

## Morphological Characteristics of Hyphal Interaction between *Grifola umbellata* and its Companion Fungus

Xiao-Ke Xing<sup>1</sup>, Shun-Xing Guo<sup>1\*</sup> and Min-Woong Lee<sup>2</sup>

<sup>1</sup>Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100094, China

<sup>2</sup>Department of Biology, Dongguk University, Seoul 100-715, Korea

(Received October 29, 2004)

Morphological characteristics of hyphal interaction between *Grifola umbellata* (Pers. Ex Fr.) Pilat and its companion fungus which related to sclerotia formation from hyphae were investigated by external observations, light microscopy and transmission electron microscopy (TEM). External observations showed that a dense antagonism line was formed by both *G. umbellata* and companion fungus after their hyphae contacted each other in dual culture. Many hyphal strands emerged on the colony of *G. umbellata* and differentiated to sclerotia from where hyphal strands crossed. Light microscope observations revealed the process of antagonism line formation. Mature antagonism with structural differentiation, was composed of three main layers: the rind, the rind underlayer and the hypha layer. TEM observations showed that after colonies hyphal contact, a series of reactions always occurred in both *G. umbellata* and companion fungus. Cells in the center of antagonism line were dead. Cells of *G. umbellata* adjacent to the antagonism line were usually large and hollow, with unilateral thickened wall, whereas those of companion fungus were empty, with thin or thick wall. Both hyphal interaction at the antagonism line may be one of the main reasons for sclerotia of *G. umbellata* differentiation from hypha.

**KEYWORDS:** Companion fungus, *Grifola umbellata*, Hyphal interaction, Morphological characteristics

*Grifola umbellata* belongs to Polyporaceae, Basidiomycetes. Sclerotia of *G. umbellata* have been used in traditional Chinese medicine for over 2000 years to cure edema (Pharmacopoeia, 2000). Recently, a polysaccharide isolated from sclerotia of *G. umbellata* has been to enhance human immunity and to inhibit the growth of malignant cells (Xu, 1997). In China, the semi-artificial cultivation of *G. umbellata* sclerotia has been practiced more than 20 years. It was well known that the sclerotia of *G. umbellata* could not be grown without an *Armillariella mellea* strain. The interaction and nutritional relationship between both fungi have been well documented (Xu and Guo, 1992; Guo and Xu, 1992, 1993). However, there were some disadvantages in the semi-artificial cultivation, such as low proliferation rate, unstable yield and a larger amount of small sclerotia used as seed for cultivation (Guo and Xu, 1991) which blocked the productive scale and lowering in product of *G. umbellata* sclerotia. Consequently, the finding of the mode of differentiation of sclerotia can be done directly from the hyphae of *G. umbellata* cultivation under artificial conditions happened to be key event for research and development in this area.

The sclerotia of *G. umbellata* grow underground. Around the sclerotia, there is a complicated microflora. The sclerotia formation under natural condition maybe associated with the microflora which just the main reason for sclerotia that could not be formed from hyphae under

artificial condition. Recently Guo *et al.* (2002) isolated a fungal strain from underground cavity associated with *G. umbellata* in field, which is stimulated to sclerotial formation from hyphae of *G. umbellata*, and called it companion fungus. The companion fungus was found to play a major role in the differentiation of *G. umbellata* sclerotia. When dual cultured with *G. umbellata* and companion fungus, many sclerotia of different size were formed on the surface of medium 60 days after inoculation. Companion fungus was considered to be a vital bio-controller capable to regulate the sclerotia formation of *G. umbellata*. Till to now, the companion fungus has not been identified. We compared the sequences of 5.8S rDNA and the flanking internal transcribed spacers (ITS1 and ITS2) of the companion fungus and *G. umbellata*. The results suggested that the companion fungus was closely related to *G. umbellata* (Xing and Guo, 2004). For exploring the mechanisms of sclerotia formation induced by companion fungus, the aim of this paper was to study the morphological characteristics of hyphal interaction between *G. umbellata* and companion fungus.

### Materials and Methods

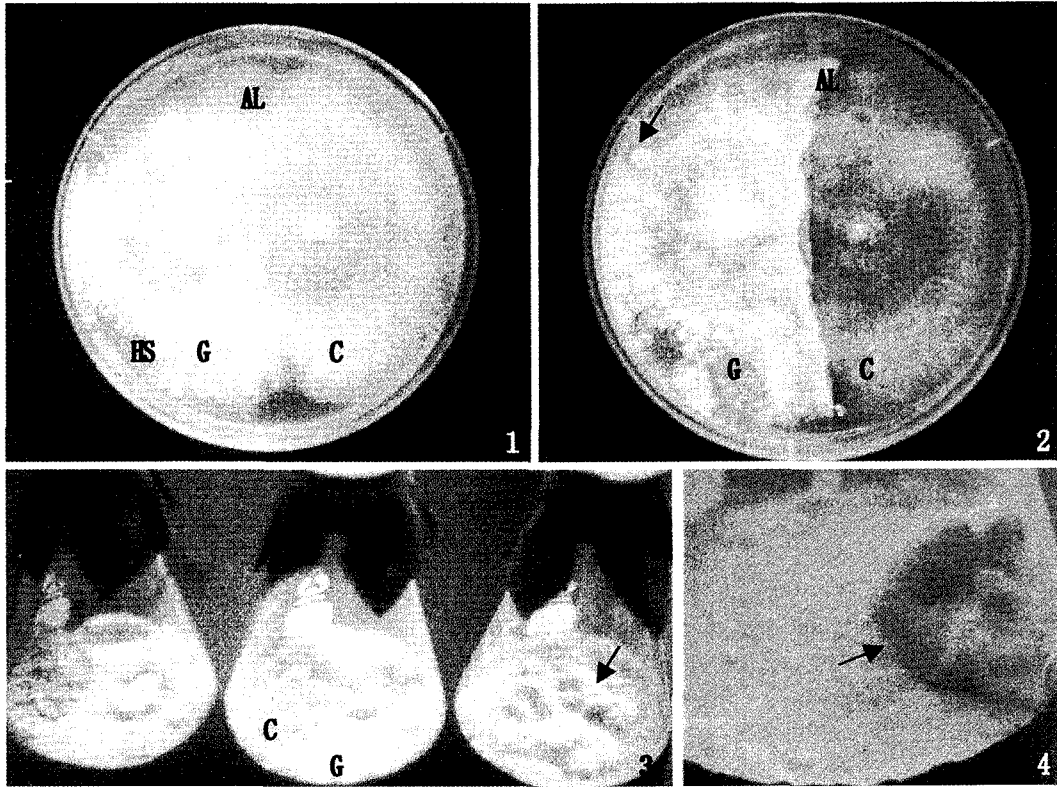
**Fungal isolates and growth.** The all of isolates of *G. umbellata* and companion fungus used in this study were isolated by Guo (2002) in ShanXi Province of China. They were maintained in Petri plates on a WBA medium (g/l: wheat bran 30, glucose 20, KH<sub>2</sub>PO<sub>4</sub> 3, MgSO<sub>4</sub> 1.5,

\*Corresponding author <E-mail: guosx2000@sohu.com>

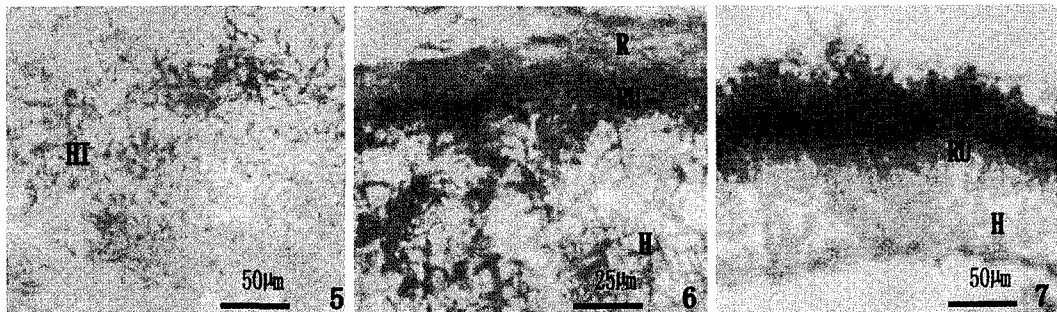
agar 15, adjust pH to 6.0) at 22 to 24°C under dark condition.

The interaction between hypha of *G. umbellata* and companion fungus was studied according to the following procedure. For short time culture, each plate (9 cm) con-

taining 20 ml of WBA was used. At the same time, 250 ml flasks containing 100 ml WBA were also prepared for long time culture. Mycelial plugs (5 mm diameter) collected from actively growing agar colonies of both fungi were placed 3.0 cm apart each other on the surface



**Figs. 1-4.** *G. umbellata* (G) and companion fungus (C) in dual inoculation 25 and 35 days after inoculation respectively, and many hyphal strands (HS) emerged on the colony of *G. umbellata*. Fig. 1. An evident antagonism line (AL) formed at the interface region. Fig. 2. The antagonism line matured 35 days after inoculation. Sclerotia initials emerged (arrow). Fig. 3. Dual incubation of *G. umbellata* and companion fungus 60 days after inoculation in 250 ml flasks, the development of sclerotia and rind differentiation (arrow) could be seen. Fig. 4. Mature sclerotia of *G. umbellata* produced on WBA medium (arrow) result from dual incubation with companion fungus.



**Figs. 5-7.** Light microscope photographs of the antagonism formation process. Fig. 5. Haphae of *G. umbellata* and companion fungus interwove (HI) at the interface region. Fig. 6. An evident antagonism line formed 25 days after inoculation. For this time, it seems that the rind was differentiating on the surface of antagonism line. After dual staining with safranin and fast green, only fast green present positive reaction. Fig. 7. Up to 35 days of incubation, the antagonism line matured entirely, composed of three main layers: the rind (R), the rind underlayer (RU) and the hypha layer (H). After dual staining epidermis was red, both epidermal and hypha layer were green. Arrays in sequence according to the number in manuscript.

of the agar and allowed to grow at 23°C under dark condition. Both hypha grew toward each other, and hyphal contact occurred by 10 to 12 days after inoculation.

**Microscopic observation.** Ten samples were collected from interface region 25 and 35 days, respectively after inoculation were fixed with FAA (formalin : glacial acetic acid : 50% ethanol 1 : 1 : 18), embedded in wax following dehydration through a graded alcohol and sectioned with a rotary microtome at 7  $\mu\text{m}$ . Embedding and staining with safranin and fast green followed the protocol of Johansen (1940). Preparations were observed and photographed under microscope (Olympus Co., Japan). For each sample, 10 sections were examined.

Ten samples collected from interface region on 25 and 35 days, respectively after inoculation were immediately fixed by immersion in 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer, pH 7.2, for 2 h at room temperature. Samples were post fixed with 1% osmium tetroxide in the same buffer for 1 h at 4°C and dehydrated in a graded ethanol series prior to being embedded in Epon 812. Thin sections (0.7  $\mu\text{m}$ ) cut from the Epon-embedded material with glass knives were mounted on glass slides and stained with 1% aqueous toluidine blue prior to examination with a Olympus microscope. Ultra thin sections (0.1  $\mu\text{m}$ ) collected on nickel grids were examined with a JEOL 100s transmission electron microscope. For each sample about 10 ultra thin sections were examined under the electron microscope (Jeol Co, Japan).

## Results

The *Grifola umbellata* strain was differentiated white villiform-like colonies that grew vigorously, covering the whole Petri dish in 24 days of incubation in monoculture of strains. It formed aerial mycelia and one month old colonies exudated brown oil-like liquid on the colony surface when the color of the media turned yellow.

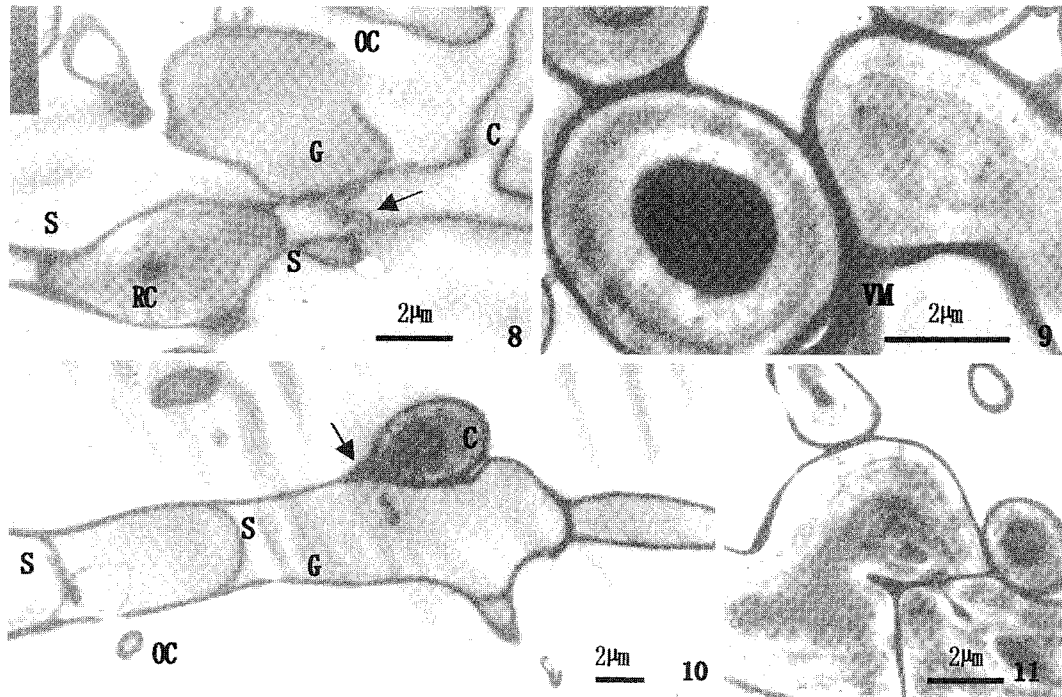
The companion fungus strain differentiated white to yellow colonies that grew slightly slower than the *G. umbellata*, covering the whole Petri dish on 28 days after incubation. Colonies formed a aerial mycelia. The color of medium turned to filemot, caused by exudation of fungus. When grown together, *G. umbellata* and companion fungus colonies established contact around 10 days after inoculation. Twenty-five days later of dual cultures, an evident antagonism line was formed at the interface region (Fig. 1). With culture time prolonged, the antagonism line became more developing and maturing, and matured 35 days after inoculation. The mature antagonism line reached 0.4 cm high like a wall in the middle between two colonies (Fig. 2). The color of media turned brown gradually. On the surface of *G. umbellata* colony, many hyphal strands emerged and crossed each other. Initial

sclerotia was formed from where hyphal strands crossed (Fig. 2 arrow). However, in monocultures of *G. umbellata*, hyphal strands and sclerotia initials could hardly be seen. The size of these sclerotia initials which obtained in Petri plates cultures no more enlarged probably because of nutrition exhaustion in the media. The further development and maturation of sclerotia in 250 ml flasks prepared were observed at the same time as Petri plates (Figs. 3, 4). With the enlargement of sclerotia size, the differentiation of rind in some sclerotia initials were observed (Fig. 3, arrow). Fig. 4 showed the mature sclerotia of *G. umbellata* resulted from dual cultured with companion fungus (arrow).

At the light microscope level, the whole process of antagonism formation was observed. The first mycelial contact between both fungal colonies was established after 10 days of incubation and both colonies hypha interwove at the interface region (Fig. 5). Then, an evident antagonism line formed 25 days after inoculation (Fig. 6). For this time, it seems that the rind was differentiating on the surface of antagonism line. After dual staining with safranin and fast green, only fast green presents positive reaction. Up to 35 days of incubation, the antagonism line matured entirely. Examination of the mature antagonism line showed that this structure was composed of three main layers: the rind, which was composed of closely arranged cells, the rind underlayer, in which the cells arranged more loosely than in the rind, and the hypha layer, which was just the active hyphal interaction region of both fungus (Fig. 7). After dual staining, epidermis was red, both epidermal underlayer and hypha layer were green. The rind underlayer was composed of compact hypha, looked like a transitional state of epidermis. However, these observations by light microscopy could not clearly differentiate hyphae of *G.umbellata* and companion fungus in the antagonism line.

A more detailed pictures of the hyphal interaction between *G. umbellata* and companion fungus were obtained through TEM observations of ultrathin sections. Examination of samples collected at 25 days of incubation revealed that hyphal interaction actively occurred on the interface of both colonies. The adherence between the mycelia of *G. umbellata* and companion fungus occurred after their contact. Electron-dense materials were observed in the adherence region, maybe related to the presence of viscous matrix or to the hyperplastic cell wall (Fig. 8 arrow).

After contact with companion fungus, a series of reactions occurred in *G. umbellata*, such as rupture of cell wall, outflow of cytoplasm, formation of septa at both sides of cracked section (Fig. 8). A series reactions also occurred in *G. umbellata* include retraction of cytoplasm and formation of septa and degradation of cell wall, the degraded cell wall existed in the hypha (Fig. 9, arrow).



**Fig. 8-11.** Transmission electron micrographs of hyphae interaction between *G. umbellata* and companion fungus. Fig. 8. After contacted with companion fungus a series of reaction also occurred in *G. umbellata* include rupture of cell wall, outflow of cytoplasm (OC) and formation of septa at both sides of cracked section. Electron dense materials existed in the adherence region (arrow). Fig. 9. After contacted with *G. umbellata*, a series of reactions occurred in companion fungus, such as retraction of cytoplasm (RC), formation of septa (S) and degradation of cell wall, the degraded cell wall existed in the hypha (arrow), and cell wall of *G. umbellata* collapsed resulted in outflow of cytoplasm (OC). Fig. 10. Adherence also occurred in hypha of companion fungus, the viscous matrix (VM) derived from the outflowing cytoplasm of cracked *G. umbellata* hypha. Fig. 11. After dual cultured with *G. umbellata*, clamp connections of the companion fungus hyphae were easily observed.

Adherence also occurred in hypha of companion fungus, the viscous matrix derived from the outflowing cytoplasm of cracked *G. umbellata* hypha (Fig. 10). After dual culturing with *G. umbellata*, clamp connections of the companion fungus hyphae were easily observed (Fig. 11), but few were seen in its monocultures.

Examination of samples collected on 35 days on incubation showed that the center of the antagonism line was composed of dead cells with thickened wall (Figs. 12, 13). Hyphal cells of *G. umbellata* and companion fungus which located on the two sides of antagonism line respectively could be clearly differentiated. Cells of *G. umbellata* adjacent to the antagonism line were usually large and hollow, with unilateral thickened wall (Fig. 14), whereas those of companion fungus were empty, with thin or thick walls (Fig. 12).

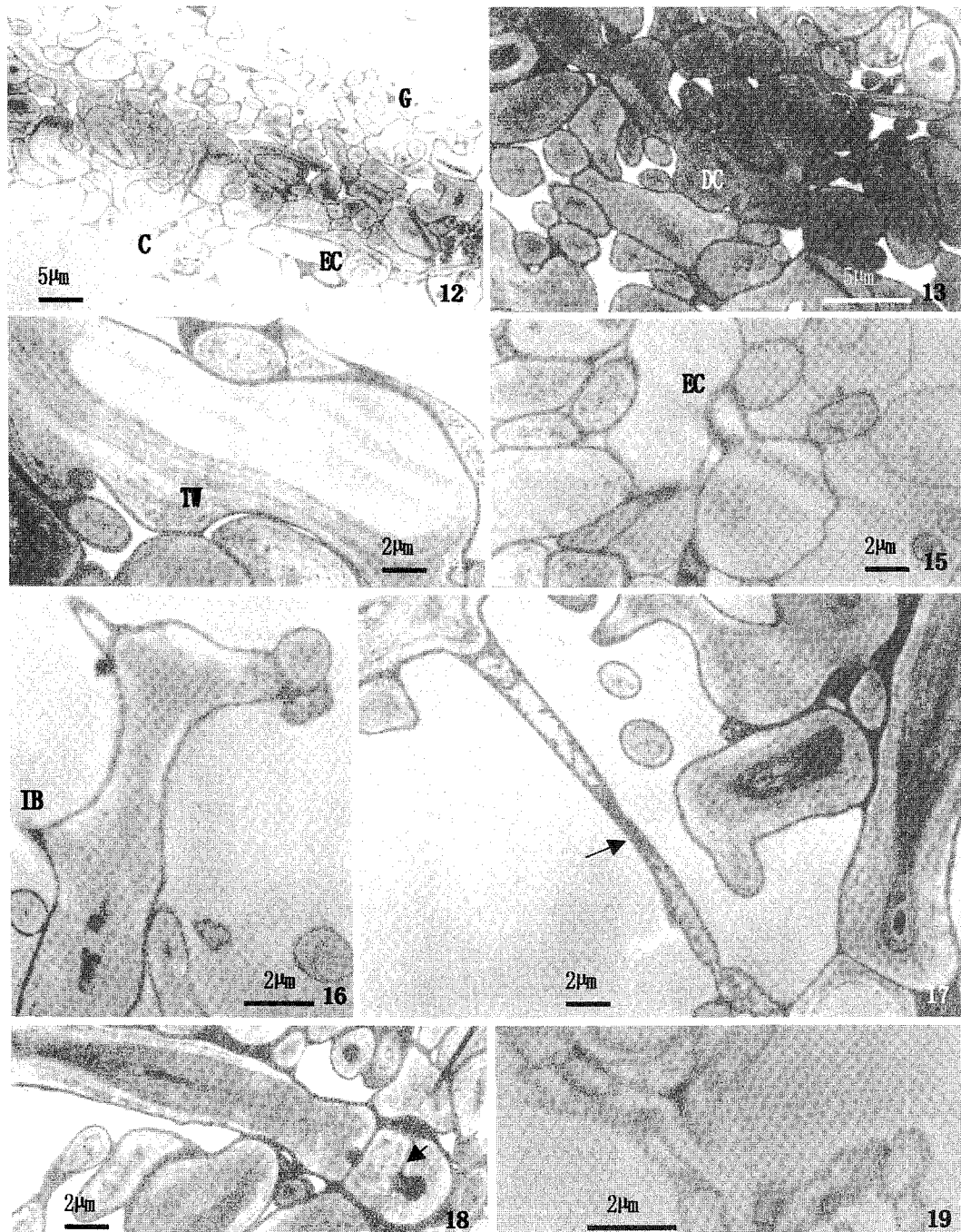
A little apart from antagonism line, the hypha of companion fungus branched irregularly (Fig. 15), and those of *G. umbellata* were made of hypha with both thin and thick wall (Fig. 16). The branches connected each other by confluence of tips, and a dense hyphal network was differentiated (Fig. 17, arrow). Hypha of companion fungus showed dolipore septa near to the antagonism line

(Fig. 18). Totally degraded cells of companion fungus also occurred inside the antagonism line (Fig. 19).

## Discussion

Most of the studies dealing with fungus-fungus relation focus on the interaction between mycoparasites and their host fungi. These interactions are usually accompanied by haustoria formation such as appressoria, hyphal coils or hook-like bodies (Dragt *et al.*, 1996; Inbar *et al.*, 1996; Li and Shen, 1996). With the *G. umbellata* and companion fungus, no haustoria type structures could be observed. The nutritional relationship concerning sclerotia formation, therefore between *G. umbellata* and its companion fungus is unclear. The interactions resulted in dense antagonism line formed at the interface region of colonies, which role is expected to protect the fungus against invasion by antagonists.

TEM revealed that both hyphae agglutinated each other. Recognition of both fungus may be made through chemical mechanisms of chemotaxis which induce the hyphae of companion fungus to adhere to *G. umbellata*. After companion fungus adhered to the hyphae of *G. umbel-*



**Figs. 12-19.** Transmission electron micrographs of antagonism line. Fig. 12. Antagonism line and hyphal cells of *G. umbellata* (G) and companion fungus (C) which located on the two sides of antagonism line, respectively. Cells of companion fungus adjacent to the antagonism line were empty (EC). Fig. 13. Center of antagonism line was composed of dead cells (DC). Fig. 14. Cells of *G. umbellata* adjacent to the antagonism line were usually large and empty, with thin or thick wall. Fig. 15. A little apart from antagonism line, hyphae of companion fungus branched irregularly (IB). Fig. 16. The irregular branches of companion fungus connected each other by confluence of tips (arrow), and a dense hyphal network was differentiated. Fig. 17. Hypha of companion fungus showed dolipore septa (arrow) near to the antagonism line. Fig. 18. Totally degraded cells also occurred inside antagonism line.

*lata*, a series of reaction occurred in both of them, such as rupture of cell walls and outflow of cytoplasm in *G. umbellata*, and collapse of cell walls, retraction of cytoplasm and formation of septa in companion fungus. A

specific lectin was found to be involved in the recognition of a fungus-fungus interaction (Barak, 1985). A series of reaction always induced by recognition in both host fungus and mycoparasites. We also discovered agglutina-



tion activity in the crude protein extraction of companion fungus. It is then hypothesized that a lectin may also be involved in the *Grifola*-companion fungus recognition, too.

Hyphal strands of *G. umbellata* were differentiated in result of the interaction. The sclerotial initials differentiated from the sites where hyphal strands crossed. The hyphal behavior of *G. umbellata* in sclerotial production was similar to the hyphal behavior of *Sclerotium rofsii* during sclerotia formation (Townsend and Willetts 1957). Chet and Henis (1975) have reviewed the factors which affected the sclerotial formation of filamentous fungi in detail. It was also noted that sclerotial production usually occurred when the mycelium reached the Petri plate walls and its linear growth was thus restricted (Henis et al., 1965; Wheeler and Sharan, 1965). When *G. umbellata* and companion fungus grew in dual cultures and established contact, the linear growth was restricted essentially to the zone of antagonism and sclerotia were differentiated in abundance, but *G. umbellata* in monoculture, no sclerotia differentiation occurred at the edges of the Petri plates. This indicates that sclerotia induction not attribute to *G. umbellata* hyphal linear growth was restricted but the presence of the companion fungus. Morphogenetic processes in fungi are often stimulated by microorganisms or by their products (Bitancourt, 1951; Manning and Crossan, 1966). Bedi (1958) also discovered that the number of sclerotia formed by *S. sclerotiorum* significantly increased when staling products of other cultures or the same fungus were stored to the growth medium. The hyphal interaction between *G. umbellata* and companion fungus resulted in a dual organism recognition. In spite of the incapacity of the hyphae of companion fungus to invade *G. umbellata* cells, the diffusion of a chemical agent all over the growing media or interaction at the interface may affected the capacity of *G. umbellata* strain to differentiate sclerotia.

This study dealt essentially with morphological changes of hyphae in the hyphal interaction between *G. umbellata* and its companion fungus. Companion fungus seemed to be acting as a bio-controller capable to regulate the sclerotia differentiation of *G. umbellata*. The mechanism of sclerotial formation and the interaction with companion fungus certainly implied diverse process, which complicated the comprehension of the phenomenon. We expect to understand the mechanism of sclerotial formation clearly in the future.

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