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ARTICLE

Mycorrhizas Affect Polyphyllin Accumulation of *Paris polyphylla* var. *yunnanensis* through Promoting *PpSE* Expression

Hailing Li[1,2], Lingfeng Xu[1], Zhuowei Li[1], Shunxin Zhao[1], Dongqin Guo[1], Lu Rui[1,*] and Nong Zhou[1,*]

[1]Chongqing Engineering Laboratory of Green Planting and Deep Processing of Famous-Region Drug in the Three Gorges Reservoir Region, College of Biology and Food Engineering, Chongqing Three Gorges University, Chongqing, 404120, China

[2]College of Pharmaceutical Sciences, Dali University, Dali, 671000, China

[*]Corresponding Authors: Lu Rui. Email: ruilu1986@163.com; Nong Zhou. Email: erhaizn@126.com

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ABSTRACT

Paris polyphylla var. *yunnanensis* is a traditional Chinese medicinal plant, in which polyphyllin as the main medicinal component is an important secondary metabolite with bioactivity. Arbuscular mycorrhizal fungi (AMF) have multiple positive effects on plants, while it is not clear whether AMF increase the content of medicinal components in medicinal plants. In this study, a total of nine AMF treatments were laid to analyze the mycorrhizal effect on polyphyllin accumulation and *PpHMGR* and *PpSE* expression of *P. polyphylla* var. *yunnanensis*. AMF increased the content of polyphyllin in the cultivated variety with low relation to the increase of inoculation intensity. Polyphyllin I, II, and VII were identified and partly improved by AMF inoculation, dependent on AMF treatments and culture environments. Similarly, the *PpHMGR* and *PpSE* expression was induced by mycorrhization, dependent on AMF species, whilst the induction was more obvious in *PpSE* than in *PpHMGR* after mycorrhization. It concluded that the symbiotic relationship between *P. polyphylla* var. *yunnanensis* and AMF increased polyphyllin content level in the plant, which was associated with the up-regulation of *PpSE* transcripts.

KEYWORDS

Paris polyphylla var. *yunnanensis*; Arbuscular mycorrhizal fungi; Polyphyllin; 3-hydroxy-3-methylglutaryl CoA reductase (HMGR); squalene epoxidase (SE)

1 Introduction

Medicinal plants are valuable natural resources with health owing to their biological activity [1]. Because the huge medicinal and economic values, people paid great concern about medicinal plants recently. Besides their medicinal usage, medicinal plants are served as food supplements, herbal teas and health care products for their benefits to human health.

Paris polyphylla var. *yunnanensis* is a perennial herb belonging to *Liliaceae*, whose dried rhizomes are widely used in the Traditional Chinese Medicine (TCM) [2]. The plant mainly distributes in tropical or cold temperate areas throughout Europe and Eastern Asia. Guizhou Province, Yunnan Province and Sichuan Province are primary distribution areas in China. Additionally, *P. polyphylla* var. *yunnanensis* is one of the original plants of *Paris polyphylla* recorded in 2020 edition of Chinese Pharmacopoeia [3]. The dry rhizome of the plant is used as medicine, which has effects on hemostasis, detoxification, detumescence

and pain relief, and is used in antitumor and antimicrobial treatments [4–6]. *P. polyphylla* var. *yunnanensis* has high medicinal and economic value as a main raw material of traditional Chinese patent medicines such as "Yunnan Baiyao" and "Gongxuening Capsule" [7]. However, habitat loss, slow growth and over-exploitation result in its wild resources to be endangered. *P. polyphylla* has been listed as national level II protected plants (http://rep.iplant.cn/). Therefore, it is particularly important to increase the production and the content of the main active ingredients of *P. polyphylla* var. *yunnanensis*.

P. polyphylla var. *yunnanensis* contains hundreds of active components such as steroidal saponins, phytosterols, flavonoids, and fatty acid ester, etc. Polyphyllin is one of the main medicinal ingredients belonging to steroidal saponins [8,9]. Polyphyllin I exhibits anticancer properties in prostate cancer, gastric cancer and ovarian cancer [10–12]. Polyphyllin II exerts a significant effect on anti-lung adenocarcinoma and anti-hepatocellular carcinoma [13,14]. Polyphyllin VII shows its activity on inducing apoptotic cell death in lung cancer cells and inhibiting reactive oxygen species production in osteoclast to attenuate its differentiation [15–17]. In addition, polyphyllin VII displays special anti-inflammatory effects *in vitro* and *in vivo* [18].

The biosynthesis of steroidal saponins in plants relies on both cytosolic mevalonate (MVA) pathway and plastidial methylerythritol-4-phosphate (MEP) pathway [19]. Although the initial chemicals in the two pathways are different, both pathways lead to the formation of iso-amyl pyrophosphate (IPP), then isomerizes into dimethylallyl diphosphate (DMAPP), which provides the backbones of steroidal aglycone [20]. The 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) is a crucial regulator, catalyzing the conversion of 3-hydroxy-3-methyl-glutaryl-CoA into mevalonate which is an irreversible and rate limiting step. The expression level of *HMGR* directly affects the content of steroidal saponin compounds [21,22]. *HMGR* was identified in various model and economic plants such as *Arabidopsis* [23], wheat [24], rice [25], coffee [26], etc., and characterized in numerous traditional Chinese medicinal materials like *Ginkgo biloba* [27], *Salvia miltiorrhiza* [28], *Paris fargesii Franch* [29] and *Panax ginseng* [30]. After IPP and DMAPP formation, DMAPP is catalyzed to synthesize squalene via geranyl diphosphate synthase (GPS), farnesyl diphosphate synthase (FPS) and squalene synthase (SQS). Squalene is subsequently converted into 2, 3-oxidosqualene under the catalytic activity of squalene epoxidase which determines the biosynthesis rate of 2, 3-oxidosqualene to a great extent, and affects the synthesis of steroidal saponins [19].

Arbuscular mycorrhizal fungi (AMF) are widespread beneficial soil microorganisms establishing interactions with more than 80% of vascular plants. During the interaction between AMF and plant roots, a symbiotic relationship is formed. AMF provide nutrition for plant roots derived from soil in exchange for sugar or other organic carbon sources [31]. It was demonstrated that plants symbiotic with AMF have improved survival rate after planting and increased production [32]. AMF also promote the absorption of water and mineral nutrients by host plants, elevating stress resistance, affecting the production of secondary metabolites of medicinal plants [33]. What's more, they improve the quality of soil and are favorable to the environment. Although numerous researches have been carried out the interaction between AMF and different host plants, including many medicinal plants, little was known about the effects on *P. polyphyll*a var. *yunnanensis* after AMF inoculation. In this study we analyzed the change in polyphyllin contents of *P. polyphylla* var. *yunnanensis* and the expression level of *PpSE* and *PpHMGR* after AMF inoculation.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

One wild variety and one cultivated variety were planted separately in two different resources base that both located in Xixiu district, Anshun City, Guizhou Province, China. The seeds of the wild variety and the cultivated variety were harvested in October 2013 from one-year-old plants of *P. polyphylla* var. *yunnanensis*

separately. All samples were stored in sand at room temperature for almost 4 months and were then identified. After removing the exocarp, the seeds were washed with distilled water and soaked in 10% NaClO for 15 min. And then seeds were washed with distilled water again to eliminate NaClO. Cultivation substrate was a mixture of vegetable garden soil and sand (soil:sand = 3:1, and was sterilized at 121°C for 2 h). Ten groups (9 AMF species inoculation groups and 1 control group) were set up with 10 replicates in each group. Fresh seeds from the cultivated and the wild variety of *P. polyphylla* var. *yunnanensis* were mixed with AMF in January 2014. Hoagland solutions were regularly watered during the growth of seedlings.

2.2 AMF Inoculation

AMF were purchased from the International Arbuscular Mycorrhizal Fungi Collection (INVAM). Mycorrhizal fungal inoculum contained cultivation substrates with spores, hyphae and infected root segments. At transplanting time, AMF inoculums were applied into the pots. A total of ten treatments were laid, as shown in Tab. 1.

Table 1: Different groups of AMF

Group name	AMF species in details
S1	*Gigaspora rosea, Gigaspora albida, Gigaspora margarita* and *Gigaspora gigantea*
S2	*Scutellospora calospora, Scutellospora pellucida, Racocetra coralloidea* and *Racocetra fulgida*
S3	*Rhizophagus intraradices, Septoglomus deserticola, Claroideoglomus claroideum* and *Rhizophagus clarum*
S4	*Gigaspora rosea, Gigaspora albida, Scutellospora pellucida, Racocetra coralloidea, Claroideoglomus claroideum* and *Rhizophagus clarum*
S5	*Gigaspora albida, Gigaspora margarita, Racocetra coralloidea, Racocetra fulgida, Rhizophagus intraradices* and *Septoglomus deserticola*
S6	*Gigaspora margarita, Gigaspora gigantea, Scutellospora calospora, Scutellospora pellucida, Septoglomus deserticola* and *Claroideoglomus claroideum*
S7	*Gigaspora rosea, Gigaspora margarita, Scutellospora pellucida, Racocetra fulgida, Rhizophagus intraradices* and *Rhizophagus clarum*
S8	*Gigaspora albida, Gigaspora gigantea, Scutellospora calospora, Racocetra fulgida, Rhizophagus intraradices* and *Claroideoglomus claroideum*
S9	*Gigaspora rosea, Gigaspora gigantea, Scutellospora calospora, Racocetra coralloidea, Septoglomus deserticola* and *Rhizophagus clarum*

2.3 Polyphyllin Extraction and Measurement

The polyphyllin I, II, and VII in *P. polyphylla* var. *yunnanensis* was extracted according to the first volume of Chinese pharmacopoeia in 2020 and determined using a HPLC assay described by Zhou et al. [34] previously.

2.4 RNA Sample Collection and RNA Extraction

Total RNA was extracted with 0.1 g per sample using SGTriEx high purity total RNA extraction kit following the manufacturer's instruction. A total of 5 μg RNA were subjected to first strand of cDNA synthesis.

2.5 Quantitative Real-Time-PCR

Analysis of gene expression were performed as described previously by Wang et al. [35]. The qRT-PCR data were subjected to standard statistical analysis. Primers of genes used in this study are: *PpSE*-F: ATGTCCCTGGTCAGAAGGTG, *PpSE*-R: AGTTATCTCCAAGTCGATCAGTT, *PpHMGR*-F: GGCAGTGCTGCGAGATGCC, *PpHMG*R-R: CTTGCAGCCCCTATTGGTG *PpCCaMK*-F: GCCGTC-GCCGTTGGCGTC, *PpCCaMK*-R: GCCGTCGCCGTTGGCGTC and *PpActin-F*: TCCATCATGAAGT-GTGATGT, *PpActin-F*: AGTGATCTCCTTGCTCATAC.

2.6 Statistical Analysis

The figures were generated using Microsoft Excel and Adobe illustrator software. Statistical analysis was performed using the SPSS 20.0 and GraphPad Prism 4 software.

3 Results

3.1 Mycorrhizal Colonization and Plant Growth

The AMF used in our trials were listed in Tab. 1. As described in details by Zhou et al. [34], AMF in our study successfully established mycorrhizal symbioses with *P. polyphylla* var. *yunnanensis* plants, showing percentages of colonized root length ranging from 91.29%~100.00%. The naturally occurring AMF showed lower mycorrhizal percentages, which ranged from 36.41%~83.7% [36]. The average intensity of the mycorrhizal colonization in the root system was 55.35%, indicating AMF had a good symbiotic relationship with *P. polyphylla* var. *yunnanensis* seedlings. However, the significant difference of colonization percentages and intensity of mycorrhizalonisation exists only between control groups and AMF treatment groups and there were no statistical differences among the treatments. Moreover, compared with the uninoculated plants, the ALP and SDH activities of mycorrhizal were enhanced, indicating that the biological activities of mycorrhizal fungi were also improved. Mycorrhization was reported to promote plant growth however its impact on *P. polyphylla* var. *yunnanensis* was not fully understood. In our previous study, applying of AMF altered the content of carotenoid, chlorophyll A and chlorophyll B and slightly increased the biomass of the plants [37].

3.2 AMF Improved Polyphyllin Content Level in P. Polyphylla var. Yunnanensis with Different Efficiency

Total polyphyllin was shown in Fig. 1. Overall, the results showed that except for some AMF treatment groups (S1 after 7 months of colonization, S2 after 19 months of colonization), mycorrhiza-based inoculation increased the total polyphyllin content in inoculated group either at 7 months post inoculation (MPI) or 19MPI, indicating AMF extensively improved polyphyllin accumulation (Fig. 1). However, the effects of different mixture of fungi species were greatly different, the applied AMF had an impact on the content of total polyphyllin, which was significantly enhanced in the plants colonized with S5 to S9 after 7 months comparing to the S1 to S4 inoculation. After 19 months of planting, polyphyllin at basal level decreased which may due to maturation of plants and reduction of biosynthesis of saponin. It revealed that after 7 months growth, polyphyllin level was relatively low in plants colonized by S1, whereas plants colonized by S1 after 19 months had a significantly higher polyphyllin content. The species in group S2 had the opposite effect to S1 on polyphyllin, suggesting the species in S2 group played an important role mainly within 7 months while after long period of AMF colonization the strains in S1 group were important factors to regulate polyphyllin accumulation.

To further investigate AMF effect on different varieties, we compared polyphyllin content level in the wild and cultivated varieties after 7 months of mycorrhization. As was shown in Fig. 1, plants had similar polyphyllin levels regardless of uninoculated group or S3 and S4 treatment. Fungi species in S5 to S9 groups also improved polyphyllin accumulation with higher efficiency which was consistent with that in the cultivated variety. Polyphyllin concentration was much significantly elevated in the cultivated

plants than the wild variety after S5, S6 and S9 inoculation, indicating the fungi in these groups were much fitter for the cultivated variety. In addition, the level of polyphyllin was not significantly increased after inoculated with S4 regardless of the time of colonization, plant varieties or planting environments.

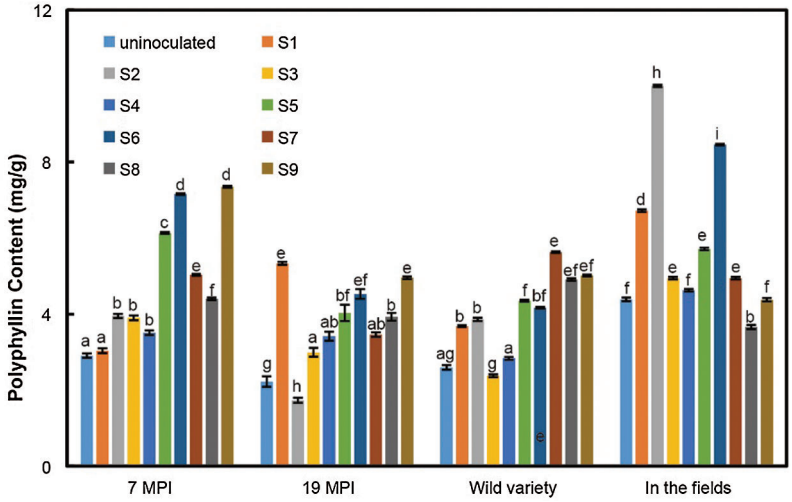

Figure 1: Total polyphyllin content in *P. polyphylla* var. *yunnanensis* after AMF inoculation in wild and cultivated varieties in different time and culturing environment

Basal polyphyllin level in the cultivated variety planted in the field after 7 months of AMF colonization was remarkably higher than that grew in the chamber, which may be caused by the presence of AMF in the soil and environment. This is not surprising as AMF occur naturally in field soils. Similarly, plants colonized by AMF had significantly higher polyphyllin concentrations except S4, S8 and S9 treatment. Specifically, S6 treatment still highly induced the accumulation of polyphyllin whereas the induction effect of the fungi in S5 were not as obvious as that in the chamber. To our surprise, the least efficient AMF strains in stimulating polyphyllin performance in the chamber were S1 and S2 which had a good induction ability in the fields. Our results suggested that in the fields, mycorrhizal fungi in group S1, S2, and S6 established a good symbiotic relationship with *P. polyphylla* var. *yunnanensis* and improved the mycorrhizal and polyphyllin content of the plant. Taken together, we assessed the effects of AMF on *P. polyphylla* var. *yunnanensis* and found applied fungi in S5, S6 and S7 improved the content of polyphyllin regardless of plant varieties, inoculation time or planting environments.

3.3 Polyphyllin VII is the Main Component of Polyphyllin

Polyphyllin I (PPI), polyphyllin II (PPII) and polyphyllin VII (PPVII) are three of the primary active components isolated from rhizoma of *P. polyphylla*, which had different concentration and biological activity. In the chamber, both in the cultivated and wild varieties, PPVII level was highest, followed by PPI and PPII (Fig. 2). However, in the fields, the proportion of PPVII and PPI was decreased with increased PPII, suggesting planting environment affected the accumulation of different active components of polyphyllin.

We further analyzed the effect of AMF on different components of polyphyllin, As shown in Fig. 3A, after 7 months, PPI was dramatically increased both in the cultivated and wild varieties after S9 colonization. After 19 months, out of nine AMF groups tested, S4 increased PPI most efficiently, followed by S9. In the comparison between the cultivated and wild varieties, The PPI of *P. polyphylla* var. *yunnanensis* seedlings were found to be significantly higher in the wild plants grown in soil inoculated with AMF in S7 and S8 group. The seedlings cultured in the presence of AMF in group S1, S2 and S6 showed the highest increasement of PPI in the plant rhizome, followed by AMF application in group S3, S4 and S5.

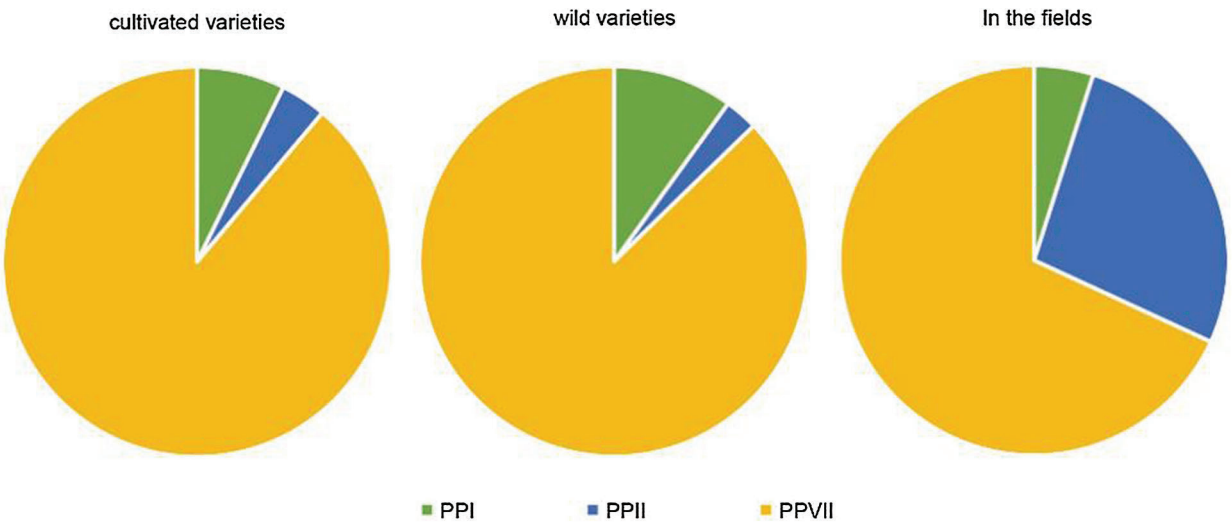

Figure 2: The percentage of PPI, PPII and PPVII in *P. polyphylla* var. *yunnanensis* after AMF inoculation was illustrated in the form of pie chart

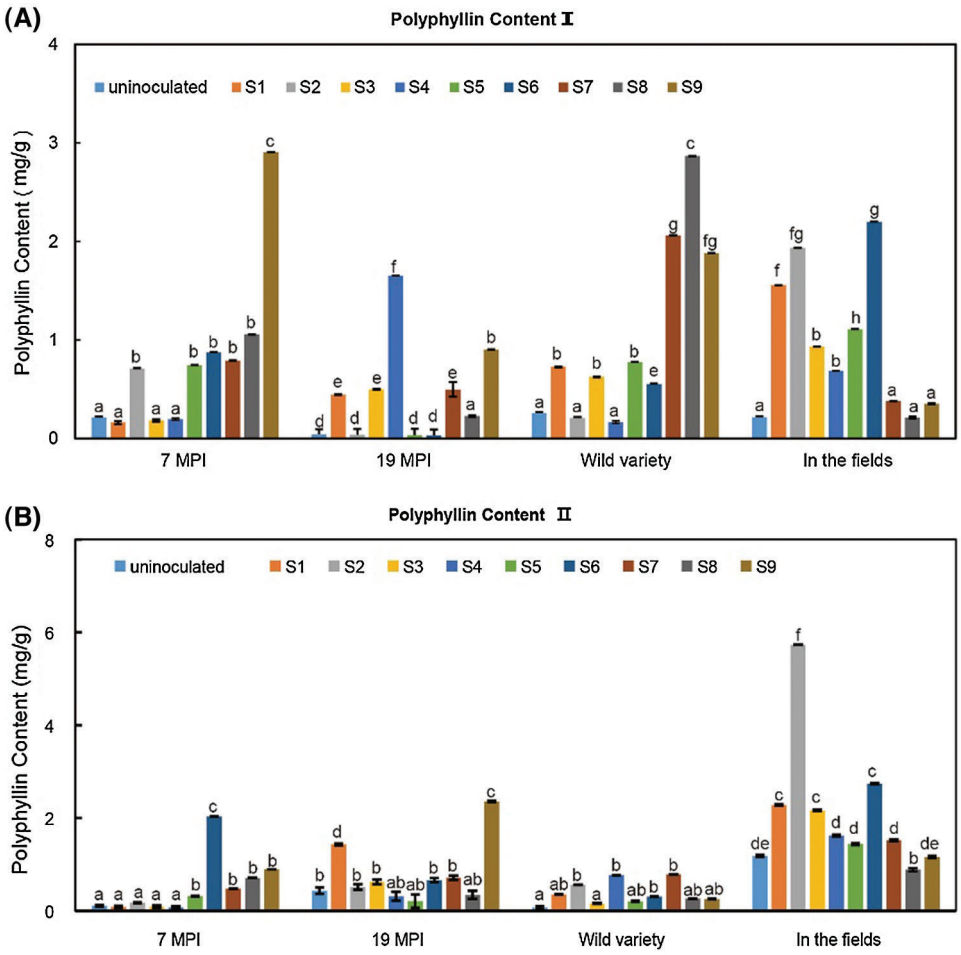

Figure 3: PPI (A) and PPII (B) levels after AMF inoculation in wild and cultivated varieties in different time and culturing conditions

S5, S6, S7, S8 and S9 mycorrhization had positive impact on the content of PPII at 7MPI, among which was especially pronounced in the differences found in the plants colonized with S6 (Fig. 3B). After 19 months, with the extension of inoculation time, most groups inoculated with AMF had not significantly increase the content of PPII, except for AMF in S1 and S9 group. It was revealed in Fig. 3B that introducing AMF inoculants were not remarkably increase PPII in wild plants. Additionally, PPII was significantly accumulated after S2 inoculation in the fields. Above all, *P. polyphylla* var. *yunnanensis* inoculated with AM inoculum resulted in an increased beneficial effect on accumulation of PPI and PPII depending on AM stains, inoculation time, plant varieties and environments.

3.4 Quantitively Real Time PCR Analyze of PpSE and PpHMGR in P. polyphylla var. yunnanensis after AMF Inoculation

Intensity of mycorrhizalonisation in the root system is one of the most important factors to determine plant biomass and secondary metabolism rate. We evaluated the correlation between intensity of mycorrhizalonisation and polyphyllin level in the plants after 7 months. As revealed in Fig. 4A, AMF in S6 significantly increased the content of polyphyllin with low inoculation intensity, suggesting the inoculation intensity was not strongly correlated with polyphyllin content level in *P. polyphylla* var. *yunnanensis*. Similar results were shown in Figs. 4B and 4D. The correlation between mycorrhizalonisation intensity and polyphyllin content was not affected by increasing inoculation time or changing planting environments. It is interesting that in the wild variety, the correlation was significantly enhanced (Fig. 4C). Overall, AMF increased the content of polyphyllin in the cultivated variety with low relation to the increase of mycorrhizalonisation intensity.

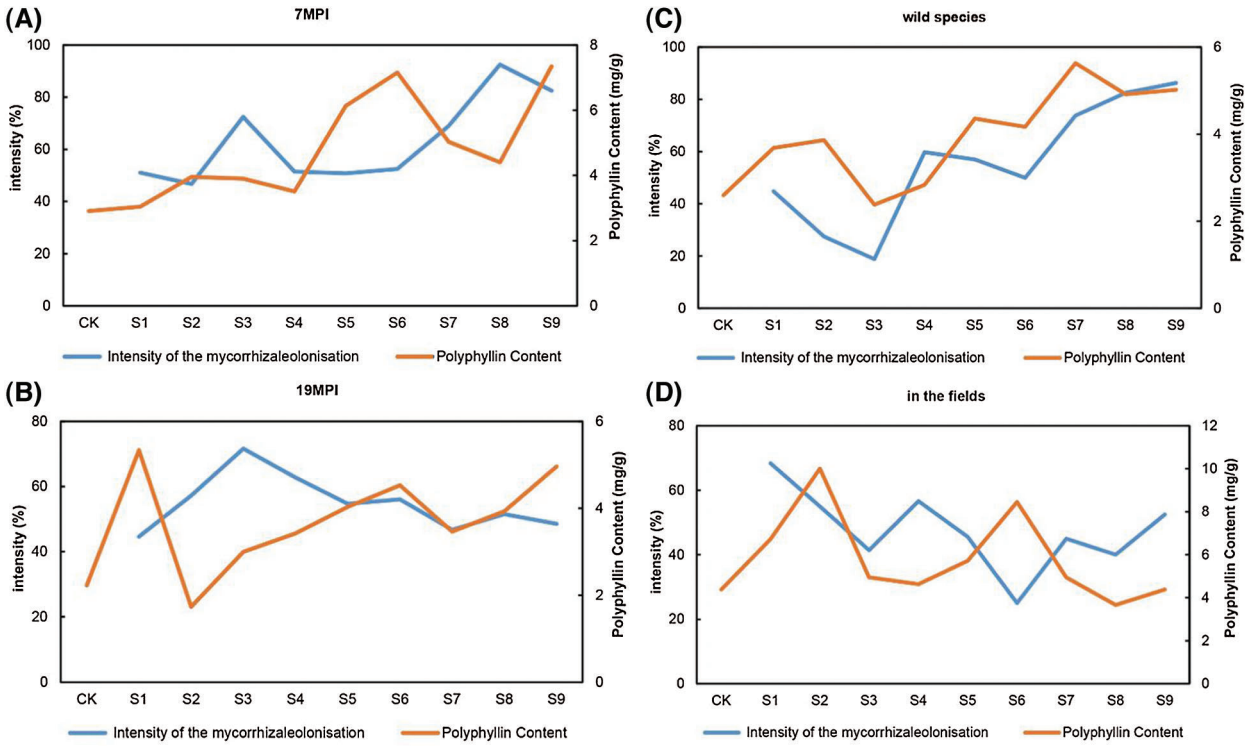

Figure 4: Intensity of mycorrhizalonisation in the root system and polyphyllin content after AMF inoculation at 7MPI (A), 19MPI (B), the wild variety (C) and in the field (D)

HMGR and SE are two rate limiting enzymes in steroidal saponins biosynthesis in plants. To further investigate the mechanism underling polyphyllin accumulation and the relationship to polyphyllin biosynthesis after AMF colonization. We detected the *PpSE* and *PpHMGR* transcripts by quantitively real time PCR analysis. After 7 months, *PpSE* was up-regulated by AMF regardless of strains (Fig. 5A). The expression levels of *PpSE* were also significantly up-regulated in the plants colonized by S1, S3, S4, S6, S7, S8 after 19 months. Prior to inoculation, *PpSE* transcriptional level was rather low in both varieties. Upon inoculation with AMF, *PpSE* transcripts accumulated at higher levels in cultivated plants than in wild plants, which was consistent with polyphyllin content level. *PpSE* expression level was remarkably up-regulated by S2 and S8 in the fields.

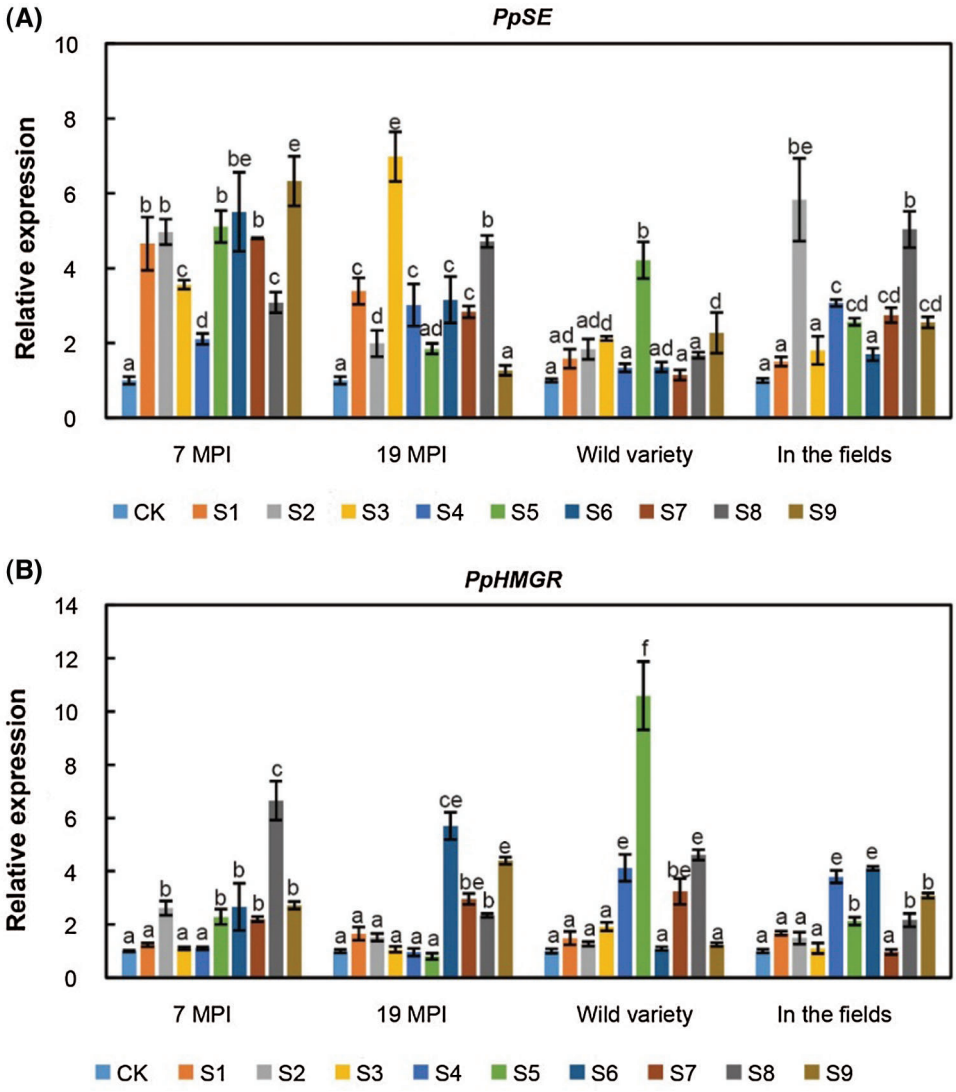

Figure 5: Relative expression level of *PpSE* (A) and *PpHMGR* (B) in *P. polyphylla* var. *yunnanensis* after AMF inoculation in wild and cultivated varieties in different time and culturing conditions

We also examined expression level of *PpHMGR*, the relative transcriptional level of this gene was lower in CK group, unlike *PpSE*, *PpHMGR* transcripts were not extensively affected by mycorrhizas (Fig. 5B).

PpHMGR transcriptional level in S8, S6, S5 AFM-inoculated plants was markedly higher than in control plants at 7MPI, 19MPI and in the wild variety, respectively. These results demonstrated that the transcriptional level of the *PpSE* gene has higher correlation with the content of polyphyllin content of *P. polyphylla* var. *yunnanensis*, suggesting up-regulating of *PpSE* led to polyphyllin content accumulation after mycorrhization (Fig. 6). *CCaMK* gene encodes a calcium and calmodulin-dependent protein kinase that is necessary for the establishment of both rhizobial and mycorrhizal symbioses, hence *PpCCaMK* was used as a negative control. There was no significant different of the expression level of *PpCCaMK* among different treatment groups (Fig. 6).

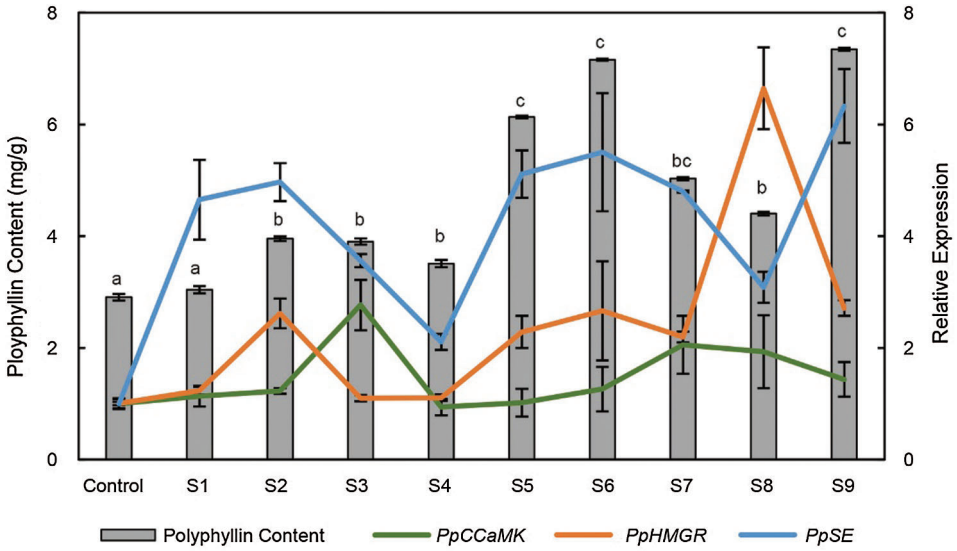

Figure 6: Relationship between polyphyllin content and the expression level of *PpSE, PpHMGR* and *PpCCaMK* after different AMF inoculation in cultivated *P. polyphylla var. yunnanensis*

4 Discussion

4.1 AMF Increased the Accumulation of Polyphyllin Content

AMF are most ubiquitously existent and of ancient origin which establish symbioses with the roots of almost all groups of land plants, with 80% of species forming these associations. The AMF-plant symbioses were found to contribute to various biological process in host plants, including improving biomass, enhancing tolerance to biotic and abiotic stress, increasing mineral uptake and stimulating photosynthesis as well as influencing secondary metabolism in plants [38,39]. Therefore, AMF are proposed to be used in agriculture to improve the yield and quality of crops and medicinal plant species [40]. AMF were found to improve plant growth in tomato [41], orange [42], maize [43], yam [44], mulberry and papaya [45], confirming that AMF possess a considerable potential for enhancing crop yield. In the impact of secondary metabolism, AMF-colonized strawberry [46] and tomato [41] exhibited beneficial effects on increased levels of secondary metabolites resulting in quality improvement. Although much has been done about the effects of AMF on plant yield, stress and secondary metabolism, little was known about the effects of AMF on polyphyllin content accumulation in *P. polyphylla* var. *yunnanensis* [47]. Until recently, few investigations focused on AM effects on the production of polyphyllin in *P. polyphylla var. yunnanensis* plants. In the present study, we demonstrated that AMF established a well symbiotic relationship with roots of *P. polyphylla* var. *yunnanensis*, showing high intensity of mycorrhizal colonization in the root system (Fig. 4). Total and three main active polyphyllin components were

successfully obtained from the plants after mycorrhization at two time points, two plant varieties and two planting environments. And we reported to the best of our knowledge for the first time, AMF was found to influence the contents of total polyphyllin (Fig. 2), polyphyllin I, polyphyllin II and polyphyllin VII (Fig. 3). AMF increased the content of polyphyllin in the cultivated variety with low relation to the increase of inoculation intensity. Polyphyllin I, II, and VII were identified and partly improved by AMF inoculation, dependent on AMF treatments and culture environments. The variability of polyphyllin concentrations we observed in plants colonized by different AMF highlighted the functional diversity that existed between fungal isolates (Figs. 1 and 3). There are also researches indicating variations in effectiveness of different AMF species in the production of active compounds [48,49]. Additional, Our data revealed that S5, S6 and S7 mycorrhizal fungi are key organisms that can be beneficial in the development of strong and healthy plants with reasonable polyphyllin level (Figs. 1 and 3). And it would be better to collect the plant tissues after 7 months of mycorrhization to obtain higher content of total polyphyllin (Fig. 1). Our results provide a theoretical basis for the application of AMF in enhancing the content of polyphyllin in *P. polyphylla* var. *yunnanensis*.

4.2 Up-Regulating of PpSE Gene Expression Increased the Polyphyllin Level

The mechanism how AMF trigger changes in polyphyllin concentration in plant might be multidirectional and is remain unclear. Some studies suggest that the essential pathway for the synthesis of steroidal saponins is the known mevalonate pathway, in which SE is one of the key rate-limiting enzymes. Regulating the biological activity of SE enzyme can directly affect the biosynthesis of squalene epoxide, and then affect the production of various steroidal saponins.

Squalene epoxidase were studied in many plants, such as *Panax notoginseng* [30], *Gynostemma pentaphyllum* [50] and *Betula platyphyll*a [51]. In addition, also a key rate-limiting enzyme, HMGR, is suggested to be a regulatory factor during steroidal saponins biosynthesis. It was reported that HMGR is the key enzyme and regulator of mevalonate pathway (MVA) in medicinal plants. The *HMGR* gene was studied in many plants, such as *Aquilaria sinensis* [52], *Glycyrrhiza* [53] and *Salvia miltiorrhiza* [28]. Understanding of the expression level of these two key enzyme genes in the biosynthesis pathway of steroidal saponins would provide a theoretical foundation for enhancing the content of effective medicinal ingredients in precious or endangered medicinal plants by manipulating biological process using molecular biological technology. In our study, the expression levels of both *PpSE* and *PpHMGR* contributed to the accumulation of polyphyllin (Fig. 5), in which the *PpSE* expression and the polyphyllin content had a higher correlation (Fig. 6), suggesting *PpHMGR*, *PpSE* played a crucial role of polyphyllin biosynthesis in *P. polyphylla* var. *yunnanensis*. Up-regulating of *PpSE* is one of the mechanisms underlying polyphyllin increasement, meaning *P. polyphylla* var. *yunnanensis* has established a symbiotic relationship with AMF to regulate the expression of *PpSE*, and further affects the level of polyphyllin. More work is needed to elucidate the mechanisms of how the *PpSE* specifically regulates polyphyllin accumulation.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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