Growth and Cultural Characteristics of *Ophiocordyceps longissima* Collected in Korea

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We investigated the effect of nutritional and environmental factors on *Ophiocordyceps longissima* mycelial growth. The longest colony diameter was observed on *Schizophyllum* (mushroom) genetics complete medium plus yeast extract, *Schizophyllum* (mushroom) genetics minimal medium, and Sabouraud dextrose agar (SDA); however, malt-extract yeast-extract agar, SDA plus yeast extract, yeast-extract malt-extract peptone dextrose agar, SDA, oatmeal agar, and potato dextrose agar showed higher mycelia density. A temperature of 25°C was optimum and 7.0 was the optimum pH for mycelial growth. Colony diameter was similar under light and dark conditions. Maltose and yeast extract showed the highest mycelial growth among carbon and nitrogen sources respectively. The effect of mineral salts was less obvious; however, K₂PO₄ showed slightly better growth than that of the other mineral salts tested. Among all nutrition sources tested, complex organic nitrogen sources such as yeast extract, peptone, and tryptone were best for mycelial growth of *O. longissima*. *Ophiocordyceps longissima* composite medium, formulated by adding maltose (2% w/v), yeast extract (1% w/v), and K₂PO₄ (0.05% w/v) resulted in slightly longer colony diameter. *In vitro* mycelial *O. longissima* growth was sustainable and the production of fruiting bodies could be used for commercial purposes in the future.

KEYWORDS : Carbon source, Growth characteristics, Mineral salt, Nitrogen source, *Ophiocordyceps longissima*

Many *Cordyceps* species grow on nymphs (larvae) of cicada (Cicadidae, Homoptera) [1-3]. One is *C. longissima* Kobayasi, which was first reported from Japan on *Tanna japonensis* nymphs [2]. This species was later confirmed in Korea in 1998 [4]. One year later, it was reported in China together with its *Hirsutella* anamorph [5]. Li et al. confirmed the anamorph as *H. longissima* Li et al. [6]. Recently, Sung et al. transferred *C. longissima* to a new genus *Ophiocordyceps* based on a phylogenetic classification and renamed it *O. longissima* (Kobayasi) Sung et al. [7].

The stromata of *O. longissima* are 5–20 cm long, sometimes much longer (Fig. 1). The stroma of *O. longissima* is characterized by a long stalk with a terminal clavate fertile part without any clear demarcation between the two parts. Perithecia are ovoid to long ovoid, with a short neck, 440–590 × 130–300 μm; asci and secondary spores measure 190–350 × 5–6 μm and 8–11 × 1–1.2 μm respectively (Figs. 2 and 3).

*Cordyceps* species, including cicadicolous fungi such as *O. sobolifera*, are regarded as medicinal mushrooms in oriental society [2, 8-11]. In this context, many researchers have begun to study cultivation characteristics of *Cordyceps* and allied species [5, 12-23]. Within the past few years, *O. longissima* specimens have been collected by the Cordyceps Research Institute (CRI), Mushtech, Korea on Mt. Halla at Jeju-do and on Mt. Duryun at Jeollanam-do Korea. In this study, we provide detailed information on mycelial growth characteristics of *O. longissima* collected in Korea for the first time.

Materials and Methods

Fungal isolates. Multi-ascospore isolates were derived from fresh *O. longissima* specimens CRI C-6764, CRI C-7080, and CRI C-8587 following the method of Sung et al. [24]. Specimen CRI C-6764 was collected on Mt. Duryun at Jeollanam-do on July 8, 2001. Similarly, two other specimens, CRI C-7080 and CRI C-8587, were collected on Mt. Halla at Jeju-do on July 10 and 12, 2001 respectively. The specimens have been preserved at CRI, Mushtech, Korea. The multi-ascospore isolates, after growing on Sabouraud dextrose agar plus yeast extract (SDAY;
Fig. 1. Various natural specimens of *Ophiocordyceps longissima* collected in Korea.

Fig. 2. Morphological characteristics of *Ophiocordyceps longissima*. A–C, Apical fertile part of stromata; D–F, Cross-section of stromata showing perithecia; G, H, Ascus heads; I, Threadlike fragmented ascospores.
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dextrose 20 g, yeast extract 5 g, peptone 5 g and agar 15 g per 1,000 mL; pH 5.6) agar plates at 24 ± 1°C for 30 days, were used in the experiment.

Effect of medium, temperature, light, and pH on O. longissima mycelial growth. Nine different types of agar media were used to observe the growth characteristics of O. longissima isolates (Table 1). Mycelial discs (5 mm) of all three isolates were inoculated in the center of the agar media and incubated at 25°C for 30 days. Water agar (2%, WA) was used as the control. Colony diameter (CD) was measured in mm and mycelial density (MD) was qualitatively graded as thin (+), moderate (++) or compact (+++) after the incubation.

Schizophyllum (mushroom) genetics minimal medium (MM) and malt-extract yeast-extract agar (MYA) showed better mycelial growth and, hence, were used for selecting the optimum temperature for growth of the O. longissima isolates. Mycelial discs were inoculated on MM and MYA agar plates and incubated at various temperatures ranging from 15–35°C at regular intervals of 5°C for 30 days. Similarly, to observe the effect of light on growth, mycelial discs were inoculated on MM agar plates and incubated under continuous light and dark conditions for 30 days at 25°C. CD and MD were recorded after the incubation, as described above.

Liquid MM (100 mL MM without agar) was prepared in 250 mL Erlenmeyer flasks. The pH of the liquid medium was adjusted from 4.0–10.0 at intervals of 1.0 before sterilization. Five mycelial discs were inoculated in the liquid medium with different pH levels and incubated on a rotary shaker at 120 rpm for 30 days at 25°C. The liquid cultures were then filtered through Whatman no. 2 filter paper, the residual mycelia were dried at 60°C for 24 hr, and the dry weight (DW) of the mycelium was measured in g.

Selection of the optimum carbon source, nitrogen source, mineral salts, and carbon/nitrogen (C/N) ratio. O. longissima isolates were grown on WA supplemented with carbon sources (2% w/v) only. Additionally, 100 mL of MM liquid medium prepared with the carbon sources

Table 1. Synthetic media composition

| Medium (g/L) | WA | OA | MYA | MM | PDA | MCM | YMA | CDA | SDAY | SDA |
|-------------|----|----|-----|----|-----|-----|-----|-----|------|------|
| Dextrose    | 4  | 0  | 20  | 20 | 20  | 10  | 20  | 20  | 20   | 20   |
| Malt extract| 10 |    | 3   |    | 3   |     |     |     |      |      |
| Sucrose     |    |    |     | 30 |     |     |     |     |      |      |
| Oatmeal flake| 30 |    |     |    |     |     |     |     |      |      |
| Potato      |    |    |     |    | 200 |     |     |     |      |      |
| Peptone     |    |    | 2   | 5  | 5   | 5   | 5   | 5   | 5    | 5    |
| Yeast extract| 4  |    | 2   | 3  | 3   |     |     |     | 3    | 3    |
| NaNO₃       |    |    |     |    |     |     |     |     | 3    | 3    |
| DL-asparagine| 2  |    |     |    |     |     |     |     |      |      |
| MgSO₄·7H₂O  | 0.5| 0.5| 0.5 | 0.5| 0.5 | 0.5 | 0.5 | 0.5 | 0.5   | 0.5   |
| KCl         |    |    |     |    |     |     |     |     | 0.01  | 0.01  |
| FeSO₄·7H₂O  |    |    |     |    |     |     |     |     | 0.46  | 0.46  |
| KH₂PO₄      |    |    | 0.46| 0.46| 0.46| 0.46| 0.46| 0.46| 0.46  | 0.46  |
| K₂HPO₄      |    |    | 1   | 1  | 1   | 1   | 1   | 1   | 1     | 1     |
| Agar        | 20 | 20 | 15  | 20 | 20  | 20  | 20  | 20  | 20   | 20   |

WA, water agar; OA, oatmeal agar; MYA, malt-extract yeast-extract agar; MM, Schizophyllum (mushroom) genetics minimal medium; PDA, potato dextrose agar; MCM, Schizophyllum (mushroom) genetics complete medium plus yeast extract; YMA, yeast-extract malt-extract peptone dextrose agar; CDA, Czapek-dox agar; SDAY, Sabouraud dextrose agar plus yeast extract; SDA, Sabouraud dextrose agar.
(2% w/v) in 250 mL Erlenmeyer flasks were inoculated with the isolates. Similarly, isolates were inoculated in WA and MM liquid media supplemented with nitrogen sources (2% w/v) only. Different mineral salts (0.05% w/v) were also tested for their effect on mycelial growth of *O. longissima* isolates in WA and MM liquid media. Agar cultures and liquid cultures were incubated as described above.

Maltose and yeast extract were the best carbon and nitrogen sources respectively for the mycelial growth of *O. longissima* isolates. Hence, they were added together in the WA and MM liquid media at different ratios of 20 : 1, 10 : 1, 5 : 1, 2 : 1, 1 : 2, 1 : 5, and 1 : 10 and inoculated with the isolates. Maltose concentration was fixed at 0.5%, 1.0%, and 2.0% (w/v) for all ratios. Agar and liquid cultures were incubated as described above, and WA medium was used as the control. CD and MD were recorded on agar cultures, and DW was measured in liquid cultures, as described above.

Comparison of *O. longissima* composite medium (OLCM) with MYA. OLCM was prepared by adding maltose, yeast extract, and K$_3$PO$_4$ at concentrations of 2% (w/v), 1% (w/v), and 0.05% (w/v), respectively, on WA. The *O. longissima* isolates were inoculated on OLCM and MYA agar plates and observed for CD and MD. WA was used as the control.

**Results and Discussion**

**Selection of optimum medium, temperature, and pH.**

The CD of *O. longissima* isolates was relatively longer on *Schizophyllum* (mushroom) genetics complete medium plus yeast extract, MM, and Sabouraud dextrose agar (SDA); however, compact or moderate density was observed on MYA, SDA, yeast-extract malt-extract peptone dextrose agar, SDA, potato dextrose agar (PDA), and oatmeal agar (Table 2). CD differed among the isolates; CRI C-7080 and CRI C-8587 grew faster than CRI C-6764. It was clear that media that induced a compact density produced a shorter CD compared to media that produced moderate density (Table 2), as shown in *C. militaris* isolates [25]. This result indicates that MD and CD of the isolates do not correlate with each other on agar culture. Czapek-dox agar (CDA) produced a thin density and the shortest CD in *O. longissima* isolates, as reported in other *Cordyceps* species [26, 27]. CDA does not contain an organic nitrogen source, which may be why it could not support rich growth of the *O. longissima* isolates. Li et al. [5, 6] showed longer CD of *O. longissima* isolates on Czapek agar than that on PDA, but did not report the MD. CD of *O. longissima* isolates in our study was generally longer than that of Li et al. [5, 6].

The longest CD was observed at 25°C (Fig. 4), agree-

![Fig. 4. Effect of temperature on mycelial growth of *Ophiocordyceps longissima* isolates cultured on *Schizophyllum* (mushroom) genetics minimal medium (MM). CRI, Cordyceps Research Institute.](image)

**Table 2. Effect of medium on *Ophiocordyceps longissima* mycelial growth**

| Medium          | Isolate No. | CD    | MD    | CD    | MD    |
|-----------------|-------------|-------|-------|-------|-------|
| MCM             | CRI C-6764  | 31    | ++    | 50    | ++    | 34    | ++    |
| MM              | CRI C-7080  | 30    | ++    | 46    | ++    | 44    | ++    |
| SDA             | CRI C-8587  | 24    | +++   | 28    | +++   | 43    | ++    |
| MYA             | CRI C-6764  | 30    | +++   | 37    | +++   | 30    | +++   |
| OA              |             | 27    | ++    | 25    | +++   | 36    | ++    |
| PDA             |             | 32    | +++   | 25    | +++   | 36    | ++    |
| SDAY            |             | 32    | +++   | 18    | +++   | 36    | +++   |
| YMA             |             | 25    | +++   | 34    | +++   | 26    | +++   |
| CDA             |             | 11    | +     | 20    | +     | 15    | +     |
| WA              |             | 11    | +     | 20    | +     | 11    | +     |

CRI, Cordyceps Research Institute; CD, colony diameter; MD, mycelial density; MCM, *Schizophyllum* (mushroom) genetics complete medium plus yeast extract; MM, *Schizophyllum* (mushroom) genetics minimal medium; SDA, Sabouraud dextrose agar; MYA, malt-extract yeast-extract agar; OA, oatmeal agar; PDA, potato dextrose agar; SDAY, Sabouraud dextrose agar plus yeast extract; YMA, yeast-extract malt-extract peptone dextrose agar; CDA, Czapek-dox agar; WA, water agar.

![Fig. 5. Effect of light on colony diameter of *Ophiocordyceps longissima* isolates cultured on *Schizophyllum* (mushroom) genetics minimal medium. CRI, Cordyceps Research Institute.](image)
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ing with previous studies [5, 14, 24, 26, 28, 29]. *O. longissima* isolates had a similar CD at 20°C and 30°C, which was similar to *Metacordyceps yongmunensis* and *O. heteropoda* [27, 29]. However, this was in contrast to *C. cardinalis* that showed no growth at 30°C and above [26]. Almost no mycelial growth of *O. longissima* isolates occurred at 15°C and 35°C (Fig. 4).

Almost no mycelial growth of *O. longissima* isolates occurred at 15°C and 35°C (Fig. 4). No obvious difference was observed in the CD of *O. longissima* between light and dark conditions (Fig. 5). The prime effect of light is the induction of pigmentation [25]. In our study, the *O. longissima* isolates produced reddish white pigmentation under light, similar to that observed by Li *et al.* [5, 6]. In addition to pigmentation, light also controls fruiting morphology, such as elongation and branching in culture [14]. Cycles of dark/light periods may be critical in some species to induce fruiting bodies [16]. A pH of 7.0 produced the highest DW, followed by pH 8.0, which was similar to previous studies (Fig. 6) [14, 26, 28].

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**Table 3. Effect of carbon source on *Ophiocordyceps longissima* mycelial growth**

| Carbon source | Isolate No. | CRI C-6764 | CD | MD | DW | CRI C-7080 | CD | MD | DW | CRI C-8587 | CD | MD | DW |
|---------------|-------------|-------------|----|----|----|-------------|----|----|----|-------------|----|----|----|
| Maltose       | CRI C-6764  | 6           | +  | 0.1200 | 14 | +  | 0.0813 | 10 | +  | 0.0983 |
| Dextrin       | CRI C-7080  | 10          | +  | 0.0519 | 8  | +  | 0.0333 | 7  | +  | 0.0360 |
| Fructose      | CRI C-8587  | 9           | +  | 0.0467 | 15 | +  | 0.0330 | 11 | +  | 0.0417 |
| Mannose       |             |             | 6  | +  | 0.0467 | 17 | +  | 0.0330 | 5  | +  | 0.0427 |
| Saccharose    |             |             | 6  | +  | 0.0467 | 16 | +  | 0.0397 | 9  | +  | 0.0403 |
| Glucose       |             |             | 7  | +  | 0.0110 | 10 | +  | 0.0113 | 7  | +  | 0.0113 |
| WA            |             |             | 7  | +  | 0.0110 | 10 | +  | 0.0113 | 7  | +  | 0.0113 |

CRI, Cordyceps Research Institute; CD, colony diameter; MD, mycelial density; DW, dry wt. of mycelium; WA, water agar.

**Table 4. Effect of nitrogen source on *Ophiocordyceps longissima* mycelial growth**

| Nitrogen source | Isolate No. | CRI C-6764 | CD | MD | DW | CRI C-7080 | CD | MD | DW | CRI C-8587 | CD | MD | DW |
|-----------------|-------------|-------------|----|----|----|-------------|----|----|----|-------------|----|----|----|
| Yeast extract   | CRI C-6764  | 26          | +++| 0.1249 | 33 | +++| 0.2556 | 28 | +++| 0.2196 |
| Peptone         | CRI C-7080  | 16          | ++ | 0.0739 | 13 | +++| 0.0592 | 15 | +++| 0.0916 |
| Tryptone        | CRI C-8587  | 19          | +++| 0.0736 | 17 | ++ | 0.0732 | 18 | +++| 0.0598 |
| NaNO₃          |             | 10          | +  | 0.0434 | 7  | +  | 0.0514 | 7  | +  | 0.0542 |
| KNO₃           |             | 14          | +  | 0.0305 | 8  | +  | 0.0603 | 9  | +  | 0.0291 |
| Glycine        |             | 7           | +  | 0.0374 | 6  | +  | 0.0459 | 6  | +  | 0.0427 |
| L-asparagine   |             | 8           | +  | 0.0366 | 9  | +  | 0.0393 | 10 | ++| 0.0392 |
| Ammonium tartrate |           |              | 14 | +  | 0.0305 | 9  | +  | 0.0328 | 8  | +  | 0.0244 |
| WA             |             | 7           | +  | 0.0114 | 10 | +  | 0.0112 | 7  | +  | 0.0113 |

CRI, Cordyceps Research Institute; CD, colony diameter; MD, mycelial density; DW, dry wt. of mycelium; WA, water agar.
and tryptone in both agar and liquid cultures (Table 4). Yeast extract, peptone, and tryptone also produced much better mycelial growth than the carbon sources. NaNO₃, KNO₃, glycine, and ammonium tartrate all produced short, thin densities, as with WA (Table 4). However, L-asparagine produced moderate density and a similar CD as NaNO₃, KNO₃, glycine, and ammonium tartrate. These results showed that complex organic nitrogen sources such as yeast extract, peptone, and tryptone sustain favorable growth of *O. longissima*, as in other *Cordyceps* and allied species [25-27, 29]. Yeast extract was selected as the best nitrogen source for mycelial growth of *O. longissima* isolates and was used for further tests. Mineral salts showed the poorest mycelial growth among all nutritional sources tested (Table 5). All mineral salts tested produced thin MD and CD, similar to WA. K₃PO₄ showed slightly better mycelial growth than that of other mineral salts. Notably, CuSO₄·5H₂O showed a rather shorter CD than that of WA in all isolates (Table 5).

*O. longissima* isolates produced compact MD at all C/N ratios (Table 6). However, C/N ratios of 2 : 1 and 1 : 1 showed the longest CD (Table 6). Nitrogen favors mycelial growth but higher nitrogen ratios slowed CD, probably due to higher nutrient concentrations; thus, increasing the water potential of the medium and consequently decreasing the amount of water available to the isolates. A 2 : 1 C/N ratio was selected with maltose and yeast extract at concentrations of 2% and 1%, respectively, to formulate the OLCM.

### Table 5. Effect of mineral salt on *Ophiocordyceps longissima* mycelial growth

| Mineral salt       | CRI C-6764 CD | CRI C-6764 MD | CRI C-7080 CD | CRI C-7080 MD | CRI C-8587 CD | CRI C-8587 MD |
|-------------------|----------------|--------------|----------------|--------------|----------------|--------------|
| NaNO₃             | 5 +            | 5 +          | 6 +            |              |                |              |
| MgSO₄·7H₂O        | 3 +            | 5 +          | 6 +            |              |                |              |
| CaCO₃             | 3 +            | 6 +          | 4 +            |              |                |              |
| K₃PO₄             | 4 +            | 5 +          | 5 +            |              |                |              |
| KH₂PO₄            | 4 +            | 5 +          | 5 +            |              |                |              |
| FeSO₄·7H₂O        | 2 +            | 4 +          | 3 +            |              |                |              |
| CuSO₄·5H₂O        | 2 +            | 2 +          | 2 +            |              |                |              |
| WA                | 3 +            | 4 +          | 3 +            |              |                |              |

CRI, Cordyceps Research Institute; CD, colony diameter; MD, mycelial density; WA, water agar.

### Table 6. Effect of carbon/nitrogen (C/N) ratio on *Ophiocordyceps longissima* isolate CRI C-7080 mycelial growth

| C/N ratio | Maltose concentration 0.5% | 1.0% | 2.0% |
|-----------|-----------------------------|------|------|
|           | CD  | MD  | CD  | MD  | CD  | MD  |
| 20 : 1    | 32  | +++ | 29  | +++ | 28  | +++ |
| 10 : 1    | 31  | +++ | 31  | +++ | 27  | +++ |
| 5 : 1     | 30  | +++ | 33  | +++ | 31  | +++ |
| 2 : 1     | 36  | +++ | 40  | +++ | 47  | +++ |
| 1 : 1     | 35  | +++ | 38  | +++ | 43  | +++ |
| 1 : 2     | 19  | +++ | 20  | +++ | 22  | +++ |
| 1 : 5     | 19  | +++ | 20  | +++ | 19  | +++ |
| 1 : 10    | 23  | +++ | 21  | +++ | 18  | +++ |

CRI, Cordyceps Research Institute; CD, colony diameter; MD, mycelial density.

### Table 7. Comparison between *Ophiocordyceps longissima* composite medium (OLCM) and malt-extract yeast-extract agar (MYA)

| Medium | CRI C-6764 CD | CRI C-7080 CD | CRI C-8587 CD |
|--------|---------------|---------------|---------------|
| OLCM   | 39 +++         | 41 +++         | 40 +++         |
| MYA    | 34 +++         | 38 +++         | 36 +++         |
| WA     | 17 +           | 14 +           | 12 +           |

CRI, Cordyceps Research Institute; CD, colony diameter; MD, mycelial density; WA, water agar.

Comparison of OLCM with MYA. Both OLCM and MYA produced compact MD (Table 7). However, OLCM produced slightly longer CD than that of MYA. A higher concentration of yeast extract is likely favorable for *O. longissima* mycelial growth, as in *C. militaris* [25]. The medium is the most important factor for mycelial growth and fruiting body formation in *Cordyceps* species. Commonly used mycological media can support profuse mycelial growth in *Cordyceps* species. In contrast, cereals such as rice, supplemented with pupal power, sawdust, peptone, and yeast extract are used for fruiting body production [5, 14]. Fruiting medium is much more complex than mycelial growth medium. Obviously, fruiting body formation is a much more complex physio- and morpho-genetic process. As with mycelial growth, nitrogen sources are likely the most important nutritional factor for fruiting body formation. Fruiting body formation of *O. longissima* has been reported by Li *et al.* [5, 6] in rice medium. It is concluded that various nutritional and environmental factors should be tested to induce profuse fruiting bodies in *O. longissima*, so that it can be commercially utilized as a health and medicinal food.

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