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Propagation of *Phellinus linteus* originated from An Giang, Vietnam

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Abstract A wild mushroom species from the highland regions of An Giang province, Vietnam, was identified as *Phellinus linteus* AG (commonly known as Sang-Hwang mushroom) through a combination of morphological characteristics and ITS sequence comparison. The ITS sequences, amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), showed a 92% similarity to those in the GenBank database. The optimal medium for the first propagation phase was potato dextrose agar (PDA), with complete hyphal growth observed after 7 days. For the second propagation phase, paddy was found to be the best medium, also achieving full hyphal growth in 7 days. The most suitable medium for producing a high yield of fruiting bodies consisted of 90% rubber sawdust + 5% rice bran + 5% corn flour. Fruiting bodies developed after 33 days, and were ready for harvest after 55 days, with a biological efficiency of approximately 0.47%.

Keywords: Mushroom, *Phellinus linteus*, Biomass propagation, Cultivation, Wild collection

Introduction

Phellinus linteus is a wood-decaying fungus that belongs to the *Hymenochaetaceae*. It is native to various regions of Asia, including Japan, Korea, and China, and has a distinctive appearance with a dark, rust-colored, glossy cap and a hard, woody texture. *P. linteus* has been used in traditional East Asian medicine for centuries, and extensive research has been conducted on its bioactive compounds and potential therapeutic properties (Chen *et al.*, 2019).

The biological value of *P. linteus* can be attributed to its rich bioactive compound profile and its associated pharmacological properties. The fungus and its constituents, such as polysaccharides, triterpenoids, and phenolic compounds, possess potent antioxidant, anti-inflammatory, and immunomodulatory activities (Chen *et al*., 2019). Numerous studies have shown that this fungus and its bioactive compounds can inhibit the proliferation, induce apoptosis, and suppress the metastasis of various types of cancer cells, making it a promising candidate

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for complementary or adjuvant therapeutic applications in oncology. *P. linteus* has also exhibited hepatoprotective and cardioprotective effects, which may be attributed to its antioxidant and anti-inflammatory properties (Ku *et al*., 2022).

Currently, the natural availability of *P. linteus* is limited, as it is primarily found in certain regions of Asia, and the natural growth and harvesting may not be sufficient to meet the increasing demand for its therapeutic applications. Controlled cultivation can ensure a more consistent and reliable supply of the desired bioactive compounds, while also addressing concerns about the sustainability of its natural supply (Min and Kang, 2021). Isolation and cultivation of *P. linteus* also allow for better control over the production process, enabling the development of standardized extracts and preparations with consistent chemical profiles and biological activities. This is essential for the reliable and effective use of *P. linteus* in various medical and nutraceutical applications. Furthermore, the ability to isolate and cultivate *P. linteus* opens up opportunities for genetic and metabolic engineering approaches, which can be employed to enhance the production of specific bioactive compounds or to develop novel strains with improved therapeutic properties (Huang *et al*., 2015).

P. linteus is a remarkable medicinal mushroom with a wide range of biological activities and potential therapeutic applications. However, the availability of this mushroom from natural resources is limited. Therefore, in the present study was undertaken the task of isolating and evaluating a *P. linteus* AG strain originating from An Giang province (Vietnam) with the aim of establishing suitable cultivation conditions for its widespread production.

Materials and methods

Mushroom

To obtain a pure strain of *P. linteus* AG, A single spore was isolated from the fruiting bodies of *P. linteus* specimens collected from An Giang province, Vietnam. This single spore was then placed on a 2% water agar medium and incubated for 2 days at a temperature of 28 ºC. Following the initial spore germination, the mycelia that had developed from the single spore were carefully transferred to a potato dextrose agar (PDA) medium. The inoculated PDA plates were then incubated for an additional 10 days at a constant temperature of 28 ºC (Lee *et al*., 2008).

Primary breeding medium

To determine the most suitable primary breeding medium for the cultivation of the *P. linteus* AG strain, the study tested four different media

formulations in a randomized experiment with ten replications for each treatment. The experiment was conducted in controlled environmental conditions to ensure consistency across all media. The first medium tested was the standard PDA (Potato Dextrose Agar) formulation, composed of 200 grams of potatoes, 20 grams of dextrose, and 20 grams of agar, dissolved in distilled water to make 1000 milliliters of medium. The second medium was a PDA-coconut water variant, in which coconut water replaced distilled water, while the other components remained the same as the standard PDA formula. The third medium, PDA-mineral salt, contained the standard PDA ingredients plus an additional 3 grams of potassium dihydrogen phosphate (KH_2PO_4), 1.5 grams of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), and 0.01 grams of vitamin B1, also dissolved in distilled water. The fourth medium was the Raper medium, consisting of 2 grams of peptone, 2 grams of yeast extract, 0.5 grams of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 1 gram of dipotassium phosphate (K_2HPO_4), 20 grams of glucose, and 20 grams of agar, all dissolved in distilled water to make 1000 milliliters of the medium.

Secondary breeding media

To explore suitable secondary breeding media for the cultivation of *P. linteus* AG, we evaluated three different grain-based substrates: rice in the husk, polished rice, and maize. The experiment was structured as a completely randomized design with three treatments and ten replications per treatment. The preparation process began with thoroughly washing the seeds to remove any impurities and surface contaminants. Following the washing step, the seeds were drained and then subjected to a standardized cooking procedure. Each type of grain was cooked separately until it just began to bloom, ensuring that the grains retained a firm texture without being overcooked. After reaching the desired cooking stage, the grains were immediately removed from the heat and allowed to cool at room temperature to prevent any further cooking.

Once the grains had cooled, they were transferred into pre-sterilized glass jars. The filled jars were then autoclaved at 121°C for 15 minutes to achieve complete sterilization, eliminating any potential microbial contaminants. This sterilization step was crucial to create a controlled environment suitable for inoculation with *P. linteus* AG mycelium.

Media for cultivating fruiting bodies

The growth performance of the *P. linteus* AG strain was evaluated. The experiment was arranged in a completely randomized design with ten replications, including four distinct treatments. The substrate formulations were

as follows: sawdust as the sole substrate; sawdust supplemented with 5% rice bran; sawdust supplemented with 5% ground maize; and sawdust supplemented with a combination of 5% rice bran and 5% ground maize. The sawdust used in these experiments was derived from rubber trees. Prior to the preparation of the growth media, the sawdust was incubated with a 3% lime water solution for a duration of 24 hours. Following the lime water incubation, the appropriate supplementary ingredients, such as rice bran and/or ground maize, were added to the sawdust in the specified proportions. The complete growth media were then transferred to plastic bags and subjected to autoclaving at a temperature of 100 ^{o}C for a period of 8 hours.

Identification of P. linteus AG

To identify the fungal species, the study extracted total genomic DNA from the specimen using a commercially available DNA preparation kit (Solgent, Daejeon, Korea). The extracted DNA was then subjected to polymerase chain reaction (PCR) amplification, targeting the internal transcribed spacer (ITS) region of the fungal ribosomal DNA. The primer pair used for the PCR amplification were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The thermal cycling conditions consisted of an initial denaturation at 95 °C for 15 minutes, followed by 35 cycles of denaturation at 95 °C for 20 seconds, annealing at 50 °C for 40 seconds, extension at 72 °C for 1 minute, and a final extension at 72 °C for 3 minutes. The amplified PCR product was then purified and subjected to DNA sequencing analysis. The obtained DNA sequence was compared against the NCBI (National Center for Biotechnology Information) nucleotide sequence database using the BLAST (Basic Local Alignment Search Tool) algorithm (Shin, 2011).

Mycelium growth rate and biological efficiency

The growth rate of the *P. linteus* AG strain was evaluated using a completely randomized design with ten replications for each treatment. The colony diameter on the surface of the growth medium over time was assessed. For quantify the biological efficiency of the *P. linteus* AG cultivation process, the researchers employed a standardized formula: Biological efficiency (%) = 100 x (Total weight of mushroom fruit bodies obtained / weight of substrate used for growing).

Statistical analysis

Statistical analysis was performed using Microsoft Excel and values are presented as mean ± standard deviation. Significance of differences was assessed by one-way ANOVA followed by Duncan's test procedure to determine statistically significant differences at the 95% confidence interval.

Results

Morphological characteristics of P. linteus AG fruit bodies

It was found that fruit bodies of *P. linteus* AG are perennial, without a stipe, and have multiple layers of tubes. The context and tube layers are a rusty brown color. The fungus grows solitarily and has a elongated, woody soft texture. The upper surface is dark brown to black, with grooves, and a hard, dark brown to black crust with relatively deep cracks. Initially, the cap is covered with a layer of golden-yellow velvety down, which gradually darkens to a blackish-brown color. The cap has numerous small protuberances and cracks into small areas. The cap margin is blunt, with a flesh-colored brownish-yellow tone when young, transitioning to a rusty brown color as the fungus matures. The fertile hymenial surface is tube-shaped, with new layers of tubes forming on top of the older ones. This detailed morphological description provides insights into the key features of the *P. linteus* AG fungal strain, including its growth habit, cap characteristics, and hymenial structure, which are important for its identification and taxonomic classification.

Comparative analysis of ITS-rDNA base sequences

The sequence comparison using the Nucleotide BLAST tool available on the NCBI website (https://www.ncbi.nlm.nih.gov/) demonstrated that the ITS (Internal Transcribed Spacer) region sequences of *P. linteus* AG exhibited a 91% similarity with the *P. linteus* strain SFC 970520-1 sequence deposited in the NCBI database (Accession number: AF534075.1). Constructing a phylogenetic tree showed that *P. linteus* AG exhibited a similarity to *P. linteus* strain SFC 970520-1 (Figure 1).

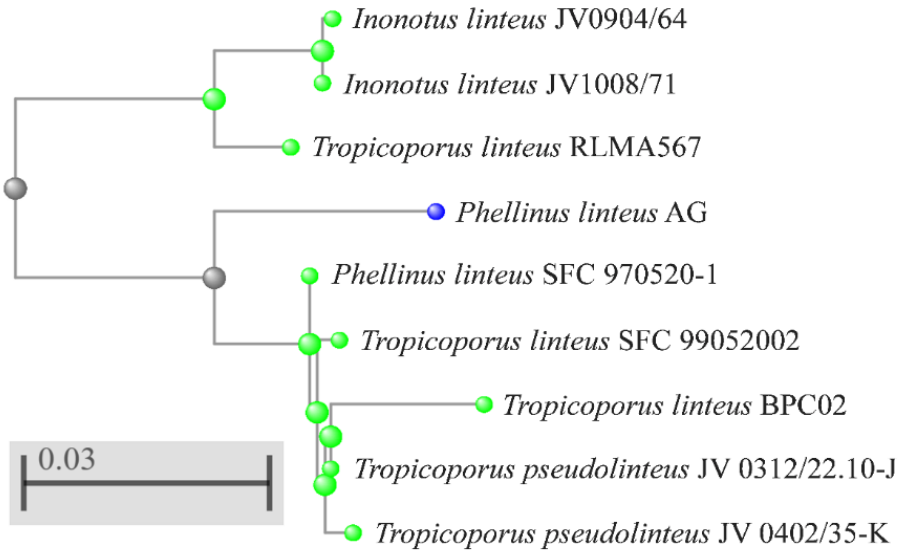

Figure 1. Phylogenetic tree using ITS sequences of *P. linteus* AG and its allied species. The tree was constructed by the Fast Minimum Evolution method

Growth of P. linteus AG on primary breeding medium

Evaluating the choice of primary breeding medium is an essential step in the development of *P. linteus*. The primary breeding medium composition can significantly impact the growth and mycelial development of *P. linteus*. Observing the mycelial growth rate of *P. linteus* AG on different breeding mediums, the results showed that the Rapper, PDA, and PDA supplemented with coconut water supported the growth of the fungal strain effectively. Statistical analysis revealed no significant differences in the mycelial growth rates among these three mediums (Table 1). In contrast, the mycelial growth of *P. linteus* AG was slower on the PDA medium supplemented with mineral salts (Table 1, Figure 2).

Table 1. Mycelial growth of *P. linteus* AG on primary breeding medium

Breeding medium	Mycelial growth rate (cm)*		
	3 days	5 days	7 days
Rapper	3.01 ± 0.31^{a}	3.74 ± 0.11^{a}	4.15 ± 0.08^{a}
PDA	2.77 ± 0.12^{a}	3.51 ± 0.21^{ab}	3.93 ± 0.11^{a}
PDA-coconut water	2.89 ± 0.17^{ab}	3.60 ± 0.09^{a}	3.99 ± 0.03^{a}
PDA-mineral salt	2.34 ± 0.55^{b}	3.13 ± 0.13^{c}	3.50 ± 0.12^{b}

*Different letters in the same column indicate significant differences at $p < 0.05$ level according to Duncan's multiple range tests.

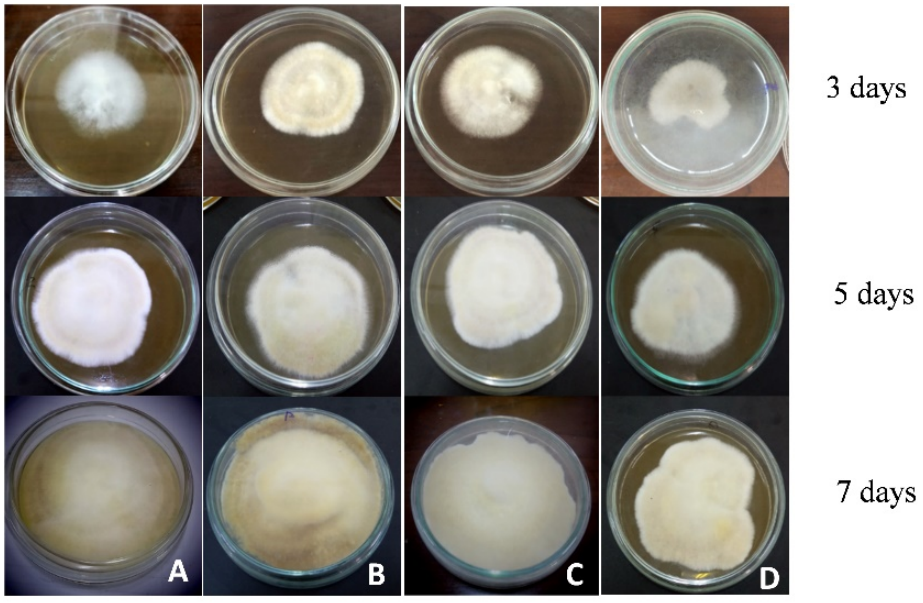

Figure 2. Mycelial morphologies of *P. linteus* AG on PDA (A), Rapper (B), PDA-mineral salt (C), and PDA-coconut water (D)

Growth of P. linteus AG on secondary breeding medium

Different substrates have varying nutrient profiles in terms of carbohydrates, proteins, lipids, and other essential compounds. The nutritional composition of the substrate can significantly impact the mycelial growth, biomass production. In the present investigation, the mycelial growth of *P. linteus* AG was evaluated on three different substrates (Figure 3).

The results indicate that the mycelial growth rate of *P. linteus* AG was more favorable on rice in the husk and rice substrates compared to maize. After seven days of cultivation, the mycelial growth rate of *P. linteus* AG on rice in the husk and rice substrates ranged from 6.01 cm to 6.42 cm. In contrast, this value was 5.24 cm for the maize substrate (Table 2).

Table 2. Mycelial growth of *P. linteus* AG on secondary breeding medium

Substrates	Mycelial growth rate (cm)*		
	3 days	5 days	7 days
Rice in the husk	2.55 ± 0.31[a]	4.55 ± 0.43[a]	6.01 ± 0.22[a]
Rice	2.80 ± 0.12[a]	4.91 ± 0.14[a]	6.42 ± 0.31[a]
Maize	1.96 ± 0.21[b]	3.67 ± 0.11[b]	5.24 ± 0.17[b]

* Different letters in the same column indicate significant differences at $p < 0.05$ level according to Duncan's multiple range tests.

Figure 3. Mycelial growth of *P. linteus* AG on different substrates: (A) maize, (B) rice in the husk, (C) rice

Medium for cultivating fruiting bodies

Replicating fruiting body culture media can facilitate the domestication and large-scale production of *P. linteus*. After being transplanted into fruiting body culture medium in the form of embryo bags, the study recorded the average number of days of mycelium spreading, fruiting body weight, and biological efficiency of *P. linteus* AG. The results showed that the number of days for mycelial growth was not significantly different on the media of sawdust, sawdust supplemented with rice bran, and sawdust supplemented with ground maize. However, the sawdust medium supplemented with both rice bran and ground maize had faster mycelial growth compared to the other media. The minimum time to develop the mycelium to reach 100% of the bag was about 33 days (Table 3). Proportional to the rate of mycelial growth, the fruiting body mass was recorded to be higher in the sawdust medium supplemented with rice bran and ground maize, reaching 5.67 ± 0.22 g. In contrast, this value was around 5.04 -

5.36 g for other media. The biological efficiency analysis also noted that the the sawdust medium supplemented with rice bran and ground maize was more effective than the other media, reaching 4.7% (Table 3).

Table 3. Growth of *P. linteus* AG on fruiting body culture media

Growing medium*	Average mycelium (day)*		Mass (g)*	Biological efficiency (%)
	50% bag	100% bag		
Sawdust	23.41 ± 1.12[a]	41.6 ± 2.17[a]	5.04 ± 0.33[b]	0.42
Sawdust + rice bran	22.9 ± 1.55[a]	38.4 ± 1.33[ab]	5.36 ± 0.78[ab]	0.44
Sawdust + ground maize	21.9 ± 1.79[a]	37.9 ± 1.11[ab]	5.19 ± 0.24[b]	0.43
Sawdust + rice bran and ground maize	17.1 ± 1.02[b]	33.7 ± 1.05[b]	5.67 ± 0.22[a]	0.47

* Different letters in the same column indicate significant differences at $p < 0.05$ level according to Duncan's multiple range tests. The sawdust used comes from rubber trees. Rice bran and ground maize are added at a dosage of 5%.

Discussion

P. linteus is a well-known medicinal mushroom that has garnered significant attention due to its diverse range of therapeutic properties (Khursheed *et al*., 2020). The present study focuses on the cultivation and analysis of the *P. linteus* AG strain, which has demonstrated promising biological activities. The isolation and characterization of the *P. linteus* AG strain provide a valuable genetic resource for further research and potential applications. The strain-specific differences in mycelial growth, fruiting body yield, and possibly the production of bioactive compounds can be exploited to expand the repertoire of *P. linteus*-based products and their therapeutic applications.

In the cultivation of *P. linteus*, the selection of primary and secondary breeding media play a crucial role in the overall success and productivity of the process. The present study's focus on evaluating different substrate compositions highlights the significance of this approach. The primary breeding medium, also known as the seed or inoculum medium, serves as the initial substrate for the mycelial growth and propagation of *P. linteus*. The composition and properties of this medium can have a profound impact on the subsequent stages of the cultivation process. Selecting an optimal primary breeding medium can ensure the production of a robust and healthy inoculum, which is essential for successful establishment and growth of the fungus in the secondary cultivation stage. Among the primary breeding media surveyed, the mycelial growth of *P. linteus* AG was satisfactory on all three media: Rapper, PDA, and PDA supplemented with coconut water. However, the PDA medium may be the preferred choice due

to its ease of use and convenience in culturing the fungus. The secondary breeding medium is the substrate on which the *P. linteus* mycelium is grown to facilitate the formation and development. In the present study, it is clear that rice is a suitable secondary breeding medium for *P. linteus* AG. The composition and characteristics of this medium can significantly affect the yield, size, and quality of the later harvested fruiting bodies. Factors such as the carbon-to-nitrogen ratio, the presence of specific growth-promoting nutrients, and the physical structure of the secondary medium can influence the mycelial growth and primordia initiation .

The results obtained from the substrate evaluation experiments indicate that the sawdust medium supplemented with both rice bran and ground maize provided the most favorable conditions for the mycelial growth and fruiting body development of *P. linteus* AG. This substrate combination facilitated faster mycelial propagation and resulted in the highest fruiting body mass compared to the other tested media. The biological efficiency analysis further corroborated the superior performance of the sawdust-rice bran-corn substrate, suggesting its potential for large-scale cultivation of this medicinal mushroom. The enhanced mycelial growth and fruiting body yield observed on the optimized substrate can be attributed to the synergistic effects of the individual components. Sawdust provides a lignocellulosic substrate for the fungus to colonize and utilize as a carbon source (Hultberg and Golovko 2024), while the supplementation with rice bran and ground corn introduces additional nutrients, such as proteins, lipids, and complex carbohydrates, that can stimulate mycelial proliferation and fruiting body formation (Omarini *et al*., 2019).

Importantly, the improved cultivation conditions not only enhance the productivity but may also influence the biosynthesis of valuable bioactive compounds in *P. linteus* AG. Previous studies have reported that *P. linteus* possesses a diverse array of secondary metabolites, including polysaccharides, triterpenes, and phenolic compounds, which contribute to its renowned therapeutic potential (Hsieh *et al*., 2013; Liu *et al*., 2020). The optimized substrate formulation developed in this study may facilitate the accumulation of these medically-relevant metabolites, thereby improving the overall biological value and medicinal properties of the *P. linteus* AG strain. The present study is demonstrated the importance of optimizing the cultivation substrate for *P. linteus* AG to enhance its growth and productivity. The findings contribute to the broader understanding of *P. linteus* cultivation and highlight the biological value of this medicinal mushroom strain, paving the way for its increased utilization in the development of novel therapeutic interventions.

The present study has successfully isolated and evaluated the cultivation potential of the *P. linteus* AG strain, a valuable medicinal mushroom originated from An Giang, Vietnam. Through the primary breeding medium survey, it was

observed that the mycelial growth of *P. linteus* AG was satisfactory on a range of media, including Rapper, Potato Dextrose Agar (PDA), and PDA supplemented with coconut water. While all three media supported good mycelial development, the PDA medium emerged as a preferred choice due to its ease of use and convenience in culturing the fungus. Furthermore, rice was a suitable secondary breeding medium for *P. linteus* AG. The investigation of medium for cultivating fruiting bodies revealed that the sawdust substrate supplemented with both rice bran and ground corn provided the most favorable conditions for the growth and fruiting body production of *P. linteus* AG. This study contributed to the broader understanding of *P. linteus* cultivation and highlights the importance of determining growing media to enhance the yield and productivity of this valuable medicinal fungus.

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