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Optimal culture conditions for mycelial growth and fruiting body formation of Ling Zhi mushroom *Ganoderma lucidum* strain GA3

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Abstract:

The objective in this study is to investigate optimal culture conditions for mycelial growth and fruiting body formation of the Ling Zhi mushroom, *Ganoderma lucidum* strain GA3. The results of the study show that the optimal media and temperature for the mycelial growth are potato, glucose, and agar (PGA) supplemented with rice bran, and 25-30^{0}C, respectively. Strain GA3 is able to grow in a wide pH range, between 4 and 12. The most favourable substrate mixture for the formation and development of the fruiting body is 87% sawdust + 4% corn powder + 8% rice bran + 1% calcium carbonate ($CaCO_3$).

Keywords: **fruiting bodies, Ling Zhi mushroom, media, mycelium.**

Classification number: **3.5**

Introduction

Ganoderma lucidum (Fr.) Karst (Polyporaceae), known as the Ling Zhi mushroom, belonging to the family Polyporaceae (or Ganodermaceae) of the order Aphyllophorales, has been recognised as one of the most highly valued medicinal mushrooms in East Asian countries for more than 2,000 years. As with other medical mushrooms, Ling Zhi is well-known for containing various chemical substances, with approximately 119 different triterpenes and several types of polysaccharides [1]. The basidiocarp, mycelia, and spores of *Ganoderma lucidum* (*G. lucidum*) are widely used in the treatment and prevention of many diseases, such as hepatitis, hypertension, hypercholesterolemia, and gastric cancer [2, 3].

Due to its bioactive components, irregular distribution in the wild, and the increasing demand for it, the Ling Zhi mushroom is artificially cultivated on various substrates for mycelial biomass and fruiting body production [4, 5]. Grain, sawdust, wood logs, and cork residues have been used as basal substrates for the artificial cultivation of *G. lucidum* [6-9]. A combination of beech sawdust supplemented with 2.5% malt extract and 10% wheat bran has been found to be the best substrate mixture for the cultivation of *G. lucidum* [10]. According to Jandaik, et al. (2013) [11], *G. lucidum* cultivated on paddy straw supplemented with wheat bran exhibited the maximum yield (82.5 g) and biological efficiency (27.5%). As previously reported by Boh, et al. (2007) [8] and Zhou, et al. (2012) [12], the biological efficiency of *G. lucidum* is strictly involved in the environmental factors such as temperature, humidity, oxygen, light, and carbon dioxide. In Vietnam, several studies have focused on the classification and distribution of the family *Ganodermataceae* [13]. Forty-three species belonging to the genus *Ganoderma* sourced from highland regions have been identified [14]. Of these, five species have

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been successfully cultivated: *G. lucidum, G. applanatum, G. australe, G. colossum,* and *G. subresinosum*. The search for *G. lucidum* strains that can possibly enhance the mushroom's disease resistance, yield, and medical value plays an essential role in its cultivation. However, at the time of writing, to our knowledge, only a few studies had selected *G. lucidum* strains that could potentially produce high yields for commercial cultivation and adapt to a broad range of climatic conditions in Vietnam. In the course of a previous investigation into strains from our mushroom resource bank with such potential, strain GA3 was found to be able to adapt better to the climatic conditions in Vietnam than were other strains. In order to achieve a high biological yield and reduce the time required to cultivate *G. lucidum*, identifying the optimal media, and chemical, physical, and biological factors is considered as among the most crucial strategies. To this end, the present study sets out to determine the optimal culture conditions for mycelial growth and fruiting body formation for strain GA3.

Materials and methods

Mushroom strain

The *G. lucidum* strain GA3 used in this study was collected in Japan. Pure mycelial cultures were isolated from internal tissue following the protocol described by Jonathan and Fasidi (2003) [15]. The culture was maintained on a PGA medium in complete darkness and stored in a refrigerator at 5-7^{0}C for further study.

Effect of different media on mycelial growth

Four different kinds of culture media - Raper, PGA, PGA supplemented with rice bran extract, and PGA supplemented with fresh oyster mushroom extract- were used to ascertain the optimal media for promoting the vegetative growth of strain GA3. To prepare the PGA, PGA supplemented with rice bran extract, and PGA supplemented with fresh oyster mushroom extract media, after peeling, potatoes were cut into small pieces, and then boiled in 500 ml distilled water for 30 minutes. Twenty grams of rice bran (PGA supplemented with rice bran extract) and 25 g of fresh oyster mushroom (PGA supplemented with fresh oyster mushroom extract) were extracted using 250 ml of warm and hot water, respectively. The crude extract obtained was filtered by means of a steel mesh. Thereafter, these two liquids were mixed thoroughly. Twenty grams of glucose and 20 g of agar were dissolved and added to the medium. The final volume of the media was increased to one litre by adding water. The media were sterilised by autoclaving at it 121^{0}C for 60 minutes. The composition of the culture media is shown in Table 1.

Table 1. Composition of various culture media for mycelial growth.

Composition of media (g/l)	Media			
	Raper	*PGA*	*PGA supplemented with rice bran extract*	*PGA supplemented with fresh oyster mushroom extract*
Glucose	20	20	20	20
Yeast extract	2	-	-	-
Peptone	2	-	-	-
Potatoes	-	250	250	250
KH_2PO_4	0.46	-	-	-
K_2HPO_4	1	-	-	-
$MgSO_4.7H_2O$	0.5	-	-	-
Rice bran	-	-	20	-
Fresh oyster mushroom	-	-	-	25
Agar	20	20	20	20
pH	7	7	7	7

Effect of temperature on mycelial growth

Following the media experiment, strain GA3 was inoculated on PGA supplemented with rice bran at pH 7 and incubated in darkness at four different temperatures (20±1^{0}C; 25±1^{0}C, 30±1^{0}C and 35±1^{0}C).

Effect of different initial pH levels on mycelial growth

The growth of *G. lucidum* strain GA3 on PGA supplemented with rice bran at 30±1^{0}C and different pH levels was tested between pH 3.0 and 12.0 in increments of 1.0 pH units. pH levels were initially adjusted by using 1M sodium hydroxide (NaOH) or hydrochloric acid (HCl).

Effect of substrate mixtures on fruiting body formation

To investigate the most favourable substrate mixtures for fruiting body formation**,** *G. lucidum* was cultivated on rubber (*Hevea brasiliensis*) wood sawdust as the basal substrate with different types of supplements added, as indicated in Table 2.

Table 2. Composition of substrate mixtures for fruiting body formation.

Composition (%)	Treatment				
	1	*2*	*3*	*4*	*5*
Sawdust	87	87	87	87	87
Corn powder	4	4	4	4	4
Rice bran	8	6	4	2	0
Wheat bran	-	2	4	6	8
$CaCO_3$	1	1	1	1	1

Data collection

Important characteristics of mycelial morphology such as texture (cottony, floccose), density (high, moderate, low), and colour (off-white, white) were recorded by means of visual observation. Diameter growth (mm) was measured at 5, 7, and 9 days after inoculation. The mycelial growth rate was calculated as follows: V = D/T, where V is the mycelial growth rate (mm/day), D is as the diameter growth (mm), and T is the duration of mycelial growth (days).

The period of surface colonisation (days) was defined as the time required for the mycelium to grow throughout the media and establish total colonisation on the bag surface. The period of primordia formation (days) was defined as the time required for the formation of primordia from the time of inoculation. The length of stalk (cm) and width of fruiting body (cm) were measured. Biological efficiency was measured as the ratio of the mass of dry fruiting body (g) per dry mass of substrate (g) and expressed as a percentage.

Statistical analysis

The data obtained were statistically analysed using GraphPad Prism (version 7.0, GraphPad Software Inc., San Diego, CA). Each treatment was replicated three times. Differences among the means of groups were assessed using two-way ANOVA followed by Tukey multiple range test, with $p<0.05$. Values in the same column in a table with different letters were considered to differ significantly.

Results and discussion

Effect of media on mycelial growth of strain GA3

Nutrients, temperature, light, and pH are known to be significant factors that noticeably influence onthe mycelial growth of mushrooms. To investigate the optimal media for rapid mycelial growth of strain GA3, diameter growth, and mycelial morphology (texture, density, and pigmentation) were recorded in the four different culture media. As shown in Fig. 1 and Table 3, strain GA3 was able to grow in all four kinds of media. Flocculence and whiteness were identified as the main mycelial morphology and pigmentation, respectively. Maximal mycelial growth was observed on PGA supplemented with rice bran extract, with an average mycelial growth rate of 9.29±0.11 mm/day. PGA was determined to be unsuitable media for mycelial growth of GA3. PGA supplemented with rice bran exhibited high mycelial density. In contrast, the mycelial density of GA3 was found to be moderate on Raper and PGA supplemented with oyster mushroom fresh but low on PGA media. These results suggest that PGA supplemented with rice bran may be considered the optimal media for mycelial growth of strain GA3 and this was therefore selected for further optimisation. Jayasinghe, et al. (2008) [16] report that Hamada (dextrose, ebiose, hyponex yeast-extract), Glucose Peptone (glucose, malt-extract, peptone, yeast-extract), Yeast-Malt extract (dextrose, malt-extract, peptone, yeast-extract), Mushroom Complete (malt-extract, peptone, yeast-extract, K_2HPO_4, $MgSO_4$, KH_2PO_4) and Lilly (asparagine, maltose, $MgSO_4$, KH_2PO_4) are suitable media for the growth of *G. lucidum*. As reported by Badalyan, et al. (2015) [17], the mycelial morphology of *G. lucidum* is white and felt/cottony, with denser aerial mycelium in the centre during the initial incubation period stage of growth.

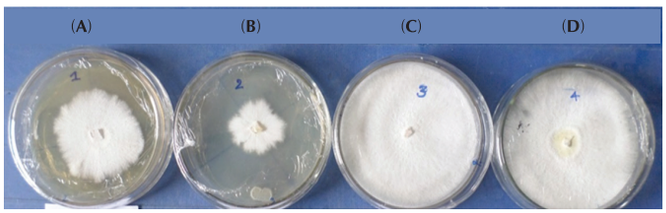

Fig. 1. Mycelial growth on different media, incubated in darkness for 9 days at 25^{0}C, pH 7. (**A**) Raper; (**B**) PGA; (**C**) PGA supplemented with rice bran; (**D**) PGA supplemented with oyster mushroom fresh.

Table 3. The influence of different culture media on the mycelial growth performance of strain GA3 at 25^{0}C, pH 7.

Media	Diameter growth (mm) after days			Mycelial characteristics		
	5	*7*	*9*	*Density*	*Texture*	*Pigmentation*
Raper	20.39±0.43^a	35.33±0.63^a	46.16±1.42^a	Moderate	Floccose	White
PGA	14.50±0.48^b	23.16±0.17^b	29.94±1.13^b	Low	Floccose	White
PGA + rice bran	24.28±0.62^c	60.5±0.82^c	83.68±1.06^c	High	Floccose	White
PGA + oyster mushroom fresh	21.28±0.22^d	57.50±0.35^d	71.39±0.11^d	Moderate	Floccose	White

Effect of temperature level on mycelial growth of strain GA3

As with the media, temperature is one of the most significant physical factors affecting both the growth of mycelium and fruiting body formation. According to Jayasinghe, et al. (2008) [16], favourable mycelial growth of *G. lucidum* was recorded at 25-30^{0}C. However, it is worth noting that a range of temperatures, between 30 and 35^{0}C, was found suitable for mycelial growth of *G. lucidum* [18, 19]. To ascertain the optimal temperature for favourable mycelial growth of strain GA3, the mycelial growth pattern was recorded at four different temperatures, 20±1^{0}C, 25±1^{0}C, 30±1^{0}C, and 35±1^{0}C for 5, 7, and 9 days. The results indicate that the incubation temperature has a significant influence on the growth of strain GA3. Optimal mycelial growth was observed at 30^{0}C, followed by 25^{0}C and 20^{0}C, as indicated in Fig. 2 and Table 4.

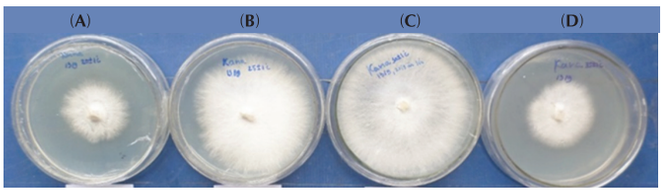

Fig. 2. Mycelium growth of strain GA3 grown in darkness on PGA medium supplemented with rice bran for 7 days at pH 7 after inoculation at various temperatures. (**A**) 20±1^{0}C; (**B**) 25±1^{0}C; (**C**) 30±1^{0}C; (**D**) 35±1^{0}C.

Table 4. The influence of different temperatures on mycelial growth performance. Strain GA3 was grown on PGA medium supplemented with rice bran at pH 7.

Temperature (^{0}C)	Diameter growth (mm) after days			Mycelial density		
	5	*7*	*9*	*Density*	*Texture*	*Pigmentation*
20±1	27.89±0.15^a	43.22±0.20^a	76.22±0.37^a	Moderate	Floccose	White
25±1	39.83±0.33^b	66.61±0.20^b	85.89±0.31^b	High	Floccose	White
30±1	48.94±0.20^c	74.94±0.48^c	90.00±0.15^c	Moderate	Floccose	White
35±1	21.05±0.28^d	39.50±0.29^d	54.39±0.42^d	Moderate	Floccose	White

Effect of different initial pH levels on mycelial growth of strain GA3

One of the most important chemical factors, pH can affect cell membrane function, the uptake of various nutrients, cell morphology and structure, the solubility of salts, the ionic state of substrates, enzyme activity, and product biosynthesis [20]. Rai (2003) [18] has reported that *G. lucidum* prefers an acidic pH for vegetative growth. In addition, a pH range from 4.0 to 6.5 was found to be the optimal initial pH for the growth of *G. lucidum*, as previously described by Veena and Pandey (2006) [19]. According to Kapoor and Sharma (2014) [21], *G. lucidum* can grow in a broad range of pH values, from 3.0 to 11.0, though the highest mycelial growth rate was observed at the pH 5.0 level. As indicated by Jayasinghe, et al. (2008) [16], the optimal pH for mycelial growth varies widely and is strongly related to the genotype of strain. Remarkably, strain GA3 is capable growing in a wide pH range, from 4 to 12, as shown in Fig. 3 and Fig. 4.

Effect of substrate mixtures on fruiting body formation of strain GA3

As previously reported, the yield and biological efficiency of *G. lucidum* relate not only to the kind of sawdust but also the supplements used [22]. Sawdust is used as the basal substrate in mixtures for cultivating *G.*

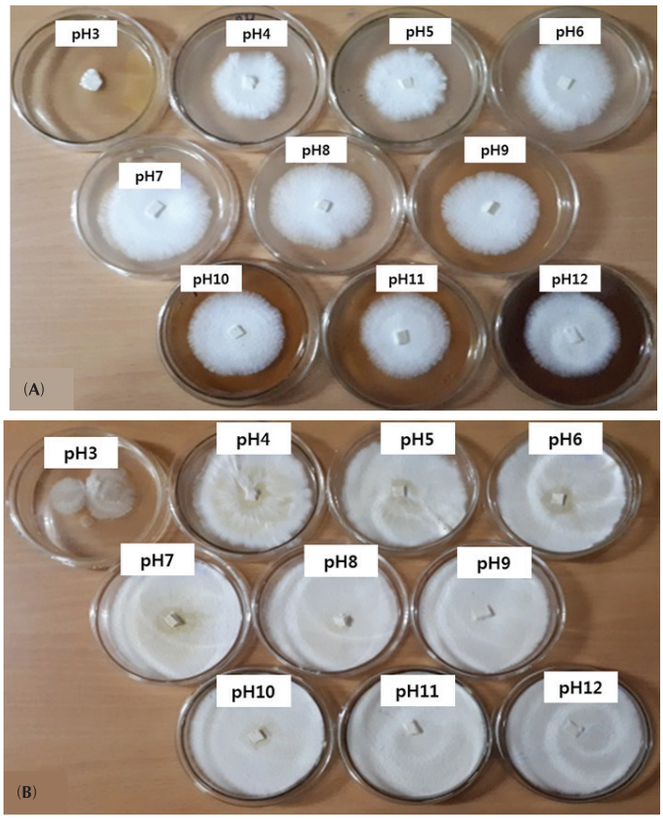

Fig. 3. Mycelium growth of strain GA3 grown in darkness on PGA medium supplemented with rice bran for 3 days (A) and 7 days (B) after inoculation at 30^{0}C.

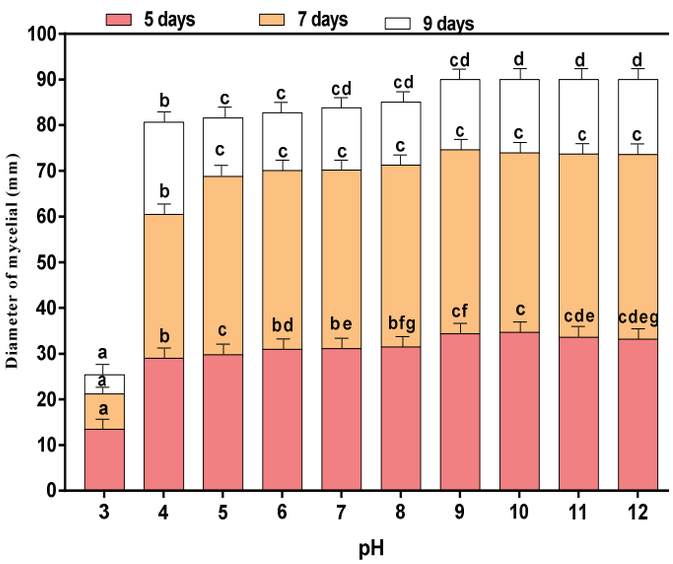

Fig. 4. Effect of initial pH on mycelial growth of strain GA3.

lucidum [23]. Compared to poplar and beech sawdust, oak sawdust was observed to support the cultivation of *G. lucidum* and produced the highest biological efficiency [22]. The effect of various kinds of substrate mixtures on fruiting body formation of *G. lucidum* was investigated in this study.

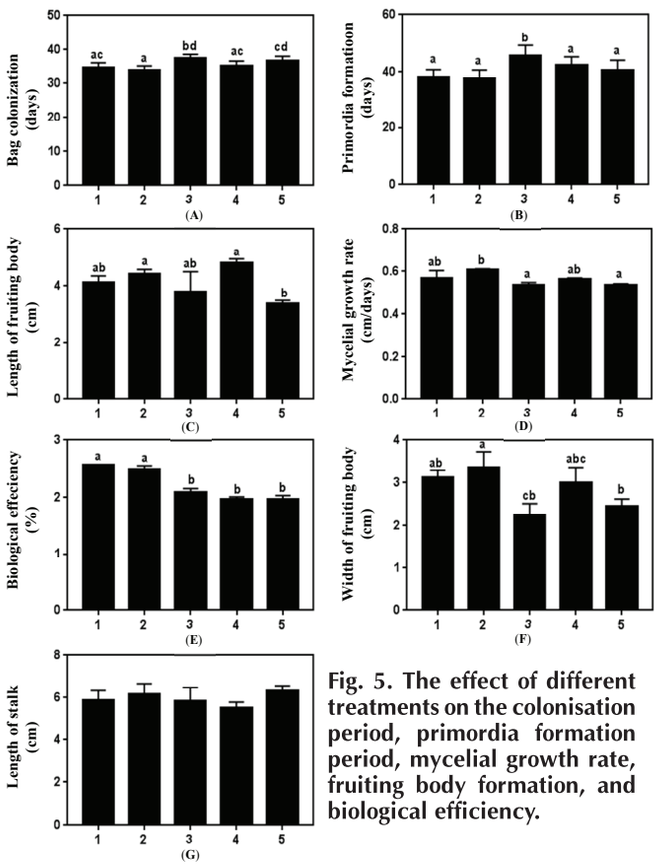

Fig. 5. The effect of different treatments on the colonisation period, primordia formation period, mycelial growth rate, fruiting body formation, and biological efficiency.

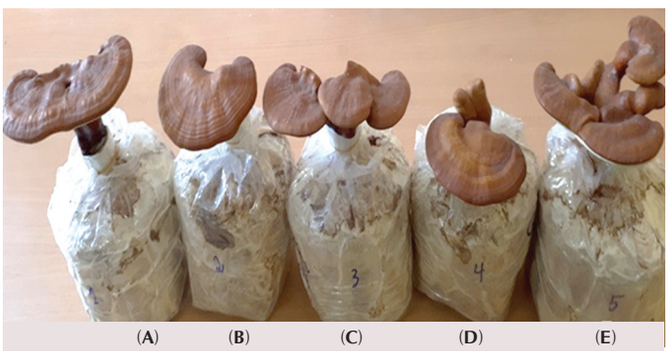

Fig. 6. Fruiting body of *Ganoderma lucidum* strain GA3 cultivated using different treatments. (**A**) treatment 1; (**B**) treatment 2; (**C**) treatment 3; (**D**) treatment 4; (**E**) treatment 5.

In this experiment, five treatments were used to in order to ascertain the most effective treatment for the growth of mycelial and for fruiting body formation. To cultivate strain GA3, corn powder, rice bran, and wheat bran were used as major nutrients to supplement the substrate. The period required for surface colonisation, primordia formation, the length of the fruiting body, the mycelial growth rate, biological efficiency, the width of fruiting body, and the length of the stalk were monitored and are shown in Figs. 5A, 5B, 5C, 5D, 5E, 5F, and 5G, respectively. The results presented in Figs. 5A, 5B indicate that strain GA3 is able to grow to form primordia in all five treatments. The pilei of strain GA3 were found to be kidney-shaped (Fig. 6). Compared with other treatments, treatment 2 (87% sawdust + 4% corn powder + 6% rice bran + 2% wheat bran + 1% $CaCO_3$) and treatment 1 (87% sawdust + 4% corn powder + 8% rice bran + 1% $CaCO_3$) reduced the time required for surface colonisation, bag colonization, and primordia formation and resulted in a significantly higher growth rate (Figs. 5A, 5B, and 5D). As expected, the greatest fresh mass (17.16 g) and biological efficiency (2.56%) were obtained with treatment 1, followed by treatment 2 (16.66 g and 2.49%) (Fig. 5E). Rice bran and corn powder are known to have high vitamin content, especially vitamin B2. This is may be due to the presence of high percentage of rice bran in the substrate mixtures of treatments 1 and 2. In contrast, treatments 4 and 5 showed a lower biological efficiency (13.13 g and 1.96%, and 13.20 g and 1.97%), respectively. Therefore, treatment 1 (87% sawdust + 4% corn powder + 8% rice bran + 1% $CaCO_3$) is considered the most suitable substrate combination for cultivating strain GA3.

Conclusions

The optimal conditions for mycelial growth of strain GA3 were observed at 25-30^{0}C on PGA media supplemented rice bran. Strain GA3 grew in a wide pH range, from 4 to 12. Of the five treatments used for cultivating of *G. lucidum*, treatment 1 (87% sawdust + 4% corn powder + 8% rice bran + 1% $CaCO_3$) was the most suitable substrate mixture for improving biological efficiency.

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The authors declare that there are no conflicts of interest regarding the publication of this article.

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