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Fructification of *Antrodia cinnamomea* Was Strain Dependent in Malt Extract Media and Involved Specific Gene Expression

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Antrodia cinnamomea is an expensive medicinal fungus that grows only inside the rotten trunk of *Cinnamomum kanehirae*. In vitro culture of *A. cinnamomea* fruiting body is difficult and, therefore, of value for further investigation. To study whether the fructification of *A. cinnamomea* is strain dependent in artificial media, we grew four different *A. cinnamomea* strains on malt extract agar (MEA) media. The standard MEA and a series of dilution of the MEA nutrient components were made to culture *A. cinnamomea*. The formation of fruiting body was determined by visual and microscopic observation on *A. cinnamomea*'s porous morphogenesis and HPLC analysis. All *A. cinnamomea* strains cultured grew best in 50% MEA, but carried different capabilities of fructification. In addition, we studied four antioxidation- or senescence-related genes, including a cytochrome P450, a glutathione-*S*-transferase, a peroxiredoxin, and a manganese superoxide dismutase. We found both cytochrome P450 and glutathione-*S*-transferase were expressed 3.66- and 2.75-fold in fruiting body compared with mycelium, respectively, and perxoiredoxin and manganese superoxide dismutase were found with similar expressions in both fruiting body and mycelium.

KEYWORDS: *Antrodia cinnamomea*; fructification; gene expression; malt extract agar; medicinal fungus

INTRODUCTION

Antrodia cinnamomea is a medicinal fungus naturally grown inside the rotten trunk of *Cinnamomum kanehirae*, a native tree species of Taiwan (*1*−*3*). Medicinal uses of *A. cinnamomea* have been reported in many studies; these include antioxidant (*4*, *5*), anticancer (*6*), antivirus (*7*), and antibiotic (*6*) activities. Because of its broad spectrum of medicinal application and extremely slow growth rate, *A. cinnamomea* is now considered to be one of the most expensive herbal medicines in the market.

The high-demand situation is also causing a serious conservation issue because wild *A. cinnamomea* fruiting body has been massively harvested by cutting off the *C. kanehirae* trunk and endangering the unique tree species in Taiwan. To solve the conservation issue and utilize the medicinal benefit, scientists from the academic and pharmaceutical areas have been intensively working to develop the products of *A. cinnamomea* in laboratories. One major approach of the laboratory study is to culture the fungus in hyphae forms and optimize the chemical composition, especially production of secondary metabolites, by culturing the hyphae in the format of a bioreactor (*5*, *8*). Several studies have shown this approach might be feasible and, therefore, many commercial products have been produced.

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Another approach is to mimic the fungal growth condition in the laboratory to culture the fungal fruiting body in solid media. This approach successfully grew *A. cinnamomea* fruiting body on *C. kanehirae* trunk (*9*) but not other plant species. In addition, Chang and Wang (*10*) reported an in vitro fruiting body formation of an *A. cinnamomea* isolate on malt extract agar (MEA) and potato dextrose agar (PDA) media. The fruiting body was also reportedly inducible through a novel wounding treatment (*9*) and contact of different varieties of *A. cinnamomea* (*10*).

Some evidence has indicated that fructification of *A. cinnamomea* in artificial media was strain dependent (*9*, *10*). To confirm this hypothesis, we tested four different *A. cinnamomea* strains on various concentrations of MEA media. In this study, we adjusted our MEA culture to search for the best MEA concentration for *A. cinnamomea* fruiting body formation. Furthermore, we analyzed several gene expression patterns which may be related to antioxidation or senescence processes, hoping to provide insight at molecular levels in *A. cinnamomea* frutification and its medicinal activities.

MATERIALS AND METHODS

Fungal Strains and Chemicals. Strains ACI3, A, and B of *A. cinnamomea* were isolated from rotten *C. kanehirae* trunks containing *A. cinnamomea* fruiting body and identified as *A. cinnamomea* on the basis of its morphological features. *A. cinnamomea* strain BCRC35398 was purchased from Food Industry Research and Development Institute, Hsinchu, Taiwan. In addition, a molecular comparison of tested strains

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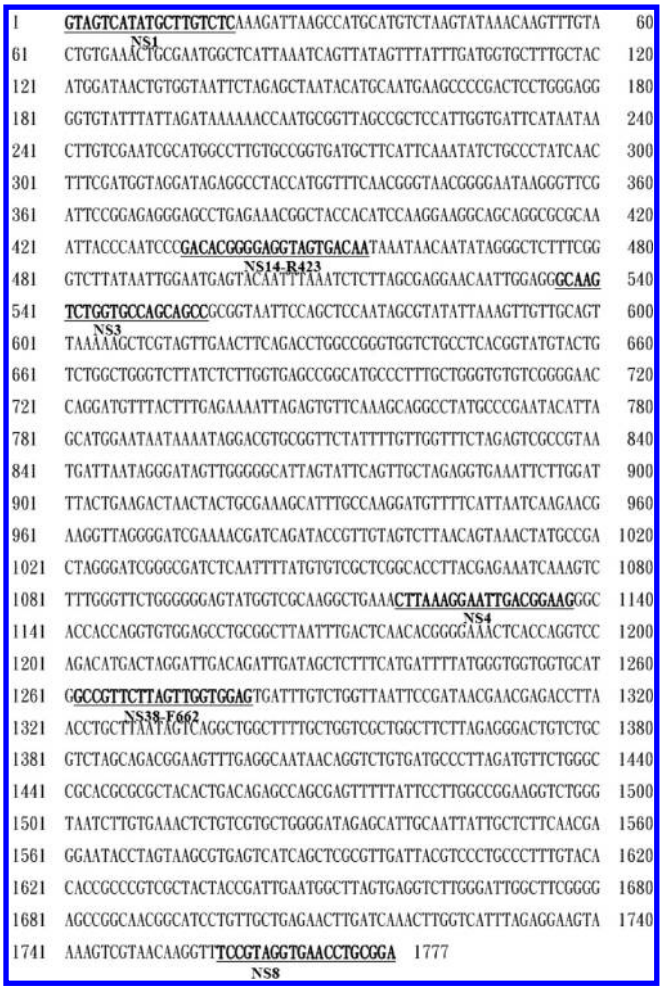

Figure 1. Partial DNA sequence of *A. cinnamomea* 18S rRNA gene obtained from PCR based on NS1, NS3, NS4 and NS8 as the PCR primers. Also, the sequences of NS14-R423 and NS38-F662 in addition to NS1, NS3, NS4, and NS8 were primers used for DNA sequencing.

was performed to confirm the taxonomic identification as described in the following section. All of the chemicals used were of analytical grade or higher.

Genomic DNA Extraction and Molecular Identification of *A. cinnamomea* Based on 18S rRNA DNA Sequences. Genomic DNA of *A. cinnamomea* was purified on the basis of the protocol of QIAamp DNA Mini Kit (catalog no. 51304, Qiagen Inc., Valencia, CA). Four universal PCR primers were synthesized for 18S rRNA DNA amplification based on the report of White et al. (*11*). The primers were NS1 (GTA GTC ATA TGC TTG TCT C), NS3 (GCA AGT CTG GTG CCA GCA GCC), NS4 (CTT CCG TCA ATT CCT TTA AG), and NS8 (TCC GCA GGT TCA CCT ACG GA). The PCR thermocycling program was 98 °C for 2 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s, and 72 °C for 2 min, and a final step of 72 °C for 10 min. The PCR products were analyzed on 1% TAE agarose gel and purified on the basis of the protocol of the Qiagen MiniElute Gel Extraction Kit (catalog no. 28606, Qiagen Inc.). The purified DNA was subjected to DNA sequencing analysis provided by Genomics BioSci and Tech (Taipei, Taiwan) using NS1, NS14-R423, NS3, NS4, NS38-F662, and NS8 as sequencing primers The primer sequences are shown in **Figure 1**. Nucleotide sequence alignment was performed using the software tools of the EMBL-EBI Web site (http://www.ebi.ac.uk).

Growth of *A. cinnamomea*. The 100% MEA plates were prepared on the basis of Blakeslee's composition (1 L of medium containing 20 g of malt extract, 20 g of glucose, 1 g of peptone, and 20 g of agar). Subsequently, 50, 25, and 10% MEA plates were prepared by reducing the nutrient components of malt extract, glucose, and peptone proportionally. The 50% MEA was found to be the best to grow all four

Table 1. Primer Sequences Used for Real-Time PCR Analysis of Selected *A. cinnamomea* Genes

gene name	primer sequence[a]
cytochrome P450 (accession no. AY632358)	F: TTG ACC CAT TCC GCT TCT CA
	R: TCC CAT GTC CGA AGG CAA T
glutathione-*S*-transferase (accession no. AY632357)	F: TTG GCG AGA CCG ATT CAA AG
	R: CGG CGA GGT ACT TCG TCT TAG A
peroxiredoxin (accession no. AY383653)	F: GAA CAT GGT GAG GTT TGC CCT
	R: ATG ACC ATT TGC ACC CGC T
manganese superoxide dismutase (accession no. AY279973)	F: GGT GTC GAT GGC TTG AAG AAG A
	R: CGG TTG TGA CAA CCT CAA GCT T
18S ribosomal RNA (accession no. EU077561)	F: CGC AAG GCT GAA ACT TAA AGG A
	R: TGT CTG GAC CTG GTG AGT TTC C

[a] F, forward primer; R, reverse primer.

A. cinnamomea strains and was used to maintain the *A. cinnamomea* mycelia. The MEA plates inoculated with *A. cinnamomea* mycelia were kept in an incubator at 25 °C.

Total RNA Extraction and Reverse Transcription Experiments. A Trizol reagent kit (catalog no. 15596-018, Invitrogen, Carlsbad, CA) was used for total RNA extraction. In short, 1.2 g (fresh weight) of *A. cinnamomea* mycelia or fruiting body was collected and ground to powder in liquid N_2. For 50 mg of sample, 1 mL of Trizol reagent was added and mixed well at room temperature. Chloroform (0.2 mL) was added and vortexed for 15 s. The sample was, then, subjected to centrifugation at 12000 rpm for 15 min, and the supernatant was collected. Half a milliliter of isopropanol was added and gently mixed. The sample was subjected centrifugation at 12000 rpm for 10 min, and the pellet was collected. The pellet was washed with 1 mL of 75% ethanol and centrifuged at 10000 rpm for 5 min. The supernatant was removed and the pellet air-dried. The pellet was then resuspended in DEPC-treated water as *A. cinnamomea* total RNA and stored at −20 °C freezer. The total RNA was then subjected to reverse transcription experiments. A Verso cDNA kit (catalog no. AB-1453, Thermo Fisher Scientific, Epsom, Surrey, U.K.) was used for reverse transcription experiments based on the manufacturer's protocol.

Real-Time PCR. Real-time PCR was performed to compare the gene expression patterns of four selected genes during the growth stages of mycelium and fruiting body. The genes are a cytochrome P-450, a glutathione-*S*-transferase, a peroxiredoxin, and a manganese superoxide dismutase. The PCR primers were designed on the basis of computer software, Primer Express version 2.0 (Applied Biosystems, Foster City, CA) and listed in **Table 1**. 18S rRNA-F and 18S rRNA-R were PCR primers for the 18S rRNA gene used as internal control (**Table 1**). The real-time PCR mix contained 2 μL of the above reverse transcription product as template, 1.8 μL of each appropriate primer pair, 12.5 μL of Absolute QPCR SYBR Green Mix (ABgene, Taipei, Taiwan), and additional H_2O to a final volume of 25 μL. The reaction condition was 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 15 min, 40 cycles at 95 °C (15 s), and 65 °C (1 min). In addition, the dissociation protocol was set at 60 °C (ABI prism 7000 sequence detection system).

HPLC Analysis of *A. cinnamomea* Methanol Extracts. *A. cinnamomea* mycelia and fruiting bodies were placed in an oven at 60 °C for 24 h to measure their dry weight. The samples were then subjected to 100% methanol extraction (*12*, *13*). The crude methanol extracts were filtered using a 2.2 μm syringe filter and then subjected to HPLC analysis. A 250 × 4.6 mm HyPURITY C18 HPLC column (ThermoHypersil-Keystone, Bellefonte, PA) with a Hitachi L-7100 HPLC pump and a 7240 UV detection system (San Jose, CA) was used for the analysis of methanol

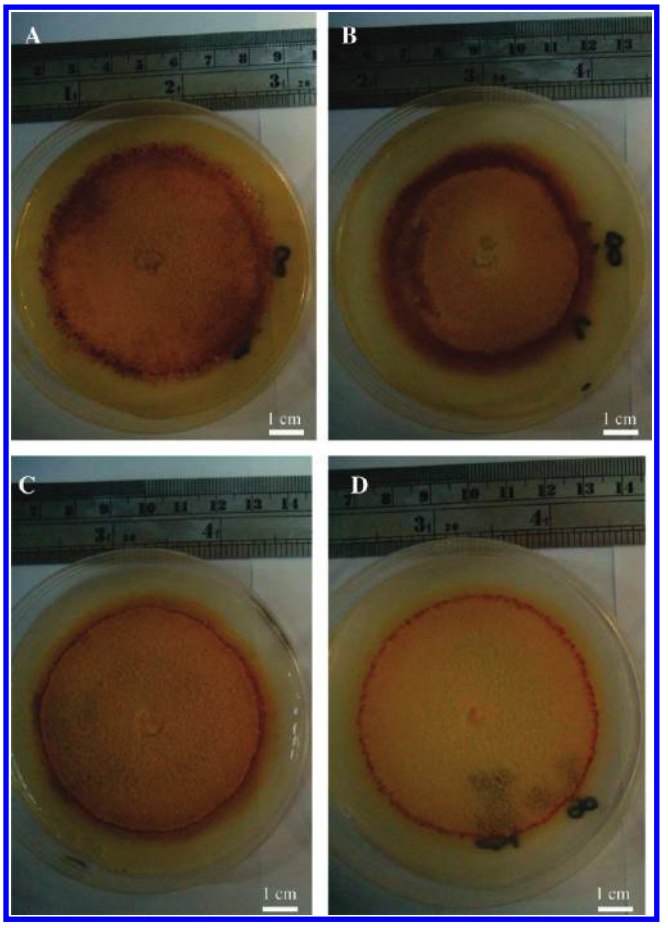

Figure 2. Growth of 6-week-old *A. cinnamomea* strain B in various concentrations of MEA media: (**A**) 100% MEA; (**B**) 50% MEA; (**C**) 25% MEA; (**D**) 10% MEA.

extracts of *A. cinnamomea* under 254 nm UV detection for secondary metabolite profiles. The mobile phase program of HPLC was 30−100% acetonitrile from 0 to 20 min, 100% acetonitrile for the next 20 min, then, linearly replacing the acetonitrile with 100% methanol for the next 10 min, and finally 100% methanol for the next 10 min.

RESULTS

18S rRNA DNA Sequence Analysis for Identification of *A. cinnamomea* Strains. Molecular identification of tested *A. cinnamomea* strains was based on 18S rRNA gene sequencing analysis. Two 18S rRNA DNA sequences were PCR amplified and sequenced with two pairs of universal PCR primers, NS1/NS4 and NS3/NS8, for fungal 18S rRNA genes. The DNA sequence is shown in **Figure 1**. All four fungal strains carried identical DNA sequences and were confirmed as *A. cinnamomea*.

Growth of *A. cinnamomea* Strains on Modified MEA Media. The four *A. cinnamomea* strains were cultured in 10, 25, 50, and 100% nutrient concentrations of MEA media. The results showed all four strains grew best in 50% MEA and also formed the best fruiting body morphology in 50% MEA as shown in **Figure 2**. In addition, we found strain B grew best among the our tested strains (**Figure 3**). The fruiting body of 8-week-old *A. cinnamomea* strain B was subjected to further microscopic observation under a stereomicroscope (**Figure 4**). A clear porous morphology on the surface of *A. cinnamomea* was found (**Figure 4B**,**D**). The longitudinal dissection of the MEA-cultured fruiting body showed, under the porous surface, the fruiting body contains two distinct zones (**Figure 4C**,**E**). The outer zone is a relatively dark brown tissue, and the inner zone is a white-yellow

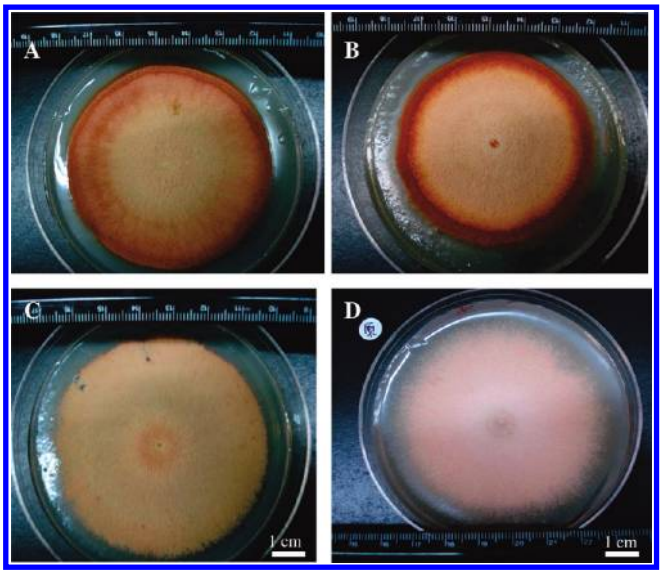

Figure 3. Growth patterns of *A. cinnamomea* strains A (**A**), B (**B**), ACI (**C**), and BCRC35398 (**D**) in 50% MEA media.

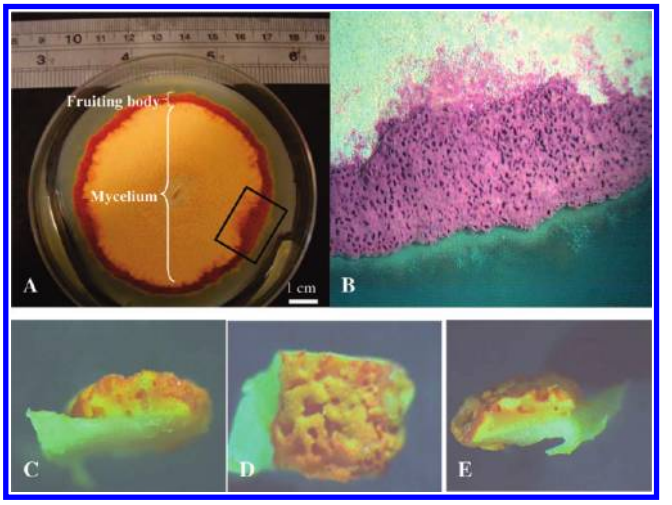

Figure 4. Dissection of fruiting body of 8-week-old *A. cinnamomea* strain B grown in 50% MEA media: (**A**) overall view (the outer dark orange ring was the fruiting body and the white-orange area inside the ring was the mycelium); (**B**) enlarged view of outlined portion of (**A**); (**C**−**E**) right, top, and left side views of dissected fruiting body under stereomicroscope.

tissue. Both structures were compact and distinct from the filamentous hyphae structure.

HPLC Analysis of *A. cinnamomea* Methanol Extracts. The MEA-cultured *A. cinnamomea* mycelia and fruiting body were extracted with methanol and subjected to HPLC analysis as described in an earlier study (*9*). The HPLC profiles were compared with known compounds purified from *A. cinnamomea*. The methanol extract HPLC profiles of both *A. cinnamomea* mycelia and fruiting body agreed with our earlier study, which found that the fruiting body contained more nonpolar compounds and the mycelia contained more polar compounds. Typical major compounds were found in the fruiting body when compared with the HPLC profiles of purified compounds, such as antcin K (t_R at 10.6 min), zhankuic acid C (t_R at 28.8 min), and zhankuic acid A (t_R at 40.1 min), confirming the MEA-cultured fruiting body also carried the same chemical properties as wild *A. cinnamomea* fruiting body (**Figure 5**).

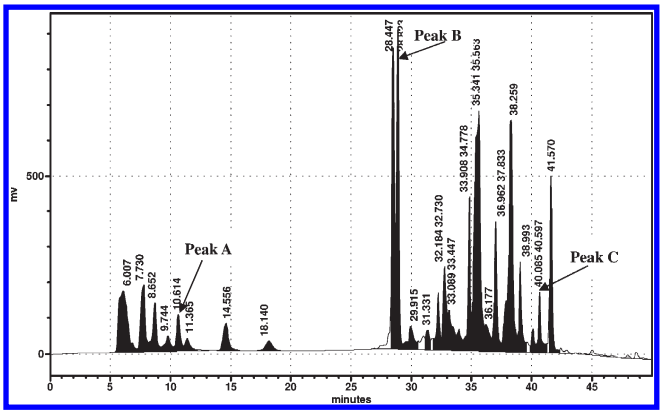

Figure 5. HPLC chromatogram of a methanol extract of 50% MEA cultured 8-week-old *A. cinnamomea* strain B fruiting body with characteristic peaks shown as peak A (antcin K), peak B (zhankuic acid C), and peak C (zhankuic acid A).

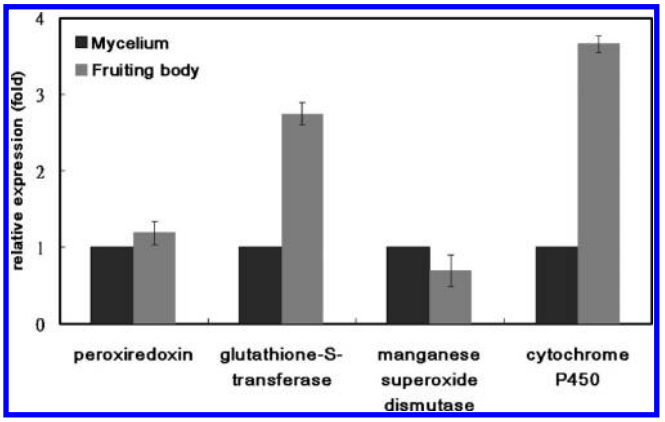

Figure 6. Comparison of selected gene expressions between mycelium and fruiting body of *A. cinnamomea* strain B grown in 50% MEA media. Data are the average ± SD of three experiments.

Selected Gene Expressions of *A. cinnamomea* Fruiting Body and Mycelia. We further investigated on the molecular level some gene activities in the MEA-cultured *A. cinnamomea* mycelium and fruiting body. Four known genes, including a cytochrome P450, a peroxiredoxin, a glutathione-*S*-transferase, and a manganese superoxide dismutase, related to antioxidation or senescence were analyzed by real-time PCR for their expression patterns in both mycelium and fruiting body. As shown in **Figure 6**, both cytochrome P450 and glutathione-*S*-transferase had higher expression rates in fruiting body than in mycelium. The peroxiredoxin and manganese superoxide dismutase genes were only slightly changed in expression in this study.

DISCUSSION

The in vitro culture of *A. cinnamomea* fruiting body has been extensively studied in recent years for its high economic value in herbal medicine (*9*, *10*). The most common method to grow *A. cinnamomea* is to inoculate the fungal hyphae in old rotten *C. kanehirae* wood blocks (*9*). This method can easily grow *A. cinnamomea* fruiting body, but endangers the *C. kanehirae*. Also, its growth is very slow. Therefore, alternative methods to culture *A. cinnamomea* fruiting body with non-*C. kanehirae* source artificial media are necessary for the production of *A. cinnamomea* fruiting body. Among the artificial media developed, MEA, PDA, and seeds of *Coix lacryma-jobi* (Job's tears) have been reported to successfully grow *A. cinnamomea* (*9*, *10*, *14*). However, the repeatability of fructification is not very good. Hence, the development of a stable in vitro culture method is needed.

MEA has been the most promising medium tested in our laboratory to grow *A. cinnamomea* fruiting body. In this study, we investigated various concentrations of MEA and found 50% MEA is best for growth of *A. cinnamomea* both in mycelium and in fruiting body. In addition, we grew four strains of *A. cinnamomea* from four different sources to determine whether fruiting body formation in MEA is strain-dependent. The result strongly supported this hypothesis. This result indicates that a good choice of *A. cinnamomea* strain combined with an optimal nutrient composition may be essential for a successful culture of *A. cinnamomea* fruiting body. A systemic investigation of optimal composition of *A. cinnamomea* strains and culture media will be necessary.

Another important issue regarding the in vitro culture of *A. cinnamomea* is the composition of its secondary metabolites, which are considered to be key to its novel medicinal effects. We analyzed the cultured fruiting body through HPLC and found it carried similar secondary metabolite profiles in methanol extract with wild-grown *A. cinnamomea*. This indicated a high potential to apply the method developed in this study for production of this expensive herbal medicine.

The genomic investigation in gene expression of *A. cinnamomea* mycelium and fruiting body has been discussed recently (*15*). However, a detailed comparison on specific gene expression in *A. cinnamomea* has not been addressed yet. In this study, for the first time, we compared four gene expressions between mycelium and fruiting body from a single culture to eliminate potential errors caused by strain specificity. This, therefore, provided more reliable data for further discussion. Cytochrome P450 is the gene found to be significantly expressed during fructification in this study. Even though cytochrome P450 is composed of a giant gene family, the most common function of cytochrome P450 is to catalyze the reaction of monooxygenation. Filamentous fungi can secrete a great diversity of compounds and perform many different complex conversions mediated by monooxygenase enzymes belonging to the cytochrome P450 superfamily (*16*). These conversions are especially obvious when the fungi need a strong metabolic flexibility. Muraguchi and Kamada (*17*) studied a cytochrome P450 gene, *eln2*, of *Coprinus cinereus* and found that mutation of *eln2* will affect fructification of *C. cinereus*. Hence, it is reasonable to find the overexpression of cytochrome P450 gene during *A. cinnamomea* fructification. Further study on the metabolism pathway of cytochrome P450 mediated conversion may be interesting to scientists to discover metabolites carrying medicinal effects.

A glutathione-*S*-transferase (GST) gene was also found to be significantly increased during fructification in this study. The GST, like cytochrome P450, is composed of a large multifunctional gene family. It plays roles in normal cellular metabolism as well as in defense mechanisms by detoxification of a wide variety of xenobiotic compounds (*18*). There was no report to describe GST might related with fungal fructification yet. However, *A. cinnamomea* fructification involved a dramatic morphogenesis changes. During the fructification, *A. cinnamomea* fruiting body emerged from inside the rotten wood to the surface of the rotten wood. The fungi will face a very different environment and therefore may initiate different metabolic pathways. Thus, it is expected that there will be a significant change in cellular metabolism. In this in vitro culture study, we found strong water depletion from the MEA medium when fructification started, indicating strong metabolic activities were ongoing.

Taken together, we found the fructification of *A. cinnamomea* is strain-dependent and that 50% MEA worked better than regular MEA for *A. cinnamomea* fructification. This result will be useful for researchers intending to culture *A. cinnamomea* fruiting body in vitro in the future. The molecular behavior of cytochrome P450 and glutathione-*S*-transferase during fructification may provide insight for basic research scientists to discuss a possible relationship between antioxidant-related genes and fungal fructification as well as the search for the major compounds related with its medicinal effects.

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