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Cultural Characteristics of *Ophiocordyceps heteropoda* Collected from 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*

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. *Usuirooosemitake*, 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

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Fig. 1. Morphological characteristics of *Ophiocordyceps heteropoda*. Various natural specimens.

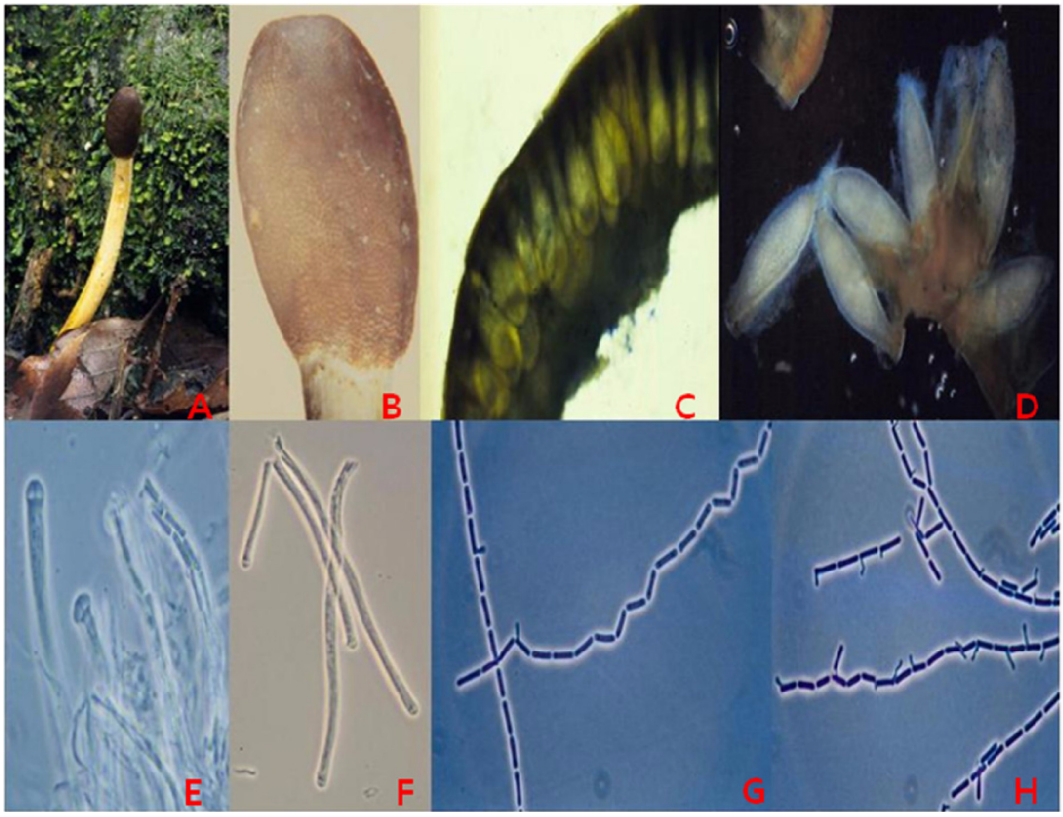

Fig. 2. Morphological characteristics of *Ophiocordyceps heteropoda*. A, Stroma; B, Magnified head; C, Immersed perithecia; D, Perithecia; E, Ascus head; F, Asci; G, Threadlike ascospores; H, Germination of ascospores.

Table 1. Culture media composition

Reagents (g/L)	Medium									
	MEA	OA	MYA	MPDA	BM	PDA	MCM	HA	CDA	SDAY
Potato						200				
Dextrose	20		4	10		20	20	20		40
Malt extract	20		10							
Sucrose					30				30	
Oatmeal		30								
Peptone	1			5			2			10
Yeast extract			4		3		2	3		10
$NaNO_3$									3	
$MgSO_4 \cdot 7H_2O$				0.5	1		0.5		0.5	
KCl									0.5	
$FeSO_4 \cdot 7H_2O$									0.01	
KH_2PO_4				1	1		0.46			
K_2HPO_4							1		1	
Hyponex								3		
Ebiose								5		

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.

media at a 2% concentration (w/v). 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_4NO_3, $(NH_4)_3PO_4$, $(NH_4)_2SO_4$, 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 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.

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	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.

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_4NO_3. $(NH_4)_2SO_4$ showed the best growth among the inorganic nitrogen sources and performed better than any of the individual amino acids (Table 4). NH_4NO_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 $(NH_4)_3PO_4$ all produced a thin MD.

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	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	++
$(NH_4)_2SO_4$	18.8	++	18.5	++	19.1	++
Ammomium tartrate	14.2	++	15.3	++	10.4	++
NH_4NO_3	11.5	++	11.3	++	11.8	++
KNO_3	10.2	++	10.3	++	10.2	++
$(NH_4)_3PO_4$	8.5	+	8.1	+	8.3	+

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

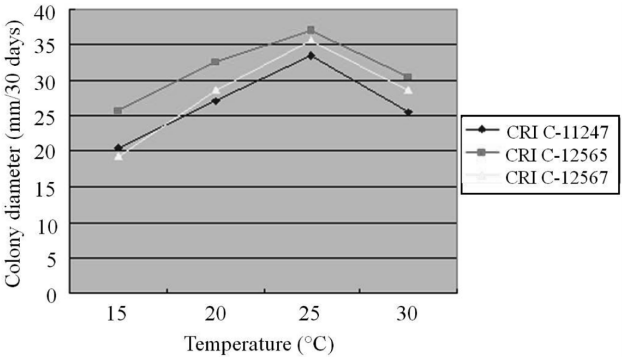

Fig. 3. Effect of temperature on *Ophiocordyceps heteropoda* mycelial growth in potato dextrose agar after 30 days of culture. CRI, Cordyceps Research Institute.

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

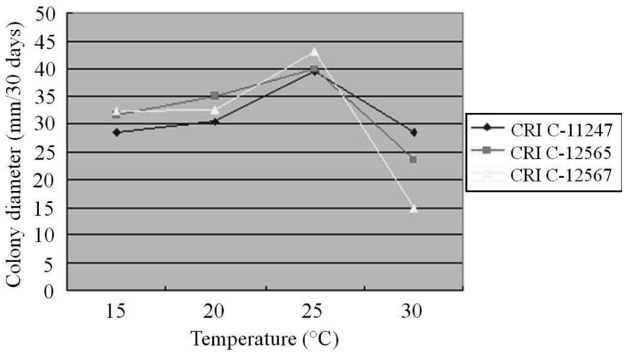

Fig. 4. Effect of temperature on *Ophiocordyceps heteropoda* mycelial growth in *Schizophyllum* (mushroom) genetics complete medium plus yeast extract medium after 30 days of culture. CRI, Cordyceps Research Institute.

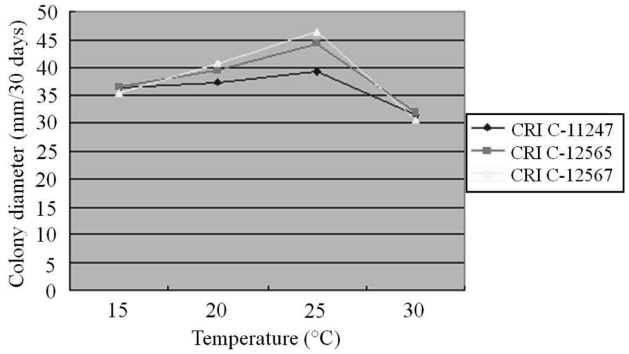

Fig. 5. Effect of temperature on *Ophiocordyceps heteropoda* mycelial growth in basal medium after 30 days of culture. CRI, Cordyceps Research Institute.

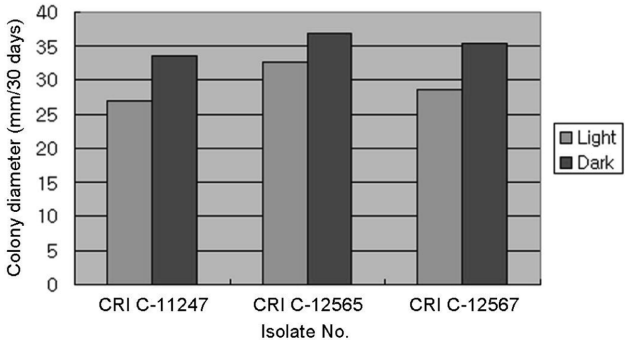

Fig. 6. Effect of light on *Ophiocordyceps heteropoda* mycelial growth in potato dextrose agar. CRI, Cordyceps Research Institute.

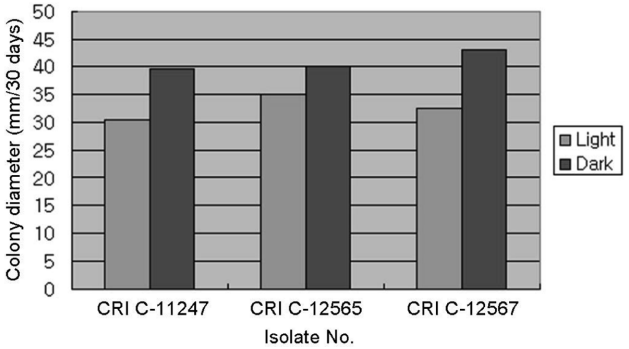

Fig. 7. Effect of light on *Ophiocordyceps heteropoda* mycelial growth in *Schizophyllum* (mushroom) genetics complete medium plus yeast extract medium. CRI, Cordyceps Research Institute.

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].

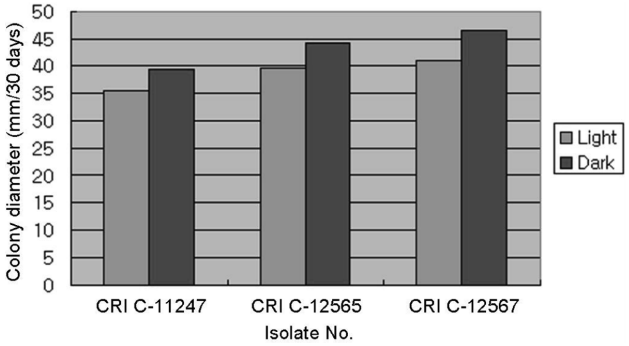

Fig. 8. Effect of light on *Ophiocordyceps heteropoda* mycelial growth in basal medium. CRI, Cordyceps Research Institute.

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