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Original Article

Penicillium sp. YJM-2013 induces ginsenosides biosynthesis in *Panax ginseng* adventitious roots by inducing plant resistance responses

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ABSTRACT

Objective: *Fusarium oxysporum* is a common pathogenic fungus in ginseng cultivation. Both pathogens and antagonistic fungi have been reported to induce plant resistance responses, thereby promoting the accumulation of secondary metabolites. The purpose of this experiment is to compare the advantages of one of the two fungi, in order to screen out more effective elicitors. The mechanism of fungal elicitor-induced plant resistance response is supplemented.
Methods: A gradient dilution and the dural culture were carried out to screen strains. The test strain was identified by morphology and 18 s rDNA. The effect of different concentrations (0, 50, 100, 200, 400 mg/L) of *Penicillium* sp. YJM-2013 and *F. oxysporum* on fresh weight and ginsenosides accumulation were tested. Signal molecules transduction, expression of transcription factors and functional genes were investigated to study the induction mechanism of fungal elicitors.
Results: Antagonistic fungi of *F. oxysporum* was identified as *Penicillium* sp. YJM-2013, which reduced root biomass. The total ginsenosides content of *Panax ginseng* adventitious roots reached the maximum (48.95 ± 0.97 mg/g) treated with *Penicillium* sp. YJM-2013 at 200 mg/L, higher than control by 2.59-fold, in which protopanoxadiol-type ginsenosides (PPD) were increased by 4.57 times. Moreover, *Penicillium* sp. YJM-2013 activated defense signaling molecules, up-regulated the expression of PgWRKY 1, 2, 3, 5, 7, 9 and functional genes in ginsenosides synthesis.
Conclusion: Compared with the pathogenic fungi *F. oxysporum*, antagonistic fungi *Penicillium* sp. YJM-2013 was more conducive to the accumulation of ginsenosides in *P. ginseng* adventitious roots. *Penicillium* sp. YJM-2013 promoted the accumulation of ginsenosides by intensifying the generation of signal molecules, activating the expression of transcription factors and functional genes.

1. Introduction

Panax ginseng C. A. Meyer, belonging to the Araliaceae family, is one of the rare traditional Chinese medicines. *P. ginseng* is widely used in the field of food and medicine because of its anti-tumor, anti-aging and anti-fatigue properties (Lee, Jung, Kim, Lee, & Chung, 2006; Lee et al., 2007). These active ingredients are mainly attributed to ginsenosides, which are structured into three groups, Rb, Rg and Ro groups (Cordell, 2001). At present, the endangered wild *ginseng*, *ginseng* field cultivation is the main source, however, the longer cultivation of *P. ginseng* growth cycle suffer the harm of environmental pollution and diseases and pests and old *P. ginseng* soil erosion damage ecological environment problems such as difficult to solve, and tissue culture has the advantages of short growth period which is not affected by the external environment, therefore, we can partially solve the problem of resource and production of medicinal active ingredients of *P. ginseng* through plant tissue culture technology.

However, compared with the roots of mother plants, active compounds in tissue culture are often lower (Jung et al., 2014). Elicitors, especially fungal elicitors, are demonstrated to be a useful tool to promote the synthesis of secondary metabolites in medical plants. There is pervasive application of fungal elicitors to enhance the yields of these valuable metabolites from tissue of numerous plant species, including *Isatis tinctoria* L. (woad) (Jiao et al., 2018), *Withania somnifera* (Ahlawat, Saxena, Ali, Khan, & Abdin, 2017), *Astragalus membranaceus* (Gai et al., 2017) etc. Similarly,

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fungal elicitors are also be used in *P. ginseng* (Le, Im, Paek, & Park, 2018), *Panax quinquefolius*, and *Panax sikkimensis* (Ban) to promote the production of ginsenosides.

With the fungal elicitors' identification by the receptors of the plant cells, the signal molecules were also be activated, such as the presence of Ca^{2+} ion fluxes, activation of phospholipase A_2 (PLA_2) and protein kinase (Zhao, Davis, & Verpoorte, 2005) etc. Then, this series of messages trigger the formation of endogenous signaling molecules including nitric oxide (NO), ethylene (ET), abscisic acid (ABA) in NO, ET, jasmonic acid (JA) and salicylic acid (SA) signaling pathway. Then, all kinds of signals are integrated into DNA transcription factors, which eventually activate key genes expression and trigger metabolic pathways of secondary metabolites (Aharoni & Galili, 2011). A schematic illustration of the sequential signaling pathways activated in elicited ginseng were listed in Fig. 1. WRKY proteins as transcription factors, play vibrant roles in many plant growth process, including resistance responses to external stress, as well as metabolism of active ingredients.

Among the current application of fungal elicitor, like endophytic fungal microorganisms that colonize healthy plant tissue, or pathogenic fungi that cause plant disease are reported to be used as elicitors to stimulate the accumulation of ginsenosides (Yu et al., 2016), pytethrin (Khan et al., 2016), vindoline (Pandey et al., 2016), asiaticoside (Jisha, Gouri, Anith, & Sabu, 2018), gymnemic acid (Netala, Kotakadi, Gaddam, Ghosh, & Tartte, 2016) etc. However, few reports have described that antagonistic fungi which was resistant to pathogens could be used as elicitors to increase the content of ginsenosides. Antagonistic fungi, known as biocontrol fungi, refers to the various microorganisms that are beneficial to the prevention of plant diseases. It is reported that biocontrol fungi can balance endogenous hormones in plants (Viterbo, Landau, Kim, Chernin, & Chet, 2010), increase the activity of host defense enzymes, induce the expression of plant disease-related protein (Chen, Harman, Comis, & Cheng, 2005), and even improve the level of plant secondary metabolites (Ponce, Scervino, Erra-Balsells, Ocampo, & Godeas, 2004). Since the elicitors promote the synthesis of secondary metabolites by plants self-defense reaction (Zhang et al., 2015), we try to use the biocontrol fungi as an inducer to study the accumulation of secondary metabolites in the adventitious roots of ginseng. Hence, we compared the effect of pathogen *F. oxysporum* and its antagonistic fungi on accumulation of biomass and ginsenosides. Moreover, the production of signal molecules, expression of PgWRKY family and functional genes were also be evaluated to comprehend the signal transduction underlying elicitation. Also, ginsenoside composition were detected by HPLC-ESI-MS^n after fungal elicitor treatment.

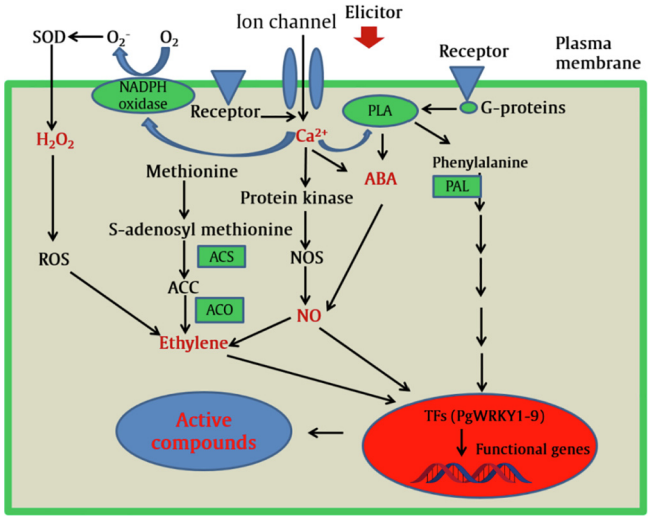


Fig. 1. A schematic illustration of sequential signaling pathways activated in elicited ginseng (Rahimi, Kim, & Yang, 2015). Enzymes were marked in green box. The signal molecules were shown in red. ABA: abscisic acid, ACC: 1-aminocyclopropane-1-carboxylic acid, ACS: 1-aminocyclopropane-1-carboxylic acid synthase, ACO 1-aminocyclopropane-1-carboxylic acid, NO: nitric oxide, NOS: nitric oxide synthase, O^{2-}: superoxide radical, H_2O_2: hydrogen peroxide, ROS: reactive oxygen species, SOD: superoxide dismutase, PLA: phospholipase, PAL: phenylalnine ammonialyase, TFs: transcription factors.

2. Materials and methods

2.1. Separating and screening of antagonistic fungi in P. notoginseng rhizosphere soil

P. notoginseng rhizosphere soil samples were collected from Guishan town, Shilin county, Kunming city ($103^0 32' 35''$ E, 1972 m ± 3.00 m, $24^0 40' 9''$ N), China. A gradient dilution method was used to screen the colony of soil (Fan et al., 2016). Antagonistic *F. oxysporum* screening experiments was carried out by using the dural culture method (Chen et al., 2016). *F. oxysporum* A549 was provided by Tianjin University of Science and Technology.

2.2. Morphological identification and 18 s rDNA identification

The activated strain was inoculated on PDA medium, and cultured at 28 °C for 6–7 d, and its morphology was observed. The genomic DNA was extracted by Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, China) and 18S ribosomal RNA (rRNA) genes were amplified by PCR using the primers ITS1 and ITS4. The PCR cycling protocol included an initial denaturation at 94 °C for 4 min, followed by 32 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. PCR products were visualized on 1% agarose gels, which were then excised and purified with Nucleo-pore PCR Clean-up Gel Extraction kit (Genetix Biotech Asia Pvt. Ltd., India). DNA sequencing was performed by GENEWIZ, Inc (Suzhou, China) and sequence similarities were determined by NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.3. Adventitious root culture

The ginseng adventitious roots were cultured in the 3/4 MS liquid medium supplement with 5.0 mg/L IBA, 0.1 mg/L KT and 4% sucrose (pH 5.8 ± 0.2). The medium was autoclaved for 25 min at 121 °C. The fresh adventitious roots of 1 g were inoculated into a 100 mL Erlenmeyer flask on a rotary shaker (130 rpm) at 25 °C in dark. After 35 d of cultivation, adventitious roots were harvested.

2.4. Preparation of elicitor

Penicillium sp. YJM-2013 and *F. oxysporum* were used in this study. They were cultivated and inoculated on PDA liquid medium for 7 d in dark at 28 °C and 150 rpm. Firstly, their mycelium was harvested and dried in drying oven at 50 °C for 48 h. Secondarily, the dry mycelium was ground to powder and dissolved in distilled water, and the concentration of this solution was 10 g/L. Finally, they were sterilized at 121 °C for 25 min, and the sterilized powder were used for the induction experiment. The elicitor concentration was determined by its carbohydrate content and the content was measured by Anthrone-sulfuric acid assay.

2.5. Elicitation treatment

Different concentrations (0, 50, 100, 200, and 400 mg/L) of *Penicillium* sp. YJM-2013 and *F. oxysporum* were appended to the 30-day-old *P. ginseng* adventitious roots. And after 5 d of elicitor treatment, the adventitious roots were harvested. The fresh weight and the content of ginsenosides were then analyzed. After obtaining the positive results, the 30-day-old adventitious roots were treated with the best elicitor concentration on different treatment time (0, 12, 24 and 48 h) to investigate the transduction of signal molecules (H_2O_2, NO, ET, PLA_2 and ABA, for Ca^{2+}, 0, 10, 20, 30 and 60 mins), the expression level of transcription factors and functional genes referred to ginsenosides biosynthesis pathway.

2.6. Quantitation of signal molecules of roots after treatment with *Penicillium sp. YJM-2013* in different time

To test Ca^{2+}, H_2O_2, ABA, PLA_2, NO, ABA and ET, adventitious roots (0.5 g, for NO and H_2O_2 assays, 0.1 g) were ground into homogenate on the rice with 4.5 mL PBS. The supernatant was collected at 4 °C for subsequent experiment after centrifugation at 3000 rpm for 20 min. Then the content of Ca^{2+}, H_2O_2, NO was measured by Calcium Assay Kit, Hydrogen Peroxide assay kit (colorimetric method) and Nitric Oxide assay kit (Nitrate reductase method) (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China), and the content of ABA, PLA_2, ET were measured by Plant Abscisic Acid Elisa Kit, Plant PLA_2 Elisa Kit and Plant ET Elisa Kit (Senbejia Nanjing Biotechnology Co., Ltd, Nanjing, China) according the manufactures' directions.

2.7. RNA isolation and quantitative real-time expression analysis

For RT-PCR, cDNA-equivalent to 200 ng total RNA was used as template in 20 μL volume and total RNA were extracted from fresh adventitious root samples using the Plant RNA Kit (OMEGA, USA), and RNA was transcribed into the first strand cDNA reversely with the use of the HiFiScript 1st Strand cDNA Synthesis Kit (CWBIO, China). Resulting reverse transcription products were stored at −80 °C until analysis. The PCR cycling protocols were as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50–58 °C for 1 min (PgWRKY8: 52 °C, GPS, FPS, SS, SE, DS, CYP716A47, CYP716A53v2, UGT74AE2, UGT94Q2, UGTPg100, UGTPg1, PgWRKY1, PgWRKY2, PgWRKY3, PgWRKY4, PgWRKY6, PgWRKY7, PgWRKY9: 56 °C, PgWRKY5, 58 °C, and 72 °C for 45 s) with a final 10 min extension at 72 °C. The relative expression value of each genes was calculated by the $^{\triangle\triangle}$Ct method. Moreover, expression levels were normed using β-actin mRNA levels. Each analysis was repeated for three times. And the primers of genes were shown in Table 1.

2.8. Quantification of ginsenoside contents

Samples are prepared according to the experimental requirements (Liu et al., 2013). To identify and quantify the ginsenosides Rg_1, Re, Ro, Rf, Rb_1, Rg_2, Rh_1, Rc, Rb_2, Rd, Rg_3, Rb_3 and Rh_2, the extract was used and all standards (purity: ≥98%) were purchased from Suzhou Star Ocean Ginseng Bio-pharmaceutical Co., Ltd. (Suzhou, China). Performed on an Agilent 1200 series HPLC instrument, the analysis samples were separated on a Kromasil C_{18} (4.6 mm × 250 mm, 5 μm) column using a gradient phase composed of CH_3-CN (A) and water (B), and the column was kept at 35 °C. The flow rate was 1.0 mL/min and the flow program was: 0–35 min, 20% A; 35–40 min, 30% A; 40–50 min, 31% A; 50–60 min, 32% A; 60–70 min, 45% A; 70–100 min, 60% A. The injected volume was 20 μL and the peaks were monitored at 203 nm.

Table 1
Sequence of primers.

Primer names	Primer sequences (5′ to 3′)
β-actin	For: CAG AAG AGC ACC CTG TTC TTT
	Rev: ATA AAT GGG GAC TGT GTG GCT
GPS	For: GTC AGA ATT GAT ATT CTT GCC CGC CC
	Rev: ATG TCT CGC ACG TGT GTC TTC T
FPS	For: CTG AAA TCC GAG CTA CTC AAC GA
	Rev: GCC ATT CAA TGC ACC AAC CA
SS	For: ATG GGA AGT TTG GGG GCA ATT CT
	Rev: GTT CTC ACT GTT TGT TCA GTA GTA GGT T
SE	For: AGC AGC AGT TGA CAA AGG
	Rev: GCC ACA TTC GTT TTG GTG AAG G
DS	For: CGG AAA CGT GTT TGG TTG CC
	Rev: CAA ACA ACA CCT ATT TCC GAT T
CYP716A47	For: ATG GTG TTG TTT TTC TCC CTA TCT
	Rev: TTA ATT GTG GGG ATG TAG ATG AAT
CYP716A53v2	For: ATG GAT CTC TTT ATC TCATCT CAA
	Rev: TTA AAG CGT ACA AGG TGA TAG ACG
UGT74AE2	For: ATG CTG AGC AAA ACT CAC ATT A
	Rev: AAC TCC CAT ATA AGC CTG CAT
UGT94Q2	For: GGT AGA ATC AGT ATA GCG TTG C
	Rev: TCT GAG TGG GAG CAT GAG ATG GGA A
UGTPg100	For: CGCGGATCCATGAAGTCAGAATTGATATT
	Rev: CCGCTCGAGTTACATAATTTCCTCAAATA
UGTPg1	For: GGA ATG GTG GAG ATG GCT A
	Rev: TTG AGT GTC TGG AGA TGG AA
PgWRKY1	For: CAAGCGAAAGTGCAGTTCAA
	Rev: CTGGGCAACCCCTTACACTA
PgWRKY2	For: TTTATCTCCTCGTTGAGTGTCG
	Rev: ACCTTCGTTTGTGCTGGTATG
PgWRKY3	For: CGGAAACCCAGACTCACG
	Rev: TCGGGACCCATTAGACATCA
PgWRKY4	For: CACCAACAGCATCAACAGCG
	Rev: ATTCGGGACCCATTAGACATCA
PgWRKY5	For: GGAGCAATAGTGGCATCAACCTG
	Rev: GAGTCGCACCAATTAAATGGAAAG
PgWRKY6	For: CCATTCCCGTCAAGTTTCCAC
	Rev: CGGCAAAGTCTATGTTGTCAGG
PgWRKY7	For: CGACATTATCTTCCTTCAACTTTA
	Rev: ACTCGCATTTGGGACGGC
PgWRKY8	For: ATGGATAACTCTCCCTCCCCTA
	Rev: CATTCTCTTCAATCTTCACTGC
PgWRKY9	For: GCAGAAAAGAGTGGTGTCCG
	Rev: AGGCTTTTGACCATACTTTC

2.9. HPLC-ESI-MS^n

The analysis were carried out by an Agilent 1260 series HPLC (Agilent 6420 Triple Quad LC/MS), and the source conditions were as follows: full scan of ions ranging from m/z 100–1400 in the negative code, capillary voltage at 4000 V, nebulizer pressure at 15 bar, dry gas flow rate of 11 L/min, and dry gas temperature at 300 °C. High-purity nitrogen and He were used as sheath and collision gases, respectively. The data were operated on the Qualitative Analysis B.06.00 software.

2.10. Statistical analysis

The fresh weight and ginsenoside contents were expressed as means ± standard deviation (SD). Statistical analyses were carried out by using SPSS17.0 (SPSS Inc., Chicago, USA). $P < 0.05$ indicated significant difference.

3. Results

3.1. Morphological characteristics and 18S rRNA gene analysis for identification and phylogenetic analysis

The morphology of the test strain on PDA medium was shown in Fig. 2A: oval, grayish green with a dense, flat surface and white edges, which is similar to that of *Penicillium*. Moreover, phyloge-

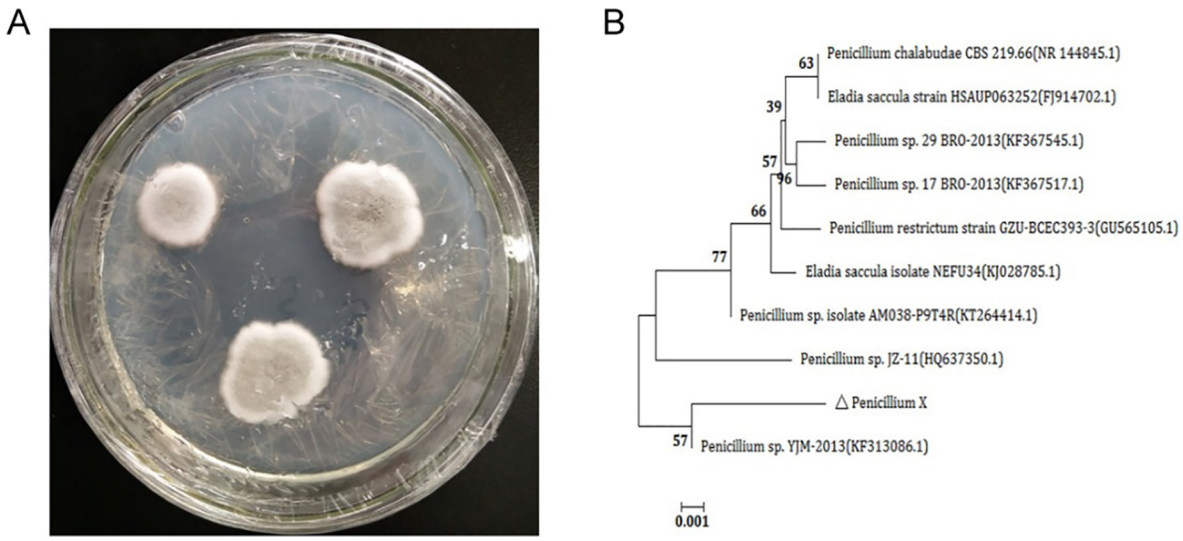


Fig. 2. *Penicillium* on PDA plate medium (A); Phylogenetic tree based on 18S rDNA gene sequences showing the phylogenetic relationships between *Penicillium* sp. YJM-2013 and closely related species (B).

netic analysis (Fig. 2B) based on 18S rRNA gene sequences indicated that the test strain was most close to *Penicillium* sp. YJM-2013 (KF313086.1, 99%).

3.2. Roots growth and ginsenoside production in P. ginseng adventitious roots following Penicillium sp. YJM-2013 and F. oxysporum A549 elicitation

Different elicitor has different influence on biomass of adventitious roots. *Penicillium* sp. YJM-2013 did not significantly decrease the fresh weight of adventitious roots at concentration of less than 50 mg/L, but significantly more than 100 mg/L (Table 2). However, *F. oxysporum* A549 could increase it at different concentrations (Table 3).

Both *Penicillium* sp. YJM-2013 and *F. oxysporum* A549 could promote the accumulation of total ginsenosides (Tables 2 and 3) with individual differences. *Penicillium* sp. YJM-2013 was optimal for Rb, Rg group and Ro at 200 mg/L, especially for Rb group, which was 4.57 fold of the control group (4.69 ± 0.47 mg/g). *F. oxysporum* A549 was optimal for Rb group and Rg group at 200 mg/L and 100 mg/L, respectively, whereas 400 mg/L was more conducive to the accumulation of Ro, and the Ro content increased 4.44-fold compared with the control group (2.32 ± 0.01 mg/g). The maximum of total ginsenosides (48.95 ± 0.97 mg/g) was obtained at 200 mg/L of *Penicillium* sp. YJM-2013, and this content was 2.59 fold higher than the control group (18.92 ± 0.74 mg/g). Therefore, *Penicillium* sp. YJM-2013 at concentration of 200 mg/L was selected for the following experiments.

3.3. Signal molecules accumulation in P. ginseng adventitious roots following Penicillium sp. YJM-2013 elicitation

Penicillium sp. YJM-2013 effectively improved the accumulation of Ca^{2+}, H_2O_2, ABA, PLA_2, NO and ET in *P. ginseng* adventitious roots.

Table 2
Effect of *Penicillium* sp. YJM-2013 elicitor concentration on biomass and ginsenoside accumulation of *P. ginseng* adventitious roots after 5 d of elicitation (mean ± SD, n = 3).

Elicitor concentrations/ (mg·L^{-1})	Fresh weight /g	Ginsenoside content /(mg·g^{-1})			
		Rb group	Rg group	Ro	Total
0	7.71 ± 0.30a	4.69 ± 0.47d	11.59 ± 0.25d	2.64 ± 0.02d	18.92 ± 0.74e
50	6.91 ± 0.36ac	10.60 ± 0.35c	13.03 ± 0.66d	3.81 ± 0.16c	27.44 ± 0.85d
100	5.86 ± 0.56bc	12.21 ± 0.73c	15.50 ± 0.61c	4.82 ± 0.12b	32.54 ± 1.23c
200	5.78 ± 0.35b	21.42 ± 1.05a	21.74 ± 0.22a	5.79 ± 0.30a	48.95 ± 0.97a
400	5.16 ± 0.33b	15.35 ± 0.28b	17.27 ± 0.53b	3.79 ± 0.46c	36.41 ± 0.20b

Note: Rb group = Rb_1 + Rb_2 + Rb_3 + Rc + Rd + Rg_3 + Rh_2; Rg group = Re + Rg_1 + Rg_2 + Rf + Rh_1. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.

Table 3
Effect of *F. oxysporum* A547 concentration on biomass and ginsenoside accumulation of *P. ginseng* adventitious roots after 5 d of elicitation (mean ± SD, n = 3).

Elicitor concentration / (mg·L^{-1})	Fresh weight/g	Ginsenoside content /(mg·g^{-1})			
		Rb group	Rg group	Ro	Total
0	6.82 ± 0.05e	6.21 ± 0.12c	11.57 ± 0.36b	2.32 ± 0.01c	20.10 ± 0.23d
50	7.33 ± 0.06d	6.06 ± 0.16c	17.07 ± 1.05a	1.90 ± 0.10c	25.03 ± 0.99c
100	8.43 ± 0.12c	9.89 ± 0.56b	17.77 ± 2.15a	8.94 ± 1.36a	36.60 ± 0.36a
200	8.94 ± 0.33b	11.44 ± 0.13a	16.75 ± 0.35a	7.13 ± 0.28b	35.31 ± 0.50a
400	10.10 ± 0.09a	9.41 ± 0.51b	11.78 ± 0.57b	10.30 ± 0.35a	31.50 ± 1.48b

Note: Rb group = Rb_1 + Rb_2 + Rb_3 + Rc + Rd + Rg_3 + Rh_2; Rg group = Re + Rg_1 + Rg_2 + Rf + Rh_1. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.

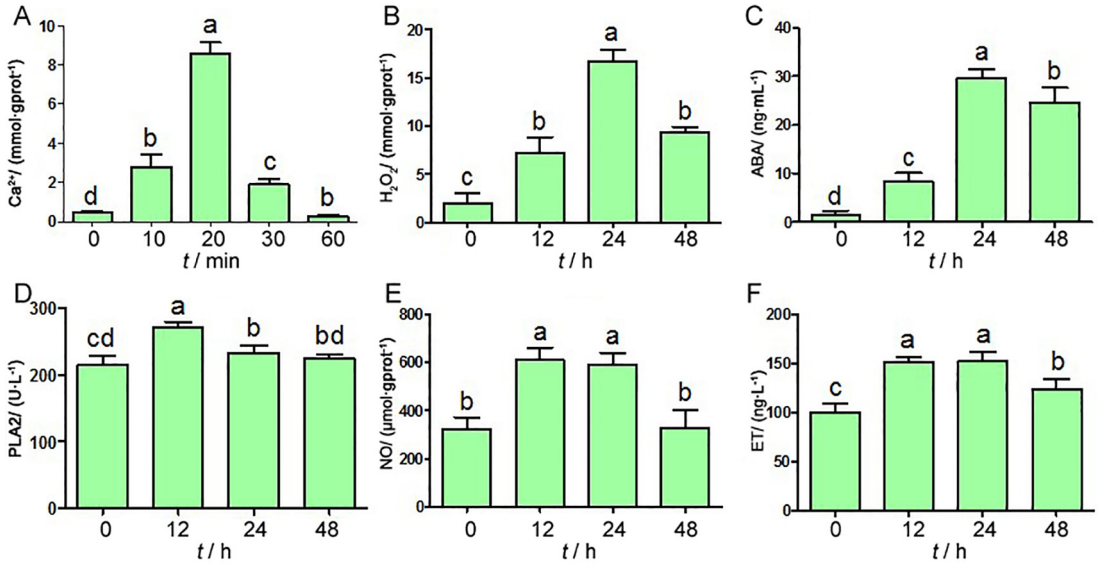


Fig. 3. Accumulation of Ca^{2+} (a), H_2O_2 (b), ABA (c), PLA_2 (d), NO (e), ET (f) in *P. ginseng* adventitious roots affected by *Penicillium* sp. YJM-2013 elicitor. Data represent as mean values ± SD of three replicates. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$.

As shown in Fig. 3A, Ca^{2+} (8.58 ± 0.37 mmol/g prot) generated immediately and reached its peak value at 20 min, H_2O_2 (16.78 ± 0.59 mmol/g prot) (Fig. 3B), ABA (29.50 ± 0.99 ng/mL) (Fig. 3C), NO (609.22 ± 25.02 μmol/g prot) (Fig. 3D) and ET (151.74 ± 5.20 ng/L) (Fig. 3E) reached the highest level at 12 h. PLA2 (233.27 ± 5. 78 U/L) (Fig. 3F) also increased after *Penicillium* sp. YJM-2013 elicitor treatment and reached its highest at 24 h.

3.4. PgWRKYs accumulation in P. ginseng adventitious roots following Penicillium sp. YJM-2013 elicitation

We implemented qRT-PCR analysis of PgWRKY1-9 at different time intervals (0, 12, 24, 48 h). Seven PgWRKY genes were significantly up-regulated, while the PgWRKY4 and PgWRKY8 genes were notably down-regulated at 48 h. As shown in Fig. 4, the expression level of PgWRKY1, PgWRKY5, PgWRKY6, PgWRKY7,

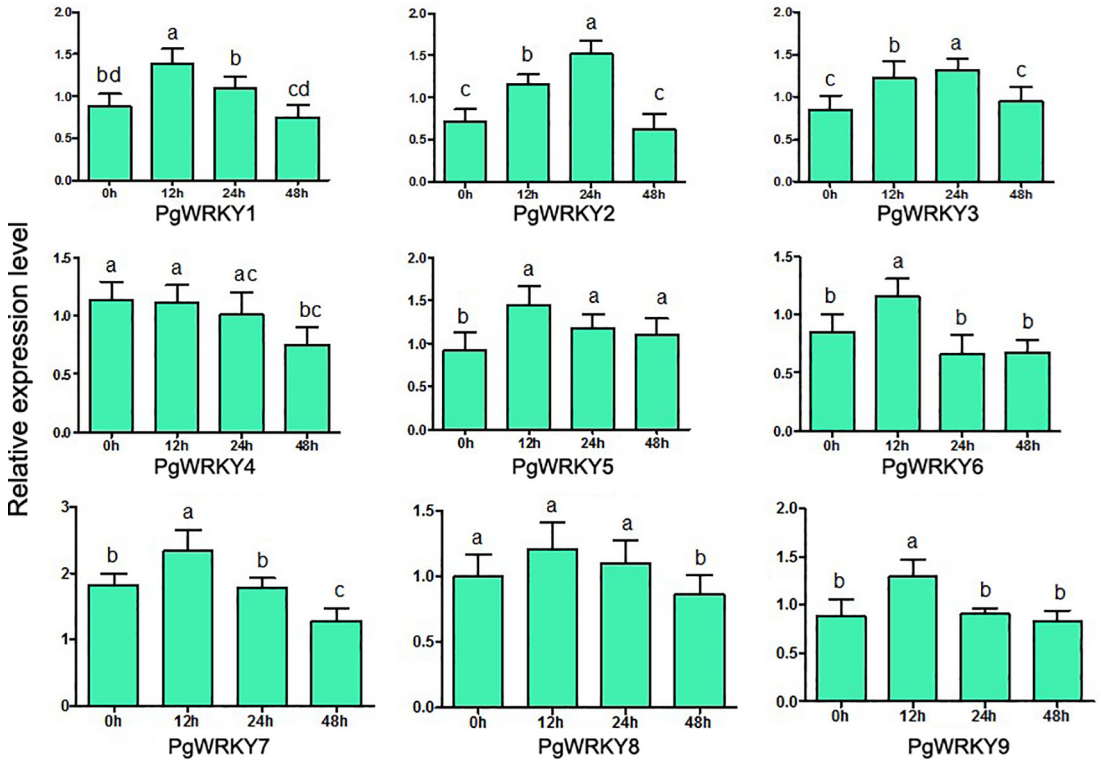


Fig. 4. Effects of *Penicillium* sp. YJM-2013 elicitor on expression of PgWRKY1-9 transcription factors via ginsenoside biosynthetic pathway in *P. ginseng* adventitious root. Data represent as mean values ± SD of three replicates. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$.

PgWRKY9 reached the maximum at 12 h, however, PgWRKY2, PgWRKY3 had the highest expression level at 24 h.

3.5. Biosynthetic genes expression and ginsenosides production in P. ginseng adventitious roots following Penicillium sp. YJM-2013 elicitation

We investigated the compound of ginsenosides and the changes in transcriptional levels of eleven genes involved in ginsenosides biosynthetic pathway at different time intervals (0, 12, 24, 48 h). As shown in Fig. 5, all tested genes were significantly up-regulated during the elicitation period, indicating that the increased accumulation of ginsenosides was due to the elevated transcription of these functional genes. Also, the expression levels of UDP-glycosyltransferases (UGT) genes UGT74AE2, UGT94Q2 and UGTpg100, reached the highest at 48 h, 12 h, 12 h differently, and they consisted in the production of monomer ginsenoside Rh_2, Rg_3 and Rh_1.

3.6. HPLC-ESI–MS^n analysis of ginsenosides

We performed secondary metabolites profiling in *Penicillium* sp. YJM-2013 treatment group and control group using HPLC-ESI-MS^n. As shown in Table 4, a total of 11 compounds were identified via comparisons with authentic standards or data found in previous reports, including Rg_1, Re, Malonyl-Rg_1, Ro, Rf, Rb_1, Rg_2, Rc, Rb_2, Rb_3, Rd, and they all existed in control group and *Penicillium* sp. YJM-2013 treatment group. And the structures of 11 kinds of identified ginsenosides were shown in Table 5.

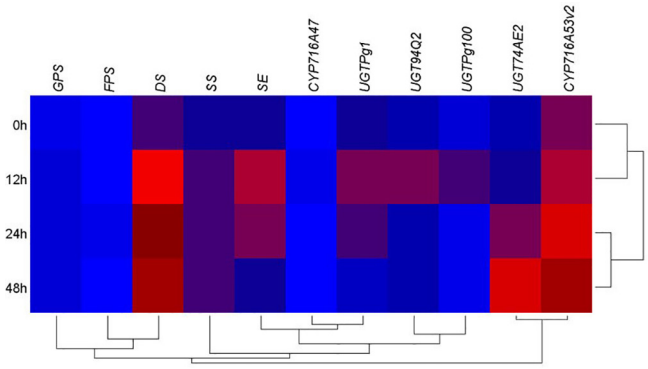


Fig. 5. Expression profile for genes involved in terpenoid biosynthesis and content of monomer ginsenoside in *P. ginseng* adventitious root elicited by *Penicillium* sp. YJM-2013 elicitor. Data represent as mean values ± SD of three replicates. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$.

4. Discussion

4.1. Effects of Penicillium sp. YJM-2013 and F. oxysporum A549 on ginsenosides accumulation

Fungal elicitors were added to the adventitious, hairy root or cell culture of *Panax* for the improvement of ginsenoside production. It is reported that treatment with the extract of pathogenic fungi *Alternaria panax* Whetz. and *Cylindrocarpon destructans* could induce the production of ginsenosides in adventitious roots culture of *P. quinquefolius* (Yu et al., 2016). However, few reports tried to compare the effects of pathogenic fungi and its antagonistic fungi on the production of secondary metabolites in plant tissue culture. This study found that the mycelium of *Penicillium* sp. YJM-2013 could increase total ginsenosides content by 2.59 times compared with control. Moreover, *Penicillium* sp. YJM-2013 had a rather significantly positive effect on the accumulation of PPD-type ginsenosides, which have drawn more and more people's attention for their better anti-tumor effect than PPT-type ginsenosides and more safe anti-depressant effects (Xu et al., 2010). It was the first time to use the antagonistic fungi as biotic elicitor to enhance ginsenosides accumulation, and this method provides a new way to substitute resources.

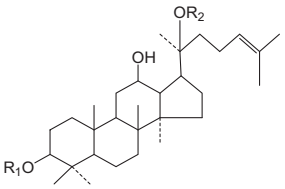
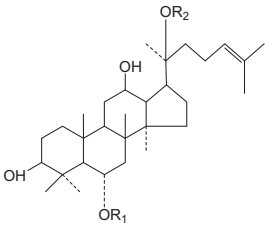
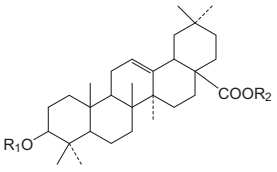
4.2. Effects of Penicillium sp. YJM-2013 on signal molecule generation

Fungal elicitors can be recognized by plant receptors located in plasma membrane or cytoplasm of plant cells, which will initiate a signal transduction network that activates plant defensive secondary metabolism. Ca^{2+} spiking, burst of ROS and NO are early physiological response of plant cells reacted to elicitors, and plays a core role in various signal transduction (Almagro, Bru, Pugin, & Pedreno, 2012). H_2O_2 was the main intermediate product in the metabolic reactions of elicitors inducing the accumulation of ginsenosides in *P. ginseng* (Hu, Neill, Cai, & Tang, 2010). Besides, ethylene was proved to be a phytohormone which involved in ginseng growth and development and mediated ginsenosides accumulation (Bae, Choi, Shin, Kim, & Kim, 2006). Moreover, ABA and PLA_2 were also involved in secondary metabolites induced by fungal elicitors, but studies on these were limited. In order to understand how signal molecules reacted to *Penicillium* sp. YJM-2013, contents of Ca^{2+}, H_2O_2, ABA, PLA_2, NO and ET in fresh root samples harvested at different time points were determined in this work. And present study showed that signal molecules of the elicitor-treated group were transiently expressed, which proved that Ca^{2+}, H_2O_2 ABA· PLA_2 NO· ET were upstream signal molecules that might contribute to gene activation involved in plant secondary metabolism for the enhanced production of ginsenosides in the culture of *P. ginseng* adventitious roots.

Table 4
HPLC-MS^n data of ginsenosides in *Penicillium* sp. YJM-2013 elicitor treated *P. ginseng* adventitious root.

No.	tR/min	Identification	MS/ (*m*/*z*)	ESI(-)MS^n	Distribution	Reference
1	36.45	Rg_1	800	835.3[M + Cl] $^-$	All	Standards
2	39.70	Re	946	981.4[M + Cl] $^-$	All	Standards
3	41.53	Malonyl-Rg_1	885	885[M − H] $^-$	All	(MacCrehan & White, 2013)
4	45.34	Ro	957	885[M − H] $^-$	All	Standards
5	52.12	Rf	801	835.3[M + Cl] $^-$	All	Standards
6	59.19	Rb_1	1108	1107.3[M − H] $^-$	All	Standards
7	60.45	Rg_2	784	819.4[M + Cl] $^-$	All	Standards
8	62.43	Rc	1078	1191.3[M-H + CH_3COONa] $^-$	All	Standards
9	65.82	Rb_2	1078	1191.4[M-H + CH_3COONa] $^-$	All	Standards
10	66.67	Rb_3	1078	1091.3[M-H + CH_3COONa] $^-$	All	Standards
11	69.21	Rd	946	981.3[M + Cl]	All	Standards

Table 5
Structures of 11 kinds of identified ginsenosides (Glc, β-*D*-glucose; Rha, α-rhamnose; Ara (p), α- *L*-arabinose (pyranose); Ara (f), α-*L*-arabinose (furanose); Xyl, β-*D*-xylose; GlcUA, β-*D*-glucuronic acid).

Chemical structures	Saponins	R_1	R_2
	Ginsenoside Rb_1	Glc(2–1)Glc	Glc(6–1)Glc
	Ginsenoside Rb_2	Glc(2–1)Glc	Glc(6–1)Ara(p)
	Ginsenoside Rb_3	Glc(2–1)Glc	Glc(6–1)Xyl
	Ginsenoside Rc	Glc(2–1)Glc	Glc(6–1)Ara(f)
	Ginsenoside Rd	Glc(2–1)Glc	Glc
	Ginsenoside Rg_1	Glc	Glc
	Ginsenoside Re	Glc(2–1)Rha	Glc
	Ginsenoside Rf	Glc(2–1)Glc	H
	Ginsenoside Rg_2	Glc(2–1)Rha	H
	Ginsenoside malonyl-Rg_1	Glc(6)Mal	Glc
	Ginsenoside Ro	GlcUA(2–1)Glc	Glc

4.3. Effects of Penicillium sp. YJM-2013 on PgWRKY1-9 expression

The activation of defense gene expression depends on the transcription factors, which can integrate multiple signaling pathways, activate functional genes and then lead the production of secondary metabolites (Aharoni & Galili, 2011). The expression of PgWRKY1-9 genes was obviously affected by MeJA, SA, ABA, and NaCl treatments (Nuruzzaman et al., 2016; Xiu et al., 2016). This study explored the obvious influence of fungal elicitors on the expression of the PgWRKY1-9, providing a comprehensive understanding of the WRKYs in *P. ginseng*. The information offered here was facilitated to explore the functions of these PgWRKYs on the production of ginsenosides.

4.4. Effects of Penicillium sp. YJM-2013 on ginsenosides biosynthetic genes expression

Fungal-induced signal transduction induce the synthesis of secondary metabolites probably by increasing the expression of genes involved in the synthesis of secondary metabolites (Du, Liang, Han, Yu, & Liang, 2015). In this study, the content of ginsenosides was changed at different time intervals, which is consistent with the expression levels of biosynthetic genes in ginsenosides biosynthesis pathway. The similar phenomenon was observed in other tissue culture *P. ginseng*, FPS, SS, SE, DS, CYP716A47 and CYP716A53v2 expression levels were significantly elevated by Tween 80 (Liang et al., 2015), MeJA (Lee et al., 2017), SA and yeast extract (Rahimi et al., 2014), resulting in ginsenosides accumulation. Studies of these regulators may elucidate ginsenoside biosynthesis mechanism and further make it possible to manipulate ginsenoside accumulation.

4.5. Effects of Penicillium sp. YJM-2013 on ginsenosides profile

Some studies have shown that induction treatment could increase the variety of secreted compounds as well as several types of phytochemicals. (Singh, 2016). Probably, it was because the existence of different elicitors would active different signaling pathways, speed up enzyme activities, thereby leading to synthesis of different components (Cheng, Yuan, & Graham, 2011). Our previous experiments also found that Rb_3 was only detected in the adventitious roots of *P. ginseng* treated with *Aspergillus niger* (Li et al., 2016). Therefore, the LC-MS^n technology was used to investigate the influence of *Penicillium* sp. YJM-2013 on ginsenoside profile. However, we found no significant differences in the composition of ginsenosides in the experiment. The content in the tissue culture can be further increased by methods of mixed induction or microbial embedding.

5. Conclusion

As biocontrol fungi, Penicillium sp. YJM-2013 was more conducive to the accumulation of ginsenosides in *P. ginseng* adventitious roots. Penicillium sp. YJM-2013 promoted the accumulation of ginsenosides by intensifying the generation of signal molecules, activating the expression of transcription factors and functional genes. This result is conducive to the industrial production of ginsenosides.

Declaration of Competing Interest

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

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