Vietnam Journal of Agricultural Sciences

ISSN 2588-1299

VJAS 2019; 2(1): 321-331
https://doi.org/10.31817/vjas.2019.2.1.03

Morphological Characteristics, Yield Performance, and Medicinal Value of Some Lingzhi Mushroom (*Ganoderma lucidum*) Strains Cultivated in Tam Dao, Vietnam

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Abstract

The aim of this study was to evaluate the biological efficiency and main bioactive components of three G. lucidum strains, viz. GA1, GA2, and GA3, cultivated in Tam Dao town. The results demonstrated that all strains were capable of growing well on PDA medium supplemented with rice bran. The time required for complete colonization was 9 days. All tested strains of *G. lucidum* were able to adapt to climate conditions and produce fruiting bodies with satisfactory yield (13-17%), and therefore, they could be considered suitable candidates for commercial cultivation of *G. lucidum* in Tam Dao. No significant differences in polysaccharide content were observed among all strains. High concentrations of lucidenic N acid (0.33 mg g^{-1}) and ganoderic acid (2.38 mg g^{-1}) were determined in strain GA3. However, the highest ganodermanontriol content was detected in the strain GA1 with 0.3 mg g^{-1}.

Keywords

Lingzhi mushroom, Polysaccharide, lucidenic N acid, ganoderic A acid, Ganodermanontriol

Introduction

Ganoderma lucidum (Fr.) Karst (Polyporaceae) has long been regarded as one of the most famous traditional medicinal herbs in the orient for more than 2000 years, and belongs to the family Polyporaceae (or Ganodermaceae) of order Aphyllophorales (Gurung *et al.*, 2012). *G. lucidum*, also known as Lingzhi in China or Reishi in Japan (Wagner *et al.*, 2003), has been reported as a source of medicinal compounds (Boh *et al.*, 2007). Therefore, the basidiocarp, mycelia, and spores of *G. lucidum* are widely utilized

Received: August 22, 2018
Accepted: March 7, 2019

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to treat and prevent more than 20 different illnesses such as hepatopathy, chronic hepatitis, nephritis, hypertension, hyperlipemia, arthritis, neurasthenia, insomnia, bronchitis, asthma, gastric ulcer, arteriosclerosis, leukopenia, diabetes, anorexia, and cancer (Stamets, 1993; Mizuno *et al.*, 1995; Wagner *et al.*, 2003). The two main groups of bioactive compounds isolated from *G. lucidum* are triterpenoids and polysaccharides (Chen *et al.*, 2012). As reported previously, the polysaccharides found in Lingzhi mushrooms are mainly composed of glucose, mannose, galactose, fucose, xylose, and arabinose (Nie *et al.*, 2013). To date, more than 150 triterpenoids as main bioactive components have been identified in *Ganoderma* spp. (Boh *et al.*, 2007). According to Nakagawa *et al.* (2018), the contents of triterpenoids and polysaccharides could be related to the growth stage. Fruiting bodies before maturity exhibit the highest amounts of total triterpenoids and total polysaccharides.

Owing to scarcity in nature, Lingzhi mushrooms are artificially cultivated in solid culture to meet demands for medicinal herbs and international markets, especially in tropical Asian countries (Boh *et al.*, 2007; Gurung *et al.*, 2012; Roy *et al.*, 2015; Ninluam *et al.*, 2016; Chang & Buswell, 2018). Grain, sawdust, wood logs, cork residues, sunflowers, and seed hulls are commonly utilized as the main substrates and nutritional sources for traditional artificial cultivation of *G. lucidum* (Riu *et al.*,1997; Chang & Buswell, 1999; Gonzalez-Matute *et al.*, 2002; Wasser *et al.*, 2005; Boh *et al.*, 2007). The biological efficiency of *G. lucidum* is strictly the result of environmental factors such as temperature, humidity, oxygen, light, and CO_2 (Boh *et al.*, 2007; Zhou *et al.*, 2012). The moisture content in the substrates should be maintained from 60 to 65% (Zhou *et al.*, 2012).

In Vietnam, the first successful artificial cultivation of *G. lucidum* was reported in 1978 (Do *et al.*, 1994). Currently, Lingzhi mushrooms are widely cultivated and contribute to improving sustainable rural development. However, to date, the Vietnamese Lingzhi mushroom industry is struggling to find potential strains that can adapt to a broad range of climatic conditions and produce high yields. In order to further improve yield, disease resistance, and medicinal value, the search for potential *G. lucidum* strains is considered to be one of the key strategies in the cultivation of mushrooms. To our knowledge, only a few studies have been carried out to select *G. lucidum* strains for commercial production. According to a paper published by Nguyen *et al.* (2013), 43 species of the *Ganoderma* genus isolated from diverse environments were classified in the Highlands of Vietnam. Among the 43 species, five species could be successfully cultivated, namely *G. lucidum, G. applanatum, G. australe, G. colossum,* and *G. subresinosum*. For this research, during the pre-screening process looking for potential strains from our mushroom resource bank, three strains (GA1, GA2, and GA3) were able to adapt better to climatic conditions in Vietnam compared to other strains and, therefore, were selected for further characterization. As one of the most difficult challenges in the cultivation of Lingzhi mushrooms, high temperatures could seriously affect fruiting body formation, yield, and economic value. Unlike other ecological areas in Northern Vietnam, the climate in Tam Dao has been classified as relatively suitable for mushroom cultivation with a temperature and humidity range of 22-28^{o}C, 85-90%, respectively in the summer. Taken together, the present communication aims to evaluate the biological yield and main bioactive components of three *G. lucidum* strains, *viz.* GA1, GA2, and GA3, cultivated in Tam Dao town.

Materials and Methods

Mushroom strains

G. lucidum strains GA2 and GA3 were collected from Thailand and Japan, respectively. Strain GA1 was a native strain isolated from Vietnam. Pure mycelial cultures were obtained from internal tissue according to the protocol of Jonathan & Fasidi (2003). The culture was maintained on PGA (potato, glucose, and agar) medium under complete darkness conditions and stored in a refrigerator at 4^{o}C for further experiments.

Culture media

During the present investigation, PGA medium supplemented with rice bran was utilized to investigate the mycelial characteristics of the strains. PGA medium was prepared with the following ingredients: 20 g L^{-1} glucose, 250 g L^{-1} potatoes, and 20 g L^{-1} agar supplemented with rice bran. The diameter of mycelial growth (mm) and morphology were monitored after 5, 7, and 9 days of incubation. The important characteristics of mycelial morphology such as texture (cottony, floccose), density (high, regular, low), and color (off-white, white) were recorded from visual observations.

Substrate preparation and cultivation

Strain GA1, GA2, and GA3 were cultivated in Tam Dao National Park (21°31′0″N 105° 33′0″E). A rubber (*Hevea brasilient*) wood substrate was prepared for the cultivation of Lingzhi mushrooms and composed as follows: 86% sawdust, 10% rice bran, 3% corn powder, and 1% $CaCO_3$. The sawdust was prepared according to the method of Nguyen *et al.* (2018). Polythene bags were filled with 1.2kg of the substrate and their mouths were plugged by inserting water absorbing cotton with plastic rings. The bags were autoclaved at 100°C for 16-17 hours. The inoculated bags were incubated at 25°C in the spawn running room under dark conditions. For fruiting body formation, the temperature and relative humidity were maintained at 25 ± 3°C and 85 ± 5%, respectively.

Mycelial growth was calculated according to the formula: V = D/T (V: mycelial growth rate (mm/day); D: the length growth of mycelium; T: duration of mycelial growth (days). The period of substrate colonization (days) was the time required for the mycelium to grow throughout the full substrate and establish total colonization on the bag's surface. The period of primordia formation (days) was the time required for the appearance of primordia after inoculation. The 1st and 2nd flushes (days) were the times required for the first and second fruiting bodies to be harvested, respectively. The biological efficiency (BE) was determined for each packet as described previously by Chang *et al.* (1981):

$$\text{BE (\%)} = \frac{\text{Fresh weight of mushrooms (FW)}}{\text{Dry weight of substrate}} \times 100$$

Quantification of triterpene content

The percentage of ganodermanontriol, ganoderic A acid, and lucidenic N acid was determined by high-pressure liquid chromatography (HPLC) following the recommended standards proposed by Ha *et al.* (2015). Fruiting bodies (200g) were sliced and extracted with 75mL of 96% ethanol at 100°C for 45min and subsequently filtered. After filtering, the remaining insoluble material was extracted with 10mL of 95% ethanol twice. The extract solutions were filtered using a 0.22µm disposable filter. HPLC was carried out on a chromatographic silica gel C-18 column (5µm. 250 x 4.6mm). The mobile phase contained acetonitrile (solvent A) and 2% acetic acid (solvent B). The flow rate and injection volume were 0.8 mL min^{-1} and 20µL, respectively. Ganodermanontriol was detected under a UV lamp with a wavelength of 243nm. The detection wavelength was set at 256nm for both ganoderic A acid and acid lucidenic N. A step gradient solvent system was followed as: 0-5min 25% solvent B; 5-20min, 25-40% solvent B; 20-40min, solvent 40% B; 40-50min 40-80% solvent B; 50-65min 80% solvent B; 65-75min 80-95% solvent B; and 70-80min solvent 95%. Mixed standard solutions including ganoderic A acid, acid lucidenic N, and ganodermanontriol (Sigma-Aldrich) were prepared.

Quantification of total polysaccharide content

The total polysaccharide content was qualified by the phenol-sulfuric acid method as described by Dubois *et al.* (1956). After treating the polysaccharides with an aqueous solution of phenol and concentrated sulfuric acid, the polysaccharides formed a stable colorimetric product and exhibited maximum absorptions at 490nm. Fruiting body powder (1g) was extracted with 100mL of 80% ethanol for 1 hour. Then, 1mL of 4% phenol was added to the 2mL sample solution followed by 7mL of

concentrated sulfuric acid at 40°C for 30min and kept in ice for 5min. The absorbance was measured using ultraviolet (UV) spectrophotometry at 490nm. Glucose (Sigma-Aldrich) was used to construct the standard curve.

Statistical methods

Data were statistically analyzed using *IRRISTAT*, version 5.0 (International Rice Research Institute, Philippines) and *GraphPad Prism*, version 7.0 (GraphPad Software, Inc., San Diego, CA, USA). Each treatment was replicated three times. Significant differences were indicated with asterisks and identified by two-way ANOVA followed by Tukey's multiple comparisons test (* P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001).

Results and Discussion

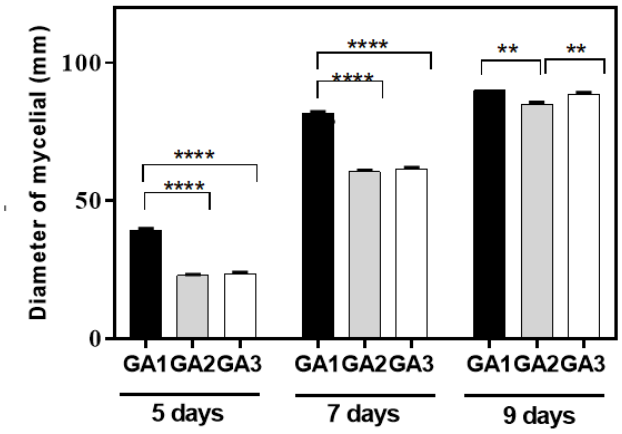
Mycelial characteristics and growth of strains grown on PDA supplemented with rice bran

As shown in **Figure 2**, floccose was identified as the main mycelial texture. In terms of pigmentation, strains GA1 and GA2 were white whereas strain GA3 exhibited white at the initial stage of growth, and then changed to off-white on the 7th day. The mycelial density of the tested strains was high except in strain GA1. All the strains were able to grow well on PDA medium supplemented with rice bran and required 9 days of incubation for complete colonization. In comparison with the other strains, strain GA1 exhibited a higher mycelial growth rate at 5 days and 7 days (**Figure 1**). However, notably, there was no statistically significant difference between strains GA1 and GA3 regarding the diameter of the mycelium on the 9th day.

In order to clarify the identity of *G. lucidum*, the identification of morphological characteristics was considered a useful general method for preliminary evaluation. The aim of this experiment was to characterize the mycelial morphology of all the strains. According to Güler *et al.* (2011), *G. lucidum* mycelium formed a white color and very solid tissue at the end of the stage of growth. As described by Badalyan *et al.* (2015), the colonies of *G. lucidum* were observed to be white, felt-like/cottony, with denser aerial mycelium in the center at the initial incubation period stage of growth.

Cultivation characteristics of Lingzhi mushroom strains

The duration of the growth cycle, data on growth, fruiting body morphological characteristics, and yield of the Lingzhi mushroom strains were recorded and are shown in **Tables 1 and 2**, and **Figures 2 and 4**.



*Note: All data are expressed as mean ± SE (Standard Error). Significant differences were indicated with asterisks (** P <0.01, **** P <0.0001).*

Figure 1. Mycelial growth of strains GA1, GA2, and GA3 on PDA supplemented with rice bran

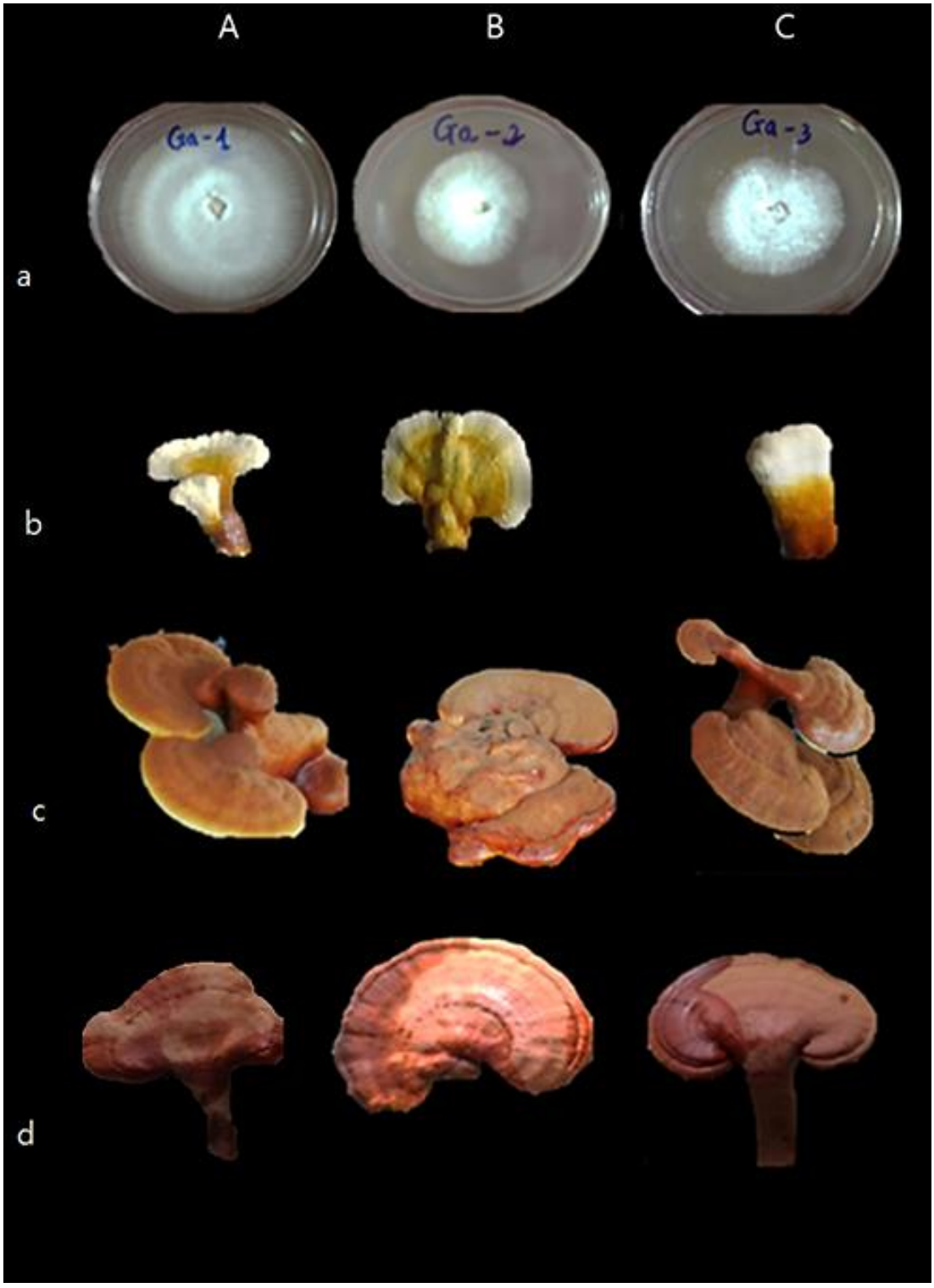


Figure 2. Mycelium (a) and the development process of fruiting bodies of strains GA1 (A), GA2 (B) and GA3 (C). Bud-developing stage (b), growth stage (c), and maturity stage (d)

The results indicated that strains GA1, GA2, and GA3 used for this study exhibited the ability to form fruiting bodies and adapt to climate conditions in Tam Dao. The highest mycelium run rate was observed for strain GA1, as indicated in **Table 1**. The mycelium color of

strains GA1 and GA2 changed according to the stage of development. The color was found to be white in the primary stage of development and then changed to yellow in the next stage.

All strains formed primordia and produced two flushing cycles during the period of the experiment as indicated in **Figure 3**. After inoculation, earlier primordia formation (43.6 ± 2.63 days) and first flush (89.6 ± 2.67 days) were found in strain GA1. Remarkably, no significant differences among strains regarding the time required for the second flush were observed. The fruiting body fresh weight in the first flush showed a range from 50.65g (strain GA1) to 70.39g (strain GA2). The obtained mushroom productivity of all the strains in the first flush was higher than the second flush. The highest biological efficiency was recorded for strain GA2 with 17%, followed by strain GA1 (13.2%) and strain GA3 (13%). According to Azizi *et al.* (2012), G. lucidum cultivated on a combination of beech sawdust with 2.5% malt extract and 10% wheat bran reached the highest yield (142.44 g kg^{-1}) and biological efficiency (18.68%). In another experiment, G. lucidum cultivated on paddy straw supplemented with wheat bran showed the maximum yield (82.5g) and biological efficiency (27.5%) (Jandaik *et al.*, 2013). Furthermore, Swietenia mahagoni supplemented with wheat bran was observed as the optimal substrate for the cultivation of G. lucidum with a yield of 235.2 g kg^{-1} and biological efficiency of 7.6% (Roy *et al.*, 2015). In comparison to previously published data, the strains GA1, GA2, and GA3 showed high yields and, therefore, could be considered as promising candidates for commercial cultivation. To improve their biological efficiencies, optimal culture conditions for mycelial growth and fruiting body formation of strains GA1, GA2, and GA3 should be determined.

Among fruiting body morphological characteristics, pileus shape is one of the most important characteristics which affects the commercial value of *G. lucidum*. Based on morphological characteristics, the pileus shape of *G. lucidum* could be divide into three groups, namely kidney type, flabelliform type, and antler type (Liu *et al.*, 2017). The pileus shape of the three strains was found to be kidney-shaped as shown in **Figure 2**. Stipe length, pileus length, and pileus width varied among the strains (**Figure 2** and **Table 2**).

Evaluation of triterpene and polysaccharide contents of Lingzhi mushroom strains

It was observed that the polysaccharides isolated from strains GA1, GA2, and GA3 showed strong UV absorptions at 490nm and matched with the standard (**Figure 6**).

Table 1. Colonization period, contamination percent, and mycelia characteristics of the Lingzhi mushroom strains

Strain	Colonization period (days)	Mycelial characteristics	
		Texture	Density
GA1	49	Floccose	High
GA2	53	Floccose	High
GA3	55	Floccose	Regular

Table 2. Fruiting body morphological characteristics of the Lingzhi mushroom strains

Strain	Stipe length (cm)	Pileus length (cm)	Pileus width (cm)	Color		Pileus morphology
				Pileal surface	Pore surface	
GA1	5.07 ± 0.38^{a}	6.41 ± 0.37^{ab}	11.27 ± 0.40^{a}	Reddish brown	White	Kidney-shaped
GA2	1.97 ± 0.13^{b}	7.47 ± 0.28^{a}	12.57 ± 0.37^{a}	Pale yellowish	Beige	Kidney-shaped
GA3	1.16 ± 0.21^{b}	5.78 ± 0.24^{b}	11.50 ± 0.44^{a}	Reddish brown	White	Kidney-shaped

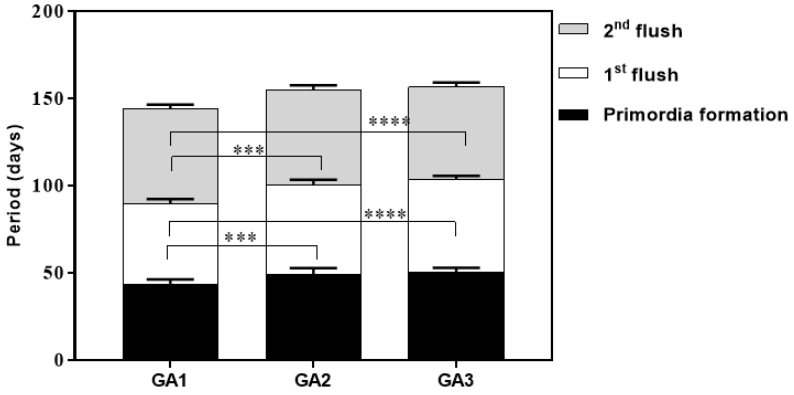
Note: Mean ± SE, different letters denote statistically significant differences at P <0.05.

A calibration curve (y = 11.777x - 0.0419) with a high coefficient of determination (R^2 = 0.9994) was determined and used to quantify the polysaccharide contents (**Figure 5**). The high polysaccharide concentrations ranged from 6.62 (strain GA3) to 7.34 mg g^{-1} (strain GA1) dry weight and were detected in all the strains as shown in **Table 3**. There were no significant differences in polysaccharides among all the investigated strains. According to Skalicka-Wozniak *et al.* (2012), the concentration of total polysaccharides of four *G. lucidum* strains cultivated on different substrates varied from 18.45 to 112.82 mg g^{-1} dry weight. The concentrations of bioactive compounds within the mushrooms were affected by different factors like cultivation substrate, the genotype of strain, and harvesting season (Ha *et al.*, 2015). Polysaccharides are considered to be bioactive components that contribute to medicinal properties such as activating the immune system and promoting the proliferation of neural progenitor cells (Nakagawa *et al.*, 2018). Nie *et al.* (2013) reported that the total polysaccharides isolated from Lingzhi mushrooms mainly contained glucose, mannose, galactose, fucose, xylose, and arabinose.

To evaluate the medical value of Lingzhi mushroom, triterpenoids could be considered as the "marker compound" (Ha *et al.*, 2015). In this study, lucidenic N acid, ganoderic A acid, and Ganodermanontriol isolated from the fruiting bodies were identified and quantified by HPLC. Based on the retention time of the standard and samples, acid lucidenic N, ganoderic A acid, and Ganodermanontriol were detected in all the strains (**Figures 7 and 8**). For a detailed comparison, strain GA3 showed higher concentrations of lucidenic N acid and ganoderic A acid as compared to the other strains. However, the highest ganodermanontriol level was present in strain GA1. As previously reported, the ganoderic acid content of *G. lucidum* cultivated under normal growth conditions of 85% humidity, at 27°C, with proper ventilation (CO2 <0.1%), and light (2.98 mmol m^{-2} s^{-1}) was found to be 0.833 mg g^{-1} dry weight (Sudheer *et al.*, 2018). Based on our obtained results, the gonoderic acid contents of strains GA1, GA2, and GA3 were higher than the commercial strain as described previously by Sudheer *et al.* (2018).

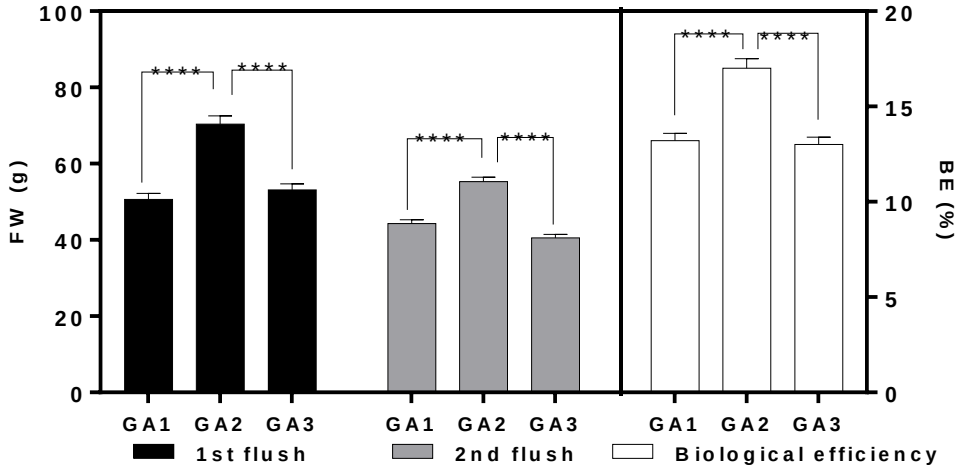
Table 3. Comparison of triterpene and total polysaccharide contents of the Lingzhi mushroom strains

Content (mg g^{-1})	lucidenic N acid	ganoderic A acid	Ganodermanontriol	polysaccharides
GA1	0.23	1.11	0.30	7.34
GA2	0.32	2.08	0.26	7.02
GA3	0.33	2.38	0.04	6.62



*Note: All data are expressed as mean ± SE (Standard Error). Significant differences were indicated with asterisks (** P <0.01, **** P <0.0001).*

Figure 3. Growth period of the Lingzhi mushroom strains



*Note: All data are expressed as mean ± SE (Standard Error). Significant differences were indicated with asterisks (** P <0.01, **** P <0.0001).*

Figure 4. The biological yield of the Lingzhi mushroom strains

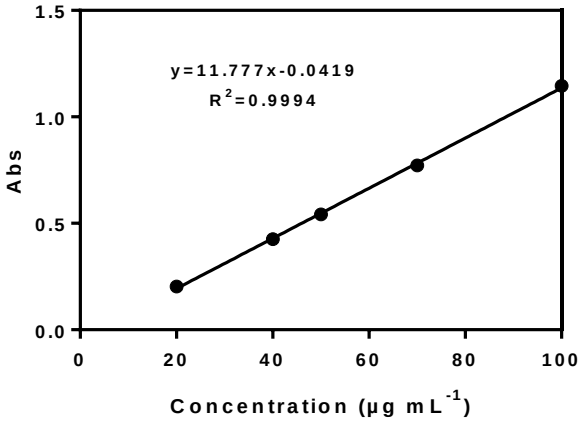


Figure 5. Glucose standard curve for the total polysaccharides assay

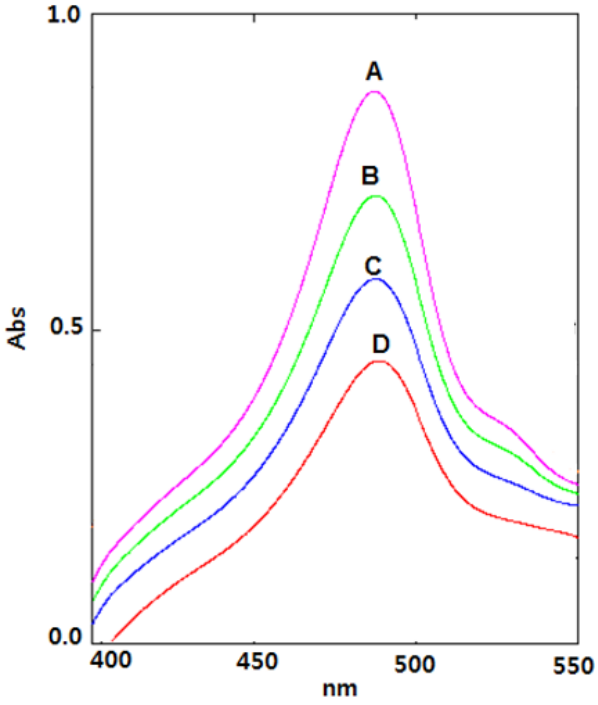


Figure 6. Absorbance spectrum of strains GA1 (A), GA2 (B), and GA3 (C), and standard glucose 40 µg mL^{-1} (D)

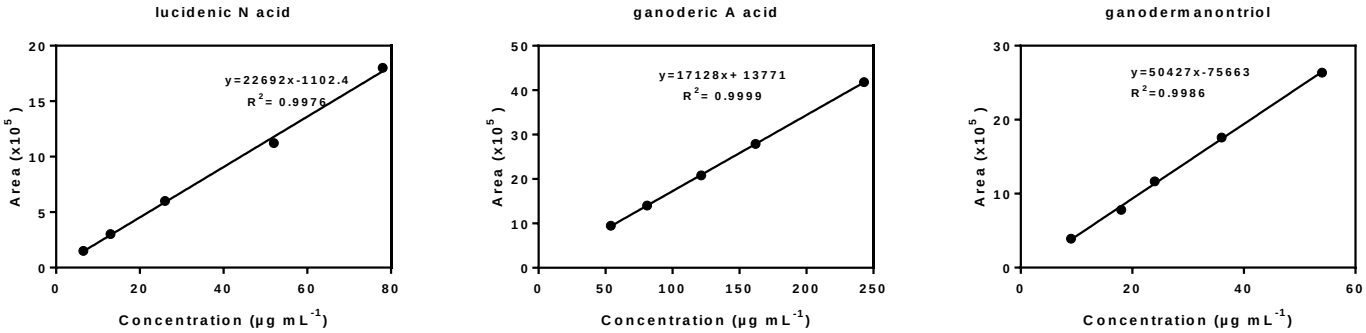


Figure 7. Standard curves of lucidenin N acid, ganoderic A acid, and ganodermanontriol

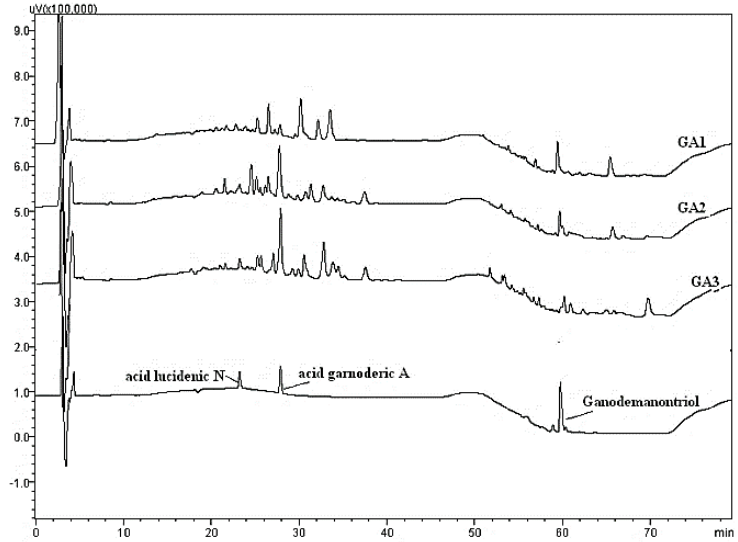


Figure 8. HPLC chromatogram of the standard and strains GA1, GA2, and GA

Conclusions

All the strains cultivated in Tam Dao were found to grow, adapt, and produce the fruiting bodies with satisfactory yield (13-17%). Regarding polysaccharide content, there were no significant differences among the tested strains. Higher contents of lucidenic N acid and ganoderic A acid were detected in strain GA3 and strain GA2 at rates of 0.33 mg g^{-1} and 0.32 mg g^{-1}, respectively, for lucidenic N acid, and rates of 2.38 mg g^{-1} and 2.08 mg g^{-1}, respectively, for ganoderic A acid. However, the highest ganodermanontriol level was present in strain GA1 at a rate of 0.3 mg g^{-1}. Based on the obtained results, strains GA1, GA2, and GA3 could be considered suitable candidates for commercial cultivation of *G. lucidum* in Tam Dao.

Acknowledgments

This study was partially funded by Vietnam National University of Agriculture as the "Research Working Group project".

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