15 and 50 d) using the E.Z.N.A.® Soil DNA Kit. 16S rRNA and ITS genes were amplified with the primer sets: 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907r (5′-CCGTCAATTCMTTTRAGTTT-3′) (Klindworth et al. 2013), ITS3-F: (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4-R: (5′-TCCTCCGCTTATTGATATGC-3′) (McKay et al. 1999), respectively, and then added with index codes. A 25 μL of reaction mixture contained 5 μL 5×reaction buffer, 5 μL 5×GC buffer, 2 μL dNTP (2.5 mM), 1 μL of each primer (10 μM), 2 μL template DNA, 0.25 μL Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA), and 8.75 μL ddH_2O. The temperature cycle consisted of 98°C for 1 min, followed by 30 cycles of 98°C for 15 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Bacterial or fungal community of each sample was measured by Illumina MiSeq System (Biozeron Biotechnology Co., Ltd, Shanghai, China). The gene sequences were picked using a 97% identity threshold after original data consolidation, as well as filtering and quality assessment by QIIME (v1.8.0, http://qiime.org/). The Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) was used for the analysis of species annotation. Filtered reads were clustered into operational taxonomic units (OTUs) assuming 97% similarity cutoff using uclust (Edgar 2010), and the OTUs whose abundances were lower than 0.0001% were eliminated (Bokulich et al. 2013).

Data analysis

Raw data were imported into SPSS Statistics for Windows version 18.0 (WinWrap Basic, Polar Engineering and Consulting) for calculating means and standard errors (SE). A phylogenetic tree was constructed using DNA sequences by software Mega X neighbor-joining method. Principal component analysis (PCA) was performed with the STAMP software based on the correlation matrix. A two-way analysis of variance (ANOVA) was employed to investigate the effects of extract kinds (no extract, LE, or RE) and pathogen (with or without) on the plant growth indices. Similarly, a two-way ANOVA was performed to test the effects of extract kinds (LE, RE) and amount (0, 10, 20, 30 g/L) on the growth and the activities of pathogenesis-related enzymes of strain D1. After that, the Bonferroni's post-hoc tests were performed. In each plot, different numbers of asterisk (*, **, ***) represent significant differences between samples at *P*-values of 0.05, 0.01, and 0.001. For some plots, different letters indicate significant differences existed at one sampling time (Duncan's multiple range test, $P \leq 0.05$). The line and column charts presented in this paper were obtained using Sigma Plot for Windows Version 10.0 (Systat Sofware, San Jose, CA, USA).

Results

Isolation and identification of fungal pathogen causing root rot disease

Root rot disease is prevalent during the continuous cropping of CA based on our field investigation. Thus, the fungal pathogen causing root rot disease is required to be isolated and analyzed for its response to LE and RE. Among the seven possible pathogenic fungi, strain D1 was selected for the strongest pathogenic effect on CA (Fig. 1A). Afterwards, strain D1 was confirmed according to Koch's postulates, and the symptom caused by D1 was in consistent with root rot disease (Fig. 1B) including round or irregular light brown lesions that progress to dark black lesions on below ground roots and stems, stunting and death (Thaines Bodah 2017). Then, strain D1 was identified as belonging to *F. solani* based on fungal ITS sequencing (Fig. 1C) and the falciform spores (Fig. 5E).

Effects of extracts and strain D1 on plant growth indices of CA

Strain D1 significantly decreased the seeds germination rate by 68.29% compared with control without D1 addition ($P < 0.001$) (Fig. 2A). LE and RE with autoclave treatment also inhibited seeds germination rates by 78.05% and 68.29%, respectively. Either LE or RE had synergistic effect with strain D1 on the seed germination (Fig. 2B). Filtration treatments of LE and RE showed the similar effects compared with the autoclave treatments. This indicated that some kinds of substances in the extract inhibiting seed germination were heat tolerant. A similar phenomenon occurred in soil through pot experiment (Fig. 2C). Neither LE nor RE could significantly affect plant length measured on day 15 (Fig. 2D). Compared with control, the addition of extract significantly decreased the dry weight of root and the aboveground parts except the RE treatment (Fig. 2E). Moreover, we also determined the changes in the contents of

photosynthetic pigments and the activities of defensive enzymes following the addition of plant extracts and strain D1. Overall, many of these data showed significantly correlated through the Pearson correlation coefficient (Table 1), among which Chl *a*:*b* exhibited a relatively good relationships with other indices. On day 0, most of the indices were similar without significant differences except Chl *a*:*b* in LE + D1, RE, and RE + D1 treatments (Fig. 3A). Data showed similar tendencies on day 15 and 50, on which LE + D1 was often the lowest. For POD activity, strain D1, LE, and RE exhibited significant increases compared with the control (Fig. 3F), however, the addition of strain D1 significantly decreased the POD values. These results showed that some heat-resistant substances in the extracts especially in LE inhibited the seed germination, plant dry weight, photosynthetic pigments, the activities of defensive enzymes, and strain D1 aggravated the impacts exerted by the extracts.

Table 1
Pearson correlation coefficients among plant growth indices

	Chla:b	Chla + b	Carotenoid	PAL	SOD	POD
Chla:b	1.00	0.84**	0.76**	0.60**	0.86**	-0.36**
Chla + b	0.84**	1.00	0.90**	0.84**	0.87**	-0.15
Carotenoid	0.76**	0.90**	1.00	0.78**	0.79**	-0.08
PAL	0.60**	0.84**	0.78**	1.00	0.81**	-0.12
SOD	0.86**	0.87**	0.79**	0.81**	1.00	-0.34*
POD	-0.36**	-0.15	-0.08	-0.12	-0.34*	1.00

* and ** indicate significant correlation between the corresponding data at 0.05 and 0.01 levels, respectively.

Effects of CA extracts on soil enzymes and abundances of strain D1 in soil

Dissimilar to plant growth related indices, the additions of the extracts and strain D1 generally increased the soil enzymes but with distinct models. Invertase activity was the fastest one responding to the additions, because LE + D1, RE, and RE + D1 significantly increased the invertase activities on day 0 (Fig. 4A). This enzymatic activity was enhanced by LE on day 50 and kept stable following D1 inoculation with time, however, it was largely boosted once inoculation with the mixture of LE and D1. Strain D1 significantly increased urease activity by 26.58% compared with control treatment only on day 15 and had no obvious effects on day 0 and day 50. LE + D1 showed different effects on urease activity with time, which was significantly inhibited only on day 50 (Fig. 4B). Similar phenomenon was found for the performance of alkaline phosphatase activity compared to the invertase activity, except that no significant differences was shown among treatments on day 50 (Fig. 4C).

Besides, the highest abundance of strain D1 on day 0 was shown in LE + D1, followed by RE + D1, and D1 treatments (Fig. 4D). The abundance of D1 was highest in LE + D1 treatment except on day 15 (Fig. 4D). This was due to the possibility of LE and RE carrying D1 into soil and their promoting effects on the propagation of D1 because of the occurrences of D1 in these treatments on day 50. These results indicated that LE and RE (especially LE) could affect the activities of soil enzymes in some degrees, as well as carry and transfer fungal pathogen and stimulate their propagations in soil system.

Effects of extracts on the growth of D1 and its activities of pathogenesis-related enzymes

Either LE or RE could significantly promote the hypha weights of strain D1 in a concentration-dependent manner, and RE exhibited a more rapid effect compared with LE (Figs. 5A and 5B). Colony diameters were slightly inhibited by LE or RE, whereas the spore numbers and percentage of spore germination were significantly promoted by the extracts especially by LE at the concentration of 30 g/L (Figs. 5C and 5D). With SEM observation, it could be clearly shown that the spore numbers and hypha diameters were significantly promoted by the extracts especially by LE30, by which the hypha diameter was increased by ~ 50% (Fig. 5E). This could be one of the reasons accounting for the increment in the hypha weight following the additions of the extracts.

Moreover, the extracts could significantly increase the activities of pectinase and cellulase of D1 in a concentration-dependent manner, and LE showed a higher promoting effect than RE (Figs. 6A and 6B). LE and RE could also promote the activities of β-glucosidase and α-amylase, while LE did not always exhibit a significant higher effect than RE and only showed the phenomena at the concentrations of 20 g/L for β-glucosidase (Figs. 6C and 6D). These results indicated that the extracts especially LE could promote both spore germination and hypha weight of strain D1 and activities of pathogenesis-related enzymes.

Contents of phenolic acids in soils and extracts

Due the possible effectors had some specific characteristics including heat resistance, increasing with years, and higher in LE than RE, we compared the contents of phenolic acids in soils with different cropping histories of *CA*, as well as the contents in LE and RE. It was shown that p-hydroxybenzoic acid and caffeic acid increased with time, and p-coumaric acid and ferulic acid were also more abundant in continuous cropping soils compared with control soil (Fig. 7A). Caffeic acid, p-coumaric acid, and ferulic acid were detected with high contents in LE but with no detections in RE, while p-hydroxybenzoic acid were higher in RE than in LE (Fig. 7B). Using the four phenolic acids, we determined their effects on the growth of strain D1 with the index of colony diameters. Caffeic acid and p-coumaric acid were shown to be capable of promoting the growth of D1(Figs. 7C and 7D), plus with their accumulation characteristics in soil with time (Fig. 7A), they were selected for further study.

Profiles of the microbial communities following LE and D1 additions

The dominant fungal species in control were *Mortierella* and *Alternaria* on day 0, and then changed to *Mortierlla* and *Pseudeurotium* on day 15 and 50 (Fig. 8A). *Filobasidium* was predominant in LE treatment on day 0, while it decreased sharply on day 15 and 50. *Fusarium* increased dramatically in LE + D1 treatment on day 15 and 50 compared with that on day 0. Besides, *Fusarium* exhibited negative relationships with other dominant fungal genera (Table 2), indicating that it could be an overwhelming preponderance versus other fungi once it breaks out in soil.

Table 2
Pearson correlation coefficients among dominant fungal genera

	Fusarium	*Mortierella*	*Pseudeurotium*	*Alternaria*	*Thielavia*	*Filobasidium*	*Unclassified*	*Clydaea*	*Leptodiscella*	*Hormi*
Fusarium	1	-0.538**	-0.672**	-0.313	-0.432**	-0.306	-0.231	-0.177	-0.140	-0.552
Mortierella	-0.538**	1	0.732**	-0.253	0.680**	-0.365*	-0.292	0.327	0.384*	0.700
Pseudeurotium	-0.672**	0.732**	1	-0.267	0.446**	-0.137	-0.175	0.449**	0.281	0.754
Alternaria	-0.313	-0.253	-0.267	1	0.188	0.355*	0.146	-0.360*	-0.288	-0.012
Thielavia	-0.432**	0.680**	0.446**	0.188	1	-0.330**	-0.252	0.053	-0.018	0.798
Filobasidium	-0.306	-0.365*	-0.137	0.355*	-0.330*	1	0.085	-0.226	-0.161	-0.383
Unclassified	-0.231	-0.292	-0.175	0.146	-0.252	0.085	1	-0.157	-0.168	-0.226
Clydaea	-0.177	0.327	0.449**	-0.360*	0.053	-0.226	-0.157	1	-0.276	0.419
Leptodiscella	-0.140	0.384*	0.281	-0.288	-0.018	-0.161	-0.168	-0.276	1	0.074
Hormiactis	-0.552**	0.700**	0.754**	-0.012	0.798**	-0.383*	-0.226	0.419*	0.074	1

* and ** indicate significant correlation between the corresponding data at 0.05 and 0.01 levels, respectively.

There were obvious differences in bacterial and fungal communities among treatments with time. Bacteria belonging to *Pseudarthrobacter*, *Pontibacter*, *Planococcus*, and *Paracoccus* were abundant in the LE treatment, however, they decreased significantly on days 15 and 50, indicating that they were not competitive in the soil. No significant differences and shifts in bacterial communities were shown in treatments with time, which were reflected in the principal component analysis (PCA) (Fig. S2). While for the fungal community, PCA analysis showed that two groups could be clearly distinguished from the horizontal axis which explained 76.19% of data variability. One group contained control and those treatments without strain D1 (except LE + D1 on day 0), and another group consisted of treatments with strain D1 (Fig. 8B). This result indicated that strain D1 played a dominant role in changing the fungal community, and LE showed a synergetic effect with D1. The top 30 dominant genera in the treatments were shown in Fig. S3, among which 20 genera were within *Ascomycota*.

Discussion

CA was widely planted in Jiangsu Province, China, especially in Yancheng City. Like some medicinal plants, CCO was also occurred in *CA* planting, and some traditional measures have been taken to resolve the issue such as soil flooding, fallow, and crop rotation. To better achieve the goals, mechanisms underlying the phenomenon need to be clearly uncovered. Among the well-known mechanisms, the increase in the abundance of fungal pathogen was an important aspect during CCO (Chen et al. 2012). In the past decades, different kinds of fungal pathogens have been revealed along with CCO process based on both culturable and unculturable strategies. For example, *F. oxysporum* and *F. solani* were bloomed during CCO of American ginseng (Liu et al. 2020). *Phoma eupyrena* and *F. culmorum* were abundant in continuous wheat cropping soil (Bateman and Kwaśna 1999). *Cylindrocarpon* sp. was the key fungal pathogen against continuous cropping of flue-cured tobacco (Wang et al. 2020). In this work, the primary fungal pathogen belonging to *F. solani* responsible for root rot disease was isolated and confirmed. *F. solani* is a common pathogenic genus in soil with CCO leading to diseases in melon (Ku et al. 2022), peanut (Oddino et al. 2008; Xie et al. 2017), and soybeans (Ranzi et al. 2017) etc., and we proved that *F. solani* strain D1 can also be an invading pathogen attacking *CA* and cause the root rot disease.

For the stress response system in *CA*, most of the indices showed positively significant relationships except a negative correlation with POD activity, which was in accordance with some studies (Boonsiri et al. 2007; Teixeira et al. 2017). Chl *a*:*b* was an indicator of the functional pigment equipment and light adaptation of the photosynthetic apparatus (Lichtenthaler and Buschmann 2001), and was sensitive in response to some environmental stresses such as drought stress (Ebrahimiyan et al. 2013), salt stress (Zhu et al. 2019), and high temperature stress (Bahrami et al. 2021), et al. In this study, it seems that Chl *a*:*b* is also an appropriate indicator for characteristic of the stress status in response to LE or RE, which is recommended to be from 0.6 to 0.8 for a healthy plant.

Most studies have pointed out that CCO was attributed to microbial communities shifts (Li et al. 2020; Chen et al. 2022b) and soil acidification (Bai et al. 2019; Li et al. 2021). We found that continuous cropping of *CA* resulted in significant decreases in pH with time (Fig. S1A), and the pH values in the treatments of LE and LE + D1 significantly decreased compared with control (Fig. S1B). However, the decrease value was less than 0.24 units, and the soil pH was always higher than 7.4 within three years of continuous cropping process. Besides, a short-term drop in pH can quickly returned to higher than 7.0 (Fig. S1B). Thus, due to the distinctly main reasons for CCO in different phases (Chen et al. 2020), we inferred that soil acidification in this phase may not be the main cause, as the changes in pH value were not dramatic and always higher than 7.0. We did not find significant changes in bacterial community following D1 and LE additions, which was different from some studies (Bai et al. 2019; Liu et al. 2020). This might be due to the different cropping time and/or different plants. *Filobasidium* was predominant in LE treatment on day 0 only, which is due to the fact that leaves or fruits are their optimal living environments (Bougreau et

al. 2019; Cureau et al. 2021). The most obvious shift in fungal community was occurred in LE + D1 treatment. The addition of LE could change the fungal community significantly with time, mainly reflected in the increase in the abundance of *Fusarium*, which was in agreement with the result that LE could promote the growth and propagation of strain D1. Moreover, the propagation of *Fusarium* in soil could inhibit some dominant fungal genera including *Mortierella*, *Pseudeurotium*, *Thielavia*, and *Hormiactis*, etc. (Table 2). Among these, *Mortierella* (Ozimek and Hanaka 2020), *Pseudeurotium* (Zhou et al. 2018), and *Thielavia* (Tõlgo et al. 2021, p. 172) were regarded as beneficial fungi because of their functions in plant growth promotion and biomass degradation. Thus, the promotion of *Fusarium* growth and propagation by LE can not only enhance its pathogenicity in plants but also decrease the proportions of potential beneficial fungal genera in soil, which synthetically leads to the CCO phenomenon. *Pseudarthrobacter*, *Pontibacter*, *Planococcus*, and *Paracoccus* were abundant in LE treatment but whereafter lost their competitions in soil, possibly because these bacteria were mainly derived from the marine (Liu et al. 2008; Balan et al. 2016; Wang et al. 2022b). *CA* planting place in Binhai county was near the Yellow Sea, and leaves probably accumulated these bacteria during the growth period. When LE was introduced into the soil far from the sea, these bacteria in it lost competitions with indigenous bacteria gradually.

In this study, for the first time we advanced a hypothesis that leaf extracts might play a key role in CCO based on the field investigation and previous studies. We found that the water extract of leaf of *CA* showed more inhibitory effects on seeds germination, seedlings growth, and soil enzymes activities compared with root extract. This was unlike the beneficial effects of leaf litter on soil properties in forest ecosystem (Li et al. 2014) or other farmland ecosystem (Chen et al. 2022a). Very few studies focused on the possible active substances in leaves of medicinal plants for harvesting tuberous roots. We found that phenolic acids including caffeic acid and p-coumaric acid accumulated much more in leaf than in root. Though the substances were abundant in LE and RE, they could be degraded in a few hours in soil (Bravetti et al. 2020), and thus were much lower in soil (Fig. 7). Phenolic acids were commonly known to be the main inhibitors in seed germination and plant growth processes (Lodhi 1975), while distinct effects were exhibited for different kinds of phenolic acids (Li et al. 2016; Bravetti et al. 2020). Caffeic acid and p-coumaric acid were often mentioned in some other studies, but how they interact with the main fungal pathogen and shift the microbial communities were unclear. Recently, a study pointed out that three phenolic acids chlorogenic acid, salicylic acid, and vanillic acid promoted a higher relative abundance of soil-borne fungi with the ability to invade plant roots in the simulated rhizosphere (Clocchiatti et al. 2021). Another study drew a similar conclusion by using Illumina MiSeq sequencing technology (Li et al. 2018). Beyond this, we further showed that LE and RE could promote the growth of strain D1 mainly reflecting in the increases in hypha weight and spore numbers (Fig. 5). Thicker hypha and more abundant spores in soil can undoubtedly enhance their competitions in microbial community. Despite that researches have indicated that some medicinal plant extracts could be antagonistic against *Fusarium* spp. (Dwivedi et al. 2015), we provide a different conclusion which is partly consistent with a study, in which they showed that pathogenic *Fusarium* spp. could consume the active substance ginsenoside (Jiao et al. 2015). This difference may be due to distinct substances used in studies. Interestingly, some pathogenesis-related enzymes were also stimulated by LE or RE (Fig. 6), which could be an important reason for the more serious impacts on *CA* seedlings growth following strain D1 invasion coupled with LE or RE. This result was consistent with some previous studies. For instance, a study showed that fungus expressed most of the extracellular biodegradative enzymes (i.e. proteases, pectinases, and cellulases) in the cultures with litter (Schneider et al. 2010). Besides, a research also indicated that *Agave* leaf extracts could induce enzymes activities in fungal strains belonging to *Fusarium* and *Lasiodiplodia* (Campos-Rivero et al. 2019). Besides, now that phenolic acids in leaves could stimulate the growth and propagation of strain D1, the positive detection of D1 in a later phase in the leaf litter treatment can be understandable (Fig. 4D).

Conclusions

In summary, the contribution of LE or RE to the CCO of *CA* seems to be comprehensive in planting system including affects seed germination and plant growth, attenuates plant immune system, regulates soil fungal communities (by increasing pathogen and decreasing beneficial fungi) and enzymatic activities, promotes the growth of a fungal pathogen strain D1 and induces the activities of pathogenesis-related enzymes, and these effects are possibly due to caffeic acid and p-coumaric acid in the extracts especially LE. As a result, leaf litters should not be discarded in soil any longer, and needs to be collected for resource utilization possibly for weed control, isolation of active substances, and preparation of functional animal feeds in the future.

Declarations

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Conflicts of interest

The authors declared no conflicts of interest.

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Figures

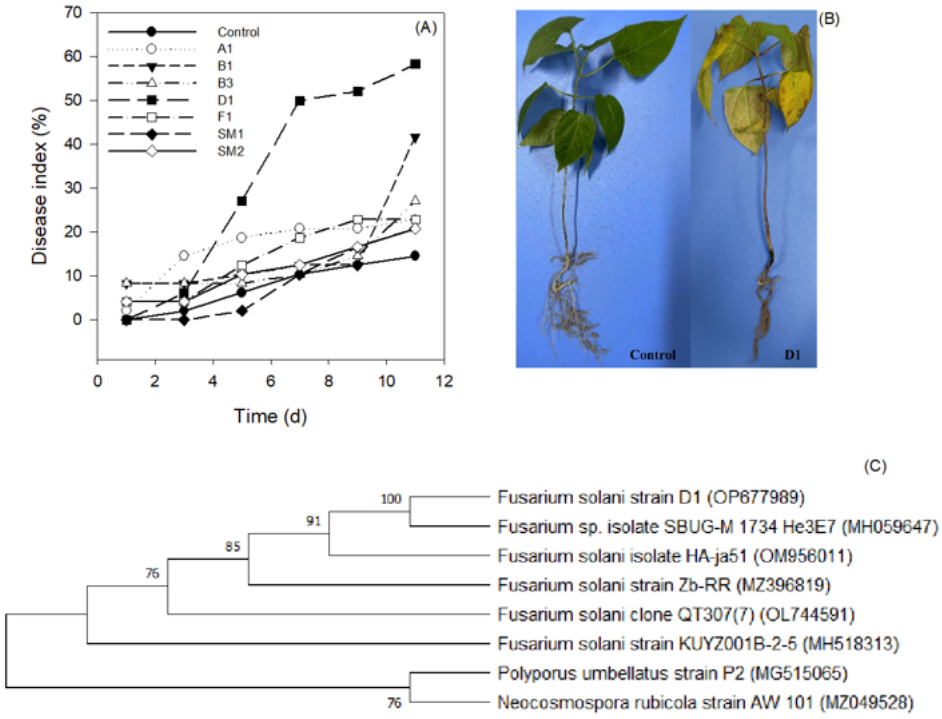

Figure 1

Isolation and identification of a fungal pathogen causing root rot disease. (A) Disease index with time after inoculation with different fungal strains at the final concentration of 10^9 CFU/kg soil. (B) Symptom of root rot disease caused by strain D1 on day 11. (C) Phylogenetic tree based on the sequences of ITS region of strain D1 and its relatives by using Neighbor-Joining method.

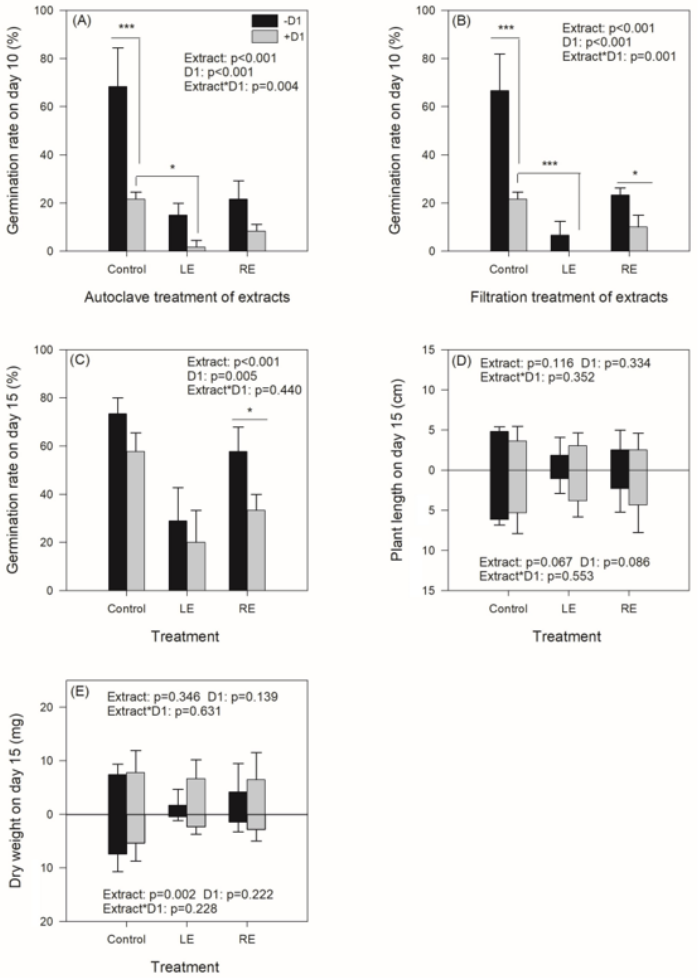

Figure 2

Effects of LE and RE on seed germination and growth indices using petri dishes (A, B) and pots (C-E) experiments, respectively. A two-way ANOVA was performed to test the effects of extract kinds (LE or RE) and D1 (with or without) on the indices, followed by Bonferroni's post-hoc tests. "-D1" and "+D1" in panels A and B indicated treatments without and with the spores of strain D1 at the final concentration of 10^6 CFU/mL. Different numbers of asterisk (*, **, ***) represent significant differences between samples at *P*-values of 0.05, 0.01, and 0.001.

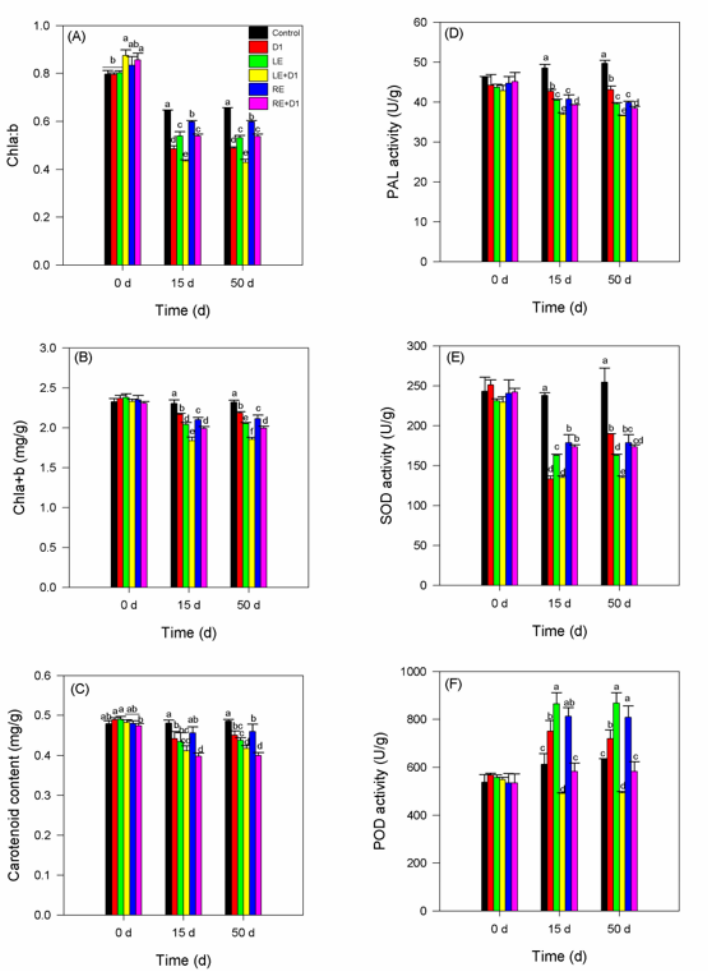

Figure 3

Plant stress responses to LE, RE, and/or strain D1. The changes in the contents of photosynthetic pigments of Chl *a*:*b* (A), Chl *a*+*b* (B) and Carotenoid content (C) in *CA* plants following the addition of plant extracts and/or strain D1. The activities of defensive enzymes following the additions of plant extracts and/or strain D1(D-F). Legends shown in panel A apply equally to panels B-F. Different letters at one sampling time point indicates significant differences among treatments (Duncan's multiple range test, $P \leq 0.05$).

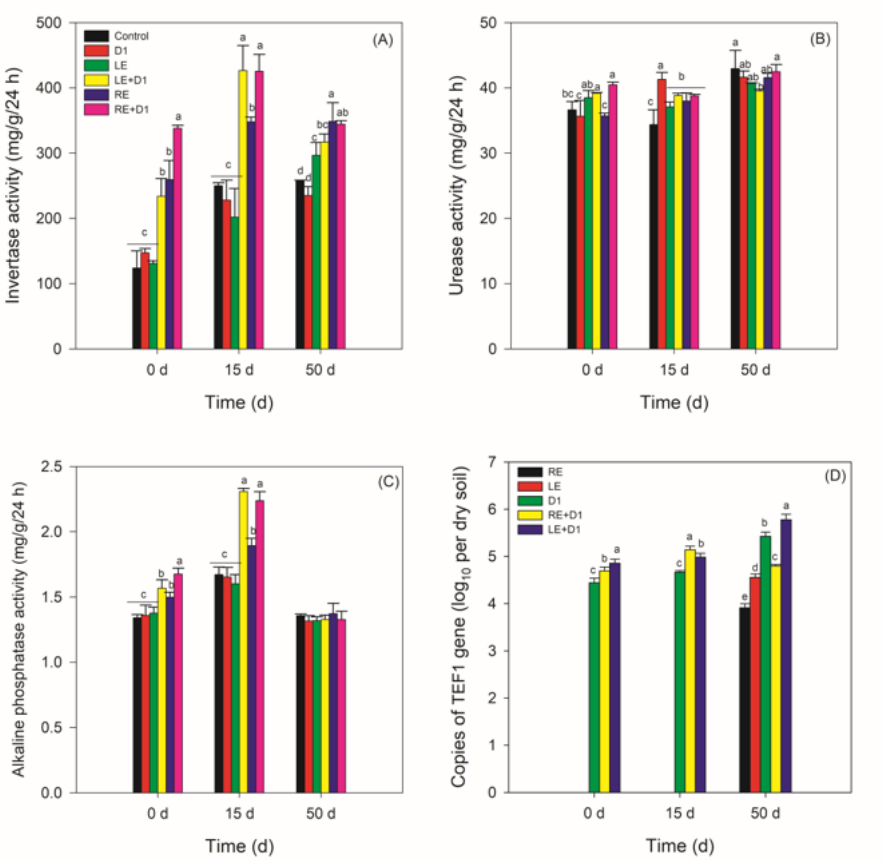

Figure 4

Effects of LE and RE on soil enzymes activities (A-C) and abundances of strain D1 using qPCR method (D). Legends shown in panel A apply equally to panels B and C. Different letters at one sampling time point indicates significant differences among treatments (Duncan's multiple range test, $P \leq 0.05$).

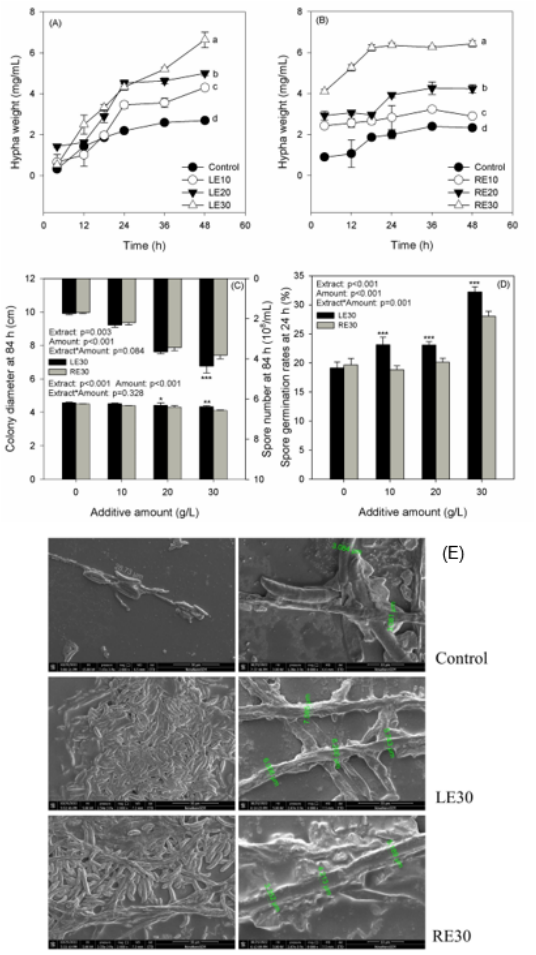

Figure 5

Effects of LE and RE on the growth of strain D1. Hypha weights in responses to LE (A) and RE (B), and the different letters on the plots indicates significant differences among treatments at 48 h (Duncan's multiple range test, $P \leq 0.05$). Effects of LE and RE on colonies diameters and spore numbers at 84 h (C), and spore germination rates at 24 h (D). (E) Morphologies of strain D1 following LE30 and RE30 treatments using SEM, and the pictures exhibiting the spores and mycelium of strain D1 were magnified by 2000 and 8000 times, respectively. Different numbers of asterisk (*, **, ***) represent significant differences between samples at *P*-values of 0.05, 0.01, and 0.001.

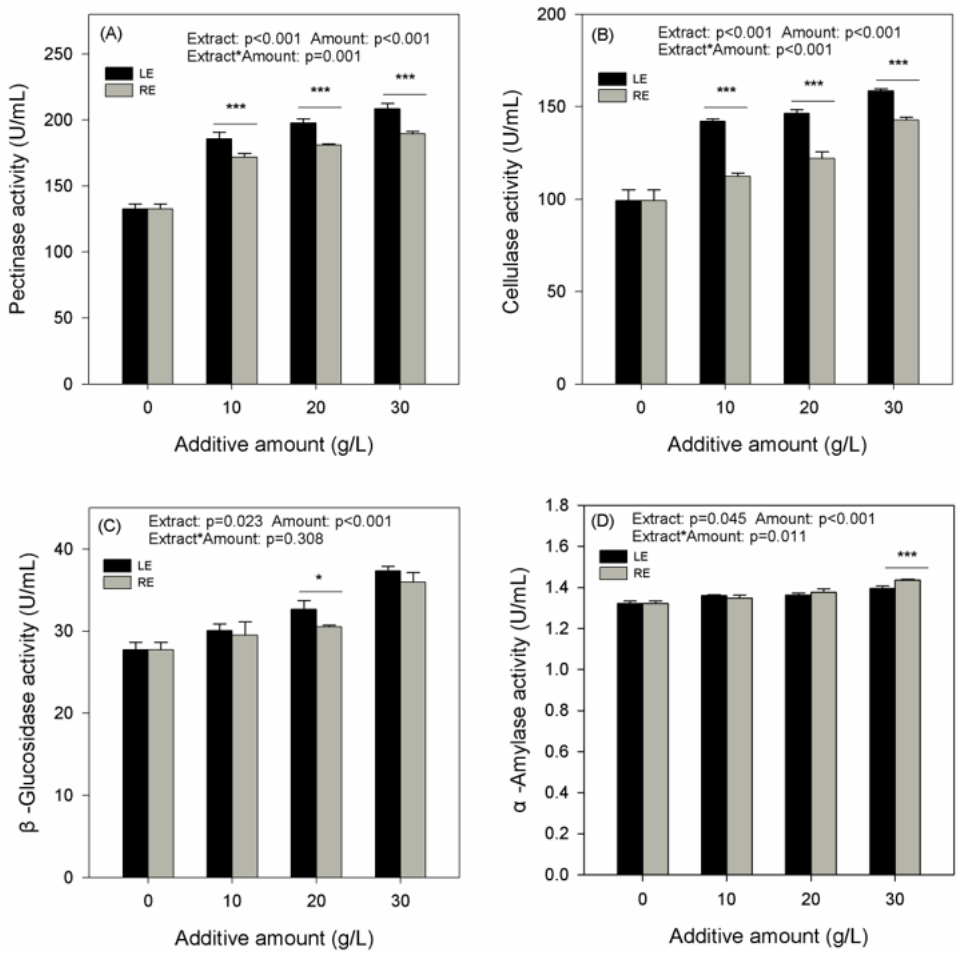

Figure 6

Effects of LE and RE on the pathogenesis-related enzymes activities of strain D1. Effects of different additives on strain D1 pectinase activity (A), β-Glucosidase activity (B), cellulase activity (C) and α-Amylase activity (D). Different numbers of asterisk (*, **, ***) represent significant differences between samples at *P*-values of 0.05, 0.01, and 0.001.

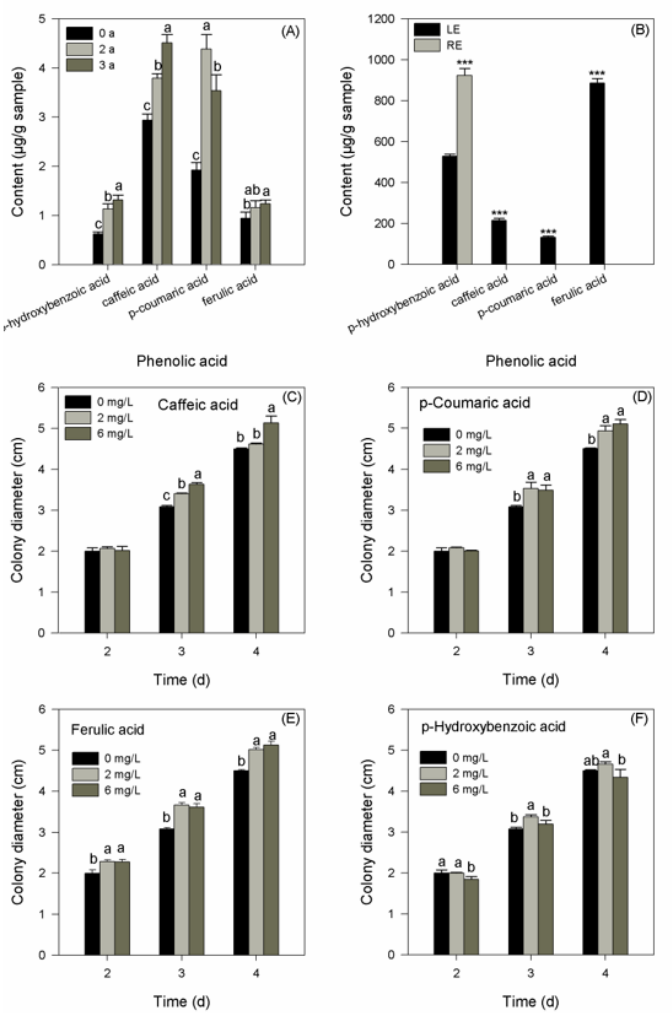

Figure 7

Contents of the main phenolic acids in soils with different *CA* cropping histories (A) and plant tissues (B), and their effects on the growth of stain D1 (C-F). In panel A, 0 a, 2 a, and 3 a indicated that soils have been planted with *CA* for 0, 2, and 3 years, respectively. Different numbers of asterisk (*, **, ***) represent significant differences between samples at *P*-values of 0.05, 0.01, and 0.001. Different letters at one sampling time indicates significant differences among treatments (Duncan's multiple range test, P≤0.05).

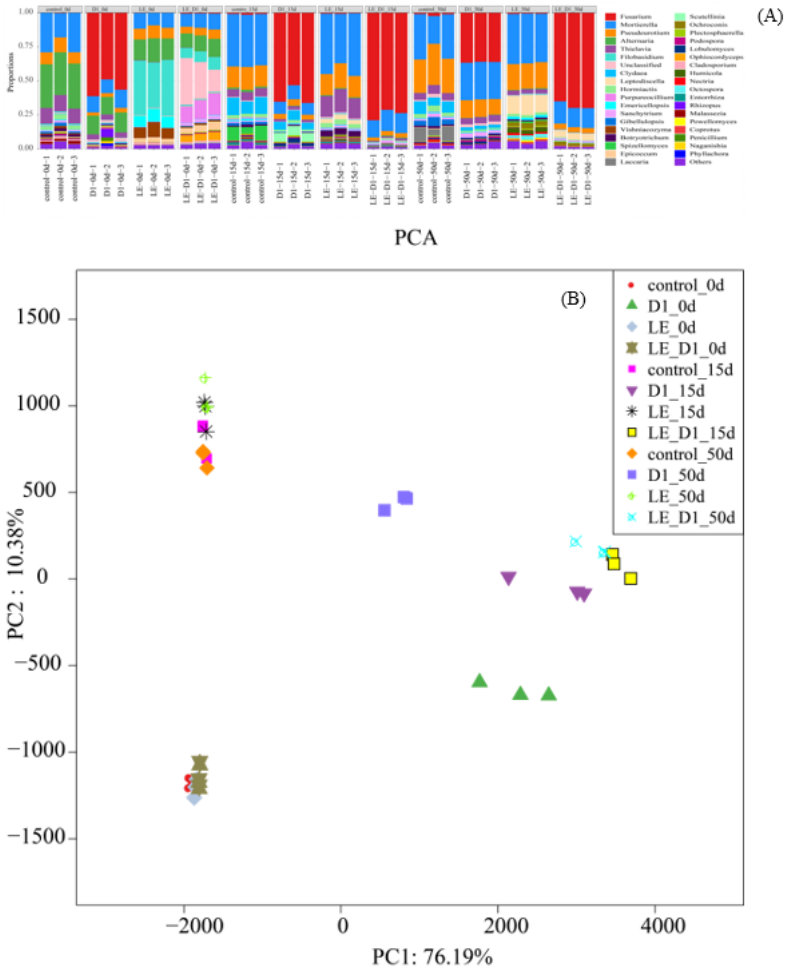

Figure 8

Effects of LE and/or strain D1 on the fungal community with time (A), and the changes in fungal composition among different treatments during 50 days using Principal Component Analysis (PCA) (B). 0d represents the initial fungal community, 15d and 50d represents day 15 and day 50, respectively. Those relative abundance lower than 1% were categorized to “others”.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialsrevisedversionwithmarks.docx