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Cultivation of fungus *Cordyceps militaris* on pupa species butterfly *Catopsilia pomona*

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Abstract Isolation of fungus *Cordyceps militaris* on Potato Glucose Agar medium supplemented with peptone and yeast extract was evaluated well with fast and strong mycelial growth both on agar and liquid medium. The process of isolating mushrooms is 7 days, level 2 propagation is 5 days, cultured on a synthetic environment under temperature conditions of 22°C and humidity of 80%, after 10 days, the mycelium has completely eaten the surface of the substrate, moving to after 3 days of growing light with light intensity of 700-900 lux, fruit bodies began to appear. On the 55th day of the growing period, harvest and evaluate a number of growth indicators such as quantity, length and diameter of the fruit bodies as well as describe the external morphological characteristics of the fruit bodies. The size and diameter of mushroom fruiting bodies also depend greatly on the amount of nutrients in the culture medium.

Keywords: *Catopsilia pomona*, *Cordyceps militaris*, adenosin, cordycepin

Introduction

Medicinal mushrooms have long been used as medicine in traditional Eastern medicine, in which *Cordyceps militaris* are highly valued because they contain many biologically active compounds (Dai D.B., 2009). Due to its high medicinal and economic value, many research projects on cultivating *Cordyceps militaris* have been published and applied to production. However, most of the publications are about cultivating *Cordyceps militaris* on synthetic medium supplemented with Silkworm pupa powder, peptone, yeast extract or growing on the host Silkworm pupae (Wang J.F, 2006).

Butterfly *Catopsilia pomona* is a species that often appears from February to April every year in large flocks and migrates from one area to another in the provinces in the Central Highlands region. They create beautiful landscapes but at the same time also have potential dangers ability to destroy forests and crops. From March to April every year, butterflies lay eggs on the *Cassia siamea* trees. The eggs hatch into larvae that eat the Cassia leaves and then develop through stages, finally into pupae and finally into adult butterflies. Currently, people here have collected pupae of this species to process as food.

This article introduces some research results on isolating *C. militaris* and cultivating it on a pupa of *C. pomona* with the desire to create a good quality Cordyceps mushroom product, similar to the product. The product is grown using the traditional substrate of Silkworm pupae.

Materials and Methods

Research subjects:

- The fruiting body (*C. militaris*) originated from *Dalat royal food* joint stock company, Vietnam.
- Pupa of the species (*C. pomona* Fabricius) in Krong Bup district, Dack Lak province. Research period: from February 2023 to April 2023.



Cordyceps militaris



Pupa of the species *Catopsilia pomona*

Research Methods:

Analysis of biochemical components of *C. pomona* pupae: according to standards of the Ministry of Health of Vietnam.

Breeding environment:

- Isolation medium: use PGA medium with the following main ingredients: 200g potatoes, 20g glucose, 5g peptone, 5g yeast extract, 20g agar and enough distilled water to make 1000ml (Nguyen L.D, 2003).
- Level 2 propagation environment: 20g/L glucose; 5 g/L peptone; 5 g/L yeast extract; 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.3 g/L KH_2PO_4 .

Varieties isolation method: The mushroom fruit bodies were washed with 70% alcohol, then the sample was rinsed with sterile distilled water twice. Use a sterile knife to cut the sample into 2×3 mm pieces, then use a sterile pint to place 1-3 of these samples on the surface of a petri dish containing PGA medium. After inoculation, culture the sample in a dark room and temperature conditions $\leq 22^{\circ}\text{C}$. Monitor the process of mycelium formation.

Research on cultivating *C. militaris* using *C. pomona* pupae according to the following formulas:

+ Recipe 1: 60g brown rice/bottle; 120 mL mineral solution

+ Recipe 2: 60g brown rice/bottle; 5% dried *C. pomona* powder + 120 mL mineral solution

+ Recipe 3: 60g brown rice/bottle; 10% dried *C. pomona* powder + 120 mL mineral solution

Mineral solution has the following ingredients: 100 mL/L coconut water; 100 mL/L potato extract; 1 g/L vitamin B1; 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.3 g/L KH_2PO_4 ; 17g/L glucose; 5g/L peptone; 5g/L yeast; 1 chicken egg/L.

All culture vessels were autoclaved at 120°C for 30 minutes.

Research on fruit body development on pupation substrate: Arrange experiments on 3 formulas. Each recipe is conducted with 20 culture flasks, culture conditions are guaranteed to be uniform and stable.

Monitor the growth and development of mushrooms at the following times: the mycelium spreads over the substrate surface in the culture vessels, the time when fruiting bodies begin to appear, the number and size of fruiting bodies in each vessel in the growing vessels. culture formula.

Light intensity was determined using a handheld light intensity meter Lux-Meter 0500 from Testo (Germany). Humidity and temperature were determined using a Thermo hygrometer TH108 from Kichitachi (Japan).

The length and diameter of the fruiting bodies were determined using a Mitutoyo caliper

Polysaccharide content in fruit bodies *C. militaris* was determined according to the phenol - sulfuric acid method, described by Nielsen (Nielsen S.S, 2010)

Determination of cordycepin and adenosine content in fruit bodies *C. militaris* by high performance liquid chromatography. Using HPLC-Shimadzu liquid chromatography system (L. Huang et al, 2009) Statistics and processing of experimental data using Microsoft Excel 2013 program (Dang V.G. 2023).

Results and Discussion

Biochemical composition of *C. pomona* pupae

The results of analyzing the main biochemical components of *C. pomona* pupae are presented Table 1.

Table 1. Biochemical composition of *C. pomona* pupae

No	Parameter	Unit	Result
1	Protein (N×6,25)	g/100g	12.69 ± 0.31
2	Nitroamine	g/100g	1.67 ± 0.03
3	Lipid	g/100g	14.64 ± 0.41

4	Total minerals	g/100g	7.21 ± 0.11
5	Phosphorus (P)	mg/100g	41.05 ± 0.25

Analysis results in table 1 show that pupae of butterfly *C. pomona* are very rich in nutrients: protein (12.69 g/100g), lipid (14.64 g/100g); nitroamine (1.67 g/100g); total minerals (7.21 g/100g) and phosphorus (41.05 g/100g). Compared with the results of Ha Thi Hong Vi research on the biochemical composition about pupae *C. pomona* collected from Chu Prong district of Gia Lai province, these results are equivalent. Notably, the protein of pupae is nearly equal to that of silkworm pupae (13.0 g/100g), which is an ideal protein source for humans (Ha T.H.V., 2021).

Currently, in Cordyceps farming technology, people are using Silkworm pupa powder as the main source of protein. The above analysis results show that it is possible to use butterfly pupa *C. pomona* powder to grow *C. militaris* fungus, partially replacing Silkworm pupa and taking advantage of this protein rich material source (Nguyen T.M.H., 2017).

Results of isolation and secondary propagation of *C. militaris* fungus

From fruiting body samples that were sterilized and cultured on PGA medium, after 24 hours, fungal mycelium began to appear from the culture samples. From 24 hours to 72 hours after inoculation, the mycelium grows relatively slowly (mycelium length is 1-1.5 cm) but begins to grow rapidly from day 4 onwards (mycelium length is 2-3 cm). At first, the mycelium is clear white, with sparse density. Later on, the mycelium density becomes thicker, bright white at the most intense stage of development, turning milky white to pale yellow when the mycelium gets old and stops growth (Figure 1).

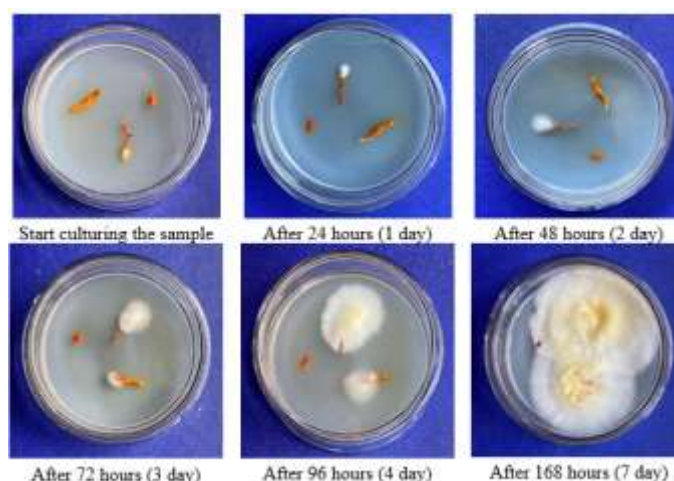


Figure 1. Isolation of *C. militaris* fungus on PGA medium

After being isolated for 6-7 days, corresponding to 144-168 hours, the fungal mycelium is cultured to the second level. Use a sterile culture knife to cut 2-3 pieces of agar (1×2 cm) in size with the same mycelium as the first level. Put into the bottle of level 2 propagation environment. Culture the sample for 4-5 days at temperature $\leq 25^{\circ}\text{C}$, low light intensity (<500 lux), humidity 60-65%, sample shaking speed 180 - 200 rpm until the mycelium spreads in the level 2 propagation medium.



Figure 2. Secondary propagation of *C. militaris* fungus

After 5 days of raising level 2 seeds, pellet density: 81/10 mL of culture medium, seed fluid is viscous, pellets have spines, little branching (Figure 2). Thus, the technique of isolating the original variety and multiplying secondary varieties of Cordyceps fungus has been developed

Cultivation of *C. militaris* fungus using pupae *C. pomona*

After level 2 propagation, put 5 mL of liquid medium into each tank containing the sterilized substrate and spread evenly on the surface. Then let all these flasks grow in a dark room at a temperature of 20-22°C and humidity of 80-85%, monitor the growth process of the mycelium until it covers the entire surface of the substrate, then transfer through the light stage to stimulate the development of fruiting bodies.

Monitoring the growth process of mycelium in dark conditions, we found that: after 2 days of culture, the mycelium began to grow on experimental formulas supplemented with pupae at different densities, while in the control group (recipe 1) the mycelium grew but very little.

By the 10th day after culture, in recipe 1 the mycelium was still growing scattered on the substrate surface, while in the experimental batches supplemented with pupa powder, the mycelium had grown to cover the substrate surface (Figure 3). Proceed to transfer the culture bottles of and recipe 3 to the light stage to develop fruit bodies.



Figure 3. Mycelium after 10 days of dark culture



Figure 4. Fungal mycelium after 2 days of light culture

After 2 days of culturing to stimulate fruiting bodies under temperature conditions of 20-22°C, humidity of 80-85% and light intensity of 700 - 900 lux, the mycelium in all culture vessels turned from white to white yellow (picture 4).

By the 3rd day of the light culture period, fruiting body sprouts appeared in the culture vessels, however in formula 3 (recipe 3) there were statistically significantly more sprouts than in formula 2.

Continue culturing until day 45 of the light cultivation period, harvest and evaluate a number of growth indicators such as quantity, length and diameter of fruit bodies as well as describe the external morphological characteristics of the fruit can (table 2).



Figure 5. Fruiting bodies begin to appear



Figure 6. Fruiting bodies after 30 days of light culture

The results in table 2 show that: the number, length and diameter of fruiting bodies were highest in the environmental formula recipe 3 and higher than that in recipe 2. This result shows that the size and diameter of mushroom fruiting bodies also depend greatly on the amount of nutrients in the culture medium. The more nutritious the environment, the larger the fruiting body size. Therefore, it is necessary to choose an environment that provides enough nutrients so that the mushrooms can grow in the most balanced size, giving the best yield and quality while reducing input material costs.

Table 2. Growth and development characteristics of fruiting bodies

C. militaris mushroom on different culture medium formulas

Experimental formula	Time for mycelium to cover the surface (date)	Time for fruiting bodies to appear (date)	Average fruiting body mass (g/rearing tank)	Average fruiting body size		Fruiting body characteristics
				Length (mm)	Diameter (mm)	
Recipe 2	10	13	51,01 ± 3,1	46,13 ± 2,4	3,2 ± 0,5	Fruiting bodies are large, moderately long, orange in color
Recipe 3	10	13	57,20 ± 2,8	47,25 ± 2,2	3,9 ± 0,5	The fruiting bodies are much larger, long, and dark orange

Biochemical composition of *C. militaris* fruiting bodies

After evaluating the growth indicators of *C. militaris* grown on different formulas, we chose fruiting bodies grown on a medium supplemented with 10% lemon butterfly pupae (recipe 3) to analyze some indicators of the main biochemical components of fruiting bodies, the results are presented in Table 3.

Table 3. Some biochemical components of Cordyceps
when grown on different substrates (dry mass)

No	Content	Analytical results
1	Protein (g/100g)	2,84
2	Nitroamine (mg/100g)	12,8
3	Phosphor (mg/100g)	143,66
4	Polysaccharide (g/100g)	4,48
5	Andenosine (mg/100g)	187
6	Cordycepin (mg/100g)	236

The protein, nitroamine and phosphorus content in fruit bodies when fed with butterfly pupae is quite high. The polysaccharide content is 4.48 g/100g, showing that pupae can be used to grow *C. militaris* mushrooms to replace silkworm pupae in mass production.

In addition to the basic biochemical components in *C. militaris* fruiting bodies including proteins, lipids, polysaccharides... compounds such as adenosine and cordycepin are considered important compounds that determine the pharmacological value of this type of mushroom. this mushroom, so cordycepin and adenosine content are considered two main indicators to evaluate the quality of *C. militaris*. Analytical results show that adenosine is 187 mg/100g and cordycepin is 236 mg/100g dry mass, which are equivalent to the research of Lo H.C. et al announced that the adenosine content in the fruiting bodies of the caterpillar fungus *Ophiocordyces sinensis* (syn. *Cordyces sinensis*) reached 245 mg/100g and the cordycepin content was 265 mg/100g (Lo H.C. et al, 2023).

Research results by Huang Lei and others in 2008 showed that the adenosine content in fruit bodies reached 180 mg/100g and in the mycelium reached 60 mg/100g, while the cordycepin content in fruit bodies reached 970 mg/100g and in mushroom mycelium is 360 mg/100g (Huang Lei et al, 2009)

Thus, the content of these two main active ingredients varies between studies, which may be due to the use of different strains and culture processes. In addition, adenosine is also a derivative and can be converted into cordycepin during the growth of *C. militaris*. Therefore, harvest time also affects the content and ratio of these two active ingredients in *C. militaris* mushroom products (Zhong J.J., 2005).

Conclusions

PGA medium supplemented with peptones and yeast extract is used to isolate level 1 varieties for healthy mycelium systems that spread evenly across the surface of the propagation medium plate.

Pupae *C. pomona* have a relatively high protein content (12.69%) and are not significantly lower than Silkworm pupae (13.00%); This is an ideal protein source to grow *C. militaris* mushroom to replace Silkworm pupae.

The fruiting body culture medium supplemented with 10% *C. pomona* pupa powder gives a biological yield equivalent to adding 5% silkworm pupa powder commonly used in mass production.

Fruiting bodies obtained from a culture medium supplemented with 10% of pupae *C. pomona* gave adenosine content of 187 mg/100g and cordycepin of 236 mg/100g of dry mass, showing that it can be applied in production.

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