Biologia (2018) 73:917–926
https://doi.org/10.2478/s11756-018-0091-9

ORIGINAL ARTICLE

Fungal elicitors stimulate biomass and active ingredients accumulation in *Dendrobium catenatum* plantlets

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Received: 20 March 2018 / Accepted: 10 July 2018 / Published online: 5 September 2018

Abstract
Endophytic fungi have been widely used as biotic elicitors to stimulate the growth and production of metabolites in plant cells, tissues and organ cultures. Here, mycelium extract (ME), supernatant liquor (SL), ethanol sediment (ES) and protein-polysaccharide fraction (PPF) were prepared from four endophytic fungi, DO14 (*Pestalotiopsis* sp.), DO18 (*Talaromyces* sp.), DO19 (*Xylariaceae* sp.) and DO120 (*Hypoxylon* sp.), and applied to their host *Dendrobium catenatum*. After 8 weeks of co-culturing, ME, ES and PPF exhibited strong stimulation on biomass yields and contents of active ingredients. Among the three elicitors, PPF was found to be the active constituent responsible for the enhanced biomass and active ingredients in *D. catenatum*. Under the treatment of 240 mg/L PPF from DO14, we achieved maximum stem fresh weight (FW) and leaf FW. However, to maximize the productions of polysaccharides, naringenin and schaftoside one need only 60 mg/L of PPF from DO14. PPF from DO18, DO19 and DO120 showed different effects. Under 30 mg/L treatment, the ethanol extractives, total flavonoids and total phenols contents increased most. These results indicate that fungal elicitor PPFs can be used for industrial production of high quality *D. catenatum* seedlings and may be served as a broad microbial fertilizer resource for other plant growth.

Keywords Active ingredient · Biomass · *Dendrobium catenatum* · Fungal elicitor · Stimulation effect

Abbreviations

ME	mycelium extract
SL	supernatant liquor
ES	ethanol sediment
PPF	protein-polysaccharide fraction
FW	fresh weight
DW	dry weight
MS	murashige and skoog
NAA	naphthalene acetic acid
PDA	potato dextrose agar medium
PDB	potato dextrose broth medium
UPLC	ultra-performance liquid chromatography
HPLC	high performance liquid chromatography
SD	standard deviations
ANOVA	one-way analysis of variance
PSF	polysaccharide fraction
MoHrip1	*Magnaporthe oryzae* hypersensitive response-inducing protein
PevD1	protein elicitor from *Verticillium dahlia*
DAMPs	damage-associated molecular patterns

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Introduction

In nature, plants maintain a complex relation with a variety of microorganisms, including mutualism with endophytes or antagonism with pathogens (Chen et al. 2016). In response to the attacks by microorganisms, plants have established a defense mechanism through producing different types of secondary metabolites, such as flavonoids, saponins, and alkaloids (Pavarini et al. 2012; Cordell 2013; Mechri et al. 2015). These secondary metabolites usually constitute the main quality indicators for many medicinal herbs (Lu et al. 2017; Rafinska et al. 2017). Therefore, it is highly valuable to develop ways to effectively stimulate the growth of metabolites in plants. Biotic elicitors, such as endophytic fungi and constituents of microbial cells, especially their carbohydrate or polysaccharide fractions, are found to meet this request (Yu et al. 2016).

Fungal elicitors often include the degradation products, metabolites, secreted substances or fermented liquid of fungi, which can also be classified into oligosaccharide, proteins and polyunsaturated fatty acids (Algar et al. 2012). It is known that fungal elicitors affect plant growth and the effects of active ingredient stimulation vary with the ingredients' chemical nature, concentrations and source species (Wang et al. 2006), but the actual stimulating effects of various fungal elicitors and their ingredients remain unknown. Furthermore, early studies of the effects of oligosaccharides and their homologous elicitors have been mainly focused on the hairy root, cell, callus, root and shoot organ in a cultured system (Sharp et al. 1984; Fry et al. 1993; Yamaguchi et al. 2000; Satdive et al. 2007). However, the results may not be applicable to a plantlet which has a systematic organ, tissue and cell structures with well-organized photosynthesis and respiration mechanisms (Masondo et al. 2015; Trendafilova et al. 2015). For field production, relevant study on plantlets is highly required as it is more conducive to understand the functional roles of endophytic fungus on host survival and fungal fertilizer application. Here, we used the aseptic plantlets of *Dendrobium catenatum* to evaluate the effects of fungal elicitors.

D. catenatum (alias *D. officinale*) is a traditional Chinese medicinal herb and bears significant advantages in relieving stomach upset, promoting the production of physical fluid and enhancing immune system, as recorded in the Chinese pharmacopoeia (Zhao et al. 2013; Pharmacopoeia Committee of the People's Republic of China 2015; Wu et al. 2015). Due to the increasing demand, the wild resource of *D. catenatum* is hardly available now. In fact, it has been listed as the key wild herb protected by the State Department in 1987 and catalogued in the Chinese Plant Red Book (Rudgers et al. 2012). In the past 15 years, various strategies have been utilized for the cultivation of *D. catenatum* and the corresponding cultivation industry has expanded quickly to over 6600 hm^2 in China. However, the yield and quality of *D. catenatum* are gradually declining due to the deterioration of present varieties. Meanwhile, variety breeding of *D. catenatum* becomes both labor intensive and time consuming (Si et al. 2013). To promote the yield and quality, we seek alternatives and evaluate elicitors' role as a potential regulator on the growth and active ingredients accumulation in *D. catenatum*. Previously, we isolated four endophytic fungi DO14 (*Pestalotiopsis* sp.), DO18 (*Talaromyces* sp.), DO19 (*Xylariaceae* sp.) and DO120 (*Hypoxylon* sp.) from the stems and roots of *D. catenatum*, and found that co-culturing them with their host plantlets promotes the plantlet growth and metabolite biosynthesis (Zhu et al. 2016). Indeed, the fungi could suppress the growth of plantlet for their overgrowth and competition for nutrition and space resources with *D. catenatum* at the later co-culturing period. To seek a long-term symbiotic fungal elicitor and better evaluate the induction effects of these fungal species on *D. catenatum*, in the present study, fungal elicitors including mycelium extract (ME), supernatant liquor (SL), ethanol sediment (ES) and protein-polysaccharide fraction (PPF) at different concentrations were prepared from DO14, DO18, DO19 and DO120, and their stimulations on growth and biosynthesis of active ingredients of *D. catenatum* were investigated.

Materials and methods

Plant and fungal strains

The aseptic plantlets of *D. catenatum* were from the Engineering and Technical Research Center of *D. catenatum* of State Forestry Administration in China. The tested fungal strains DO14 (*Pestalotiopsis* sp.), DO18 (*Talaromyces* sp.), DO19 (*Xylariaceae* sp.) and DO120 (*Hypoxylon* sp.) were prepared from *D. catenatum* as described in our previous study (Zhu et al. 2016).

Equipment and tools

A high-handed sterilization pan (SANYO Electric Co. Ltd., Japan) was used for culture medium preparation. SW-CJ-1C vertical flow clean bench (Shanghai Kanglu Biotechnology Co. Ltd., China), MJP-250S fungal incubator (Shanghai Senxin Biotechnology Co. Ltd., China), and HYG-A shaker (Jiangsu Taicang Biotechnology Co. Ltd., China) were used for fungi cultured. An MGC-300A botanic incubator (Ningbo Laifu Co. Ltd., China) was used for plant cultured. Alpha 1–2 LDplus lyophilizer (Marin Christ Co. Ltd., Germany), DGG-9070 drying oven (Shanghai Senxin Biotechnology Co. Ltd., China), RE-3000A rotary evaporator (Shanghai Yarong Co. Ltd., China) and 1-16 K centrifuge (Sigma-Aldrich Co. Ltd., USA) were used for elicitors preparation. ME104E electronic scales (METTLER TOLEDO Co. Ltd., Switzerland) and LS02-A tiny pulverizer (Hangtai Instrument Co. Ltd., China) were used for sample preprocessing and biomass determination. Milli-Q academic pure water filter system (Millipore Co. Ltd., USA), ultraviolet-uisible spectrophotometer (Shanghai Third Instrument Co. Ltd., China), KQ5200DE aqueous ultrasonic cleaning system (Kunshan Ultrasonic Instrument Co. Ltd., China), 1260 Infinity high performance liquid chromatography (HPLC) system (Agilent Technologies Co. Ltd., USA), and ultra-performance liquid chromatography (UPLC) with Acquity system and PDA detector (Waters Co. Ltd., USA) were used for extraction and determination of the active ingredients.

Culture medium and condition

The modified Murashige and Skoog (MS) medium was used for tissue culture and co-culture at 25 °C with 10 h/d of light. The medium contained (per liter): 5.15 g of agar, 30.0 g of sugar, 75.0 g of banana, 185 mg of $MgSO_4·7H_2O$, 825 mg of NH_4NO_3, 950 mg of KNO_3, 85 mg of KH_2PO_4, 166 mg of $CaCl_2$, 0.83 mg of KI, 6.2 mg of H_3BO_3, 16.94 mg of $MnSO_4·4H_2O$, 8.6 mg of $ZnSO_4·7H_2O$, 0.25 mg of $Na_2MoO_4·2H_2O$, 0.025 mg of $CuSO_4·5H_2O$, 0.025 mg of $CoCl_2$, 100 mg of inositol, 2 mg of glycine, 0.1 mg of VB_i, 0.5 mg of VB_b, 0.5 mg of niacin, 37.3 mg EDTA-Na_2, 27.8 mg of $FeSO_4$, 0.5 mL of Naphthalene Acetic Acid (NAA) (pH at 7.08~7.09).

Two media compositions and conditions were used: potato dextrose agar medium (PDA) at 25 °C in the dark for solid fungal culture, containing (per liter): 200.0 g of potato, 20.0 g of glucose, 15.0 g of agar (pH at nature), and potato dextrose broth medium (PDB) at 25 °C with shaking (180 rev/min) for liquid fungal culture, containing (per liter): 200.0 g of potato, 20.0 g of glucose (natural pH).

Elicitors preparation and induction

Several fungal elicitors were prepared and applied for induction. The endophytic fungus was incubated in PDA medium and separated into small cycloidal pieces (5 × 5 mm), then incubated into 250 mL Erlenmeyer flasks, each containing 100 mL of PDB medium. The mycelium was collected at various time intervals (0, 4, 8, 12, 16 and 20 days) and autoclaved at 121 °C for 20 min, then washed with distilled water for three times, resuspended and filtered through two pieces of filter paper under vacuum. The filtrate was obtained as the ME and stored at 4 °C before induction. The ME dose was expressed via the soluble sugar content determined by the anthrone colorimetry method using sucrose as the standard (Sinopharm Co. Ltd., China) (Khoo et al. 2005). MEs of the four tested fungi containing 300 mg/L soluble sugar content (ME-300) or 600 mg/L soluble sugar content (ME-600) were respectively added into the co-culture medium. Meanwhile, plantlets cultured in the co-culture medium with addition of the same volume of distilled water were designed as controls (CK1). All the induction experiments were performed in triplicate.

The ES, SL and PPF were prepared from 25 l of ME using the chemical separation method (Ming et al. 2013). ME was concentrated at 80 °C in a low pressure rotary evaporator and precipitated with 95% ethyl alcohol for 48 h. The solution was centrifuged at 8000 rpm for 5 min, then was separated and freeze-dried as the ethanol sediment (ES) and supernatant liquor (SL). Then ES was dissolved with some distilled water and deproteinized using Sevage reagent, then was centrifuged at 4000 rpm for 5 min, the liquid supernatant was dialyzed with distilled water in a regenerated cellulose membrane tubing (2000 Da) for 48 h. The rest liquid was freeze-dried as the protein-polysaccharide fraction (PPF).

ES at 30 mg/L (ES-30), SL at 30, 60, 240 mg/L (SL-30, SL-60, SL-240) and PPF at 30, 60, 120, 240 mg/L (PPF-30, PPF-60, PPF-120, PPF-240) from DO14, PPF at 30 mg/L from DO18, DO19 and DO120 were added into the co-culture medium, respectively. DO14ME, DO18ME, DO19ME and DO120ME denoted ME prepared from DO14, DO18, DO19 and DO120 respectively. Similarly, DO14PPF, DO18PPF, DO19PPF and DO120PPF denoted PPF prepared from DO14, DO18, DO19 and DO120 respectively. Controls 2 (CK2) refer to the plantlets cultured in the co-culture medium without fungal elicitors. All assays were performed in triplicate.

Biomass determination

After 8 weeks co-culturing in elicitor-added media, plantlets of *D. catenatum* were collected and washed with distilled water. The stems and leaves were then separated and their FW were determined using an analytical scale. The assays were performed in triplicate.

Extraction of the active ingredients

After biomass determination, the stems and leaves were dried to constant weight at 60 °C in an oven and smashed into powder in a tiny pulverizer respectively. The polysaccharides in the stems were extracted with distilled water (2 mg/mL, DW) in a water bath at 100 °C for 110 min. The stem ethanol-soluble fraction was extracted with 95% ethanol (20 mg/mL, DW) in a water bath at 80 °C for 60 min. The total flavonoids and total phenols in the leaves were extracted with 80% methanol (20 mg/mL, DW) and sonicated in an ultrasonic water bath with 100 W of power at 25 °C for 30 min. Then the solution was filtered by 0.45 μm microsieve in a syringe as the schaftoside extract. The naringenin in the stems was extracted with neat methanol (10 mg/mL, DW) in a water bath at 80 °C for 60 min, then was filtered and evaporated to dry. After that, the extract was dissolved with distilled water and leached by ethyl acetate for three times, then dried by evaporation prior tore-dissolve with methanol in a volumetric flask. All experiments were performed in triplicates.

Determination of the active ingredients

The polysaccharides content was determined by the phenol-sulfuric acid method using glucose as the standard (Ming et al. 2013). The ethanol-soluble extractives content was determined by the hot-dipping method (Zhu et al. 2016). The total flavonoids and phenols contents were determined by the ultraviolet spectrophotometry method using rutin and gallic acid as the reference standard respectively (Jimenez-Zamora et al. 2016; Wang et al. 2017). The naringenin content was analyzed by UPLC and performed on a Waters ACQUITY system using BEH C_{18} chromatographic column (2.1 × 150 mm, 1.7 μm) at 30 °C (λ = 325 nm) with a ammonium acetate (0.02 mmol/L) (A)/ acetonitrile (B) gradient. The schaftoside content was analyzed by HPLC and performed on an Agilent-1260 system using XB C_{18} chromatographic column (4.6 × 250 mm, 0.5 μm) at 30 °C (λ = 335 nm) with a H_2O (+0.2% CH_3COOH) (A)/ acetonitrile (B) gradient. Naringenin and schaftoside contents were identified by comparison with the available standards. The reference standards were obtained from Shanghai Yuanye Biotechnology Co. Ltd., China. All experiments were performed in triplicates.

Experimental design and data analysis

The numerical results were presented as their mean values and standard deviations (SD). Pair-wise multiple comparisons of the biomass and active ingredients under the studied conditions were conducted using one-way analysis of variance (ANOVA) with SPSS 17.0 software. The statistical analyses were further performed using Graph Pad Prism 5.0 software while the letters have been used to denote the significant differences for which P was<0.05.

Results

Effects of ME on biomass

Soluble sugar was determined to sign the dose of MEs since the carbohydrate is the main potential active ingredient of fungal elicitors (Zhai et al. 2017). The standards information and their curve equations were shown in Table 1. Here, we found that the soluble sugar contents of ME peaked at 4 d with 1.530 ± 0.124, 3.484 ± 0.142, 1.188 ± 0.085 and 3.015 ± 0.277 mg/mL when incubated with DO14, DO18, DO19 and DO120 respectively (Table 2). Therefore, we chose at day 4 to add ME-300 and ME-600 to the co-culture media. After further 8 weeks of co-culturing, a significant stimulation of *D. catenatum* biomass was observed. Stem FW and leaf FW reached maxima when treated with ME-300 and ME-600 prepared from the fungal strain DO14, which were 1.55-fold (39.61 ± 3.89 g vs 25.51 ± 4.90 g, $P < 0.05$) and 1.42-fold (32.14 ± 5.12 g vs 22.64 ± 1.86 g, $P < 0.05$) of control respectively (Table 3).

Effects of ME on active ingredients

For the plantlets treated with MEs, a massive induction of polysaccharides, ethanol extractives, total flavonoids, total phenols, naringenin and schaftoside was observed (Table 3). Polysaccharides content reached a maximum and was 1.84-fold that of control (5.76 ± 0.24% vs 3.09 ± 0.14%, $P < 0.05$) when treated with DO14ME-600. Additionally, maximum elicitation of ethanol extractives (20.62 ± 0.18%), total flavonoids (7.4045 ± 0.1240 mg/g DW), total phenols (7.0123 ± 0.1101 mg/g DW), naringenin (0.0454 ± 0.0006 mg/g DW) and schaftoside (0.4224 ± 0.0279 mg/g DW) production were observed under the treatment of DO120ME-600, DO19ME-300, DO19ME-300, DO19ME-600 and DO14ME-600 respectively. They were all significantly higher than the control ($P < 0.05$).

Effects of SL, ES and PPF on biomass

To ascertain the main active constituents of ME responsible for the stimulation of growth and active ingredients of *D. catenatum*, ES, SL and PPF at various concentrations from ME of DO14 were added into the co-medium. The results showed that the stem FW and leaf FW were significantly increased under the treatments with ES and PPF elicitors when compared with the control cultures. However, no significant difference was observed with SL treatment, even its concentration was increased from 30 mg/L to 60 mg/L and 240 mg/L (Fig. 1a, b). However, the effect of PPF concentration on the increases of stem FW and leaf FW was marginal. At PPF concentrations of 30, 60, 120 and 240 mg/L, stem FW was 1.21-fold, 1.22-fold, 1.23-fold, 1.23-fold that of control, while leaf FW was 1.25-fold, 1.28-fold, 1.35-fold, 1.19-fold, respectively ($P < 0.05$, Fig. 1c, d).

Table 1 Standard curve equations of the content determination trials

Ingredients	Standards (batch number)	Equations	R^2	Linear range (μg/mL)
Soluble sugar	Sucrose (40159760)	Y = 8.7171X + 0.0147	0.9998	5.0–50
Polysaccharides	Glucose (MSN016)	Y = 13.777X-0.0079	0.9999	5.0–50
Total flavonoids	Rutin (B20771)	Y = 0.0116X + 0.0104	0.9996	4.48–71.68
Total phenols	Gallic acid (B20851)	Y = 87.0125X-0.0027	0.9998	1.0–10
Naringenin	Naringenin (B21596)	Y = 68.318X-1.9854	0.9998	0.5–100
Schaftoside	Schaftoside (B21349)	Y = 13,851X-3.1718	0.9997	2.5–15

Y denotes the OD values or peak areas. X denotes the concentration of standard

Table 2 The soluble sugar contents of mycelium extract (ME) at different time intervals

Time intervals (d)	Soluble sugar content (mg/mL)			
	DO14	DO18	DO19	DO120
0	0.207 ± 0.026	0.207 ± 0.042	0.597 ± 0.064	0.356 ± 0.021
4	1.530 ± 0.245	3.484 ± 0.651	1.188 ± 0.099	3.015 ± 0.512
8	nd	1.857 ± 0.512	0.708 ± 0.085	0.618 ± 0.057
12	nd	1.355 ± 0.245	0.756 ± 0.123	1.437 ± 0.115
16	nd	0.866 ± 0.042	0.932 ± 0.110	0.681 ± 0.068
20	nd	0.765 ± 0.035	0.929 ± 0.084	0.481 ± 0.048

Values are presented as mean ± standard. Data are means of three replicates. DO14ME, DO18ME, DO19ME and DO120ME denote mycelium extract (ME) prepared from DO14 (*Pestalotiopsis* sp.), DO18 (*Talaromyces* sp.), DO19 (*Xylariaceae* sp.) and DO120 (*Hypoxylon* sp.) respectively

nd, not detected since the mycelium cannot be collected

Effects of SL, ES and PPF on active ingredients

Polysaccharides and schaftoside accumulation were significantly stimulated by ES and PPF elicitors at 30 mg/L ($P < 0.05$). However, no significant increase of polysaccharides or schaftoside contents was found under all the SL treatments (Fig. 1e, f). Studies indicated that elicitor concentration was an important factor for plant metabolite accumulation (Nair et al. 2013). To examine if this is the case for PPF, 30 mg/L, 60 mg/L, 120 mg/L and 240 mg/L PPF were separately applied to the *D. catenatum* plantlets. After 8 weeks, the contents of polysaccharides, schaftoside and naringenin were measured and found dramatically increased. At a dose of 60 mg/L, the contents of polysaccharides, schaftoside and naringenin reached to maxima, and were 1.25-fold, 1.59-fold and 1.22-fold that of the control respectively (Fig. 1g–i).

Comparison of the effects of PPF from various fungal species

The stem FW and polysaccharides content under treatment of DO14PPF (30 mg/L) were 1.30-fold and 1.57-fold that of the control respectively (12.87 ± 0.85 g vs 9.92 ± 0.51 g, $P < 0.05$; 9.26 ± 0.10% vs 5.88 ± 0.42%, $P < 0.05$) (Fig. 2a, c). Additionally, the leaf FW and ethanol extractives content were also significantly increased under the treatment with DO120PPF, which were 1.20-fold and 1.18-fold that of the control respectively (7.54 ± 0.30 g vs 6.26 ± 0.16 g, $P < 0.05$; 22.05 ± 1.70% vs 18.74 ± 0.31%, $P < 0.05$) (Fig. 2b, d). Moreover, the content of the total flavonoids reached a maximum when treated with DO18PPF (8.8367 ± 0.7206 mg/g DW versus the control of 7.9067 ± 0.4055 mg/g DW, $P < 0.05$) (Fig. 2e). The contents of the total phenols and naringenin reached maximum when treated with DO19PPF (8.9503 ± 0.2458 mg/g DW and 0.0388 ± 0.0008 mg/g DW versus the controls of 7.7702 ± 0.5305 mg/g DW and 0.0347 ± 0.0020 mg/g DW, respectively, $P < 0.05$) (Fig. 2f, g). In addition, the maximum elicitation of schaftoside accumulation was observed when treated with DO14PPF, which was 1.23-fold that of the control (0.2746 ± 0.0033 mg/g DW vs 0.2238 ± 0.0039 mg/g DW, $P < 0.05$) (Fig. 2h).

Discussion

Employment of fungal preparations as elicitors has become one of the most important and successful measures to enhance growth and secondary metabolite production in plant cell cultures (Li et al. 2011). However, there have been few reports regarding the effects of these endophytic fungi elicitors on their host plants. Here, we evaluated the induction effects of various fungal species on their host plant *D. catenatum* and ascertained the main active constituents of the fungal elicitors. Our results revealed that fungal elicitors also behaved as a strong growth promoter on the whole plantlets, consistent with early reports that raw elicitors prepared from endophytic fungi could induce multiple responses in host plant suspension cell cultures and hairy roots, leading to enhanced plant growth (Wang et al. 2012). However, the effects of growth enhancement varied with the ingredients' chemical nature of fungal elicitors. ES and PPF were found as the effective elicitor to stimulate growth in *D. catenatum* plantlets (Fig. 1a–d). When comparing the effects of ES and PPF, it is concluded that PPF is one of the main active constituents responsible for promoting growth in *D. catenatum*. Polysaccharide fraction (PSF) was found to be more capable to promote *Salvia miltiorrhiza* hairy root growth than supernatant fluid (SF) despite both were prepared from *Trichoderma atroviride* (Ming et al. 2013). Additionally, some protein elicitors, such as protein elicitor from *Verticillium dahlia* (PevD1), and *Magnaporthe oryzae* hypersensitive response-inducing protein (MoHrip1) prepared from *M. oryzae*, could promote the growth of Arabidopsis and rice respectively (Liu et al. 2016; Lv et al. 2016). Although the compositions of different fungal elicitors

Table 3 Biomass and active ingredients accumulation of *D. catenatum* stimulated by the mycelium extract (ME) from DO14 (*Pestalotiopsis* sp.), DO18 (*Talaromyces* sp.), DO19 (*Xylariaceae* sp.) and DO120 (*Hypoxylon* sp.) at different concentrations

Treatments	Stem fresh weight (g)	Leaves fresh weight (g)	Polysaccharides content (% DW)	Ethanol extractives content (% DW)	Total flavonoids content (mg/g DW)	Total phenols content (mg/g DW)	Naringenin content (mg/g DW)	Schaftoside content (mg/g DW)
DO14ME-300	39.61 ± 3.89*	26.23 ± 3.22*	3.83 ± 0.28*	18.56 ± 0.02	6.7430 ± 0.2067	5.7699 ± 0.9131*	0.0295 ± 0.0019*	0.3542 ± 0.0518
DO14ME-600	36.67 ± 4.30*	32.14 ± 5.12*	5.76 ± 0.24*	18.75 ± 0.11	6.6190 ± 0.2068	6.6200 ± 0.5888	0.0436 ± 0.0066*	0.4224 ± 0.0279*
DO18ME-300	28.99 ± 4.20*	21.40 ± 6.30	3.71 ± 0.07*	17.86 ± 0.04*	6.8670 ± 0.0827*	5.6699 ± 1.0602*	0.0356 ± 0.0043	0.3425 ± 0.0167
DO18ME-600	30.56 ± 5.30*	23.14 ± 3.21	4.95 ± 0.37*	17.59 ± 0.45*	6.4261 ± 0.3929	6.8777 ± 0.2712	0.0322 ± 0.0021*	0.3272 ± 0.0114
DO19ME-300	34.27 ± 2.30*	26.96 ± 4.08*	5.13 ± 0.18*	18.24 ± 0.14	7.4045 ± 0.1240*	7.0123 ± 0.1101*	0.0194 ± 0.0003*	0.2569 ± 0.0255*
DO19ME-600	35.18 ± 3.80*	27.91 ± 3.12*	4.60 ± 0.17*	19.60 ± 0.62	6.3985 ± 0.8282	6.8777 ± 0.4443	0.0454 ± 0.0006*	0.2947 ± 0.0320
DO120ME-300	31.33 ± 4.20*	25.52 ± 2.98*	3.65 ± 0.26*	16.55 ± 0.47*	6.1091 ± 0.3103	6.6738 ± 0.0696	0.0254 ± 0.0004*	0.3813 ± 0.0118*
DO120ME-600	32.41 ± 3.00*	27.91 ± 1.99*	4.25 ± 0.31*	20.62 ± 0.18*	6.5363 ± 0.1240	6.2353 ± 0.0705	0.0242 ± 0.0007*	0.3361 ± 0.0060
Control	25.51 ± 4.90	22.64 ± 1.86	3.09 ± 0.14	18.92 ± 0.44	6.3571 ± 0.1864	6.6161 ± 0.0520	0.0382 ± 0.0019	0.3235 ± 0.0053

ME-300 and ME-600 denote 300 mg ME l^{-1} and 600 mg ME l^{-1} treatments, respectively. Values are presented as mean ± standard, data are means of three replicates

* indicates significant differences ($P < 0.05$) with control group using one way analysis of variance. DW denotes dry weight. Control was added with the same volume of distilled water

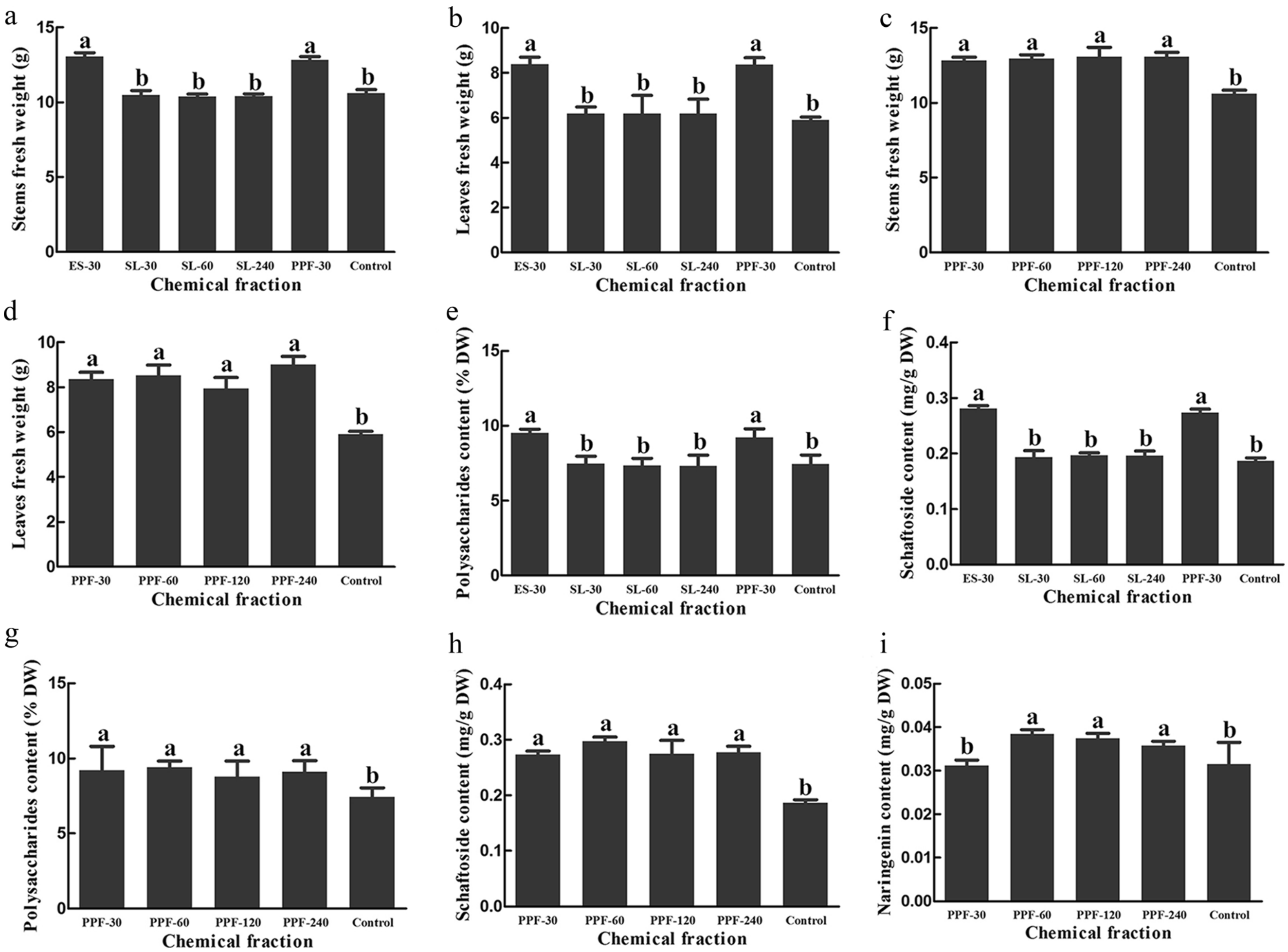

Fig. 1 Effects of ethanol sediment (ES), supernatant liquor (SL) and protein-polysaccharides fraction (PPF) elicitors from DO14 at different concentrations on biomass and active ingredients accumulation of *D. catenatum*. ES-30 denotes ES treatment at 30 mg/l, SL-30, SL-60 and SL-240 denote SL treatments at 30 mg/l, 60 mg/l and 240 mg/l, PPF-30, PPF-60, PPF-120 and PPF-240 denote PPF treatments at 30 mg/l, 60 mg/l, 120 mg/l and 240 mg/l, respectively. Values are presented as mean ± standard, data are means of three replicates. Different letters indicate significant differences ($P < 0.05$) within groups using one way analysis of variance. Control refers to the plantlets cultured in the co-culture medium without fungal elicitors

are different, saccharides especially oligosaccharide remain as the main common active ingredients (Zhai et al. 2017). This agrees with our results that PPF elicitor, mainly contains some polysaccharides and proteoglycan, can significantly promote the growth of *D. catenatum* plantlets. However, we did not observe a concentration dependent effect (Fig. 1c, d). Possibly, 30 mg/L PPF was a saturated concentration on the growth stimulation of *D. catenatum*. PPF concentration (lower than 30 mg/L) may be further optimized for maximal growth with minimal input.

Stems are often regarded as the medicinal part of *D. catenatum*, and early studies indicated that in the stems, polysaccharides, ethanol extractives, and naringenin contents are responsible for various pharmacological actions, therefore, they become the main quality indicators (Rudgers et al. 2012; Zhou et al. 2013). Moreover, the leaves of *D. catenatum* also contain a large group of flavonoids constituents such as schaftoside which shows activities in antisepsis, antioxygenation, anti-inflammatory (Erlund 2004; Jimenez-Zamora et al. 2016; Wang et al. 2017). Thus, we respectively use the contents of polysaccharides, ethanol extractives, naringenin in stems and the contents of total flavonoids, total phenols, schaftoside in leaves to evaluate the quality of *D. catenatum* plantlets. Effects of fungal elicitors on plant cell accumulation of secondary metabolites have been considered to be related to their chemical nature (Zhai et al. 2018). Among four fungal elicitors in this study, ME, ES and PPF were found to significantly promote the active ingredients accumulation in *D. catenatum* plantlets with PPF as the leading active constituent (Table 3, Fig. 1e–i).

Fungi and oomycetes are major plant pathogens that cause devastating diseases in crops (Bacete et al. 2018). Pathogens need to be recognized in a timely manner by the host in order to activate the proper defenses that restrict invasion and

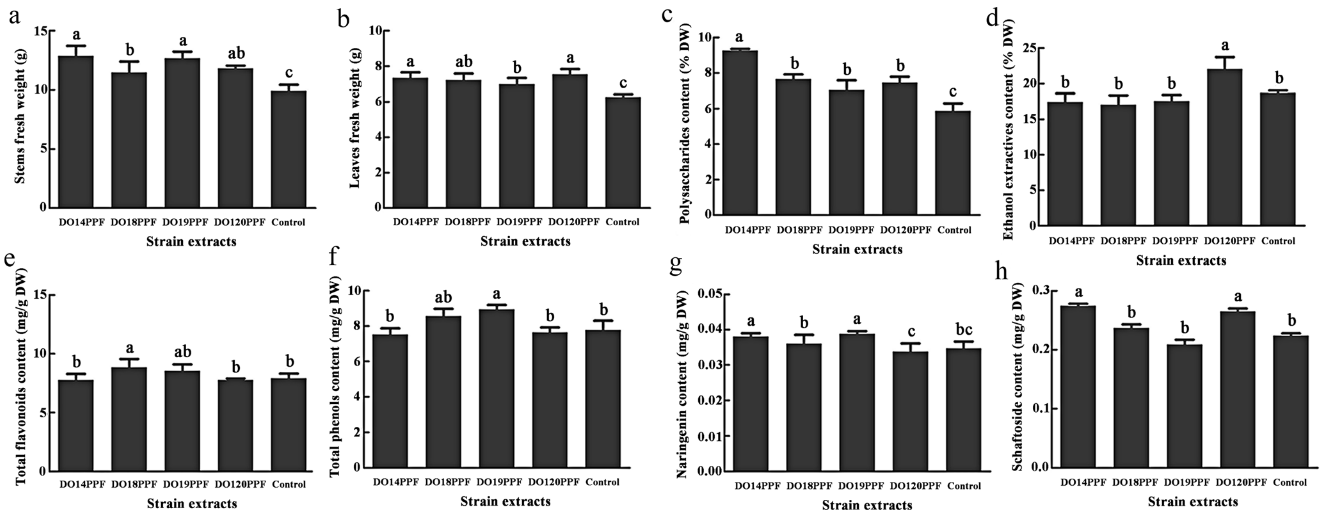

Fig. 2 Effects of PPFs from DO14, DO18, DO19 and DO120 on biomass and active ingredients accumulation of *D. catenatum*. DO14PPF, DO18PPF, DO19PPF and DO120PPF denote PPF prepared from DO14, DO18, DO19 and DO120 treatments respectively. Values are presented as mean ± standard, data are means of three replicates. Different letters indicate significant differences ($P < 0.05$) within groups using one way analysis of variance. Control refers to the plantlets cultured in the co-culture medium without fungal elicitors

colonization. A crucial feature of the innate immune system in plants is the ability to sense a potential danger through the recognition of molecules that alert the cell. Molecules associated with pathogenic microbes (microbe-associated molecular patterns), like chitin from fungi, and glucans from the cell wall of oomycetes are specifically sensed by the host cells and trigger an immune response. Endogenous molecules with elicitor activity are released from cellular components during pathogen attack or abiotic stresses, and have been indicated as damage-associated molecular patterns (DAMPs) in plants, where they have also been called alarmins (Bianchi 2007). Oligosaccharides as well as their derivatives are probably the best characterized plant DAMPs and elicit in several plant species a wide range of defense responses, including accumulation of phytoalexins, glucanase, and production of reactive oxygen species such as flavonoids compounds (Galletti et al. 2011; Mélida et al. 2018). Fungal cell wall is a semirigid and dynamic structure composed primarily of polysaccharides, like chitin and glucans, and highly glycosylated proteins (Latgé and Calderone 2006). Polysaccharides have roles in signaling transduction systems that regulate both plant defensive and developmental processes, consequently stimulating growth and metabolite accumulation (And and Farmer 1991; Veloso and Diaz 2013; Srivastava and Srivastava 2014). When DO14, DO18, DO19 and DO120 were colonized on the tissue of *D. catenatum*, PPF on the surface, mainly contain some types of polysaccharides which may be recognized as an exogenous additive and degraded to small molecules like oligosaccharides. Oligosaccharides may further elicit chemical defense response and stimulate the accumulation of growth regulators such as auxin, and antimicrobial ingredients such as naringenin and schaftoside (Zhao et al. 2005; Silipo et al. 2010; Jeyadevi et al. 2013; Ogawa et al. 2017). Despite the detailed mechanisms of the effects of fungal elicitors remain to be explored, it is also necessary to clarify which type of polysaccharide from PPF is the active constituent responsible for the enhanced growth and active ingredients accumulation of *D. catenatum*.

However, no system has been found to respond to all elicitors and no elicitor has been found to have a common effect on many culture systems (Singh et al. 2017). It is essential to investigate the elicitors from various fungal species for a specific system for production of a desirable compound. In the present study with the PPFs from various fungal species, we found PPF prepared from *Pestalotiopsis* sp. (DO14) was more suitable as an elicitor for the productions of polysaccharides, naringenin, and schaftoside in *D. catenatum* plantlets (Fig. 2a–h). PPFs prepared from *Talaromyces* sp. (DO18) (Fig. 2e), *Xylariaceae* sp. (DO19) (Fig. 2f) and *Hypoxylon* sp. (DO120) (Fig. 2d) were more suitable for the productions of total flavonoids, total phenols and ethanol extractives. The specific and diverse effects of fungal elicitors, as observed in this study, are most likely to be implicated with unique modes of recognition upon interactions with fungi and the complexity of elicitor signal transduction, resulting in plant defense response (Simic et al. 2015). Some endophytic fungal species may be susceptible to certain or specific chemical components. Upon contact with fungus, host plants may synthesize certain compounds to inhibit the growth of fungus (Abreu et al. 2012; Hartley et al. 2015). This may explain why, in the present study, PPFs from different fungal species influence differently on the active ingredients in *D. catenatum*. In any case, all these studies high-lights the specificity of the varied fungal strains for the production of high quality *D. catenatum* plantlets. This is the first report concerning the functional role of fungal elicitors prepared from *Pestalotiopsis* sp., *Talaromyces* sp., *Xylariaceae* sp. and *Hypoxylon* sp. on plant cultures.

To the best of our knowledge, there were no previous reports on the use of extracts from endophytic fungi as elicitors for efficient promoting growth and active ingredients production of *D. catenatum*. Without obvious changes in the appearance of the plantlets, the exogenous fungal mycelia crude extracts effectively promote biomass and active ingredients accumulation of *D. catenatum*, and the promotion effect was closely aligned with the elicitor origin, along with its chemical nature. Moreover, PPF was found as the leading active constituent responsible for the enhancement. Among the strains in this study, PPF from DO14 was most effective for biomass, polysaccharides, naringenin and schaftoside enhancement while PPFs from DO18, DO19 and DO120 were more suitable for the productions of total flavonoids, total phenols and ethanol extractives. As the PPF can be readily prepared and easily administered to the plant cultures, it should be helpful for practical application in the laboratory or large-scale production of high quality *D. catenatum* plantlets in the future. Our results provide operational guidelines for effective utilization of endophyte resources and fungal fertilizer exploitation.

Acknowledgments This work was funded by the National Natural Science Foundation of China (31600259, 81630105), and Opening Project of Zhejiang Provincial First-rate Subject (Chinese Traditional Medicine), Zhejiang Chinese Medical University (Ya2017001).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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