Mycobiology 38(3) : 189-194 (2010)
DOI:10.4489/MYCO.2010.38.3.189

Cultural Characteristics of *Shimizuomyces paradoxus* Collected from Korea

Gi-Ho Sung[1], Bhushan Shrestha[2], Ki-Byung Park[3] and Jae-Mo Sung[4]*

[1]*Mushroom Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Suwon 441-707, Korea*
[2]*Green Energy Mission/Nepal, Anam Nagar, Kathmandu, P.O. Box 10647, Nepal*
[3]*GEO Environ Seongnam, Seongnam 463-825, Korea*
[4]*Cordyceps Institute of Mushtech, Chuncheon 200-936, Korea*

(Received July 5, 2010. Accepted July 27, 2010)

This study investigated the cultural characteristics of *Shimizuomyces paradoxus* in different nutritional and environmental conditions. The highest mycelial growth was observed in *Schizophyllum* (mushroom) genetics complete medium plus yeast extract agar medium, and the optimal temperature and pH were 25°C and pH 8.0, respectively. The optimal carbon and nitrogen sources were 1% dextrose and 1% peptone in agar. However, in liquid culture the highest dry mycelium weight was found for the potato dextrose agar and potato sucrose agar broths. The optimum inoculum size was five mycelial discs (5 mm) per 100 mL of broth, and the optimum liquid culture period was 25 days. This is the first ever report of *S. paradoxus* cultural characteristics.

KEYWORDS : Agar medium, Environmental factors, Liquid medium, Nutrition sources, *Shimizuomyces paradoxus*

Shimizuomyces has been described as a new genus by Kobayasi [1] from Japan, of which stromata grow on plant fruits. *Shimizuomyces* is morphologically similar to *Cordyceps* and has been placed phylogenetically in Clavicipitaceae [1-3]. *S. paradoxus*, the type species, grows on *Smilax sieboldi* fruits. *S. kibiana*, which was also described by Kobayasi [4], grows on *Smilax china* seeds. Besides Japan, *S. paradoxus* has been reported only from Korea [5]. The stromata of *S. paradoxus* from Korea are slightly larger than those reported from Japan; however, the perithecia and ascospores of Japanese specimens are bigger [1, 5]. The stromata are clavate, grow gregariously on host fruits, and can be distinguished by an apical head and basal stipe (Fig. 1A). The perithecia are slightly emerged, ovate, and densely distributed in the head. Ascospores are fusiform, multiseptate, but not disarticulating, usually with a dilated cell in the middle. The host fruits are covered with white mycelial outgrowths.

Much interest has been generated to culture *Cordyceps* and allied species [6-13]. This study reports the cultural characteristics of *S. paradoxus* in different media. Culturing *S. paradoxus* has opened the door for future applications. However, the culturability of *S. kibiana* is not yet known.

Materials and Methods

Fungal specimen and isolates. A specimen of *Shimizuomyces paradoxus* EFCC C-5280, collected from *Mt.* Chundeung at Chungcheong-do on July 23, 2000, was used in this study. Heads and stipes of the specimen were 3~17 mm and 4~38 mm long, respectively. The perithecia were 300~500 × 150~300 μm (Fig. 1B). Asci were 4~8 spored and 90~130 μm long; ascospores were 60~75 μm long (Fig. 1C and 1D). For multi-ascospore isolation, ascospores were discharged from fresh specimen over 2% water agar plates. Small agar blocks containing ascospores were then cut and transferred to Sabouraud dextrose agar plus yeast extract (SDAY) agar plates of half strength (dextrose 20 g, yeast extract 5 g, peptone 5 g and agar 15 g per 1,000 mL; pH 5.6) and incubated at 20°C under continuous light condition for 30 days. The specimen and isolates were preserved at the Entomopathogenic Fungal Culture Collection (EFCC) of Cordyceps Institute of Mushtech, Chuncheon, Korea.

Selection of optimum nutrition, temperature, and pH. The isolate was inoculated on 11 different agar media and incubated at 20 ± 1°C for 55 days. Growth characteristics such as colony diameter, mycelial density, and colony pigmentation were observed on each medium. The composition of most of the agar media followed Shrestha *et al*. [13] (Table 1). Agar was added at a concentration of 20 g/L for all media. The isolate was also incubated at different temperatures ranging from 15~35°C on *Schizophyllum* (mushroom) genetics complete medium plus yeast extract (MCM) agar medium and observed for growth characteristics. Five mycelial discs (5 mm) of the isolate were inoculated in 100 mL of MCM broth (MCM without agar), adjusted to different pH levels from 4.0 and then steril-

*Corresponding author <E-mail : cordyceps@hanmail.net>

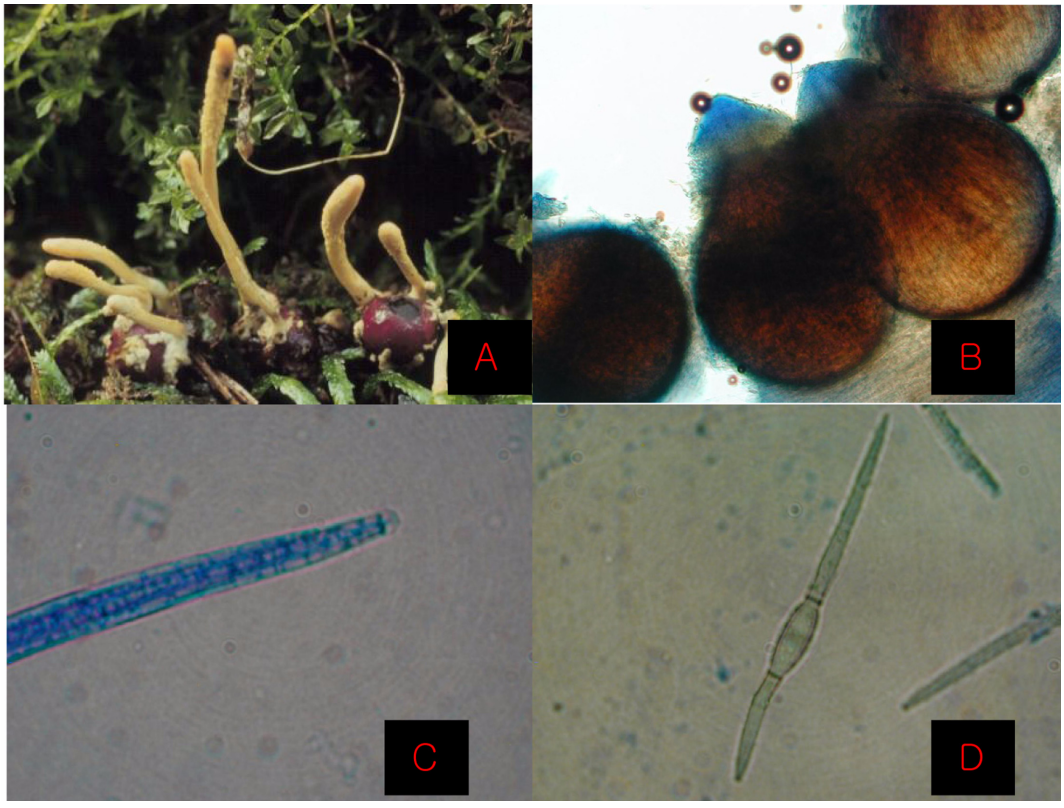

Fig. 1. Morphological characteristics of the *Shimizuomyces paradoxus*. A, Natural specimens; B, Perithecia; C, Ascus; D, Ascospores.

Table 1. Agar media composition

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

PDA, potato dextrose agar; PSA, potato sucrose agar; MA, malt agar; MYA, malt-extract yeast-extract agar; HA, Hamada agar; SDAY, Sabouraud dextrose agar plus yeast extract; YMA, yeast-extract malt-extract peptone dextrose agar; BM, basal medium agar; MPDA, Martin's peptone dextrose agar; MCM, *Schizophyllum* (mushroom) genetics complete medium plus yeast extract; CDA, Czapek-dox agar.

ized and incubated at 25°C for 20 days. The broth cultures were filtered through Whatman no. 2 filter paper and dried at 60°C for 24 hr to measure dry weight.

To understand the effect of carbon and nitrogen sources on mycelial growth, nine different types of carbon sources and 12 different types of nitrogen sources were used in the Martin's peptone dextrose agar (MPDA) medium at a concentration of 10 and 5 g/L, respectively. Similarly, 12 different types of mineral salts were added to the MPDA at a concentration of 0.5 g/L. Controls without carbon, nitrogen, or mineral salts were used for each experiment. The growth characteristics were observed after 55 days of

incubation at 25°C. Furthermore, dextrose, peptone, and K_2HPO_4 were added to MPDA at different concentrations of 0~7%, 0~3.5%, and 0~0.1%, respectively, to select optimum concentrations for mycelial growth. The growth characteristics were observed after 30 days of incubation at 25°C.

All experiments were conducted under continuous white fluorescent light conditions. The colony diameter was measured in mm. The mycelial density was noted as thin (+), moderate (++), or compact (+++). The colony pigmentations of white, yellowish white (YW), pale yellow, light yellow, grayish yellow, yellowish grey, yellowish brown, olive brown, brown, brownish orange, orange grey, and grayish orange followed Kornerup and Wanscher [14].

Selection of optimum liquid culture conditions. Five mycelial discs (5 mm) were inoculated in 100 mL of each broth media and incubated at 25°C for 20 days. To select the optimum inoculum size, one to eight mycelial discs (5 mm) were inoculated in 100 mL of potato dextrose (PD) broth (potato dextrose agar [PDA] without agar) and incubated at 25°C for 20 days. To determine the optimum culture period, five mycelial discs (5 mm) were inoculated in 100 mL of PD broth and incubated at 25°C for 5 to 40 days. Mycelial dry weight was measured as mentioned above for all experiments.

Results and Discussion

Selection of optimum nutrition, temperature, and pH. The greatest colony diameter was observed on MCM, followed by malt-extract yeast-extract agar (MYA), and MPDA (Table 2). However, compact mycelial density was found on MCM and others such as Czapek-dox agar (CDA), PDA, SDAY, basal medium agar (BM), and potato sucrose agar (PSA) (Table 2). In general, wider colonies produced less mycelial density, except on MCM, which agreed with the results obtained by Shrestha *et al*. [13]. Yeast-extract malt-extract peptone dextrose agar produced the darkest colony pigmentation, followed by Hamada agar, SDAY, PDA, PSA, and MCM. Similar to *Cordyceps militaris* [13], the isolate did not produce any pigmentation on CDA, probably due to the lack of an organic nitrogen source. Both PDA and PSA produced compact mycelia and yellowish grey pigmentations. However, dextrose showed faster growth than sucrose. It was unclear why MPDA produced the second least pigmentation, which was similar to malt agar (MA), despite its high peptone content. Taken together, MCM produced the best mycelial growth and moderate pigmentation. The colony diameter was greatest at 25°C, after which growth decreased rapidly (Fig. 2). Mycelia did not grow at 35°C. At all temperatures, except 35°C, MCM produced compact mycelial density and grayish yellow pigmentation. Dry mycelial weight increased as the pH level increased from 4.0 to 8.0, but decreased rapidly there after indicat-

Table 2. Effect of medium on mycelial growth of *Shimizuomyces paradoxus* isolate EFCC C-5280

Medium	Colony diameter (mm)	Mycelial density	Colony pigmentation
MCM	38	+++	GY
MYA	36	+	PY
MPDA	35	++	YW
CDA	33	+++	W
MA	31	+	YW
PDA	29	+++	YG
HA	27	++	OB
SDAY	27	+++	YB
BM	26	+++	PY
YMA	26	++	B
PSA	23	+++	YG

MCM, *Schizophyllum* (mushroom) genetics complete medium plus yeast extract; MYA, malt-extract yeast-extract agar; MPDA, Martin's peptone dextrose agar; CDA, Czapek-dox agar; MA, malt agar; PDA, potato dextrose agar; HA, Hamada agar; SDAY, Sabouraud dextrose agar plus yeast extract; BM, basal medium agar; YMA, yeast-extract malt-extract peptone dextrose agar; PSA, potato sucrose agar; GY, grayish yellow; PY, pale yellow; YW, yellowish white; W, white; YG, yellowish grey; OB, olive brown; YB, yellowish brown; PY, pale yellow; B, brown.

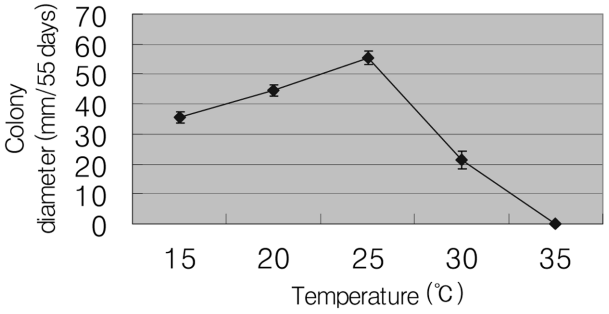

Fig. 2. Effect of temperature on colony diameter of *Shimizuomyces paradoxus* isolate EFCC C-5280 on *Schizophyllum* (mushroom) genetics complete medium plus yeast extract agar medium.

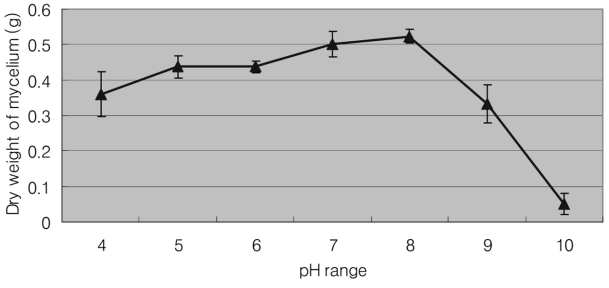

Fig. 3. Effect of pH on *Schizophyllum* (mushroom) genetics complete medium plus yeast extract broth on mycelial dry weight of *Shimizuomyces paradoxus* isolate EFCC C-5280.

Table 3. Effect of carbon source on mycelial growth of *Shimizuomyces paradoxus* isolate EFCC C-5280

Carbon source	Colony diameter (mm)	Mycelial density	Colony pigmentation
Dextrose	52	+++	YW
Sucrose	50	+++	PY
Dextrin	48	+	BO
Fructose	46	+++	YG
Mannose	43	+++	YG
Lactose	43	++	GY
Maltose	42	+++	GY
Starch	38	+	OG
Xylose	11	+	LY
Control	35	+	BO

YW, yellowish white; PY, pale yellow; BO, brownish orange; YG, yellowish grey; GY, grayish yellow; OG, orange grey; LY, light yellow.

Table 4. Effect of nitrogen source on mycelial growth of *Shimizuomyces paradoxus* isolate EFCC C-5280

Nitrogen source	Colony diameter (mm)	Mycelial density	Colony pigmentation
Peptone	56	+++	YW
KNO_3	55	+++	YG
$C_4H_6O_6 \cdot 2H_3N$	54	++	YG
$NaNO_3$	50	++	YG
Yeast extract	46	++	GY
$(NH_4)_3PO_4$	46	++	YW
Tryptone	40	++	YG
Alanine	40	++	GY
NH_4NO_3	37	++	GO
Asparagine	35	++	YG
$(NH_4)_2SO_4$	34	++	GY
Glycine	26	++	GY
Control	46	+	W

YW, yellowish white; YG, yellowish grey; GY, grayish yellow; GO, grayish orange; W, white.

ing that an acidic to slightly alkaline condition was most favorable for mycelial growth (Fig. 3).

Dextrose, sucrose, and dextrin produced the greatest colony diameters, followed by fructose, mannose, and lactose (Table 3). However, dextrin produced thin mycelial density, which was similar to the control. Starch produced a similar colony diameter and density as the control. Xylose, not only produced thin mycelial density, but also strongly inhibited mycelial growth when compared to the control (Table 3). In general, carbon sources helped to increase mycelial density (Table 3). All carbon sources produced less colony pigmentation than the control, except dextrin and starch, which produced the same level of pigmentation as the control. Coincidently, carbon sources that produced thin mycelial density also produced the same types of pigmentation. It was also observed that compact mycelia produced less pigmentation. Dextrose (1%) resulted in the largest colony diameter (Fig. 4). Mycelial density was compact and the pigmentation was YW at all dextrose concentrations.

Peptone and potassium nitrate produced the largest colony diameter and most compact mycelial density (Table 4). The mycelial density was lowest in the absence of a nitrogen source, indicating that both carbon and nitrogen sources are necessary for greater mycelial density. Tryptone, alanine, NH_4NO_3, asparagine, $(NH_4)_2SO_4$, and glycine produced smaller colony diameters compared to the control but produced greater mycelial density. These results showed that colony diameter and mycelial density are usually inversely related to each other. Ammonium nitrate produced the densest grayish orange pigmentation, followed by potassium nitrate, ammonium tartrate ($C_4H_6O_6 \cdot 2H_3N$), sodium nitrate, tryptone, and asparagine. Inorganic nitrogen sources and amino acids produced denser pigmentations than complex organic nitrogen sources such as peptone and yeast extract. This was just the opposite of the pigmentation characteristics of *C. militaris*, which prefer complex organic nitrogen sources to inorganic ones to produce denser pigmentations [13]. This experiment also showed that a nitrogen source is required for colony pigmentation in *S. paradoxa* isolates, as the control did not produce any pigmentation similar to *C. militaris* isolates [13]. The optimal concentration of peptone was 1% (Fig. 5).

All of the mineral salts produced moderate mycelial density, except, K_2HPO_4, which promoted mycelial density (Table 5). Even the control produced moderate myce-

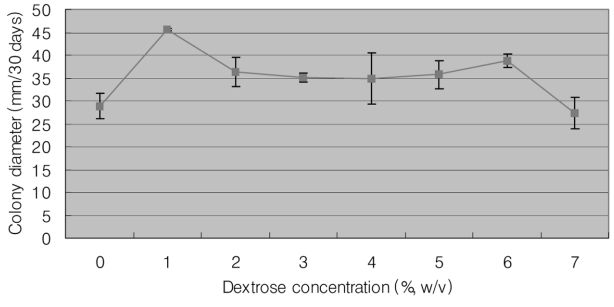

Fig. 4. Effect of dextrose concentration on colony diameter of *Shimizuomyces paradoxus* isolate EFCC C-5280.

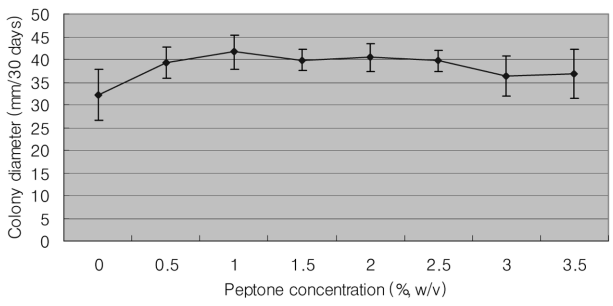

Fig. 5. Effect of peptone concentration on colony diameter of *Shimizuomyces paradoxus* isolate EFCC C-5280.

Table 5. Effect of mineral salts on mycelial growth of *Shimizuomyces paradoxus* isolate EFCC C-5280

Mineral salt	Colony diameter (mm)	Mycelial density	Colony pigmentation
KH_2PO_4	58	++	YG
K_2HPO_4	57	+++	YG
$MnSO_4$	52	++	YG
$CaCO_3$	51	++	YG
KCl	50	++	YG
$MgSO_4 \cdot 7H_2O$	50	++	GY
$CaCl_2$	48	++	OB
$NaSO_4$	48	++	YG
NaCl	48	++	YG
$CaSO_4 \cdot 1/2H_2O$	42	++	YG
$ZnSO_4 \cdot 7H_2O$	39	++	OB
$FeSO_4 \cdot 7H_2O$	39	++	YB
$CuSO_4 \cdot 5H_2O$	34	++	OB
Control	48	++	YG

YG, yellowish grey; GY, grayish yellow; OB, olive brown; YB, yellowish brown.

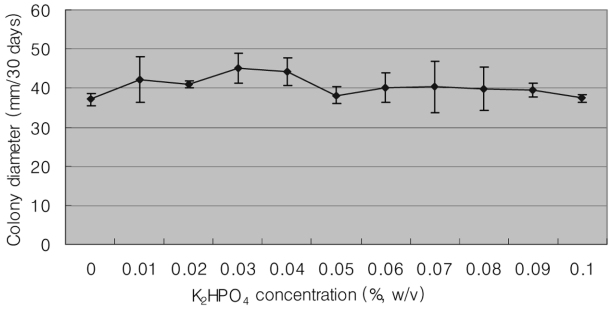

Fig. 6. Effect of K_2HPO_4 concentration on colony diameter of *Shimizuomyces paradoxus* isolate EFCC C-5280.

lial density, showing that mineral salts do not increase mycelial density. $CaSO_4 \cdot 1/2H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $CuSO_4 \cdot 5H_2O$ slowed mycelial growth, compared to the control. However, three of them, including $CaCl_2$, produced denser pigmentation than the control. Many other mineral salts produced the same pigmentation as the control. K_2HPO_4 (0.03~0.04%) resulted in a slightly greater colony diameter than the remaining concentrations (Fig. 6).

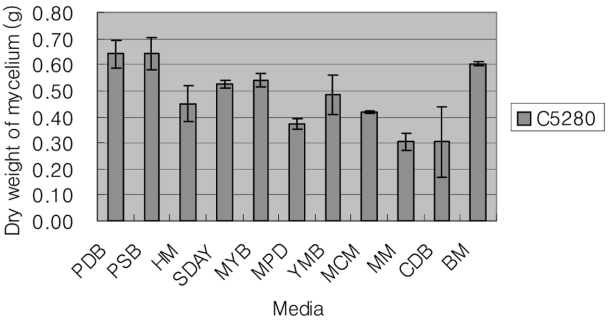

Fig. 7. Effect of liquid medium on mycelial dry weight of *Shimizuomyces paradoxus* isolate EFCC C-5280.

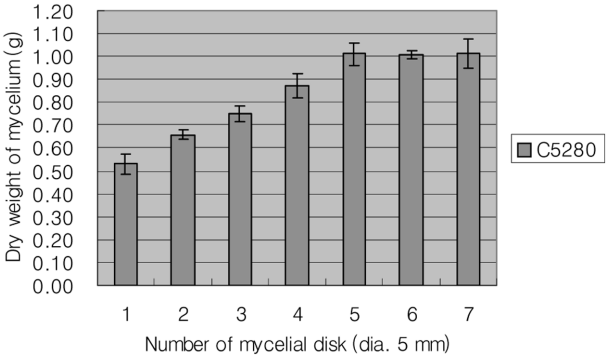

Fig. 8. Effect of inoculum size on mycelial dry weight of *Shimizuomyces paradoxus* isolate EFCC C-5280 in potato dextrose agar broth.

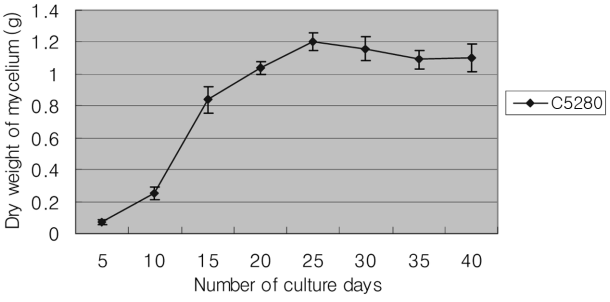

Fig. 9. Effect of culture period on mycelial dry weight of *Shimizuomyces paradoxus* isolate EFCC C-5280 in potato dextrose agar broth.

Optimum conditions for liquid culture. Mycelial growth was different in liquid culture than agar culture. The PDA and PSA broths resulted in the highest mycelial dry weight, followed by the BM, MYA, and SDAY broths (Fig. 7). In agar culture, PDA, PSA, and BM resulted in compact mycelial density, but the colony diameter was shorter than other media. However, MYA resulted in thin mycelial density on agar culture but produced higher mycelial dry weight in liquid culture, after PDA, PSA and BM, suggesting that the fungus grew faster on MYA broth. MCM resulted in the highest growth rate and compact mycelial density in agar culture but produced much less dry mycelial weight in broth culture. The most surprising difference was obtained with CDA. CDA showed one of the best mycelial growths in agar culture but resulted in the lowest mycelial dry weight in broth. MA showed the lowest mycelial growth both in agar and liquid cultures. Mycelial growth in liquid culture increased as the number of mycelial discs increased up to five, but more than five discs did not increase the dry weight (Fig. 8). Thus, five mycelial discs were found to be optimum for 100 mL of liquid culture. Also, mycelial growth con-

tinued for up to 25 days of culture, after which growth started decreasing (Fig. 9).

References

1. Kobayasi Y. Revision of the genus *Cordyceps* and its allies 1. Bull Natl Sci Mus Tokyo Ser B 1981;7:1-13.
2. Sung GH, Hywel-Jones NL, Sung JM, Luangsa-ard JJ, Shrestha B, Spatafora JW. Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Stud Mycol 2007; 57:5-63.
3. Johnson D, Sung GH, Hywel-Jones NL, Luangsa-Ard JJ, Bischoff JF, Kepler RM, Spatafora JW. Systematics and evolution of the genus *Torrubiella* (Hypocreales, Ascomycota). Mycol Res 2009;113:279-89.
4. Kobayasi Y. Miscellaneous notes of fungi (4). J Jpn Bot 1984;59:31-2.
5. Sung JM. The insects-born fungus of Korea in color. Seoul: Kyohak Publishing Co., Ltd.; 1996.
6. Sung JM, Choi YS, Shrestha B, Park YJ. Cultural characteristics of mycelial growth by *Cordyceps militaris*. Kor J Mycol 2002;30:1-5.
7. Park GB, Park GB, Shrestha B, Sung JM. Investigation on favorable condition for mycelial growth of *Paecilomyces tenuipes*. J Mush Sci Prod 2004;2:21-7.
8. Sasaki F, Miyamoto T, Tamai Y, Yajima T. Isolation of vegetable wasps and plant worms, *Cordyceps nutans*, from fruitbody tissue. J Invertebr Pathol 2004;85:70-3.
9. Sasaki F, Miyamoto T, Tamai Y, Yajima T. Optimum temperature and pH for mycelial growth of *Cordyceps nutans* Pat. (Ascomycetes). Int J Med Mush 2005;7:301-4.
10. Shin JC, Shrestha B, Lee WH, Park YJ, Kim SY, Jeong GR, Kim HK, Kim TW, Sung JM. Distribution and favorable conditions for mycelial growth of *Cordyceps pruinosa* in Korea. Kor J Mycol 2004;32:79-88.
11. Xiao JH, Liu ZL, Liu JW, Xiao Y, Fang N, Qi Y, Liang ZQ. Studies on optimization conditions of liquid culture of *Cordyceps pruinosa*. Food Sci 2004;25:113-8.
12. Leung PH, Zhang QX, Wu JY. Mycelium cultivation, chemical composition and antitumor activity of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. J Appl Microbiol 2006;101:275-83.
13. Shrestha B, Lee WH, Han SK, Sung JM. Observations on some of the mycelial growth and pigmentation characteristics of *Cordyceps militaris* isolates. Mycobiology 2006;34:83-91.
14. Kornerup AM, Wanscher JH. Methuen handbook of colour. 3rd ed. London: Eyre Methuen; 1978.