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SUMMARY OF DOCTORAL THESIS

Name: Rudy Hermawan

Title: Study on hyphal bacteria from several strains of ectomycorrhizal mushroom and their effect on mycelial growth of host mushrooms

Bacteria are ubiquitous microorganisms present almost everywhere. They can live in fungal mycelia, such as exo-hyphal or endo-hyphal bacteria. The interaction between the fungal mycelium as the host and the bacterium as the endosymbiont is mostly reported as a mutualistic symbiosis. Fungal-bacterial endosymbiosis encompasses mutualistic symbiosis between a fungus as a host and intracellular bacterial species residing within the fungus. Endosymbiosis by a bacterium is widespread in fungi; however, it mostly comes from several reports of endosymbionts in the Zygomycota phylum and arbuscular mycorrhizal fungi. Few reports have been published on phylum Ascomycota and Basidiomycota. Endosymbiont bacteria have also been isolated as an isolated culture from fruiting bodies, not only from the mycelium. In this study, I explored the endosymbiont bacteria (exo- and endo-hyphal bacteria) from several mushroom strains belonging to ectomycorrhizal mushroom strains.

Endosymbiont-hyphal bacteria are defined as those that live outside (on the surface) or inside hyphal fungi. Bacteria that live on the hyphal surface are called exo-hyphal bacteria, whereas those that live inside the hyphae are called endo-hyphal bacteria. In some cases, bacteria can live as exo- or endo-hyphal bacteria. Similar to endo-hyphal bacteria, exo-hyphal bacteria can also penetrate hyphae. In the second section, bacteria (hyphal bacteria) were detected using SYTO staining. This study defined all mushroom species into three strain groups: Hyphae1, Hyphae2, and Hyphae 3. Strain Hyphae1 was the original hyphae from the deposited strains Strain Hyphae2 was strain Hyphae1, which was treated with exo-hyphal bacterial removal. The strain Hyphae2 was then treated with four kinds of antibiotics to produce strain Hyphae3. Several strains of Hyphae1 were successful in detecting the bacterial presence in the mycelium. Hyphae2 and Hyphae3 were also detected. Some mushroom species (*Laccaria bicolor* TUFC 101044, *L. amethystina* TUFC 100722, and *L. vinaceoavellanea* TUFC 101211) showed bacterial presence only in strain Hyphae1 and Hyphae2, not in the strain Hyphae3. In contrast, several mushroom species, such as *Rhizopogon roseolus* TUFC 10010, *Suillus bovinus* TUFC 31988, *S. granulatus* TUFC 31903, *Boletus bainiugan* TUFC 10018, *Russula bella* TUFC 101232, and *Amanita manginiana* TUFC 100797, revealed the presence of bacteria in Hyphae3. However, some species, such as *Lyophyllum semitale* TUFC 100632, *Ly. shimeji* TUFC 30169, *Boletus subtomentosus* TUFC 100970, *Psilothus arhizus* TUFC 101385, *Lactarius akahatsu* TUFC 101127, and *Hebeloma spoliatum* TUFC 100927, were not detected in the original hyphae (strain Hyphae1). The mycelial growth of strain Hyphae1, strain Hyphae2, and strain Hyphae3 were also significantly different. Three results were obtained from this experiment. Hyphae1 showed more vigorous growth than Hyphae3. The second was the reverse of the first response, as strain Hyphae3 showed more robust growth than strain Hyphae1. The third result showed no difference in growth between strain Hyphae1 and strain Hyphae3 (hyphal treatment showed no effect).

As described in another chapter, the experiment was continued to isolate and identify exo- and endo-hyphal bacteria. In this study, I isolated bacteria from the

fungal sources as strain Hyphae1 and strain Hyphae2 of each mushroom species. A total of 23 and 17 bacterial strains were found in Hyphae1 and Hyphae3, respectively. Bacterial strains were grouped and classified based on characteristic similarities in their morphologies. In total, 17 and 6 strains were selected as endo-hyphal and exo-hyphal bacteria, respectively. Identification was performed using a molecular approach. Genomic DNA was amplified using 16S rRNA genes with 27F and 1429R primers. A phylogenetic tree was constructed using the randomized axelarated maximum likelihood method. The most common endo-hyphal bacterium was *Paenibacillus chitinolyticus* (bacterial strains MBLb1a, MBLa2a, MBLa2b, MBGl4b, MBBb8a and MBRb12a). Then, other species as *Bacillus tequilensis* (strain MBRr6a) from *Rhizopogon roseolus*, *B. subtilis* (strain MBRr6b) from *R. roseolus*, *Kytococcus sedentarius* (strains MBLv13b and MBAm16b) from *Laccaria vinaceoavellanea* and *Amanita manginiana*, *Dietzia timorensis* (strain MBLv13a) from *L. vinaceoavellanea*, *Aneurinibacillus migulans* (strain MBGl4a) from *Gyrodon lividus*, and *B. pseudomycoides* (strain MBSg7b) from *S. granulatus* were the first record as new information as the endo-hyphal bacteria from some ectomycorrhizal mushroom strains. While two species of *Staphylococcus* such as *Staphylococcus warneri* (strain MBSb5a, MBSb5b and MBAm16a) and *S. pasteuri* (strain MBSb5c) were also found in this study. All the strains were Gram-positive bacteria. This indicates that they struggled with stress or harsh conditions during the cultivation or maintenance of the isolate.

As a final experiment, this study also investigated the role of the endo-hyphal bacteria on their host mushroom growth. In some cases, each member of a relationship benefits from symbiosis. The endo-hyphal bacteria are useful for activating host mushroom growth. Hence, mushroom strains can be used as model species to investigate the biological interactions with their endo-hyphal bacteria as symbionts. The investigation used two assays: a dual-culture assay and a bi-plate VOC assay. The dual-culture assay showed the different results for different media. However, some pairings between the bacterium and their host mushroom showed consistent results. The bi-plate VOC assay showed that all pairings stimulated mycelial growth, except for the pairing between *S. granulatus* and *B. pseudomycoides* strain MBSg7b. The potential pairing of host mushrooms and the bacteria came from pairing *R. roseolus* with the *B. tequilensis* strain MBRr6a and *R. roseolus* with *B. subtilis* strain MBRr6b. In contrast, the mycelial growth of strain MBRr6b paired with a non-host mushroom (*H. spoliatum* TUFC 100927) showed a reverse response. Significant stimulation of mycelial growth of *H. spoliatum* resulted from pairing with the *B. tequilensis* strain MBRr6a, *A. migulanus* strain MBGl4a, and *P. chitinolyticus* strain MBGl4b.

This study detected, isolated, and identified exo- and endo-hyphal bacteria in several strains of ectomycorrhizal mushrooms. Not only that, this study also provides the first record of endo-hyphal bacterial species from several strains of ectomycorrhizal mushrooms that have never been reported. In addition, the investigation of the effect of endo-hyphal bacteria on their host and non-host mushroom (*H. spoliatum*) mostly showed a stimulatory effect. Hence, I also proposed some potential bacteria, such as *B. tequilensis* strain MBRr6a, *B. subtilis* strain MBRr6b, and *B. pseudomycoides* strain MBSg7b, to continue the investigation in future experiments. I believe that the bacteria would also promote the mycelial growth of host and non-host mushrooms to make a fruiting body or biomass production. The other abilities of the bacteria that produce beneficial metabolites are also very attainable to enrich the nutrient in the mushroom fruiting or biomass. The mechanisms associated with these potential bacteria to promote the mushroom mycelial growth remain to be better understood and may lead to successful cultivation for other mushrooms (non-host mushrooms). Because of that, the future studies should investigate the relationship between the potential bacteria with ectomycorrhizal mushrooms (their non-host mushrooms).