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Morphological Characteristics of Pseudosclerotia of *Grifola umbellata* in *In Vitro*

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The present study was carried out to investigate morphological characteristics of pseudosclerotia of *Grifola umbellata* formed by artificial cultures. Isolate *G. umbellata* DUM GUS-01 was obtained from sclerotium cultivated in field. The fungal isolate was cultured on PDYM broth, PDYMA (potato dextrose yeast malt agar) and oak sawdust media at 20°C under the dark condition. *G. umbellata* DUM GUS-01 showed a volumetric increment of fungal lumps rather than mycelial growth. Particularly, *G. umbellata* DUM GUS-01 produced a large amount of melanin pigments in all culture treatments. The color of the fungal mass has been changed into grey gradually, and then formed melanized rind-like structure on its superficial part. The fungal structures which were covered with melanized rind-like layer were named as pseudosclerotia of *G. umbellata*. The pseudosclerotia of *G. umbellata* DUM GUS-01 formed a new white mycelial mass, which was swollen out of the melanized rind structure for its volumetric increment. When the pseudosclerotia were sectioned, their structure was discriminated from two structures such as a melanized rind-like structure layer formed by aggregation of aged mycelia and a white mycelial mass with high density. As results of scanning electron microscopic examination, the pseudosclerotia of *G. umbellata* DUM GUS-01 which were formed in *in vitro* conditions were similar to the sclerotia of *G. umbellata* cultivated in natural conditions except for the crystals formed in medula layer of natural sclerotia. Although size, solidity of rind structure and mycelial compactness of pseudosclerotia were more poor than those of natural sclerotia, the morphological structure and growth pattern of pseudosclerotia were very similar to those of natural sclerotia. Therefore, it is probable to induce pseudosclerotia to sclerotia of *G. umbellata* in *in vitro* conditions. Consequently, it seems that the induced pseudosclerotia can be used as inoculum sources to substitute natural sclerotia in field cultivation.

KEYWORDS: Culture characteristics, *Grifola umbellata*, Morphological characteristics, Pseudosclerotia

Grifola umbellata (Per. : Fr.) Pilt belongs to the family *Polyporaceae* of Basidiomycetes, and forms an underground sclerotium which derives fruiting-body under favorable condition in its life cycle (Imazeki and Hongo, 1989). The sclerotium of *G. umbellata* has been known to possess an anti-tumor activity (Azuhata, and Sugiyama, 1994; Yang, 1991), immunopotentiation ability (You *et al.*, 1994) and antimicrobial activity against *Helicobacter pylori* (Ha, 2001). Namely, substances of polysaccharide such as β-glucan which was isolated from sclerotia of *G. umbellata* have been known to exhibit an outstanding effectiveness for curing human diseases. Nowadays, the active substances of sclerotium have been studied intensively (Guo *et al.*, 1992; Kang and Ko, 1975; Lee *et al.*, 2002). With these verifications of medicinal properties, the sclerotia of *G. umbellata* have been used as one of the important herbal medicines in Korea.

Although the production of sclerotia has been achieved by inoculating slices of sclerotium on wood logs colonized by rhizomorphs of *Armillaria mellea* in field (Lee *et al.*, 2000), it is no doubt that the production of valuable sclerotia has been known to require some conditions such as a long term more than 1 year, a large number of wood logs for their inoculation, and somewhat wide areas for their mass production. Therefore, it is necessary that a lot of sclerotia must be secured as an inoculum source to accomplish its mass production. Since *G. umbellata* has been distributed at the natural habitats of an upland more than 1500 meters, it is impossible to secure natural sclerotia of *G. umbellata* in field of Korea. It is obvious that such a geographical handycap has induced the cause for importing a lot of sclerotia from China. As one of alternative methods to overcome this difficulty, our research was focused to identify the possibility which the mycelial tuft of *G. umbellata* could form its pseudosclerotia and finally develop sclerotia in *in vitro* conditions. To compare fungal masses of *G. umbellata* with natural sclerotia, its mycelial tuft was induced to pseudosclerotia (such as fungal masses) in *in vitro* conditions. Therefore, this investigation was carried out to confirm if morphological characteristics of fungal masses were corresponded with those of natural sclerotia.

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Materials and Methods

Isolates. Isolate of *G. umbellata* DUM GUS-01 was obtained from its sclerotium cultivated artificially in field. Sclerotium of *G. umbellata* was washed with distilled water and then its surface was sterilized with 3% sodium hypochlorite (NaClO) solution. After then, sclerotial tissues were cut into 0.5×0.5 cm, transferred on PDA and 1.5% water agar, and incubated for 3 weeks at 20°C under the dark condition. Fungal isolates were incubated on PDA at 25°C under the dark condition and then maintained at 4°C. Isolate of *Armillaria mellea* DUM-007 was isolated from its rhizomorphs and used for this study.

Culture of *G. umbellata* DUM GUS-01. To culture fungal isolates, three types of cultures were performed in this experiment. First, isolate *G. umbellata* DUM GUS-01 was cultured on potato dextrose yeast malt agar (PDYMA) at 23°C for 45 days. Second, *G. umbellata* DUM GUS-01 was cultured on PDYM broth at 23°C for 45 days without shaking. Third, *G. umbellata* DUM GUS-01 was inoculated on oak sawdust media (oak sawdust : wheat : rice bran = 4 : 1 : 1) with moisture content of 70%, cultured at 18°C for 30 days, and cultured at 23°C for 30 days under the dark condition.

Dual culture. To induce sclerotial development, *G. umbellata* DUM GUS-01 was inoculated in glass tubes (24×150 mm) filled with oak sawdust medium of about 20 mm in height and then cultured at 18°C for 45 days under the dark condition. After then, the sawdust culture of *G. umbellata* DUM GUS-01 was transferred into petri-dish which *A. mellea* DUM-007, fungus symbiotic to *G. umbellata* was cultured on PDA at 23°C for 2 weeks. The petri-dish was re-cultured at 23°C for 20 days.

Microscopical observation. To perform light microscopic observation, pseudosclerotia of *G. umbellata* DUM GUS-01 were washed twice with 0.1 M sodium phosphate buffer (pH 7.2), cut into small pieces of about 1 cm^3, embedded in a freezing tissue embedding medium, and then thin sectioned in the range of 7 to 10 μm thickness at –20°C with a cryomicrotome (Leica CM 1900, Germany). Sectioned specimens were stained by using water-iodine solution (25% iodine in distilled water) and melzer's reagent (1.5% iodine and 5% potassium iodide in distilled water), and then examined under a light microscope (Optiphot-2; Nikon, Japan). To perform scanning electron microscopic (SEM) examination, the specimens were fixed overnight at 4°C in 2.5% glutaraldehyde, rinsed twice for 30 minutes in 0.1 M sodium phosphate buffer (pH 7.2) and post fixed at 4°C for 2 hours with 1% osmium tetroxide (OsO_4). The specimens double fixed were rinsed 3 times with 0.1 M sodium phosphate buffer (pH 7.2), and then dehydrated with a graded ethanol series such as 50%, 70%, 80%, 90% and 100%. The ethanol was substituted by isoamyl acetate. The specimens were dried by using a critical point dryer (Hitachi HCP-2, Japan), and then coated by using a sputter coater (Hitachi E-1010, Japan). As described on some reports (Choi *et al.*, 2002; Zarani and Christias, 1997), the specimens were examined under a scanning electron microscope (Hitachi S-2380N, Japan).

Results

Culture characteristics. Cultural characteristics of *G. umbellata* DUM GUS-01 were an unique growth pattern such as a volumetric increment of irregular mycelial mass rather than mycelial growth. Fungal colony of *G. umbellata* DUM GUS-01 grew slowly with high mycelial density on the surface of PDYMA medium and penetrated into the medium (Fig. 1A and B). Aerial mycelia of *G. umbellata* DUM GUS-01 have been changed gradually into grey or light brown and produced dark melanin pigments on their superficial part of the fungal colony. Particularly, new white mycelia grew out on the periphery of the aged mycelial mat and developed a new fungal structure such as swelling form on the surface of mycelial mat (Fig. 1A). In broth culture, the white mycelial mass of *G. umbellata* DUM GUS-01 was aggregated on the surface of PDYM broth in early culture, changed into grey or light brown and produced a large amount of dark pigments in PDYM broth at old culture (Fig. 1C).

Based on sawdust media, *G. umbellata* DUM GUS-01 was colonized on sawdust media and aggregated irregularly with white and compact mycelial mass. After then, the aggregated mycelia of *G. umbellata* DUM GUS-01 were conglomerated with melanin pigments and formed a black rind-like structure on the surface of its aged mycelial mass (Fig. 1D).

Inducement of pseudosclerotia. In dual culture of both *G. umbellata* and *A. mellea*, the fungal mass of *G. umbellata* DUM GUS-01 which was colonized fully on the oak sawdust medium increased its mycelial density. Also, the surface of fungal mass has been changed gradually into grey or light brown. The superficial part of *G. umbellata* DUM GUS-01 which was cultured on sawdust media was surrounded by grey or light brown rind-like structure. As a result of mycelial growth and rhizomorph development of *A. mellea* DUM-007, morphological characteristics of *G. umbellata* DUM GUS-01 which was cultured on sawdust media were discriminated clearly into rind-like layer and inner layer with white compact mycelial mass, and white mycelia of *G. umbellata* DUM GUS-01 were grown out newly to the opposite direction of *A. mellea* DUM 007 (Fig. 1E). The mycelial aging or color change of *G.*

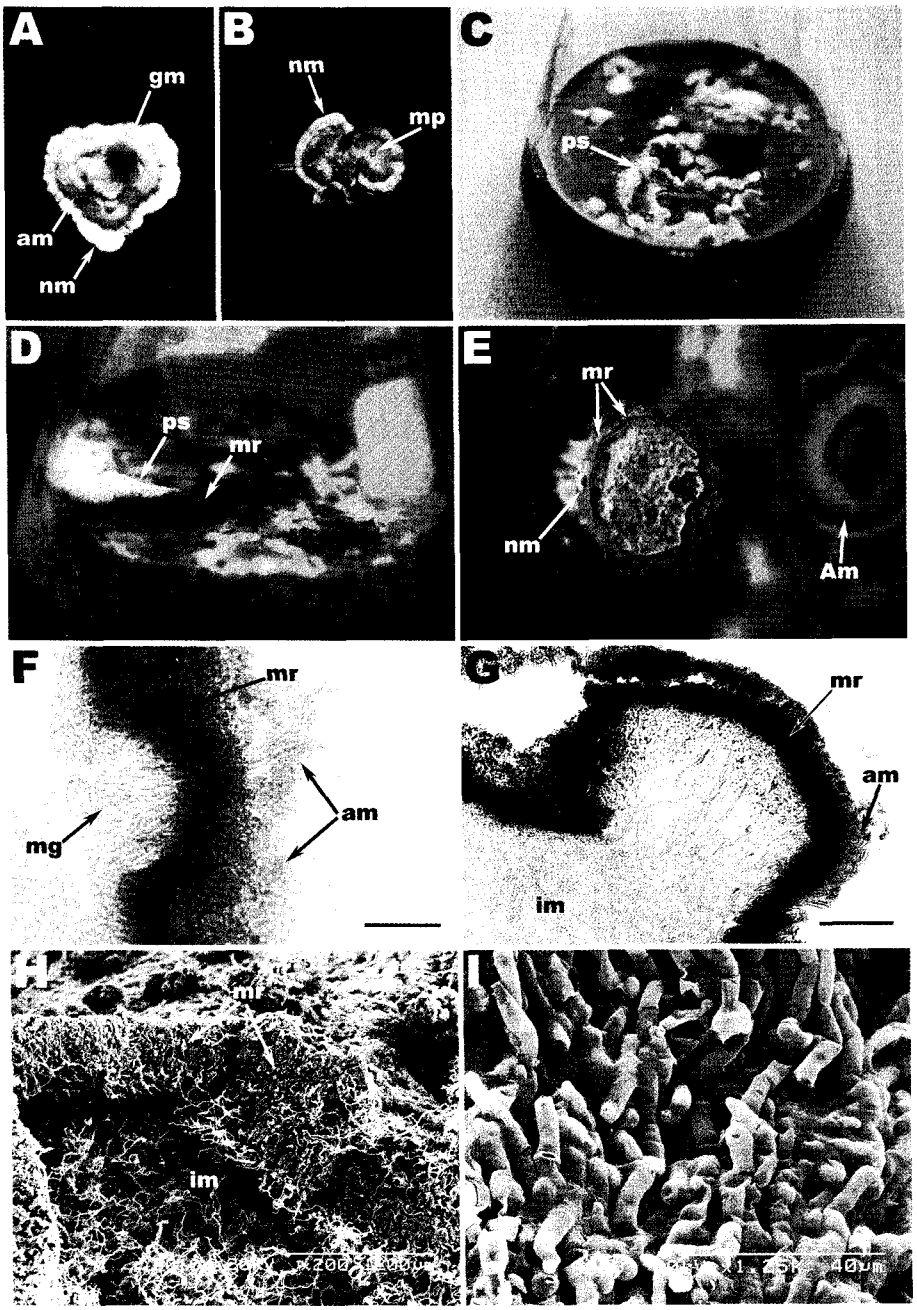

Fig. 1. Pseudosclerotia of *Grifola umbellata* DUM GUS-01 formed in artificial media and their morphological characteristics. **A**, Forward view of fungal colony of *G. umbellata* DUM GUS-01 cultured on PDYMA medium. **am**: aged mycelial mat, **gm**: globular mycelial structure swollen on the aged mycelial mat, **nm**: new mycelial growth on the periphery of aged mycelial mat. **B**, Reverse view of fungal colony of *G. umbellata* DUM GUS-01 cultured on PDYMA medium. **nm**: new mycelial growth on the periphery of aged mycelial mat, **mp**: melanin pigments produced on the aged mycelial mat. **C**, Pseudosclerotia of *G. umbellata* DUM GUS-01 formed in PDYM broth medium. **ps**: pseudosclerotium. **D**, Pseudosclerotia of *G. umbellata* DUM GUS-01 formed in oak sawdust medium. **mr**: melanized rind-like structure, **ps**: pseudosclerotium. **E**, Dual culture of both *G. umbellata* DUM GUS-01 and *Armillaria mellea* DUM-007. **Am**: *A. mellea*, **mr**: rind-like structure accumulated with melanin pigments, **nm**: fungal structures of pseudosclerotium developed newly for its volumetric increment. **F**, Early stage of globular structure developed on the superficial part of rind-like layer for its volumetric growth in dual culture. **am**: aged mycelia, **mg**: mycelial mass developed newly with high mycelial density, **mr**: rind-like layer accumulated with melanin pigments. (Scale bar=50 μm). **G**, Later stage of globular fungal structure. **am**: aged mycelia, **im**: inner mycelial layer of the fungal structure aggregated with high mycelial density, **mr**: rind-like layer accumulated with melanin pigments. (Scale bar = 100 μm). **H**, Cross section of pseudosclerotium of *G. umbellata* DUM GUS-01. **im**: inner mycelial layer of the pseudosclerotium, **mr**: melanized rind-like layer. **I**, Superficial structure of rind-like layer aggregated with deformed mycelial mass.

umbellata DUM GUS-01 were not observed in the inner mycelial layer protected by rind-like structure. Particularly, it was observed that *G. umbellata* DUM GUS-01 developed a new white mycelial mass on the superficial part of rind-like structure (Fig. 1E). Gradually, this fungal mass has swelled and increased its volume.

Morphological characteristics of pseudosclerotia. It was observed that *G. umbellata* DUM GUS-01 formed its pseudosclerotia in all culture treatments. The white mycelia of *G. umbellata* DUM GUS-01 grew compactly in the initial stage. Aged mycelia of *G. umbellata* DUM GUS-01 produced melanin pigments and formed a rind-like structure in the matured stage. In dual culture, new fungal masses of *G. umbellata* DUM GUS-01 were developed on the rind-like layer of pseudosclerotium colonized on the oak sawdust medium. As a result of light microscopic observation, the new fungal mass was not developed on the surface of the existing rind-like layer but grown by pushing out the rind-like layer with compact mycelia. In early stage, the rind-like layer of the new fungal mass was swollen slightly (Fig. 1F). Later, volume and size of fungal mass were increased by increasing mycelial density and then developed into a globular form (Fig. 1G). This fungal mass was distinguished distinctly between the rind-like structure and compact mycelial layer. Particularly, melanin pigments were heavily accumulated and coagulated with aged mycelial mass on the rind-like structure (Fig. 1G). In SEM examination, the pseudosclerotia of *G. umbellata* DUM GUS-01 was clearly discriminated from a melanized rind-like structure and an inner compact mycelial layer (Fig. 1H). The structure of rind-like layer was assembled solidly with aged mycelial mass, and its thickness was from 67 to 133 μm. The superficial part of the rind-like layer was twined and aggregated with aged mycelia which were deformed and wrinkled (Fig. 1I).

Discussion

Sclerotia of fungi usually consist of a medulla of pseudoparenchymatous tissue, a cortex of close-fitting hyphae and melanized cells known as a rind which forms on the outer surface and encases a broad medulla of interwoven hyphae (Chet and Henis, 1975; Willetts, 1978). In spite of the morphological diversity of fungal sclerotia, their anatomy, physiology and biochemistry are basically similar (Willetts and Bullock, 1992). Like other fungi, sclerotia of *G. umbellata* have the same maturation processes divided into three arbitrary stages: white sclerotia developing into full size, grey sclerotia pigmented slightly and black-matured sclerotia (Choi *et al.*, 2002).

In this study, *G. umbellata* DUM GUS-01 grew very slowly with compact mycelial aggregation and produced melanin pigments in PDYM broth and PDYMA media. The surface of its mycelial mat has been changed gradually into grey or light-brown, and then the superficial part has been transformed into a solid structure. Fungal mass of *G. umbellata* DUM GUS-01 was seemed to be developed gradually into sclerotial structures which were named as pseudosclerotia. Fungal mass which was pigmented with melanins showed resting phase. After then, white mycelia grew newly on the periphery of the aged mycelial mat and formed the globular structure on the surface of melanized rind-like structure. It is likely to suggest the fact that *G. umbellata* DUM GUS-01 has been grown continuously by repetition of this procedure for increasing its size. This growth pattern of *G. umbellata* DMU GUS-01 was very similar to that of natural sclerotia in field. When the pseudosclerotia of *G. umbellata* DUM GUS-01 were sectioned, their structure was discriminated from two structures such as a medullary tissue with a compact mycelial mass and melanized rind-like layer formed by aggregation of aged mycelia. The rind-like layer seemed to be formed by aggregation of aged and deformed mycelia with melanin pigments. Except for the formation of crystals, the morphological characteristics of pseudosclerotia were similar to those of grey sclerotium described by Choi *et al.* (2002).

G. umbellata DUM GUS-01 produced a large amount of melanin pigments in all cultures. Particularly, melanins were heavily accumulated in the rind-like layer. In sclerotial development of higher fungi, a pigmented deposit may be also accumulated between cells and on the outer surface of the sclerotium. Melanins have been known to be important in increasing fungal propagules resistant to adverse environmental condition and to attack by microorganisms (Bell and Wheeler, 1986; Huang *et al.*, 1993). Particularly, when melanins are complex with chitin of fungal wall, they play roles as an inhibitor against polysaccharases and increase protective effects against enzymes produced by the fungus itself and other antagonistic microorganisms (Bull, 1970; Willetts and Bullock, 1992). Therefore, melanin production seemed to be an important characteristic for formation of rind and protection of medullary tissue in sclerotial development of *G. umbellata*. In dual culture, the white mycelial mass of *G. umbellata* DUM GUS-01 which was protected by melanized rind-like structure was grown compactly without color change and pigment deposit. These results seemed to suggest that a melanized rind-like structure of pseudosclerotia plays the same role as that of sclerotia in nature.

Once the sclerotium has been delimited by the rind, enlargement of the medullary tissue generates internal pressures which stretch the peripheral layer and may lead to rupture of individual rind cells. Expansion of the rind to accommodate an increase in surface area of the sclerotium could be achieved by enlargement of individual cells and by incorporation of new tips into the layer from

underlying hyphae (Willetts and Bullock, 1992). This phenomenon was also observed in dual culture of both *G. umbellata* DUM GUS-01 and *A. mellea* DUM-007. *G. umbellata* DUM GUS-01 formed a new fungal structure swollen on the melanized rind-like layer.

As a result of light microscopy, this fungal structure was very similar to the sclerotial primordium of natural sclerotia which was formed on the matured sclerotium and developed into white sclerotium (Choi *et al.*, 2002). It was supposed that the pseudosclerotium of *G. umbellata* DUM GUS-01 developed the new fungal mass on the rind-like layer for its continuous growth. This process seemed to agree with that of the sclerotial development of *G. umbellata* in field.

Although size, solidity of rind structure and mycelial compactness of pseudosclerotia were more poor than those of natural sclerotia, the morphological structure and growth pattern of pseudosclerotia were very similar to those of natural sclerotia. Therefore, it is probable to induce pseudosclerotia to sclerotia of *G. umbellata* in *in vitro* conditions. Consequently, it seems that the induced pseudosclerotia can be used as inoculum sources to substitute natural sclerotia of *G. umbellata* in field cultivation.

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