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Full Length Article

Castanea henryi Roots Serve as Host for *Ganoderma lucidum*

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Abstract

A new fungal strain was isolated from a fruiting body of *Ganoderma* Karst. grown in Chongyi county, Jiangxi Province, China. Morphological characterization and ITS rDNA sequence analysis suggested that the strain belongs to *Ganoderma lucidum* Karst., and we designated it as strain Gl8. Methods for culturing Gl8 and *Castanea henryi* (Skan) Rehd. Et Wils cuttings were established, and the ability of *C. henryi* cuttings serve as host for *G. lucidum* was studied *in vitro*. Ten days after inoculation, Gl8 grew freely on the living roots of *C. henryi* and the co-cultured mycelium formed fruiting body primordium, primitive stalk and primitive cap compared to free living mycelia. In inoculated cutting roots, transmission electron microscopy (TEM) showed that hyphae colonized the epidermal and cortical cells which retained their intact cell wall, cytoplasm, and organelles. There was no cell disintegration observed, and no hyphae were found in the apoplastic spaces. No mature fruiting bodies were observed in co-cultured and free-living mycelium at the end of the trial. TEM also showed that co-cultured roots had obvious cytoclasis. Our results indicated that Gl8 could inoculate living *C. henryi* roots and live endophytically in early stages, and *C. henryi* could promote Gl8 differentiation into the primordium stage of fruiting body development. © 2019 Friends Science Publishers

Keywords: Fungi; Fruiting body development; Cuttings; Saprophytic; Parasitic

Introduction

Ganoderma Karst. is a genus of Basidiomycota including both saprophytic and parasitic fungi, and is distributed worldwide (Loyd *et al*., 2018a; Tchoumi *et al*., 2018). To date, 250 *Ganoderma* species have been described all over the world (Zhou, 2017). *Ganoderma lucidum* is a species of *Ganoderma* with medicinal value. It was first recorded approximately two thousand years ago in 'The Shen Nong Herbal', a classical textbook of oriental medical science, and is now recognized by the Therapeutic Compendium and the American Herbal Pharmacopoeia (Sanodiya *et al*., 2009; Zhang *et al*., 2017). In recent years, more attention has been paid to its medicinal and biotechnological effects, since species of the genus *Ganoderma* can act as biofactories for producing pharmacologically active secondary metabolites such as bioactive triterpenoids, polysaccharides, oligosaccharides, and ganoderic-acid (Shiao, 2003; Hajjaj *et al*., 2005; Shi *et al*., 2010; Kues *et al*., 2015; Zhang *et al*., 2017; 2018a,b), and act as producers of ligninolytic enzymes applicable in numerous processes (Dias *et al*., 2010; Liu *et al*., 2012; Ćilerdžić *et al*., 2016). To meet market and research demands, artificial cultivation of *Ganoderma* has gradually spread from China, Japan, and the United States to all over the world (Zhou, 2017). All cultivation methods are based on its saprophytic lifestyle. Though cultivation methods have improved constantly, almost all growers now prefer to adopt wood-log cultivation or substitute cultivation to produce fruiting bodies of *G. lucidum*. Production of fruiting bodies involves many steps, including tree harvest, preparation and sterilization of substrate, bagging and sterilization, spawn development and embedding in substrate, and transfer into mushroom house for maturation (Zhou, 2017). However, *Ganoderma* species are primarily white rot fungi found on dead trees and on live trees (Loyd *et al*., 2018b; Xing *et al*., 2018) which indicates that we can grow *G. lucidum* using live trees based on the parasitic lifestyle of the fungus.

A study of the collections of laccate species of *Ganoderma* in the U.S. showed that the most frequent host (68%) is hardwood. Among hardwoods, *Castanopsis fargesii*, *C. sclerophylla*, and *C. carlesii* of Fagaceae family are the most suitable species for *Ganoderma* cultivation (Loyd *et al*., 2018a). *Castanea henryi* is an important non-wood species of the *Castanea* genus in the Fagaceae family and Fagales order. *C. henryi* is used for timber and starch production and is widely distributed in southern China with one million hectares of cultivated area (Yang *et al*., 2013; Fan *et al*., 2017; Xiong *et al*., 2018a, b). This hardwood species has

been increasingly used as a woody grain since the nut has several favorable characteristics, including a high starch content of 47.58–56.94% (Zheng *et al*., 2002), a high mineral nutrition content, and 18 amino acids (Fan *et al*., 2015). Additionally, successful cultivation of *Dictyophora echinovolvata*, an edible fungus belonging to family Phallaceae has been reported in *C. henryi* forest land. This new practice of agroforestry is considered a sustainable form of land management that optimizes the use of natural resources (Santiago-Freijanes *et al*., 2018; Wu *et al*., 2018; Yao *et al*., 2018). However, other studies have shown that some *Ganoderma* species could also act as a pathogen causing root and butt-rot disease, for they were regularly seen attached to the base of dying trees (Taylor, 1969; Wood and Ginns, 2006; Tchoumi *et al*., 2018).

In order to provide a higher biomass production per unit of land and save the cost of artificial cultivation, rooted cuttings of *C. henryi* were used as the host to cultivate *G. lucidum in vitro.* Thus, the goal of this research is to study the feasibility of a new cultivation model of *G. lucidum*. The major objectives were 1)- to determine whether *G. lucidum* could inoculate living *C. henryi* roots and 2)- to investigate the relationship between *G. lucidum* and *C. henryi* when co-cultured.

Materials and Methods

Isolation of the Fungi

Fruiting bodies were collected from roots of *Castanopsis chunii* Cheng tree in Shiluo Forestry Station, a natural preserve of broad-leaved trees in Chongyi county, Jiangxi Province, China. At an altitude of 480 m, the thickness of soil humus was 2–3 cm. *Castanopsis* and *Choerospondias* (30–40 years old) were the dominant tree species, and *Castanea*, *Schima*, and *Quercus* as updated species, with a canopy density between 0.6 and 0.7. Collected fruiting bodies were placed in plastic bags and stored at 4°C until later use of fungal isolation.

Fungi were isolated from fruiting bodies by direct plating. The collected sample was soaked in cool tap water and washed gently to remove excess soil. The sample surface was sterilized with 75% ethanol for 10 s, and then rinsed four times in sterile distilled water. Using an inoculation loop, small pieces of fruiting bodies were collected from the inside of the stalk, plated on potato dextrose agar (PDA; 200 g/l potato, 20 g/l glucose, 3 g/l KH_2PO_4, 1.5 g/l $MgSO_4$. $7H_2O$; 7 g/l agar), and cultured in the dark at 28°C for several days (Chen *et al*., 2017). Plates were observed periodically and selected filamentous fungal colonies were re-inoculated at least five times to remove contaminants or undesirable colonies.

Morphological Identification

Morphological characteristics including colony diameter, color, thickness, texture, and pigmentation, and the colony color on the reverse side were examined after the fungus was cultured on PDA for 7 days in the dark at 28°C.

Molecular Identification

The internal transcribed spacer (ITS) region was amplified using the ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers with thermocycling conditions of an initial step of 95°C for 3 min followed with 35 cycles of 95°C for 40 s, 54°C for 45 s, and 72°C for 1 min, and a last step of 72°C for 10 min. PCR products were purified and sequenced at Majorbio co. (Shanghai, China), using forward and reverse PCR primers.

Phylogenetic Trees

The G18 sequence was submitted to the NCBI database and sequences of most closely matching the G18 sequence were selected using the Basic Local Alignment Search Tool (BLAST). Sequences including Gl8 and closely-related fungi from NCBI database were analyzed by neighbor joining method using distances from Kimura's two-parameter model with the MEGA X.0 software system which performed 1,000 bootstrap replicates to assess support for nodes (Zhang and Yao, 2019).

Plant Material and Growth Conditions

Seeds of *Castanea henryi* cv. Huali 1 were collected from a chinquapin experimental field at Central South Forestry University of Science and Technology in Rucheng county, Chenzhou city, Hunan province (25°33′N, 113°45′E). Seeds were surface sterilized, and part of the cotyledons were excised before seed embryos were placed on aseptic MS (Murashige and Skoog, 1962) medium in glass culture tubes under white fluorescent lamps (50 mol/m^2 s) with a photoperiod/dark period of 14/10 h at a temperature of 25 ± 2°C (Xiong *et al*., 2018b). The culture conditions (light and temperature) were kept constant for all the following described experiments. Three weeks later, seedlings of truncated hypocotyls were then transferred to MS medium containing 1.5 mg/l indole butyric acid for four weeks to induce adventitious roots (Xiong *et al*., 2018b).

Plant/Fungus Co-cultures

Rooted cuttings were transferred to conical flasks containing solidified agar (12 g/l agar) of low-carbon medium (PAD/4 + MS/4) and a pH 5.8. Mycelium of Gl8, cultured on PDA for 1 week, was collected using a sterile 5-mm cork borer. Two or three mycelium disks per flask were used to inoculate roots of chinquapin cuttings. Ten flasks were inoculated with Gl8, ten were used as control without inoculation (free living cuttings), and ten were used as control without cuttings (free living mycelium). The

lower parts of all flasks were covered with silver paper to protect the roots from light. The experiment was arranged as a randomized completed block design with ten replications (one plate is one replication).

Microscopic Observation

In order to ascertain the relationship between Gl8 and *C. henryi*, root segments surrounding the mycelium were fixed in 2.5% (w/v) glutaraldehyde in phosphate-buffered saline (PBS; pH 7) overnight at 4°C. Using the paraffin sectioning method, cut sections were observed using an optical microscope (BX-51, Olympus, Tokyo, Japan) and transmission electron microscopy (TEM; Hitachi TEM System 7700, Japan) (Gao *et al*., 2018).

Results

Morphological Characteristic

Gl8 formed a thin white colony after incubation in the dark at 28°C for 7 days on PDA medium. Colony diameter ranged from 45 to 50 mm (Fig. 1A). Generative hyphae were colorless, thin-walled, with lots of clamp connections, occasionally branched, and 2–4 μm in diameter (Fig. 1B and 1C). Hyphae tips with treelike branches were viewed under the microscope (Fig. 1B and 1D).

Molecular Characteristics

Molecular analysis of Gl8 resulted in a 599 bp ITS rDNA sequence (Fig. 2). The ITS rDNA sequence was compared to available sequences obtained by BLAST from the GenBank database. The neighbor-joining phylogenetic tree showed that the sequence has 99.9% identity to the sequence of *G. lucidum* strain P2 and strain C6, which is supported by a bootstrap of 100% (Fig. 3).

Development of the Fungi when Co-cultured with Cuttings

After 10 days of co-culture, roots of inoculated *C. henryi* cuttings were wrapped by hyphae, cuttings were healthy with green leaves with an increased height of approximately 4 cm (Fig. 4A), and with special hyphae structures on the wrapped roots (Fig. 4B–C). Stereomicroscope observations showed that hyphae was at the mulberry stage of fruiting body development, as there were a large number of white and brown particles called fruiting body primordium shaped like mulberries (Fig. 4D). Stereomicroscope observations also showed the hyphae were at the coral period of development where parts of the primordium continued to grow while other parts were shrinking. The growth primordium, that was thick on top and thin below, developed into primitive stalks shaped like coral. Meanwhile, the growth primordium shaped like a fan appearing at the top is called the primitive cap (Fig. 4E–F).

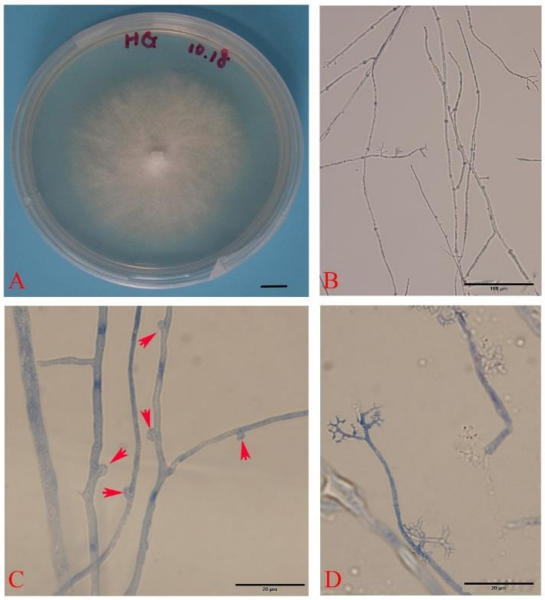

Fig. 1: Morphological characteristics of the fungus Gl8. **A**: Colony of Gl8 cultured on PDA medium for 7 days. **B**: Hyphae morphology and branching. **C**: Hyphae present at clamp connections (arrows). **D**: Hyphae tips with treelike branches. Scale bars: 1 cm (**A**), 100 μm (**B**), 20 μm (**C, D**)

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1   AGCCGTCGCTTGACGGGTGTAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCAC
61  TCTACACCTGTGCACTTACTGTGGGCTTCAGATTGCGAGGCACGCTCTTTACCGGGCTTG
121 CGGAGCATATCTGTGCCTGCGTTTATCACAAACTCTATAAAGTAACAGAATGTGTATTGC
181 GATGTAACACATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAA
241 GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
301 TGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAAT
361 CTTCAACCTACAAGCTTTTGTGGTTTGTAGGCTTGGACTTGGAGGCTTGTCGGCCGTTAT
421 CGGTCGGCTCCTCTTAAAATGCATTAGCTTGGTTCCTTGCGGATCGGCTCTCGGTGTGATA
481 ACGTCTACGCCGCGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCTTATAAGACAGCTT
541 TATGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAAAAG
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Fig. 2: ITS rDNA sequence of Gl8

Anatomical Structure of Inoculated Roots

No hyphae were visible in the free-living root cuttings (Fig. 5A). Ten days after inoculation, microscopic observations of inoculated *C. henryi* cuttings showed that roots were infected by hyphae with intracellular hyphae growth observed in epidermal and cortical cells (Fig. 5B). TEM observations also showed that the hyphae inoculated the epidermal and cortical cells which maintained an intact cell wall, cytoplasm, and organelles, and no cellular disintegration (Fig. 5C and 5E); while the root hair cells were becoming deformed (Fig. 5D). Hyphae were observed to cross the cell membrane into another cell for proliferation, while no hyphae were observed in the apoplastic spaces, and the host cell walls were intact (Fig. 5E). Thirty days after inoculation, TEM showed that the hyphae colonized the intra- and intercellular spaces, and that root cells have obvious cytoclasis (Fig. 5F).

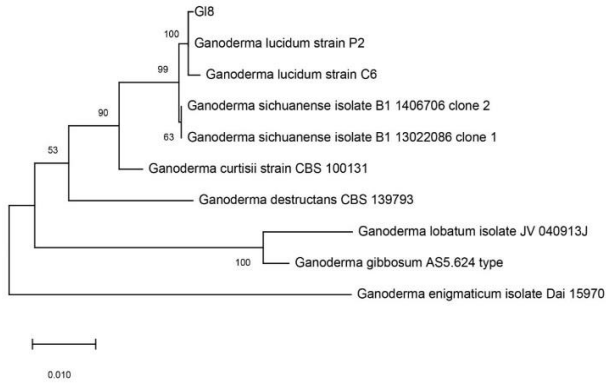

Fig. 3: Phylogenetic tree based on ITS rDNA sequence data of Gl8. The phylogenetic tree was constructed using the neighbor-joining method within MEGA software (version X). Numerical values above the branches indicate bootstrap percentiles from 1000 replicates, bootstrap numbers over 50% are indicated

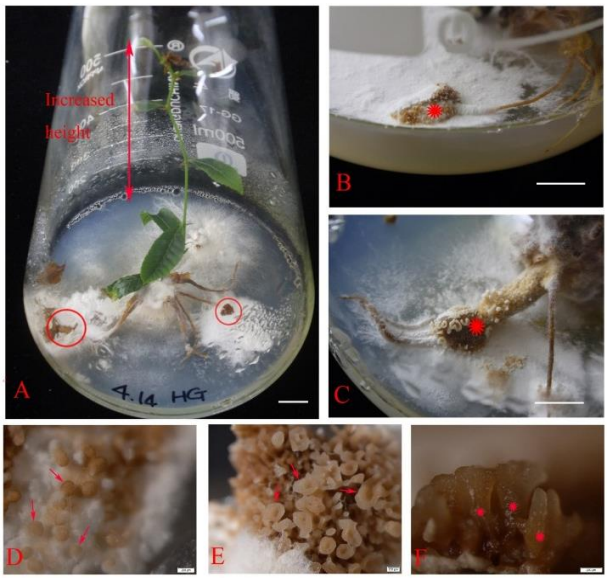

Fig. 4: Photographs illustrating the fungus Gl8 development process when co-cultured with *C. henry* cuttings. **A**: Rooted cuttings were transferred to conical flasks, and their roots were inoculated by Gl8 mycelium disks. 10 days later, the seedlings have grown taller (increased height) and with special mycelium structure wrapped around the roots (circle). Scale bar: 1 cm. **B** and **C**: Closeup of the special mycelium structure (stars) shown in Figure 4A. Scale bars: 1 cm. **D**: Additional enlargement of the region circled in Figure 4A showing the fruiting body primordium shaped like mulberries (arrows). Scale bar: 200 μm. **E**: Additional enlargement of the region circled in Figure 4A showing the primitive cap (arrows). Scale bar: 200 μm. **F**: A medium longitudinal section of the region shown in Figure 4E showing the primitive stalk shaped like coral (stars). Scale bar: 200 μm

Discussion

The isolated strain reproduced using clamp connections, which were a hyphal protrusion that develop during cell

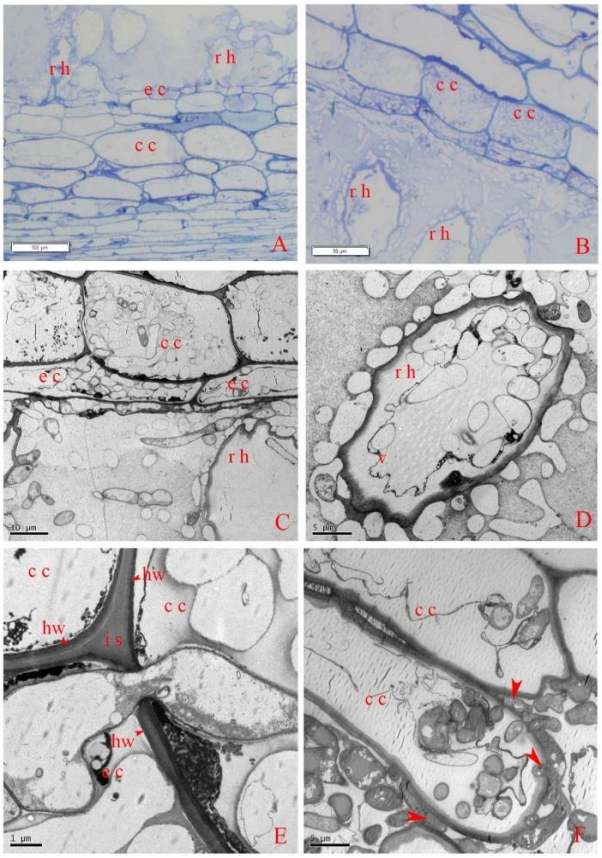

Fig. 5: Anatomical structures of *C. henryi* root cuttings inoculated with Gl8. **A**: Control roots with no hyphae inoculation. Scale bar: 100 μm. **B**: 10 days after inoculation, microscopic observation of hyphae-inoculated epidermal cells (ec) and cortical cells (cc). Scale bar: 50 μm. **C**: TEM of hyphae-inoculated epidermal cells (ec) and cortical cells (cc). Scale bar: 10 μm. **D**: hyphae-inoculated root hair cells (rh). Scale bar: 5 μm. **E**: An enlargement of the section shown in C. Hyphae crosses the cell membrane into another cell; hyphae branched inactively; only one septum in the whole area visible and no hyphae visible in the intercellular space (is). Host wall (hw; arrows) is intact. Scale bar: 1 μm. **F**: As the infection progresses, there is widespread attack of the host cell walls (arrows) from intracellular and transmural hyphae. The hyphae colonize intra- and intercellular, root cells have cytoclasis

division to maintain the dikaryon condition (Krings *et al*., 2011; Taylor *et al*., 2014; Wan *et al*., 2017). ITS rDNA analysis showed a high degree of sequence identity (99%) to *G. lucidum* strain P2 and C6 reported by Chen *et al.* (2017) which places the strain in one cluster in the neighbor joining phylogenetic tree. Thus, the strain was named *G. lucidum* Gl8.

Ganoderma species growth consists of several different stages, such as mycelium, primordium, and mature fruiting body (Zhou, 2017). *C. henryi* cuttings promoted the Gl8 mycelium differentiation into the primordial stage of fruiting body development compared to the free-living *C. henryi* cuttings. Thus, *C. henryi* cuttings could supply some

necessary substance for Gl8 mycelium differentiation. However, further studies are needed to determine which substances from *C. henryi* contributed to mycelium differentiation.

The *in vitro* nutritional source for Gl8 development could be both *C. henryi* cuttings and the medium. On one hand, TEM indicated that the inoculated Gl8 epidermal and cortical cells had intact cell walls. Thus, it is very likely that the hyphae multiplied using nutrients from *C. henryi* and lived endophytically in *C. henryi*. This is supported by Abdullah (2000) who suggested that *G. boninense* is an endophyte in coconuts and by Panchal and Bridge (2005) who detected *Ganoderma* DNA in oil palms (*Elaeis guineensis* Jacq.) exhibiting no symptoms. On the other hand, TEM indicated that the root hair cells were becoming deformed. This phenomenon could be similar to the ectomycorrhizal fungi infecting the host roots causing root hair decay, and the fungi cells could replace root hair cells to absorb nutrients from the medium for plant and mycelium growth (Horan *et al*., 1988; Ditengou *et al*., 2000; Vayssières *et al*., 2015).

Despite reports of some *Ganoderma* species causing wood decay and can be found on dead and living trees, there were many reports demonstrating that some *Ganoderma* species also act as a plant pathogen causing root and butt rot diseases on trees with high economic value such as oil palm, eucalyptus (*Eucalyptus pellita* Muell.), and ornamental forest trees (Paterson, 2007; Gill *et al*., 2016; TchotetTchoumi *et al*., 2018). Ten days after inoculation, TEM indicated that hyphae inoculated the epidermal and cortical cells with no cell disintegration and that host cell walls were still intact. Moreover, the inoculated cuttings had green leaves and an average height increase of approximately 4 cm indicating that Gl8 had not caused any disease. Therefore, Gl8 did not act as a plant pathogen at the early stages of *C. henryi* inoculation. Nevertheless, no fruiting bodies were observed in co-cultured or in free-living mycelium at the end of the study. Thirty days after inoculation, we observed that leaves were turning yellow, and TEM showed that hyphae colonized the intra- and intercellular spaces, and that root cells had obvious cytoclasis, which seemed to indicate that Gl8 can act as a saprophyte or pathogen at this stage. Results in this study were similar to that by Rees *et al*. (2009) on the development and progress of basal stem rot in oil palm roots. Martin *et al*. (2015) found that 75% of the endophytic species in the wood of the genus *Hevea* were in the order Polyporales, which includes *Ganoderma* sp. Others reported that *G. zonatum* can live endophytically in palm trees; however, when tissues become weakened and susceptible to decay, *G. zonatum* would act as a latent saprophyte or pathogen (Martin *et al*., 2015; Song *et al*., 2017).

No mature fruiting bodies were observed in co-cultured and free-living mycelium at the end of the trial. The most logical reason could be that the nutrients and volume in the conical flask are limiting factors for *C. henryi* cuttings and Gl8 primordium growth. In addition, the environment including light, humidity, and temperature might not be suitable for Gl8 to form fruiting bodies. Zhou (2017) reported that each development stage of *G. lucidum* has a unique set of requirements. Tree species produce and release antimicrobial compounds, such as phenolic compounds, resins, and tannins to resist decay caused by fungi (Scheffer and Cowling, 1966; Deflorio *et al*., 2008; Rees *et al*., 2009). The biosynthesis of antimicrobial compounds might be impeded under limited nutrition conditions. In addition, the roots of *C. henryi* cuttings are surrounded by a tough mycelium, which released plant cell wall degrading enzymes such as cellulase, laccases, and manganese peroxidases that are involved in the degradation of host cell wall (Rees *et al*., 2009).

There were no reports on taxa of *Ganoderma* causing *C. henryi* death in nature; however, Gl8 almost acted as a pathogen on *C. henryi in vitro* at the end of this study. Boddy (2000) demonstrated that competition for allocation of a substrate by wood degrading fungi can occur in several ways including: chemical antagonism, mycoparasitism, and biological incompatibility. All these strategies can result with the displacement of a "vulnerable" fungus, or the hindrance of normal physiological function of a given fungus (Boddy, 2000). In *C. henryi* forested land in nature, there are many types of soil microbes, including wood decaying fungi, pathogenic fungi, and mycorrhizal fungi. The order Fagales is likely to be the oldest angiosperm ectomycorrhizal (ECM) group, and > 80% of the Fagales genera are ECM (Larson-Johnson, 2015; Tedersoo and Brundrett, 2017). Furthermore, there were reports showing that many *Castanea* species are ECM plant, including *C. mollissima* (Wan *et al*., 2016), *C. sativa* (Martins *et al*., 1996; Acioli-Santos *et al*., 2008) and *C. henryi* (Liu *et al*., 2016). ECM enhance nutrient uptake and increase host resistance to plant pathogens (Harely and Smith, 1983; Marx, 1969; Pfabel *et al*., 2012). Therefore, Gl8 should not act as a pathogen on *C. henryi* in nature. All the results indicated that further studies are need to better understand the relationship between *C. henryi* and Gl8, and the conditions required for cultivation of Gl8 under *C. henryi* forested land.

Conclusion

The present study isolated a new *G. lucidum* strain from fruiting bodies, which grown under mixed forests with Fagaceae as dominant tree species in south China, and we named it Gl8.The Gl8 was able to colonize the living roots of *C. henryi*, and the colonization permitted the Gl8 to form fruiting body primordium, primitive stalk, and primitive cap. This means that *C. henryi* live trees could potentially be used as a host of *G. lucidum* to improve the cultivation methods, as a new practice of agroforestry.

Acknowledgments

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