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Original Article

Trichoderma species associated with green mold disease of *Ganoderma lingzhi* in Thailand♣

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Abstract

Green mold disease caused by *Trichoderma* species is a serious problem for Lingzhi growers. Recently, green mold disease of lingzhi mushroom caused by unknown species of *Trichoderma* was found in lingzhi farm at Songkhla province, Southern Thailand. Therefore, the aim of this research was to identify the species of the causal agent. *Trichoderma* species were collected from *G. lingzhi* spawn to isolate and identify based on morphological characteristics and DNA sequence of the internal transcribed spacer regions (ITS). The phylogenetic tree was constructed by maximum likelihood analysis base on the ITS sequence comparison with sequences of ex-type culture from closely related taxa. A total of three *Trichoderma* species were identified as *T. harzianum*, *T. pleuroticola* and *T. reesei*. *T. pleuroticola* and *T. reesei* are the first report of green mold disease of *G. lingzhi* in Thailand.

Keywords: lingzhi mushroom, identification, internal transcribed spacers

1. Introduction

Lingzhi or reishi mushroom (Genus: *Ganoderma*, Family: Ganodermataceae, Phylum: Basidiomycota) is a high value medicinal mushroom in China, Japan, and other countries in Asia. It is mainly cultivated for used as herbal medicine due to their medicinal metabolites that include polysaccharides, triterpenes, lucidenic acids, adenosines, ergosterols, glucosamines and cerebrosides. Modern medicinal studies indicated that these metabolites have medicinal properties in the prevention and treatment of diseases such as cancer, chronic bronchitis, diabetes mellitus, hyperlipidemia and hypertension, as well as improvement of immunity and anti-aging (Dai, Yang, Cui, Yu, & Zhou, 2009, Lin, 2009). Lingzhi has long been considered as *G. lucidum* until the morphological and molecular studies revealed that *G. lucidum* distribution in East Asia was actually *G. lingzhi* (Cao, Wu, & Dai, 2012). In Thailand, Lingzhi are popular and cultivated throughout the country. The occurrence of fungal diseases on this mushroom has also been increased. Green mold disease caused by *Trichoderma* species is a serious problem, and its incidence and severity have increased.

Several *Trichoderma* species such as *T. aggressivum*, *T. atroviride*, *T. harzianum T. longibrachiatum T. pleurotum* and *T. pleuroticola* have been reported as green mold pathogen in commercial mushroom farms worldwide (Choi, Hong, & Yadav, 2003; Hatvani, *et al*., 2012; Samuels, Dodd, Gams, Castlebury, & Petrini, 2002). Recently, green mold disease of lingzhi mushroom caused by several species of *Trichoderma* was found in lingzhi farm at Songkhla province, Southern Thailand. Therefore, the aim of this research was to identify the species of the causal agent.

2. Materials and Methods

2.1 Sample collection and isolation

Trichoderma species were isolated from *G. lingzhi* that rubber wood (*Hevea brasilensis*) sawdust was used as the main substrates at lingzhi farm in Songkhla province southern Thailand. *Trichoderma* mycelium on basidiomata and spawn

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bags of *G. lingzhi* showing green mold disease was directly picked up with sterilized needle to suspend in 10 ml sterile distilled water containing 0.2 ml Tween 20. A ten-fold serial dilution was done for single spore isolation. A hyphal tip germinated from single spore was transferred to PDA (potato dextrose agar, HiMedia) and incubated at room temperature (28–30 °C) for seven days. All *Trichoderma* isolates were deposited in the Culture Collection of Pest Management Department, Faculty of Natural Resources, Prince of Songkla University (PSU), Thailand.

2.2 Morphology characterization and observation

Cornmeal dextrose agar (CMD; Himedia supplemented with 20 g of dextrose per liter), PDA and *spezieller nährstoffarmer Agar* (SNA; 1.0 g of KH_2PO_4, 1.0 g of KNO_3, 0.5 g of $MgSO_4 \bullet 7H_2O$, 0.5 g of KCl, 0.2 g of glucose, 0.2 g of sucrose, 20 g of agar, 1 L of distilled water) were used for colony observation (Jaklitsch, 2009). Morphological characteristics, which were such as chlamydospores, conidiophore, conidia and phialides were observed under the light microscope (Leica DM750) and measured with Leica application suite (LAS software version 4.9.0). Identifications were performed by using the identification keys provides by Barnett and Hunter (1909), Domsch, Gams, and Anderson (1980) and Samuels and Hebbar (2015).

2.3 DNA isolation, PCR amplification and sequence analysis

Fungal cultures were grown on PDA at room temperature for two days. Three pieces of mycelia with agar medium were excised with 5 mm cork borer and transferred to a microtube for DNA extraction. Fungal genomic DNA was extracted from mycelium by the mini preparation method (Saitoh, Togashi, & Arie, 2006). The internal transcribed spacer regions (ITS) were amplified using the primer pair ITS1/ITS4 (White, Bruns, Lee, & Taylor, 1990) by polymerase chain reaction technique (PCR) in 25 µL reaction volume containing 10 pmol of each primer, 2× Dreamtaq Green PCR Master Mix (Thermo Scientific) and 50 ng of DNA template using a T100TM thermocycler (BIO-RAD, USA). The amplification procedures were as follows: initial denaturation 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, with the final extension of 10 min at 72 °C. PCR products were purified and sequenced by Macrogen Corporation (South Korea).

Sequences obtained were compared with known sequences available in Genbank (The National Center of Biological Information) using BLASTN and the reference databases of *Trich*OKEY (ISTH, 2008). For phylogenetic analyses, the nucleotide sequences were aligned with sequences of ex-type cultures from closely related taxa (*Trichoderma* Harzianum and Longibrachiatum clades) (Visagie *et al*., 2015) using MUSCLE of the MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura., 2018) and manual adjustments were applied when necessary. Further, the data were analyzed using the maximum likelihood (ML) with partial deletion of gaps and 1,000 bootstrap replicates.

3. Results and Discussion

3.1 Morphology identification

Basidiomata and spawn bags of *G. lingzhi* were initially colonized by white mycelium of *Trichoderma* species, which later conspicuously visible mass of green conidia on the surface (Figure 1A, B) and the infected basidiomata were withered or dried with hollow inside. (Figure 1C).

Twenty-four isolates of *Trichoderma* species were isolated from basidiomata and spawn bags of *G. lingzhi* that were covered with green conidia of *Trichoderma* species. Based on morphological characteristics described by Kubicek and Harman (1998) and Samuels and Hebbar (2015), the *Trichoderma* isolates were divided into two sections. Sixteen isolates were considered as *Trichoderma* species in section *Pachybasium* and the remainder in section *longibrachiatum*. Two morphologically distinct types of colony color and conidial shape have been found in the isolated *Trichoderma* section *Pachybasium*, whereas the morphological differences of all isolates in section *longibrachiatum* were no discovered (Table 1). The results revealed that the causal agent of green mold disease on *G. lingzhi* was probably caused by three species of *Trichoderma* but the accurate identification to the species level could not be done. Species identification of *Trichoderma* using morphological characters was difficult due to the high similarity of morphological characters. However, currently the molecular methods based on DNA sequence analysis were developed to identify every *Trichoderma* isolate at the species level (Lee, Jung, Hong, Choi, & Ryu, 2020; Tomah, Alamer, Li, & Zhang, 2020).

All *Trichoderma* isolates were then deposited in Culture Collection of Pest Management Department, Faculty of Natural Resources, Prince of Songkla University, Thailand with accession number PSU-T-GL01 to PSU-T-GL24. The bold isolates in Table 1 (T-GL1, T-GL6 and T-GL15) were used as the representative isolates for identification by DNA sequencing.

Figure 1. Symptom of green mold disease caused by *Trichoderma* species. (A) mycelium of *Ganoderma lingzhi* in spawn covered with white mycelium and green conidia of *Trichoderma* species, (B) green conidia of *Trichoderma* species on basidiocarp, (C) the infected *Ganoderma lingzhi* showing withered basidiocarp with hollow inside

Table 1. Colony and microscopic characteristics of *Trichoderma* species

Isolates code*	Colony morphology and microscopic observation	Section
T-GL4, **T-GL15**, T-GL16, T-GL21, T-GL22, T-GL 24	Colony; yellowish-green to dark green. Conidiophores comprised of 3 branches. Phialides ampulliform to lageniform. Conidia; subhyaline to pale green, 1 – celled, subglobose to obovoid, 2.5–3.5 x 2.0–2.6 µm.	sect. *Pachybasium*
T-GL1, T-GL2, T-GL3, T-GL5, T-GL8, T-GL9, T-GL10, T-GL11, T-GL20, T-GL 23	Colony; green to dark green. Conidiophores comprised of 2–4 branches. Phialides ampulliform. Conidia; subhyaline to green, 1–celled, ellipsoidal to obovoid, 2.7–4.1 x 1.72–2.3 µm.	sect. *Pachybasium*
T-GL6, T-GL7, T-GL12, T-GL13**,** T-GL14, T-GL17, T-GL18, T-GL19	Colony; pale yellowish-green. Conidiophores sparingly branches. Phialides cylindrical. Conidia; pale green, 1–celled, ellipsoid, 3.6–4.6 x 2.32–3.12 µm.	sect. *Longibrachiatum*

* The representative isolates used in molecular identification by DNA sequence are marked in bold.

3.2 Molecular identification and phylogenetic analyses

Molecular identification of *Trichoderma* species was performed in the *Trich*OKEY, combined with traditional blast searches in the GenBank. The T-GL1, T-GL6 and T-GL15 isolates were identified with high-confidence by *Trich*OKEY and 99% identity in BLAST search as *T. pleuoticola*, *T. reesei* and *T. harzianum*, respectively. The ITS sequences were deposited into GenBank with accession numbers LC596097, LC596098, LC596099 (Table 2) and morphological characteristics of the representative isolates of *Trichoderma* species were shown (Figures 2, 3, 4).

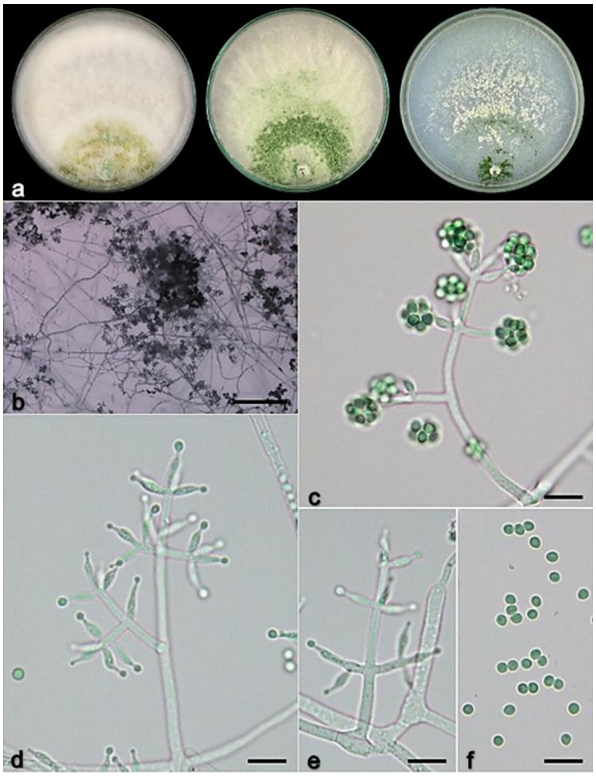

Figure 2. Morphology of *Trichoderma harzianum* T-GL15. (a) Seven-day-old cultures incubated at room temperature (left: on PDA; middle on CMD; right on SNA). (b) conidiation on growth plate (SNA, 7 days). (c-e) conidiophores. (f) conidia. Scale bars: b = 1 mm. c-f =10 µm

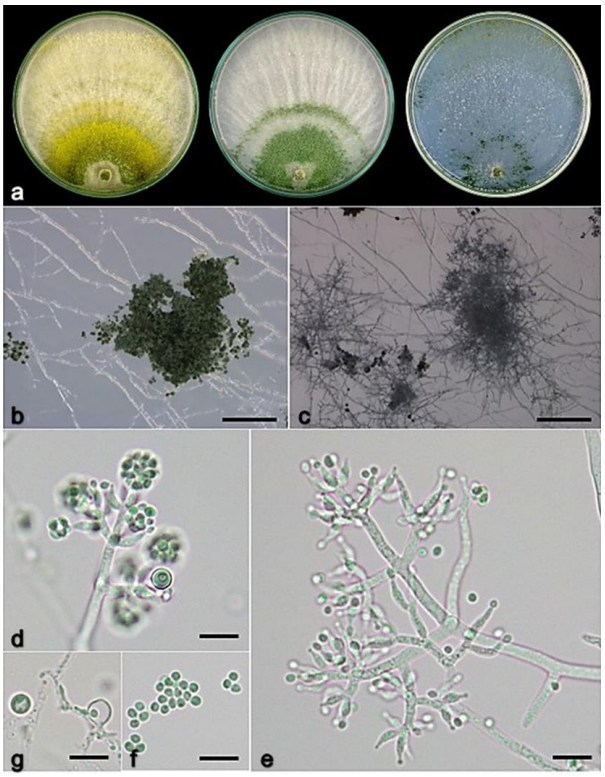

Figure 3. Morphology of *Trichoderma pleuroticola* T-GL1. (a) Seven-day-old cultures incubated at room temperature (left: on PDA; middle: on CMD; right: on SNA). (b) conidiation pustules on growth plate (SNA, 7 days). (c) conidiation on growth plate (SNA, 7 days). (d, e) conidiophores. (f) conidia. (g) chlamydospores. Scale bars: b, c = 1 mm. d-g =10 µm

According to the phylogenetic relationships, our *Trichoderma* species T-GL1, T-GL6 and T-GL15 were clearly confirmed with ex-type as *T. pleuroticola* closely related to *T. pleuroticola* CBS 124383, *T. reesei* CBS 383.78 and *T. harzianum* CBS 226.95, respectively (Figure 5). Previously, *T. atroviride*, *T. harzianum*, *T. hengshanicum*, *T. Longi brachiatum* have been reported as green mold pathogen on *G. lingzhi* in China (Cai, Idrees, Zhou, Zhang, & Xu, 2020; Lu, Zuo, Liu, Feng, & Wang, 2016; Yan, Zhang, Moodley, Zhang, & Xu, 2019; Zhang, Lu, Zhangchunlan, & Jize, 2019), whereas *T. pleuroticola* has been reported as green mold

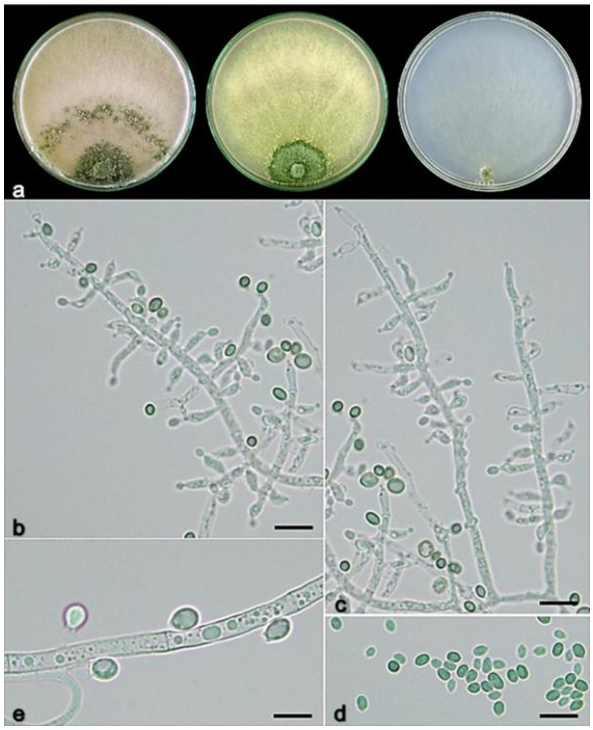

Figure 4. Morphology of *Trichoderma reesei* T-GL6. (a) Seven-day-old cultures incubated at room temperature (left: on PDA; middle: on CMD; right: on SNA). (b, c) conidiophores. (d) conidia. (e) chlamydospores. Scale bars: b-e =10 µm

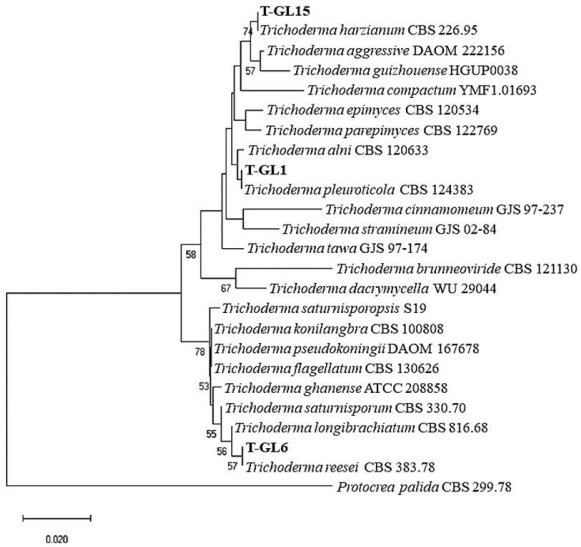

Figure 5. Maximum likelihood (ML) phylogenetic tree of *Trichoderma* including related species from GenBank, based on the internal transcribed spacer sequences. Bold letters indicate our isolates. *Protocrea pallida* CBS 299.78 is used as outgroup. ML bootstrap support ≥ 50%

pathogen on *Agaricus bisporus* and *Pleurotus ostreatus* in Europe (Kredics *et al*., 2009, 2010). There have been no reports of *T. pleuroticola* and *T. reesei* causing green mold disease on *G. lingzhi* in Thailand or anywhere else, so this is the first such report.

4. Conclusions

The present study revealed that green mold disease on *G. lingzhi* in Thailand caused by *T. harzianum*, *T. pleuroticola* and *T. reesei*. In order to control the green mold pathogens, the physiology of the three species of *Trichoderma* pathogens and the origin of the disease at mushroom farms will be examined to design the efficient control strategies and preventive measures. *T. harzianum* was the most popular antagonistic fungi for controlling plant disease in Thailand. Therefore, farmers should use the antagonistic *Trichoderma* species with caution.

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Table 2. Species identification, accession number and figures of the representative isolates of *Trichoderma* species in this study

Isolate	Section	Suggested Species	ITS GenBank Accession No.	Figure
T-GL4	sect. *Pachybasium*	*Trichoderma harzianum*	LC596097	2
T-GL1	sect. *Pachybasium*	*Trichoderma pleuroticola*	LC596098	3
T-GL6	sect. *Longibrachiatum*	*Trichoderma reesei*	LC596099	4

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