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Microbial diversity in *Paris polyphylla* var. *yunnanensis* rhizomes of varying ages

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ABSTRACT. Endophyte microorganisms live inside plants without causing them any apparent damage. Recently, endophytic microorganisms have attracted attention because they can produce bioactive compounds of biotechnological interest. The endophytic microorganisms in *Paris polyphylla* var. *yunnanensis* (Liliaceae) - a species used since antiquity in traditional Chinese medicine - are under scrutiny because they may be responsible for producing the bioactive metabolites associated with the plant. The levels of bioactive metabolites in the rhizomes of *P. polyphylla* increase with rhizome age. To elucidate the roles played by endophytes in the accumulation of bioactive metabolites, we investigated the community structure and diversity of the endophytic microorganisms in *P. polyphylla* rhizomes of different ages (4, 6, and 8 years) using 16S rRNA and internal transcribed spacer (ITS) sequence analysis. 16S rDNA amplicon pyrosequencing revealed that the number of operational taxonomic units was lower in the 8-year-old samples than in the other samples. A total of 28 phyla were observed in the *P. polyphylla* samples and the predominant

bacteria were of the Cyanobacteria and Proteobacteria phyla. Moreover, the percentage of Cyanobacteria increased with rhizome age. Similarly, ITS1 amplicon pyrosequencing identified developmental changes in the most abundant fungal classes; some classes were more prevalent in the 8-year-old rhizomes than in younger rhizomes, indicating the importance in secondary metabolism in older rhizomes. Our study showed that endophyte microorganism diversity and prevalence depend on *P. polyphylla* rhizome age. There was also an indication that some endophyte microorganisms contribute to the higher saponin content in older *P. polyphylla* specimens.

Key words: Endophytic microorganism; *Paris polyphylla*; HiSeq 2000 sequencing

INTRODUCTION

Endophytic microorganisms live within plant tissues without causing any apparent harm to their host. They spend the whole or part of their lifecycle in the inter-and/or intra-cellular colonization of the healthy tissues of the host plant (Tan and Zou, 2001). They are also an important and novel source of natural bioactive compounds, and have potential applications in agriculture, medicine, and the food industry (Gunatilaka, 2006; Verma et al., 2009; Zhou et al., 2010). Consequently, an increasing number of scientists are studying endophytes as potential producers of novel and bioactive compounds. To date, large numbers of endophyte species have been isolated from hundreds of plants, some of which have been found to produce bioactive substances that arise from the relationships between endophytes and their hosts. Endophytes are ubiquitous among terrestrial plants (Gond et al., 2007; Li et al., 2007; Verma et al., 2007; Zhang et al., 2013; Ling et al., 2014; Saini et al., 2015). However, it has been estimated that only 6-7% of the endophytes in existence have been described (Hawksworth, 2001). Therefore, it is necessary to investigate the remaining 93% of these microorganisms in other plant species.

Paris polyphylla (Liliaceae) is a highly valued medicinal plant. It is mainly distributed in southwestern China, particularly in the Yunnan and Sichuan provinces (Zhang, 2007). In India, it is found in Manipur, Uttarakhand, Himachal Pradesh, and in the Lushai and Aka Hills (Tiwari et al., 2010). The rhizomes of *P. polyphylla* have been used in traditional Chinese medicine for the treatment of various inflammations and injuries (Zhao et al., 2010). They are also an important ingredient of certain Chinese patent medicines such as ‘‘Biyan Qingdu Keli’’, which is widely used in southern China for the treatment of chronic rhinitis and nasopharyngeal cancer (Guo et al., 2006). Recent pharmacological studies have also demonstrated that the plant imparts hemostatic, antitumor, uterine contractile, analgesic, and sedative effects (Guo et al., 2008). The rhizomes of *P. polyphylla* have also been used as an anthelmintic and vermifuge folk remedy in Nepalese communities (Devkota, 2005), and the powdered roots are an ethnopediatric treatment for diarrhea in Garhwal Himalaya, Uttarakhand, India (Tiwari et al., 2010). The main active ingredients of the plant are steroidal saponins (Zhang, 2007); at least 30 steroidal saponins have been isolated through phytochemical methods (Liu et al., 2006; Xu et al., 2007; Zhao et al., 2009). In China, the demand for this species has increased in recent years. However, its slow growth and excessive harvesting in recent years mean that it is significantly less common and is currently facing the risk of extinction (Zhang et al., 2011). Therefore, there is an urgent need to discover an alternative way of

producing active steroidal saponins, such as microbial fermentation, to meet the increasing demand.

Previous researchers investigated the diversity of the endophytic fungi in *P. polyphylla* using cultural methods, and found limited fungal diversity in a rhizome of unknown age (Jing et al., 2008). *P. polyphylla* rhizomes of different ages contain different amounts of saponins; older rhizomes have a higher saponin content than younger ones (Xia et al., 2011). Furthermore, the bacterial diversity in *P. polyphylla* has not been studied until now. Pyrosequencing is a high-throughput technique that has revolutionized microbial detection, including that of rare species (Huse et al., 2008). For example, Sogin et al. (2006) performed 118,000 sequence reads and found more than 3000 bacterial operational taxonomic units (OTUs) present in a water sample. Pyrosequencing has also been used to examine bacterial communities in plant samples (Romero et al., 2014; Yamamoto et al., 2014), revealing an unprecedented level of microbial diversity.

In recent years, medicinal plants have received increasing attention owing to the growing demand for green chemistry and sustainable practices. The quest for novel antibiotics that are able to tackle the increasing multidrug resistance of pathogenic bacteria has also fueled demand. In spite of the high relevance of medicinal plants, to the best of our knowledge, very little, if anything, is known about the endophytic bacterial communities isolated from medicinal plants. For this reason, in this work we made a preliminary characterization of the endophytic community of *P. polyphylla* from a taxonomical viewpoint. In this study, we used the first known pyrosequencing analysis to examine the bacterial and fungal endophyte community in *P. polyphylla* rhizomes of different ages. We attempted to determine the relationship between the broad diversity of endophytic microorganisms and the existence of steroid saponins in *P. polyphylla* by studying the endophyte diversity change during *P. polyphylla* rhizome development.

MATERIAL AND METHODS

Plant materials and treatment

Healthy *P. polyphylla* rhizomes (4, 6, and 8 years old named C1, C2, and C3, respectively; three plants for each year) were collected in Kunming, China in August 2014, and stored in sealed plastic bags at 4°C. The taxonomical identification of the plant material was carried out by Prof. Zhongjun He of Yunnan Agriculture University. The voucher specimen has been deposited in the Faculty of Agronomy and Biotechnology, Yunnan Agriculture University. The rhizome samples were rinsed under running tap water for 10 min, immersed in 70% ethanol with shaking for 3 min, followed by fresh sodium hypochlorite solution (2.5% available Cl^-) for 5 min, and 70% ethanol for 30 s, and finally washed three times with sterile water. Aliquots of the final rinsing water were spread on nutrient agar solid medium plates and cultured for 3 days at 28°C for detection of bacterial colonies to examine the effect of the surface sterilization. The uncontaminated samples were used for further DNA-based analyses.

Plant metagenomic DNA extraction, and retrieval of 16S rDNA and internal transcribed spacer (ITS) sequences

A Qiagen Stool Kit (Qiagen Inc., USA) was used to extract total metagenomic DNA from the *P. polyphylla* samples following the manufacturer instructions for microorganism metagenomic DNA extraction. To amplify 16S rDNA and ITS, the extracted metagenomic DNA (combining one

sample for each year) was used for polymerase chain reaction (PCR). The universal primers, 16Sf 5'-GATCCTACGGGAGGCAGCA-3' and 16Sr 5'-GCTTACCGCGGCTGCTGGC-3', were used to amplify bacterial 16S rDNA fragments, and ITSf 5'-TGGTCATTTAGAGGAAGTAAAA-3' and ITSr 5'-GCTGCGTTCTTCATCGATGC-3' were used for fungal ITS fragments (de la Cerda et al., 2007). The reaction program was as follows: 5 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at 55°C, 90 s at 72°C, maintained at 4°C in a 50-µL PCR volume. The PCR products were examined on a 1% agarose gel, and the main amplicon band was excised using a razor blade. The PCR amplicon was purified using a QIAGEN Gel & PCR Purification Kit (Qiagen Inc.) and sequenced using an Illumina paired-end protocol from BGI (Beijing Genomics Inst., China) to generate millions of reads.

Statistical analysis

For bacterial diversity analysis, the 16S rDNA sequences generated from the RNA-seq sequencer were processed by the QIIME (quantitative insights into microbial ecology) pipeline. Briefly, sequences, incorrect primer sequences, or more than one ambiguous base were discarded. Denoising of the pyrotag sequences was performed using DENOISER v.0.9.1, as implemented in the QIIME platform. Chimeric sequences were removed using ChimeraSlayer. Sequences were clustered into OTUs at the threshold of 97% sequence similarity. A Venn diagram was generated using custom Perl scripts to identify the number of core OTUs. For fungal diversity analysis, generated ITS sequences were excluded from analysis after quality trimming. Raw sequences without pyrotags and forward primer sequences, as well as non-fungal ITS sequences, were excluded from the analysis. Removal of ITS chimeric sequences was performed using a Basic Local Alignment Search Tool (BLAST)-based open source ChimeraChecker software package. The resultant sequences for individual samples were subjected to the pyrosequencing analysis pipeline at the UNITE database. The rarefaction analysis for both bacterial and fungal data was performed using ANALYTIC RAREFACTION v.1.4, and the calculation of Shannon richness and Chao1 diversity indices was performed using ESTIMATES v.8.0. The core community was analyzed based on the species present in individual rhizomes.

RESULTS AND DISCUSSION

Medicinal plants are attracting the attention of many researchers owing to the presence of compounds that constitute a large fraction of the current pharmacopoeias. *P. polyphylla* is an important plant in Chinese and Indian traditional medicine; the main active ingredients derived from it are steroidal saponins, which have various activities. Owing to the important role played by endophytes in plant secondary metabolism, our major goal was to develop effective strategies for the manipulation of microbial communities to increase the overall content of saponins in *P. polyphylla*. Hence, an in-depth knowledge of the bacterial diversity and the principles governing microbial community assembly were the first steps needed to achieve our long-term goals. Previous research employed relatively shallow sampling to detect the fungal diversity in *P. polyphylla* rhizomes (Li et al., 2008), and none of these studies assessed bacterial diversity. These limitations have been overcome to a certain extent in this study by our sampling and sequencing efforts. Here, we studied the endophyte diversity change among rhizomes of varying ages using culture-independent methods on a HiSeq 2000 platform, because previous studies have shown that older *P. polyphylla* specimens contain more saponin (Conglong et al., 2011). The 16S rDNA and ITS was

amplified from the extracted DNA samples using universal 16S rDNA and ITS primers to reveal genomic information about the dominant endophytic bacteria and fungi responsible for saponin accumulation during *P. polyphylla* growth.

For the bacteria, a total of 2,847,490 raw reads were found. Of these, 1297 reads were found to be chimeric and were removed from further analyses. After quality filtering, 2,675,295 reads were used for microbiome analysis. Good's coverage values, calculated at a 97% similarity cut-off, indicated that the number of pyrosequencing reads obtained was sufficient to capture the microorganism diversity in the *P. polyphylla* samples (Figure 1A). To examine the diversity of the microbial community in the *P. polyphylla* samples, OTUs were identified in the microbiome of *P. polyphylla*. The average numbers of high-quality OTUs were as follows: C1, 2742; C2, 1762; and C3, 1211, which are in close agreement with the Chao1 and abundance-based coverage estimators (ACE) estimates (Table 1). Both Chao1 and ACE species richness indices at the same sequencing depth for each sample showed that the 4-year-old rhizomes expressed the highest richness (Table 1). Additionally, Simpson and Shannon indices of diversity showed that the 4-year-old rhizomes had the highest bacterial diversity (Table 1). This pattern of colonization suggests that young rhizomes provide a more favorable environment for the establishment of endophytic bacteria. Numerous biotic and abiotic factors are involved in endophytic infection and colonization, including the chemistry and structure of the host tissue and the environmental conditions to which the plant is exposed (Sanchez-Azofeifa et al., 2012). Therefore, it may be that the higher secondary metabolism with antifungal and anti-herbivore substances in older rhizomes affect the abundance of endophytic bacteria. Hierarchical clustering was performed on a subset of OTUs selected from rhizome communities of different ages, which were segregated into three clearly different clusters.

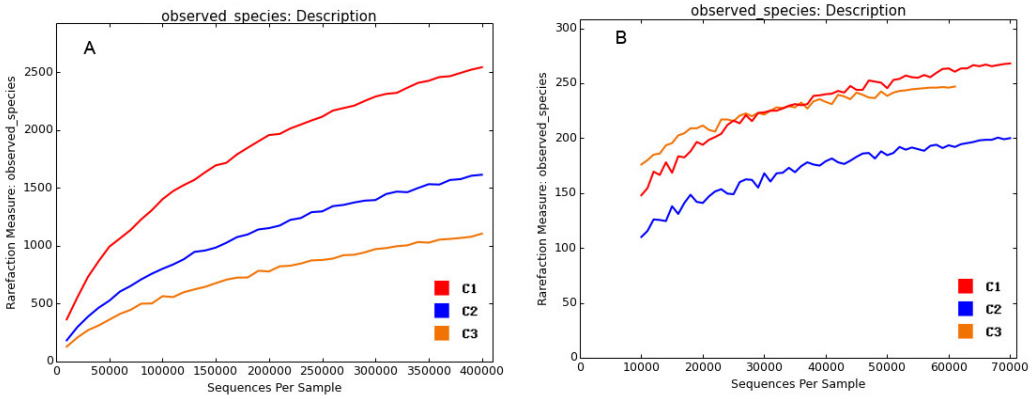

Figure 1. Rarefaction analysis curves of bacterial 16S rDNA (**A**) and internal transcribed spacer (ITS) sequences (**B**). Sequences were associated with operational taxonomic units (OTUs) using a pairwise sequence similarity threshold of 97%.

The total abundance of the observed OTUs distributed across different rhizome ages is indicated in a Venn diagram (Figure 2A). The overlapping OTU clusters among the treatments (C1, C2, and C3) were calculated. C1 and C2 showed the largest OTU overlap among the different degrees of development compared with C3 (Figure 2A). At the OTU level, a total of 544 OTUs

(15.2%) were common to the three different degrees of development, and 3033 OTUs (84.8%) were identified in all samples, indicating that the microbial diversity was significantly different during *P. polyphylla* development.

Table 1. Summary of the 16S rRNA sequencing data obtained from the *Paris polyphylla* samples.

Samples	Number of reads	Number of OTUs[a]	Good coverage[a]	Richness estimator[a]		Diversity index[a]	
				Chao1	ACE	Shannon	Simpson
C1	1,005,494	2742	0.99	3382.15	3637.26	1.12	0.16
C2	823,099	1762	0.99	2554.96	2673.19	0.44	0.06
C3	846,702	1211	0.99	1853.57	1943.44	0.35	0.05

OTUs = operational taxonomic units; ACE = abundance-based coverage estimators. [a]Calculated at a 97% sequence similarity cut-off.

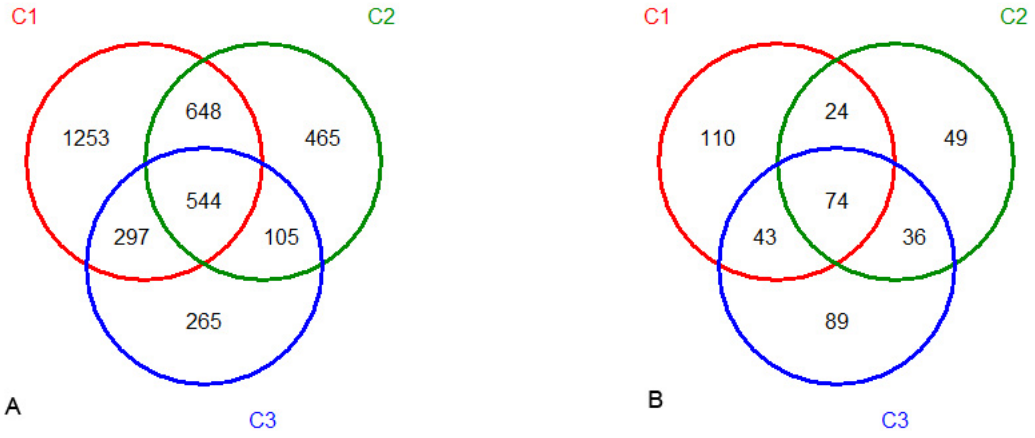

Figure 2. Venn diagram showing the distribution of all operational taxonomic units (OTUs) identified by 16S rDNA (**A**) and internal transcribed spacer (ITS) sequences (**B**). Sequencing among the three trichome samples.

Additionally, a total of 28 phyla were distributed throughout three sample types, and the most abundant bacteria were members of the Cyanobacteria (96%) and Proteobacteria (3%) phyla. The relative abundance of these taxonomic phyla varied among the sample ages. Cyanobacteria was the most abundantly represented phylum among the different rhizome ages. Cyanobacteria are a diverse group of oxygenic photosynthetic prokaryotes that occur globally in marine, aquatic, and terrestrial environments (Rippka et al., 1979). Some cyanobacteria have the ability to live in association with a wide range of plants from the divisions Bryophyta (liverworts and hornworts), Pteridophyta (genus *Azolla*), gymnosperms (family *Cycadaceae*), and angiosperms (family *Gunneraceae*) (Table 1) (Rai et al., 2002). Cyanobacteria are also important as bioinoculants for enhancing fertility, and improving soil structure and crop yields in rice fields (Prasanna et al., 2012). In addition to the highly abundant Cyanobacteria, the endophytic community also included a small proportion of Proteobacteria (3%) and other phyla (1%). Proteobacteria were more prevalent in the 4-year-old rhizomes, and there was a notable difference in species in the older rhizomes. The phylum Proteobacteria comprises several species that promote plant growth and also act as biological control agents of different diseases (Bulgarelli et al., 2013). A study of cultivable bacteria

associated with tomato leaves also revealed the presence of Proteobacteria in both greenhouse- and field-grown plants (Enya et al., 2007). However, the phylum Proteobacteria also comprises several species that are pathogenic to plants (Mansfield et al., 2012).

The remaining phyla, including Actinobacteria, Bacteroidetes, Verrucomicrobia, Acidobacteria, Chloroflexi, etc., were much less abundant. In addition, some sequence reads (about 0.03%) were classified as unknown bacterial phyla. Taxonomic groups, such as Cyanobacteria (95.82%), Proteobacteria (3.48%), Actinobacteria (0.25%), and Bacteroidetes (0.25%) phyla, were found in all three rhizome ages. The Proteobacteria, Actinobacteria, and Bacteroidetes phyla were most prevalent in the 4-year-old rhizomes, while Cyanobacteria had the greatest prevalence in the 8-year-old rhizomes compared with the younger rhizomes. The community of endophytic bacteria in *P. polyphylla* rhizomes shares several similarities with endophytic communities in other plant species in terms of phylum composition, suggesting that members of these phyla are adapted to the particular conditions required for the colonization of this habitat. At the genus level, Pseudomonas (0.19%), Flavobacterium (0.1%), and Sphingobium (0.09%) were most abundant in the 4-year-old rhizomes, but Steroidobacter (0.09%) was most abundant in the 8-year-old rhizomes. Therefore, further investigation should be conducted on the roles of these genera in secondary metabolism in *P. polyphylla* rhizomes.

For fungi, a total of 214,550 ITS sequences passed the various quality control steps and the numbers of reads per sample ranged from 61,767 to 82,192. The rarefaction curves indicated that a high level of fungal diversity was also obtained for the subsequent analysis of different rhizome ages (Figure 1B). The resultant sequences were clustered into OTUs at a 97% similarity level. Upon removal of singletons and non-fungal sequences, the average numbers of OTUs were: C1, 251; C2, 183; and C3, 242, which is in close agreement with the Chao1 estimate. The rarefaction and species richness indices for fungi diversity also indicated that our deep sequencing was enough for a comprehensive understanding of fungal diversity in *P. polyphylla* rhizomes. Our sampling efforts revealed a rich fungal community, and more than 180 OTUs were identified for each *P. polyphylla* rhizome. These results contradicted the previous findings, which reported lower species richness (10 OTUs) using traditional study methods (Li et al., 2008). It is also noteworthy that the saturation of rarefaction curves and fungal diversity indices indicated an effective description of the entire fungal community in our study. The overlapping OTU clusters among the treatments (C1, C2, and C3) indicated that C1 and C3 showed the largest OTU overlap compared with C2 (Figure 2B). The three different developments had a total of 74 OTUs (17.4%) in common, and 425 OTUs (82.6%) were identified in each treatment, indicating that the fungal diversity was significantly different during *P. polyphylla* development.

In contrast to our bacterial community results, the oldest rhizomes had the highest fungal species richness and diversity in 4-year-old rhizomes (Table 1). Most of the fungal OTUs identified in the rhizomes were affiliated with the phyla Ascomycota, except for a few reads that were ascribed to the Basidiomycota fungal lineage (Table 2). The relative abundances of Ascomycota made an approximately equal contribution to the development of the three different rhizome ages, but in the 8-year-old rhizomes, Ascomycota made the highest contribution. Species in the phyla Ascomycota are well known for the production of bioactive metabolites that play a role in the mycoparasitic or entomopathogenic behavior of the fungi, as well as in resistance in the plant hosts (Vinale et al., 2008).

At the species identification level, 251 species were found in the 4-year-old rhizomes, 183 species in the 6-year-old rhizomes, and 242 species in the 8-year-old rhizomes. Of all the fungal

species identified, only 74 were found to be in the core fungal community among the three sample types in this study (Figure 2B). The majority of core fungal species were members of the Nectriaceae and Plectosphaerellaceae families. At the species level of the core fungal community, high abundances of Fusarium and Plectosphaerella were found in all three samples, but Fusarium (>10%) was highest in the 4-year-old rhizomes, and Plectosphaerella (>2%) was highest in the 8-year-old rhizomes. Fusarium from an endophytic fungus of corn (*Zea mays*), has been reported to metabolize 2-benzoxazolinone (BOA) and 6-methoxy-benzoxazolinone (MBOA) (Yue et al., 1998). The study also showed that *Fusarium* sp may cause *in vitro* wilting by colonizing the xylem conductor system in *Hyptis marrubioides* roots (Annis et al., 1997), although further studies are definitely required. Our fungal diversity data highlight the need for further studies on the fungi that colonize *P. polyphylla* rhizomes and their roles in secondary metabolism. To our knowledge, this is the first exploration of the entire fungal community in *P. polyphylla* rhizomes. These findings suggest that rhizome fungi may have a potential role in promoting secondary metabolism. Therefore, the ecological relationship between bacteria and fungi in *P. polyphylla* rhizomes requires further investigation.

Table 2. Summary of the internal transcribed spacer (ITS) sequencing data obtained from the *Paris polyphylla* samples.

Samples	Number of reads	Number of OTUs[a]	Good coverage[a]	Richness estimator[a]		Diversity index[a]	
				Chao1	ACE	Shannon	Simpson
C1	70,419	251	0.99	286.83	288.12	3.11	0.78
C2	82,192	183	0.99	210.85	217.52	3.14	0.78
C3	61,767	242	0.99	249.89	257.61	4.45	0.92

OTUs = operational taxonomic units; ACE = abundance-based coverage estimators. [a]Calculated at a 97% sequence similarity cut-off.

Conglong et al. (2011) showed that older *P. polyphylla* specimens have a higher saponin content. Increased saponin concentration is associated with the prevalence of most endophytic fungi. Even though the evidence is still not incontrovertible, the possibility that the bioactive metabolites found in medical plants may derive from the activity of endophytic microorganisms cannot be excluded; endophytes may promote plant health and growth, elicit plant metabolism, or directly produce biotechnologically relevant compounds (Brader et al., 2014). Moreover, Zhao et al. (2010) found that the metabolites derived from the endophytes found in *Pichia guilliermondii* were potential antimicrobial agents, which confirms our understanding of the relationship between the endophytes and their host. In this context, the characterization of uncultivatable microorganism communities is a fundamental step towards the production of natural antimicrobial agents because it paves the way to understanding the important role played by endophytes in plant metabolism. In our opinion, the data obtained in this work offer a preliminary but very promising example of the biotechnological potential of microorganisms. In conclusion, this is the first study to characterize concurrently bacterial and fungal communities in *P. polyphylla* rhizomes of varying age using 16S rRNA pyrotag sequencing. Further investigation focusing on the functional characteristics of microbiota is critically important for understanding the composition and activity of the intestinal microbes associated with ageing in *P. polyphylla* rhizomes. Furthermore, the isolation of beneficial prokaryotes and eukaryotes may prove useful in the development of *P. polyphylla* rhizome metabolism studies.

Conflicts of interest

The authors declare no conflict of interest.

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