Original Article

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

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**A B S T R A C T**

**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 (48.75 ± 0.97 mg/g) treated with Penicillium sp. YJM-2013 at 200 mg/L higher than control by 2.59-fold, in which protopanaxadiol-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 more efficiently promoted the accumulation of ginsenosides by intensifying the generation of signal molecules, activating the expression of transcription factors and functional genes.

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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,
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 signaling networks 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), pythethrin (Khan et al., 2016), vindoline (Pandey et al., 2016), asiaticoside (Jisha, Gouri, Anith, & Sabu, 2018), gynnemic acid (Netala, Kotakadi, Gaddam, Ghosh, & Tartte, 2016), etc. However, few reports have described that antagonistic fungi or pathogenic fungi that cause plant disease are reported to be biocontrol fungi, refers to the various microorganisms that are beneficial to the prevention of plant diseases. It is reported that 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. However, few reports have described that antagonistic fungi or pathogenic fungi that cause plant disease are reported to be 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\(^{a}\) after fungal elicitor treatment.

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°32’ 35° E, 1972 m ± 3.00 m, 24°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 dual 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 kit, and 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. Secondly, 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 was used for the induction experiment. The elicitor concentration was determined by its carbohydrate content and the content was measured by Anthrone-sulfuric acid assay.
Penicillium sp. YJM-2013 in different time the expression level of transcription factors and functional genes

Rg1, Re, Ro, Rf, Rb1, Rg2, Rh1, Rc, Rb2, Rd, Rs2, Rb3 and Rh2, the expression levels were normed using

repeated for three times. And the primers of genes were shown in Table 1.

levels were normed using

l 70 min, 45% A; 70–100 min, 60% A. The injected volume was

35–40 min, 30% A; 40–50 min, 31% A; 50–60 min, 32% A; 60–

rate was 1.0 mL/min and the flow program was: 0–35 min, 20% A;

CN (A) and water (B), and the column was kept at 35

method) and Nitric Oxide assay kit (Nitrate reductase method)

Calcium Assay Kit, Hydrogen Peroxide assay kit (colorimetric

To test Ca2+, H2O2, ABA, PLA2, NO, ABA and ET, adventitious

roots were harvested. The fresh weight and ginsenoside contents were expressed as

2.9. HPLC-ESI-MSn

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-
nomic 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$_2$O$_2$, ABA, PLA$_2$, NO and ET in *P. ginseng* adventitious roots.

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**Table 2**

| 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 ± 0.566c   | 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 = Rb1 + Rb2 + Rb3 + Rg3 + Rg4; Rg group = Re + Rg1 + Rg2 + Rf + Rh2. 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**

| 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 ± 0.05a   | 9.41 ± 0.51b                        | 11.78 ± 0.57b | 10.30 ± 0.35a | 31.50 ± 1.48b | |

Note: Rb group = Rb1 + Rb2 + Rb3 + Rg + Rg3 + Rg4; Rg group = Re + Rg1 + Rg2 + Rf + Rh2. Mean values followed by the same letters within a column are not significantly different according to Duncan’s multiple range test at 5% level.
As shown in Fig. 3A, Ca²⁺ (8.58 ± 0.37 mmol/g prot) generated immediately and reached its peak value at 20 min, H₂O₂ (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,
PgwRK9Y reached the maximum at 12 h, however, PgwRK2, and PgwRK3 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 UGTp100, reached the highest at 48 h, 12 h, 12 h differently, and they consisted in the production of monomer ginsenoside Rh2, Rg3 and Rh1.

3.6. HPLC-ESI–MSn analysis of ginsenosides

We performed secondary metabolites profiling in Penicillium sp. YJM-2013 treatment group and control group using HPLC-ESI–MSn. As shown in Table 4, a total of 11 compounds were identified via comparisons with authentic standards or data found in previous reports, including Rg1, Re, Malonyl-Rg1, Ro, Rf, Rb1, Rg2, Rc, Rb2, Rb3, 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 ≤ 0.05.](image)

### Table 4

HPLC-MSn data of ginsenosides in Penicillium sp. YJM-2013 elicitor treated P. ginseng adventitious root.

| No. | tR/min | Identification | MS/ (m/z) | ES- ESn | Distribution | Reference |
|-----|--------|----------------|-----------|----------|--------------|-----------|
| 1   | 36.45  | Rg1            | 800       | 835.3[M + Cl] | All          | Standards |
| 2   | 39.70  | Re             | 946       | 981.4[M + Cl] | All          | Standards |
| 3   | 41.53  | Malonyl-Rg1    | 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  | Rb1            | 1108      | 1107.3[M – H] | All          | Standards |
| 7   | 60.45  | Rg2            | 784       | 819.4[M + Cl] | All          | Standards |
| 8   | 62.43  | Rc             | 1078      | 1191.3[M-H + CH3COONa] | All          | Standards |
| 9   | 63.82  | Rb2            | 1078      | 1191.4[M-H + CH3COONa] | All          | Standards |
| 10  | 66.67  | Rb3            | 1078      | 1091.3[M-H + CH3COONa] | All          | Standards |
| 11  | 69.21  | Rd             | 946       | 981.3[M + Cl] | All          | Standards |
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 (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.

4.5. 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 Rb3 was only detected in the adventitious roots of P. ginseng treated with Aspergillus niger (Li et al., 2016). Therefore, the LC-MSⁿ 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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