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Endophytic Fungi Accelerate Leaf Physiological Activity and Resveratrol Accumulation in *Polygonum cuspidatum* by Up-Regulating Expression of Associated Genes

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Abstract: *Polygonum cuspidatum* Sieb. et Zucc. is a major raw material for the extraction of drugs such as resveratrol, while the over-exploitation of *P. cuspidatum* decreases the yield and drug components. The purpose of this study was to analyze the effect of inoculation with root endophytic fungi *Funneliformis mosseae* and *Piriformospora indica* singly or in combination in biomass production, physiological activities (e.g., chlorophyll, soluble protein, and gas exchange) and main medicinal ingredients of *P. cuspidatum*, accompanied by the expression levels of associated genes in resveratrol biosynthesis. Single and co-inoculation with *P. indica* significantly improved shoot and root biomass production, and single and co-inoculation with *F. mosseae* and *P. indica*, especially single *P. indica*, significantly promoted leaf chlorophyll and soluble-protein concentrations and improved leaf gas exchange, including photosynthetic rate, transpiration rate, stomatal conductance, and intercellular CO_2 concentration. The application of endophytic fungi increased resveratrol and polydatin concentrations, while it affected chrysophanol, emodin, and physcion concentrations in a complex manner. In addition, *F. mosseae* inoculation and co-inoculation induced the expression of *PcCRS1*, *PcRS11*, *PcRS*, and *PcSTS*, and only single *F. mosseae* and *P. indica* inoculation up-regulated the expression of *PcCHS1* and *PcCHS2*. It was concluded that endophytic fungi accelerated biomass production, leaf physiological activity, and resveratrol accumulation in *P. cuspidatum*, which was associated with the up-regulation of related gene expression in resveratrol biosynthesis.

Keywords: arbuscular mycorrhiza; medicinal plant; *Piriformospora indica*; resveratrol; resveratrol synthase

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1. Introduction

Polygonum cuspidatum Sieb. et Zucc. is a perennial herb with medicinal components mainly containing anthraquinones, stilbenes, flavonoids, tannins, and polysaccharides [1]. *P. cuspidatum* is used in China and Japan to treat inflammation, infection, jaundice, skin burns, and hyperlipemia [2]. Among secondary metabolites of *P. cuspidatum*, resveratrol is a non-flavonoid polyphenolic compound containing a terpene structure, which is a

phytoalexin in response to biotic or abiotic stresses, as well as possessing antitumor, antioxidant, and antibacterial effects [3]. The resveratrol and anthraquinones in *P. cuspidatum* are from the metabolic pathway of phenylpropanoids [4]. These secondary metabolites of *P. cuspidatum* have shown strong clinical medical benefits, such as being anti-coronavirus and anti-hepatitis B virus [5]. Therefore, it is very important to increase the contents of the main medicinal components in *P. cuspidatum*.

In nature, endophytic fungi do not result in any harm to the host, and they can promote the production of bioactive secondary metabolites in the host [6]. Among them, arbuscular mycorrhizal (AM) fungi can form a symbiont with the roots of terrestrial plants [7]. AM fungi help the host plant to obtain more water and mineral nutrients for the improvement of plant growth, and in turn, the host plant has to provide AM fungi with sugar substances and fatty compounds [8]. It has been documented that AM fungi could influence the accumulation of bioactive secondary metabolites in *Paris polyphylla* var. yunnanensis [9]. In a study conducted by Mandal et al. [10], AM fungal inoculation in *Artemisia annua* plants promoted the synthesis and accumulation of the medicinal component artemisinin, which was associated with the up-regulated expression of key genes in the artemisinin synthesis pathway. Similarly, AM fungal inoculation also stimulated the accumulation of glycyrrhizin and liquiritin in *Glycyrrhiza uralensis* plants under drought stress and the concentrations of essential oils in oregano [10–12]. These results suggest that AM fungi improve the concentrations of intrinsic medicinal components in medicinal plants, which may be linked to the change in the differential expression of genes.

Piriformospora indica is an endophytic fungus isolated from the rhizosphere of desert plants in India and has similar functions to those of AM fungi [13]. Previous studies also showed that *P. indica* colonization significantly altered the contents of secondary metabolites in the host, such as increasing aristolochic acid in *Aristolochia elegans*, promoting artemisinin content in *Artemisia annua*, and increasing oil content in sunflower [14–17]. The change in the secondary metabolism of plants caused by the endophytic fungus was associated with different expressions of genes related to chlorophyll and cysteine in host plants [18].

The concentrations of secondary metabolites such as resveratrol and polydatin in *P. cuspidatum* are influenced by planting techniques and seasons. Moreover, the over-exploitation of *P. cuspidatum* decreases the yield and drug components. Since AM fungi and *P. indica* collectively have positive effects on plant growth and secondary-metabolite production, we hypothesized that AM fungi and *P. indica* could promote the contents of the main medicinal components in *P. cuspidatum* and that the change could be associated with the expression of genes related to the synthesis of medicinal components induced by endophytic fungi. To confirm the above hypothesis, an AM fungus (*Funneliformis mosseae*) and an endophytic fungus (*P. indica*) were inoculated into *P. cuspidatum* singly or in combination to analyze their effects on biomass production, physiological activities (chlorophyll, soluble protein, and gas exchange), and medicinal ingredient contents, along with the changes in the expression of six genes associated with the resveratrol synthesis pathway.

2. Materials and Methods

2.1. Plant Culture and Endophytic Fungal Inoculation

The seeds of *P. cuspidatum* were surface-disinfected with 75% alcohol for 10 min and then sown in an autoclaved mixture of soil and sands (3:1, *v*/*v*). One month later, we transplanted the *P. cuspidatum* seedlings with four leaves into a plastic pot containing 1.5 kg of an autoclaved mixture of soil and sands (5:2, *v*/*v*). Meanwhile, the inoculation with endophytic fungi was also performed.

An endophytic fungus, *P. indica,* and an AM fungus, *F. mosseae* (BGC XZ02A), were selected. Among them, *F. mosseae,* which was provided by the Bank of Glomeromycota in China (BGC; Beijing, China), was allowed to proliferate with white clover as a trap plant. The mycorrhizal inoculum included root segments infected by AM fungi, spores (23/g), sporocarps, and growth substrate. *P. indica* was provided by Professor Zhi-Hong Tian of Yangtze University. The proliferation of *P. indica* was carried out as per the protocol

described by Yang et al. [19]. The obtained spore suspension had the concentration of 2.85×10^8 CFU/mL by colorimetry at 600 nm.

For AM fungal inoculation, 100 g of *F. mosseae* inoculum was applied to a pot; for *P. indica* treatment, 30 mL of spore suspension of *P. indica* was inoculated into a pot; for dual inoculation of *F. mosseae* and *P. indica*, 100 g of *F. mosseae* inoculum and 30 mL of spore suspension of *P. indica* were applied simultaneously to a pot. The treatment without fungal inoculation received equal amounts of autoclaved *F. mosseae* inoculum and equal volumes of autoclaved spore suspension of *P. indica*, plus 2 mL of filtrate (30 μm) of *F. mosseae* inoculum. After the completion of inoculation, the treated plants were grown under controlled environmental conditions, the details of which were described by Yang et al. [19]. The plants did not receive any additional fertilizer during the experiment.

2.2. Experimental Design

The experiment consisted of four treatments: single inoculation with *F. mosseae*; single inoculation with *P. indica*; double inoculation with both *F. mosseae* and *P. indica*; no inoculation with either *F. mosseae* or *P. indica*. Each treatment had five replicates, and each replication included 2 pots (two seedlings per pot), for a total of 40 pots. The plants were randomly placed in the climatic chamber and changed weekly to eliminate environmental effects.

2.3. Determination of Root Fungal Colonization and Biomass Production

Eleven weeks after inoculation with endophytic fungi, the experiment was terminated. The roots were harvested, cut into 1 cm long root segments and stained for the colonization status of endophytic fungi in the roots using the method described by Meng et al. [20]. The frequency of root fungal colonization was the percentage of the number of root segments infected with endophytic fungi versus the total number of root segments observed. The treated plants were divided into shoots and roots and were oven-dried to a constant weight at 70 °C for 48 h; their dry weights were determined separately.

2.4. Determination of Chlorophyll, Soluble Protein, and Gas Exchange

Chlorophyll concentration in leaves was measured according to the method described by Lichtenthaler and Wellburn [21], using an 80% acetone solution for extraction. Leaf gas exchange was measured on the top fifth fully expanded leaves of the plants using a Plant Gas Exchange Measurement (LI-6400; LI-COR Inc., Lincoln, NE, USA) from 9:00 a.m. to 11:00 a.m. on the day before plant harvest. Soluble protein concentration was measured according to the method of Bradford [22], after homogenization in 5 mL of distilled water with 0.2 g of leaf samples and centrifugation at 4000× *g* for 10 min at 4 °C.

2.5. Determination of Medicinal Components in Leaves

High-performance liquid chromatography (HPLC) was used to determine the contents of six medicinal components in the leaves from *P. cuspidatum*, namely, aloe-emodin, chrysophanol, emodin, physcion, polydatin, and resveratrol. The extraction and assay conditions for these medicinal components of *P. cuspidatum* referred to the method described by Sun et al. [23] in detail under HPLC (LC-20AT; Shimadzu, Tokyo, Japan) conditions.

2.6. Determination of Relative Expression Levels of Resveratrol-Synthesis-Related Enzyme Genes

Leaf total RNA was extracted using a Quick RNA Isolation Kit (ZH120) (Huayueyang Biotechnology (Beijing) Co., Ltd., Beijing, China) according to the user's guidelines. The extracted RNA was analyzed using a Bio Photometer Plus 6132 ultramicro spectrophotometer (Eppendorf, Hamburg, Germany) for RNA purity and reversely transcribed using a TRUE 1st Strand cDNA Synthesis Kit with gDNA Eraser (PC5402) (Aidlab Biotechnologies Co., Ltd., Beijing, China).

Six genes associated with resveratrol biosynthesis in *P. cuspidatum*, including *resveratrol synthase* (*PcRS*), *stilbene synthase* (*PcSTS*), *supposed stilbene synthase 1* (*PcCRS1*), *chalcone*

synthase 1 (*PcCHS1*), *chalcone synthase 2* (*PcCHS2*), and *resveratrol-forming stilbene synthase 11* (*PcRS11*), were obtained through the NCBI database (http://www.ncbi.nlm.nih.gov, accessed on 16 October 2021). The primer sequences of these genes (Table 1) were designed using Primer Premier 5.0 software, and qRT-PCR amplification was performed using the reverse-transcribed cDNA of each treatment as the template. Using β-actin of *P. cuspidatum* as an internal gene, real-time fluorescence expression analysis was performed using the CFX96 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and a fluorescent dye method (2× AceQ qPCR SYBR Green Master Mix; Aidlab, Beijing, China). Each treatment was replicated three times. The $2^{-\Delta\Delta Ct}$ method [24] was used to calculate the relative expression of genes with the non-fungi inoculation as the control.

Table 1. Primer sequences of associated genes in resveratrol biosynthesis.

Genes	Gene ID	Forward Sequence (5′→3′)	Reverse Sequence (5′→3′)
PcCHS1	AB019030. 1	GTAGCTGCCGAATCTTCTACTG	TGTCGTAGCATCGTCCTTTG
PcCHS2	EU647246. 1	GAAGCTTAAGGCGACTAGACAA	CAACCGACTTCTTCCTCATCTC
PcCRS1	DQ459350. 1	TGAGCGAGTACGGGAATTTG	CCTTCTCCAGTCGTCTTCTTAC
PcRS11	EF117977. 1	GATGAGATGATGAAGGCACAAAC	GGAAGTAGAAGTCGGGAAAGTC
PcRS	DQ900615. 1	GAGATGACGAAGGCACTAACA	GGAAGTAGAAGTCGGGAAAGTC
PcSTS	EU647245. 1	GAAGAGATGATGAAGGCACAAAC	GGAAGTAGAAGTCGGGAAAGTC
PcActin	MK288156. 1	TACAATGAGCTTCGGGTTGC	GCTCTTTGCAGTTTCCAGCT

2.7. Statistical Analysis

Experimental data were analyzed by analysis of variance using SAS® Software (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was utilized to compare the significant difference among treatments at the level of 0.05.

3. Results

3.1. Changes in Root Fungal Colonization Frequency and Biomass Production

No fungal colonization was observed in the seedlings inoculated without any fungi, while the seedlings with *F. mosseae*, *P. indica*, and *F. mosseae* + *P. indica* had 58.75 ± 4.15%, 39.57 ± 3.18%, and 28.09 ± 2.27% of root fungal colonization frequency, respectively. The application of endophytic fungi dramatically improved the growth of *P. cuspidatum* (Figure 1a). Among them, *F. mosseae*, *P. indica*, and *F. mosseae* + *P. indica* significantly increased shoot biomass by 40%, 212%, and 102%, respectively (Figure 1b), and also significantly improved root biomass by 8%, 40%, and 25%, respectively (Figure 1c), compared with the non-fungi control.

3.2. Changes in Leaf Chlorophyll and Soluble-Protein Concentrations

Compared with the uninoculated control, the inoculation with single *F. mosseae*, single *P. indica*, and dual *F. mosseae* + *P. indica* significantly increased leaf chlorophyll concentration by 44%, 107%, and 79% (Figure 2a) and also distinctly elevated leaf soluble-protein concentration by 38%, 162%, and 110% (Figure 2b). Significantly higher leaf chlorophyll and soluble-protein concentrations were ranked as *P. indica* > *F. mosseae* + *P. indica* > *F. mosseae* > non-fungi control in decreasing order.

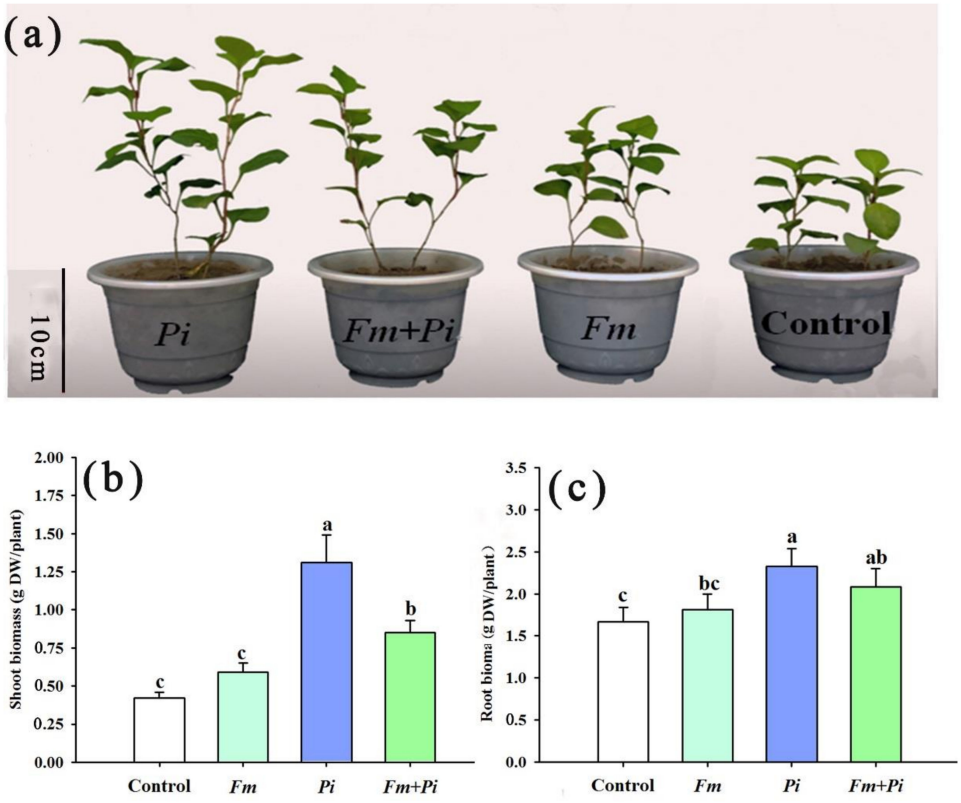

Figure 1. Effects of single and co-inoculation with *Funneliformis mosseae* (*Fm*) and *Piriformospora indica* (*Pi*) on plant growth (**a**), shoot biomass (**b**), and root biomass (**c**) in *Polygonum cuspidatum*. Data (means ± SD, $n = 5$) followed by different letters above the bars indicate significant ($p < 0.05$) differences among treatments.

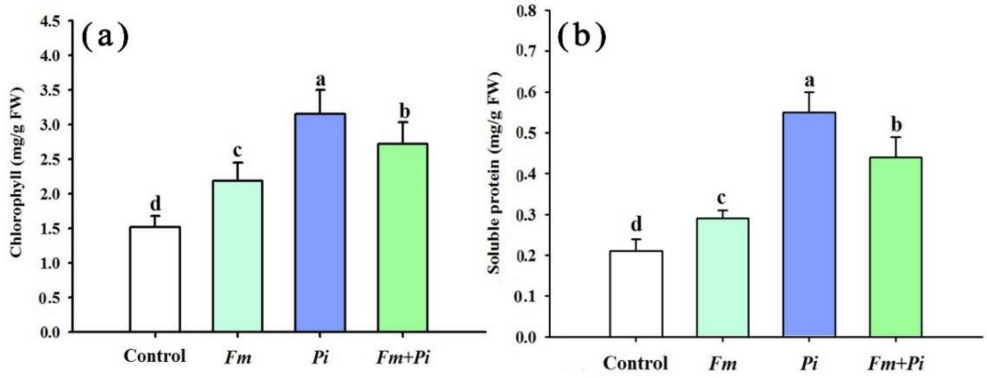

Figure 2. Effects of single and co-inoculation with *Funneliformis mosseae* (*Fm*) and *Piriformospora indica* (*Pi*) on leaf chlorophyll (**a**) and soluble-protein (**b**) concentrations in *Polygonum cuspidatum*. Data (means ± SD, $n = 5$) followed by different letters above the bars indicate significant ($p < 0.05$) differences among treatments.

3.3. Changes in Leaf Gas Exchange

The endophytic fungal treatments improved leaf gas exchange in *P. cuspidatum* (Figure 3a–d). Compared with the uninoculated control, single *P. indica*, single *F. mosseae*, and dual *F. mosseae* + *P. indica* significantly increased the photosynthetic rate, transpiration rate, and stomatal conductance, reaching 174%, 86% and 113% higher values of photosynthetic rate (Figure 3a); 90%, 22% and 55% higher values of transpiration rate (Figure 3b); and 100%, 50% and 75% higher values of stomatal conductance (Figure 3c), respectively. In addition, single *P. indica* and single *F. mosseae*, but not double inoculation with *F. mosseae* and

P. indica, significantly increased intercellular CO_2 concentration by 30% and 18%, compared with the non-fungi control (Figure 3d).

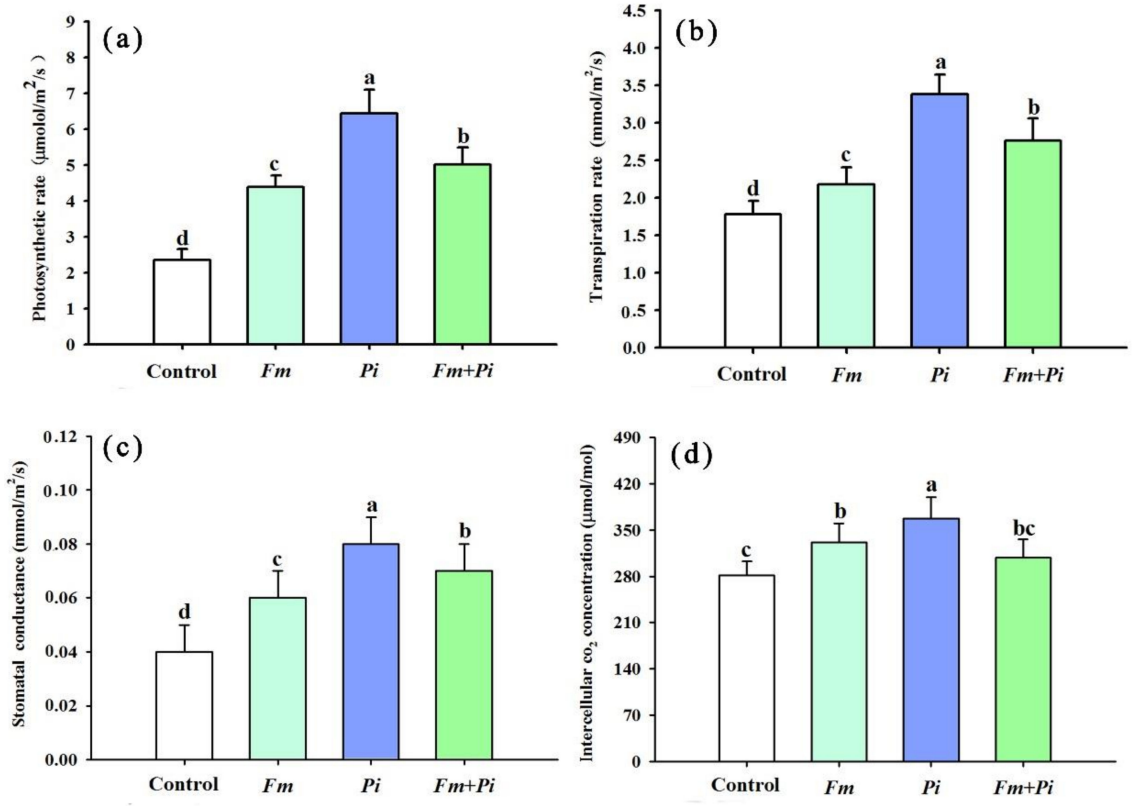

Figure 3. Effects of single and co-inoculation with *Funneliformis mosseae* (*Fm*) and *Piriformospora indica* (*Pi*) on leaf photosynthetic rate (**a**), transpiration rate (**b**), stomatal conductance (**c**), and intercellular CO_2 concentration (**d**) in *Polygonum cuspidatum*. Data (means ± SD, n = 5) followed by different letters above the bars indicate significant ($p < 0.05$) differences among treatments.

3.4. Changes in Leaf Medicinal Components

Of the six medicinal components in *P. cuspidatum*, chrysophanol, emodin, physcion, polydatin, and resveratrol were detected, and aloe-emodin was not detected within the range of concentrations detected in this study (Table 2). Single *F. mosseae*, single *P. indica*, and dual *F. mosseae* + *P. indica* significantly increased leaf polydatin and resveratrol concentrations, with the corresponding increases of 52%, 150%, and 134% in polydatin and the corresponding increases of 220%, 137%, and 80% in resveratrol. Compared with the non-fungi control, single *F. mosseae* and dual *F. mosseae* + *P. indica* significantly reduced emodin concentration by 11% and 32%, respectively. No significant changes were observed in chrysophanol concentration after inoculation with endophytic fungi. Regarding physcion, *P. indica* significantly increased it, whereas dual *F. mosseae* + *P. indica* decreased it.

Table 2. Effects of single and co-inoculation with *Funneliformis mosseae* (*Fm*) and *Piriformospora indica* (*Pi*) on leaf chrysophanol, emodin, physcion, polydatin, and resveratrol contents (mg/g DW) in *Polygonum cuspidatum*.

Treatments	Chrysophanol	Emodin	Physcion	Polydatin	Resveratrol
Control	0.28 ± 0.03 ab	0.62 ± 0.05 a	0.10 ± 0.01 b	1.01 ± 0.11 c	0.30 ± 0.04 d
Fm	0.32 ± 0.02 a	0.55 ± 0.03 b	0.07 ± 0.01 c	1.54 ± 0.14 b	0.96 ± 0.09 a
Pi	0.26 ± 0.02 b	0.57 ± 0.04 ab	0.13 ± 0.01 a	2.52 ± 0.25 a	0.71 ± 0.08 b
Fm + *Pi*	0.27 ± 0.02 b	0.42 ± 0.04 c	0.07 ± 0.01 c	2.36 ± 0.20 a	0.54 ± 0.06 c

Data (means ± SD, n = 3) followed by different letters in the row indicate significant ($p < 0.05$) differences among treatments.

3.5. Changes in Expression of Associated Genes

Endophytic fungal application altered the expression levels of genes encoding resveratrol biosynthesis (Figure 4a–f). Compared with the non-fungi control, inoculation with *F. mosseae* significantly induced the relative expression levels of leaf *PcCHS1*, *PcCHS2*, *PcCRS1*, *PcRS11*, *PcRS*, and *PcSTS*; inoculation with *P. indica* stimulated the expression levels of *PcCHS1*, *PcCHS2*, and *PcRS*; double inoculation also up-regulated the expression levels of *PcCRS1*, *PcRS11*, *PcRS*, and *PcSTS*, accompanied by the suppression of *PcCHS2* (Figure 4a–f).

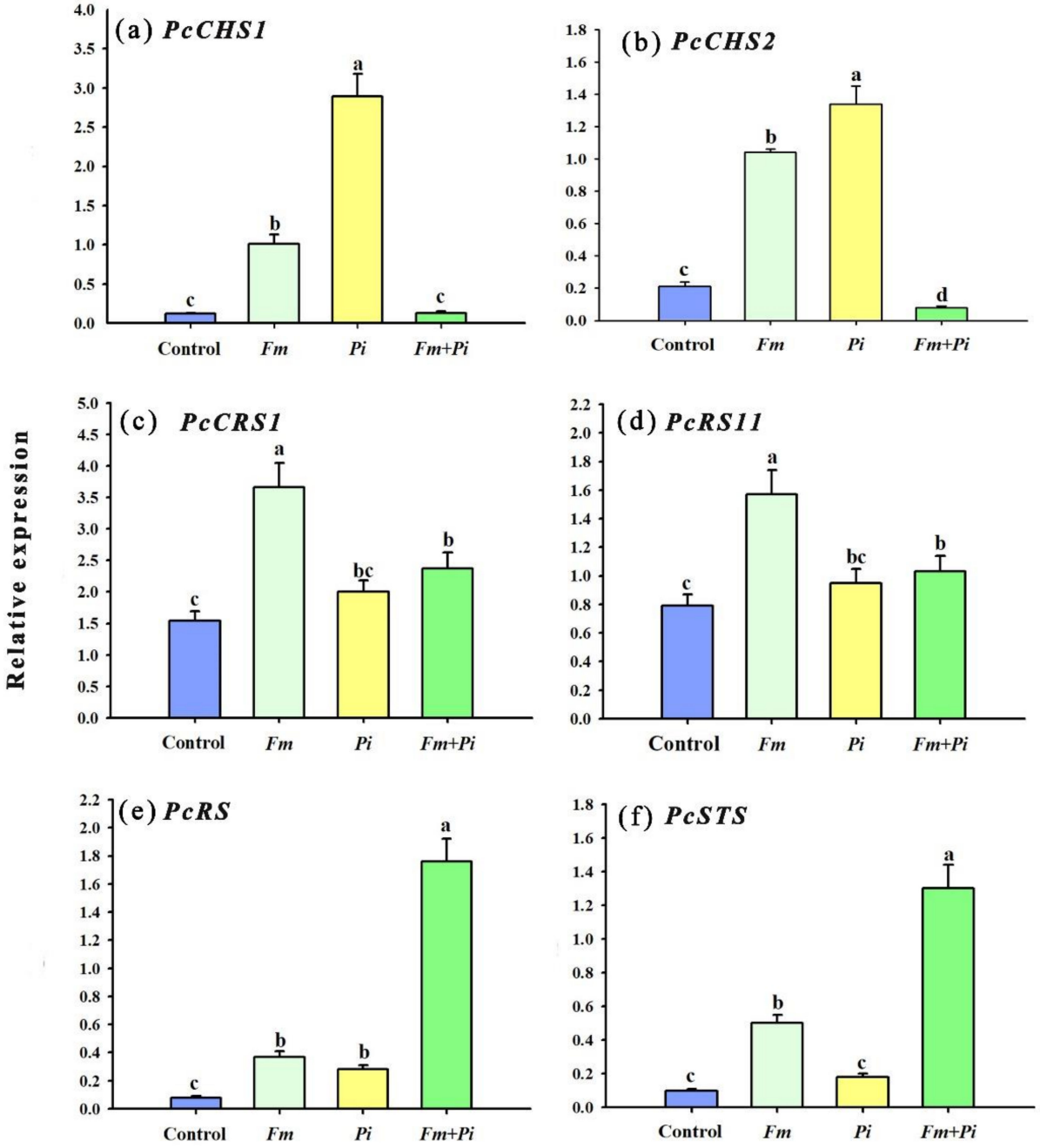

Figure 4. Effects of single and co-inoculation with *Funneliformis mosseae* (*Fm*) and *Piriformospora indica* (*Pi*) on the relative expression of *PcCHS1* (**a**), *PcCHS2* (**b**), *PcCRS1* (**c**), *PcRS11* (**d**), *PcRS* (**e**), and *PcSTS* (**f**) in *Polygonum cuspidatum*. Data (means ± SD, $n = 3$) followed by different letters above the bars indicate significant ($p < 0.05$) differences among treatments.

4. Discussion

In our study, single *P. indica* and dual *F. mosseae* + *P. indica* collectively improved shoot and root biomass production, while single *F. mosseae* did not alter shoot and root biomass, suggesting that increased biomass of *P. cuspidatum* was more dependent on *P. indica* than *F. mosseae*. Earlier studies have also revealed improved growth of *Withania somnifera*, *Spilanthes calva*, *Coleus forskohlii*, and *Adhatoda vasica* [25,26]. However, Meng et al. [20] reported that single or dual inoculation with *F. mosseae* and *P. indica* considerably improved shoot and root biomass in trifoliate orange, but the increase in root biomass was more important in *F. mosseae*-inoculated than in *P. indica*-inoculated trifoliate orange. These results suggest that the improvement of host growth by endophytic fungi such as *F. mosseae* and *P. indica* depends on the compatibility between host plants and endophytic fungi. The increase in plant growth caused by endophytic fungi may be due to a combination of nutrient and water promotion, phytohormone production, root morphological improvement, and reduced amount of ethylene [27].

Previous studies have shown that leaf chlorophyll and soluble-protein concentrations were increased by endophytic fungi [28–30]. Our study indicated that *P. indica* and *F. mosseae*, singly or in combination, distinctly increased leaf chlorophyll and soluble-protein concentrations in *P. cuspidatum*. Among them, the significant increase among endophytic fungal treatments presented the trend of *P. indica* > dual *F. mosseae* + *P. indica* > *F. mosseae*. This was in line with the effect of endophytic fungal inoculation on the frequency of root colonization and the improved biomass production. Similar results were observed in *Aloe vera* infected with *P. indica* [31]. Pandey and Banik [32] also reported higher shoot and root biomass production in *P. indica*- than in *Glomus sp.*-inoculated *A. vera*. As a result, the endophytic fungi used in this experiment, particularly *P. indica*, were able to accelerate the synthesis of chlorophyll and proteins, as reported by Yaghoubian et al. [28]. Since endophytic-fungus-inoculated *P. cuspidatum* plants were recorded to have a higher chlorophyll concentration, inoculated plants represented greater leaf gas exchange than non-inoculated plants. Khalvandi et al. [30] concluded that an AM fungus, *P. indica*, and their co-inoculation improved the function of the plant photosynthetic apparatus, such as ΦPSII, for stimulating the capacity of light harvesting and electron transfer to plastoquinone.

There are four secondary metabolites with potential pharmacological value in the extract from *P. cuspidatum*, namely, resveratrol, polydatin, emodin, and physcion [33]. We detected five secondary metabolites in the leaves from *P. cuspidatum*, namely, chrysophanol, emodin, physcion, polydatin, and resveratrol, while aloe-emodin was below the detection line. The five secondary metabolites were heavily influenced by root endophytic fungi. Our study indicated that the application of endophytic fungi significantly increased leaf polydatin and resveratrol concentrations, whilst the highest polydatin concentration was observed in *P. indica*-inoculated plants, and the highest resveratrol concentration was recorded in *F. mosseae*-inoculated plants. In *Vitis vinifera* cvs Pinot Noir and Divico, ten days after infection with *Plasmopara viticola*, *Rhizophagus irregularis*-inoculated plants presented a considerably higher resveratrol concentration than non-inoculated plants [34]. In *Fallopia japonica*, mycorrhizal fungi induced the accumulation of resveratrol-glucoside content in roots [35]. In *Rumex gmelini* seedlings, inoculation with AM fungi increased resveratrol concentration after 60 days [36]. It is well-known that resveratrol is found in grapes, *P. cuspidatum*, pine trees, cassia seeds, peanuts, and other natural plants, among which the content of resveratrol in *P. cuspidatum* is the highest [37]. Resveratrol is a type of non-flavonoid polyphenol compound, which has a variety of physiological activities, such as tumor inhibition and anticancer activity [38]. Polydatin is a natural precursor of resveratrol, which has positive effects on bone health and related diseases alone or in combination with resveratrol [39]. Therefore, it is speculated that the root endophytic fungi strongly promoted the biosynthesis of resveratrol in *P. cuspidatum*. On the other hand, resveratrol as an antioxidant also scavenges reactive oxygen species under biotic and abiotic stresses [3]. AM fungi and *P. indica* collectively mitigated oxidative damage in host plants exposed to stress environments [40,41]. This suggests that the *P. cuspidatum* plants inoculated

with endophytic fungi have higher stress resistance than uninoculated plants, but further experiments are needed to confirm it.

In addition to polydatin and resveratrol, endophytic fungi also affected the concentrations of emodin and physcion in *P. cuspidatum* leaves, and most of them showed an inhibitory effect. For example, *F. mosseae* and co-inoculation significantly inhibited the concentrations of emodin and physcion, and only *P. indica* significantly increased the concentration of physcion. Chrysophanol concentration in *P. cuspidatum* was not significantly affected by endophytic fungi. However, in *Rumex gmelini* and *Ophiopogon japonicus* plants, AM fungi dramatically increased chrysophanol content, and this was dependent on inoculated times [36,42]. These results indicate that the effects of endophytic fungi on some secondary metabolites of medicinal plants depend on environmental conditions, host plant types, endophytic fungi species, and secondary-metabolite species.

Resveratrol is synthesized through the phenylpropanoid metabolic pathway, in which phenylalanine is deaminated into cinnamic acid under the catalysis of phenylalanine ammonia-lyase; then, cinnamic acid is changed into coumaric acid by cinnamate-4-hydroxylase, which is catalyzed into p-coumaryl-coA by coumaryl-CoA-ligase [43]. P-coumaryl-CoA and malonyl-CoA as substrates synthesize resveratrol under the action of RS [43]. Therefore, RS is the key enzyme required in the resveratrol synthesis pathway [44]. STS is a type III polyketide synthase, which is only found in plants that can synthesize resveratrol and related compounds [43]. The results of our study indicate that *F. mosseae* and co-inoculation induced *PcCRS1*, *PcRS11*, *PcRS*, and *PcSTS* gene expression, thus inducing more accumulation of resveratrol. *P. indica* only up-regulated *PcRS* gene expression to activate resveratrol biosynthesis. In grapevines, AM fungi induced *STS* gene expression in the Pinot Noir and Divico varieties under *Plasmopara viticola* infection conditions and in the Chasselas variety under *Botrytis cinerea* infection conditions [34]. In *Paris polyphylla* var. *yunnanensis*, the application of mixed AM fungi promoted the expression of the *squalene epoxidase* gene to induce the accumulation of polyphyllin [45]. The accumulation of saponins in *Centella asiatica* was increased by the colonization with *P. indica*, coupled with the increased transcription levels of the *squalene synthase* gene and *β-amyrin synthase* gene in the saponin synthesis pathway [46]. This suggests that endophytic fungi may have their independent system to regulate resveratrol biosynthesis in *P. cuspidatum* by up-regulating the expressions of different genes associated with resveratrol biosynthesis, which still needs to be further studied.

Interestingly, single inoculation with *F. mosseae* and *P. indica*, but not co-inoculation, significantly promoted the expression of the *PcCHS1* and *PcCHS2* genes. CHS is an enzyme that plays a major role in the biosynthesis of flavonoids in plants [44]. P-coumaryl-coA and malonyl-coA as substrates also synthesize naringin under the action of CHS into the flavonoid synthesis pathway. Therefore, CHS and RS compete for the same substrate, causing different catalytic mechanisms [47]. Although this experiment did not measure the changes in flavonoids, it also revealed that endophytic fungi may potentially regulate flavonoid synthesis in *P. cuspidatum*.

5. Conclusions

Single or combined inoculation with *F. mosseae* and *P. indica* in *P. cuspidatum* promoted biomass production, especially shoots, and also enhanced leaf physiological activities, such as chlorophyll and soluble-protein syntheses and gas exchange. Inoculation with endophytic fungi mainly increased the concentrations of resveratrol and polydatin in *P. cuspidatum* leaves, which was closely related with the induced expression of genes in resveratrol biosynthesis by endophytic fungi. Such endophytic fungi inoculation provides a technique for accelerating the production of resveratrol and polydatin in *P. cuspidatum* in the future.

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