Cultural Characteristics of *Shimizuomyces paradoxus* Collected from Korea

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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 *Shimi- zuomyces 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-
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

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 | 20  | 20 | 10   | 10  | 10 | 20   | 20  | 20  |
| Sucrose                | 20  | 20  | 30 | 30  |    |      |     |    |      |     |    |
| Malt extract           | 20  | 20  | 20 | 3   |    |      |     |    |      |     |    |
| Potato                 | 200 | 200 |    |     |    |      |     |    |      |     |    |
| Peptone                |     |     |    |     | 3  | 3    | 3   | 3  | 2    |     |     |
| Yeast extract          | 2   | 3   | 5  | 5   | 5  | 2    |     |    |      |     |     |
| NaNO₃                  |     |     | 1  | 0.5 | 0.5| 0.5  |     |    |      |     |     |
| MgSO₄·7H₂O             |     |     | 1  | 1   | 0.46|      |     |    |      |     |     |
| K₂HPO₄                 |     |     | 1  | 1   | 1  | 1    |     |    |      |     |     |
| KCl                    |     |     |    |     |    | 0.5  |     |    |      |     |     |
| FeSO₄·7H₂O             |     |     |    |     |    | 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.

Fig. 1. Morphological characteristics of the *Shimizuomyces paradoxus*. A, Natural specimens; B, Perithecia; C, Ascus; D, Ascospores.
incubation at 25°C. Furthermore, dextrose, peptone, and K,HPO₄ 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 pigmentation included white, yellowish white (YW), pale yellow, light yellow, grayish yellow, yellowish grey, yellowish brown, olive brown, brown, brownish orange, orange grey, and grayish orange following 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 pigmentation. 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 Shimizuumyces 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 Shimizuumyces 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 Shimizuumyces paradoxus isolate EFCC C-5280.
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. 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, NH₄NO₃, asparagine, (NH₄)₂SO₄, 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 (C₄H₆O₆·2H₃N), 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 K₂HPO₄, which promoted mycelial density (Table 5). Even the control produced moderate myce-

**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                  |
| KNO₃            | 55                   | +++              | YG                  |
| C₆H₁₂O₇·2H₂N    | 54                   | ++               | YG                  |
| NaNO₃           | 50                   | ++               | YG                  |
| Yeast extract   | 46                   | ++               | GY                  |
| (NH₄)PO₄        | 46                   | ++               | YW                  |
| Tryptone        | 40                   | ++               | YG                  |
| Alanine         | 40                   | ++               | GY                  |
| NH₄NO₃         | 37                   | ++               | GO                  |
| Asparagine      | 35                   | ++               | YG                  |
| (NH₄)₂SO₄       | 34                   | ++               | GY                  |
| Glycine         | 26                   | ++               | GY                  |
| Control         | 46                   | +                | W                   |

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

**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.
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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 |
|-------------------|----------------------|------------------|---------------------|
| $\text{KH}_2\text{PO}_4$ | 58                   | ++               | YG                  |
| $\text{K}_2\text{HPO}_4$ | 57                   | +++              | YG                  |
| MnSO$_4$          | 52                   | ++               | YG                  |
| CaCO$_3$          | 51                   | ++               | YG                  |
| KCl               | 50                   | ++               | YG                  |
| MgSO$_4$$\cdot$7H$_2$O | 50       | ++               | GY                  |
| CaCl$_2$          | 48                   | ++               | OB                  |
| NaSO$_4$          | 48                   | ++               | YG                  |
| NaCl              | 48                   | ++               | YG                  |
| CaSO$_4$$\cdot$1/2H$_2$O | 42      | ++               | YG                  |
| ZnSO$_4$$\cdot$7H$_2$O | 39       | ++               | OB                  |
| FeSO$_4$$\cdot$7H$_2$O | 39      | ++               | YB                  |
| CuSO$_4$$\cdot$5H$_2$O | 34       | ++               | OB                  |
| Control           | 48                   | ++               | YG                  |

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

Fig. 6. Effect of $\text{KH}_2\text{PO}_4$ concentration on colony diameter of Shimizuomyces paradoxus isolate EFCC C-5280.

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

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