

ENDOPHYTIC FUNGI MEDIATE *CAMELLIA OLEIFERA* C. ABEL. (THEACEAE) GROWTH AND PROMOTE ACCUMULATION OF THE SECONDARY METABOLITES

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Abstract

Endophytic fungi are generally found in plants as beneficial microbial flora, establishing a symbiotic relationship with them and enhancing their resistance and promoting growth. To study the effect of endophytic fungi on *Camellia oleifera* C. Abel. growth, four endophytic fungi strains were isolated from rhizomes of *C. oleifera* and co-cultured with *C. oleifera* seedlings individually in sterile soil for 49 days: *Didymella* sp. (DS), *Fusarium* sp. (FS), *Penicillium* sp. (PS), and *Clonostachys rosea* (CR). The experimental results showed that bioactivities of four fungal stains differed, but all exhibited the promotion of quercetin accumulation and reduction of quercetin glycosides after co-culture with *C. oleifera* seedlings. DS, FS and PS significantly increased the leaf area of *C. oleifera*, and all of the experimental groups were more than 50% heavier than the Control (CON). Our results demonstrate the prospective utility of endophytic fungi in *C. oleifera* production with the capacities to enhance productivity and accumulation of plant metabolism products.

Key words: *Camellia oleifera*, Endophytic fungi, Secondary metabolism.

Introduction

From the early nineteenth century, when plant endophytes were discovered, people have been fascinated by this interesting thing (Rayner, 1925). In 1991, endophytes were redefined to include all organisms inhabiting plant organs that at some time, they can colonize internal plant tissues without causing apparent harm to it (Petrini, 1991). The symbiotic linkage between host plants and microorganisms was gradually reawakened. Subsequently, a number of researches showed that interactions between plant and microbe affect growth and development of plant, health, and crop yield (Khare *et al.*, 2018; Leadbetter & Greenbegr, 2000; Yan *et al.*, 2018). It has been reported that plant-endophytic fungi-plant host interactions also clearly elaborate on this concept. Usually, Host plants offer protection and nutrition to endophytic fungi, and in return, endophytic fungi can enhance host plant endurance to abiotic and/or biotic stresses, including the assault by herbivorous insects or causative agent, or stress conditions (e.g. drought or salinity) (González-Teuber *et al.*, 2017; Gupta *et al.*, 2020; Khare *et al.*, 2018; Latz *et al.*, 2018). These metabolites can promote plant growth and bio-active metabolite accumulation, which can enormously enhance the host plant vigour (Christian *et al.*, 2019).

Camellia oleifera C. Abel. Of the family Theaceae an endemic species of woody oil in China, is one of mainly oilseed woody plants, with high economic and medicinal values (Wang *et al.*, 2016). Its main product, tea oil, is an internationally recognized healthy vegetable oil, rich in functional active ingredients, including tea polyphenols, squalene, vitamin E, and polyphenolic compounds, which have positive effects on preventing cardiovascular diseases, enhancing immunity, and lowering blood lipids (Ke *et al.*, 2019; Xiong *et al.*, 2017; Ye *et al.*, 2017; Luan *et al.*, 2020), and tea oil's antibacterial activity is confirmed, and terpinen-4-ol is the main contributor to the

antibacterial activity (Li *et al.*, 2016). Some researchers have analyzed tea oil cake meal, *C. oleifera* seed hulls and other trimmings for substance extraction and found that the extracts contained a variety of antioxidant and antitumor-active substances (Ma *et al.*, 2013; Zhang *et al.*, 2020).

Many culturable microorganisms in healthy *C. oleifera* plants have been isolated and identified, but major researches have reported on *C. oleifera*'s microbe diversities and its surroundings (Liu *et al.*, 2020; Zhou *et al.*, 2014; Zhang *et al.*, 2020), nitrogen-fixing bacteria (Cai *et al.*, 2011), phosphate-solubilizing bacteria (Liu *et al.*, 2016; Wang *et al.*, 2015), Y13 strain (*Bacillus subtilis*) capable of antagonizing *C. oleifera* anthracnose (Bu *et al.*, 2012; Liu *et al.*, 2020), *Beauveria bassiana* for the *C. oleifera* pest's toxicities (Cai *et al.*, 2013; Deng *et al.*, 2012) and AM fungi, which can help growth of plant and improve resistance (Tan *et al.*, 2022; Wang *et al.*, 2011), but relatively little has been done on function of individual endophytic fungi in *C. oleifera*. Therefore, research evaluation of *C. oleifera* endophytic fungi to reveal function of individual endophytic fungi in *C. oleifera* and to explore their potential application in the *C. oleifera* industry has the potential to provide new thinking for the *C. oleifera* industry development.

In this study, to learn how microorganisms mediate *C. oleifera* growth, we established a model of plant-microbe interactions, co-cultured seedlings with each of four fungal strains, controls for abiotic factors and measured plant physiological indicators, morphological indices and secondary metabolites. This allowed us to visualize the specific effects of endophytic fungi on *C. oleifera* growth and to identify interaction between microorganisms and *C. oleifera*. Secondary metabolism may be essential for the establishment of communication between fungi and plants and is critical for sustaining the growth of *C. oleifera*, providing a promising ecological strategy for developing more resilient and productive crops.

Material and Methods

Fungal strains: The surface soil of fresh *C. oleifera* rhizomes was cleaned with a brush and cut into 4~5 cm segments and rinsed under running water for 2 h. Then, the surface was sterilized: using sterile water (2~3 times), 30 s soaking in 75% (v/v) alcohol, then sterile water (2~3 times), soaking in 0.2% HgCl₂ (m/v) for 12 min, and finally 4~5 times with sterile water. Under aseptic conditions, the epidermis of the rhizome was cut off using a sterile scalpel, and the central part was evenly cut into small pieces of about 5 mm along the growth direction and laid flat on PDA medium containing 0.2% streptomycin. Placed in a constant temperature incubator for 7 d in the dark culture at 28 °C. The obtained colonies were purified and cultured to obtain individual strains.

Sterile seedlings: *C. oleifera* seeds were sterilized in 75% alcohol for 30 s, then HgCl₂ (0.2%, w/v) for 8 min, and finally cultured in sterile soil until seedlings (sterile soil consisted of humus: vermiculite = 3:1, remove impurities using a 2 mm sieve, then placed in glass jars and autoclaved 3 times at 121°C for 20 min). Culture conditions were 25°C, light intensity 1500-2000 lx, 12 h of light /12 h of darkness.

Hyphae suspension preparations and a co-culture system establishment of *C. oleifera* and endophyte: The fungal spores or aerial hypha growing on PDA medium were picked using a dissecting needle into sterile water placed in a 50 mL centrifuge tube and incubated in a shaking incubator at 30°C, constant temperature for 24 h. The spore concentration was adjusted to 1.0×10^4 CFU/mL by hemocytometer observed under a microscope, and *C. oleifera* seedlings of uniform growth condition were selected, and mycelial suspension (1 mL) was added under aseptic conditions along the seedlings were selected and mycelial suspension (1 mL) was added to sterile seedlings along the *C. oleifera* seedling roots under aseptic conditions, while sterile water (1 mL) was placed in the control group, thus establishing a co-culture system of *C. oleifera* and a single endophytic fungus.

Morphological parameter determination: Seedlings of *C. oleifera* were rinsed to remove soil under running water, and filter paper was used to absorb surface water after 49 days. Then, total fresh weight, rootstock length and plant height of *C. oleifera* seedlings were determined, and total leaf area was measured using indirect representation of $0.69 \times \text{leaf length} \times \text{leaf width}$. Each group is allocated 3 parallels.

Determination of malondialdehyde concentrations in *C. oleifera*: Each group was allocated 3 parallels; the parameters were measured every 7 days after inoculation of the hyphae suspension until day 49. The top mature leaves were crushed. A 100 mg of fresh leaves was homogenized using trichloroacetic acid (10 mL, 10%) and subsequently centrifuged at 4000 rpm for 10 min, then, which supernatant (2 mL) with thiobarbituric acid (2 mL, 0.6%) was added. The temperature of the mixture rised to 100°C, retained 15 min, followed by rapid cooling on ice, then centrifugated as

described previously. The supernatant absorbance at 450, 532 and 600 nm was recorded and the concentration of malondialdehyde (MDA) in the extract was calculated according to the following equation:

$$\text{CMDA } (\mu\text{mol/L}) = [6.45 \times (A_{532} - A_{600}) - [0.56 \times A_{450}]]$$

The activity determination of antioxidant enzymes in *C. oleifera*: Each group was allocated 3 parallels; the parameters were measured every 7 days after inoculation of the hyphae suspension until day 49. Superoxide dismutase (SOD) activities were analyzed using nitroblue tetrazolium (NBT). Fresh leaf tissue (100 mg) was pulverised with saline-buffered saline (PBS) (0.05 mM) at pH 7.8, then centrifuged for 10 min (4000 rpm) and aspirated the supernatant as the enzyme solution. The reaction system was as Table 1. After the reaction mixture was exposed at fluorescent light (4000 lx) for 20 min, the reaction mixture absorbance was determined at 560 nm to calculate SOD activity (based on inhibition rate):

$$\text{Inhibition rate} = [(A_0 - A_i) / (A_0)] \times 100\%$$

A₀ indicates control reaction volume absorbance; A_i indicates treated reaction volume absorbance. SOD activity's one unit (1 U) was defined as 50% inhibition concentration.

Table 1. Reaction system for SOD enzyme measurement.

Reagents	Dosage(mL)	Final concentration (when colorimetric)
Phosphate buffe	1.5	
Methionine (Met) solution	0.3	13.0 mmol
NBT	0.3	75.0 μmol
EDTA-Na	0.3	10.0 μmol
Riboflavin solution	0.5	2.0 μmol
Enzyme solution	0.1	
Water	0.5	
Toatal volume	3.5	

The activity of peroxidase (POD) was measured by grinding fresh leaves (100 mg) with 40 mM PBS (5 mL) at pH 6.0, the mixture was homogenized for 15 min, then, and centrifuged at 4000 rpm. Supernatant (100 μL) was added to a mixture including 2 μM H₂O₂ and 9 mM guaiacol and adjusted with PBS at pH 6.0 to 5 mL (total volume). Absorbance changes at 470 nm were recorded every 30 s for 3 min. The activity of POD enzyme was expressed as activity units (increasing 0.01 per minute at A₄₇₀ represented one enzyme unit (U).

Quercetin and its glucosides HPLC analysis: Each group was allocated 3 parallels; the parameters were measured every 7 days after inoculation of the hyphae suspension until day 49. Extraction, identification and quantification of quercetin and its glycosides were done: Fresh leaves sliced into shreds and 0.1 g of the rhizome was placed in a 20 mL of sample bottle holding 2 mL methanol, followed by extraction of ultrasound-assisted for 30 min. Then filtered the extract through a 0.2 μm membrane filter, and the filter liquor was collected into a 2 mL bottle of brown

glass. All samples were placed refrigerator at 4°C was performed. Isoquercitrin, quercetin and quercitrin were determined by high-performance liquid chromatography (HPLC), which include HPLC instrument (LC-20AT; Shimadzu, Japan) a reversed-phase column (SHIM-PACK C18 CLCDS, 150×6.0 mm), mobile phase: (methanol: acetonitrile = 5:11 (v/v)) (solvent A) and (0.1% formic acid (v/v)) (solvent B). The program of specific gradient elution was referenced from our research report (Ye *et al.*, 2021).

Results

Isolation and pathogenicity of fungi isolated from *C. oleifera*: Four different endogenous fungal strains were isolated from fresh *C. oleifera* rhizomes. The rRNA ITS DNA sequences were compared with those in the GenBank database and identified as (100% identity) *Didymella* sp. (DS), *Fusarium* sp. (FS), *Penicillium* sp. (PS), *Clonostachys rosea* (CR) ([https:// www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/)) (Fig. 1).

Effect of endophytic fungal infiltration on the morphology of *C. oleifera*: Inoculation with different types of fungi affected the morphology of *C. oleifera*. DS, FS and PS all significantly increased the leaf area of *C. oleifera* by > 50%, wider than the control (CON) *C. oleifera* seedlings (234.5 mm²), 338.6 mm², 335.9 mm² and 372.9 mm² respectively, the leaf area of CR was 236.6 mm² and it had no significant impact on the leaf area of *C. oleifera* (Fig. 2a). CR (105 mm) significantly increased plant height, 10% higher than CON (95 mm), which was not significant in other groups (Fig. 2b). FS (229.5 mm) significantly increased root length, 15% longer than CON, which was not significant in other treatment groups (Fig. 2c). DS (0.5510 g), FS (0.6235 g) and PS (0.6186 g) remarkably increased the fresh weight of *C. oleifera* compared to that of CON (0.3882 g) (Fig. 2d). Except CR, dry weight of DS (0.191 g), FS (0.275 g), PS (0.196 g) remarkably increased the dry weight of *C. oleifera* compared to that of CON (0.161 g) (Fig. 2e).

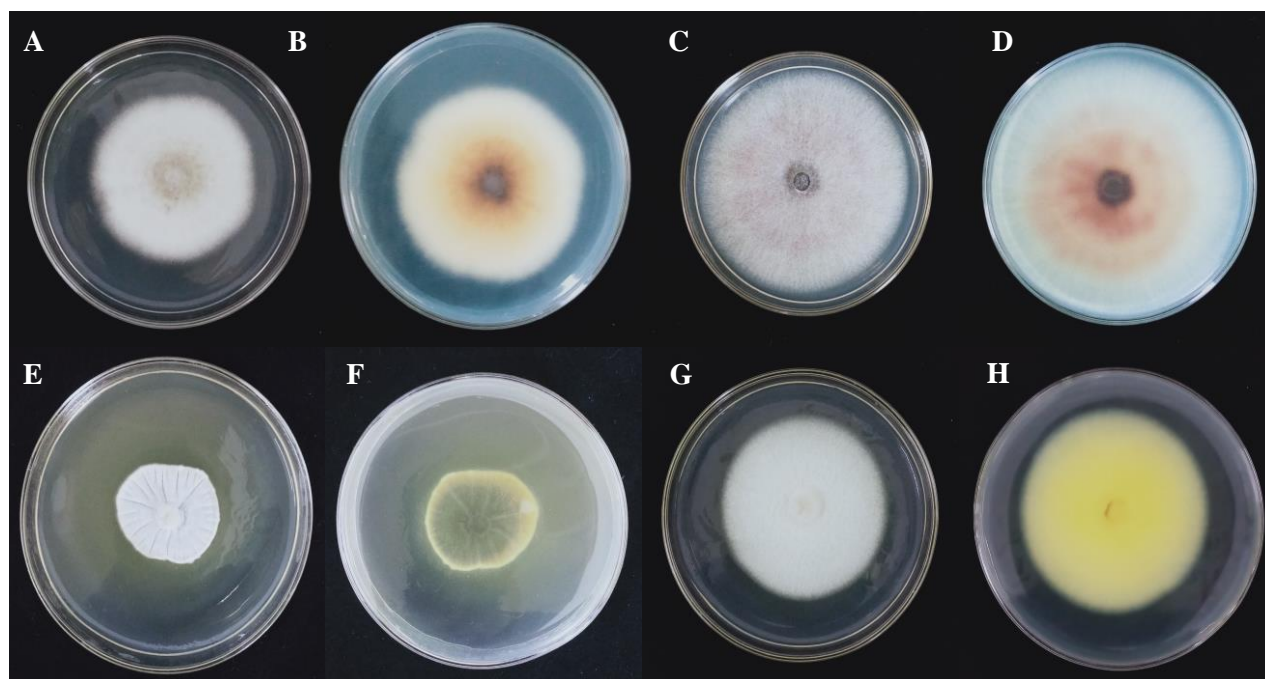


Fig. 1. Endogenous fungi isolated from fresh *C. oleifera* rhizomes: (A) DS surface side, (B) DS reverse side, (C) FS surface side, (D) FS reverse side, (E) PS surface side, (F) PS reverse side, (G) CR surface side, (H) CR reverse side.

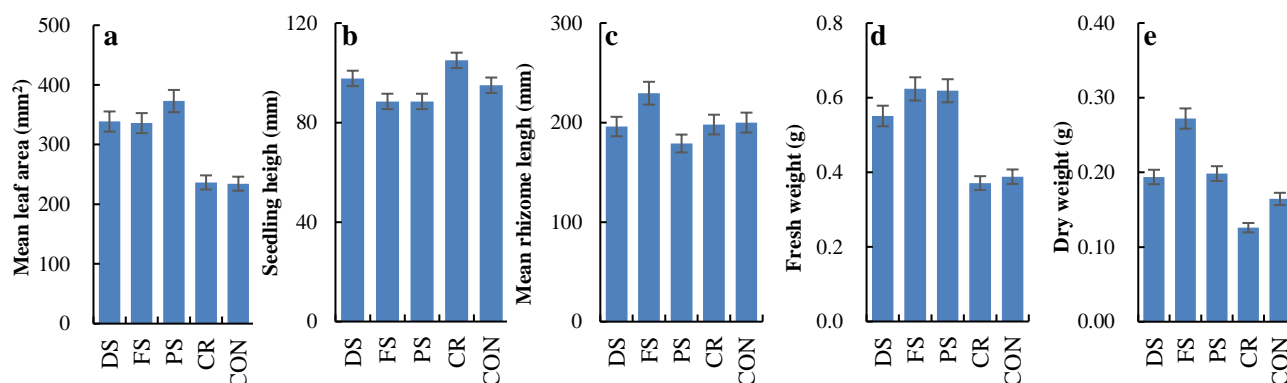


Fig. 2. Effect of inoculation with endophytic fungi on the morphology of *C.oleifera*. Note: Values indicate mean ± standard deviation ($n = 3$). CON = control DS = *Didymella* sp., FS = *Fusarium* sp., PS = *Penicillium* sp., CR = *Clonostachys rosea*.

Physiological effects of endophytic fungi on *C. oleifera* seedlings: The POD values of PS and CR were increased with time and significantly greater than those of CON (Fig. 3c and d), while the POD values of DS and FS did not change noticeably with time and were always significantly smaller than those of CON (Fig. 3a and b). The impacts of four fungi on the *C. oleifera* seedling SOD activities were significantly smaller than those of CON in the early stage and showed a gradual increase with time (Fig. 3e-h). The trend of MDA over time showed that four fungi contributed to the resistance of *C. oleifera* seedlings to membrane lipid peroxidation and adversity damage (Fig. 3i-l).

Effect of endophytic fungi on quercetin and its glycosides: We found that the fresh leaves of *C. oleifera* contained quercetin and its glycosides, quercitrin, and isoquercitrin, by HPLC. As quercetin is known as a natural flavonoid that promotes normal plant growth and development[52], we measured these three compound contents to reveal the extent of the differential impacts of inoculation with different fungi on the contents of secondary metabolites of *C. oleifera* (Fig. 4).

The levels of quercetin in CON were found to remain stable by 49-day follow-up measurements (Fig. 4i-l), while the levels of quercitrin and isoquercitrin accumulated gradually over time (Fig. 4a-h). All four fungal strains promoted the accumulation of quercetin, which was significantly greater than that of CON (Fig. 4a-h), and showed a negative effect on the accumulation of quercitrin and isoquercitrin (Fig. 4i-l).

The correlation coefficient analysis between different physiological indicators: The Pearson correlation coefficient heatmap revealed that leaf area of *C. oleifera* seedlings was extremely significantly positively correlated with fresh weight ($p < 0.01$), leaf POD was significantly negatively correlated with leaf MDA ($p < 0.05$), and plant height was significantly negatively correlated with leaf SOD ($p < 0.05$). Quercetin content in *C. oleifera* leaves was positively correlated with leaf area, fresh weight, plant height and leaf SOD activity, while it was negatively correlated with quercitrin and isoquercitrin (Fig. 5).

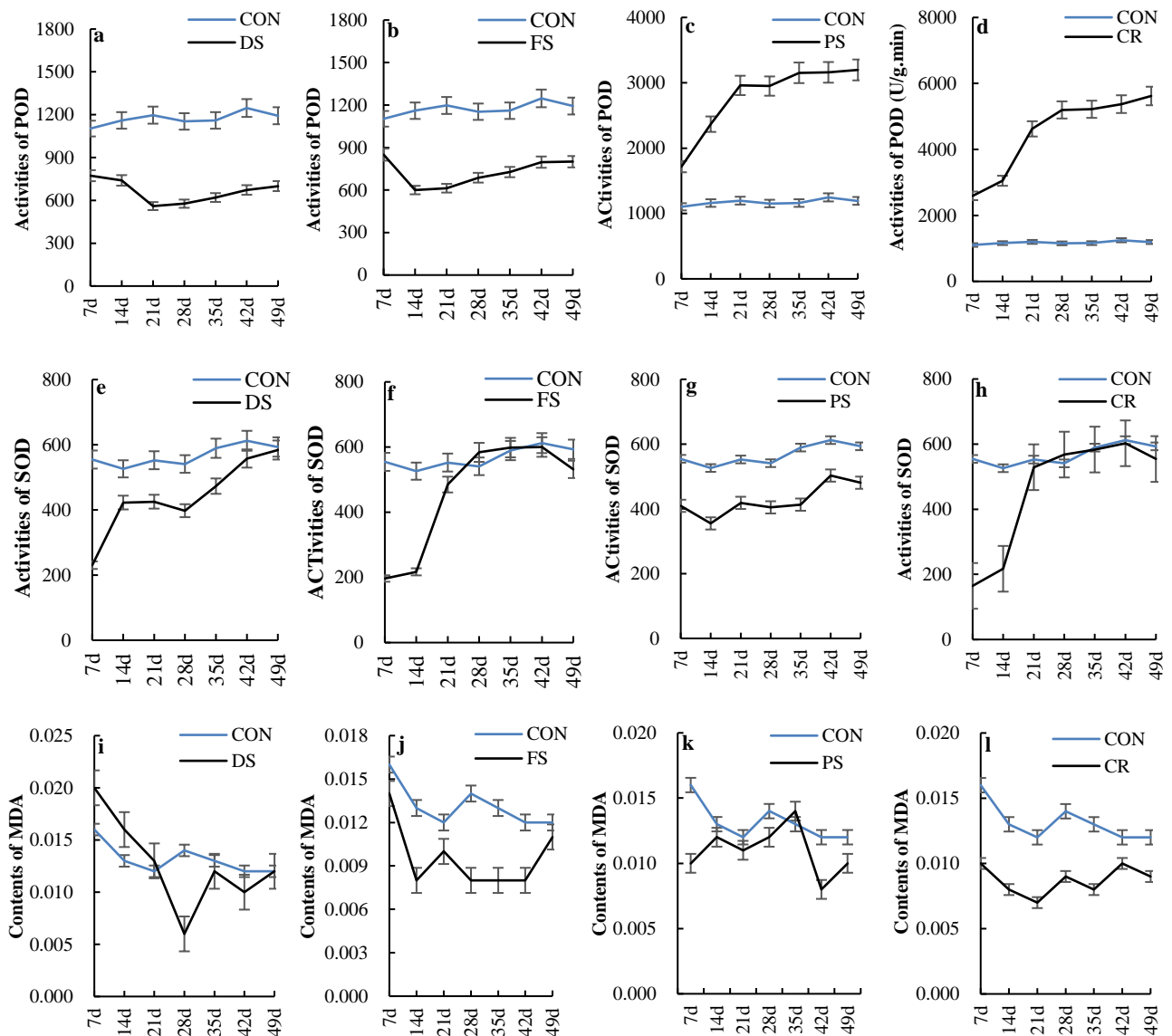


Fig. 3. Effects on physiological indices of *C. oleifera* inoculated with endophytic fungi. (Note: Values represent means \pm standard deviation ($n = 3$). CON = control DS = *Didymella* sp., FS = *Fusarium* sp., PS = *Penicillium* sp., CR = *Clonostachys rosea*).

Discussion

In general, normally by changing the growing conditions, the crop yield was improved or the accumulation of certain secondary metabolites in crops was regulated are rarely mentioned (Gomez-Del-Camp *et al.*, 2017; Liu *et al.*, 2020). While plant biomass is an important indicator of its growth status, the study showed that four fungi isolated from *C. oleifera* were able to promote the increase of leaf area and fresh weight; significantly affect the formation and accumulation of secondary metabolites and were able to influence the enzyme activities in the plant. The similar studies have been reported that, for example, endophytic fungi can induce changes in key enzyme activities and thus regulate the secondary metabolite accumulation such as *p-tyrosol* and *salidroside* by inducing activation of the host signaling molecular network system (Wang *et al.*, 2015); that endophytic fungi in *Salvia miltiorrhiza* roots stimulate hairy root growth and tanshinone biosynthesis (Ming *et al.*,

2013); *Bletilla striata* co-culture with endophytic fungi can have a regulatory effect on the accumulation of active ingredients of *B. striata*, and different strains have different accumulation levels of different compounds (Gomez-Del-Camp *et al.*, 2017); The plant growth-promoting rhizobacteria can promote the growth and carbon and nitrogen metabolites of *Pseudostellaria heterophylla* and increase its resistance to adversity (Liu *et al.*, 2020).

DS and FS often appear as plant disease fungi, *Didymella bryoniae* leads to Gummy stem blight in watermelon and cucurbits (Bahu *et al.*, 2015), *D. pinodes* is one of the most important fungal diseases of peas in the worldwide (Barilli *et al.*, 2016), *D. bellidis* and *D. bellidis* are pathogenic fungi that cause leaf spot of the tea tree (Ren *et al.*, 2019); The genus *Fusarium* includes many agriculturally important pathogens of plant, fungal toxin producers and opportunistic fungal pathogens of human (Ren *et al.*, 2019). For example *F. oxysporum* is the pathogen of wilt diseases or root rot in some plant species (e.g., banana, tomato and asparagus) (Berrocal-Lobo & Molina, 2013).

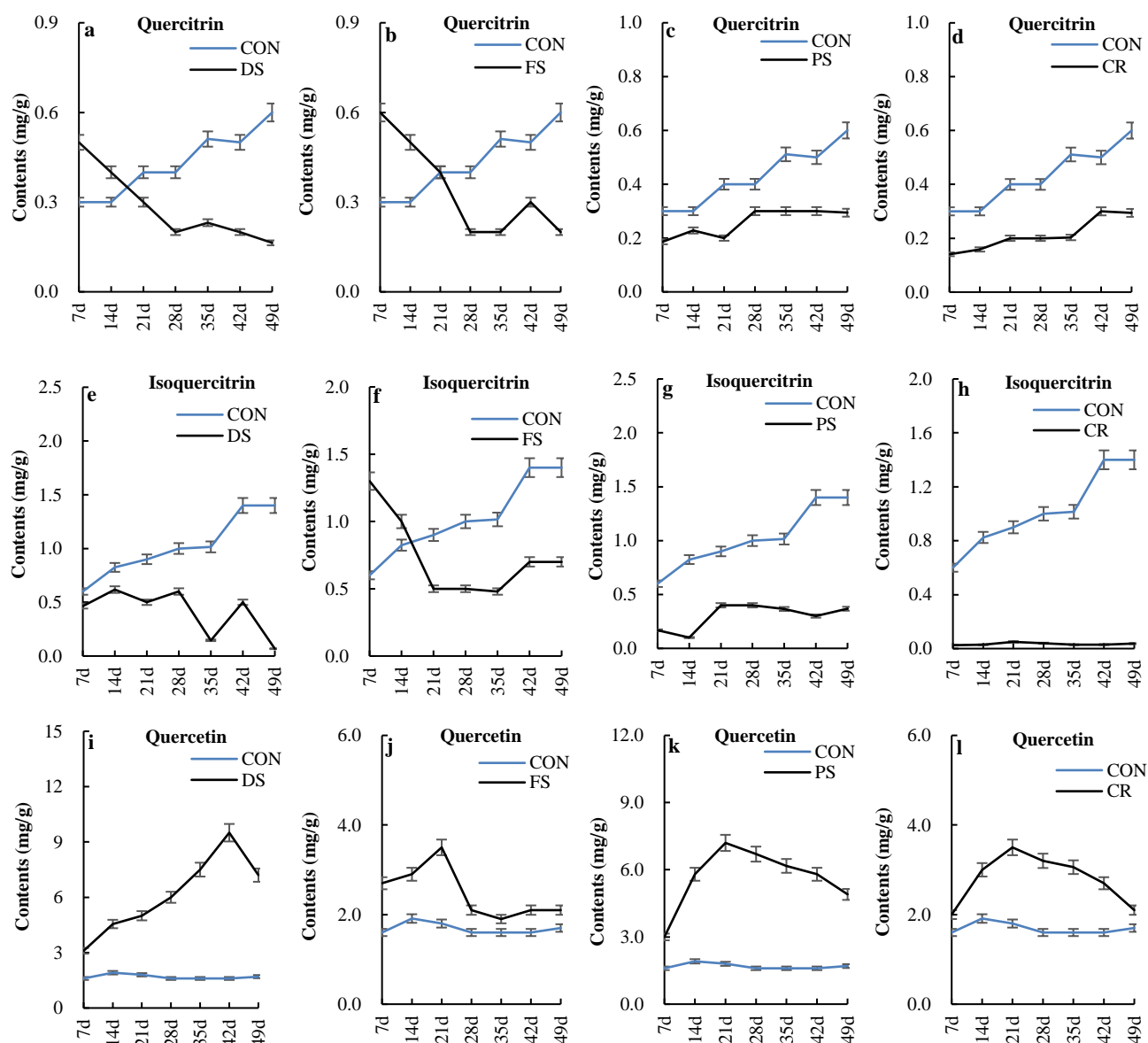


Fig. 4. Effects on quercetin, quercitrin and isoquercitrin of *C. oleifera* inoculated with endophytic fungi. (Note: values represent means ± standard deviation (n = 3). CON = control, DS = *Didymella* sp., FS = *Fusarium* sp., PS = *Penicillium* sp., CR = *Clonostachys rosea*).

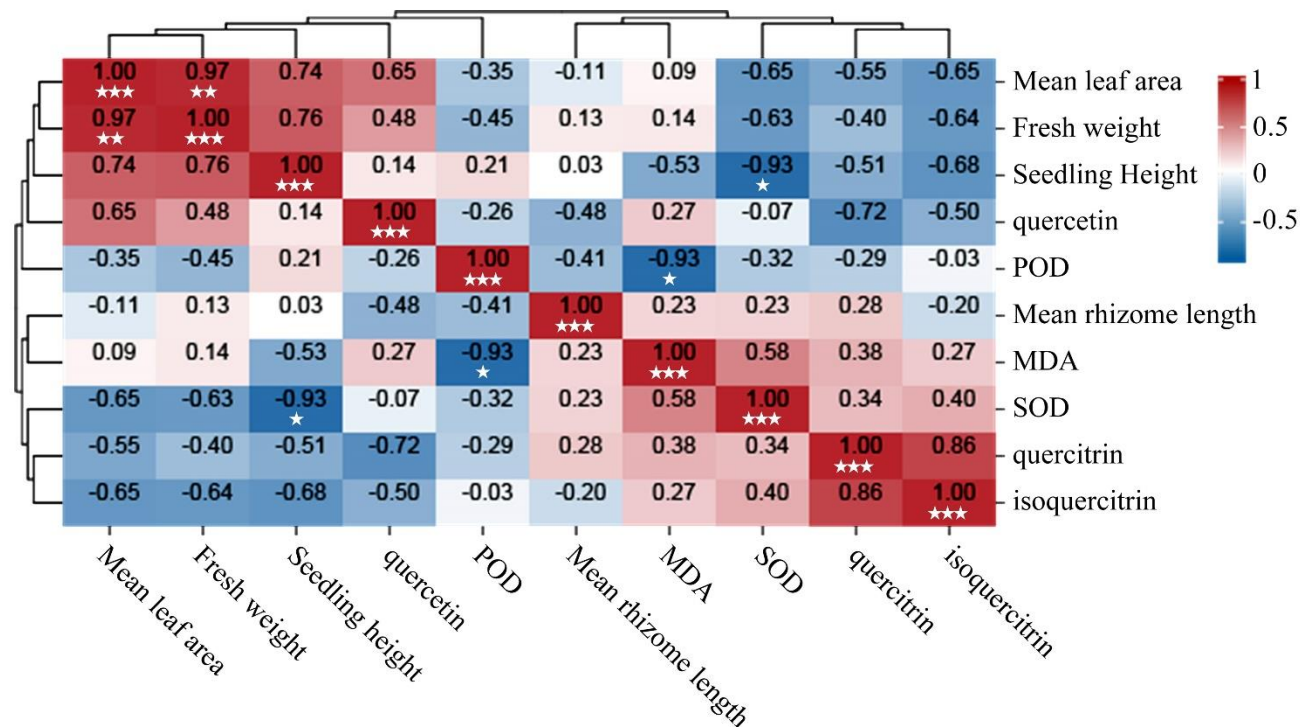


Fig. 5. The Pearson correlation coefficient heatmap for various indicators of *C. oleifera* seedlings. (Note: 1. all of which are derived from day 49. 2. ** indicate $0.01 < p < 0.05$, *** indicate $0.001 < p < 0.001$, **** indicate $p \leq 0.0001$).

The members of the genus *Penicillium* often cause decay of food crops (Assaf *et al.*, 2020), there are also strains of *Penicillium* sp. that produce gibberellins, which regulate plant growth and development, and production of bioactive gibberellins as a fungus response that makes plants to resist salinity stress (Leitao *et al.*, 2016), and some study reported that *P. citrinum* in *Swertia chirayita* was active in limiting the plant pathogen growth (Sharma *et al.*, 2021). *Clonostachys rosea* often appears as a beneficial plant fungus with good biological control of many fungal pathogens of plants (Wang *et al.*, 2021), and has been reported as a promising saprophytic filamentous fungus with not only significant biocontrol activity but also for biodegradation of plastic waste, bioactive compound biotransformation, fermentation and bioenergy (Sun *et al.*, 2020), when applied to important economic crops, both *C. rosea* strains ACM941 and 88-710 have been recognized as beneficial microorganisms for control of their plant disease and growth promoting characteristics (Demissie *et al.*, 2021).

The bioactivities of the four fungal strains differed, but after co-cultivation with *C. oleifera* seedlings all showed a promotion of quercetin accumulation and abatement of quercetin glycosides, and correlation analysis also showed that quercetin content was negatively correlated with quercitrin and isoquercitrin content, accompanied by a gradual increase in SOD activity and a continuously low MDA content compared to the control group, which may be related to the antioxidant activity of quercetin, and the quercetin antioxidant activity (Singh *et al.*, 2021). Quercetin as a natural flavonoid, can promote a variety of plant physiological processes (e.g., photosynthesis, pollen growth, seed germination, antioxidant mechanisms and induce normal growth and development of plant, in

addition, it can effectively provide plants with resistance to biotic- and abiotic-stresses (Singh *et al.*, 2021), while glycosylation of quercetin can decrease quercetin antioxidant activity (Brown *et al.*, 1998), which might be the reason for the gradual accumulation of quercetin and the gradual rebound of SOD activity in *C. oleifera* seedlings. Other studies have shown that flavonoids can alter growth hormone transport and signaling to aid plant adaptation, growth and development and defense against external stresses (Brown *et al.*, 2001; Peer & Murphy, 2007), confirming our results. In addition, the abundant and diverse secondary metabolites in fungi can manipulate the dynamics of plant community due to promoting or inhibiting the symbiotic organism establishment, and affect the structure of plant metabolites, may also be responsible for the secondary metabolite accumulation in *C. oleifera* (Bills *et al.*, 2016; Etalo *et al.*, 2018; Keller, 2019; Rangel *et al.*, 2021; Spatafora and Bushley, 2015; Yan *et al.*, 2019). Unlike FS and CR, the POD of oilseed tea seedlings was consistently significantly lower than that of controls after inoculation with DS and FS, possibly related to their phytopathogenic properties (Ma *et al.*, 2013; Ren *et al.*, 2019; Wang *et al.*, 2021).

In the current study, we used four endophytic fungi strains isolated from the *C. oleifera* rhizosphere in co-culture with *C. oleifera* sterile seedlings, which positively affected the concentrations of secondary metabolites and growth of *C. oleifera* (Hardoim *et al.*, 2015; Rodriguez *et al.*, 2009; Yan *et al.*, 2019). However, we only tested the effects of fungus individually on *C. oleifera* growth and secondary metabolites under the pot experiment. We will investigate the molecular mechanisms of endophytic fungi in mediating the accumulation of secondary metabolites in our future studies.

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