（Format No. 13）

SUMMARY OF DOCTORAL THESIS

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Title: Study on ectomycorrhizal formation of *Pinus thunbergii* seedlings inoculated with edible mushroom, *Rhizopogon roseolus*: a three-dimensional analysis of cell structure and a comparative analysis of helper bacteria in symbiotic interactions
（食用きのこショウロを接種したクロマツにおける外生菌根形成に関する研究：細胞の３次元解析と共生関係におけるヘルパー細菌の比較解析）

Ectomycorrhizal (ECM) fungi are believed to have a beneficial impact on plant nutrition and growth by forming symbiotic associations with plant roots. ECM fungi are critical and important microbes for plant growth and survival because they can facilitate nutrient and water uptake. There are various kinds of ECM, including the basidiomycete *Rhizopogon roseolus* (Corda) Th. M. Fr. (=*R. rubescens* Tull. & Tul.), an edible ECM mushroom referred to as 'shoro' in Japanese, which is an important symbiont of *Pinus thunbergii*. However, this mushroom is difficult to cultivate. Studies on investigation cultivation techniques for boost yields are limited in *R. roseolus* fruiting bodies. A group of bacterial strains known mycorrhizal helper bacteria (MHB), which inhabit the mycorrhizospheres, play an important symbiotic role in promoting mycorrhiza formation. They have a positive interaction with the functional symbiosis, including improving the recognition process between roots and fungi, modifying the rhizosphere to make it more conducive for mycorrhizal infection, stimulating fungal growth before symbiosis, and inducing fungal spore germination. In the recent study, bacteria were isolated from fruiting bodies of *R. roseolus*, and showed stimulatory effect on mycelial growth as well as basidiospore germination. To date, few studies have examined bacteria from fruiting bodies of *R. roseolus*. Therefore, this study was carried out to reveal following aspects: 1) ectomycorrhizal microstructure using ImageJ software 3D image analysis 2) microscopic characterization and molecular identification of bacterial strains isolated from fruiting bodies, and evaluation of the stimulatory effects of bacteria on mycelial growth, through combined inoculation techniques under axenic conditions, and 3) investigation of the role of the bacteria on ectomycorrhizal formation and growth on *P. thunbergii* seedlings under diverse microcosms.

The ectomycorrhizal structure has been found to beneficial for the host plant. The Hartig net is believed to contribute to stimulate drought tolerance and grow in polluted soil in the plant roots. However, the intricate details of this ectomycorrhizal microstructure have not yet been fully examined. Therefore, in this study, I utilized three-dimensional (3D) image analysis to investigate the microstructure of the ectomycorrhizas formed in *P. thunbergii* roots inoculated with *R. roseolus*. When *P. thunbergii* seedlings were artificially inoculated with dichotomous *R. roseolus*, ectomycorrhizae appeared in seedling roots at four weeks after inoculation. These ectomycorrhizas examined by light microscopy. Using serial sections, 3D images of Hartig net cells were constructed. Results showed a highly branched structure, with certain cells common in both the mantle sheath and the Hartig net. The 3D image of the mantle sheath revealed that the outermost cells were cylindrical, and the innermost cells were irregularly shaped. Cell volume measurements from the 3D image analysis revealed that the single cell volume of the most exterior and interior mantle sheaths, and the Hartig net measured 365, 452, and 1,516 μm^3, respectively. Moreover, cell volume of the interior mantle cells was more variable than that of the exterior mantle cells. Despite extensive efforts to extrapolate fungal biomass, the details and volume of fungal cells in the ectomycorrhiza remain unclear, even though the structures have been recognized as important tissues in the symbiotic relationship. Compared to traditional methods of observations of microstructure under a light microscope, using the Fiji package; images software enabled us to easily quantify and clarify morphological changes in cell structures. Compared to scanning electron microscopy

(SEM), this package allows simple and easy observation of ECM fungal characteristics. This study is the first report on 3D analysis of cell volume in the mantle and Hartig net cells in ectomycorrhizas.

I also studied how bacterial strains promoted the development of mycorrhizal symbiosis by isolating certain bacteria from fruiting bodies of *R. roseolus* that stimulated mycelial growth. The bacterial strains were identified, characterized by SEM, and evaluated for stimulatory effects on mycelial growth through co-cultivation techniques under axenic conditions. Of the nineteen cultivable bacterial strains from the fruiting body of *R. roseolus*, six stimulated mycelial growth. BLAST analysis of these six strains revealed that they belonged to Proteobacteria, and they were identified as *Paraburkholderia fungorum*, *Caballeronia sordidicola*, *Janthinobacterium agaricidamnosum*, *Paraburkholderia caledonica*, *Novosphingobium rosa*, and *Rhodobacter azotoformans*. The remaining thirteen bacterial strains had either mildly negative effects or no effect on mycelial growth in *R. roseolus*. Previous studies also found slightly negative effects of some bacterial species, suggesting that competing for resources, e.g. nutrient, may promote antifungal effects. Furthermore, *Burkholderiaceae* proved to be the major group of bacteria, which promoted the mycelial growth of filamentous fungi. These bacteria are often found in environments conducive to fungal growth, indicating that they play a role related to the fruiting bodies of ectomycorrhizal mushrooms. Ultrastructural morphologies of the three strains were revealed by SEM observations and exerted a positive influence on mycelial growth in *R. roseolus* by the dual culture method with bacteria. However, the mechanism by which this occurs is not yet completely understood. The signal molecule associated with interaction between ECM and the bacteria will be require to establish a means of effectively cultivating ECM mushrooms in the future research.

The final portion of this study examined the effects of isolated bacteria from the *R. roseolus* fruiting body on *P. thunbergii* seedling growth and mycorrhization. Here, differing inoculation conditions with three strains of bacteria isolated from fruiting bodies; *P. fungorum* (GIB024), *C. sordidicola* (GIB028), and *J. agaricidamnosum* (GIB029) were investigated at three inoculation time treatments, viz., pre-bacterial inoculation, simultaneous inoculation, and post-bacterial inoculation and two bacterial concentrations, viz., low (5.0×10^6 CFU/ mL), and high (1×10^7 CFU/ mL) to determine the effect of mycorrhization on *P. thunbergii* roots and their growth after inoculation with the bacterial isolates in combination with *R. roseolus*. Mycorrhizal synthesis between *R. roseolus* and *P. thunbergii* seedlings was successful. The combined inoculation bacteria and *R. roseolus* showed significant stimulation of the thicker lateral roots and/or branching roots formations with a whitish coloration. Gradual increase in mycorrhization with time was found in *P. thunbergii* roots in the treatment of bacterial pre inoculation and simultaneous inoculation with *R. roseolus*. Post-bacterial inoculation of strain GIB029 after *R. roseolus* resulted in great variations between the *P. thunbergii* mycorrhizal formation after 1 month (39%) and 2 months (21%) of incubation. However, significant increase in mycorrhization at 2 months was observed in the pine seeding inoculated with GIB024 or GIB028 after *R. roseolus*. When low concentration of bacterial suspension was used as inoculum, mycorrhization at 2 months was decreased than that at 1 month. When high concentration of bacterial suspension was used as inoculum, mycorrhization at 2 months was increased that that at 1 month. Survival of introduced bacterium in the substrate after combined inoculation at 2 months rapidly decreased in all treatments. Seedling growth between control and other treatments after inoculation for 2 months showed no significant difference. These results indicate that effect of bacterial inoculation on mycorrhization was dependent on strain of bacteria and inoculation timing, and show that post bacterial inoculation with GIB024 or GIB028 after *R. roseolus* was effective to increase mycorrhization of *R. roseolus*.

This study revealed the 3D-structure of ECM cells, indicating that 3D construction method using Fiji package is useful to understand the interaction between host plant and ECM fungi. In addition, taxonomic and cytological study provided basic information on helper bacteria isolated from fruiting body of *R. roseolus*. A comprehensive study on the effects of bacterial colonization on ectomycorrhizal formation and the host pine growth could deepen our understanding of the functional significance and provided application ideal of bacteria in the establishment of ECM cultivation.