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Optimal Conditions for the Mycelial Growth of *Coprinus comatus* Strains

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The principal objective of this study was to acquire basic data regarding the mycelial growth characteristics for the artificial cultivation of *Coprinus comatus*. 12 URP primers were employed to evaluate the genetic relationships of *C. comatus,* and the results were divided into three groups. Among six kinds of mushroom media, MYP medium was selected as the most favorable culture medium for *C. comatus.* The optimal temperature and pH ranges for the mycelial growth of *C. comatus* were 23~26°C and pH 6~8, respectively. The carbon and nitrogen sources for optimal mycelial growth were sucrose and tryptone, respectively.

KEYWORD : *Coprinus comatus*, Cultural characteristics, Mycelial growth, RAPD

Coprinus comatus is a member of the Agaricales family. The Agaricales is a common fungus frequently seen on lawns, along gravel roads, and in waste areas all over the world (Park and Lee, 2005). As they age, they deliquesce--i.e. auto-digest themselves--from the bottom of the cap upwards, eventually turning into black ink. Sporophores aid digestion, and have been utilized for the treatment of piles. Its inhibition rates against Sarcoma 180 and Ehrlich carcinoma have been reported as 100% and 90%, respectively (Ying *et al*., 1987). *C. comatus* has been identified as containing 'superior'(1 → 3)-β-glucan contents (Yang *et al.*, 2003), and were determined to harbor ergothioneine, a thiol compound with antioxidant properties (List, 1957). The expected anti-oxidant activity was later confirmed (Badalyan *et al.*, 2003). Additionally, *C. comatus* has been shown to harbor compounds that kill nematodes (Li and Xiang, 2005), and the mushroom is a delicious and nutritious agaric (Luo *et al.*, 1991) that has been designated as natural, nutritious, and healthy by both the Food and Agriculture Organization and the World Health Organization (Liu and Zhang, 2003). 13 varieties of *C. comatus* were previously reported in a catalog of books in China, and the optimal growth temperature has been reported as 25°C (Zhou, 2007). Additionally, many farmers in China cultivate *C. comatus,* which is also referred to by names such as "shaggy ink cap", "lawyer's wig", and "shaggy mane" (Chen, 2000). Dong *et al.* (2006) previously reported that the optimal liquid medium composition of *C. comatus* was (adjusted to pH 8.0): sucrose 3%, corn powder 2%, wheat bran 4%, KH_2PO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%. Additionally, methods for its cultivation include bag cultures (Luo and Qian, 1999; Zhu, 1998) and bed cultures (Zhu, 1998), and the substrates used for its cultivation include cotton waste, corn cobs, rice straw, urea, ox manure, lime, etc. (Yang and Xue, 2000). Recently, efforts to improve the cultivation techniques for our farm and automated facilities have resulted in an increase in total mushroom production; 55,274 M/T in 1990, and 146,346 M/T in 2007 (Ministry for Food, Agriculture, Forestry and Fisheries in Korea, 2007). How-

Fig. 1. Fruit-body of *Copinus comatus*.

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ever, only about 10 species of mushrooms are currently cultivated on a large scale in Korea. Therefore, we should develop new revenues by assessing the adaptability of mushrooms that are cultivated in China, and use the results as a foundation for the enhancement of international competitiveness in the mushroom industry.

Materials and Methods

Strains and RAPD analysis. We previously collected a total of 7 varieties of *C. comatus* at the Edible Fungi Institute, Liaoning Academy of Agricultural Sciences, from 1996 to 2006; the mycelia of each isolate were grown on PDA. For RAPD analysis, we performed a static culture of the mycelia into PDB, and then eradicated the media by vacuum culturing the mycelium on Whatman No. 2 filter paper, pouring liquid nitrogen into the sample after lyophilization, and pulverizing it. We then added 1 *ml* of nucleic lysis buffer and 10 μl of proteinase K (25 mg/*ml*) into 0.2 g of pulverized. Mycelium, and allowed the reaction to proceed for 1 hour at 60°C after 2~3 minutes of vortexing, and then divided it into four 1.5 *ml* microtubes to generate 250 μl supernatants after 10 minutes of centrifugation at 14,000 rpm. We collected the supernatants from the columns after centrifugation at 14,000 rpm on repeat for 4 1-minute cycles in a spin column after mixing 500 μl of DNA binding buffer with the sample. Then, in sequential order, we rinsed the sample with 800 μl of 75% ethanol, applied 1 minute of centrifugation at 14,000 rpm, rinsed again with 300 μl of 75% ethanol, applied 1 additional minute of centrifugation at 14,000 rpm, 2 more minutes at 14,000 rpm after the addition of 100 μl of elution buffer, and finally assessed the DNA concentration via electrophoresis. 12 types of primer were utilized, and PCR was conducted as follows: preheating at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes and maintenance at 4°C after a final extension step at 72°C for 2 minutes after a total of 35 cycles. The amplified fragments were then electrophoresed at 100 V in 1.8% agarose gel, dyed for 10 minutes with EtBr (ethidium bromide) and investigated for bands using a UV transilluminator lamp. We conducted cluster analysis via UPGMA (unweighted paired group methods with arithmetic average) for RAPD analysis.

Selection of favorable culture media. Six different culture media were utilized to assess the mycelial growth of *C. comatus* (Table 1). The media were adjusted to pH 6 prior to the autoclaving process. After autoclaving (1.5 kg/cm^2) for 15 minutes at 121°C, 20 *ml* of each medium was poured aseptically into a petri dish. A 5 mm cork borer of the inoculum was removed from a 10-day-old culture of *C. comatus* grown on PDA, and placed in the centre of agar plates, each containing one of six different culture media. After 9 days of incubation at 25 ± 1°C, the mycelial growth of *C. comatus* was evaluated.

Effect of temperature for mycelial growth. In order to determine the ideal temperature for the mycelial growth of *C. comatus*, 6 different temperatures (17, 20, 23, 26, 29 and 32°C) were adopted. A 5 mm diameter agar plug was removed from 10-day-old cultures grown on PDA and positioned in the centers of agar plates, each filled with 20 *ml* of PDA. After 9 days of incubation at 6 different temperatures, the mycelial growth of *C. comatus* was evaluated.

Effect of pH for mycelial growth. A 5 mm diameter agar plug was removed from 10-day-old cultures grown on PDA, then added to 250 *ml* Erlenmeyer flasks filled with 100 *ml* of PDB. The medium was adjusted to pH values of 4, 5, 6, 7 and 8 with the addition of 0.1 N-NaOH or 0.1 N-HCl and incubated for 14 days at 25°C, 125 rpm after inoculation. We filtered the liquid with fil-

Table 1. The composition of used media

Ingredients	Concentration (g/*l*)					
	PDA	MEA	MCM	Czapek-Dox	GPYM	MYP
Potato dextrose	24					
Dextrose		20	20	30		
Glucose					10	
Peptone		1	2		10	5
Malt extract		20			15	3
Yeast extract			2		10	3
K_2HPO_4			1	1		
KH_2PO_4			0.46			
$MgSO_4 \cdot 7H_2O$			0.5	0.5		
$NaNO_3$				2		
KCl				0.5		
$Fe_2SO_4 \cdot 7H_2O$				0.01		
Agar	20	20	20	20	20	20

ter paper (Whatman No.2) and air-dried the samples at 50°C for 48 hours for dry weight measurements of the mycelial samples after 14 days.

Effect of carbon and nitrogen sources for mycelial growth. The mycelial growth of *C. comatus* was assessed on each of the basal media supplemented with 10 carbon and eight nitrogen sources, respectively. The basal media for carbon source selection were composed of 11 carbon sources (30 g), tryptone (1 g), $MgSO_4 \cdot 7H_2O$ (5.0 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), KH_2PO_4 (1.0 g), agar (20 g), and distilled water (1000 *ml*). The basal media for nitrogen source selection were composed of 8 nitrogen sources (30 g), sucrose (30 g), $MgSO_4 \cdot 7H_2O$ (5.0 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), KH_2PO_4 (1.0 g), agar (20 g), and distilled water (1000 *ml*). In both cases, the basal medium was adjusted to pH 6 and autoclaved for 15 minutes on 121°C poured plates. The inoculated dishes were replicated four times and incubated for 14 days at 25°C in darkness. Mycelial growth was measured as previously described.

Results and Discussion

RAPD analysis. PCR band phase was analyzed with 12 URP primers (Fig. 2), and resulted in seven varieties of different bands at URP 3; in particular, the URP 5 and 11 primers tended to stain similarly, which is characteristic of intraspecificity among equal groups. The RAPD analysis of the collected varieties was divided into 3 groups according to the results of the band phases of URP 3, 5 and 11; however, we noted no morphological differences among strains (Fig. 3).

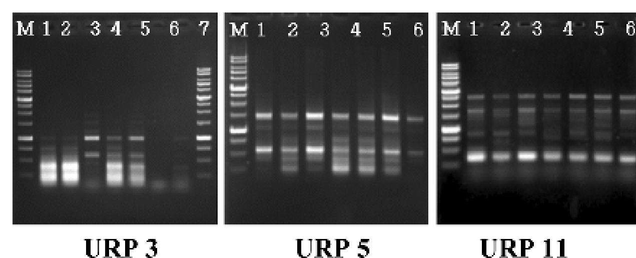

Fig. 2. Random amplified polymorphic DNA patterns by primer URP3, URP5 and URP11. M: 1 kb ladder; lane 1, GMCC54002; lane 2, GMCC54003; lane 3, GMCC54004; lane 4, GMCC67001; lane 5, GMCC67002; lane 6, GMCC67003; lane 7, GMCC67004.

Selection of favorable culture media. Six different culture media were utilized to determine the optimal mycelial growth of seven different strains of *C. comatus*. Among the 6 culture media utilized, MYP medium was shown to be exceedingly favorable in promoting the mycelial growth of *C. comatus,* whereas Czapek-Dox medium performed extremely poorly; additionally, among 7 variants of *C. comatus,* GMCC 54001 evidenced the highest levels of mycelial growth length on MCM medium (Table 2). Chaiyma *et al*. (2007) previously reported favorable mycelial growth of *C. comatus* cultivated on Malt extract agar. However, Chaiyma *et al*. (2007) did not make use of MYP in his experiments. Therefore, we concluded that MYP was the optimal medium for the mycelial growth of *C. comatus*.

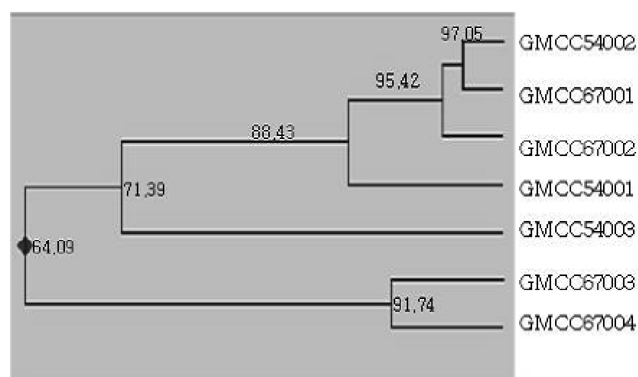

Fig. 3. Grouping of *C. comatus* strains by URP 3, URP 5 and URP 11 primers. The numbers above each branch indicate the coefficient value. The right number indicates the strain number.

Table 2. Effect of media on mycelial growth of *C. comatus* strains

GMCC strain	Mycelial growth (mm/9 days)					
	PDA	MEA	MCM	Czapek-Dox	GPYM	MYP
54001	46 ± 2.6	59 ± 1.2	81 ± 1.2	22 ± 2.5	53 ± 3.8	75 ± 0
54002	50 ± 0	60 ± 2.0	67 ± 1.5	22 ± 2.0	49 ± 1.2	76 ± 1.7
54003	50 ± 5.0	55 ± 3.0	67 ± 2.1	13 ± 4.2	51 ± 0.6	74 ± 1.7
67001	62 ± 2.6	66 ± 1.2	73 ± 7.9	22 ± 2.1	53 ± 1.2	79 ± 1.2
67002	47 ± 4.4	52 ± 2.5	68 ± 1.5	22 ± 2.5	44 ± 1.7	72 ± 2.5
67003	43 ± 3.2	55 ± 2.0	67 ± 2.9	26 ± 4.6	47 ± 1.5	74 ± 1.2
67004	46 ± 3.8	53 ± 2.5	68 ± 2.9	23 ± 2.3	47 ± 3.6	77 ± 1.7
Mean[a] ± SD	49d ± 6.1	57c ± 4.8	70b ± 5.4	22e ± 4.0	49d ± 3.3	75a ± 2.3

[a]Values followed by the same letter do not differ significantly at $p > 0.05$ according to Duncan's multiple range test.

Table 3. Effect of temperature on mycelial growth of *C. comatus* strains

GMCC strain	Mycelial growth(mm / 9days)					
	17°C	20°C	23°C	26°C	29°C	32°C
54001	23 ± 2.0	30 ± 5.0	54 ± 1.0	44 ± 6.0	35 ± 3.0	21 ± 1.0
54002	24 ± 2.5	29 ± 4.9	51 ± 2.8	47 ± 4.2	35 ± 3.0	27 ± 2.0
54003	19 ± 1.2	24 ± 3.0	43 ± 3.1	47 ± 3.8	30 ± 3.6	23 ± 2.1
67001	24 ± 3.2	32 ± 2.8	54 ± 2.0	63 ± 1.0	43 ± 5.0	35 ± 0.7
67002	16 ± 1.0	23 ± 1.5	45 ± 4.2	44 ± 2.1	34 ± 9.8	30 ± 0.7
67003	19 ± 1.2	25 ± 2.0	45 ± 3.5	56 ± 3.5	32 ± 4.2	27 ± 4.2
67004	18 ± 1.0	30 ± 2.6	55 ± 3.5	54 ± 2.1	46 ± 2.5	29 ± 4.0
Mean[a] ± SD	20d ± 3.2	28c ± 3.4	49a ± 5.1	51a ± 7.2	36b ± 5.8	27c ± 4.4

[a]Values followed by the same letter do not differ significantly at $p > 0.05$ according to Duncan's multiple range test.

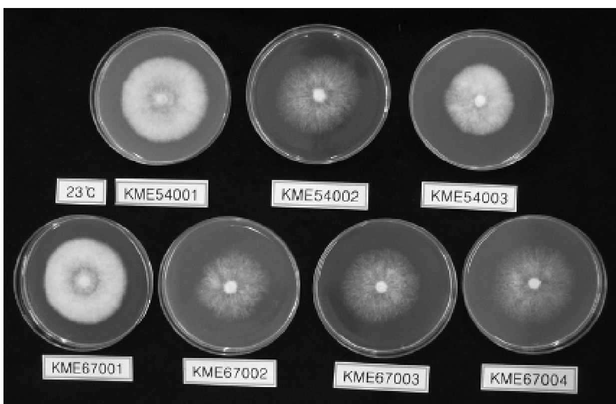

Fig. 4. Configuration on mycelial growth of *Copinus comatus* strains incubated at 23°C for 9 days.

Effect of temperature on mycelial growth. The suitable temperature for the mycelial growth of *C. comatus* was observed at 23~26°C and GMCC 67001 evidenced the highest levels of mycelial growth at 63 mm at 26°C (Table 3, Fig. 4). However, at temperatures above 29°C or below 20°C, seven strains of *C. comatus* evidenced poor mycelial growth. With regard to the optimal culture temperature range of *C. comatus*, Luo and Qian (1999) and Kim (2001) reported it as 25~27°C, Yang and Xue (2000) as 24~28°C and Shiyong and Zaipei (2007) as 22~28°C. The above results were consistent with the results of this study. Consequently, we selected 26°C as the optimal mycelial growth temperature of *C. comatus*.

Effect of pH for mycelial growth. Favorable mycelial growth of *C. comatus* was observed in a pH range of 6~8 (Table 4). Among the seven strains, the optimal mycelial growth was noted at a pH of 7. GMCC 67002 and GMCC 67004 had the highest mycelial dry weight at 75 mm at a pH of 7. Since the mycelial growth of *C. comatus* was generally favorable in a pH range of 6.5~7.5 (Chen and Yang, 2000; Shiyong and Zaipei, 2007). We observed the highest mycelial dry weight at pH 7. Therefore, it can be seen that the results shown above are similar with the results of the present study.

Effect of carbon and nitrogen sources for mycelial growth. The carbon sources that most effectively promoted the mycelial growth of *C. comatus* were maltose and sucrose in disaccharide, and starch in polysaccharide (Table 5). Among the 10 carbon sources utilized herein, GMCC 67001 evidenced the highest mycelial growth (74 mm) in sucrose. Chaiyama *et al*. (2007) identified the optimal culture carbon sources of *C. comatus* as mannose and maltose. However, we did not use mannose in this experiment. So, the mycelial growth of *C. comatus* at

Table 4. Effect of pH on mycelial growth of *C. comatus* strains

GMCC strain	Dry weight of mycelial (mg/14 days)				
	pH 4	pH 5	pH 6	pH 7	pH 8
54001	55 ± 3.7	59 ± 5.3	69 ± 25.4	67 ± 8.9	65 ± 4.6
54002	60 ± 5.7	63 ± 1.4	64 ± 3.9	65 ± 9.2	64 ± 4.1
54003	60 ± 9.5	59 ± 13.0	63 ± 11.2	65 ± 3.6	62 ± 2.4
67001	56 ± 5.1	65 ± 1.8	67 ± 5.1	69 ± 5.7	63 ± 7.1
67002	44 ± 14.8	47 ± 10.9	59 ± 5.7	75 ± 6.6	65 ± 11.2
67003	56 ± 7.7	61 ± 11.1	64 ± 7.0	72 ± 6.5	59 ± 11.1
67004	59 ± 6.7	61 ± 8.7	64 ± 3.2	75 ± 5.9	62 ± 6.7
Mean[a] ± SD	56c ± 5.6	59bc ± 5.6	64b ± 3.2	70a ± 4.4	63b ± 2.1

[a]Values followed by the same letter do not differ signification at $p > 0.05$ according to Duncan's multiple range test.

Table 5. Effect of carbon source on mycelial growth of *C. comatus* strains

GMCC strain	Mycelial growth (mm/14 days)									
	Glu[a]	Gal[a]	Fru[a]	Xylo[a]	Mal[a]	Suc[a]	Lac[a]	CM[a]	Xyla[a]	Sta[a]
54001	48 ± 1.3	50 ± 1.3	39 ± 0.6	24 ± 0.7	54 ± 0.9	64 ± 1.7	22 ± 0.6	41 ± 0.7	43 ± 0.6	60 ± 1.6
54002	51 ± 3.0	49 ± 1.9	47 ± 0.7	27 ± 1.3	58 ± 1.5	65 ± 2.1	26 ± 1.2	54 ± 3.9	44 ± 2.4	62 ± 1.8
54003	52 ± 3.7	47 ± 0.8	42 ± 1.5	24 ± 4.1	63 ± 2.0	64 ± 0.2	22 ± 0.7	47 ± 2.2	48 ± 0.8	69 ± 3.3
67001	5 6± 1.6	54 ± 1.8	48 ± 1.2	30 ± 0.5	64 ± 1.0	74 ± 1.0	22 ± 0.5	50 ± 1.1	48 ± 1.5	72 ± 2.6
67002	54 ± 4.9	48 ± 2.0	46 ± 3.4	32 ± 1.2	66 ± 3.0	65 ± 0.7	26 ± 0.6	54 ± 4.7	45 ± 1.8	64 ± 2.4
67003	57 ± 3.9	48 ± 2.1	46 ± 2.4	32 ± 1.5	66 ± 1.3	71 ± 2.3	25 ± 0.5	49 ± 2.2	43 ± 1.8	62 ± 4.6
67004	52 ± 4.7	47 ± 0.8	46 ± 4.5	30 ± 0.9	61 ± 3.5	68 ± 1.3	28 ± 0.4	53 ± 3.3	43 ± 1.5	68 ± 0.8
Mean[b] ± SD	53c ± 3.3	49c ± 2.4	45d ± 3.3	28e ± 3.6	62b ± 4.4	67a ± 3.9	24f ± 2.2	50c ± 4.5	45d ± 2.4	65ab± 4.6

[a]Glu: glucose, Gal: galactose, Fru: frutose, Xylo: xylose, Mal: maltose, Suc: sucrose, Lac: lactose, CM: CM-cellulose, Xyla: xylan, Sta: starch.
[b]Values followed by the same letter do not differ significantly at $p > 0.05$ according to Duncan's multiple range test.

Table 6. Effect of nitrogen source on mycelial growth of *C. comatus* strains

GMCC strain	Mycelial growth (mm/14 days)[a]							
	$NaNO_2$	$NaNO_3$	NH_4NO_3	NH_4Cl	peptone	tryptone	urea	Yeast extract
54001	–	13 ± 0.5	10 ± 0	12 ± 2.0	55 ± 3.2	63 ± 0.9	51 ± 3.4	49 ± 1.0
54002	–	18 ± 0.7	13 ± 1.1	14 ± 1.3	64 ± 2.4	58 ± 2.5	56 ± 7.9	49 ± 1.0
54003	–	16 ± 3.0	11 ± 0.1	14 ± 1.6	67 ± 1.0	71 ± 0.6	49 ± 2.2	63 ± 1.1
67001	–	18 ± 1.0	12 ± 1.5	13 ± 0.4	66 ± 1.6	68 ± 1.1	46 ± 7.6	63 ± 1.3
67002	–	17 ± 1.4	13 ± 0.8	11 ± 0.7	66 ± 1.8	72 ± 1.4	62 ± 3.8	47 ± 5.5
67003	–	18 ± 0.4	14 ± 1.7	15 ± 4.3	23 ± 1.0	65 ± 1.7	57 ± 3.0	48 ± 2.5
67004	–	18 ± 1.6	14 ± 1.2	14 ± 2.7	23 ± 1.2	66 ± 2.7	59 ± 1.3	51 ± 3.4
Mean[b] ± SD	–	17c ± 1.7	12c ± 1.2	13c ± 1.3	52b ± 20.2	66a ± 4.5	54b ± 5.7	53b ± 7.0

[a]Growth rate.
[b]Values followed by the same letter do not differ significantly at $p > 0.05$ according to Duncan's multiple range test.

amaltose was comparatively good.

The nitrogen source that most effectively promoted the mycelial growth of *C. comatus* was tryptone in organic nitrogen (Table 6). Among the eight nitrogen sources utilized herein, GMCC 67002 evidenced optimal mycelial growth (72 mm) in tryptone. However, $NaNO_2$ in mineral nitrogen depressed the mycelial growth of *C. comatus,* and mycelial growth was weak in $NaNO_3$, NH_4NO_3, and NH_4Cl. Thus, on the basis of our results, we identified tryptone as the optimal nitrogen source for the mycelial growth of *C. comatus.*

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