Journal of Mushrooms 한국버섯학회지 Research Article

Effect of growth condition on mycelial growth and fruiting body cultivation of *Cordyceps militaris* wild strain

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ABSTRACT: *Cordyceps militaris* is widely used in China, Korea, and other Asian countries as both a traditional medicinal ingredient and an edible fungus. This study aimed to optimize the growth conditions and fruiting body production of *C. militaris* by investigating various culture media and physical parameters such as pH, aeration, illumination, temperature, spawn materials, and oat–sawdust-based substrate formulations. After a 7-day incubation period, oats with a pH of 6.0, under sealed and illuminated conditions at 32°C, demonstrated the most effective mycelial growth. Substrates consisting of 70% oat and 30% sawdust had the shortest incubation time of 30.5 days for fruiting body formation. The basidiospores showed a typical germination pattern where the sporidium produced a single germ tube that elongated, and branched to form monokaryotic primary mycelia. In conclusion, using oats as a substrate in the cultivation of *C. militaris* could reduce production costs and help protect the environment.

KEYWORDS: *Cordyceps militaris*, Cultivation phases, mycelia growth, wild mushrooms

INTRODUCTION

Cordyceps militaris contains a wide array of bioactive compounds such as polysaccharides, cordycepin, and ergosterol, which exhibit notable pharmacological properties (Abdullah and Kumar, 2023). Recent research has led to the purification of haemagglutinin (Jung et al., 2007) and a cytotoxic antifungal protease from the dry fruiting body of *C. militari*. Cordycepin, a nucleoside analog derived from the culture broth of *C. militaris*, has garnered significant attention (Wang et al., 2022). It has demonstrated antitumor (Zheng et al., 2020), antiviral (Chanda et al., 2015), and has shown efficacy in the treatment and prevention of obesity.

J. Mushrooms 2024 September, 22(3):81-86
http://dx.doi.org/10.14480/JM.2024.22.3.81
Print ISSN 1738-0294, Online ISSN 2288-8853

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Received July 22, 2024
Revised September 5, 2024
Accepted September 23, 2024

On this scale, it is crucial to continuously seek out wild genetic resources of fungi as foundational elements for cell lines, enabling more sustainable research efforts aimed at their wise utilization and conservation. Several mushrooms with nutraceutical potential have been preserved from forest ecosystems, and effective cultivation techniques have been developed (Khatun et al., 2012). Among these, *C. militaris* stands out as an excellent candidate for cultivation due to its abundance of biologically active metabolites (Chen et al., 2020). *C. militaris* symbolizes good fortune and longevity, earning it the nickname "mushroom of immortality" (Shevchuk et al., 2023).

Recent research has concentrated on advancing cutting-edge cultivation technology for *C. militaris* at the laboratory level. However, there is a gap in the current understanding of the physiological processes related to its growth morphology, which is crucial for developing cost-effective large-scale production methods (Posch and Herwig, 2014). Smith et al. (2002) highlighted the importance of extracting every part of the mushroom, from spores to the fruiting body, for health benefits (Kontogiannatos et al., 2021). Moreover, it's for underscoring the need to identify optimal nutritional and physical conditions for basidiospore germination, mycelial growth, and basidiomata formation of *C. militaris* (Deshmukh and Bhaskaran, 2024). Additionally, Vlasova et al. (2016) pointed out that despite species similarities,

growth performance can vary significantly among different strains. Sun et al. (2017) investigated the morphological and molecular characteristics of *C. militaris*. Similarly, Shih et al. (2007) examined different substrate compositions for *C. militaris* production. Technological advancements have also enabled the controlled cultivation of *C. militaris* on lignocellulosic substrates, such as agro-industrial residues, which support its growth, development, and fruiting (Pilafidis et al., 2022). Selecting the correct cultivation parameters is vital for developing industrial-scale cultures of *C. militaris* (Kontogiannatos et al., 2021).

Therefore, it is crucial to design an optimal production medium and establish ideal process operating conditions. Based on the preceding review, elucidating the biophysiological profile of *C. militaris* strains, with a particular focus on their morphogenesis, will serve as a benchmark for developing successful production technology for its utilization in the country.

MATERIALS AND METHODS

Source of strain

A wild strain of *C. militaris* basidiocarp was collected from Hwasan farm (441-12, Bangi-dong, Songpa-gu, Seoul, Republic of Korea) and brought to the laboratory for mycelial rescue. For comparison with this strain, we used *C. militaris* (KCCM NO. 60304) from the Korean Microbial Conservation Center. Internal tissues from the immature basidiocarp were excised and inoculated onto potato dextrose agar (PDA) plates containing 0.1 mg/mL. The culture plates were incubated at room temperature for 14 days to allow for mycelial growth.

Evaluation of the mycelial growth

The growth performance of secondary mycelia was assessed using various indigenous culture media, specifically oats, rice, and brown rice. The prepared decoctions were diluted with water to make up 1 L and then reboiled. These culture media were sterilized at 15 psi and 121°C for 15 minutes, then aseptically poured into sterile Petri dishes. Mycelial discs, 5 mm in diameter, from a 7-day-old pure culture of the secondary mycelia, were centrally inoculated onto the plated culture media. The plates were sealed with parafilm and maintained at room temperature under a constant day and night cycle. The effect of different indigenous culture media on the mycelial growth rate of *C. militaris* was monitored every 24 hours for 7 days using a digital Vernier caliper. The media containing mycelia were melted in an oven for 30 seconds, and the mycelia were gently removed using tweezers and rinsed in two containers of distilled water. The mycelia were then oven-dried at 40°C for 30 minutes and weighed using an analytical balance. Oats were adjusted to different pH levels (6.0, 6.5, 7.0, 7.5, and 8.0) using 0.1M NaOH or 0.1M HCl before sterilization. Each plated medium was inoculated with a 5 mm diameter fungal disc from a 7-day-old pure culture, incubated, and evaluated. For the optimal pH level, inoculated plates were sealed with parafilm, while others remained unsealed to assess the impact of aeration. Fungal discs inoculated on oats at pH 6.0 were subjected to both light and total darkness (with plates covered in aluminum foil for complete darkness) to determine the illumination requirement. Finally, the optimal nutritional and physical factors identified were used to evaluate the favorable temperature for mycelial growth. Plates were incubated under various temperature conditions: growth chamber (32°C), air-conditioned room (23°C), and refrigeration (8°C). We assumed that the environment in which mushrooms are cultivated cannot always be the same as in the laboratory, which means that we needed to evaluate mushroom cultivation in a wide range of extreme environments. Therefore, we chose this temperature range. Mycelial growth on agar plates, influenced by these physical parameters, was measured based on mean radial mycelial growth and biomass over ten trials.

Mother spawn production

The mycelial growth performance of *C. militaris* on a variety of spawning materials, such as oat, was assessed. The granular spawning materials were boiled, drained, and maintained at 60% moisture content. 20 g of each substrate were dispensed into a sterile bottle, cotton-plugged, wrapped in paper, and sterilized at 15 psi and 121°C for 40 minutes. After cooling, a 5 mm fungal disc was inoculated and incubated at room temperature to allow mycelial colonization. The total number of days required for complete mycelial colonization of each substrate was recorded. The substrate that exhibited the densest mycelial growth and achieved full colonization in the shortest time was selected as the optimal spawning material.

For basidiocarp formation, corn grit, identified as the most favorable grain spawn from the previous evaluation, was aseptically inoculated into sterilized polypropylene bags containing different formulations of oat straw and sawdust (pine (*Pinus densiflora*) wood waste) substrates as follows: T1: 100% oat (control), T2: 70% oat + 30% sawdust, T3: 50% oat + 50% sawdust, T4: 30% oat + 70% sawdust, and T5: 100% sawdust. Sterile polypropylene bags (250×380 mm) containing 20 grams of this material were inoculated

with 1 g of mycelial. The fruiting bags were incubated at room temperature until complete mycelial colonization. The fully colonized bags were then opened to allow primordia to develop into mature basidiocarps.

Effect of preservation on fruiting body induction

The production of fruiting bodies under optimized preservation conditions was studied to ascertain the best preservation duration. The fungal samples cultivated under these optimized conditions were separately stored at 4°C or at room temperature for various preservation durations (i.e., 0.5, 1, or 3 months). At the conclusion of each period, the fungal samples were utilized for fruiting body induction, and fresh biomass along with morphological characteristics was evaluated. Each preservation period was tested in duplicate.

Statistical Analyses

The data is expressed as the mean ± standard deviation (n = 3). Statistical analyses were conducted at a significance level of 5% using the Statistical Analysis System software (SAS Institute, Inc., Cary, NC, USA). Variations among group means were evaluated through Duncan's multiple-range test employing SAS.

RESULTS AND DISCUSSION

Influence of culture media

Table 1 displays the mycelial growth performance and biomass of *C. militaris* across five different indigenous culture media. Oat demonstrated the fastest mycelial growth, achieving the highest mean radial mycelial growth and biomass of 45.12 mm and 0.51 g, respectively, significantly surpassing all other treatments. Likewise, among the five-culture media tested, oat plates exhibited very dense and luxurious mycelial growth. While *C. militaris* mycelia spread quickly on plates containing rice and brown rice, their respective mycelial biomasses were notably low.

The capacity of oats to promote rapid mycelial growth of *C. militaris* can be attributed to their rich nutritional profile. Fresh oats are both clean and nutritious. The liquid endosperm of oat grains contains essential minerals and nutrients necessary for morphogenesis induction. In a previous study, oats were innovatively utilized as a medium for the biotechnological cultivation of other edible mushrooms (Ritota and Manzi, 2019). These findings were similar to those of KCCM 60304 from the Korea Microbiome Conservation Center, which was used for comparison with the strain in this study (not shown).

Table 1. Mycelial growth and biomass of *C. militaris* on the different indigenous culture media.

Culture media	Radial mycelial growth (mm)	Mycelial biomass (g)
Oat	45.12 ± 1.00 a	0.51 ± 0.00 a
Rice	35.16 ± 1.10 b	0.34 ± 0.00 b
Brown rice	32.61 ± 1.00 c	0.26 ± 0.01 c

Values are the Mean ± SD. Means within a column having the same letter of superscripts are insignificantly different from each other at 0.05 level of significance using t-test. Growth condition: pH 7, darkness, sealed and 32 °C growth chamber.

Table 2. Mycelial growth and biomass of *C. militaris* as influenced by the different pH factors

Physical factors	Radial mycelial growth (mm)	Mycelial biomass (g)
pH 6.0	50.24 ± 1.00 a	0.49 ± 0.01 a
pH 6.5	43.11 ± 1.03 ab	0.44 ± 0.01 ab
pH 7.0	42.09 ± 1.13 b	0.40 ± 0.00 bc
pH 7.5	40.74 ± 0.81 c	0.39 ± 0.01 cd
pH 8.0	37.33 ± 1.52 d	0.38 ± 0.02 cd

Values are the Mean ± SD. Means within a column having the same letter of superscripts are insignificantly different from each other at 0.05 level of significance using t-test. Growth condition: oat medium, darkness, sealed and 32 °C growth chamber.

Influence of physical factors

1. pH

Radial mycelial growth tended to decrease with increasing pH, i.e., in alkaline media. This was a significant difference at a p-value of 0.05. Mycelial biomass showed the same trend as radial mycelial growth, with higher weights being found in more slightly acidic conditions. The optimal pH range for the mycelial growth of *Cordyceps* species has been reported to be between 5.0 and 6.0 (Adnan et al. 2017), aligning with recent findings that a pH of 6.0 supports favorable mycelial growth of *C. militaris*, achieving a maximum average dry weight of 0.4740 g within 5 days. However, in this study, *C. militaris* exhibited nearly identical mycelial growth and biomass at both pH 6.0 and 6.5 (Table 2). Additionally, Shrestha et al. (2006) noted that Korean strains of *C. militaris* could grow across a wide pH range (5.5–6.0) but showed efficient mycelial growth at pH 5.0. Furthermore, Park et al. (2001) found maximum mycelial growth of *C. militaris* at an initial pH of 6.0. Mushrooms release enzymes to break down organic matter into absorbable forms for their vegetative structure, and enzyme activity is significantly influenced by pH. Mushroom

species have adapted to function optimally in their specific environmental pH. In this study, the enzyme activity of *C. militaris* decreased with increasing pH, indicating that a more basic medium can denature the cells and impair their functionality.

2. Aeration

The experiment revealed that parafilm sealing had a significant impact on the colonization rate, as illustrated in Table 3. The sealed plates exhibited a higher mean radial mycelial growth of 51.31 mm, compared to 43.45 mm in unsealed conditions. This finding indicates that aeration is a crucial physical factor to consider for optimizing mycelial growth of *C. militaris*.

3. Illumination

The evaluation of illumination as a crucial physical factor is detailed in Table 4. Plates incubated under light conditions exhibited a mean radial mycelial growth of 49.11 mm and a biomass of 0.47 g, significantly higher than those grown in complete darkness. In summary, the presence of light, combined with the carbon and nitrogen sources in oats, acts as a signal for *C. militaris* to stimulate rapid mycelial growth, achieving maximum biomass within 7 days. Additionally, light exposure during mycelial growth is thought to promote the accumulation of fungal food reserves like glycogen and lipids, which are essential for the development of macroscopic mushrooms from microscopic mycelium (Salimian et al., 2022). Furthermore, light, an essential abiotic factor, triggers sporulation (Mieslerová Mieslerová et al. 2022). This study also found that light during mycelial growth enhanced hyphal branching and conidiation of *C. militaris* on oats.

Table 3. Mycelial growth and biomass of *C. militaris* as influenced by the different aeration factors

Physical factors	Radial mycelial growth (mm)	Mycelial biomass (g)
Sealed	51.31 ± 1.33 a	0.49 ± 0.01 a
Unsealed	43.45 ± 1.07 b	0.42 ± 0.01 b

Values are the Mean ± SD. Means within a column having the same letter of superscripts are insignificantly different from each other at 0.05 level of significance using t-test. Growth condition: oat medium, pH 6, darkness, and 32 ℃ growth chamber.

Table 4. Mycelial growth and biomass of *C. militaris* as influenced by the different illumination factors

Physical factors	Radial mycelial growth (mm)	Mycelial biomass (g)
Lighted	49.11 ± 0.15 a	0.47 ± 0.01 a
Darkness	43.15 ± 0.70 b	0.32 ± 0.00 b

Values are the Mean ± SD. Means within a column having the same letter of superscripts are insignificantly different from each other at 0.05 level of significance using t-test. Growth condition: oat medium, pH 6, sealed, and 32 ℃ growth chamber.

4. Temperature

Generally, the cardinal temperatures for the mycelial growth and biomass of *C. militaris* ranged from a maximum of 32°C. Mycelial growth was most rapid at 32°C, significantly outperforming all other treatments. Conversely, mycelial growth was markedly inhibited at 8°C, correlating with the temperature decrease. This inhibition is due to the denaturation and inactivation of essential enzymes that catalyze the metabolic processes of *C. militaris*. The optimal dry growth weight was recorded at 32°C, with an average biomass of 0.53 g in 7 days (as shown in Table 5). Similarly, Kim and Yun (2005) reported that the optimal temperature for the mycelial growth of Korean strains of *C. militaris* was 30°C.

5. Evaluation of Spawn

Spawn quality is a critical factor in the cultivation of medicinal mushrooms (Shirur Shirur et al., 2021). This sub-study was conducted to evaluate the mycelial growth of *C. militaris* on various granulated materials. The oats were fully colonized by the ninth. The results clearly indicated the fastest growth rate was observed in oats. The high carbohydrate, fatty acid, and protein content of oats likely promote fruiting. Additionally, larger surface area and pore size of substrates enhance spawn run, which could explain the results observed with oats. Based on the recorded results, basidiocarps of *C. militaris* can be effectively cultured using agro-industrial residues in combination with locally available granular spawning materials. Towards the end of the vegetative spawn run, mycelia began to form constricted growth loops in response to environmental stimuli. Subsequently, the

Table 5. Mycelial growth and biomass of *C. militaris* as influenced by the different temperature factors

Physical factors	Radial mycelial growth (mm)	Mycelial biomass (g)
Air-conditioned (23°C)	15.17 ± 1.01 b	0.22 ± 0.02 b
Refrigerated (8°C)	5.37 ± 0.07 c	0.07 ± 0.00 c
Growth chamber (32°C)	52.11 ± 0.15 a	0.53 ± 0.13 a

Values are the Mean ± SD. Means within a column having the same letter of superscripts are insignificantly different from each other at 0.05 level of significance using t-test. Growth condition: oat medium, pH 6, sealed, and lighted.

production of basidiocarps of *C. militaris* was assessed using oat and sawdust from agro-industrial residues as base substrates.

Fruiting Development and Production

Morphogenesis initiates at the center of the constricted mycelial growth, gradually forming an amorphous primordial mass that eventually develops into a distinct primordium. This primordium appears as a whitish-rounded mound on the substrate's surface. It then elongates perpendicularly into an antler-like structure. Under optimized growth conditions, the antler-like basidiocarp expands horizontally, forming an undeveloped pileus. The pileus subsequently develops into a typical bean shape. As the pileus reaches full development, the margin begins to yellow. Full maturity is indicated when the pileus turns fully maroon or the undifferentiated white growth at the edge of the basidiocarp disappears. Spores are shed on the top of the pileus. Less visible but crucial is the differentiation of a fertile layer called the hymenium beneath the mushroom cap. The hymenium contains long fecund tubes, visible as pores, where basidiospores are produced. The cultivation phases of *C. militaris* are depicted in Table 6. It takes approximately about 60 days from primordial formation to fruiting body maturation. Substrates with 70% oats + 30% sawdust had the shortest fructification period of 30.50 days, followed by 50% oats + 50% sawdust, taking 35 days. In terms of yield, 70% oats + 30% sawdust produced the heaviest weight (25.96 g), similar to 50% oats + 50% sawdust (20.33 g).

Fruiting body induction affected by preservation

Fruiting body induction was further examined to evaluate preservation periods under optimized conditions. Mycelial plugs were stored at 4°C or room temperature with or without a protective agent (e.g., peptone) for various durations. The

Table 6. Fructification, yield and biological efficiency of *C. militaris* on the oats-sawdust based substrate

Substrate formulations	Fructification (day)	Yield (g)
100% oats	52.00 ± 0.50a	19.71 ± 0.17bc
70% oats + 30% sawdust	30.50 ± 1.00d	25.96 ± 0.31a
50% oats + 50% sawdust	35.00 ± 1.00c	20.33 ± 0.27b
30% oats + 70% sawdust	47.50 ± 1.00b	18.31 ± 1.05c
100% sawdust	48.50 ± 2.00b	16.19 ± 0.02d

Values are the Mean ± SD. Means within a column having the same letter of superscripts are insignificantly different from each other at 0.05 level of significance using t-test. Growth condition: pH 6, sealed, lighted, and 32 °C.

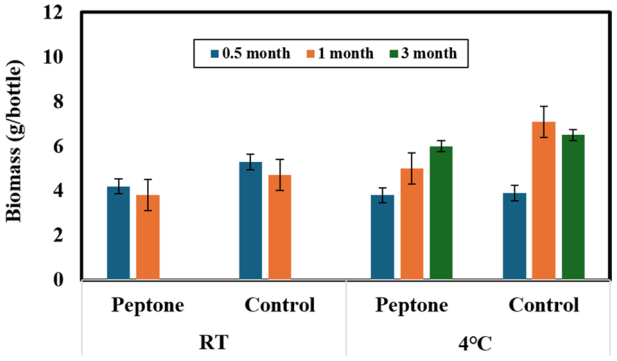

Fig. 1. Fruiting body production and preservation term under the optimized conditions. Biomass of fruiting bodies induced by strains treated by the optimized preservation conditions.

morphologies and biomass of fruiting bodies induced on the artificial medium were recorded and measured (Fig. 1). Abnormal fruiting bodies or stroma with fewer perithecia developed after half a month or one month of preservation (Fig. 1). Additionally, strains preserved at room temperature for over a month were more susceptible to contamination compared to those stored at 4°C. This contamination affected fungal preservation, leading to failures in artificial inoculation and fruiting body production. Consequently, subsequent investigations were conducted solely at 4°C. In cultures with three months of preservation, fruiting bodies or stroma were induced and developed, but the perithecia produced by the fungus preserved with the protective agent were fewer and underdeveloped (Fig. 1). The fruiting bodies induced by strains preserved for three months at 4°C without protective agents consistently showed higher biomass and abundantly developed perithecia (Fig. 1).

Fruiting bodies of *C. militaris* are widely recognized for their edible and medicinal value, leading to thriving industrial production. The biomass and quality of these fruiting bodies are crucial factors impacting industrial yield and production. The results of this study will be used to inform the cultivation of *Cordyceps* fruiting bodies.

ACKNOWLEDGEMENTS

This study was carried out with the support of ´R&D Program for Forest Science Technology (Project No. " 2023478B10-2425-BC0361382116530002")´ provided by Korea Forest Service (Korea Forestry Promotion Institute).

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