Cultural Characteristics of *Ophiocordyceps heteropoda* Collected from Korea

Gi-Ho Sung¹, Bhushan Shrestha², Sang-Kuk Han¹ and Jae-Mo Sung*¹

¹Mushroom Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Suwon 441-707, Korea
²Green Energy Mission/Nepal, Anam Nagar, Kathmandu P.O. Box 10647, Nepal
³Division of Forest Biodiversity, Korea National Arboretum, Pocheon 487-820, Korea
⁴Cordyceps Institute of Mushtech, Chuncheon 200-936, Korea

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Isolates of *Ophiocordyceps heteropoda* (Kobayasi) collected from Mt. Halla on Jeju-do, Korea were tested for mycelial growth on different agar media and in the presence of different carbon and nitrogen sources. Similarly, isolates were also incubated at different temperatures as well as under continuous light and dark conditions. Growth was better on Hamada agar, basal medium, and malt-yeast agar, but poor on Czapek-Dox agar. Different carbon sources such as dextrin, saccharose, starch, lactose, maltose, fructose, and dextrose resulted in better growth. Complex organic nitrogen sources such as yeast extract and peptone revealed the most effective growth. Mycelial growth was best at 25°C. The growth rate was faster in the dark than the light, but mycelial density was less compact in the dark.

**KEYWORDS**: Carbon source, *Cordyceps heteropoda*, Medium test, Nitrogen source, *Ophiocordyceps heteropoda*

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*Ophiocordyceps heteropoda* is a relatively rare species that was first described by Kobayasi as *Cordyceps heteropoda* from north Japan, growing on hypogaeous cicada nymphs (Figs. 1 and 2) [1]. It was then reported from the Congo, a central African country [2]. Besides Japan, it has been recently reported from other east Asian countries such as Korea and China [3-5]. *C. heteropoda* var. *haiirooosemitake* and two form species, *C. heteropoda* f. sp. *tsutsunagaoosemitake* and *C. heteropoda* f. sp. *Usuiroonoosemitake*, were also reported by Shimizu [6]. A different variety, *C. heteropoda* var. *langyashanensis*, and its anamorph, *Hirsutella heteropoda*, have been reported recently from China [4]. Korean *C. heteropoda* is more similar to the Japanese species [1, 3]. This species has a very patchy distribution, as it has been reported only from east Asia and central Africa, probably due to a lack of exploration. *C. heteropoda* was previously confused with another *Cordyceps* species, *C. scottianus* [1, 7, 8]. *Cordyceps* species growing on cicadas, including *C. heteropoda*, have been explicitly described, beautifully illustrated, and well reviewed in different pictorial books [1, 3, 5-6, 8-11]. Recently, *C. heteropoda* was transferred to *Ophiocordyceps* by Sung et al. [12], hence, it was renamed *O. heteropoda* (Kobayasi) Sung et al. [12]. This species is particularly characterized by the epigaeous part of its stem, which is distinct from the hypogaeous part. The head is oval to spherical and is quite distinct from the stem. This fungus produces anti-bacterial and anti-fungal compounds [13]. In the context of growing studies on *Cordyceps* and allied species [14-23], isolates of this species were tested for growth on different agar media, at different temperatures, and under light and dark conditions. This species showed moderate growth on agar media with the possibility of growing to a larger scale under optimum cultural conditions and nutrition sources.

**Materials and Methods**

**Fungal isolates.** Multi-ascospore isolates of *O. heteropoda* specimens CRI C-11247, CRI C-12565, and CRI C-12567, which were preserved at the Cordyceps Research Institute (CRI), Mushtech, Korea, were used. The isolates were grown on Sabouraud dextrose agar plus yeast extract (SDAY; dextrose 40 g, yeast extract 10 g, peptone 10 g, and agar 20 g per 1,000 mL; pH 5.6) plates at 25°C for 30 days and were used for further experiments. Specimen CRI C-11247 was collected on May 21, 2004. Similarly, two other specimens, CRI C-12565 and CRI C-12567, were collected on May 20, 2005. All specimens were collected from Mt. Halla on Jeju-do.

**Effect of medium on *O. heteropoda* mycelial growth.** Ten different types of agar media, including malt-extract agar, oatmeal agar (OA), malt-yeast agar (MYA), Martin’s peptone dextrose agar (MPDA), basal medium (BM), potato dextrose agar (PDA), *Schizophyllum* (mushroom) genetics complete medium plus yeast extract (MCM), Hamada agar (HA), Czapek-Dox agar (CDA), and SDAY were used to observe the effect of medium on *O. heteropoda* mycelial growth (Table 1). Agar was added to all
Fig. 1. Morphological characteristics of *Ophiocordyceps heteropoda*. Various natural specimens.

Fig. 2. Morphological characteristics of *Ophiocordyceps heteropoda*. A, Stroma; B, Magnified head; C, Immersed perithecia; D, Perithecia; E, Ascus head; F, Asci; G, Threadlike ascospores; H, Germination of ascospores.
In Vitro Growth of *Ophiocordyceps heteropoda*

Mycelial discs (5 mm) were cut from the isolates and were inoculated on all experimental agar plates. The agar plates were then incubated at 25°C for 30 days under white fluorescent light and were observed for colony diameter (CD) and mycelial density (MD).

Different carbon and nitrogen sources were tested for their effect on *O. heteropoda* mycelial growth. Ten different carbon sources, including arabinose, dextrin, dextrose, fructose, galactose, lactose, maltose, saccharose, starch, and xylose were individually added to 2% water agar (WA) at a 2% concentration (w/v). Similarly, ten different nitrogen sources, including NH$_4$NO$_3$, (NH$_4$)$_3$PO$_4$, (NH$_4$)$_2$SO$_4$, ammonium tartrate, KNO$_3$, arginine, asparagine, glycine, peptone, and yeast extract were individually added to WA at a 1% concentration (w/v). The isolates were inoculated on WA plates supplemented with carbon and nitrogen sources and incubated at 25°C for 30 days under white fluorescent light. CD was measured in mm and MD was qualitatively categorized as thin (+), moderate (++), or compact (+++).

### Effect of temperature and light on *O. heteropoda* mycelial growth.

The isolates were inoculated on PDA, MCM, and BM agar plates and incubated at 15–30°C at intervals of 5°C for 30 days under white fluorescent light. Similarly, the isolates were inoculated on PDA, MCM, and BM agar plates and incubated at 25°C for 30 days under white fluorescent light as well as under dark conditions. CD and MD were observed.

### Results and Discussion

CD was longer on HA, BM, and MYA followed by MPDA, MCM, SDAY, and PDA (Table 2). The isolates produced compact to moderate MD on all media, except CDA in which a thin MD was produced (Table 2). CDA also resulted in the shortest CD. The major difference between CDA and other media is that the former does not contain any organic nitrogen source. OA and PDA also do not contain an extra organic nitrogen source, but oatmeal and potato are complex organic substances that contain organic nitrogen sources. However, all remaining media were supplemented with either peptone, yeast extract, or both. From

### Table 1. Culture media composition

| Reagents (g/L) | MEA | OA | MYA | MPDA | BM | PDA | MCM | HA | CDA | SDAY |
|---------------|-----|----|-----|------|----|-----|-----|----|-----|------|
| Potato        |     |    |     |      | 200| 20  | 20  | 20 | 40  | 40   |
| Dextrose      | 20  | 4  | 10  | 20   | 20 | 20  | 20  | 20 | 40  | 40   |
| Malt extract  | 10  |    |     | 10   |    |     |     |    |     |      |
| Sucrose       |     | 30 |     |      |    |     |     |    |     |      |
| Oatmeal       |     |    | 30  |      |    |     |     |    |     |      |
| Peptone       | 1   | 5  |     | 2    | 3  | 3   | 3   | 3  | 10  | 10   |
| Yeast extract | 4   | 3  |     | 2    | 3  | 3   | 3   | 3  | 10  | 10   |
| NaNO$_3$      |     | 0.5|     | 0.5  |    | 0.5 | 0.5 |    | 3   |      |
| MgSO$_4$.7H$_2$O | 1  | 1  |     | 0.46 |    | 1   | 1   | 1  | 0.01|      |
| KCl           |     | 0.5|     |      |    |     |     |    | 3   |      |
| FeSO$_4$.7H$_2$O |     |    |     |      |    |     |     |    | 1   |      |
| KH$_2$PO$_4$  | 1   | 1  |     | 0.46 |    | 0.46| 1   | 0.46| 3   |      |
| K$_2$HPO$_4$  |     |    |     |      |    |     |     |    | 1   |      |
| Hyponex       |     |    |     |      |    |     |     |    | 1   |      |
| Ebiose        |     |    |     |      |    |     |     |    | 3   |      |
| MEA, malt-extract agar; OA, oatmeal agar; MYA, malt-yeast agar; MPDA, Martin’s peptone dextrose agar; BM, basal medium; PDA, potato dextrose agar; MCM, *Schizophyllum* (mushroom) genetics complete medium plus yeast extract; HA, Hamada agar; CDA, Czapek-Dox agar; SDAY, Sabouraud’s dextrose agar plus yeast extract.

### Table 2. Effect of medium type on *Ophiocordyceps heteropoda* mycelial growth

| Medium         | Isolate No. | CRI C-11247 | CRI C-12565 | CRI C-12567 |
|----------------|-------------|-------------|-------------|-------------|
|                | CD | MD | CD | MD | CD | MD |
| HA             | 40.7 | +++ | 45.5 | +++ | 40.5 | ++ |
| BM             | 35.5 | ++ | 39.5 | ++ | 40.8 | ++ |
| MYA            | 35.0 | +++ | 43.8 | +++ | 40.0 | +++ |
| MPDA           | 32.5 | +++ | 36.8 | ++ | 35.5 | +++ |
| MCM            | 30.5 | +++ | 35.0 | +++ | 32.6 | +++ |
| MEA            | 29.0 | +++ | 32.8 | +++ | 33.5 | +++ |
| PDA            | 27.3 | +++ | 32.5 | +++ | 35.5 | +++ |
| SDAY           | 25.3 | +++ | 29.5 | +++ | 36.0 | +++ |
| OA             | 25.0 | ++ | 34.0 | ++ | 33.2 | ++ |
| CDA            | 8.8  | + | 8.2  | + | 8.0  | + |

CRI, Cordyceps Research Institute; CD, colony diameter; MD, mycelial density; HA, Hamada agar; BM, basal medium; MYA, malt-yeast agar; MPDA, Martin’s peptone dextrose agar; MCM, *Schizophyllum* (mushroom) genetics complete medium plus yeast extract; MEA, malt-extract agar; PDA, potato dextrose agar; SDAY, Sabouraud’s dextrose agar plus yeast extract; OA, oatmeal agar; CDA, Czapek-Dox agar.
this observation, it can be concluded that organic nitrogen sources are the most important factor for rich mycelial growth in *O. heteropoda*. Besides CDA, OA produced shorter CD as well as a moderate MD. Dextrin, saccharose, starch, lactose, maltose, fructose, and dextrose resulted in better growth than the remaining carbon sources both in terms of CD and MD (Table 3). In general, dextrin, saccharose, and starch were more favorable carbon sources. The results showed that carbon sources alone could not sustain growth when compared to growth on complete media (Tables 2 and 3). However, it was unclear why CDA performed worse than the simple carbon sources despite being supplemented with inorganic nitrogen sources and mineral salts.

Complex organic nitrogen sources resulted in better growth than inorganic nitrogen sources (Table 4). Furthermore, complex organic nitrogen sources, such as yeast extract and peptone, performed better than amino acids (Table 4), as shown by previous studies [24, 25]. The mycelial growth patterns revealed that only yeast extract and peptone resulted in a compact MD. It was obvious that yeast extract and peptone consisted of many types of amino acids and, hence, resulted in better growth than that provided by individual amino acids. Among the amino acids tested, asparagine was the best and resulted in growth similar to ammonium tartrate and NH$_4$NO$_3$. (NH$_4$)$_2$SO$_4$ showed the best growth among the inorganic nitrogen sources and performed better than any of the individual amino acids (Table 4). NH$_4$NO$_3$ and KNO$_3$ also resulted in better mycelial growth than glycine and arginine both in terms of CD and MD (Table 4). Glycine, arginine, and (NH$_4$)$_3$PO$_4$ all produced a thin MD.

The effect of temperature on *O. heteropoda* mycelial growth differed from medium to medium. All isolates showed their longest CD at 25°C on PDA, followed by 20°C, 30°C and 15°C (Fig. 3). Similar to PDA, the longest CD was observed at 25°C on both MCM and BM, whereas the least growth was observed at 30°C (Figs. 3, 4 and 5). In general, mycelial growth occurred at all the

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

| Carbon source | Isolate No. | CRI C-11247 | CRI C-12565 | CRI C-12567 |
|---------------|-------------|-------------|-------------|-------------|
|               | CD          | MD          | CD          | MD          |
| Dextrin       | 12.8 ++     | 12.5 ++     | 14.3 ++     |
| Saccharose    | 12.3 ++     | 13.5 ++     | 12.8 ++     |
| Starch        | 13.0 ++     | 11.7 ++     | 12.5 ++     |
| Lactose       | 11.8 ++     | 10.4 ++     | 12.5 ++     |
| Maltose       | 12.5 ++     | 11.8 ++     | 11.0 ++     |
| Fructose      | 9.0 +       | 13.7 +++    | 10.6 ++     |
| Dextrose      | 11.5 ++     | 10.5 ++     | 9.0 ++      |
| Galactose     | 8.5 +       | 8.8 +       | 10.5 ++     |
| Arabinose     | 8.5 +       | 8.0 +       | 8.0 +       |
| Xylose        | 7.3 +       | 8.0 +       | 8.3 +       |

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

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

| Nitrogen source | Isolate No. | CRI C-11247 | CRI C-12565 | CRI C-12567 |
|-----------------|-------------|-------------|-------------|-------------|
|                 | CD          | MD          | CD          | MD          |
| Yeast extract   | 22.3 +++    | 28.8 +++    | 26.5 ++     |
| Peptone         | 21.5 +++    | 22.1 +++    | 21.0 ++     |
| Asparagine      | 15.3 ++     | 14.5 ++     | 12.4 ++     |
| Glycine         | 10.3 +      | 9.8 +       | 9.3 +       |
| Arginine        | 8.1 +       | 8.0 +       | 10.3 ++     |
| (NH$_4$)$_2$SO$_4$ | 18.8 ++   | 18.5 ++     | 19.1 ++     |
| Ammonium tartrate | 14.2 ++   | 15.3 ++     | 10.4 ++     |
| NH$_4$NO$_3$    | 11.5 ++     | 11.3 ++     | 11.8 ++     |
| KNO$_3$         | 10.2 ++     | 10.3 ++     | 10.2 ++     |
| (NH$_4$)$_3$PO$_4$ | 8.5 +     | 8.1 +       | 8.3 +       |

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

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Fig. 3. Effect of temperature on *Ophiocordyceps heteropoda* mycelial growth in potato dextrose agar after 30 days of culture. CRI, Cordyceps Research Institute.

Fig. 4. Effect of temperature on *Ophiocordyceps heteropoda* mycelial growth in *Schizophyllum* (mushroom) genetics complete medium plus yeast extract medium after 30 days of culture. CRI, Cordyceps Research Institute.
In Vitro Growth of Ophiocordyceps heteropoda

Temperatures tested ranging from 15–30°C. A 25°C temperature has been reported as optimum for Cordyceps species [25-27].

All isolates had a longer CD in the dark than the light (Figs. 6, 7 and 8). However, a difference in MD was observed. PDA and MCM resulted in a compact MD in the light but a moderate density in the dark. Moreover, BM resulted in moderate density in the light, but thin density in the dark. This result was very similar to that of Shrestha et al. [24]. Isolates of O. heteropoda produced yellowish white to yellow colonies with reddish pigmentation on the medium, as shown by Li et al. [4]. However, the growth rate was faster in this study than that of Li et al. [4]. But, the growth rate of O. heteropoda was slower, than that of C. militaris and Metacordyceps yongmunensis [24, 25].

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