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Suitable Conditions for Mycelial Growth of *Phellinus* spp.

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The fungus *Phellinus* is a mushroom that is widely used medicinally. The optimal conditions for mycelial growth of 13 strains of the fungus were investigated. Mycelial growth was optimal at 25°C and was uniformly minimal at 15°C and 35°C. Growth was optimal at pH 6~7. The mycelial phenotype was best promoted by growth using Potato Dextrose agar, Hamada, Glucose peptone, and Yeast-Malt media, whereas Czapek Dox, Hennerberg, and Lilly media were the most unfavorable for the mycelial growth of *Phellinus* spp. Glucose, sucrose, fructose, and dextrin were the most suitable carbon sources for mycelial growth, while lactose, maltose, and galactose were unsuitable. Among tested nitrogen sources, ammonium phosphate, potassium nitrate, and arginine best promoted mycelial growth, while alanine, urea, and histidine least promoted mycelial growth.

KEYWORDS : Favourable condition, Filamentous growth, *Phellinus* spp., Supplemented nutrition

The genus *Phellinus* is a medicinal mushroom that is widely used in Korea, Japan and China. Typically, it grows naturally on dead mulberry trees. *P. linteus*, which is commonly known as song-gen in China, sang-hwang in Korea, and meshimakobu in Japan, exhibits anti-tumor properties on skin, lung, and prostate cells (Han *et al*., 1999; Park *et al*., 2003; Li *et al*., 2004). *Phellinus* is also used a traditional Kampo medicine for the treatment of diarrhea. The major compounds of the therapeutic preparations are polysaccharides, aminoacids, ã-aminobutyric acid, vitamins, and sugar. Polysaccharides and proteoglycan isolated from fruiting body of *P. linteus* are cytotoxic to tumor cells (Kim *et al*., 2004) and induce functional maturation of murine dendritic cells (Park *et al*., 2003). The aqueous extract of *P. linteus* inhibits immunoglobulin E-dependent mouse triphasic cutaneous reactions (Inagaki *et al*., 2005), and stimulates antibody production (Song *et al*., 1995) by an as yet unknown mechanism.

Immunodeficiency caused by anti-tumor drugs such as mitomycin C poses a serious problem for cancer patients. Mitomycin C has strong anti-tumor activity but also restricts bone marrow activity (Ohtake *et al*., 2000). In this case, the fungal water extract plays an important role in promoting immune activity (Edwards *et al*., 1984). Since *Phellinus* spp. are widely used medicinally, scaling-up of the commercial production of the fungus would be advantageous for its desseminated use in cancer therapy. To this end, the present study sought to clarify cultural parameters that promote mycelial growth of Korean and foreign isolates of *Phellinus* spp.

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Materials and Methods

Fungal strains. The mycelial cultures of 13 strains of *Phellinus* spp. were obtained from the Culture Collection and DNA Bank of Mushrooms (CCDBM) in the Department of Biology, University of Incheon, Korea (Table 1). The strains were transferred to Potato Dextrose agar (PDA) plates and incubated at 25°C in the dark until they showed a full growth, after which they were maintained at 4°C. Unless otherwise stated, all experiments were done a minimum of four times.

Effects of temperature and pH on vegetative growth. To ascertain the optimum temperature for mycelial growth, temperatures of 15°C, 20°C, 25°C, 30°C, and 35°C were used. A 5 mm diameter agar plug removed from 10-day-old PDA cultures was placed in the centre of a Petri

Table 1. *Phellinus* spp. used in this study

Strain No.	Scientific name	Location
IUM 3149	*P. chrysoona*	Czech Republic
IUM 3150	*P. conchaatus*	Czech Republic
IUM 3151	*P. alni*	Czech Republic
IUM 3153	*P. lundellii*	Czech Republic
IUM 3155	*P. populicola*	Czech Republic
IUM 3156	*P. popaceus*	Czech Republic
IUM 3157	*P. trenulae*	Czech Republic
IUM 3158	*P. toruluae*	Czech Republic
IUM 3159	*P. linteus*	Korea
IUM 3161	*P. linteus*	Korea
IUM 3163	*P. vorax*	Czech Republic
IUM 3164	*P. cavicola*	Czech Republic
IUM 3169	*P. baumi*	Czech Republic

dish containing 20 *ml* of solidified PDA. The medium was adjusted to pH 6 and incubated for 10 days at the selected temperature. Radial growth of mycelia on each Petri dish was measured in three horizontal directions and the average value was calculated. To calculate the final mean value of mycelial growth of each strain, four replications were used. To assess the influence of pH, a plug similarly recovered from PDA was placed on fresh PDA. The medium was adjusted to pH 4, 5, 6, 7, 8, or 9 with the addition of 1 N NaOH or HCl, and incubated for 10 days at 25°C. The measurement of mycelial growth was ascertained as just described.

Effects of culture media on the vegetative growth. Nine different agar culture media (Czapek Dox, Hamada, Hennerberg, Glucose peptone, Glucose tryptone, Lilly, Mushroom complete, PDA, and Yeast Malt extract (YM)) were prepared (Table 2). The media were adjusted to pH 6 before autoclave sterilization. All media were similarly inoculated as described above. After 10 days of incubation at 25°C, measurement of mycelial growth was performed as described above.

Effects of carbon and nitrogen sources on the vegetative growth. Basal medium (0.05 g $MgSO_4$, 0.46 g KH_2PO_4, 1.0 g K_2HPO_4, 120 μg thiamine-HCl, and 20 g agar in 1 *l* of distilled water; Sung *et al.*, 1993) was separately supplemented with 10 carbon sources (dextrin, fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, sucrose, and xylose) or 10 nitrogen sources (alanine, ammonium acetate, ammonium phosphate, arginine, calcium nitrate, glycine, histidine, methionine, potassium nitrate, or urea). To screen for the carbon source most favorable for mycelial growth, each carbon source with 5 g of peptone was added to the basal medium separately at a concentration of 0.1 M and mixed thoroughly (Shim *et al.*, 1997). Each nitrogen source with 20 g of glucose was supplemented to the basal medium at a concentration of 0.02 M. In both cases, the basal medium was adjusted to pH 6 before autoclaving. To measure the colony diameter on the media, all plates were incubated for 10 days at 25°C. Radial growth of mycelia was measured as described above.

Table 2. Composition of growth media

Composition	Media (g/*l*)								
	Cza	Ham	Hen	GP	GT	Lil	MC	PDA	YM
Agar	20	20	20	20	20	20	20	20	20
Asparagine						2			
Dextrose		10						20	10
Ebiose		5							
Hyponex		3							
Glucose			50	10	5				
Malt-extract				15			20		3
Maltose						10			
Peptone				10			2		5
Potatoes								200	
Sucrose	30								
Triptone					10				
Yeast-extract		3		10	3		2		3
$NaNO_3$	3		2						
K_2HPO_4	1						1		
$MgSO_4$	0.5		0.5			0.5	0.5		
KCl	0.5								
$FeSO_4$	0.01								
$CaCl_2$			0.1						
KH_2PO_4			1			1	0.5		
KNO_3			2						

Cza: Czapek Dox, Ham: Hamada, Hen: Hennerberg, GP: Glucose peptone, GT: Glucose tryptone, Lil: Lilly, MC: Mushroom complete, PDA: Potato dextrose agar and YM: Yeast-malt extract agar.

Results and Discussion

Effects of temperature on the vegetative growth. Of the tested temperatures, 25°C generally proved optimal for mycelial growth of the 13 isolates of *Phellinus* spp. Mycelial growth of isolates IUM3155 and IUM3169 was highest at 20°C, while 30°C was best for isolate IUM3159. Mycelial growth was poorest at 15°C or 35°C. In almost every case, mycelial growth was measured at 20°C and 30°C (Table 3). The optimal temperature of 25°C differs from the 30°C optimum previously reported for *P. linteus* (Hur, 2008). The dichotomy may be due to the different isolates used in the previous and present studies.

Effects of pH on vegetative growth. Among the 13 tested strains, the best mycelial growth was found at pH 7 for IUM 3153, IUM 3155, IUM 3156, IUM 3159, and

Table 3. Effect of temperature on mycelial growth of tested *Phellinus* spp.

IUM strain	Scientific name	Temperature				
		15°C	20°C	25°C	30°C	35°C
		Mycelial growth (mm)				
IUM3149	*P. chrysoona*	1.9	6.6	10.8	6.5	3.2
IUM3150	*P. conchaatus*	5.5	16.5	29.5	20.1	5.1
IUM3151	*P. alni*	4.5	14.4	30.0	21.8	8.6
IUM3153	*P. lundellii*	4.9	14.8	21.4	19.4	6.0
IUM3155	*P. populicola*	6.0	22.7	21.5	15.7	5.5
IUM3156	*P. popaceus*	7.2	23.3	25.0	19.5	4.5
IUM3157	*P. tremulae*	6.0	13.9	21.0	11.8	4.7
IUM3158	*P. toruluae*	9.7	22.5	31.0	23.8	8.5
IUM3159	*P. linteus*	1.2	5.9	6.8	6.9	2.0
IUM3161	*P. linteus*	1.8	6.2	9.0	5.8	2.5
IUM3163	*P. vorax*	2.1	7.4	11.8	6.7	3.4
IUM3164	*P. cavicola*	3.4	15.4	20.4	13.1	5.8
IUM3169	*P. baumi*	9.6	32.9	32.0	26.5	5.9
Mean		4.9	15.6	20.8	15.2	5.0

Temperature effect was assessed using PDA.

Table 4. Effect of pH on mycelial growth of tested *Phellinus* spp.

IUM strain	Scientific name	pH					
		4	5	6	7	8	9
		mycelial growth (mm)					
IUM3149	*P. chrysoona*	11.5	11.5	12.3	11.5	8.1	8.8
IUM3150	*P. conchaatus*	18.5	21.9	26.3	23.1	22.5	22.1
IUM3151	*P. alni*	11.1	28.0	26.9	25.0	23.3	19.5
IUM3153	*P. lundellii*	17.3	17.0	20.1	20.2	15.9	12.5
IUM3155	*P. populicola*	12.4	18.0	16.8	22.0	15.3	15.0
IUM3156	*P. popaceus*	28.1	27.5	29.4	29.4	23.5	22.5
IUM3157	*P. tremulae*	12.9	8.3	5.8	5.3	4.6	4.6
IUM3158	*P. toruluae*	13.5	9.3	12.1	7.5	12.1	13.4
IUM3159	*P. linteus*	8.8	7.3	6.8	12.3	10.3	10.3
IUM3161	*P. linteus*	25.0	27.5	34.6	28.4	24.4	21.5
IUM3163	*P. vorax*	13.9	13.6	13.0	14.3	14.0	12.6
IUM3164	*P. cavicola*	21.0	21.2	21.5	19.4	19.0	21.2
IUM3169	*P. baumi*	16.8	31.5	29.3	29.2	24.6	25.0
Mean		16.2	18.6	19.6	19.0	16.7	16.1

Effect of pH was assessed using PDA.

IUM 3163; at pH 6 for IUM 3149, IUM 3150, IUM 3161, and IUM 3164; at pH 5 for IUM 3151 and IUM 3169; and at pH 4 for IUM 3157 and IUM 3158. A pH exceeding 7 did not support robust mycelial growth, with the poorest mycelial development being evident at pH 8 and 9 (Table 4). Other studies have reported that pH 6 is optimal for mycelial growth of *P. linteus* (Chi *et al*., 1996; Hur 2008). These and the present results indicate that *Phellinus* spp. including *P. linteus* optimally produce mycelia at neutral or acidic pHs.

Effects of various media on the vegetative growth. Of the 13 tested strains, mycelial growth was greatest when cultivated on PDA (n = 6), Hamada (n = 4), and Glucose peptone (n = 2), with strain IUM3153 responding best to growth on YM (Table 5). For all strains, the lowest growth of the filamentous rim was measured following growth on Czapek Dox, Lilly, and Hennerberg (Table 5). These results differ from the previous finding that Mushroom Complete medium supports excellent mycelial growth of *P. linteus* (Hur, 2008). In case of *Hericium erinaceus*, the highest filamentous growth was observed in PDA, YM, Hamada, and Glucose peptone, with mycelial growth being poorest during growth on Czapek Dox, Hoppkins, Lilly, and Hennerberg (Imtiaj *et al*., 2008).

Effects of carbon sources on the vegetative growth. While the results were varied for the tested strains, in general, glucose, fructose, sucrose, and dextrose were comparatively better than the other carbon sources used (Table 6). Lactose, maltose, and galactose were less effective in the enhancement of mycelial growth (Table 6). With exception of mannose, the present results agree with those of Hur (2008).

Effects of nitrogen sources on the vegetative growth. Ten of the 13 strains showed optimal mycelial growth when the nitrogen source was ammonium phosphate, potassium nitrate, or arginine. However, the other nitrogen sources also facilitated considerable mycelial growth of *Phellinus* spp. Comparatively, alanine, urea, and histidine produced the poorest mycelial growth (Table 7). Glycine is the most favorable and histidine, arginine and ammonium oxalate are the most unfavorable nitrogen sources for the mycelial growth of *Macrolepiota procera* (Shim *et al*., 2005), while potassium nitrate and sodium

Table 5. Effect of media on mycelial growth of tested *Phellinus* spp.

IUM strain	Scientific name	Media[a]								
		Ham	Hen	GP	GT	Lil	MC	PDA	YM	Cza
		mycelial growth (mm)								
IUM3149	*P. chrysoona*	12.3	4.4	18.0	11.3	3.0	7.5	10.8	11.9	4.6
IUM3150	*P. conchaatus*	31.8	11.8	25.0	23.0	16.5	28.3	29.5	24.5	15.5
IUM3151	*P. alni*	30.5	17.8	26.0	26.8	23.5	29.8	30.0	32.0	16.6
IUM3153	*P. lundellii*	22.8	14.3	19.8	15.8	19.5	26.0	21.4	18.8	3.0
IUM3155	*P. populicola*	17.3	10.8	20.0	16.8	3.0	11.0	21.5	22.8	7.8
IUM3156	*P. popaceus*	25.5	18.5	19.5	16.8	28.5	19.8	25.0	20.0	7.5
IUM3157	*P. tremulae*	15.0	12.0	20.0	18.8	8.0	15.8	21.0	20.3	19.0
IUM3158	*P. toruluae*	28.8	15.5	25.3	29.5	30.5	28.5	31.0	25.0	18.5
IUM3159	*P. linteus*	7.5	8.5	8.8	7.8	6.5	6.0	6.8	7.0	3.8
IUM3161	*P. linteus*	18.5	5.8	15.9	3.5	6.0	9.0	9.0	8.0	3.0
IUM3163	*P. vorax*	14.3	10.3	10.5	9.5	11.5	13.0	11.8	10.5	4.0
IUM3164	*P. cavicola*	21.3	11.9	21.5	19.0	18.0	20.3	20.4	19.4	3.5
IUM3169	*P. baumi*	34.3	18.0	33.8	27.7	31.9	26.5	32.0	30.8	26.0
Mean		21.5	12.3	20.3	17.4	15.9	18.6	20.8	19.3	10.2

[a]Cza: Czapek Dox, Ham: Hamada, Hen: Hennerberg, GP: Glucose peptone, GT: Glucose tryptone, Lil: Lilly, MC: Mushroom complete, PDA: Potato dextrose agar and YM: Yeast-malt extract agar.

Table 6. Effect of carbon sources on mycelial growth of tested *Phellinus* spp.

IUM Strain	Scientific name	Carbon sources[a]									
		Dex	Fr	Ga	Gl	Lac	Mal	Man	Sor	Suc	Xy
		mycelial growth (mm)									
IUM3149	*P. chrysoona*	9.6	7.5	5.0	15.8	7.6	22.5	15.8	10.8	17.8	16.4
IUM3150	*P. conchaatus*	15.4	17.6	16.8	17.3	13.8	12.3	15.5	5.8	11.8	8.5
IUM3151	*P. alni*	20.9	18.6	18.6	19.5	15.0	14.6	13.6	15.8	15.6	12.4
IUM3153	*P. lundellii*	21.3	17.8	14.1	21.9	10.3	10.9	8.5	5.6	15.8	11.5
IUM3155	*P. populicola*	15.8	17.0	18.6	15.9	15.8	26.4	29.8	28.4	31.3	31.1
IUM3156	*P. popaceus*	20.5	22.4	21.9	20.8	20.6	30.9	33.9	31.8	34.0	32.4
IUM3157	*P. tremulae*	6.0	5.5	3.8	16.7	4.1	6.2	6.8	7.3	6.1	4.8
IUM3158	*P. toruluae*	7.2	9.5	7.7	6.7	7.0	12.5	10.8	9.0	9.5	13.7
IUM3159	*P. linteus*	7.8	17.0	17.6	16.2	6.6	12.7	20.2	21.7	6.6	13.2
IUM3161	*P. linteus*	30.2	36.7	13.3	33.6	22.1	33.5	33.7	34.5	33.0	33.8
IUM3163	*P. vorax*	4.4	4.2	3.1	3.6	2.8	18.7	14.2	5.5	13.3	13.6
IUM3164	*P. cavicola*	24.7	20.2	19.7	21.0	20.1	17.7	19.5	15.2	25.0	17.0
IUM3169	*P. baumi*	25.6	36.3	28.7	33.7	50.8	12.1	31.8	24.1	31.2	20.7
Mean		16.1	17.7	14.5	18.7	15.1	17.8	19.5	16.6	19.3	17.6

[a]Dex: Dextrin, Fr: Fructose, Ga: Galactose, Gl: Glucose, Lac: Lactose, Mal: Maltose, Man: Mannose, Sor: Sorbitol, Suc: Sucrose and Xy: Xylose. Each carbon source was added to the basal medium at the concentration of 0.1 M.

Table 7. Effect of nitrogen sources on mycelial growth of tested *Phellinus* spp.

IUM strain	Scientific name	Nitrogen sources[a]								
		Gly	PN	Ur	His	Met	AA	Ala	Arg	AP
		mycelial growth (mm)								
IUM3149	*P. chrysoona*	3.5	4.0	3.3	3.0	7.7	3.0	3.0	12.5	8.6
IUM3150	*P. conchaatus*	14.5	5.5	3.3	3.0	5.3	3.5	3.0	12.5	22.0
IUM3151	*P. alni*	6.8	11.3	4.3	3.3	4.0	10.0	3.0	8.3	18.3
IUM3153	*P. lundellii*	2.0	6.0	3.0	2.5	5.0	3.5	3.0	9.5	20.6
IUM3155	*P. populicola*	3.5	12.8	3.8	3.0	5.3	10.0	3.0	5.0	15.3
IUM3156	*P. popaceus*	20.8	13.7	8.4	4.3	7.8	4.0	5.5	7.5	22.3
IUM3157	*P. tremulae*	3.5	5.3	3.5	4.0	6.0	6.0	4.3	4.3	4.8
IUM3158	*P. toruluae*	3.0	3.0	2.3	5.5	5.3	3.3	3.5	2.8	10.8
IUM3159	*P. linteus*	8.8	17.8	14.5	9.3	10.2	15.3	13.5	5.5	15.3
IUM3161	*P. linteus*	11.3	5.8	19.8	3.5	7.0	14.0	4.0	17.1	26.8
IUM3163	*P. vorax*	3.5	4.8	3.0	4.0	4.0	5.0	5.0	5.5	13.0
IUM3164	*P. cavicola*	3.0	3.5	5.3	4.0	13.3	5.3	3.0	3.5	14.3
IUM3169	*P. baumi*	8.0	24.0	3.0	5.0	9.3	12.5	11.5	10.0	28.5
Mean		7.1	9.0	6.0	4.2	6.9	7.3	5.0	8.0	17.0

[a]Ala: Alanine, AA: Ammonium acetate, AP: Ammonium phosphate, Arg: Arginine, Gly: Glycine, His: Histidine, Met: Methionine, PN: Potassium nitrate and Ur: Urea. Each nitrogen source was added to the basal medium at the concentration of 0.02 M.

nitrate support the highest mycelial growth of *P. linteus* (Hur, 2008). Therefore, it is concluded that most of the nitrogen sources used in this experiment except alanine, urea, and histidine are suitable for the vegetative growth of the *Phellinus* spp.

Outlook. Future studies need to examine the dichotomies between the present and previous observations, with the aim of best characterizing the cultural conditions that will support the large-scale production of mycelia. Furthermore, the micro-scale procedures employed to date will need to be scaled up. For the present, however, this study advances our knowledge of the cultural conditions that promote growth and mycelium production by *Phellinus* spp.

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