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)

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Chapter 1

General introduction

In the symbiotic association with plant roots, ectomycorrhizal (ECM) fungi have a useful influence with host plants such as *Fagaceae, Betulaceae, Salicaceae,* and *Pinaceae* (Smith and Read, 2008). ECM fungi colonize host-plant roots and gain nutrients and photosynthetic creations from their hosts through this symbiotic relationship (Simard et al., 1997; Teramoto et al., 2016). Obtaining nutrients like nitrogen and phosphorus from mineral and organic matter in soil via the exudation of various extracellular enzymes and allotting these nutrients with their hosts, ECM fungi expand hyphae from the ECM roots in response (Read et al., 2004). Accordingly, mycorrhizal fungi change the natural, biological and biochemical features of the surrounding soil and create a separate ecosystem called mycorrhizosphere in which microbial populations vary from rhizosphere populations and other areas of the soil (Izumi et al., 2006). ECM associations are distinguished by the presence of the mantle, a matrix of hyphae covering the root cells of the epidermis; the Hartig net, a system that results from the hyphal development of the intercellular spaces of the epidermis and the cortex; and a network of mycelial filaments mediating the connection of mycorrhizas to the soil and the fruiting bodies. The establishment of root hairs, which are functionally replaced by the fungal hyphae, is limited by the development of ectomycorrhizas. The secretion of indole composites by the fungus, such as indoleacetic acid and hypaphorine, responsible for controlling the root morphogenesis is involved in such limitation (Ditengou et al., 2000). ECM fungi also interact with soil microorganisms inhabited in the mycorrhizosphere as well as their symbiotic relations with host plants (Reddy and Satyanarayana, 2006). Through such complicated exchanges with host plants and other organisms for successful propagation under natural conditions, ECM fungi propagate mycelia and produce fruiting bodies.

Mycorrhizal helper bacteria interact physically and metabolically in their natural environment with soil bacterial populations that are useful, neutral or incompatible for the fungus. Specific focus has been given since the 80s to bacterial strains that aid the creation of ectomycorrhizal symbiosis, so-called mycorrhiza helper bacteria (MHB). Including the gram-negative belonged to phylum Proteobacteria, as *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas*, and *Rhizobium*. Gram-positive belonged to phylum Firmicutes, as *Bacillus*, *Brevibacillus*, and *Paenibacillus*, and phylum actinomycetes, as *Rhodococcus*, *Streptomyces*, and *Arthrobacter,* the roots of MHB recognized thus far originate from various groups and bacterial genera. Besides, MHB isolates have been inhabited from mycorrhizospheres, the substrate of extraradical hyphae, the fruiting bodies of ectomycorrhizal fungi, or as endobacteria in the cytoplasm, as described in literature (Frey-Klett et al., 2007; Bonfante and Anca, 2009)

MHBs are not plant-specific, but obviously selective about the fungal species, so the expression fungus-specific can be utilized (Garbaye, 1994). Only basidiomycetes were identified as interacting with MHB among ectomycorrhizal fungi (Frey-Klett et al., 2007). Studies have revealed the indirect beneficial effect of ectomycorrhizal symbiosis on the selective pressure of bacterial populations. Ectomycorrhizal symbiosis decides the composition of *Pseudomonas fluorescens* populations and selects the strains possibly useful for symbiosis and to the plant, as shown by Frey-Klett et al. (2005). At present, many MHB are thought to be plant growth promoting rhizobacteria (PGPR), including *Pseudomonas* sp. (Preston et al., 2001; Jaleel et al., 2007; Shilev et al., 2007). These classifications will intersect owing to the dominance of *Pseudomonas* and *Bacillus* in both classes, as reported by Fitter and Garbaye (1994). Analyses with PGPR commonly exclude the assessment of mycorrhization, which is a further factor that complicates the division of the two terms (PGPR and MHB) (Probanza et al., 2001). Still, it is worth observing that certain fungal signaling pathways are controlled by certain rhizobacteria, while some are similar to certain MHBs (Deveau et al., 2007).

Though not plant-specific, MHBs are fungus-specific. To investigate the specificity of the interaction between MHB and fungi as well as between MHB and the symbiont plant, various studies have been done, with dissimilar findings gained (Duponnois and Plenchette, 2003; Aspray et al., 2006b). As noted by Bending (2007), the production of the metabolite auxofuran by *Streptomyces* sp. AcH505 and its selective effect on the development of *Amanita muscaria* could support the proposition of specificity between certain MHB and mycorrhizal fungi due to being a specific interaction between the microorganisms.

Increased germination of fungal spores and/or survival, stimulus of the pre-symbiotic expansion of the mycelium, a boost in the receptivity to fungal signals of the short root, a stimulus of the root mycelium recognition and an alteration in the physicochemical properties that would aid in the mycorrhizal formation were included in the mechanism of action and functions of MHB (Arora and Riyaz-Ul-Hassan, 2019). The investigated factors were the possible underlying physiological mechanisms in terms of nutrient cycling, such as mineral uptake and the exchange of metabolites (Riedlinger et al., 2006; Uroz et al., 2007; Deveau et al., 2010), as well as molecular mechanisms were among the examined aspects such as observing changes in gene profiles (Becker et al., 1999; Schrey et al., 2005). Conversely, whether these findings were the result of individual micro-organisms or complex interactions was unknown. Afterward, it was shown that the microflora reaction of the soil for ectomycorrhiza was because of separate strains of bacteria (Oliveira and Garbaye, 1989).

Recently, several specific bacteria that were determined to promote basidiospore germination under high saline conditions were obtained from the fruiting body of *Rhizopogon roseolus*. Certain bacteria play a complementary protective role for the host fungus and/or host plant under biotic stress (Pakvaz and Soltani, 2016), assist in the activation of genes involved in the recognition processes, transcription activity, and production of the primary metabolism proteins (Deveau et al., 2010). Moreover, the bacteria help to control key host reproductive mechanism and are responsible for the synthesis of phytohormone

and phytotoxins such as rhizoxin production (Partida-Martinez and Hertweck, 2005; Hoffman et al., 2013; Mondo et al., 2017).

According to Frey-Klett et al. (2005), the physical and chemical interactions between ectomycorrhizal fungi and soil may dramatically alter the structure of bacterial species by choosing strains that may be potentially beneficial to symbiosis and plant growth. This study revealed that *Pseudomonas* communities in the symbiosis bacteria-fungus are quantitatively and qualitatively monitored, as the genetic diversity of cultivable *P. fluorescens* in the *Laccarius bicolor-Pseudotsuga menziesii* mycorrhizosphere was greater than in bulk soil. The majority of the mycorrhizosphere-isolated bacterial strain was able to solubilize inorganic phosphate. Further, this feature was not found in most soil bacteria. This capacity is likely to prefer plant growth in symbiosis. The mycorrhizosphere also yielded *P. fluorescens* isolates with antagonism against of phytopathogens antagonism than other isolates from the remainder of the soil. Suggesting symbiosis *L. bicolor*, this function was not selected by *P. menziesii*, there was no significant difference in the proportion of *P. fluorescens* capable of fixing nitrogen between mycorrhizosphere and bulk soil. This fact stands in contrast with the study by Rózycki et al. (1999), which resulted in an increase in the mycorrhizosphere of pine and oak in nitrogen fixing bacteria, primarily *Pseudomonas*. The existence of nitrogen fixing bacteria in various ectomycorrhizal interactions shows the potential of MHB to help in the nutrition of the related plant, as offered by Frey-Klett et al. (2007).

Helper bacteria in stimulating ectomycorrhizal involvement with plant symbionts, improved nutrition due to the degradation of complex poly-carbohydrates by lignocellulosic enzyme activity, the use of volatile organic compounds (VOCs) blocking mushroom fructification by bacteria and secretion of antibiotics to suppress competing fungi or offer defense against crop parasites comprise some of the advantages for fungi in forming mutualistic links with bacteria (Pudelko, 2014; Antunes et al., 2016; Vos et al., 2017; Pandin et al., 2017). Thus, improved dispersion and development on fungal exudates also helps bacteria (Warmink and Van Elsas, 2009). Still, fungi can also consume bacteria

and assimilate bacterial carbon and nitrogen as a means of nutrition (Vos et al., 2017; Kertesz and Thai, 2018).

According to Deveau et al. (2007) *Pseudomonas fluorescens* strain BBc6R8 was the only one that produced a survival, hyphae apex length, branching angle and radial development of *Laccaria bicolor* strain S238N. The morphological modifications were associated with changes in the transcriptome of *L. bicolor* that varied throughout the interaction. The authors reported that some responsive genes were partly unique to the association with *P. fluorescens* BBc6R8, which indicates specificity of the relationship between MHB and the mycobiont. Overall, the impact of MHB concerns fluctuations in the fungal anabolism and catabolism of lipids that could cause enhanced lipids production needed for greater fungal growth rates, as offered by existing information.

A transition from the saprophytic to the pre-symbiotic condition, the morphological variations of the mycelium in vitro may also be valuable to host root contamination by the fungus. Interestingly, not all bacterial strains are capable of inducing these improvements in the fungus. In fact, other mechanisms, not restricted to the increase of growth rate, are involved in the promotion of mycorrhization. Although some bacterial strains able to provoke variations in growth and morphology of ECM roots, while only MHB increased the development of the ECM colony and angle of branching at the pre-contact stage (Schrey et al., 2005). Much needs to be explained about the effects of the MHB-associated fungi activity. Valuable data indicating the degree of activity is that the MHB is capable of changing the mechanism.

Studies on ectomycorrhizal formation of *P. thunbergii* seedlings inoculated with *R. roseolus* may generate more interesting understanding of the activity between these organisms and the other components of the environment. Particularly, MHB research is important to promote knowledge of how integrated microbial communities induce mycorrhizal development. In *R. roseolus*, studies involving MHB remain limited, and those covering isolated bacteria from their fruiting bodies usually involve only their role in supporting plant growth without evaluating the impact on mycorrhizal production. 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.

Chapter 2

Three-dimensional analysis of ectomycorrhizas formed in *Pinus thunbergii* roots inoculated with *Rhizopogon roseolus*

2.1 Introduction

Rhizopogon is one of the large genera of hypogenous basidiomycotina. Its species are important ectomycorrhizal symbionts of native and introduced *Pinaceae* globally (Molina and Trappe, 1994). They form mantles surrounding the outer layer (epidermis) of the root and penetrate the cortical layers to form a Hartig net comprised of thick mycelium and intracellular hyphae (Brundrett et al., 1996). The ectomycorrhizal structure is beneficial for the host plant in that the Hartig net helps plant roots to tolerate drought and grow in polluted soil (Lee et al., 2000). However, the detailed microstructure of this ectomycorrhizal structure has not yet been fully revealed. The three-dimensional (3D) arrangement of plant anatomical structures is becoming an increasingly popular and powerful approach to investigate cell development; such investigations are typically conducted using microtomy techniques (Moreno et al., 2006; Bassel, 2015). For example, a cytological study in the Arabidopsis embryo revealed a previously undescribed mechanism governing 3D growth patterns in multicellular plant organs (Bassel et al., 2014). With an increase in the use of 3D image analyses, standards to ensure the accuracy and reproducibility of these data are important. New software has been necessary to visualize, process, and analyze this new type of data. ImageJ software with the Fiji plugin was therefore developed to permit 3D analysis. The Fiji plugin is a simple and easy-to-install ImageJ package that includes numerous plugins for 3D manipulation and visualization (Andrey and Boudier, 2010) In this study, the use of Fiji software to determine the detailed cellular microstructure of ectomycorrhizas formed in *Pinus thunbergii* roots inoculated with *R. roseolus* is described.

2.2 Materials and methods

2.2.1 Preparation of mycorrhizal seedling

Seeds of *P. thunbergii* were sterilized by washing in detergent for 30 min followed by rinsing in running water overnight. Disinfection was performed with 30% hydrogen peroxide for 20 min followed by two sterile distilled water rinses in a laminar flow chamber. The seeds were germinated in 90 mm circular Petri dishes with water agar at 25 °C in the dark. The vertical plate technique was employed with the following modifications. Under sterile conditions, a five-fold dilution of Modified Melin-Norkrans (⅕MMN) nutritive medium, modified and supplemented with micronutrients in order to obtain the following concentrations (in mg L^{-1}): KH_2PO_4, 100; $CaCl_2.2H_2O$, 10; $(NH_4)_2HPO_4$, 200; $MgSO_4.7H_2O$, 40; $FeCl_3$, 2.4; NaCl, 5; $C_6H_{12}O_6$, 2000; malt extract 1000; Thiamine, 0.02. Note that 15 g L^{-1} of agar were added after pH level adjustments (5.8 ± 0.05) in each case. Each vertical plate (90 mm diameter × 1.5 mm) contained 30 mL of solidified media with half removed to create a headspace for the seedling shoot. Following germination, 14-day old seedlings of *P. thunbergii* were transferred carefully onto the half-agar plates such that each plate contained only 1 seedling at the center of the cutting surface. The seedlings were incubated in a growth chamber at 25 ± 1 °C under a 16 h photoperiod with a light intensity of 2,000 lux provided by cool-white fluorescent lamps.

After 1-month incubation, the roots of the seedlings were inoculated with *R. roseolus* (TUFC10010). For inoculation into vertical plates, *R. roseolus* cultures were grown in ⅕MMN agar at 25°C for 3 weeks. The fungal inoculum consisted of 4 plugs with a dimeter of 5 mm cut from the growing margin of the colony. The disks were placed upside-down along each side of the root. The Petri dishes were sealed with Parafilm®. Three plates per treatment were set upside down at an angle of approximately 80° so that the root grew down against the lid. Seedlings were harvested at around 30 days to observe ectomycorrhizal formation.

2.2.2 Preparation for microscopic specimen

The roots of pine seedlings were aseptically inoculated with *R. roseolus* mycelia. The resulting ectomycorrhizas were fixed with 2% glutaraldehyde, dehydrated in a graded ethanol series, transferred to propylene oxide, and embedded in Spurr's resin. Light microscopic observations were performed using serial sections obtained using a diamond knife on an MT-7000 ultramicrotome (RMC, Tucson, AZ, USA). The thickness of one section was 0.5 μm, and the total depth was 20 μm. The ectomycorrhizal sections were stained with 0.5% toluidine blue O in 1% sodium borate. The stained sections were observed using an Eclipse 80i light microscope (Nikon, Tokyo, Japan), and cell images were captured with a DS-L2 digital camera (Nikon, Tokyo, Japan).

2.2.3 Image acquisition and analysis

The workflow employed for modeling the 3D microstructure of the ectomycorrhizal cells from serial images of cross sections and integrative cell visualization processes is shown in Fig. 2.1. The images were then batch processed and structure-volume measurements were performed using the ImageJ 3D viewer plugin in Fiji to analyze and visualize the 3D biological images.

In the processing step, the size of the image depended on the size of the sections, but ranged typically from 1280 × 960 pixels. First, as shown in Fig. 2.1A, images were processed by inputting serial images and setting characterization parameters. The images were then subjected to a rigid-body registration process to register each image to the previous image of the block (StackReg). Secondly, as shown in Fig. 2.1B, separation of foreground and background was performed for every stack image. The root cells and mycelial hyphae were selected and colored by inversion. Thirdly, as shown in Fig. 2.1C, in the segmentation step, split channel and image threshold were used. The contours of the structures of interest are represented in white. Despeckling was performed to reduce noises and the images were saved in TIFF format and grouped as serial images. Fourthly, as shown in Fig. 2.1D, the registered output from the ImageJ 3D viewer plugin was used to visualize volume data, orthogonal slices, and surface at the same time.

Transparencies were adjusted between the various representations. Lastly, the 3D visualization was animated, recorded and saved as AVI movies.

2.3 Results

When *P. thunbergii* seedlings were artificially inoculated with *R. roseolus*, dichotomous ectomycorrhizas were apparent in the roots of seedling at 4 weeks after inoculation. The resulting ectomycorrhizas were used to prepare specimens for observation under a light microscope. The proximal regions of dichotomous ectomycorrhizas were subjected to microscopic observation in this study. Figures 2.2-29 show the serial semi-thin section images of an ectomycorrhizal, with individual Hartig net cells colored green.

Using the serial sections, 3D images of Hartig net cells were constructed. The Hartig net cells were highly branched, and certain cells were common in the mantle sheath and the Hartig net (Fig. 2.30A). A 3D image of mantle cells revealed that the outermost mantle cells were cylindrical and the innermost mantle cells were irregularly shaped (Fig. 2.30B). Measurement of cell volumes using the 3D images revealed that the volumes of a single cell in the outermost mantle sheath, in the innermost mantle sheath, and in the Hartig net were 365, 452, and 1,516 μm^3, respectively (Fig. 2.31). The cell volume of the Hartig net was approximately 3-4 times larger than that of the mantle sheath cells. The cell volume of innermost mantle was more variable than that of outer mantle.

2.4 Discussion

Despite efforts to extrapolate fungal biomass in ectomycorrhiza, the detail and volume of fungal cells in the ectomycorrhiza has remained unclear, even though the structures have been recognized as important tissues in the symbiotic relationship (Stögmann et al., 2013; Balestrini and Bonfante, 2014). Compared to traditional methods involving mere observations of microstructure under a light microscope, using the Fiji package enabled us to easily quantify and clarify the morphological changes in cell structures. Compared to scanning electron microscopy, this method provides a simpler alternative to observe these fungal

characteristics. This is the first report to describe the 3D analysis of the cell volume in the outermost mantle cells, the innermost mantle cells, and Hartig net cells in ectomycorrhizas.

The 3D ultrastructure of organisms has recently been examined by array tomography (Wacker and Schroeder, 2013). The approach is a relatively straightforward and powerful method that relates molecular details to ultrastructure. Ultrathin sections are stained with fluorophores and then imaged by light, followed by electron microscopy to obtain a correlated view of the ultrastructure and specific staining of the region of interest. Ultrastructural features of ectomycorrhizas aseptically synthesized between *R. roseolus* and *P. thunbergii* have been described using transmission electron microscopy (Shimomura et al., 2010). However, to date, no information is available on the 3D ultrastructure of ectomycorrhizas. These findings are believed to useful for deepening our understanding of the function of symbiotic cells, such as the molecular exchange between plant and fungi. Future cytological studies should examine the 3D ultrastructure of the Hartig net and mantle cells using array tomography.

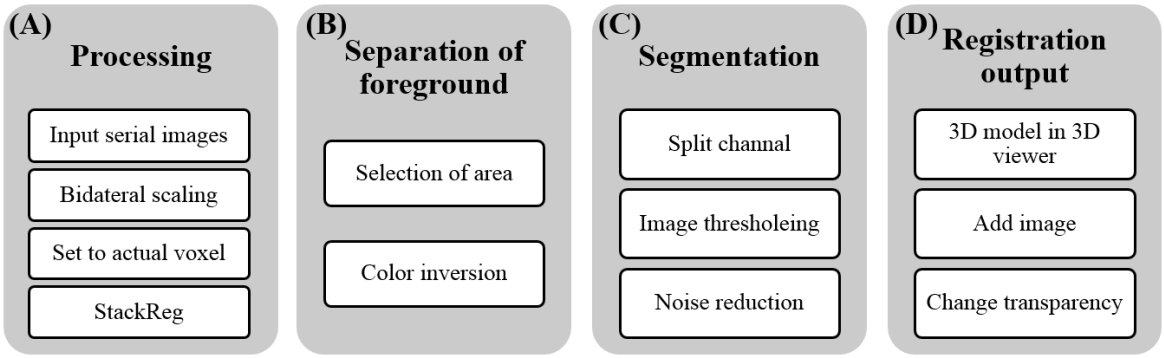
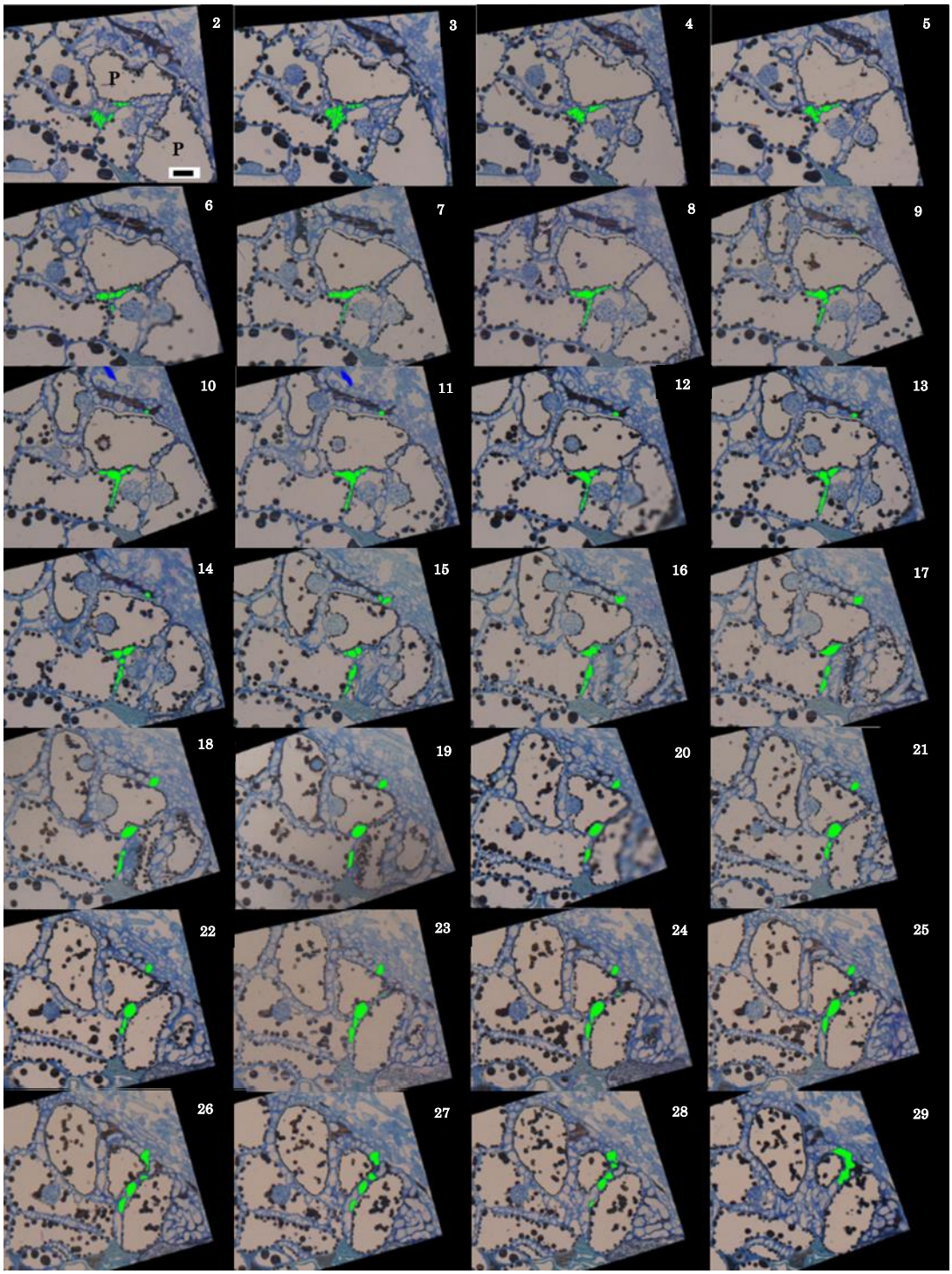


Fig. 2.1 Basic workflow of 3D ectomycorrhizal cell structure modeling using Fiji software



Figs. 2.2-2.29 Serial cross sections of ectomycorrhizal roots of *Pinus thunbergii* inoculated with *Rhizopogon roseolus*. Green: mycelial cytoplasm. P: plant cell. Bar: 10 μm

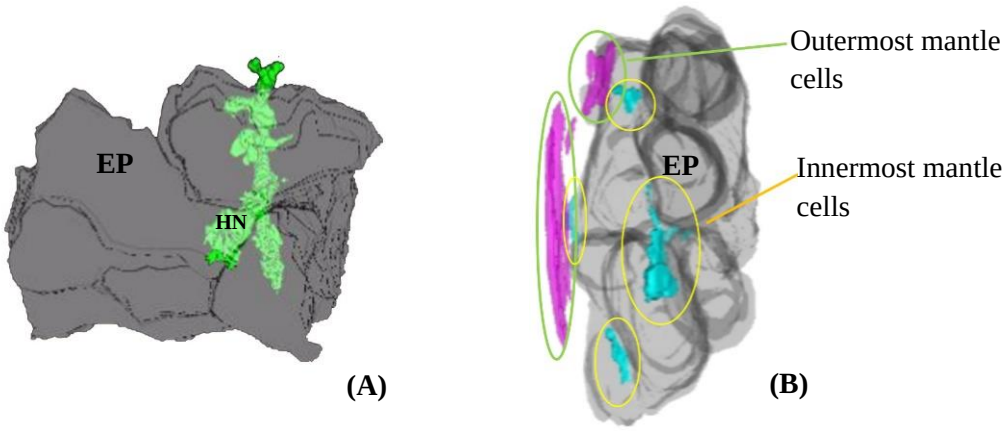


Fig. 2.30 Estimated 3D image of ectomycorrhizal cells of *Pinus thunbergii* inoculated with *Rhizopogon roseolus*. (A) Highly branched Hartig net (HN) cell penetrated into the root epidermis; (B) Outermost mantle cells and innermost mantle cells. EP: plant epidermis cell.

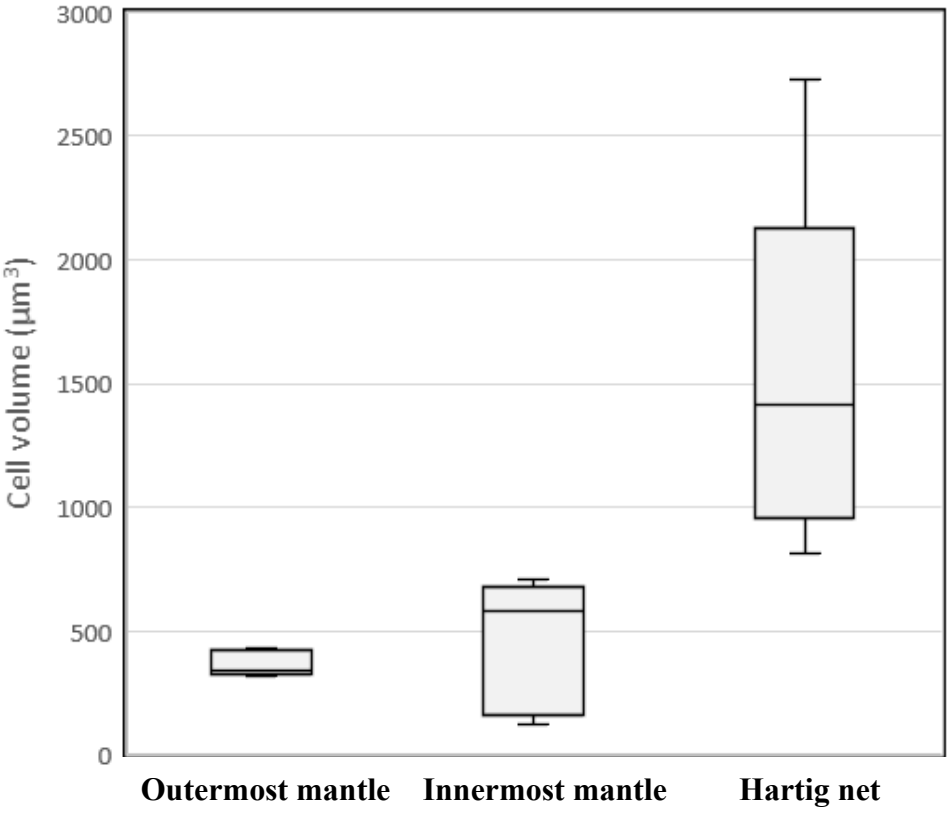


Fig. 2.31 The cell volume of the outermost mantle, the innermost mantle, and Hartig net observed and quantified using the method described in this study.

Chapter 3

Isolation of bacteria from fruiting bodies of *Rhizopogon roseolus* and their effect on mycelial growth of host mushroom

3.1 Introduction

One of the symbionts of *Pinus thunbergii* is *Rhizopogon roseolus* (Corda) Th. M. Fr., an ectomycorrhizal mushroom (ECM). The *P. thunbergii* tree can be found along the Japanese coast, and it plays host to the edible mushroom, referred to as 'shoro' in Japanese (Molina and Trappe, 1994; Shimomura et al., 2012). This mushroom is traded at a high price ($350/Kg) in local Japanese markets. However, it has not been feasible to cultivate this mushroom. Studies investigating cultivation techniques or strategies to boost yields are limited in the context of *R. roseolus* fruiting bodies.

On the other hand, *R. roseolus* mushrooms are found in the sandy soil of the black pine forest in seashore habitats. Coastal areas often have high salt levels along the shoreline, which restricts the growth of the fungus (Dixon et al., 1993). Kernaghan et al. (2002) observed that 100 mM and 200 mM sodium chloride can inhibit *R. roseolus* growth completely. Therefore, the salt-tolerant trait has been desired for a better breeding strategy in the case of mushrooms (Nakano et al., 2015; Gao et al., 2017). We isolated homokaryotic strains from basidiospore suspensions from fruiting bodies, because they are essential to construct heterokaryotic strains by cross breeding. During our breeding study, we accidentally identified interesting bacteria that stimulate basidiospore germination and subsequent colony formation in this mushroom (unpublished).

In this study, bacterial strains from fruiting bodies were isolated, evaluated for stimulatory effects on mycelia growth through co-cultivation techniques under axenic conditions, microscopically characterized, and taxonomically identified.

Furthermore, the roles of the bacteria in mushroom development and utilization for cultivation were discussed.

3.2 Materials and methods

3.2.1 Mushroom strain used in this study

R. roseolus, ectomycorrhizal fungus strain TUFC10010, a reference strain from the Fungal/Mushroom Resource and Research Center at Tottori University, was used in this study. Inoculation of the strain was plated out 5 times, diluted with Modified Melin-Norkrans agar medium (⅕MMN), and incubated at 25°C. The mycelium was maintained by sub-culturing every month.

3.2.2 Sampling and isolation of bacteria

Bacteria isolated from the fruiting bodies of *R. roseolus* were collected from 2 locations in Japan: The Arid Land Research Center, Tottori University in Tottori (N35° 32' 1.6", E134° 12' 58.5") and a public plantation in Ishikawa (N36° 52' 6.3", E136° 45' 35.9"). The fruiting body surfaces were carefully cleaned with 70% ethanol to remove the soil. Bacteria were then isolated from the gleba of *R. roseolus*, diluted in 15 mL of sterile distilled water, and pulverized with sterile forceps. The separation of spores and bacteria from the gleba tissue was performed using a 100 µm mesh cell strainer (Corning®; USA). Next, 100 µL of spore suspensions were spread on Petri dishes containing Potato dextrose agar (PDA, Nihon Seiyaku) and ⅕MMN agar, and incubated at 25°C for 4-7 days. To obtain a pure culture, each isolate was sub-cultured onto PDA and ⅕MMN agar, and colonies were grouped according to their morphology, growth rate, and color. The single representatives of the groups were picked off using a sterile loop and sub-cultured onto a PDA and ⅕MMN agar medium. A total of 19 strains were selected and maintained at 4°C in a sterile storage system.

3.2.3 Screening of isolated bacteria and their effect on ECM mycelial growth under axenic conditions

A total of 19 bacterial strains, obtained from the fruiting bodies of *R. roseolus*, were used for single plate co-culture experiments. The *R. roseolus* strain (TUFC10010) was incubated on ⅕MMN agar medium for 1 month at 25°C. Mycelial disks containing growing mycelia were cut from the edge of the fungal colony using a 5-mm cork borer. Each disk was placed individually on the center of a 9-cm plastic plate containing 20 mL of ⅕MMN agar medium without glucose, and incubated for 1 week. Bacterial cells of each strain, were then incubated on ⅕MMN agar medium for 2 weeks and one loopful of bacteria was streaked following a circular path, 25 mm away from the margins of the fungal colony (Fig. 3.1). Four replications were prepared in each treatment and the plates were incubated at 25°C in the dark. The radii of mycelial colonies on each plate were then measured daily for 5 days and the daily average of the four-directional radii was calculated. In the control, no bacterial cells were applied to the culture plates.

3.2.4 DNA extraction of bacteria and BLAST algorithm

Total bacterial DNA was extracted using a bead-beating method according to Fujimoto et al. (2004) The bacterial suspension was then washed with Tris-EDTA (TE) buffer pH 8.0 and bead-beaten at the set speed of 680 G for 30 s. The PCR premix containing 1 µL of extracted DNA, 1 µL small subunit ribosomal DNA (16S rDNA) using the primers 8F (5'- AGAGTTTGATCCTGGCTCAG)-3'), 1492R (5'- GGTTACCTTGTTACGACTT -3') and 16 µL of distilled water were used for amplification. PCR products were checked using 1% agarose gel electrophoresis and purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The sequencing reaction was performed by Eurofin Genomics (Tokyo, Japan). Sequences were edited and analyzed with Genetyx® (Japan) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Bacterial strain identifications were used with the BLAST algorithm to search for homologous sequences in the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences generated from the analysis

were deposited in GenBank under the accession number NR114134.1. Cluster analysis with bacterial sample units was performed using the TreeDyn online software using a presence binary table generating a dendrogram by the neighbor-joining method.

3.2.5 Scanning electron microscope (SEM) analysis

For SEM images, the bacterial sample preparation was grown in a ⅕MMN agar medium and incubated for 5 days. Bacterial samples were fixed in 1% osmium tetroxide at 25°C for 2 h. After loading approximately 100 µL of the bacterial sample onto the nano-percolator filter, and attaching the 10 mL syringe to the filter holder, specimens were gently sucked through the filter and dehydrated using an ethanol series and *t*-butyl alcohol. After substitution to *t*-butyl alcohol at -4°C, specimens were dried using a freeze-drier for approximately 3 h. Platinum coating of dried samples was carried out using a Hitachi E1045 ion sputter and examined using a Hitachi SU8020 field-emission scanning electron microscope.

3.2.6 Data analysis

The data obtained were initially tested for normality and homogeneity of variance and two-way ANOVA was performed for each dependent variable versus the independent variables. Duncan's multiple range Test (DMRT) was undertaken to determine any significant differences in mycelial growth between the groups. The differences between means were considered to be statistically significant at a 5% confidence level ($p<0.05$).

3.3 Results

3.3.1 Screening of bacteria and their effect on *R. roseolus* mycelial growth

When the basidiospore suspension of *R. roseolus* was incubated in agar media containing NaCl, colony formation of the basidiospore was significantly inhibited. However, colony formation of basidiospores was stimulated by contamination of certain bacteria, while no bacterial colony was observed. Figure 3.2 shows the effect of bacterial contamination on the colony formation of basidiospores on medium containing NaCl at 300 mM. Interestingly, mycelial growth of *R. roseolus* was stimulated when the mycelium was artificially co-cultured with the bacteria. Therefore, the bacteria from fruiting bodies of *R. roseolus* were isolated, and their stimulatory effect on mycelial growth were evaluated through co-cultivation techniques under axenic conditions. A total of 19 cultivable bacterial strains were isolated from *R. roseolus* fruiting bodies from a single plate co-culture experiment. Thirteen bacterial strains had a significant neutral effect on mycelial growth, while 6 bacterial strains significantly stimulated the mycelial growth ($p < 0.0001$), in comparison to the control (Fig. 3.3). The bacterial strains exhibiting stimulation on *R. roseolus* mycelial growth were identified as GIB024, GIB028, GIB029, KN1, ALRC1 and ALRC6.

3.3.2 Isolation and identification of bacteria

A total of 19 cultivable bacterial strains were obtained from 44 fruiting bodies of *R. roseolus*. Six bacterial strains enhanced the growth of *R. roseolus* strain TUFC10010. A molecular method was then employed to corroborate the bacterial strains based on 16S rDNA gene sequences. Blast results revealed that the bacterial strains GIB024, GIB028, GIB029, KN1 and ALRC1 were 99% identical to *Paraburkholderia fungorum*, *Caballeronia sordidicola*, *Janthinobacterium agaricidamnosum*, *Paraburkholderia caledonica* and *Novosphingobium rosa*, respectively, whereas ALRC6 was 73% identical to *Rhodobacter azotoformans* (Table 3.1).

16S rDNA sequences analysis for six bacterial isolates revealed a total of 6 different species of bacteria. Figure 3.4 shows a dendrogram of similarity comparing the ribosomal banding profiles. Most of the 16S rDNA sequences had a high sequence similarity to the species identified above and the final identification was assigned based on the phylogenetic tree. All of the bacterial species were found to belong to Proteobacteria.

3.3.3 Morphological analysis by scanning electron microscopy

The morphologies of 3 strains of the dominant bacteria isolated from fruiting bodies of *R. roseolus* that had a significant effect on promoting mycelial growth were examined using SEM (Fig. 3.5). The cells of strain GIB024 were rod-shaped, with a length and width of 0.9–1.5 × 0.3–0.4 μm respectively (Fig. 3.5 A and B). The cells of strain GIB028 were rod-shaped, with a length and width of 0.7-1.5 × 0.3-0.5 μm (Fig. 3.5 C and D). The median length of the cells of strain GIB028 was the shortest. The GIB029 cells were also rod-shaped, with a length and width of 1.5–3.5 × 0.3–0.5 μm (Fig. 3.5 E and F). The median length of the cells of strain GIB029 was the longest.

3.4 Discussion

The findings in this study show that the bacterial strains linked to the ECM mushroom fruiting bodies of *R. roseolus* observed within a pine forest of semi-natural origins were able to promote mycelial growth under axenic conditions. The capacity to stimulate ECM mycelial growth may prove to be a significant characteristic of the isolated bacteria. The work of Cruz and Ishii (2012) revealed that one probable bacterial type isolated from *Gigaspora margarita* spores, which was able to enhance hyphal growth, is *Paenibacillus rhizosphaerae*. This bacterium is likely responsible for hyphal growth, as indicated in studies in vitro and in vivo (Horii and Ishii, 2006; Fernández et al., 2016). This finding adds to the list of beneficial bacteria that have been isolated from fruiting bodies. Prior research has indicated that the bacteria linked to the growth of mushrooms will also have a positive effect upon the physiology of the mushrooms (Bowen and

Theodorou, 1979; Frey-Klett et al., 2007). Oh et al. (2018) reported that the growth-promoting bacteria that were isolated from the fruiting bodies of *Tricholoma matsutake* belonged to the genera *Ewingella, Dietzia, Paenibacillus, Pseudomonas, and Rhodococcus*. These isolated bacteria were shown to be neutral in terms of mycelial growth, which differs from the findings in the current research in which six bacterial strains did serve to enhance mycelial growth. Rangel-Castro et al. (Rangel-Castro et al., 2002) reported that the bacteria might be making use of the nutrients obtained from the fungi, including sugars such as trehalose and mannitol, or amino acids, rather than destroying the cell walls of the fungus. Furthermore, growth-promoting bacteria have positive interactions, which can generate compounds including auxofuran, thiamine, ketones, and methylketones (Weise et al., 2012). A majority of the bacteria, however, had either mildly negative or no effects on mycelial growth in *R. roseolus*. A number of previous studies also found slightly negative effects of certain bacterial species, arguing that in competing for resources, they have antifungal effects (de Boer et al., 2005; Zarei et al., 2011; Nimaichand et al., 2013).

During this research, I identified an interesting bacterial strain, ALRC6, which was 73% identical to *Rhodobacter azotoformans*, implying a new bacterial species. However, further taxonomical experiments will be required to confirm the occurrence of a new species involved with mushroom propagation, because there was no positive evidence in our study. On the other hand, *Burkholderiaceae* proved to be the dominant bacterial group, comprising the *Paraburkholderia*, *Caballeronia* and *Janthinobacterium*, which were isolated from the *R. roseolus* fruiting bodies. These bacteria are often found in environments conducive to fungal growth (Stopnisek et al., 2016), indicating that these bacteria are likely to play a specific role related to the fruiting bodies of ectomycorrhizal mushrooms. Furthermore, they may have developed traits that are ideal for hyphal dominant habitats at the genus level. While a majority of the principal bacteria are from the same family, it was shown in our study that those strains from the *Burkholderiaceae* family are renowned for their production of a variety of secondary metabolites. Although these are not essential in the reproduction or

growth of microbes, they have a number of critical survival functions in the natural environment (Martín et al., 2005). It was revealed that the effects of bacteria on mycorrhiza formation in plants result from the effects of the volatile compounds produced by the bacteria (Tylka et al., 1991). The role played by the bacteria may be indirect, in detoxifying the culture medium for fungal growth (Duponnois and Garbaye, 1990); this lowers the restriction upon hyphal growth, which is caused by the build-up in the medium of fungal biosynthetic metabolites (Levy et al., 2003). There is no clear evidence that *R. roseolus* and the bacteria of the Burkholderiales family have any kind of symbiotic relationship, but it is possible that the bacterial access to fungal hyphae serves to enable movement into the fruiting body. *Burkholderia* is considered to be typically a free-living specie, and this might allow the bacterial communities within the soil to play a role in the make-up of bacterial communities living inside the fruiting bodies (Antony-Babu et al., 2014). However, the uptake of bacteria from the environment is comparatively low in the case of mycorrhizal fungi.

On the other hand, bacteria that limit the mycorrhizosphere and promote ectomycorrhizal formation are known as 'mycorrhiza helper bacteria' (MHB) (Garbaye, 1994; Schrey et al., 2012). This description was later supported by Frey-Klett et al. (2007) who noted that the advantages conferred upon ECM by MHB serve to not only aid mycorrhizal formation, but also lead to positive interactions that support existing symbiosis. The main functional categories are represented by different microbial groups, but MHB can be used as a term to describe the activities of all types.

Studies involving ECM have shown that the stimulation of fungi is the principal consequence of MHB activity (Rigamonte et al., 2010). A number of different bacterial types have been observed in the fruiting bodies of the mushroom, and these have been demonstrated to exert no adverse effect upon mushroom growth (Mano and Morisaki, 2008). Furthermore, they assist in supporting acclimatization to salt stress. MHBs have the ability to colonize the hyphal surface or the cytoplasm, which enables them to alter the structure of the

mycorrhizas or aid its formation (Frey-Klett et al., 2011; Dhawi, 2016). Bacteria that serve as useful mycorrhizal associates are of diverse types and include *Pseudomonas* spp., *Paenibacillus* spp., *Burkholderia* spp., and *Streptomyces* spp. These types are understood to be widely distributed (Poole et ai., 2001). During the growth phase, interaction with plants is helpful in producing better quality of nutrition from the fruit and the leaves through triggering secondary metabolite production. These play a key role as plants interact with their surroundings, and can also provide protection against herbivores and other pathogens, while various environmental stresses are also negated (Jefferies et al., 2003; Toussaint et al., 2007; Akula and Ravishankar, 2011; Frey-Klett et al., 2011). This study, therefore, sought to isolate those bacteria that promote growth for the purposes of their identification. However, there is no evidence regarding the effect of bacteria isolated in this study on ECM formation of *R. roseolus*. If a positive effect of the bacteria was detected on ECM formation with host pine tree root, the bacteria observed in this study could be categorized as MHB. Therefore, further research on the effect of the bacteria on ECM formation is required.

It can be concluded that there is a positive influence exerted upon mycelial growth in *R. roseolus* by size of the examined species of bacteria. However, the mechanisms by which this occurs are not completely understood, while some of the metabolites between ECM and the bacteria must be further investigated in order to establish a means of effectively cultivating ECM mushrooms. It is also necessary to establish the role played by the bacteria in the formation of ECM mushrooms as well as the influence upon the symbiotic relationship with the host plant, particularly in terms of plant growth and the uptake of nutrients under axenic and natural stress-inducing conditions, e.g., salt stress. In addition, the development of bacterial strains will also be significant in order to improve the future potential for the effective cultivation of ECM mushrooms.

Table 3.1 BLAST results of six bacteria isolated from fruiting bodies of *Rhizopogon roseolus*.

Strain code	Reference taxon (strain)	NCBI accession numbers	Query coverage	Identity
GIB024	*Paraburkholderia fungorum* (NBRC 102489)	NR114118	100%	99%
GIB028	*Caballeronia sordidicola* (S5-B)	NR104563	100%	99%
GIB029	*Janthinobacterium agaricidamnosum* (NBRC 102515)	NR114134	100%	99%
KN1	*Paraburkholderia caledonica* (NBRC 102488)	NR114117	100%	99%
ALRC1	*Novosphingobium rosa* (NBRC 15208)	NR104206	99%	99%
ALRC6	*Rhodobacter azotoformans* (JCM 9340)	NR113300	80%	73%

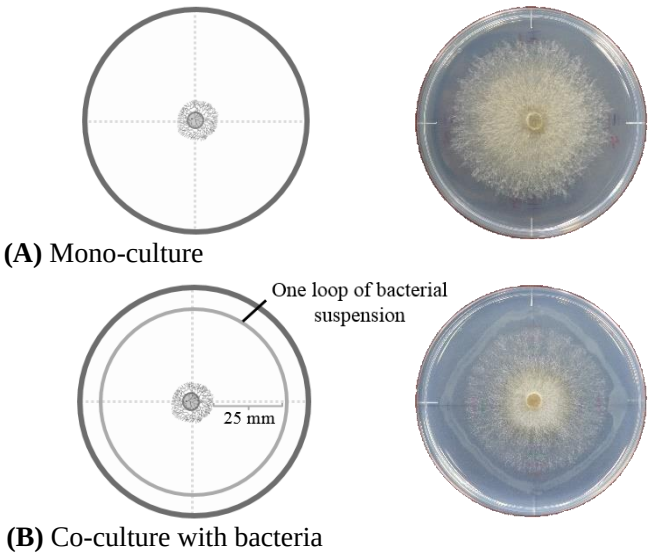


Fig. 3.1 Experimental design for evaluation of effect of bacteria on mycelial growth of *Rhizopogon roseolus.* (A) Mono-culture experiment of *Rhizopogon roseolus* mycelia without bacteria. (B) Co-culture experiment of *Rhizopogon roseolus* mycelia with bacteria. One loopful of bacterial suspension was streaked following a circular path, 25 mm away from the margins of the fungal colony. After incubation, rates of mycelial growth of *Rhizopogon roseolus* were compared.

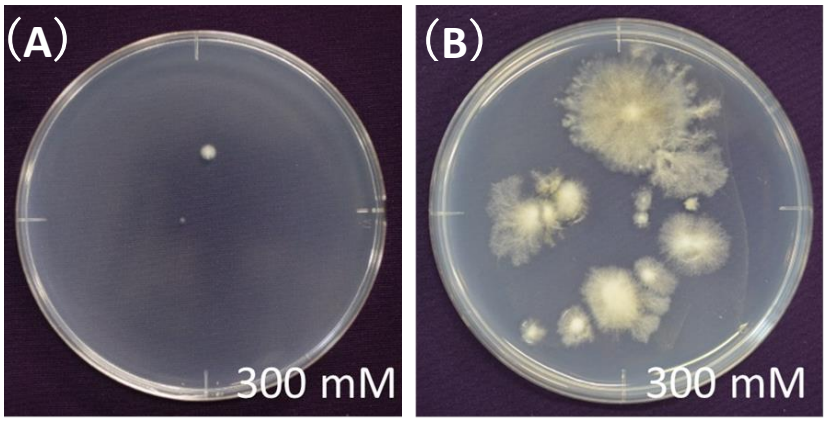


Fig. 3.2 Effects of bacterial contamination on colony formation of *Rhizopogon roseolus* basidiospores growing on agar medium with an NaCl at a concentration of 300 mM.
(A) Without bacterial contamination. (B) Bacterial contamination.

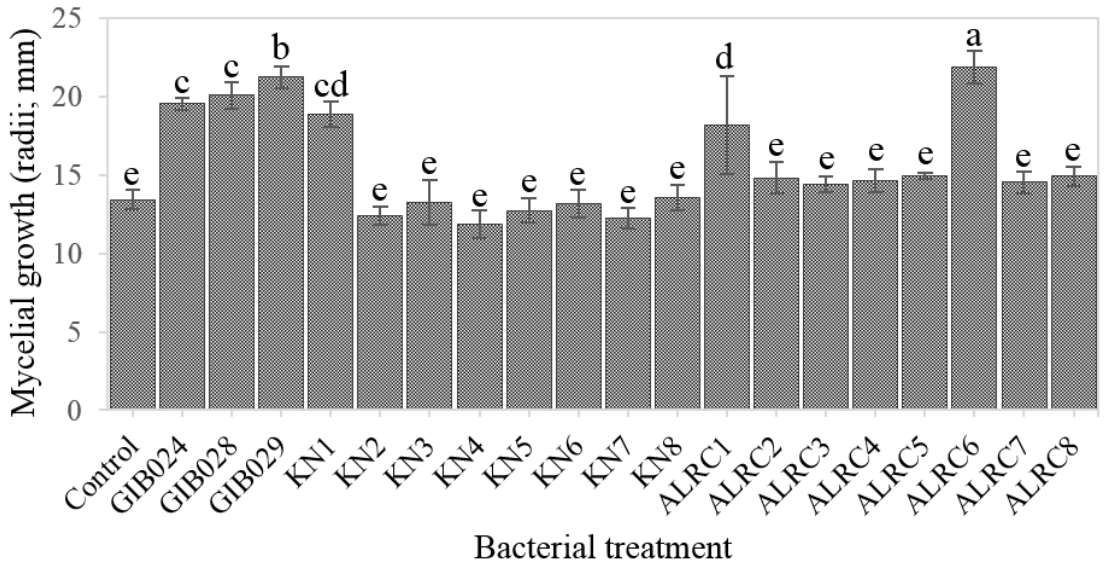


Fig. 3.3 Effects of bacteria on growth of *Rhizopogon roseolus* mycelium. Each bar represents mean ± SE. Columns with different letters differed significantly according to DMRT at $p < 0.05$. n = 8.

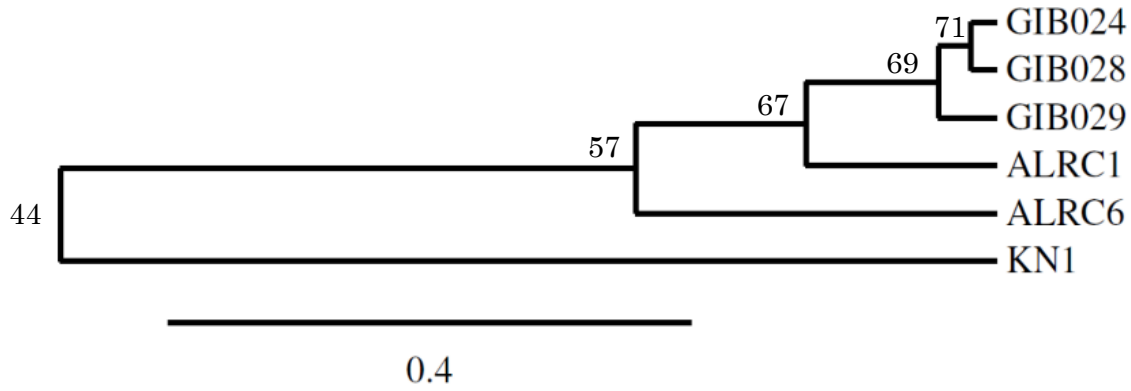


Fig. 3.4 Cluster analysis using dendrogram neighbor-joining method, based on 16S rDNA gene sequences of bacterial communities from endophytic bacteria of fruiting bodies of *Rhizopogon roseolus*. The scale bar indicates the percentage of similarity. Numbers at nodes represent the cophenetic correlations coefficient values, which estimate the consistency of each sub-cluster.

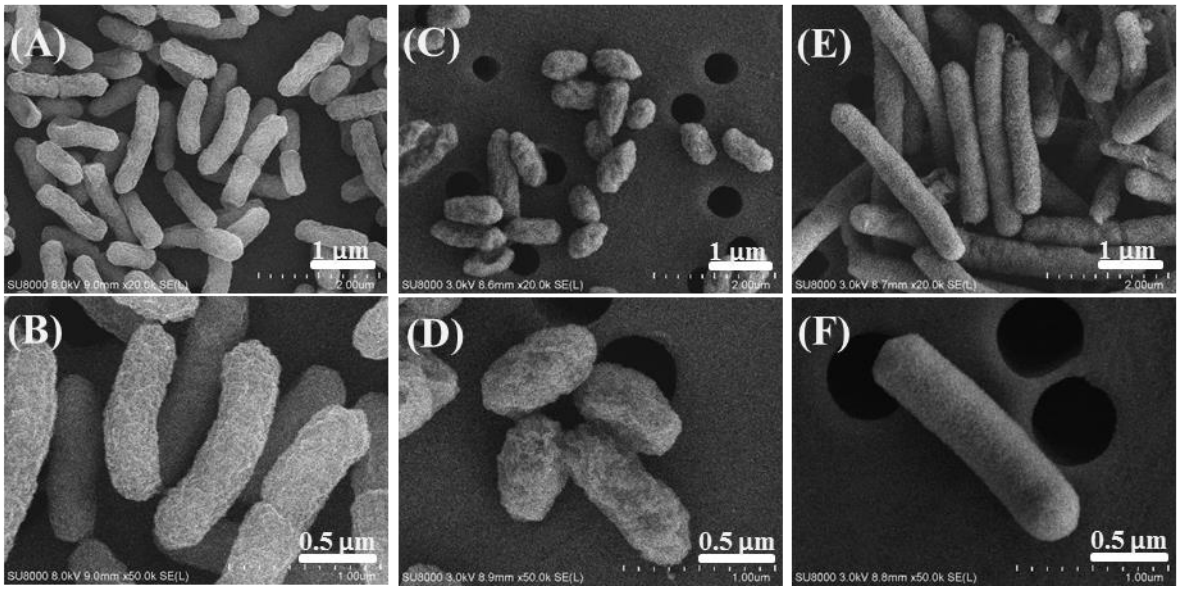


Fig. 3.5 Morphological analyses by scanning electron microscopy of bacteria isolated from fruiting bodies of *Rhizopogon roseolus*. Bacterial strains: GIB024 (A and B), GIB028 (C and D) and GIB029 (E and F).

Chapter 4

Effects of bacteria isolated from the *Rhizopogon roseolus* fruiting body on *Pinus thunbergii* seedling growth and its mycorrhization

4.1 Introduction

Ectomycorrhizal (ECM) fungi are important symbiotic partners of tree roots and are ubiquitous in forest systems (Smith and Read, 2008). Several bacteria associated with ECM fungi play a significant role in the symbiotic relationship (Cumming et al., 2015). Bacteria are widespread colonizers in ectomycorrhizal structures and occur on the mycorrhizal mantle and Hartig net (Nurmiaho-Lassila et al., 1997). Recently, functions of culturable bacteria from the mycorrhizosphere have been examined for several bacterial strains including members of *Pseudomonads* and *Bacillales*. Some of these bacteria have been found to affect fungal spore germination, hyphal growth, colonization of roots, and development of ECM symbiosis (Deveau, and Labbé, 2016; Oh and Lim, 2018), they have been termed mycorrhization helper bacteria (MHB) (Garbaye, 1994; Schrey et al., 2012). ECM fungi do not appear to be the main factor influencing the mycorrhizosphere bacterial community but may contribute to the creation of a preferred environment for specific bacteria (Shirakawa et al.,2019).

Rhizopogon roseolus (Corda) Th. M. Fr. is an edible ectomycorrhizal mushroom referred to as “shoro” in Japanese and a specific symbiont of *Pinus thunbergii* (Molina and Trappe, 1994; Shimomura et al., 2012). Recently, Pramoj Na Ayudhya et al. (2020) found that bacterial strains belonging to the family Burkholderiaceae that were isolated from the *R. roseolus* fruiting body could stimulate the mycelial growth of their host fungi under in vitro conditions. Frey-Klett et al. (1999) reported that the bacteria isolated from sporocarp had a significant effect on the mycorrhizal establishment when inoculated at different

densities. However, information concerning *R. roseolus* cultivation or improving mycorrhization using specific bacteria remains largely unknown. Therefore, the study of inoculation conditions with beneficial fruiting body bacteria for ECM fungi aims to improve mycorrhization. Here, inoculation rates and inoculation time periods with the ECM fungi *R. roseolus* were evaluated to determine the role of isolated bacteria in enhancing ectomycorrhizal formation and the growth of *P. thunbergii* seedlings under diverse microcosms.

4.2 Materials and methods

4.2.1 Fungal and bacterial strains

An H1 strain of *R. roseolus* that was selected through salt tolerance and deposited in culture collection of Tottori University, Japan (Gao et al., 2017) was used in this study. The inoculum of this fungi was cultured five times, diluted with modified Melin-Norkrans (⅕MMN) liquid medium (Brundrett et al., 1996). Culture flasks were incubated at 25±1°C, for 30 days. The mycelial inoculum was prepared by rinsing the mycelium once with sterile deionized (DI) water, then mixing the mycelium in DI water (1:4 w/v), followed by its homogenization.

Three strains of bacteria isolated from the gleba of *R. roseolus* fruiting bodies, *Paraburkholderia fungorum* (GIB024), *Caballeronia sordidicola* (GIB028) and *Janthinobacterium agaricidamnosum* (GIB029), were used. The bacterial inoculum was picked from a potato dextrose agar (PDA) medium and suspended in DI water. Bacterial concentration was determined using a hemocytometer to adjust to a standard density of 5×10^6 cell/mL.

4.2.2 Establishment of mycorrhizal seedling under microcosm

Seeds of *P. thunbergii* were sterilized by washing in detergent for 30 min and sequentially rinsed in running water overnight. Seeds were immersed in 30% hydrogen peroxide for 20 min before rinsing them twice with sterile distilled water in a laminar flow chamber. Ten seeds were allowed to germinate in one 90 mm circular Petri dish with water agar (WA) at 25°C in the dark.

The plant substrate consisted of peat moss and vermiculite mixed at 1:1 v/v was used. DI water was added to the mixture substrate (2:1 v/v), which was thoroughly mixed and packed into 90 mm diameter Petri dishes left half unfilled to create a headspace for the seedling shoot (Fig. 4.1). The peat-vermiculite substrate was autoclaved at 121°C for 40 min and the transplanted 14-day old seedlings with well-developed tap-roots were laid on the center surface of the medium individually. All treatments were inoculated with 1 mL of a homogenized slurry culture of *R. roseolus*. The mycelium suspension and bacterial inoculum were pipetted around the rhizosphere. Each Petri dish lid was sealed with Transpore™ White surgical tape (3M, Japan). Eight plates per treatment were placed in propagators. The plates were set upside down at an angle of approximately 80° horizontally so that the roots grew down against the lid. Seedlings were incubated in a growth chamber at a temperature of 25±1°C, under a 16-hour photoperiod with a light intensity of 2,000 lux provided by cool-white fluorescent lamps. The seedlings were neither fertilized nor irrigated during incubation period. Details of the microcosm setup were modified as per Timonen et al. (1997). The combined inoculation was used to assess the influence of the inoculation conditions of isolated bacterial inoculum.

4.2.3 Evaluation of mycorrhization and bacterial detection

After combined inoculation for 1 and 2 months, the seedlings were carefully extracted from the plates, and the roots were cleaned and rinsed with distilled water. The health status of the plants was determined by visualization. The plant root system was examined with a stereomicroscope to estimate mycorrhization levels as follows: roots were placed on a plate containing water, the total number of root tips per plant and the number of those that had been colonized by *R. roseolus* were counted, and the levels of plant mycorrhization were estimated as percentage of total root tips colonized by *R. roseolus*.

Quantitative detection of bacteria by plate counts as colony forming units (CFUs) were carried out for the plant substrates. CFUs were determined on standard agar plates after an appropriate dilution in sterile DI water. Three plates

per dilution were prepared and incubated at 25°C for 2-4 days. CFUs were then calculated.

4.2.4 Evaluation of seedling growth

Whole parts of the seedlings were collected and divided into shoot and root parts. The seedlings were dried at 65°C until the dry weight stabilized in the hot air oven, and then weight indexes, such as shoot dry mass, root dry mass and total dry mass were collected individually. Ratios of root/shoot (Rt/St) were calculated using the formula: Rt/St = root dry mass/shoot dry mass.

4.2.5 Statistical analysis

Microsoft Office Excel 2010 and SAS Analysis software were used for data calculation and statistical analysis. One-way ANOVA and completely randomized design (CRD) were used to identify significant difference. Mean values of levels within factors were compared by Duncan's Multiple Range Test (DMRT). A P-value of less than 0.05 was considered statistically significant.

4.3 Results

4.3.1 Effect of bacterial inoculation time condition

Three inoculation conditions and three strains of bacteria were used. Fungal and bacterial inoculations were applied at different time period conditions following three described procedures; 1) inoculation of bacteria 1 month before ECM fungi, pre-bacterial inoculation, 2) inoculation of bacteria-ECM fungi at the same time; simultaneous inoculation, and 3) inoculation of bacteria 1 month after ECM fungi; post-bacterial inoculation.

Mycorrhizal synthesis between *R. roseolus* and *P. thunbergii* seedlings was successful. The combined inoculation showed significant stimulation of the thicker lateral roots and/or branching roots formations with a whitish coloration (Fig. 4.1). The combined inoculation treatments significantly affected the presence of mycorrhizal formation for any of the time periods. Post-bacterial inoculation with bacterial strain GIB029 (procedure 3) showed great variations between the *P.*

thunbergii mycorrhizal formation after 1 month (39%) and 2 months (21%) of incubation. Simultaneous inoculation with bacterial strain GIB024 (procedure 2) showed greatly improved mycorrhizal formation (35%) after 1 month of incubation and increased mycorrhizal formation (75%) after 2 months of incubation (Fig. 4.2). Ectomycorrhizal formation was proved to be time-dependent, and inoculations with bacteria after inoculation with *R. roseolus* were more effective at increasing mycorrhizal root levels.

The population density of the bacteria introduced in the soil substrate was detected by the dilution-planting method. Results showed that for all treatments, bacterial density decreased after inoculation, except for the population density of bacterial strain GIB028 under combined inoculation with *R. roseolus* at the same time, which showed better survival ability (Table 4.1). After 2 months of incubation, factors such as bacterial inoculation time period and bacterial strain had no significant effect on seedling growth, dry biomass, Rt/St ratio, and root length for all treatments (Table 4.1).

4.3.2 Effect of bacterial inoculation dose

The bacterial inoculum was pipetted around the root system 1 month after it was inoculated with fungal inoculum. Each bacterial strain was inoculated with three different bacterial densities: 0 (control), 5.0×10^6 (low), and 1×10^7 (high) CFU/mL inoculum. Four replicate experiments were performed for each strain.

A positive influence of combined inoculation and extended bacterial inoculation dose on mycorrhizal formation was confirmed ($P = 0.004$). At the end of the first month, the low dose of bacterial inoculum significantly increased mycorrhizal formation, especially for bacterial strains GIB024 and GIB028. However, all low doses declined 2 months after inoculation and inoculation with bacterial strain GIB024 and GIB029 at high dose showed success in mycorrhizal formation when compared with the control (Fig. 4.3).

Survival of introduced bacteria in the substrate after combined inoculation rapidly declined for all treatments after 2 months of incubation (Fig. 4.4). Seedling

growth between control and other treatments after inoculation for 2 months showed no significant difference in terms of dry biomass, Rt/St ratio, and root length between plant treatments (data not shown). However, root length was slightly stimulated when inoculated with bacterial strain GIB024 at high dosage (Table 4.2).

4.4 Discussion

The present study, using in vitro experiments, provides evidence that bacteria isolated from the ECM fruiting body affect mycorrhizal formation and host plant growth through the ECM species *R. roseolus* H1 strain in both positive and negative ways. The affinity between fungal and bacterial strains is likely to underline this interaction.

The results confirm the efficacy of selective bacteria in enhancing the effect of ectomycorrhizal inoculation, in terms of increased mycorrhiza formation. It was evidenced that bacteria had a contrasting effect on mycorrhiza formation when inoculation was done with different bacterial species and at different inoculation conditions, such as concentration and time period. Post-bacterial inoculation of strain GIB029 after *R. roseolus* resulted in great variations between the *P. thunbergii* mycorrhizal formation after 1 month and 2 months of incubation (39% and 21%, respectively). 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. Thus, in the context of our study, bacteria increase the efficiency of mycorrhizas. However, the first stage of inoculation period suggests that the high dose of bacteria inoculum inhibits mycorrhizal development. According to Aspray et al. (2006a) a higher density of *Paenibacillus* sp. EJP73 and *Burkholderia* sp. EJP67 had a detrimental effect on either the plant or the fungus. This diverse

effect depending on inoculum dosage suggests that different mechanisms operate for these two bacteria. Duponnois et al. (1993) reported that high bacterial inoculum inhibited mycorrhizal development. Moreover, colonizing competition between fungi and bacteria can affect the degree of mycorrhization (Duponnois et al., 1993). Brulé et al. (2001) observed dominance of bacteria over fungi in rich growth media and stated that inoculation success depended on the survival of the fungal inoculum in soil during pre-symbiotic life. The ECM fungus is comparatively slow growing in saprophytic conditions (Mediavilla et al., 2016), as *Pseudomonas fluorescens* and *Boletus edulis* compete for nutrients during the pre-symbiotic period and, consequently, *B. edulis* gains no benefit from the bacteria before mycorrhization. Kurth et al. (2013) suggested that competition for resources between fungus and bacteria inhibited mycorrhizal development. Similar to a few reports that mention the appropriate time period for inoculation, our study revealed that bacterial inoculation after ECM inoculation is an ideal condition to prevent antagonism between microbes and promote the formation of mycorrhizal infection. Thus, the development of new methods for utilizing beneficial bacteria and ECM fungi is necessary for further investigation.

Here, I analyzed plant growth parameters influenced by the interaction between the ectomycorrhizal fungus *R. roseolus* and bacterial strains under different inoculation conditions. 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*. Several bacteria such as *Janthinobacterium*, *Pseudomonas*, and *Stenotrophomonas* have been found in mushroom fruiting bodies, and their community composition appears to be different when compared with soil bacteria found in the mycorrhizosphere (Li et al., 2016). Optimal plant growth parameters were observed for plants that were cultivated with *P. fungorum* for 2 months. In the past few years, a number of novel genera belonging to the *Burkholderialares* family have been reported to fix

nitrogen. These have shown promise as candidates for applications in biocontrol, biofertilization, and bioremediation to promote plant growth and degrade recalcitrant compounds (Suárez-Moreno et al., 2012; Estrada-de los Santos et al., 2013; Gao et al., 2015; Eberla and Vandamme, 2016). However, plants inoculated with GIB029 (*Janthinobacterium* sp.) also exhibited a good growth. The *Janthinobacterium* species were found in high abundance in the crop plant growth system and may serve as targeted genus for plant growth promoting rhizobacteria (PGPR) isolation. These bacteria are used in the production of the antibiotic violacein, a known inhibitor of viruses, fungi, and nanoflagellates (Anderson and Habiger, 2012). Additionally, Frey-Klett et al. (1999) reported that bacteria exert two opposite effects on plant growth: the obvious beneficial ones, independent of cell density, and some detrimental ones toward the plant or fungus at high cell densities. The result found that although the percentage of mycorrhizal formation inoculation with ECM fungi combined with bacterial strain GIB029 showed no significant difference from or all other treatments, the ratio between mycorrhizal roots and common roots (data not shown) as well as root length were higher in other treatments. Therefore, the bacterial strain GIB029 is considerate to be useful to promote not only ECM formation, but also host plant growth in terms of biofertilizer capability. Thus, further research should be continued the elaborated research on bacterial selection and its application methods for the best utilization.

Mycorrhizal synthesis between *P. thunbergii* seedlings and *R. roseolus* was achieved successfully using bacteria isolated from the *R. roseolus* fruiting bodies. The results in this study confirmed the beneficial effects of bacteria in enhancing the mycorrhization compared to inoculation with *R. roseolus* alone. The effects of bacterial aggregates on ECM fungi mycorrhization will allow us to gain a more precise understanding of their functional significance in the establishment of ECM symbiosis. Moreover, bacteria inoculated after the ECM fungi inoculation and in small amounts improved the level of mycorrhization. These results indicate that bacterial application maybe important for producing mycorrhizal plants inoculated with valuable ECM fungi in the future.

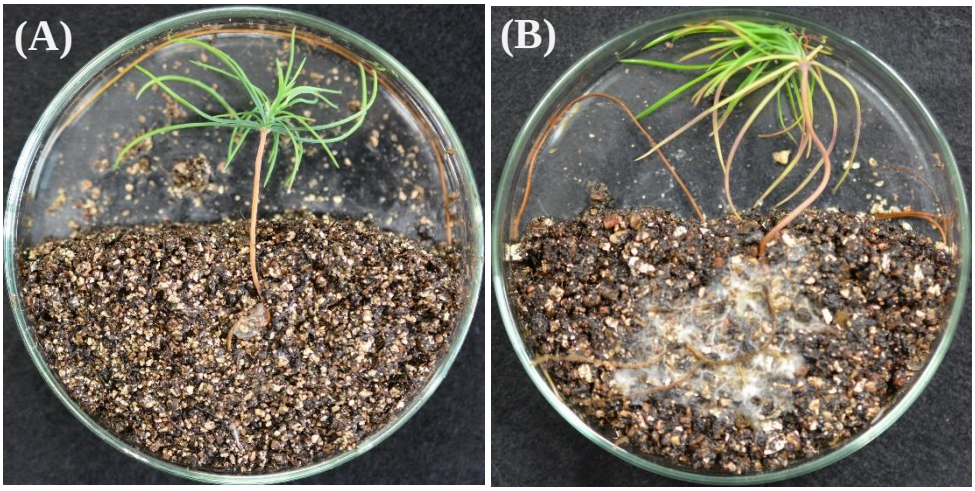


Fig. 4.1 General aspects of the *Pinus thunbergii* seedling combining inoculation with ECM species *Rhizopogon roseolus* strain H1 and bacteria strain GIB024, after 2 months of in vitro cultivation.

(A) Control, no inoculation seedling; (B) ECM inoculated seedling.

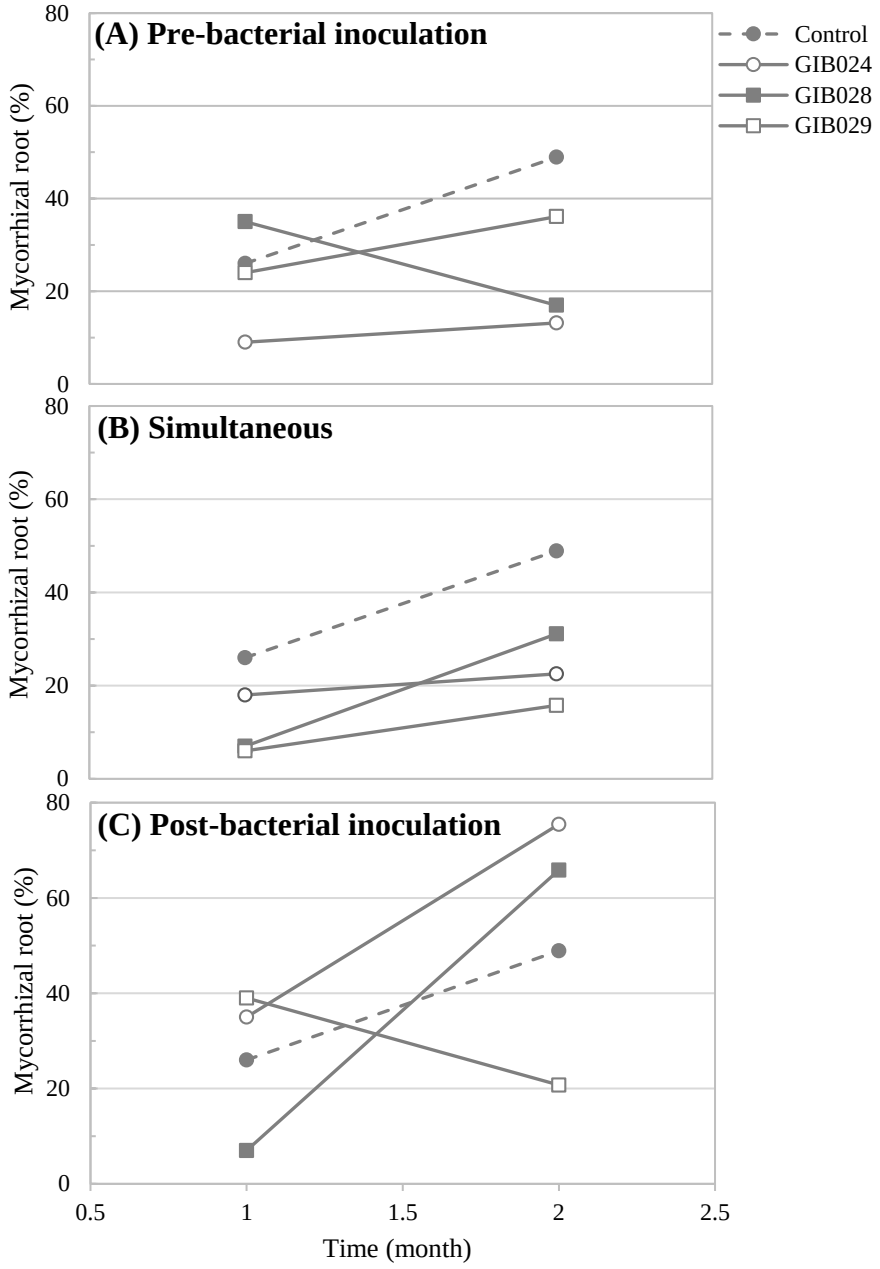


Fig. 4.2 Mycorrhizal formation percentage for *Rhizopogon roseolus*, after 1 month and 2 months of incubation, following inoculation with bacterial strains GIB024, GIB028 and GIB029, under different time period conditions of inoculation: A) Inoculation of bacteria before ECM inoculation (Pre-bacterial inoculation); B) Inoculation of bacteria and ECM fungi at the same time (Simultaneous); C) Inoculation of bacteria after ECM inoculation (Post-bacterial inoculation).

Table 4.1 Biometric parameters of shoot dry mass, root dry mass, total dry mass, root/shoot (Rt/St) ratio and root length of *Pinus thunbergii* seedlings, and bacterial density after inoculation, under different time period conditions of bacterial inoculation for 2 months of incubation.

Treatment		Bacteria (cfu/mL)	Dry biomass (g.)			Rt/Sh ratio	Rt length (cm)
Inoculation	Time period		Shoot	Root	Total		
Control	Control	-	0.05	0.05	0.10	0.96	109.0
H1	Control	-	0.06	0.04	0.09	0.71	88.5
GIB024	Control	58×10^{3}e	0.04	0.04	0.07	0.93	82.3
GIB028	Control	69×10^{7}b	0.03	0.03	0.06	0.82	79.9
GIB029	Control	21×10^{3}e	0.04	0.04	0.08	0.96	98.9
H1×GIB024	Pre-inoc.	24×10^{3}e	0.04	0.04	0.08	0.96	98.9
	Simultaneous	18×10^{4}e	0.04	0.04	0.08	1.08	116.1
	Post-inoc.	27×10^{4}e	0.05	0.05	0.10	0.89	176.0
H1×GIB028	Pre-inoc.	28×10^{7}c	0.07	0.05	0.12	0.86	101.1
	Simultaneous	16×10^{8}a	0.04	0.04	0.07	0.96	90.6
	Post-inoc.	65×10^{4}e	0.06	0.04	0.10	0.69	189.5
H1×GIB029	Pre-inoc.	13×10^{6}d	0.05	0.05	0.10	1.01	109.5
	Simultaneous	19×10^{3}e	0.06	0.05	0.11	0.83	108.7
	Post-inoc.	25×10e	0.05	0.03	0.08	0.54	169.2
CV.		28.17	35.29	24.46	28.21	25.52	21.73
p-Value		*	ns	ns	ns	ns	ns

Pinus thunbergii seedlings inoculated with *Rhizopogon roseolus* H1 strain and bacterial strains; GIB024, GIB028 and GIB029, under different time period conditions of inoculation: Inoculation of bacteria before ECM inoculation (Pre-bacterial inoculation); Inoculation of bacteria and ECM fungi at the same time (Simultaneous); Inoculation of bacteria after ECM inoculation (Post-bacterial inoculation). Each value is the average of four replications.

Control represents no inoculation.

Data with different letters in the same columns are significantly different by DMRT test at $P < 0.05$.

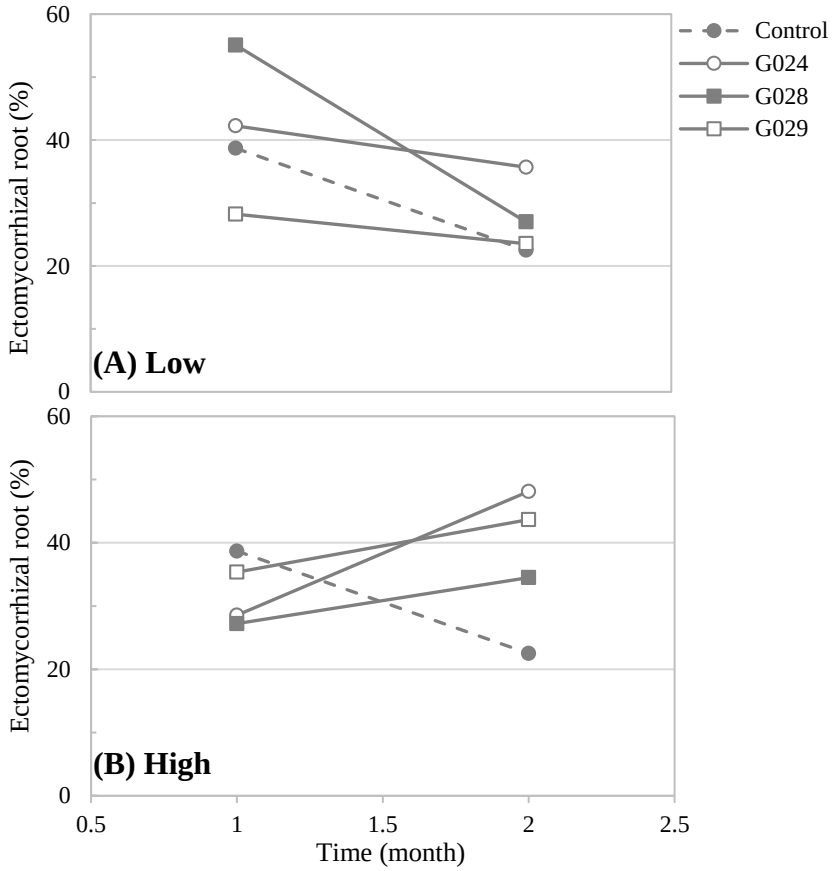


Fig. 4.3 Mycorrhizal formation percentage for *Rhizopogon roseolus*, after 1 and 2 months of incubation, under different doses of bacterial inoculum: A) Low (5×10^6 CFU/mL); B) High (1×10^7 CFU/mL).

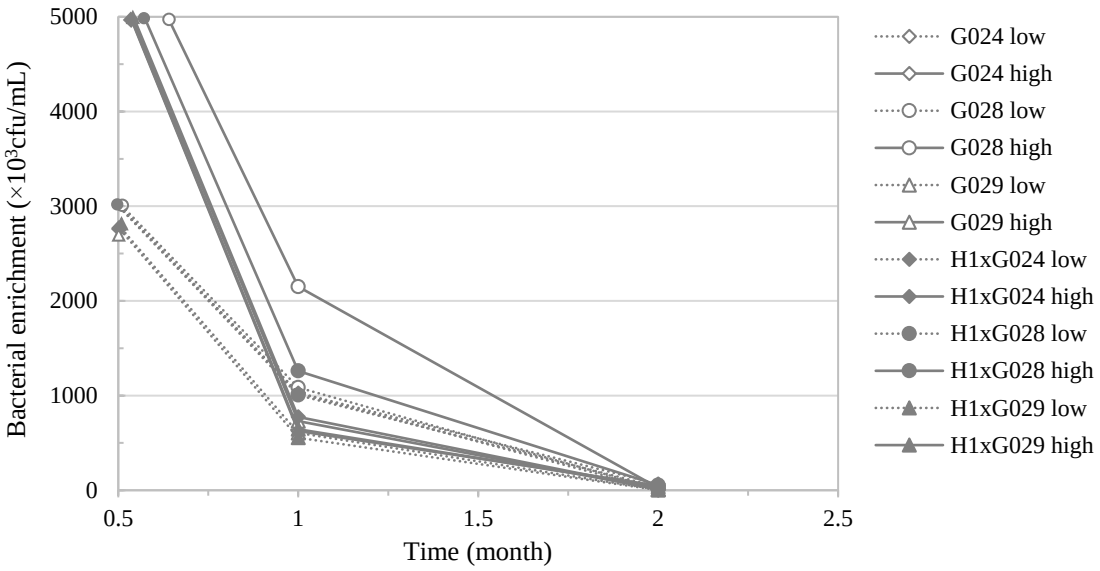


Fig. 4.4 Bacterial enrichment in soil substrates during incubation, under different doses of bacterial inoculum: low (5 ×10^6 CFU/mL) and high (1 ×10^7 CFU/mL), after 1 and 2 months of incubation. Each point represents the proportion of bacterial enrichment.

Table 4.2 Root length of *Pinus thunbergii* seedlings under different doses of bacterial inoculum, after 2 months of incubation.

Treatment	Bacterial concentration	
	Low	High
Control	43.7	43.7
GIB024	51.1	53.9
GIB028	42.4	44.7
GIB029	39.7	49.6
H1	70.2	70.2
H1×GIB024	56.7	71.9
H1×GIB028	50.0	45.6
H1×GIB029	63.2	67.1
CV.	33.69	33.48
p-Value	ns	ns

Seedlings inoculated with *Rhizopogon roseolus* H1 strain, and bacterial strains GIB024, GIB028 and GIB029, under different doses of bacterial inoculum: low (5×10^6 CFU/mL) and high (1×10^7 CFU/mL). Each value is the average of four replications.

Control presents no inoculation

Chapter 5

General discussion and conclusion

Ectomycorrhizas are established by mycelia, or "higher fungi". Phylogenetically, these almost exclusively nest in Basidiomycota and Ascomycota. Over the last 180 million years these mycobionts have evolved through transitioning from a saprophytic lifestyle multiple times (Kohler and Martin, 2017). This information on the symbiotic evolution is essential to help understand the development and architecture of ectomycorrhizal fungi (Balestrini and Kottke, 2017). Despite efforts to extrapolate fungal biomass in ectomycorrhiza, the detail and volume of fungal cells in the ectomycorrhiza have remained unclear, despite their structures being recognized as important tissues in the symbiotic relationship (Stögmann et al., 2013; Balestrini and Bonfante, 2014).

Ectomycorrhiza development was believed to begin with contact between the fungi and the roots. Establishment of the symbiosis may be controlled by the genes of both partners (Smith and Read 2008). Malajczuk et al. (1990) reported that there are five stages of development before the symbiotic function begins : i) Pre-infection, characterized by hyphal contact with the root; ii) Symbiotic initiation, characterized by fungal attachment to the epidermis; iii) Fungal colonization, with hyphal penetration between epidermal cells and the formation of the initial mantle layers; iv) Symbiotic differentiation, in which the Hartig net proliferates and there is a rapid buildup of mantle hyphae; and v) Symbiotic function, meaning the end of Hartig net growth and the development of a consistent mantle, tightly pressed against the epidermal cells. Massicotte et al. (1986) reported that the Hartig net formation is the first step to begin ectomycorrhizal relationships and indicates the existence of a true ectomycorrhizal association. This is the contact zone between the symbionts, at which the interchange of nutrients between fungi and host plant is produced. The structure typically forms from the inner part of the root to the outer. Fungal

hyphae penetrate between the epidermal cells, which become progressively more radially enlarged from the intracellular structure. By contrast, the present study (chapter 2) found that the Hartig cells were highly branched, and certain cells were common in the mantle sheath. According to Agerer (2006), in gymnosperms, the Hartig net typically penetrates beyond the epidermis to enclose several layers of cortical cells, sometimes even extending to the endodermis.

While the study of exploration types requires morphological observation of mycorrhizas, extraradical mycelial study is far more difficult to conduct using observational techniques. Methods for studying the structure and function of extraradical mycorrhizal mycelia include biochemical and DNA-based markers, in vitro and in soil observation, and root-free hyphal compartmentalization (Parladé et al., 2014). Compared to traditional methods involving only microstructure observations under a light microscope, the Fiji package allowed us to easily quantify and clarify morphological cell structure changes. In comparison to scanning electron microscopy, this method provides a simpler alternative to observe these fungal characteristics. This is the first report to describe a 3D analysis of the cell volume in the outermost mantle cells, the innermost mantle cells, and Hartig net cells in ectomycorrhizas.

The 3D ultrastructure of organisms has recently been examined by array tomography (Wacker and Schroeder, 2013). The approach is a relatively straightforward and powerful method that relates molecular details to ultrastructure. Ultrathin sections are stained with fluorophores and then imaged by light, followed by electron microscopy to obtain a correlated view of the ultrastructure and specific staining of the region of interest. Ultrastructural features of ectomycorrhizas aseptically synthesized between *R. roseolus* and *P. thunbergii* have been described using transmission electron microscopy (Shimomura et al., 2010). However, to date, no information is available on the 3D ultrastructure of ectomycorrhizas. I consider these findings to be useful to deepen understanding of the function of symbiotic cells, such as the molecular exchange

between plant and fungi. Future cytological studies should use array tomography to examine the 3D ultrastructure of the Hartig net and mantle cells.

In chapter 3, culturable bacterial strains linked to the ECM mushroom fruiting bodies of *R. roseolus* observed within a *P. thunbergii* forest of semi-natural origins were able to promote mycelial growth under axenic conditions and were dominated by Gram negative Proteobacteria. It was evidenced that mycorrhizospheres have a strong influence on culturable soil bacterial communities and *Paraburkholderia* was the major genus associated with ECM basidiospore. Previous studies reported that *Burkholderia* was among the predominant genera in the mycorrhizospheres of several pine tree species. In recent years, many species of *Burkholderia* have been reclassified as *Paraburkholderia* or *Caballeronia* (Dobritsa and Samadpour, 2016). Hence, previous studies have confirmed that many species commonly found in the mycorrhizosphere of *Pinus* trees are now considered to be *Paraburkholderia* (Shirakawa et al., 2019). These findings suggest that all bacteria isolated from fruiting bodies of *R. roseolus* are gram negative bacteria since they were not sensitive to the antibacterial activity of ECM fungi. These differences between gram negative and positive bacterial sensitivities may be attributed to differences in their membrane structures. Gram negative bacteria possess an outer membrane and a periplasmic space, whereas gram-positive bacteria do not (Brown et al., 2015). Furthermore, the expression of drug efflux pumps (Dzidic et al., 2008) and secretion of an exopolysaccharide matrix as a primary barrier against antimicrobial agents (Lewis, 2008) by gram negative bacteria may also account for these antimicrobial sensitivity difference.

The capacity to stimulate ECM mycelial growth may prove to be a significant characteristic of the isolated bacteria. The design of these experiments suggests that antifungal activity on the co-cultured ECM fungus and the inhibitory effects of the bacteria on the ECM fungus may be related. The antifungal activity of fruiting body bacteria is also dependent on the nutritional condition of the medium (Oh and Lim, 2018). Severe antifungal activity in *R.*

roseolus may be due to a bacterial response to nutrient limitation. However, there are several other mechanisms that can be involved in antagonistic relationship between bacteria and fungi such as pH alteration in a growth medium (Frey-Klett et al. 2011). Mycorrhizosphere bacteria produce soluble compounds as well as volatile organic compounds (Frey-Klett et al., 2007); however, our experiments are only indicative of effects mediated by soluble compounds.

The last experiment (chapter 4), to investigate the suitable inoculation conditions, various hypotheses concerning the mechanisms that underpin the associations between MHB, fungi, and plants have been suggested based on results of in vitro bioassays and cultures (Deveau et al., 2007). I revealed that *P. fungolum* is a fungus-specific MHB that produces fungus growth regulators and affects plant health and development. When the tree roots received a combined inoculation with a suspension of *R. roseolus* mycelia and certain bacteria, significant stimulation of mycorrhiza formation was observed. In the pine root system, a slight increase in the number of mycorrhizas was evident when the pine seedlings were inoculated with *P. fungolum*. The present study further revealed the potential of bacterial strain on its performance in a peat-vermiculate substrate and shows that *P. fungolum* benefits in the presence of mycorrhizal fungus.

As *R. roseolus*–bacterial interactions have not been previously studied in vitro, I selected to study two bacterial doses (5×10^6 and 1×10^7 CFU/ml) to determine their effect on bacterial interactions. In the first month, I observed low bacterial dose stimulating mycorrhiza formation over the control, but it was possible to detect a negative effect on mycorrhiza formation when using a higher bacterial dose. Which was caused by a high density of helper bacteria at the early inoculation stage may have a detrimental effect on either the plant or fungus (Frey-Klett et al., 1999). Similarly, Aspray et al. (2013) reported that the presence of MHB with low specificity could have unpredictable consequences for the composition of ectomycorrhizal fungal communities colonizing roots and plant growth, either in nature or under greenhouse conditions.

The soil microbe filtrate inhibited *P. thunbergii*, potentially due to resource or space competition, or alternatively because of antagonism (Frey-Klett et al., 2011). Competitive inhibition is perhaps more likely, for instance Schrey et al. (2012) obtained evidence that *P. croceum* may be particularly tolerant to antagonistic metabolites of *Streptomycete* isolates from Norway spruce in an experiment conducted to determine the in vitro activity of *Piloderma* sp. mycorrhizas against seven fungi, in which *P. croceum* was found to be the least severely affected fungus. In this study, *P. fungolum* only affected the growth of *R. roseolus* when the influence of the microbial filtrate. This indicates that communities of soil microbes conduct a multitude of small-scale processes that can affect bacterium-fungus interactions (de Boer et al., 2005, Walder et al., 2012).

At present, the detailed mechanisms behind plant symbiosis are not well known. It is therefore obvious that the mechanisms of multiple simultaneous interactions between microbes and plant are more complicated and even less understood. In the present study, I investigated the effect of interactions with certain bacteria and *R. roseolus* fungi on the growth and development of black pine seedlings during the first two months of symbiosis formation under controlled microcosm. Despite the relatively short experiment duration and the restricted number of simultaneously tested symbiotic organisms, slight, non-significant differences were found but in the growth of black pine seedlings that were dependent on the symbiont or the combination of specific symbionts, indicating a possible role in seed establishment and early development of black pine seedlings.

The morphology of mycorrhizal roots is known to be drastically different from that of non-mycorrhizal roots. Ectomycorrhizal roots, particularly those of *Pinus* spp., are characterized by a lack of root hairs, thick mantle, and extensive dichotomous branching of short roots (Norman et al., 1996; Sen, 2001). The ability of MHB to change the extent of dichotomous root branching has been reported by Aspray et al. (2013) who conducted a *L. rufus* experiment, indicating that the effects on mycorrhiza architecture were fungus-specific since only MHB strains EJP67 and EJP73 changed the extent of dichotomous branching. Phytohormones,

including auxins, cytokinin, and ethylene have been implicated in producing morphological root changes during mycorrhiza formation, including in the formation of lateral roots and dichotomous branching of short roots (Kaska et al., 1999; Barker and Tagu 2000). Although dichotomous short root branching can occur spontaneously in some *Pinus* sp., the regulation of auxin concentration and distribution in the mycorrhiza appears to be important for inducing changes to the dichotomous branching of short roots (Gay et al., 1994). As many bacteria produce auxins, this suggests a mechanism by which MHB could alter dichotomous root branching (Costacurta and Vanderleyden, 1995).

In conclusion, three experiments were undertaken in this study: 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 (Figure 5.1). 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.

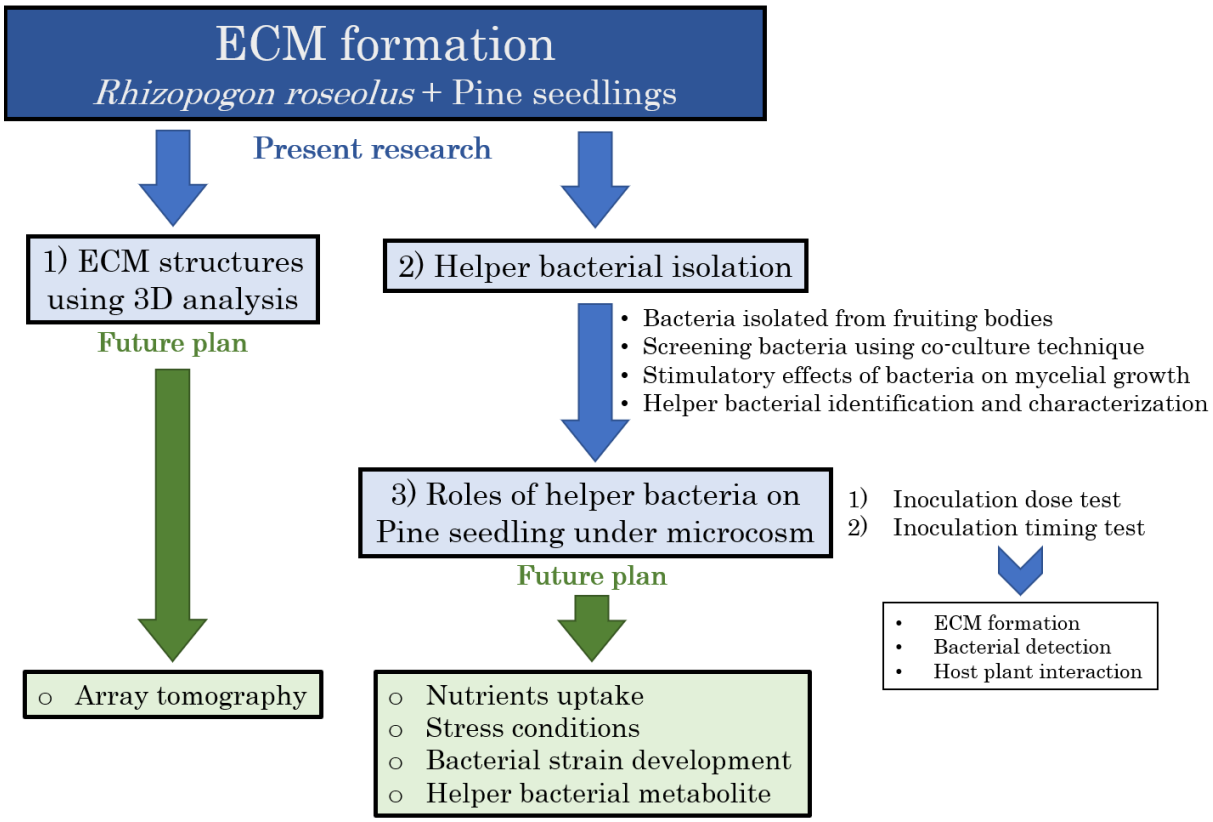


Figure 5.1 Research outline in this study and future tasks toward establishment in the cultivation process in the ectomycorrhizal fungus *Rhizopogon roseolus*

Summary

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

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 been 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, three-dimensional (3D) image analysis was used 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*, ectomycorrhizas appeared in seedling roots at four weeks after inoculation. These ectomycorrhizas were 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 average single cell volume of the outermost and innermost mantle sheaths, and the Hartig net measured 365, 452, and 1,516 μm^3, respectively. Moreover, cell volume of the innermost mantle cells was more variable than the outermost 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 more than at 1 month. When high

concentration of bacterial suspension was used as inoculum, mycorrhization at 2 months was increased more than at 1 month. Survival of introduced bacterium in the soil 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 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.

和文摘要

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

外生菌根菌は植物の根に感染して共生することにより，栄養の吸収や生育を促進する効果を有することが知られている．外生菌根菌ショウロ *Rhizopogon roseolus* はクロマツ *Pinus thunbergii* と共生するきのこ種である．本きのこの子実体を形成させるためには宿主クロマツとの共生関係を構築することが必要であり，人工栽培をすることが難しいきのこ種である．本きのこの人工栽培に関する研究が進められ，人工条件下での子実体形成に成功したものの，収量性が低いことから実用的な生産技術の確立には至っていないのが現状である．一方，菌根共生を促進するヘルパー細菌 mycorrhizal helper bacteria が近年発見されている．これらの細菌系統は，菌根共生，菌糸体の生育促進，胞子発芽促進等の作用を有する．このような背景から，これらのヘルパー細菌を上手に利用することで，本きのこの生産技術の向上が望めると考えた．そこで，本研究では，まず，外生菌根菌の実体を明らかにすること，および，ヘルパー細菌のきのこ栽培への利用を目指し，１）ショウロ―クロマツの外生菌根から得られた連続準超薄切片像を用いた外生菌根細胞の３次元解析，２）ショウロ子実体からの細菌の分離，分子生物学的同定そして微細構造の解析，３）クロマツ実生へのショウロ菌根形成およびクロマツの生育に及ぼす分離細菌系統の投与効果，について研究した．

先ずは，ショウロ菌を接種したクロマツ実生における外生菌根の細胞構造の詳細について 3 次元解析した．クロマツ実生にショウロ菌を接種すると接種後 4 週間で二叉分岐する外生菌根が形成した．形成した外生菌根から顕微鏡試料を作製し光学顕微鏡で観察した．連続準超薄切片像を用いてハルティヒネット細胞の 3 次元構造を構築したところ，本細胞は多分岐し，ある細胞は菌鞘とハルティヒネットと共通であった．菌鞘細胞の 3 次元次元像を構築したところ，外側細胞は円柱状であったが，内側細胞は起伏に富んでいた．3 次元像から細胞体積を計測したところ，

外側菌鞘細胞，内側菌鞘細胞およびハルティヒネット細胞の体積はそれぞれ，365, 452 および 1,516 μm^3 であった．本報は，外生菌根の外側菌鞘細胞，内側菌鞘細胞およびハルティヒネット細胞の体積を 3 次元解析で明らかにした初めての報告である．

次に，ショウロは食用の外生菌根菌である．菌根共生を促進する細菌がしばしば根圏から分離される．一方，ショウロ子実体から細菌が分離され，その分離細菌が宿主菌糸体の生育を促進する能力を有することを近年見出した．本研究では，ショウロ子実体から様々な細菌を分離し，ショウロ菌糸体生育に及ぼす効果について二員培養を用いて調査した．ショウロ子実体から分離した 19 細菌系統の内，６系統が特異的にショウロ菌糸体の生育を促進した．ブラスト検索をした結果，これらの細菌は，*Paraburkholderia, Caballeronia, Janthinobacterium*, *Novosphingobium* および *Rhodobacter* 属に属すると推定できた．さらに，主要な３細菌系統の微細構造を走査型電子顕微鏡観察で明らかにした．以上の結果から，ショウロ子実体形成に特定の細菌系統が特異的に関与している可能性が考えられた．

最後に，分離同定した細菌系統のショウロークロマツ菌根共生や宿主クロマツの生育に及ぼす，３系統の細菌の影響について調査した．本調査をするにあたり，ショウロ菌接１か月前に細菌を接種する細菌前接種区，ショウロ菌と細菌を同時に接種する同時接種区，そして，ショウロ菌を接種した後に細菌を接種する，細菌後接種区の３接種試験区を設定した．また，接種する細菌の濃度として低濃度と高濃度の２濃度を用いた．ショウロ菌と細菌系統を接種したクロマツ実生を育成した後，根茎における菌根形成率を調査した．その結果，細菌の前接種，ショウロ菌との同時接種において 1 か月目よりも，２か月目に菌根形成率が穏やかに増大する傾向が認められた．しかし，ショウロ菌接種後に細菌系統 GIB029 を接種すると，1 か月後には 39%，2 か月後に 21%と，時間とともに菌根形成率が減少した．しかし，ショウロ菌接種後に細菌系統 GIB024 または GIB028 を接種すると菌根形成率が，1か月目より2か月目に著しく増大した．また，低濃度の細菌系統を用いると菌根形成率は 1 か月目より 2 か月目に減少するが，高濃度細菌を用いると 1 か月目より 2 か月目に上昇した．細菌接種土壌から細菌の検出を試みたところ，細菌接種後

２か月目で著しく減少した．一方，クロマツ実生の生育について調査したが，著しい差異は認められなかった．以上の結果から，クロマツ実生におけるショウロの菌根形成に及ぼす効果は用いる細菌系統と接種のタイミングで異なり，ショウロ菌を接種した後に細菌系統 GIB024 または GIB028 を接種することが有効であると考えられた．

本研究において，ショウロ菌を接種したクロマツ実生の外生菌根細胞の３次元構造を，Fiji ソフトを用いて明らかにすることで，本手法は宿主植物を外生菌根菌との相互作用関係を解析する上で有効であることを明らかにした．また，本細菌系統を分類学的・細胞学的に解析した研究は，本細菌系統の実体等の基礎的知見を提案するものである．また，宿主クロマツおける外生菌根形成や生育に及ぼす細菌系統の効果に関する比較研究は，本細菌系統の機能についての知見を深めるとともに，ショウロ外生菌根菌の栽培技術の確立における本細菌系統の活用に関する着想を提案するものである．

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List of related publications

1. Sawithree Pramoj Na Ayudhya, Shin Sugimura, Yuma Ozaki, Tadanori Aimi and Norihiro Shimomura: Three-dimensional analysis of ectomycorrhizas formed in *Pinus thunbergii* roots inoculated with *Rhizopogon roseolus*, Mushroom Science and Biotechnology 27 (3), 93-97 (2019) (The corresponding content is in chapter 2)

2. Sawithree Pramoj Na Ayudhya, Rini Riffiani, Yuma Ozaki, Yukiko Onda, Shota Nakano, Tadanori Aimi and Norihiro Shimomura: Isolation of bacteria from fruiting bodies of *Rhizopogon roseolus* and their effect on mycelial growth of host mushroom, Mushroom Science and Biotechnology 27 (4), 134-139 (2020) (The corresponding content is in chapter 3)