A stable beneficial symbiotic relationship between endophytic fungus

Schizophyllum commune* and host plant *Panax ginseng

Xin Zhai^{1#}, Ling Chen^{1#}, Min Jia¹, Changhui Li², Hui Shen¹, Bingzhu Ye¹, Luping Qin^{1,3*}, Ting Han^{1*}

¹ Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, China

² Department of Pharmacognosy, School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, China

³ School of Pharmacy, Zhejiang Chinese Medical University, Hangzhou 310053, China

[#] These authors contributed equally to this manuscript.

**Corresponding author:*

E-mail addresses: than927@163.com (T. Han); Tel/fax: +86 21 81871306

Co-corresponding author:

E-mail address: ginsmmu@126.com (L.-P Qin); Tel/fax: +86 21 81871309

Abstract: Endophytes and plants can establish specific long-term symbiosis through the accumulation of secondary metabolites. Interactions between microbial inhabitants represent a novel area of study for natural products research. In this study, a strain of endophyte 3R-2 that can enhance the biomass and contents of ginsenoside Rc, ginsenoside Rg2 and ginsenoside Rg3 of *Panax ginseng* hairy roots was screened out via HPLC, which was identified as *Schizophyllum commune* through the morphological and molecular identification. On the base, we found the infection of the endophyte were obviously observed widely in the *P. ginseng* and the strain formed a stable relationship with *P. ginseng* hairy roots in parenchyma cells around through tissues embedding slicing, HE ammonium silver staining and immunofluorescence staining. On the other hand, elicitors of fungus 3R-2 can also significantly promote hairy root growth and contents of several ginsenosides, even several times higher than

3R-2 mycelium did. Moreover, *S. commune* 3R-2 mycelium and its elicitor could enhance the transcriptional activity of key genes during the ginsenosides biosynthetic pathway dramatically. Thus, endophyte *S. commune* 3R-2 and its elicitor change the chemical substance content by regulating the expression of genes involved in the secondary metabolite biosynthetic pathway.

Key words: *Schizophyllum commune*; endophytic fungus, infection way; expression of genes; secondary metabolite biosynthetic

Introduction

Panax ginseng (Araliaceae) is a traditional oriental herb that has been used to treat various diseases in East Asian countries (Mizikar 2011). *P. ginseng* has aroused great interest of researchers as a result of its different pharmacological and therapeutic effects on central nervous system, cardiovascular system and immunomodulation function (Hofseth and Wargovich 2007, Chen et al. 2008). The root of *P. ginseng* plays an important role in traditional medicine and its main component is considered to be a triterpenoid saponin of dammarane-type (Wang et al. 2001, Yue et al. 2007). As aglycones of dammarane-type triterpenes, protopanaxadiols (PPD) and protopanaxatriols (PPT) are the main part of these triterpene compounds in *P. ginseng*. As reported, Ginsenosides-Rg3, -Rd, -Rc, -Rb1, and -Rb2 were typical PPDs. The sugar moieties are connected to the ring of the triterpene dammarane at the three-position in PPD while attached at the six-position in PPT such as ginsenosides-Rg1, -Re, and -Rg2 (Sun et al. 2007). As the main PPD ginsenosides, Ginsenoside Rg2 could reduce memory impairment via anti-apoptosis (Zhang et al. 2008) and Ginsenoside Rc exhibited highest inhibitory activity against the expression of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and interferons (IFNs). (Jung et al. 2001, Choi et al. 2003). However, they are all present at low concentrations in ginseng, which is a trouble for pharmacology application (Kim et al. 2003).

Plants form various kinds of relationships with all kinds of microbes, such as mutually-beneficial symbiosis with endogenous or against pathogens in nature. Endogenous fungi are microorganisms that live within the plant's internal tissue and do not cause any direct or obvious negative effects (Rodriguez et al. 2009). Indeed, some endophytes, such as *Piriformospora indica*, *Trichoderma atroviride* or other

60 growth promoting endophytes can do good to plants(Ming et al. 2013). In addition,
 61 long-term colonization can lead to the accumulation of various secondary metabolites
 62 in the host(Ludwig-Müller 2015). Schulz et al. believe that the counterbalanced
 63 antagonism hypothesis can account for the relation between plants and endophytes
 64 (Schulz et al. 1999). As reported, secondary metabolites produced by host plants are
 65 likely to be the key to maintaining a balance between endophytes and host plants.
 66 Meanwhile, endogenous fungi are considered to be a powerful means to stimulate
 67 plant secondary metabolites for human use. What's more, the effect of endogenous
 68 fungi on secondary metabolism of plants will help to target drugs through
 69 bioengineering(Ludwig-Müller 2015).

70 *Agrobacterium rhizogenes*-mediated transformation of hairy roots, as a quick and easy
 71 method for introducing and expressing foreign genes can be used to synthesize
 72 specific secondary metabolites in plant cells, thus providing a quick and easy method.
 73 This method not only has a higher security level, a higher growth rate, frequent
 74 branch, genetic and biochemical stability, but also improve the cosmetics, medicines
 75 and food additives content of secondary metabolites(Schulz et al. 1999). Since the
 76 growth rate of *P.ginseng* is slow, the vitro culture system was selected to obtain the
 77 indeterminate root. The hairy root culture of *P.ginseng* has become a useful platform
 78 for the research of metabolic engineering, which can replace the whole
 79 plant(Yoshikawa and Furuya 1987, Srivastava and Srivastava 2007). The present
 80 research took advantage of the platform of *P. ginseng* hairy root to study the
 81 endophyte infection ways and the function of endophytic fungus 3R-2 on *P. ginseng*,
 82 which indicated 3R-2 was a beneficial effective endophyte.

83 In the previous study, our research group separated the endophyte *Trichoderma*
 84 *atroviride* D16 from *Salvi miltiorrhiza* and found it could promote the tanshinones
 85 content obviously in access of various chemical elicitors, which gained wide
 86 attention(Ming et al. 2013). Endophytic fungus opened up a new direction for the
 87 cultivation and active substances obtaining from medical plants. As we all know, the
 88 cultivation puzzle of *Panax ginseng* (Araliaceae) and the high demand, low output of
 89 Ginsenoside Rg2, Rg3, Rc had evolved into a large restriction for pharmaceutical
 90 development. Therefore, our group isolated dozens of endopytic fungus strains and
 91 obtained an effective fungus 3R-2, which was capable of leading to an enhancement
 92 of root growth and several important ginsenosides accumulation in *P. ginseng* hairy

roots. To the authors' knowledge, there have been no reports about the effects of ginseng plant-derived endophytic fungi on the secondary metabolism of *P.ginseng*. In this study, the identification of this endophytic fungus, as well as its infection way and effects on the root growth and bio-synthesis of ginsenosides in *P. ginseng* were researched for understanding the role of endophytes in host plant survival.

Materials and methods

Materials and media

Fresh roots of *Panax ginseng* C. A. Mey. were collected from the city of Tonghua (JilingProvince), People'sRepublicof China. The roots were obtained from the soil and then transported to the School of Pharmacy, Second Military Medical University, Shanghai, China.

The *Panax ginseng* hairy roots used in this work were donated by School of Life Sciences in Central South University and cultured in 250 ml conical flasks including 100 ml of liquid half-strength MS medium in an orbital shaker at 25 °C and 135 rpm. The hairy roots of 1.0 g fresh weightwas culturedinevery flask for 3weeks. The furry roots were harvested in a few weeks, washed three times with distilled water, dried with paper towels, and then dried in the oven for 50°C until a constant dry weight (DW) was observed.

The reference standards of ginsenoside Rc、Rb₁、Rb₂、Re、Rd、Rg₁、Rg₂、Rg₃ and Rh₂ used were from Chengdu Mansite Pharmaceutical CO. LTD., Chengdu (Sichuan Province), People's Republic of China.

Potato dextrose agar (PDA) medium included 200 g potato, 20 g dglucose, 15 g agar, 1000 ml deionized water and was used to isolate and culture endophytic fungi. Whileas, 1/2MS medium contained 2.215 g MS powder, sucrose 30 g, 1000 ml deionized water and the PH was 6.0, which was applied to theculture of *Panax ginseng* hairy roots. Both types of media were sterilized for 30 min.

Isolation and culture of endophytic fungi

The roots of *Panax ginseng* C. A. Mey. were washedthoroughly in the tap water

123 and then the soil attaching to the root was removed with the ionized (DI) water,
 124 followed by being cut into small 15 mm- long root section with a disinfected scissors.
 125 Subsequently, those segments were surface-sterilized by using continuous immersion
 126 of 75% ethanol immersion for 30 s, sodium hypochlorite (3% available chlorine) for 3
 127 min, 75% ethanol for 30s and finally rinsed three times in disinfectant water. After the
 128 excess water is daubed on a steroidal filter paper, the dried segments were cut into
 129 small pieces about 2mm thickness and placed evenly in a petri dish containing
 130 100mg·l⁻¹ penicillin to eliminate bacterial growth. After the petri dishes were sealed
 131 with a Parafilm (Pechiney, Chicago, IL), they were cultured at 26±2 °C in the
 132 incubator until fungus growth was obvious. From the root segment, the hyphae was
 133 isolated after two or three weeks and transferred to new pure PDA. Each fungus was
 134 added to the liquid half-strength MS medium, which cultured three-week-old hairy
 135 root while the blank treatment was added with no hyphae to the fresh liquid
 136 semi-strength MS medium. In the final, the roots were collected at different intervals
 137 separately at 0, 3, 6, 9, 12, 15, and 18d.

138

139 *Identification of endophytic fungus 3R-2*

140 Endogenous fungi 3R-2 grew on the PDA medium for 7 days at 28 °C and
 141 photographed its morphological characteristics. The DNA of mycelium is extracted
 142 using CTAB method. According to the previous report (Ming et al. 2013), the sequence
 143 of endogenous fungi was determined according to the sequence of its 5.8 S and
 144 ITS with primer ITS 4 and ITS 5, which were compared with GenBank reference
 145 sequence. Finally, we utilized CLUSTAL X software to construct phylogenetic tree by
 146 1000 bootstraps.

147 *HPLC analyses*

148 The hairy roots were dried at 50 °C in an oven until dry weight (DW) was
 149 constant. Then these samples were ground into powder and extracted with methanol
 150 (15 mg of roots ml⁻¹) under sonication for 60 min. With the help of high-performance
 151 liquid chromatography (HPLC) system, secondary metabolites of the extract were
 152 analyzed on an Agilent-1100 system using a ZORBAX SB-C18 chromatographic
 153 column (250 mm×4.6 mm, 5 μm) at 35 °C with elution
 154 of [time(min):D(HCN)]=[0.0:19%]-[35.0:19%]-[55.0:29%]-[70.0:29%]-[100.0:40%]-[

155 120.0:85%]. Ginsenosides of Rc, Rb1, Rb2, Re, Rd, Rg1, Rg2, Rg3 and Rh2 in the
156 methanol extract were identified compared with the available standards [Chengdu
157 Mansite Pharmaceutical Co. Ltd., Chengdu (Sichuan Province), PR China].

158

159 *Paraffin embedding and slicing of hairy root tissues*

160 The fresh tissues were fixed in 4% paraformaldehyde for more than 24h. After
161 that, these tissues were removed from the fixed liquid, pruned with scissors in the
162 fuming hood and put into the dehydration box. Then the dehydration box was placed
163 in the basket in the gradient alcohol successively for dehydration: 75% alcohol for 4h,
164 85% alcohol for 2h, 90% alcohol for 2h, 95% alcohol for 1 h, anhydrous ethanol I for
165 30 min, anhydrous ethanol II for 30 min, alcohol benzene for 5-10 min, xylene I for
166 5-10 min, xylene II for 5-10 min, wax I for 1h, wax II for 1h and wax III for 1h. The
167 immersed tissues were embeded in the melting wax on the embedding machine before
168 it solidified. After cooling at -20 degree centigrade, wax coagulation was removed
169 from embedding box and dressed. And the dressed wax block was cut into slices in
170 paraffin wax slicing machine with 4 µm thickness, which were floated and spread on
171 warm water at 40 degree centigrade, dried at 60 degree centigrade in oven and
172 preserved under normal temperature.

173

174 *Ammonium silver staining and Immunofluorescence staining*

175 The sections were put into xylene I for 20 min, xylene II for 20 min, anhydrous
176 ethanol I for 10 min, anhydrous ethanol II for 10 min, 95% alcohol for 5 min, 90%
177 alcohol for 5 min, 80% alcohol for 5 min, 70% alcohol for 5 min in turn and washed
178 in distilled water. The work preparation was blended of ammonium silver stock
179 solution (20 ml), distilled water (15 ml) and 5% sodium tetraborate (2 ml)
180 successively and preheated at the 60 degree centigrade in the oven. Meanwhile, these
181 sections were placed into 1% periodic acid 15 to 20 min for oxidation. Afterwards,
182 they were rinsed with tap water for 5 min, washed with distilled water twice, circled
183 with resistance pen, added with preheating work preparation and sealed in the wet
184 box. After 40 minutes, the staining sections could be observed on the microscope until
185 satisfied result of black fungal was obtained. Subsequently, they were treated with 5%

186 sodium thiosulfate for 2 min, rinsed in tap water for 5 min, re-stained with eosin for 5
187 min, dehydrated through successive immersion of 95% alcohol I for 5 min, 95%
188 ethanol II for 5 min, anhydrous ethanol II for 5 min, anhydrous ethanol II for 5 min,
189 xylene I for 5 min, xylene II for 5 min, dried and sealed with neutral balsam for image
190 collection and analysis under microscope.

191 The sections were put into xylene I for 15 min, xylene II for 15 min, anhydrous ethanol
192 I for 5 min, anhydrous ethanol II for 5 min, 85% alcohol for 5 min and 75% alcohol
193 for 5 min and washed with distilled water. Antigen retrieval of the tissue sections were
194 performed in boiling EDTA buffer (PH 8.0) for 5 min in the microwave. After natural
195 cooling, the slides were washed three times (5 min each time) in PBS (PH 7.4) at the
196 decoloring shaking bed. Then the target organization was circled with resistance pen,
197 covered uniformly with 3% BSA and sealed for 30 min at room
198 temperature. Afterwards, the sections were dipped with primary antibody of certain
199 proportion and incubated at 4 degree centigrade in wet box overnight. Then the slides
200 were washed three times (5 min each time) in PBS (PH 7.4) at the decoloring shaking
201 bed. After dried slightly, the sections were covered with secondary antibody of a
202 corresponding species with primary antibody and incubated in dark at room
203 temperature for 50 min. Then the slides were washed three times (5 min each time) in
204 PBS (PH 7.4) at the decoloring shaking bed. After dried slightly, the sections were
205 added with DAPI staining solution and incubated in dark for at room temperature 10
206 min. Then the slides were washed three times (5 min each time) in PBS (PH 7.4) at the
207 decoloring shaking bed. After dried slightly, the sections were sealed off with
208 anti-fluorescence-quenching agent for image collection under nikon inverted
209 fluorescence microscope.

210

211 *RNA isolation and real-time quantitative PCR analysis*

212 3R-2 hyphae was added into the liquid half-strength MS medium with 3-week-old
213 cultured hairy roots at a concentration of 10 µl while controls were treated with fresh
214 liquid half-strength MS medium. Hairy roots were collected at different intervals (0, 3,
215 6, 9, 12, 15 and 18 d) and then stored at -80 °C. With RNA Kit II (Genebase Bioscience
216 Co., Ltd.), total RNA was extracted from *P. ginseng* hairy root samples. The quality

217 and concentration of RNA were examined through ethidium bromide-stained agarose
218 gel electrophoresis and spectrophotometric analysis.

219 Total RNA was reverse transcribed by using the RevertAid™ First Strand cDNA
220 Synthesis Kit (Fermentas) to generate cDNA according to the manufacturer's
221 instruction. The gene expressions of *pgHMGR*, *pgSS*, *pgSE*, and *pgSD* were detected,
222 respectively. The realtime PCR amplification was performed in a Lin-Gene
223 FQD-33A detection system (Bioer) with UltraSYBR Mixture kit (CW BIO). Each
224 reaction included a mixture of 5 µl of SYBR Green I PCR Master Mix (ShineGene,
225 China), 0.2 µl of forward primer (10 µM), 0.2 µl of reverse primer (10 µM), 1 µl
226 of diluted cDNA and 3.6 µl of RNase-free H₂O. The reaction mixture was incubated
227 for 10 min at 94°C, and for 40 cycles of 20s at 94°C, 15s at 60°C and 20s at 72°C. The
228 relative gene expression was quantified by the comparative CT method.

229

230 *Data analysis*

231 All experiments were analysed as their mean values and standard deviations
232 (SD) in triplicate, including both control and different treatments of hairy root cultures,
233 HPLC analysis, and semi-quantitative real-time PCR. The standard deviation was
234 represented with the error bars in biological triplicates and the statistical significance
235 of the differences was analysed by one-way analysis of variance (ANOVA) with
236 SPASS software. Nevertheless, the statistical significance of differences in gene
237 transcripts was analysed by one-sample t-test.

238

239 **Results**

240 *Identification of fungal strain 3R-2*

241 In the PDA medium, there is no sporophore and the colony is white, fluffy and the
242 back of the colony is white too (Figure 3). Based on 3R-2 morphological
243 characteristics and molecular analysis of ITS rDNA (ITS 1, ITS2, and 5.8 S rRNA
244 genes), the fungus had been identified as *Schizophyllum commune*. The acquired
245 ITS-5.8 S rDNA sequence has been deposited as accession number KU042974 in
246 GeneBank. With the neighbor-joining method, we carry on phylogenetic tree after

1000 bootstrap and identification at the species classification level is based on $\geq 100\%$ similarity (Figure 4). In addition, the plant endogenous fungi have been collected and deposited in the Chinese General Culture Collection Center (CGMCC) in Beijing, China as 11009 (Figure 3). As we know, there is no report that *Schizophyllum commune* was able to increase plant growth and active substances.

The observation of 3R-2 mycelia in P. ginseng hairy roots

The existence of endophytes and the morphology of endophytes were obviously observed in *P. ginseng* hairy roots through *P. ginseng* tissues embedding slicing, HE ammonium silver staining and immunofluorescence staining. Compared with 4R-2 strain, more 3R-2 mycelia inoculated in *P. ginseng* hairy root and 4R-4 had the same inoculation capacity with 3R-2, whereas 4R-4 constructed hyphae web throughout root cells (Fig. 6). HE ammonium silver staining illustrated the main infection sites were found in parenchyma cells around (Fig. 7). HE ammonium silver staining represented the infection stable state. Significantly, intracellular hyphae remained staying in parenchyma cells and enveloped by host cell membrane, which was for longer periods not just for infection. The means of endophytic fungi infection on plant tissues may provide some experimental basis for the future research.

Effects of 3R-2 mycelium on the biomass and ginsenoside contents of P. ginseng hairy roots

Eighty three endophytic fungal strains were isolated from the roots of *Panax ginseng* on the basis of morphology. A strain of endophyte 3R-2 that could increase the contents of ginsenoside Rc and ginsenoside Rg₂ was screened out by using HPLC. Compared with other endophytic fungi, 3R-2 has exceptional effect on the growth and bioactive substances accumulation of *P. ginseng* hairy roots (Fig. 1). Endophyte 3R-2 can promote biomass of *P. ginseng* hairy roots by 16% and the content of Rg₂ and Rc were 11.74 fold and 2.75 fold separately under the treatment of 3R-2 in comparison with control group, which indicated 3R-2 was an effective beneficial endophyte for *P. ginseng*.

277 The endophyte 3R-2 hyphae was added into of *P. ginseng* hairy root cultures by
 278 means of punching, the effects of endophyte 3R-2 hyphae on the growth and
 279 secondary metabolic biosynthesis of *P. ginseng* hairy roots were studied. Results show
 280 that the endophyte 3R-2 has no obvious effects on the biomass and contents of
 281 ginsenoside Rc and ginsenoside Rg₂ of *P. ginseng* hairy roots within seven days,
 282 whereas it significantly promoted the biomass and contents of ginsenoside Rc,
 283 ginsenoside Rg₂ and ginsenoside Rg₃ of *P. ginseng* hairy roots within 14-21 days; the
 284 content of ginsenoside Rc reached the highest in 14th days after endophyte 3R-2
 285 hyphae co-culture with *P. ginseng* hairy roots (Fig. 2).

286

287 *Effects of Schizophyllum commune on the expression of genes in the ginsenoside*
 288 *biosynthetic pathway in P. ginseng hairy roots*

289 The RT-PCR of four key enzyme genes HMGR, SS, SE and DS in the secondary
 290 metabolic pathways of ginsenosides was carried out, and the results showed that key
 291 enzyme genes SS of the 3R-2 mycelium group is significantly higher than the control
 292 group and reached the highest in 6 days, which promoted the expression of SS by 3.86
 293 fold in 6 days. From 12th to 18th day, 3R-2 mycelium had an obvious effect on
 294 increasing the expression of HMGR and DS (Fig. 7A,D). And the expression of SE
 295 was increased from 9th to 18th day (Fig. 7C), whereas 3R-2 mycelium cause a
 296 distinct boost on the expression of SS from 15th to 18th day (Fig. 7B). While the key
 297 enzyme genes HMGR, SE and DS of the 3R-2 elicitor groups reached its highest in 15
 298 days and were 90 times, 20 times and 120 times higher than control group,
 299 respectively (Fig. 7). The elicitor of 3R-2 promoted the expression of HMGR in the
 300 late days from 15th to 18th day. Nevertheless, 3R-2 elicitor had a tremendously greater
 301 increase on the expression of HMGR, SS, SE, DS by tens times.

302

303 Discussion

304 Ginseng has been known for thousands of years in the Far East as a precious
 305 medicinal herb. In recent years it has attracted interest in western countries. It was
 306 widely used and extracted for pharmacological and therapeutic usage, and now
 307 restricted by low output of *P. ginseng* root and low content of ginsenosides. In this

study, endophytic fungi from *P. ginseng* were chosen as research objects to study the relationship with the host plant, aiming to promote ginseng growth.. We adopted the *P. ginseng* hairy root system as the research platform and screened out an endophyte 3R-2, which could promote the growth of host plants and accumulate the effective ginsenosides contents in *P. ginseng* hairy roots. Through the morphological and molecular identification, the target endophyte was identified as *Schizophyllum commune*. There are some reports about endophyte *Schizophyllum commune*, which is a common "miscellaneous fungus" all around the world, especially in tropical and subtropical miscellaneous tree forests. *Schizophyllum commune* was an vigorous strain and mycelium extracts contain active substances, exhibiting obvious inhibitory effects on *Staphylococcus aureus*, *Escherichia coli*, *Dysentery bacilli*, *Bacillus subtilis* and *Salmonella paratyphi B* as reported(Wang et al. 2001). Endophyte *Schizophyllum commune* not only promoted the growth of *P. ginseng* hairy root but also increased contents of ginsenoside Rc and ginsenoside Rg2, which indicated *Schizophyllum commune* 3R-2 was an efficient endophytic fungus for *P.ginseng*, and the active substance of 3R-2 could be a great study point (Kei et al. 2016, Jaber and Enkerli 2017).

Through tissues embedding slicing, HE ammonium silver staining and immunofluorescence staining, we found the endophytes were obviously observed in fresh roots, stems, leaves and fruits of cultivated *P. ginseng* . The mycelium in leaves and fruits of *P. ginseng* observed under the microscope is the most obvious, while endophytic fungi were distributed in root and stem sparsely (Fig.5). As researched, the hyphae should break through the root periderm from soil at the first time and then enter into the epidermal cells, which represented the interaction between mycelia and live plant cell(Kei et al. 2016). Subsequently, endophyte may transfer and spread through vascular bundles to stems, leaves and fruits (Sesma and Osbourn 2004). As revealed, the environment of fruits and leaves was more favorable to endophytic fungi survival. After that, the infection situations of several strains important endophytic fungi in *P. ginseng* hairy root tissues were observed by using the same method (Fig.6). Compared with 4R-2 strain, more 3R-2 mycelia inoculated in *P. ginseng* hairy root, which indicated that 3R-2 was more inclined to colonize the root and develop a biotrophic relationship with the *P.ginseng* (Fig. 6). Obviously, 4R-4 had the same inoculation capacity with 3R-2, whereas 4R-4 constructed hyphae web throughout root cells(Fig. 6). The 4R-4 manifested obvious aggressiveness than 3R-2 and did not

enhance the content of ginsenoside Rc, Rg2 and Rg3 (Fig. 1), which illustrated reciprocal stable colonization state established on less offense and more reside. To study the inoculation means, HE ammonium silver staining was applied into 3R-2 colonization and it signified that parenchyma cells was the main resident interactional place (Fig. 7). Intracellular hyphae lived in parenchyma cells and was enveloped by host cell membrane for longer periods (Bonfante and Genre 2010). As author's knowledge, it was the first time to investigate the infection way in *P. ginseng*.

In our following study, the biomass of *P. ginseng* hairy roots were tested and it was promoted by endophyte 3R-2 greatly. While most studies on the interactions between plants and endophytic fungal have so far focused on the benefits of such interactions to host plants through increased tolerance and resistance to diseases, only a handful of studies to date have investigated the potential role of endophytic fungal as plant growth promoters(Elsharkawy et al. 2012). Exploring the full potential of interactions between plants and fungal could facilitate a more effective use of these fungi for biocontrol strategies, which could present possible explanations for the lack of consistency in the plant growth promotion obtained by the inoculation of fungi(Chandanie et al. 2009). On the 21th day after inoculation of 3R-2, the biomass of *P. ginseng* hairy roots was increased by 1.6 fold, which has revealed its potential for application as a plant growth-promoting mycorrhizal fungus for realizing the targeted improvement in the production of medical plants *P. ginseng*. According to the reports, endophytic fungi ameliorated the plant growth by secreting gibberellins(GA), indole 3-acetic acid (IAA) (Abdul et al. 2012) and other signalling molecules generally (Straub et al. 2013)(Cavalcante et al. 2007) or increased the photosynthesis efficiency (Harman 2011). It was considerably difficult for endophytic fungi to form plant-fungus symbiosis(Rodriguez and Redman 2008), let alone colonize in the root for a long period to increase the growth of plant(Bae et al. 2009). Only highly effective strains can open the entrance door to initiate colonization with the invertase key (Vargas et al. 2009) and interact with the host through sucrose-independent network and chemical communicants releasing (Shoresh et al. 2010, Vargas et al. 2011). *P. ginseng* was hard to build symbiotic association with fungi, which illustrated *Schizophyllum commune* 3R-2 was a highly efficient strain for *P. ginseng*. As to the growth-promoting mechanism, it can be researched further in the next experiment.

And the change of the active ingredients in *P. ginseng* hairy roots were tested by

high performance liquid chromatography technique during the whole coculturing period. The contents of ginsenoside Rc, ginsenoside Rg₂ and ginsenoside Rg₃ of *P. ginseng* hairy roots were greatly enhanced within 14-21 days and the content of ginsenoside Rc reached the highest in 14th days after endophyte 3R-2 hyphae co-culture with *P. ginseng* hairy roots. When 3R-2 was inocubed into *P.ginseng* hairy root, the hyphae may have to adapt to the cultivated condition through several days. After adaptation phase, 3R-2 gradually interacted with *P.ginseng* hairy root and form a beneficial mutualistic relationship. The ginsenosides may be one of interaction factors and metabolic flow was strengthen in order to reach a balanced system(Berg et al. 2016). Finally, the change of key enzyme genes in ginsenosides metabolic pathways were also tested by RT-RCR techniques. We expect to provide some experimental basis and scientific evidence to reveal how endophytic fungi affect quality of Chinese medicinal plants *P. ginseng* in the preliminary study(Ghaffari et al. 2016). Ginsenosides belong to triterpenoid compounds and of the biosynthetic pathways of them have been preliminary studied, which can be divided into three stages: (1) The biosynthesis of IPP and DMAPP; (2) Biosynthesis of 2, 3-oxidosqualene; (3) oxidation of oxidosqualene and modification of the complex functional groups. In order to further analysis the influence of 3R-2 mycelium on the ginsenosides biosynthesis, we selected the four key enzymes genes HMGR, SS, SE and DS of ginsenosides biosynthetic pathways in RT-PCR studies. These key enzyme genes expressions of ginseng hairy roots were quantitatively analyzed when cocultured with endophyte 3R-2 in 0, 3, 6, 9, 12, 15 and 18 days. In this study, the results were shown in fig. 6. Strain of 3R-2 significantly promoted the expression of gene SS in the 6th days, and significantly promoted the expressions of HMGR, SE and DS in the 15th days. The strain of 3R-2 promoted the expressions of these key enzymes, which were consistent with the results of HPLC. On the other hand, in order to remove the virulence of 3R-2, we investigated the activity of the fungi elicitors, and the effect was several times higher than 3R-2 mycelium.

Similarly, we also obtained the same results in metabonomics studies. A large number of compounds were found greatly increased except the several increased ginsenosides in HPLC test(Sun et al. 2009). Next step is to identify these compounds and establish the compound libraries for the purpose of providing the basis for the production and application in the future. As studied, the results suggested that

408 *Schizophyllum commune* could cause chemical defence responses in the host when
 409 confronting pathogens(Tenkanen and Siikaaho 2000, Tan and Zou 2001). This
 410 endophyte changed the content of chemical composition in the host plant by affecting
 411 the expression of genes related to the secondary metabolite biosynthesis pathway. As
 412 a result, the addition of *Schizophyllum commune* can be considered as an effective
 413 approach for the large-scale production of ginsenosides in *P. ginseng* hairy root
 414 culture systems or exploit 3R-2 function to manufacture into biotic fertilizer for high
 415 production and superior quality (Tkacz and Poole 2015). However, the active
 416 substances responsible for the stable relationship between *Schizophyllum commune*
 417 and *P. ginseng* still keeps unclear and the reason of promoting hairy root growth and
 418 stimulating the biosynthesis of ginsenosides in the hairy root culture by
 419 *Schizophyllum commune* will be clarified in future studies.

420

421 Reference

- 422 **Abdul K., Muhammad H., Kang S. M., Yoon-Ha K., Hee-Young J., Joong-Hwan L. and**
 423 **In-Jung L.** (2012) Endophytic fungal association via gibberellins and indole acetic acid can
 424 improve plant growth under abiotic stress: an example of *Paecilomyces formosus* LHL10. *Bmc*
 425 *Microbiology* **12**: 3-10.
- 426 **Bae H., Sicher R. C., Kim M. S., Kim S. H., Strem M. D., Melnick R. L. and Bailey B. A.**
 427 (2009) The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and
 428 delays the onset of the drought response in *Theobroma cacao*. *Journal of Experimental Botany* **60**:
 429 3279-3295.
- 430 **Berg G., Rybakova D., Grube M. and Köberl M.** (2016) The plant microbiome explored:
 431 implications for experimental botany. *Journal of Experimental Botany* **67**: 995.
- 432 **Bonfante P. and Genre A.** (2010) Mechanisms underlying beneficial plant fungus interactions in
 433 mycorrhizal symbiosis. . *Nat.Comm.* **1**: 48-54.
- 434 **Cavalcante J. J. V., Vargas C., Nogueira E. M., Vinagre F., Schwarcz K., Baldani J. L.,**
 435 **Ferreira P. C. G. and Hemerly A. S.** (2007) Members of the ethylene signalling pathway are
 436 regulated in sugarcane during the association with nitrogen-fixing endophytic bacteria. *Journal of*
 437 *Experimental Botany* **58**: 673-682.

- 438 **Chandanie W. A., Kubota M. and Hyakumachi M.** (2009) Interactions between the arbuscular
439 mycorrhizal fungus *Glomus mosseae* and plant growth-promoting fungi and their significance for
440 enhancing plant growth and suppressing damping-off of cucumber (*Cucumis sativus* L.). *Applied*
441 *Soil Ecology* **41**: 336-341.
- 442 **Chen C. F., Chiou W. F. and Zhang J. T.** (2008) Comparison of the pharmacological effects of
443 *Panax ginseng* and *Panax quinquefolium*. *Acta Pharmacologica Sinica* **29**: 1103-1108.
- 444 **Choi S. S., Han E. J., Han K. J., Lee H. K. and Suh H. W.** (2003) Antinociceptive effects of
445 ginsenosides injected intracerebroventricularly or intrathecally in substance P-induced pain model.
446 *Planta Medica* **69**: 1001-1021.
- 447 **Elsharkawy M. M., Shimizu M., Takahashi H. and Hyakumachi M.** (2012) The plant
448 growth-promoting fungus *Fusarium equiseti* and the arbuscular mycorrhizal fungus *Glomus*
449 *mosseae* induce systemic resistance against *Cucumber mosaic virus* in cucumber plants. *Plant &*
450 *Soil* **361**: 397-409.
- 451 **Ghaffari M. R., Ghabooli M., Khatabi B., Hajirezaei M. R., Schweizer P. and Salekdeh G. H.**
452 (2016) Metabolic and transcriptional response of central metabolism affected by root endophytic
453 fungus *Piriformospora indica* under salinity in barley. *Plant Molecular Biology* **90**: 699-717.
- 454 **Harman G. E.** (2011) Multifunctional fungal plant symbionts: new tools to enhance plant growth
455 and productivity. *New Phytologist* **189**: 647-658.
- 456 **Hofseth L. J. and Wargovich M. J.** (2007) Inflammation, cancer, and targets of ginseng. *Journal*
457 *of Nutrition* **137**: 183-194.
- 458 **Jaber L. R. and Enkerli J.** (2017) Fungal entomopathogens as endophytes: can they promote
459 plant growth? *Biocontrol Science & Technology* **6**: 27-38.
- 460 **Jung S. Y., Choi S., Ko Y. S., Park C. S., Oh S., Koh S. R., Oh U., Oh J. W., Rhee M. H. and**
461 **Nah S. Y.** (2001) Effects of ginsenosides on vanilloid receptor (VR1) channels expressed in
462 *Xenopus oocytes*. *Molecules and Cells* **12**: 342-354.
- 463 **Kei H., Nina G., Soledad S., Thomas N. R., Stéphane H., Barbara K., Ulla N., Diana R.,**
464 **Marcel B. and O'Connell R. J.** (2016) Root endophyte *Colletotrichum tofieldiae* confers plant
465 fitness benefits that are phosphate status dependent. *Cell* **165**: 464-478.
- 466 **Kim D. H., Moon Y. S., Jung J. S., Min S. K., Son B. K., Suh H. W. and Song D. K.** (2003)
467 Effects of ginseng saponin administered intraperitoneally on the hypothalamo-pituitary-adrenal
468 axis in mice. *Neuroscience Letters* **343**: 62-76.

- 469 **Ludwig-Müller J.** (2015) Plants and endophytes: equal partners in secondary metabolite
470 production? *Biotechnology Letters* **37**: 1325-1334.
- 471 **Ming Q., Su C., Zheng C., Jia M., Zhang Q., Zhang H., Rahman K., Han T. and Qin L.** (2013)
472 Elicitors from the endophytic fungus *Trichoderma atroviride* promote *Salvia miltiorrhiza* hairy
473 root growth and tanshinone biosynthesis. *Journal of Experimental Botany* **64**: 5687-5694.
- 474 **Mizikar A.** (2011) Encyclopedia of Dietary Supplements (2nd edition). Reference Reviews **25**:
475 920-934.
- 476 **Rodriguez R. and Redman R.** (2008) More than 400 million years of evolution and some plants
477 still can't make it on their own: plant stress tolerance via fungal symbiosis. *Journal of*
478 *Experimental Botany* **59**: 1109-1114.
- 479 **Rodriguez R. J., Jr J. F. W., Arnold A. E. and Redman R. S.** (2009). Fungal endophytes:
480 diversity and functional roles, Blackwell Publishing Ltd.
- 481 **Schulz B., Römmer A., Dammann U., Aust H. and Strack D.** (1999) The endophyte-host
482 interaction: a balanced antagonism? *Mycological Research* **103**: 1275-1283.
- 483 **Sesma A. and Osbourn A. E.** (2004) The rice leaf blast pathogen undergoes developmental
484 processes typical of root-infecting fungi. *Nature* **431**: 582-586.
- 485 **Shores M., Harman G. E. and Mastouri F.** (2010) Induced systemic resistance and plant
486 responses to fungal biocontrol agents. *Annual Review of Phytopathology* **48**: 21-27.
- 487 **Srivastava S. and Srivastava A. K.** (2007) Hairy root culture for mass-production of high-value
488 secondary metabolites. *Critical Reviews in Biotechnology* **27**: 29-43.
- 489 **Straub D., Yang H., Yan L., Tsap T. and Ludewig U.** (2013) Root ethylene signalling is
490 involved in *Miscanthus sinensis* growth promotion by the bacterial endophyte *Herbaspirillum*
491 *frisingense* GSF30T. *Journal of Experimental Botany* **64**: 4603-4609.
- 492 **Sun B. S., Gu L. J., Fang Z. M., Wang C. Y., Zhen W., Mira L., Zheng L., Li J. J. and**
493 **Changkeun S.** (2009) Simultaneous quantification of 19 ginsenosides in black ginseng developed
494 from *Panax ginseng* by HPLC-ELSD. *Journal of Pharmaceutical and Biomedical Analysis* **50**:
495 15-24.
- 496 **Sun J., Hu S. and Song X.** (2007) Adjuvant effects of protopanaxadiol and protopanaxatriol
497 saponins from ginseng roots on the immune responses to ovalbumin in mice. *Vaccine* **25**:
498 1114-1120.
- 499 **Tan R. X. and Zou W. X.** (2001) Endophytes: a rich source of functional metabolites. *Natural*
500 *Product Reports* **18**: 448-459.

501 **Tenkanen M. and Siikaaho M.** (2000) An α -glucuronidase of *Schizophyllum commune* acting on
502 polymeric xylan. *Journal of Biotechnology* **78**: 149-161.

503 **Tkacz A. and Poole P.** (2015) Role of root microbiota in plant productivity. *Journal of*
504 *Experimental Botany* **66**: 2167-2172.

505 **Vargas W. A., Crutcher F. K. and Kenerley C. M.** (2011) Functional characterization of a
506 plant-like sucrose transporter from the beneficial fungus *Trichoderma virens*. *New Phytologist*
507 **189**: 777-789.

508 **Vargas W. A., Mandawe J. C. and Kenerley C. M.** (2009) Plant-derived sucrose is a key
509 element in the symbiotic association between *Trichoderma virens* and maize plants. *Plant*
510 *Physiology* **151**: 792-808.

511 **Wang J. H., Li W., Sha Y., Tezuka Y., Kadota S. and Li X.** (2001) Triterpenoid saponins from
512 leaves and stems of *Panax quinquefolium* L. *Journal of Asian Natural Products Research* **3**:
513 293-307.

514 **Yoshikawa T. and Furuya T.** (1987) Saponin production by cultures of *Panax ginseng*
515 transformed with *Agrobacterium rhizogenes*. *Plant Cell Reports* **6**: 449-453.

516 **Yue P. Y. K., Ki M. N., Cheng Y. K., Wah L. K., Bun N. T., Fan D. T. P., Wing Y. H. and Shun**
517 **W. R. N.** (2007) Pharmacogenomics and the Yin/Yang actions of ginseng: anti-tumor,
518 angiomodulating and steroid-like activities of ginsenosides. *Chinese medicine* **2**: 6-15.

519 **Zhang G., Liu A., Zhou Y., San X., Jin T. and Jin Y.** (2008) *Panax ginseng* ginsenoside-Rg2
520 protects memory impairment via anti-apoptosis in a rat model with vascular dementia. *Journal of*
521 *Ethnopharmacology* **115**: 441-450.

522

523

524

Figure 1. Effects of 3R-2 mycelium on the biomass, Rg₂ content and Rc content of *P. ginseng* hairy roots (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ vs control ; n=3)

Figure 2. Effects of 3R-2 mycelium on the green weight and biosynthesis of Rg₂, Rg₃ and Rc in *P. ginseng* hairy roots (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ vs control ; n=3)

Figure 3. The morphology of endophytic fungi 3 R-2 mycelium on PDA (A) Colony positive photo, (B) Colony opposite photo and (C) Micrograph of 3R-2.

Figure4. The phylogenetic tree of the endophytic fungus 3R-2, *Chaetomium cucumericola* is used as outgroup

Figure 5. Endophytic fungi distribution in different tissues of *P. ginseng* after immunofluorescence staining (blue-fluorescence and red triangle represented the mycelia, amplification factor : 400×)

Figure 6. Different endophytic fungi in *P. ginseng* hairy roots after immunofluorescence staining (blue-fluorescence represented the mycelia, amplification factor : 400×)

Figure 7. The endophytic fungi *P. ginseng* hairy roots after ammonium silver staining (the arrows indicate the mycelia)

Figure 8. Effects of endophyte 3R-2 and elicitor from 3R-2 on the expression of genes in the ginsenoside biosynthetic pathway in *p. ginseng* hairy roots. (A) HMGR, hydroxymethylglutaryl-CoA reductase; (B) SS, squalene synthetase; (C) SE, squalene epoxide enzyme; (D)

DS,darnmarediol synthase.(* : $P < 0.05$ 、 ** : $P < 0.01$ 、 *** : $P < 0.001$ vs control ; n=3)