The impact of host plant (*Pinus thunbergii*) on the mycelial features of the ectomycorrhizal mushroom *Rhizopogon roseolus*

外生菌根菌ショウロの菌糸形状に及ぼす宿主クロマツの影響

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Chapter 1
General introduction

Ectomycorrhiza (ECM) is one of the types of mycorrhizal association between fungi and plant roots in which both the partners are benefited and appears to be significant for both symbionts (Smith and Read 2008). They are ubiquitous in forest ecosystems, from temperate to tropical regions. Ectomycorrhiza are hosted by various higher plants in the family of Pinaceae, Fagaceae, Dipterocarpaceae, and Myrtaceae (Brundrett and Tedersoo 2018; Corrales et al. 2018). Ectomycorrhizal symbioses are occurred predominantly on the fine root tips of the host. These ECM organs are defined by the presence of a mantle/fungal sheath, consisting of interlaced hyphae on the root surface, and a labyrinth of highly branched hyphae between cells of the root epidermis or cortex to form the Hartig net (Smith and Read 2008; Brundrett and Tedersoo 2018). In addition, the mycelium which appears as multi-hyphal linear aggregates formed from the ECM mantle and serving as exploratory and exploitation organs (Cairney 1991) in the environment.

Ectomycorrhizal fungi are acknowledged for their foraging capacity of soil resources for the plant host (Tibbett and Sanders, 2002; Köhler et al., 2018) facilitated by their extraradical mycelia. They improve the access of plant to nutrients with a range of macronutrients, including phosphorus, potassium, calcium, magnesium, sulphur, and micronutrients, such as iron, zinc, copper, and manganese (Read et al. 2004; Smith and Read 2008). They also transport for water to plant roots (Read et al. 2004; Smith and Read 2008), with the roles the fungal aquaporins (Xu and Zwiazek 2020). Moreover, the mycobiont also protects the host from pathogens and pests by covering them with sheath. In addition, they improve plant health by enhancing resistance to diverse stresses like drought, salinity, and heavy metals (Jones et al. 2009; van der Heijden et al., 2015). In return, carbon (C) resources are transferred from the host to the fungus (Smith and Read 2008), estimated a third or more of tree photosynthate to ECM associates (Nehls et al., 2010).

The ECM symbiosis forming some compartments that alter the morphological features of both symbionts and mostly notably on plant organs (Peterson et al. 2004). Morphological characters of the root structure of ECM host plants are dramatically altered root architecture, with a strong short root formation and differentiation of colonized cells (Ditengou et al. 2000). The ECM symbiosis stimulates more formation of lateral roots (Felten et al. 2009), increased the volumes of root cell (Luo et al. 2009), and inhibition of root hair production

(Ditengou et al. 2000). The remarkable modification of the secondary roots including dichotomous, coralloid or short monopodial roots that are prominently different from non-mycorrhized roots can be observed (Peterson et al. 2004). The secondary plant roots become thicker due to the formation of fungal mantle on the surface of short roots (Peterson et al. 2004). However, the morphological and cytological changes of the fungal features in ECM symbiosis have less been paid with scarce information compared to the plants. The change in fungal morphology was generally only known from the hyphae at intercellular plant roots develop into the labyrinth tissue formation which leading to the Hartig net formation, and extent to form a sheath outside the cell (Smith and Read 2008).

To deepen the current knowledge of ECM symbiosis, more studies of fungal morphological changes during the ECM symbiosis needs a warrant. In addition, since ectomycorrhizal fungi are also known as facultative saprotroph (Baldrian 2009; Cullings and Courty 2009, Vaario et al. 2011; Smith et al. 2017; Tedersoo et al., 2010), it is also important to compare the morphological and cytological aspects of ECM fungi during the symbiosis with host or in the absence of the host. To date, the majority of mycorrhizal studies has been less focused on the biotic and abiotic impact to the fungal structures. Furthermore, the cytological comparison between ECM fungi with the occurrence or absence of their host has never been done. In fact, many magnificent progresses in mushroom science are the direct contribution of the work of countless of basic mycological works on understanding of the fundamental information of fungal features. However, it is impractical to fully observe the morphogenesis or cytological behavior of ECM fungi in its natural environment. To perform such experiment, in vitro studies in the laboratory cultures is feasible to investigate the cytological plasticity of the fungal structures. In addition, the non-obligate ECM fungi should be used as the fungal object to understand the mycelial adaptation during the occurrence or absence of the host. One of the ECM fungus matches with such criteria is *Rhizopogon roseolus*.

Rhizopogon roseolus (Corda) Th. M. Fr. (*Rhizopogon rubescens* Tul. & C. Tul.) is an hypogeous ectomycorrhizal fungi that are mostly restricted to the Pinaceae (Massicotte et al. 1994). *Rhizopogon roseolus* belongs to Boletales (Agaricomycetes) and comprise seven varieties worldwide (http://www.indexfungorum.org/Names/Names.asp). This ECM fungus produces basidiomata that are partially or completely immersed in soil (Shimomura et al. 2012a). In Japan, *R. roseolus* is called “shoro” and the fruiting bodies of this ECM fungus are commonly found in the sandy soils near the *Pinus thunbergii* Parl. in seashore area of Japan (Kawai et al. 2008). *Rhizopogon roseolus* also acknowledged

as a high price edible mushroom in Japan (Kawai et al. 2008). However, the market supply is dependent on the production of the fruiting bodies in the field (Kawai et al. 2008). Prior report showed that *R. roseolus* is a prospective ECM fungus species for the nursery mycorrhization due to its promising capacity for colonizing young pine seedling roots (Shimomura et al. 2012a). Therefore, more basic biological research should be performed.

The basic mycological works will ensure the better understanding of many fundamental information of fungal features and can be used for the variety of application purposes. For example, monokaryotic mycelia of *R. roseolus* developed from basidiospores with combinations of compatible mating type. Therefore, it is important to obtain basidiospores from diverse parental strains. As the spore can only be collected from the fruiting body which depends on the production on the field, the formation of basidiospores in agar medium will contribute to development of cross breeding of *R. roseolus.*

Chapter 2
Cytological comparison of mycelial aggregates of *Rhizopogon roseolus* with and without the ectomycorrhiza host

2.1 Introduction

Ectomycorrhiza (ECM) symbiosis is one of the major components of the ecosystem, which has essential advantages to both symbionts. The vast majority of ECM phytobionts found globally belong to the Pinaceae (Smith and Read 2008). Ectomycorrhizal features can be recognized at least based on three criteria. In ECM, the fungal hyphae infiltrate inwards between the cells of the root to form an aggregate intercellular system, which appears as a network of hyphae in a section called the Hartig net (Smith and Read 2008). The fungus also forms a structure called the mantle (or sheath), which encloses the rootlet. From the mantle, the hyphal elements or extraradical mycelia radiate outward into the substrate. Those vegetative mycelial exploratory organs tend to grow as a group of linear hyphal systems.

Different names, including rhizomorph, mycelial strands, and mycelial cords, have been used to refer to this hyphal system and are often inconsistently used by mycologist (Townsend 1954; Watkinson 1971; Thompson and Rayner 1983). This hyphal system, which generally appear as mycelial aggregates, is one of the paramount structures of many higher fungi that are involved in nutritive exploration and initiation of symbiosis (Cairney 1992; Moore 1995; Smith and Read 2008). Boddy (1993) suggested that mycelial cords formation in many Basidiomycota was affected by several factors, such as nutrient, mycelial age, and other environmental stimuli. However, the effect of host on the mycelial aggregates formation and its characters has not been described. In addition, according to previous reports, the mycelial aggregates have a lower degree of being produced in agar and only few references from laboratory studies were found (Jennings and Watkinson 1982; Unestam & Sun 1995; Kwaśna et al. 2001; Yafetto 2018).

The mycelial aggregates of ECM is cytologycally less observed, despite their important role for colonization strategies in time and space. Consistently, the study of mycelial aggregates response to host in ECM symbiosis is generally neglected. The prime example of prior reports related to the morphological changes in ECM fungi focused only on environmental factors, such as organic matter (Wallander and Pallon 2005) and water stress (Leyva-Morales et al. 2019). To date, no previous report has described the effect of the host on the structure of the fungal

mycelial aggregates. Hence, there is a need to reveal the phenomenon to get a better understanding on how fungal mycelial aggregates respond to the presence of their host in ECM symbiosis.

Rhizopogon roseolus, also known as 'shoro' in Japanese, is an important mycobiont partner to several species of Pinaceae (Molina and Trappe 1994). The mentioned species is a non-obligate ECM mycobiont that is easy to isolate into pure culture in artificial medium. These characteristics met the requirement to study the morphological responses of the mycelial aggregates under the laboratory conditions. Previous cytological studies mostly focused on the plant features of the ECM. As a result, less attention has been paid to the fungal structures. In a review by Leake et al. (2002), they assumed that ECM fungal mycelia produced in pure culture are certainly different in structure and function than those formed with the host. However, no subsequent evidence was presented in their review. The morphological response of the mycelium with the occurrence or absence of the host has never been reported. Understanding this structural response is however essential for deepening the current knowledge on ECM symbiosis and its specific roles. Therefore, this study aimed to evaluate the morphological characteristics of *R. roseolus* mycelial aggregates during the occurrence or absence of their host (*P. thunbergii*) under controlled laboratory conditions.

2.2 Materials and methods

2.2.1 Fungal material

The ECM fungal species used was *R. roseolus* (Corda) Th.M. Fr. (= *R. rubescens* Tul. & C. Tul.) strain number TUFC10010 (Collection of the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Japan). The strain was prepared on malt extract agar (MEA) comprising of 20 gr malt extract, 20 gr agar, and 1 L tap water with the final media pH of 5.5. The fungi were then incubated for 3 weeks at 25 °C under dark conditions.

2.2.2 Plant material

Pinus thunbergii (Japanese black pine) was used as a host tree for *R. roseolus.* The pine seeds were imbibed for 24 hour in water and then surface-sterilized in 30% hydrogen peroxide for 20 minutes. The seeds were rinsed 3 times with sterile distilled water. Pine seeds were aseptically inoculated into water agar medium and germinated at 25 °C for 1–2 weeks. Pine seedlings with fine lateral roots were transferred to half size of a five-fold dilution of Modified Melin and

Norkrans (1/5 MMN) medium in (90x20) mm Petri dish (modified from Bailey and Peterson 1988). Each Petri dish had one seedling in a vertical plate technique of 30 mL of solid 1/5 MMN medium. The Petri dish was sealed with 3M^{TM} transpore surgical tape and incubated in a controlled chamber at 25 °C, 50% relative humidity, and 16-h day at 5000 lx.

2.2.3 Morphological assessment of explorative mycelial aggregate

Mycelial inocula with a diameter of seven millimeters were collected using sterile plastic straws and used for inoculation. After 4 weeks of host incubation, *P. thunbergii* lateral roots were inoculated with six fungal inoculums in half size of 1/5 MMN medium (FIG. 2.1A) on a 90×20 mm Petri dish. The Petri dish was incubated for an additional 24 weeks in a controlled chamber at 25 °C, 50% relative humidity, and 16-h day at 5000 lx. In the absence of the ECM host, the mycelial inoculum was inoculated on 1/5 MMN medium minus host seedlings in 90×15 mm Petri dish. Each medium contained either one or three inocula of *R. roseolus* and was incubated at 25 °C for 24 weeks (FIG. 2.1B).

The morphology of explorative mycelia was observed after they produced the mycelial aggregates (12–24 weeks). The basic mycological characters of the explorative mycelial aggregates were evaluated and compared with and without the host. A total of 50 plates of *R. roseolus* with host (w/ host) and without host (w/o host) were assessed. The mycelial aggregates were first observed using a Leica EZ4 stereo microscope. The samples were then fixed in fixative solution (99.5% ethanol:acetic acid, 3:1, respectively) and the air was subsequently removed. Samples were then stained with lactophenol cotton blue and observed using an Eclipse 80i light microscope (Nikon, Tokyo, Japan). The images were captured with a DS-L2 digital camera (Nikon). In addition, the mycelial aggregates with the host were evaluated according to Agerer (2006). The septa length of tubular hypahe conjuction w/ host and w/o host were subjected to an analysis of variance (one way ANOVA), and mean values were ranked by the Student-Newman-Keuls test at $P < 0.05$.

2.3 Results

2.3.1 Morphological observation of explorative mycelial aggregates using stereomicroscope

Observation of the mycelial aggregates on the MMN medium revealed several features: the fungal inoculum produced striking hyphal cords. Further, well developed-ramified mycelial aggregates were present all over the media when inoculated w/ host (FIG. 2.2A), which contrasted to the fungi w/o host (FIG. 2.2B) of the same age. The mycelial cords extended from the dichotomous roots and fungal inoculum (FIG. 2.2A). Strands formed with numerous hyphae grew in parallel direction and were attached to each other. On the contrary, the mycelial aggregates without host had a relatively lower number of strands but had a prominent apically diffused edge (FIG. 2.2C). The mycelial aggregates w/ host and w/o host appeared white to cream, cylindric, and had a smooth surface. The mycelial cords sometimes carried the thromboplerous hypha on their surface (FIG. 2.3A). The thromboplerous hypha (FIG. 2.3B) can also be found on the mycelia of *R. roseolus*. The mycelial aggregates diameters were 48.78 µm ±16.44 (range 23.27–79.76 µm, n=53) w/ host and 30.75 µm ±16.52 (range 14.73-75.39 µm, n=50) w/o host.

2.3.2 Morphological observation of explorative mycelial aggregates using light microscopy

Generally, the mycelial aggregates of *R. roseolus* w/ host and w/o host had a common longitudinal direction and had a spiral-like to pararel hyphal orientation within the aggregate (FIG. 2.4A). The joined branching hyphae generated a complex layer of mycelia (FIG. 2.4B) that often contained empty central tubular-hyphae with thick walls (FIG. 2.4C). The mycelial aggregates were mostly formed by 2–4 tubular hyphae (FIG. 2.4D) and were enclosed within a sheath of slender hyphae. The mycelial aggregates w/ host and w/o host consisted of 2 types of hyphae: (1) undifferentiated and colourless hyphae forming the main strands of the mycelial aggregates, sometimes with trumpet-like inflations at the hyphal conjunction, septa often present, partially or completely dissolved septa, scarce clamp connection, uneven and enlarged hyphae in some parts, and (2) thromboplerous hyphae with mainly a homogenous shape, brown coloured, melanized, sometimes branched, and inflated at the end point.

In the w/ host and w/o host mycelial aggregates, the hyphae had mostly thin

walls, except the tubular hyphae, which had thick-walled cells (FIGS. 2.5C and D), clamp connections at few numbers of hyphae and mainly not located in the center of the w/ host mycelial aggregates. The tubular hyphae were empty (FIGS. 2.4C and D; 2.5A and B), sometimes had the clamp connection (FIG. 2.9A) and melanized (FIGS. 2.9B and 2.10A), typically found in the center position, with partially (FIGS. 2.6 A and B) and completely (FIGS. 2.6C and D) dissolved septa w/ host and w/o host. The tubular hyphae (length × diameter) w/ host was 71.13 µm ± 17.94 (range 52.30–110.23 µm, n=11) × 5.49 µm ± 1.25 (range 2.84–9.53 µm, n=74), while that for w/o host was 98.02 µm ± 46.97 (range 45.22-211.48 µm, n=13) × 9.33 µm ± 3.09 (range 3.86-16.02 µm, n=78) (Table 2.1). The tubular hyphae were unbranched and mainly dispersed over the mycelial aggregates length. The septa diameter of the w/ host aggregates was consistently shorter than that of w/o host at the conjunction ($P<0.05$), and both had a narrow-shape (FIG. 2.7) for some septa.

The w/ host and w/o host mycelial aggregates bore the thromboplerous hyphae (FIG. 2.8A). Thromboplerous hyphae were common and easily differentiated from other hyphae based on the following features: extremely melanized, typically smooth on the surface with small indentation in some parts and inflated at the base. The general shape of the thromboplerous hyphae w/ host was mainly straight, while that w/o host was twisted, semi-twisted, and branch structure. The cells were mainly longer than 100 µm and up to 200 µm. A plethora of oil drops could be observed on the vicinity of this type of hypha. Thromboplerous hyphae were frequent and largely accumulated near the fungal inoculum (FIG. 2.8B) for w/o host and w/ host. This hypha was slightly differentiated (Table 2.2) by the size (length × diamater) between w/ host and w/o host (260.41µm ± 73.47, range 178.16–319.53 µm, n=3) × (3.96 µm ± 0.7, range 3.1- 5.03 µm, n=9) and (154.52 µm ± 81.32, range 57.94–330.92 µm, n=10) × (4.47 µm ± 1.62, range 1.72–7.65 µm, n=23), respectively (FIGS. 2.8C and D). Some thromboplerous hyphae w/o host had a strikingly larger diameter (FIG. 2.8C), clamp connection (FIG. 2.10B), incomplete septa, and sometimes branching. the tro,bhoplerous hyphae turned dark blue and sometimes green when stained with lactophenol cotton blue.

The peripheral hyphae w/ host and w/o host had similar features. They were thin-walled, branching in some points, anastomosed, clamped, and longitudinally but sub-irregularly arranged. Some peripheral hyphae also carried the thromboplerous hyphae. Both colourless and melanized hyphae could also be observed in some parts of the mycelial aggregates. Following the rhizomorph description by Agerer (2006), the assessment of the w/ host mycelial aggregates in

this study revealed non-amyloid hyphae, small needle-like crystal, boletoid, cystidia lacking or as short hyphae-like ends, emanating hyphae with rare clamp connection, anastomosed, hydrophobic extraradical mycelia, clustered and brownish ECM roots.

2.4 Discussion

2.4.1 Mycelial aggregates comparison with and without host

In the current study, I refer to the mycelial aggregates of *R. roseolus* w/ host and w/o host as mycelial cords as they appeared as apically diffuse mycelial aggregates based on Rayner et al. (1985) description. The mycelial cords of *R. roseolus* w/ host and w/o host are rather similar, with some cytological differences. The characteristics were consistent in other strains of *R. roseolus* (data not shown). The most notable features are the complexity of the aggregates, the tubular hyphae features (including size, partially and completely dissolved septa, the conjunction septa shape), and the thromboplerous hyphae.

The results showed that *R. roseolus* produced the mycelial cords w/ host or w/o host. In this study, the mycelial cords w/o host were less complex than those w/ host in topology and less apically dominant. In contrast, the mycelial cords w/ host were scattered all over the medium surface with extensive anastomosing aggregates, which interconnected from roots and the inoculum plugs. According to Agerer (2001, 2006), the mycelial cords w/ host in the current study were identical to the long-distance exploration type with smooth surface and highly differentiated rhizomorph of type F. The higher numbers and more complex strands of mycelial cords w/ host indicated an impact of host occurence to the fungal morphology in ECM symbiosis.

The mycelial cords w/ host and w/o host had a longitudinal orientation composed of several tubular/vessel hyphae which are enclosed by the smaller hyphae. This is consistent with the finding of Schweiger et al. (2002) who visualized a maximum of four inflated hyphae of rhizomorph of *Paxillus involutus* using a laser scanning confocal microscope. The number of inflated hyphae varies with the thickness of the rhizomorph (Schweiger et al. 2002).

2.4.2 Tubular hyphae comparison with and without host

The diameter of tubular hyphae was larger in the mycelial cords w/o host than w/host. The maximum size of the tubular hyphae diameter of mycelial aggregates was identical to that in the rhizomorph of *R. roseolus* colected from field (authors personal observation). In this study, I showed the direct evidence of the

chain of tubular hyphae which distributed over the length of the mycelial cords, w/o host than w/host. This finding strengthens the function of tubular hyphae as a solute translocation organ in long distance movement as highlighted by Cairney (1992).

My results prove that the tubular hyphae of *R. roseolus* had the approximately similar architecture w/ host and w/o host. Cairney (1991) suggested that the rhizomorph structure in wood decay and ECM fungi has a similar structure to the equivalent function as exploratory and migratory organ. In this study, I confirmed that the empty-tubular hyphae of w/o host (saprobic phase) had longer length and larger diameter than those w/ host. Previously, several other reports reported that the diameter of the tubular hyphae of saprobic fungi species are generally bigger than those of ECM fungi species (Duddridge et al. 1980; Jennings and Watkinson 1982; Thompson and Rayner 1983). In addition, the diameter septa of tubular hyphae conjunction w/ host were consistently shorter than those w/o host. Thus, the current result highlights the different diameters of tubular hyphae and the diameter of hyphal septa, which may correlate with solute transport in ECM and saprobic fungi. The plausible assumption might be related to the carbon translocation inside the hyphae. Finlay and Read (1986) indicated that the requirement for translocated carbon would diminish as sufficient carbon becomes available from the mycorrhizal symbiosis. Conversely, the saprotrophic fungi will require a high inoculum potential fuelled by carbon translocated from the rhizomorph base for establishment when invading the substrate (Garrett 1960).

The size of the tubular hyphae may impact the flow rate inside the hyphae (Heaton et al. 2010); however, the size of the septa and the septal dissolution associated with cord formation is more critical (Agerer 2006, 2007). I suggest that the septa diameter differences at the tubular hypha conjunction may also be related to the solute flow inside the tubular hyphae. The shorter diameter of septa of the tubular hyphae w/ host in this study is likely impede the translocated carbon flow in mycorrhizal symbiosis, as suggested by Finlay and Read (1986).

2.4.3 The dissolved tubular hyphae with and without host

The tubular hyphae septa that were either partially or completely dissolved with the occurrence or absence of the host were evident in this study. A recent study suggested that the septal dissolution may be directly impacted by the solute flows within the hyphae, as mechanical forces are large enough to degrade septa (Pieuchot et al. 2015). The dissolved tubular septa of *R. roseolus* have never been documented in previous studies. The dissolved septa were occasionally observed on the mature

strands both in w/ host and w/o host mycelial cords. Duddridge et al. (1980) and Brownlee et al. (1983) suggested that the loss of the cross walls of tubular hyphae reduces the flow resistance of solutions, resembling the vessels (xylem) of angiosperms.

According to Agerer (2006), the type of dissolved septa w/ host and w/o host of this study has the characteristic in boletoid rhizomorphs, which is poorly understood. The boletoid rhizomorph type has complex and highly differentiated tubular hyphae structure, including partially and completely dissolved septa (Agerer 2006). Furthermore, some of the observed tubular hyphae septa w/ and w/o host tended to form a narrower morphological shape in this study. I do not have prior knowledge and evidence regarding these narrower septa structure in fungal tubular hyphae. This finding might be related to the septum dissolution process in tubular hyphae. However, I need further evidence to support this hypothesis. Clamp connections were never found in mature tubular hyphae w/ host and only few (1–2 in 100 observation) in tubular hyphae w/o host. On the other hand, the clamp connection is commonly found in peripheral hyphae of mycelial cords. Agerer (2006) revealed the lack of clamps of emanating hyphae of *Rhizopogon*. However, information on the cytology and physiology of clamped tubular hyphae in ECM is scarce.

2.4.4 The comparison of thromboplerous hyphae with and without host

Thromboplerous (Th) hyphae (or generally noted as oleiferous hyphae) with brown intracellular content, are prominent and distributed all over the mycelial cords. The present study showed the cytological characteristics of Th hyphae and the host impact on this structure. Previously, most studies on Th hyphae are only on the occurrence or absence of this structure (Wartchow and Cortez 2016; Assis et al. 2018; Gelardi et al. 2019), without the information on its cytological aspects. Numerous oil drops can be spotted near this type of hyphae. Cytological features of thromboplerous hyphae were quite similar w/ host and w/o host. The most notable differences were the diameter and cell shape. Some thromboplerous hyphae w/o host had an exceptional large diameter compare to that w/ host. Lentz (1954) considered that the thromboplerous hyphae have an important physiological role that remains unclear. Recently, Clémençon (2005) argued that thromboplerous hyphae are used as a reservoir for material reserves (food or non-food) retrieved through the intrahyphal hyphae of saprobic fungi *Ossicaulis lignatilis*. However, this proposed role needs to be further confirmed, especially in relation to its function

in ECM symbiosis. In addition, no intrahyphal hyphae structure was found in the thromboplerous hyphae w/ and w/o host in this study.

2.4.5 Thromboplerous hyphae formation with and without host

The Th hyphae were found on fungal mycelia and on the surface of mycelial cords at the center and periphery of the fungal colonies, both with and without host. These findings are in contrast with those of Clémençon (2003) who reported the presence of Th hyphae in the depths of the agar medium. Previously, only Miller et al. (1983) described the 'melanized' hyphae from *R. roseolus* cultures with thick-walls, usually non-septated, occasionally swollen, and refractive features. However, I found that those hyphae were less refractive in the present study. In addition, I noted the half melanizing tubular hyphae w/ host and w/o host, which resembled the thromboplerous hyphae.

Clémençon (2005) previously hypothesized that the thromboplerous hyphae originates from the development of tubular hyphae. The tubular hyphae accumulate the cytoplasmic material and become dense and thick. Clémençon (2005) proposed that Th hyphae function as energy reservoirs in fungi as he observed the presence of intrahyphal hyphae inside Th hyphae. While no intrahyphal hyphae were found inside Th hyphae in the present investigation. However, this study suggests that Th hyphae play an essential role for *R. roseolus* as it is produced in all stages of development of the fungal colony. While further investigation is needed to confirm this, it may explain why larger Th hyphae were found accumulated near the fungal isolate than in any other part of the colony in this study. Substrate colonization and initiation of symbiosis require a high amount of energy from the saprophytic or mycorrhizal fungi (Cairney 1992; Smith and Read 2008).

I also found the tubular hyphae in process of melanizing with the accumulation of dense material. Based on its diameter and cytological features, I cogently hypothesize that the melanizing tubular hyphae develop the thromboplerous hyphae. This phenomenon is the evidence of the initial development of thromboplerous hyphae in *R. roseolus*. In addition, the presence of incomplete septa and clamp connection in the thromboplerous hyphae in this study aligned with the findings of Clémençon (2005). In this study, only thromboplerous hyphae of mycelia cords w/o host had septa and clamp connection. However, the present study found many smaller hyphae melanizing to Th hyphae than the tubular hyphae. Therefore, the results suggest that Th hyphae production is dominated by vegetative mycelia and not by the tubular hyphae.

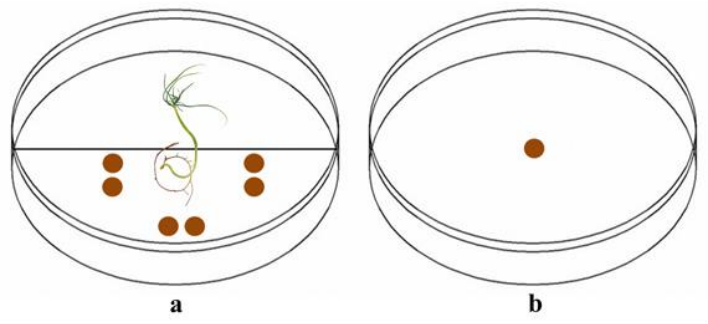

Figure 2.1 The experimental system. *Pinus thunbergii* inoculated with *Rhizopogon roseolus* in half size of a five-fold dilution of MMN medium (a). *R. roseolus* without host in same medium (b).

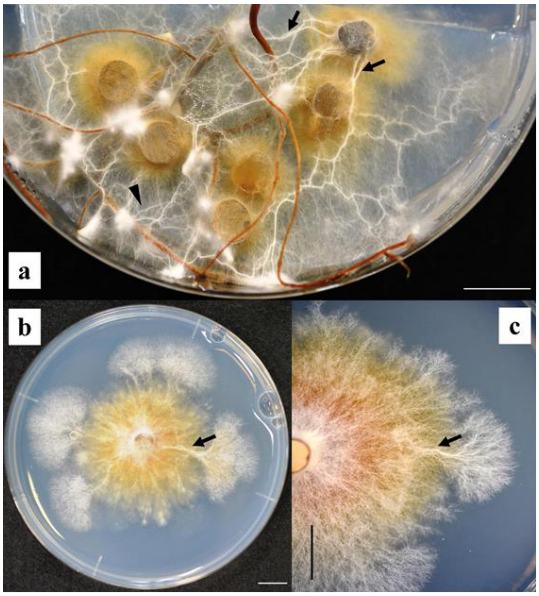

Figure 2.2 Comparison of mycelial aggregates of *Rhizopogon roseolus.* With host, extending mycelial networks from fungal colony (arrow) and roots (arrowhead) distributed over the medium surface (a). Without host, mycelial cords (arrow) only produced at some parts of the medium with prominent apical diffusion (b–c). Bars = 1 cm.

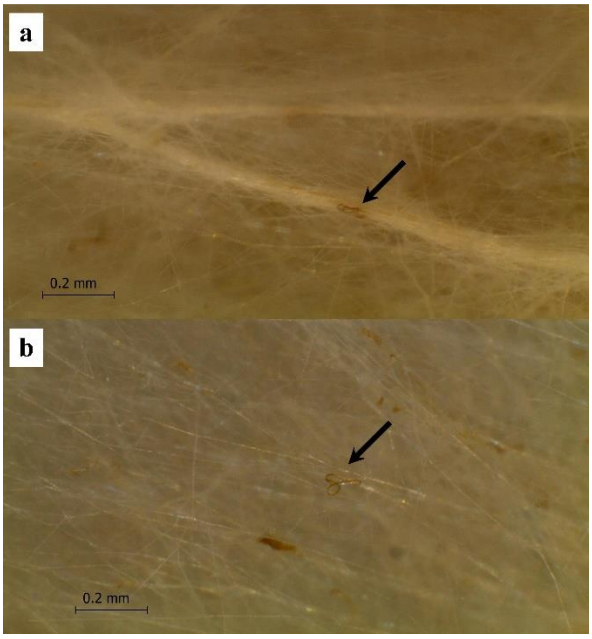

Figure 2.3 Mycelial aggregates and thromboplerous hypha. Typical mycelial aggregates of *Rhizopogon roseolus* with host and without host (a). Thromboplerous hypha (arrow) evident on the surface of mycelial aggregates (a) and mycelia (b).

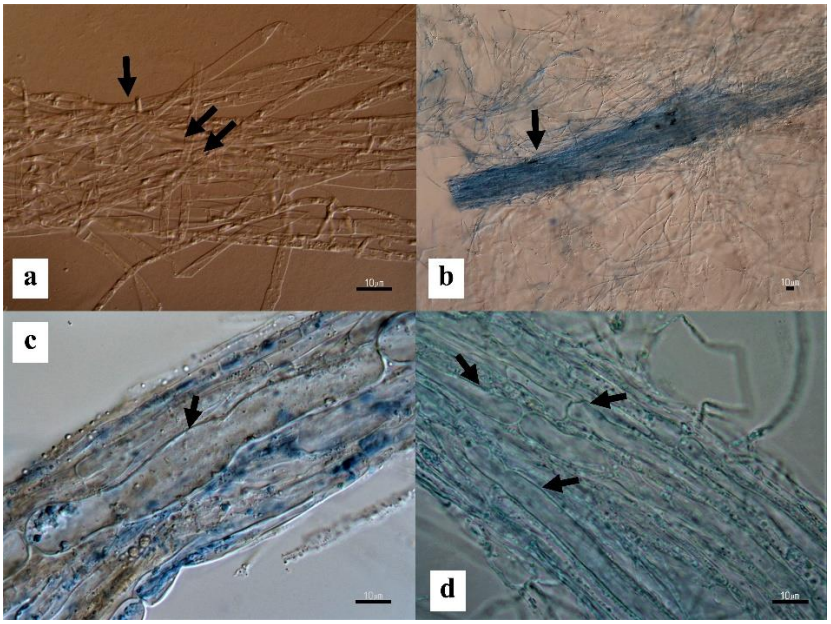

Figure 2.4 The characteristics of mycelial aggregates of *Rhizopogon roseolus.* General longitudinal direction (arrow) and spiral-like to pararel hyphal orientation (double arrows) of mycelial aggregates with host and without host (a). Typical complex layers (arrow) of mycelial aggregates with host and without host (b). Empty central vessel hypha of the mycelial aggregates with thick cell wall (arrow) (c). The layers (2–4) of tubular hyphae (arrows) of the mycelial aggregates (d). Bars = 10 µm.

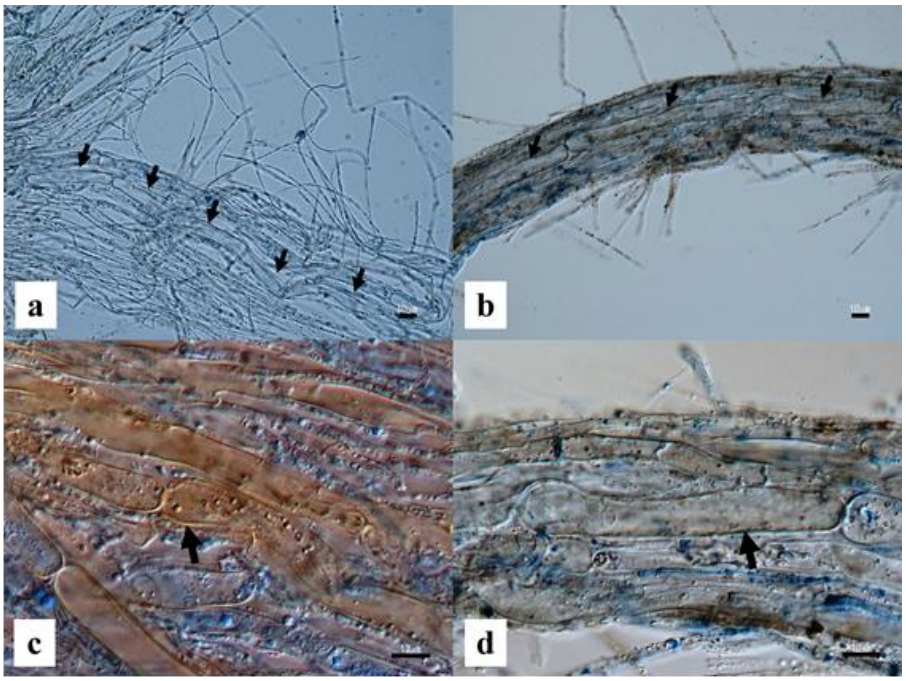

Figure 2.5 The characteristics of vessel hyphae of *Rhizopogon roseolus* with host and without host. Empty and connected (arrows) vessel hyphae with host (a) and without host (b). Thick-walled cells (arrows) of tubular hyphae with host (c) and without host (d). Bars = 10 µm.

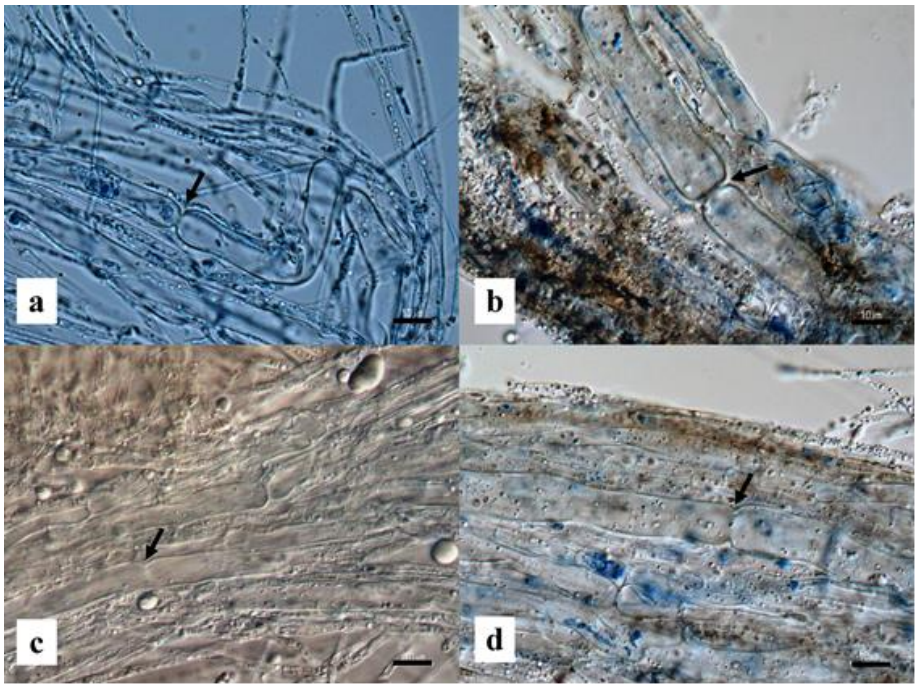

Figure 2.6 The dissolve septa of tubular hyphae of *Rhizopogon roseolus.* Partially (arrow) dissolved septa of tubular hyphae with host (a) and without host (b). Completely (arrow) dissolved septa of tubular hyphae with host (c) and without host (d). Bars = 10 µm.

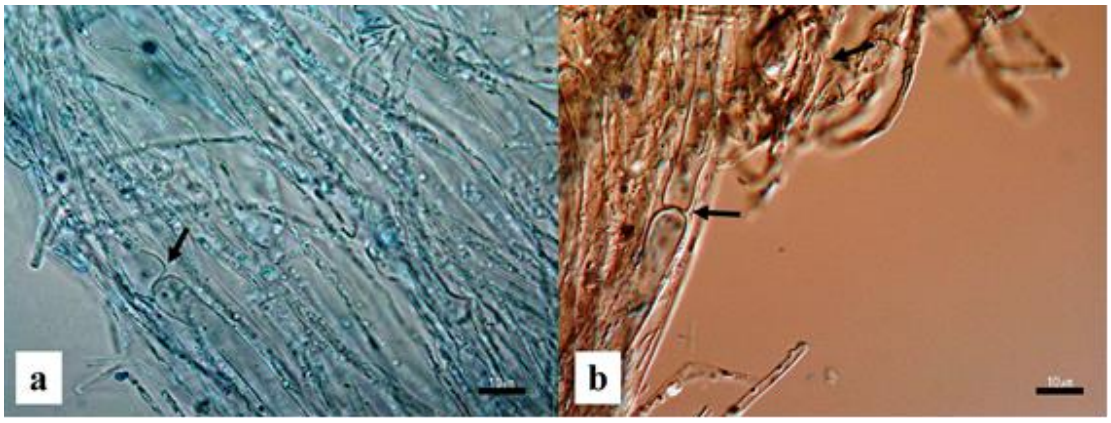

Figure 2.7 The shape of the adjacent septa of tubular septa of *Rhizopogon roseolus.* The narrow (arrow) shape of septa of vessel hypha with host (a) and without host (b). Bars = 10 µm.

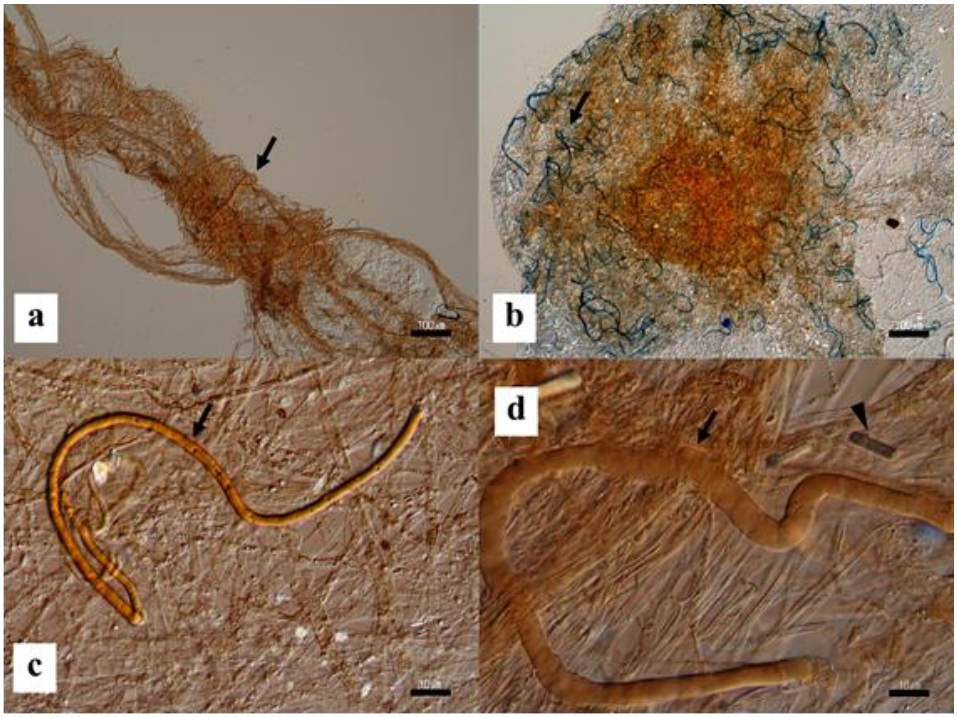

Figure 2.8 The thromboplerous hyphae of *Rhizopogon roseolus.* The mycelial aggregates bore the thromboplerous hypha (arrow) (a). Numerous thromboplerous hyphae (arrow) largely accumulated near the fungal inoculum (b). The general size of thromboplerous hypha (arrow) with host and without host (c). The exceptional large size of thromboplerous hypha (arrow) w/o host (note the needle-like crystals, arrowhead) (d). Bars = a–b = 100 µm, c–d = 10 µm.

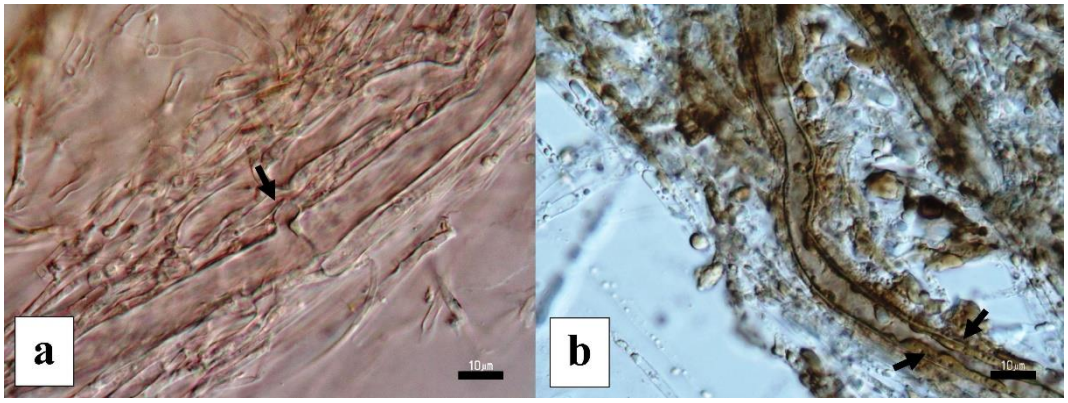

Figure 2.9 The unusual tubular hypha of *Rhizopogon roseolus*. The tubular hyphae without host with clamp connection (arrow) (a). The tubular hypha with thickened cell wall and accumulated cytoplasm materials (arrows) w/ host and w/o host (b). Bars = 10 µm.

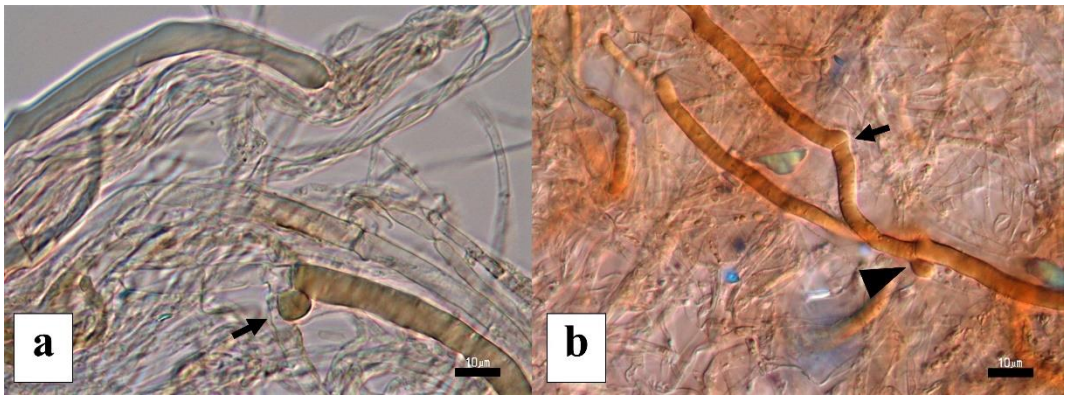

Figure 2.10 The unusual tubular hypha and thromboplerous hyphae of *Rhizopogon roseolus* without host. The clamped (arrow) tubular hypha melanizing into thromboplerous hypha by the condensed cytoplasm (a). The thromboplerous hyphae with septa (arrow) and clamp connection (arrowhead) (b). Bars = 10 µm.

Table 2.1 Comparison of the mycelial aggregates of *Rhizopogon roseolus* with or without host.

Characters	Fungi with Host	Fungi without Host
Mycelial cords	Produced	Produced
Rhizomorph-like type (Agerer 2006)	Boletoid	Boletoid
Mycelial cord orientation	Longitudinal	Longitudinal
Mycelial cord diameter (µm)	48.78 ± 16.44 (range 23.27–79.76, n=53)	30.75 ± 16.52 (range 14.73–75.39, n=50)
Tubular/vessel hyphae length (µm)	71.13 ± 17.94 (range 52.30–110.23, n=11)	98.02 ± 46.97 (range 45.22–211.48, n=13)
Tubular/vessel hyphae diameter (µm)	5.49 ± 1.25 (range 2.84–9.53, n=74)	9.33 ± 3.09 (range 3.86–16.02, n=78)
Tubular hyphae septa conjunction length (µm)	2.53 ± 0.89 (range 0.74–4.95, n=84)	5.12 ± 1.47 (range 2.77–9.28, n=60)
Tubular hyphae feature	Empty or semi-dense cytoplasm	Empty or semi-dense cytoplasm
Tubular hyphae distribution	Dispersed over the entire mycelial cords	Dispersed over the entire mycelial cords
Tubular hyphae with dissolved septum	Yes	Yes
Adjacent shape between tubular hyphae	Mostly flat but narrowed in some parts	Mostly flat but narrowed in some parts
Peripheral hyphae features	Thin-walled, branching in some points, anastomosed, clamped, longitudinally but subirregularly arranged, dense cytoplasm	Thin-walled, branching in some points, anastomosed, clamped, longitudinally but subirregularly arranged, dense of cytoplasm
Peripheral hyphae size (length × diameter) (µm)	47.55 ± 14.25 (range 24.49–78.34, n=13) × 5.58 ± 1.9 (range 1.79–8.99, n=25)	38.84 ± 17.25 (range 11.58–75.84, n=17) × 4.58 ± 1.65 (range 1.69–7.83, n=26)
Peripheral hyphae septa length (µm)	3.29 ± 1.11 (range 1.7–4.89, n=11)	3.00 ± 0.9 (range 1.9–4.65, n=11)
Clamp connection	Generally, on the peripheral hyphae	Generally, on the peripheral hyphae

Table 2.2 Comparison of the thromboplerous hyphae of *Rhizopogon roseolus* with or without host.

Characters	Fungi with Host	Fungi without Host
Thromboplerous hyphae	Produced, mainly near the fungal inoculum, only few near the roots	Produced, mainly near the fungal inoculum
Oil drop near the thromboplerous hyphae	Dense	Dense
Thromboplerous hyphae distribution on the mycelial cords	Yes	Yes
Thromboplerous hyphae distribution on the peripheral hyphae	Yes	Yes
Thromboplerous hyphae features	Extremely melanized, inflated at base, rarely branched	Extremely melanized, and inflated at the base, sometimes with clamp connection, septa, and branched
Thromboplerous hyphae shape	Mostly straight/elongated	Elongated, twisted, semi-twisted, and trichome-like structure
Thromboplerous hyphae size (length × diameter) (µm)	260.41 ± 73.47 (range 178.16–319.53, n=3) × 3.96 ± 0.7 (range 3.1–5.03, n=9)	154.52 ± 81.32 (range 57.94–330.92, n=10) × 4.47 ± 1.62 (range 1.72–7.65, n=23)
Thromboplerous hyphae septa length (µm)	Not found	4.53±1.72 (range 3.12–6.91, n=6)
Thromboplerous hyphae with clamp connection	No	Evident
The melanizing tubular hyphae into thromboplerous hyphae	Evident	Evident

Chapter 3
The impact of host plant (*Pinus thunbergii*) on the mycelial features of the ectomycorrhizal fungus *Rhizopogon roseolus*

3.1 Introduction

Ectomycorrhizal symbiosis has long been known to provide various benefits for plant and its fungal partners (Smith and Read 2008). Fungi play an essential role in the search for water and nutrients, thereby supporting the growth and development of their host plants (Tibbet and Sanders 2002; Smith and Read 2008). Ectomycorrhizal symbiosis affects the character of host plant organs, such as changes in the morphology and physiology of plant roots (Martin and Hilbert 1991; Smith and Read 2008). Conversely, the impact of host plants on the morphological characteristics of the mycelia of ECM fungi has rarely been reported. In addition, most prior studies sought to quantify and characterize mycorrhizal fungi primarily from arbuscular mycorrhizae.

Morphological characterization and quantification of mycelia are essential to obtain a better biological understanding of mycelial response to ectomycorrhiza (ECM) symbiosis and its cultivation aspects (Shimomura et al. 2012). The morphological parameters which are commonly considered in mycorrhizal forming fungi assessment include hyphal growth, hyphal length, hyphal anastomosis, and the number of branches near the hyphal tips (Declerck et al. 2004; Giovannetti et al. 2004; De La Providencia et al. 2005; Sbrana et al. 2007; De Novais et al. 2013; Sbrana et al. 2020), with no attention to the sclerotium production in agar medium. However, prior reports on those aspects of ECM fungi are scarce from both field and laboratory studies. In addition, the effect of the host on mycorrhizal fungi has only been reported at a higher biological level, where alterations occurred in the scope of the community trophic structure in arbuscular mycorrhizae (Eom et al. 2000) and ectomycorrhiza (Hoeksema et al. 2018). Reports on the impact of host plants on vegetative organs of ECM fungi are also scarce and thus warrant more assessments.

The hyphae of ECM fungi are the most poorly understood component of a symbiosis (Staddon et al. 2003; Cairney 2005; Anderson and Cairney 2007). Chen et al. (2013) revealed that an assessment of hyphae production is impractical to execute in the field. Hence, to understand mycelial response to host occurrence or absence, an in vitro approach is needed for the assessment of the morphological characteristics of ECM fungi and its quantification aspect. *Rhizopogon roseolus* is

one of the important edible ectomycorrhizal fungi in Japan, and research on its biological characteristics is ongoing (Shimomura et al. 2012). The fungi is suitable for use as a model for ECM forming fungi as it is easy to isolate and grow on laboratory media. Information regarding the impact of the presence or absence of the host on the mycelia characteristics of ECM forming fungi has never been reported. In addition, their quantification is essential to deepen current knowledge on ectomycorrhizal symbiosis and optimize its function. Hence, this chapter aimed to compare and quantify some of the mycelial features of ECM forming fungi with and without the ECM host.

3.2 Materials and methods

3.2.1 Biological materials and experimental settings

Rhizopogon roseolus (Corda) Th.M. Fr. (= *R. rubescens* Tul. & C. Tul.) TUFC10010, obtained from Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Japan, was employed as the ECM fungal species in this study. Briefly, the fungus was propagated on malt extract agar (MEA) containing 20 gr malt extract, 20 gr agar, and 1 L tap water at a final media pH of 5.5. Thereafter, the fungus was stored for 21 days at 25 °C in the dark before use. *Pinus thunbergii* was used as the plant host in this study. Surface-sterilized seeds of *P. thunbergii* were germinated in water agar at 25 °C for 1-2 weeks. Pine seedlings with fine lateral roots were transferred to a one fifth dilution of Modified Melin and Norkrans (1/5 MMN) medium with the addition of glucose and malt (rich medium) or without the addition of glucose and malt (poor medium) in a (90x10 mm) Petri dish (Figure 3.1). A layer of sterile cellophane membrane was placed on the surface of the medium (except the 40 plates for colony diameter assessment). Each Petri dish had one plug of 7-mm round inoculum and one seedling in 30 mL of solid 1/5 MMN medium. Host-free media were prepared with the same conditions. The combinations used in this study were fungi with host (WIH) on rich medium, fungi without a host (WOH) on rich medium, fungi WIH on poor medium, and fungi WOH on poor medium. The Petri dish was sealed with parafilm and incubated in a controlled chamber at 25 °C, 50% relative humidity, and 16-h d at 5000 lx, one month for colony diameter assessment, and three months for other observations.

3.2.2 Morphological assessment and quantification of mycelial features

A total of 120 plates of *R. roseolus* WIH and WOH were assessed. The mycelial features were observed using stereo and optical microscopes. The assessment of basic morphological features included anastomosis formation, hyphal branch, and chlamydospores-like structure. Colony growth was observed directly from plates. Anastomosis formation, hyphal branch, and chlamydospores-like structure were observed using an eclipse 80i light microscope at low magnification (Nikon, Tokyo, Japan). Details of anastomosis, chlamydospores-like structure, and branch of the hyphal tip structures were confirmed at higher magnification using slide cultures (Nugent et al. 2006) with modification of the medium. Images were captured with a DS-L2 digital camera (Nikon).

Mycelial features, including colony diameter, hyphal length, anastomosis number, and branch number of the hyphae, were quantified. Colony diameter was observed every five days for one month and ten plate replicates of each treatment were considered. Mycelial growth was determined using a ruler across the plate and the average of the vertical and horizontal colony diameter was calculated. Hyphal length was observed using a light microscope at 100x magnification. A total of 200 photos were obtained and used for hyphal length quantification using Hylength software (Cardini et al. 2020). Anastomosis numbers per hyphal length were determined using a light microscope at 100x magnification. A total of 600 photos were taken, and 40 photos per combination were selected. Anastomosis formation was counted, and hyphal length at the occurrence of anastomosis was assessed using Hylength software. Branch numbers were observed on the maximum length of 700 um hyphae from the tips. Tip length was confirmed using ImageJ. The data were subjected to analysis of variance (one-way ANOVA), and mean values were ranked by the Student-Newman-Keuls test at $P < 0.05$.

3.2.3 Investigation of sclerotium of *Rhizopogon roseolus* on poor medium

The scleroti produced on the explorative mycelia in 1/5 MMN-GM medium were observed using a Leica EZ4 stereo microscope. The sclerotia were then squashed, stained with lactophenol cotton blue, and examined using an Eclipse 80i light microscope (Nikon, Tokyo, Japan). Images were obtained using a Digital Sight DS-L2 digital camera (Nikon). The surface sterilized sclerotia were used to performed the spore isolation. Suspected spores of sclerotia were isolated using a single spore isolation method (Zhang et al. 2013) with some modification. The

suspected spores suspension containing 60% sterile glucose (or distilled water) was centrifuged at 3000 × g for 3 min and poured into TM7 medium (Shimomura et al. 2012c). The TM7 detection medium with suspected spores suspension was incubated for 5 days. Spore germination on the TM7 medium was observed every 24 h. The bacterial colonies that appeared after 4 days of incubation were transferred to the LA medium which generally used for bacterial cultivation and endobacteria isolation. The colonies from the TM7 and LA media were then Gram stained and observed under a light microscope.

3.3 Results

3.3.1 Morphological observation of the mycelial features

The use of cellophane membrane in this experimental setting allowed me to observe the mycelial characters in the loose growth area of hyphae via optimal imaging. Observations of anastomosis on rich media were only performed at the tip region (maximum 1 mm from hyphal tips) of growing fungal colonies; due to the thick hyphal growth occurred at the center of the colonies growing on rich media WIH or WOH, preventing the detection of anastomosis. On the other hand, observation of anastomosis in colonies growing on poor media (WIH and WOH) could be performed on all colony parts. However, to synchronize the data obtained, anastomosis observations were only made at the tip of the colony. In all experimental settings, anastomosis, which refers to the fusion of two parallel hyphae, was observed (Fig 3.2).

Cytologically, there was no difference between anastomosis characters under all experimental settings. Generally, anastomosis was initiated from two fused branches of hyphae. Some anastomosis initiations were also formed from only one branch of the hyphae (one way) that led to other hyphae without the formation of a branch or peg on the hyphae of its destination (Fig 3.3). Lateral branching of hyphae was dominant compared to apical branching in all assessments (Fig 3.4). Anastomosis never occurred from the apical branching of hyphae. Approaching hyphal tips from lateral branching generally formed branches with hyphae that are not straight/slightly crimped (Fig 3.4), never swollen, and led to other contact hyphae. The initiation of anastomosis with hyphal tips pointing toward each other was observed, depicting the growth orientation and suggesting hyphal signaling (but not yet fusion). Hyphal anastomosis was characterized by a short to medium length bridge of two parallel, thin, large trunk of hyphae in all experimental settings (Fig 3.5). In addition, hyphal fusions between two adjacent hyphae were

sometimes evident (Fig 3.5).

The chlamydospores-like structure was formed in the entire experimental set after three months of incubation (Fig 3.6). These structures were round, ovoid, and pyriform in shape and were generally found in the middle of the hyphae and clusters but were also found in a terminal position in some hyphae. This chain structure at the beginning of its formation resembles moniliform growth of hyphae. The structures with an ovoid shape had thinner walls than those with a round shape (Fig 3.7). Round and ovoid structures comprised one or two layers of cell walls. The two-layered cell wall layer was hyaline or brownish (melanizing) and thickened, with uneven thickening observed in some of the structures found. The structure had a whole/dense or empty cytoplasm with few spherical vacuoles/oils. These structures either possessed or did not possess septa when connected to vegetative hyphae.

3.3.2 Quantification of mycelial features

Fungi WIH consistently had higher colony diameter and growth per day both on poor and rich media in all observations (per 5 days monitored) than fungi WOH throughout the incubation period (Fig 3.8; Table 3.1). The colony diameter ranged from 3.22 ± 0.87 cm to 7.00 ± 2.02 cm, while growth per day ranged from 0.10 ± 0.04 to 0.23 ± 0.04. Fungi WIH on rich medium produced the longest diameter of the colony after one month compared to fungi subjected to the other treatments (Table 3.1). The colony diameter of fungi WIH and WOH on rich medium did not significantly differ from each other after one month. However, host occurrence impacts the diameter of fungal colonies on poor medium ($P<0.05$). Fungal colonies WIH on poor medium had diameters closer to those of fungal colonies WIH or WOH on rich medium. Fungi WIH on poor medium showed asymmetrical colony growth with dominance in one direction compared to fungi WIH on rich medium.

The diameter of the fungal colonies was confirmed by measuring the length of the hyphae using *Hylength* software. The colony diameter was found to be consistent with hyphal length, where fungi WIH on rich medium had higher hyphae lengths (76.44 ± 30.71, range 30.85-139.38, n=50) than fungi WOH on rich medium (64.85 ± 19.03, range 36.82 -118.97, n=50). Fungi WIH on rich medium had the longest hyphae length among all experimental sets compared with fungi WOH on poor medium, which had the shortest hyphae length (35.96 ± 12.62, range 20.68-71.88, n=50). Meanwhile, fungi WIH on poor medium had hyphae length

(51.31 ± 22.42, range 23.21-104.69, n=50) with values close to fungi WOH on rich medium. Statistically, hyphal length was significantly affected by host occurrence on both media (one-way ANOVA, $P<0.05$).

Anastomosis was observed in all experimental settings. The average number of anastomoses per hyphal length in fungi WOH was slightly higher than the number WIH on rich and poor MMN media. There was no significant difference between anastomosis with the host or without a host on both media ($P>0.05$). Thus, the occurrence of the host had no obvious impact on the anastomosis number. The average anastomosis number ranged from 14.2 to 15.13. Hyphal length during anastomosis ranged from 12.69 mm – 15.48 mm, and anastomosis per hyphal length (mm) ranged from 1.05 ± 0.45 mm^{-1} to 1.26 ± 0.72 mm^{-1}.

The average number of branches per hyphal length was higher WOH than WIH on poor and rich media. The branch number on poor medium was higher than that on rich medium. The occurrence of the host had a significant impact on branch formation on poor medium ($P=0.02$) compared with rich medium ($P=0.52$). The average branch number ranged from 3.28 to 3.52 per hyphal length in all experiments. The average hyphal length during branch formation ranged from 402.69 um – 492.29 um. The branch number per hyphal length (um) ranged from 7.82×10^{-3} um^{-1} to 11.87×10^{-3} um^{-1}. The diameter of the chlamydospore-like structure ranged from 9.85 ± 5.01 um to 22.08 ± 7.57 um. Further, this structure had a larger size with the host than without host on both poor and rich media. Fungi WIH on poor media had the highest diameter in all experiments. The occurrence of the host had a significant impact on the diameter of the structure on both poor and rich media ($P< 0.05$).

3.3.3 Investigation of sclerotium of *Rhizopogon roseolus* in agar medium

A limited number of sclerotia (1–2) were produced per Petri dish after 2 months of incubation (Fig 3.9); only 5 out of 20 Petri dishes contained the sclerotia. The sclerotia produced in each Petri dish appeared on the surface of the extraradical mycelia. The sclerotia in the current study were globose, light brown, rough in surface, and had an average diameter of 0.27 mm. Light microscopy revealed that the inner tissue of the sclerotia was light brown, uniform, pseudoparenchymatous, and contained thin-walled hyphae. Several small structures similar to spores were found in the sclerotium tissue. These structures were ellipsoid, hyaline, smooth at the surface and 1–1.5 μm in size.

When the sclerotium and these suspected spores inoculated to TM7 medium, there were no spore germination in the incubation span. Interestingly, the bacterial colonies which connected to the sclerotium hyphae were emerged after 4 days of incubation (Fig 3.10). They were then transferred to the LA medium. The bacterial colonies isolated from the TM7 and LA media in this study were round, white to cream-colored, smooth at the surface, entire edge, and convex in elevation. The examination under light microscopy followed by Gram stained, revealed the ellipsoid cells as the Gram-positive bacteria (Figure 3.11). They were of almost the same size as the spore-like structures inside the sclerotia (Fig 3.8; 3.10); these cells were evident from both TM7 and LA media.

3.4 Discussion

3.4.1 Colony diameter and hyphal length WIH and WOH

One-way ANOVA indicated a significant effect of the presence of the host on the colony diameter of *R. roseolus* on poor medium only. Previously, no information regarding a comparison of mycelia growth (colony diameter) of ECM fungi WIH or WOH is available. Smith and Read (2008) suggested that mycorrhizal symbiosis in nutrient-rich media did not provide significant benefits to the host; but no details of its impact on the growth of fungal hyphae. Here, I presented evidence of a significant effect of host on mycelia growth (radial colony) on poor medium, and not in nutrient-rich medium. Gavito and Olsson (2008) argued that mycorrhizal mycelium growing in nutrient-rich medium in plates is likely foraging for carbon, instead of nutrients or water. My result might be indicated that carbon is an important limiting resource which related to the host impact to fungal colony diameter in present study.

Radial growth is a good measurement approach for fungal colony. However, it does not consider the fungal vertical growth or the increase in density in the Petri dish (Miyashira et al. 2010). My data confirmed that the colony diameter assessment had the same pattern as hyphal length when quantified using Hylength software. The results revealed a significant impact of the host on the hyphal length of *R. roseolus* on both rich and poor media. The hyphal length WIH was consistently higher than that WOH on both media. The present study proved that *R. roseolus* produced higher hyphae during ECM symbiosis than without the host. Prior knowledge of hyphae production by mycorrhizal-forming fungi during symbiosis was incomplete. Chen et al. (2016) revealed that ECM tree can improve its foraging strategy by producing more hyphae (extraradical mycelia) from the mantle structure.

However, the regulation of fungal hyphae production by ECM fungi when growth WIH and WOH is remain unclear. External factors, such as nutrition, may influence the fungal growth and the production of mycorrhizal-forming fungal hyphae (Gavito and Olsson 2008). The results obtained in this study need to be tested with other fungal ECMs to determine whether this is common for other ECM fungal species.

3.4.2 Anastomosis comparison WIH and WOH

Most reports on the assessment of hyphal anastomosis were mainly focused on arbuscular mycorrhiza (Giovanetti et al. 2001; 2004; De La Providencia et al. 2004; Pepe et al. 2016), instead of ECM fungi, with the report by Sbrana et al. (2007) as the prime example. This study revealed that anastomosis formed in ECM forming fungi had similar characteristics both WIH and WOH. According to my results, the fusion process leading to anastomosis formation belonged to tip-to-side and tip-to-tip morphological types following the description of Hickey et al. (2002), both WIH and WOH. Several previous reports confirmed that the anastomosis of hyphae is strongly influenced by fungal species (De La Providencia et al. 2004) and plant species (Giovanetti et al. 2004). However, those observations were derived from arbuscular mycorrhiza.

This study is the first attempt to quantify the anastomosis number in ECM fungi. The use of cellophane allowed us to detect and count anastomosis in the loose growth area of hyphae and obtain optimal imaging. The number of anastomoses formed per hyphal length (mm) did not significantly differ WIH and WOH. This result was in contrast to previous reports which indicated the significant plant effect to the anastomosis formation (Giovanetti et al. 2004; Sbrana et al. 2011). In this study, the number of anastomoses per hyphal length markedly varied compared to that found in other studies by Giovanetti et al. (2001; 2004) and De La Providencia et al. (2005). The anastomosis number WOH was slightly higher than WIH in both media, eventhough they had a lower diameter colony and hyphal length number. This contrasting pattern suggests that a higher number of anastomoses might not always be positively correlated with hyphal production of ECM fungi with and without a host. Purin and Morton (2013) indicated that hyphal anastomosis contributes to colony formation of mycorrhiza fungi and may differ in function at the symbiotic and asymbiotic phase.

3.4.3 Hyphal branching comparison WIH and WOH

Lateral hyphal branching was dominant in comparison to apical branching in all set of experiments. According to Morrison and Righelato (1974), colony radial growth and hyphal density are affected by hyphal branching, which may be a determinant of the width of the peripheral growth zone. Besides contributing to colony formation, hyphal branching is also an essential process in anastomosis (Dikec et al. 2020). In present study, there was no significant impact of the host on the rich medium's branch formation, in contrast to that of poor medium. Akiyama et al. (2005) reported the effect of the host in triggering the formation of extensive hyphae branches in arbuscular mycorrhizae, with strigolactones as the compound that facilitates the process. However, in this study, poor medium WOH led to higher branches than WIH, and the reason is remained unclear. Harris (2008) explained that the interaction with a plant is an external factor that regulates branching formation in fungi, which results in induction or suppression of the branch. Further observation on the regulation of branch formation by ECM fungi, especially *R. roseolu*s WIH or WOH, is needed. Although they had the highest branch number and anastomosis values, fungi WOH had the lowest hyphal length and colony diameter values. This finding might be due to their thinner colonies than those WIH.

3.4.4 Chlamydospore-like structure formation

Of note, I reported for the first time the hyphal pattern of chlamydospore-like structure of *R. roseolus*. Some of the hyphal patterns are known to be modified by the nutritional state of the medium on which the fungal colony is growing (Ritz et al. 1996). In addition, the swollen hyphae can be induced by environmental factors, such as acid media (Bent and Morton 1963) or the induction of certain chemical compounds (Nonomura et al. 1996). However, in this study, chlamydospore-like structures were naturally produced on rich and poor media, with or without host. This structure had a larger diameter than that without host when associated with the host on both media, and is statistically affeced by the presence of the host. I argue that this structure is helpful for ectomycorrhizal symbiosis in the field as part of the survival mechanism in sandy soil (author's personal observation). The structures observed in this study share some characteristics with the germ vesicles of spores of *R. roseolus* reported by Martin and Gracia (2000), especially in smaller structures with a thin cell wall layer and a pyriform shape. Meanwhile, on some extent, the larger structures have similarities

to the chlamydospores.

3.4.5 The unusual sclerotium formation of *Rhizopogon roseolus* in agar medium

During the assessment of mycelial cords of *Rhizopogon roseolus* on poor nutrient of Modified Melin-Norkrans (MMN) medium, I found some sclerotia produced on the surface of extraradical mycelia. The sclerotia were 0.27 mm in average of diameter and produced after 2 months of incubation. The host-free media in the current experimental setting could be another factor for the induction of sclerotium development. Sclerotia contribute to survival in stress conditions such as the absence of a host (Smith et al. 2015). The absence of sclerotia on the rich MMN medium (with glucose and malt) with or without the host was also confirmed.

The current knowledge defined the sclerotium as mass of hyphae and normally having no spores in or on it. However, I found and suspected the small structures like spores (1-1.5 um) inside the sclerotium. These structures were ellipsoid, hyaline, with the smooth surface. In this study, the small ellipsoid structures were found inside the sclerotium. Initially, they were suspected to be the spores of *R. roseolus.* Further investigations confirmed that these structures were identical in shape to the spores of *R. roseolus* observed in the field but were smaller in size (Shimomura et al. 2012a).

I then incubated the sclerotium and these small structures on TM7 detecting medium whether they can produce the secondary mycelia of *R. Roseolus*, but no germination was observed. Interestingly, the bacterial colonies which connected to hyphae of sclerotium were appeared. The colonies were transferred to Luria agar (LA) medium. The morphological observation of bacterial cells from TM7 and LA confirmed that they were the same as small structures inside the sclerotium. This is the first information on production of unusual sclerotium of *R. roseolus* in pure cultures. Further study is required to reveal the role of bacteria on production of sclerotium of *R. Roseolus*

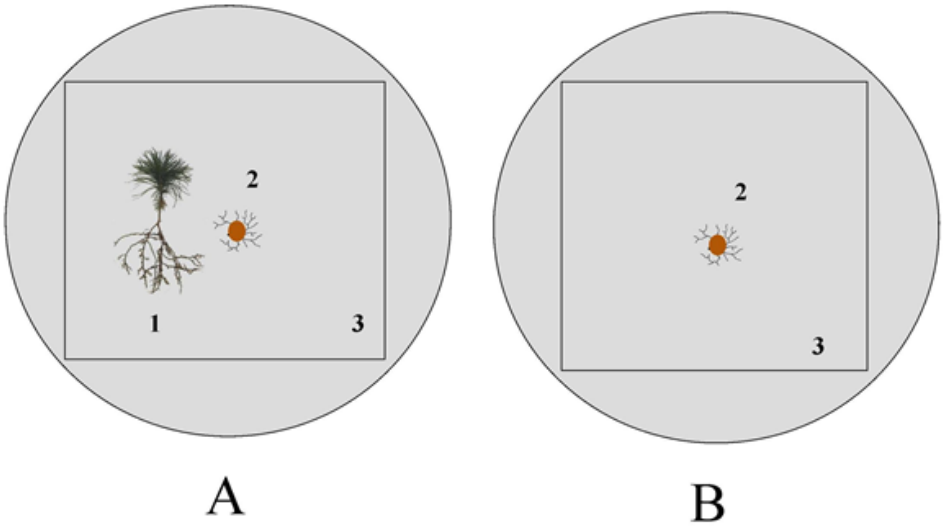

Figure 3.1 The experimental system. *Pinus thunbergii* (1) inoculated with *Rhizopogon roseolus* (2) in 1/5 dilution of MMN medium with or without glucose and malt (A). A layer of sterile cellophane membrane (3) was placed on the surface of medium. *Rhizopogon roseolus* (2) WOH in 1/5 dilution of MMN was prepared under the same condition (B).

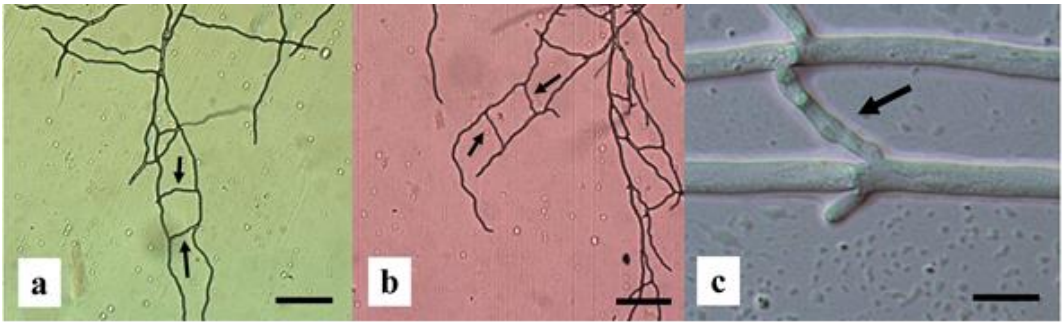

Figure 3.2 Anastomosis formation (arrows) of *Rhizopogon roseolus*. Anastomosis formation at lower magnification (a-b) and higher magnification (c). Bars: a-b = 100 µm, c = 10 µm.

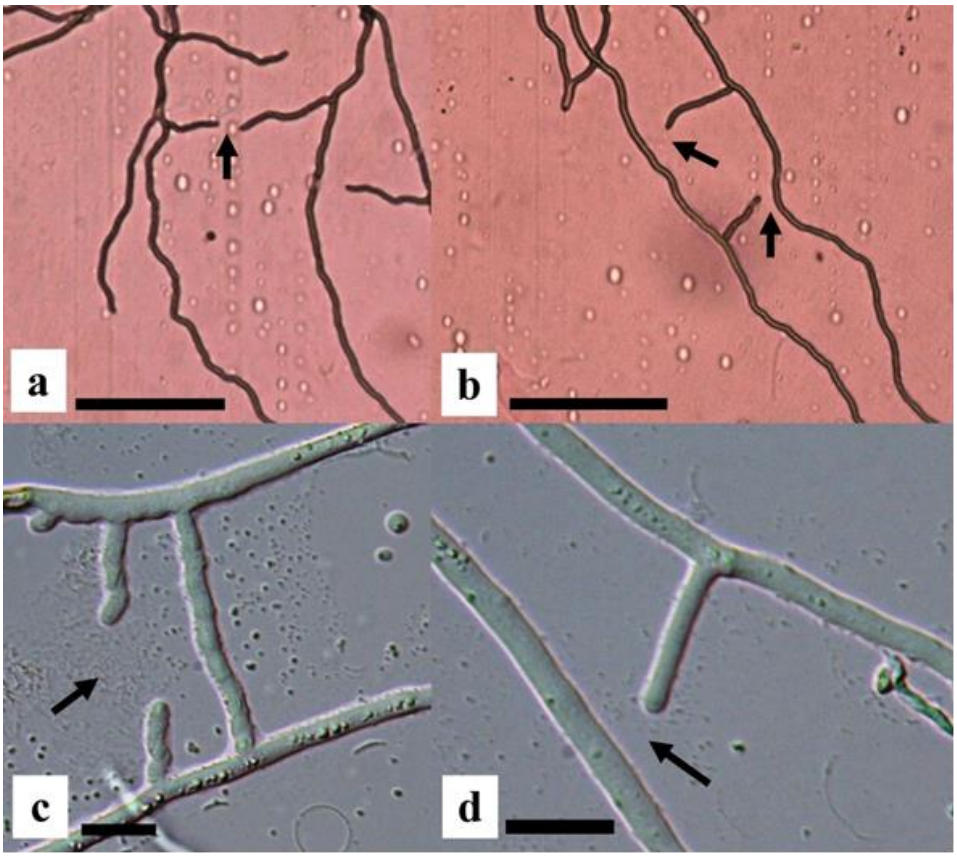

Figure 3.3 Initial anastomosis formation (arrows) of *Rhizopogon roseolus*. Tip-to-tip anastomosis formation (a & c). Tip-to-side anastomosis formation (b & d). Bars: a-b = 100 µm, c-d = 10 µm.

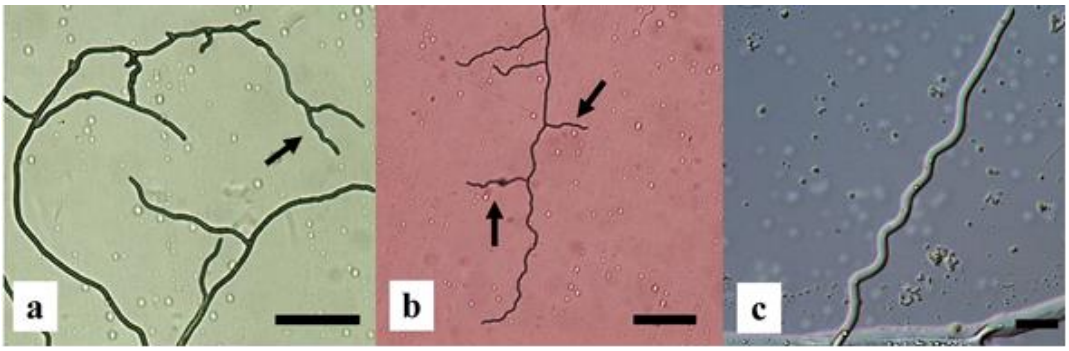

Figure 3.4 Hyphal branching (arrows) of *Rhizopogon roseolus*. Apical branching (a). Lateral branching (b). Crimped branching of hyphae (c). Bars: a-b = 100 µm, c= 10 µm.

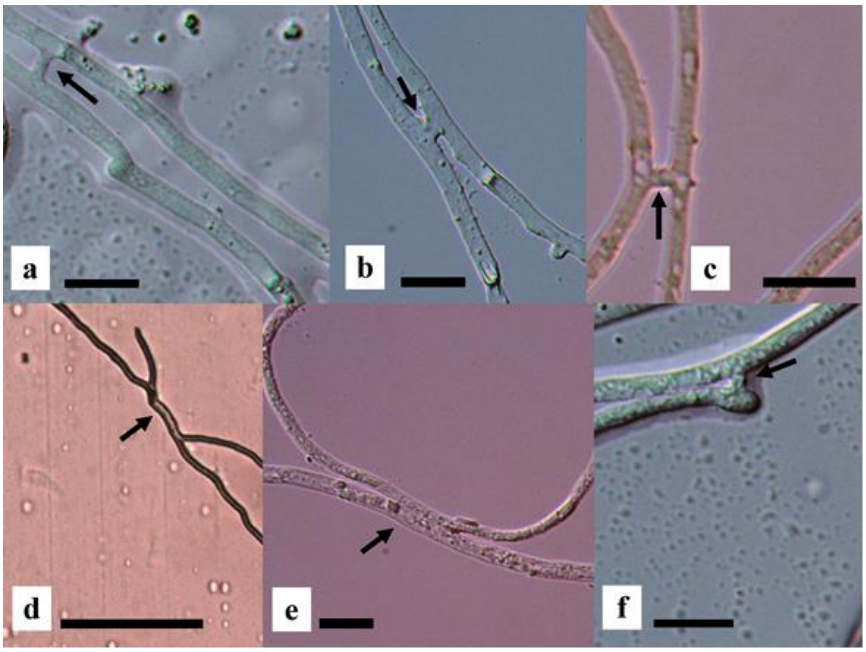

Figure 3.5 Anastomosis of *Rhizopogon roseolus*. Anastomosis bridges (arrows) of two hyphae (a-c). Hyphal fusion (arrows) of two adjacent hyphae (d-f). Bars: a-c, e,f = 10 µm, d = 100 µm.

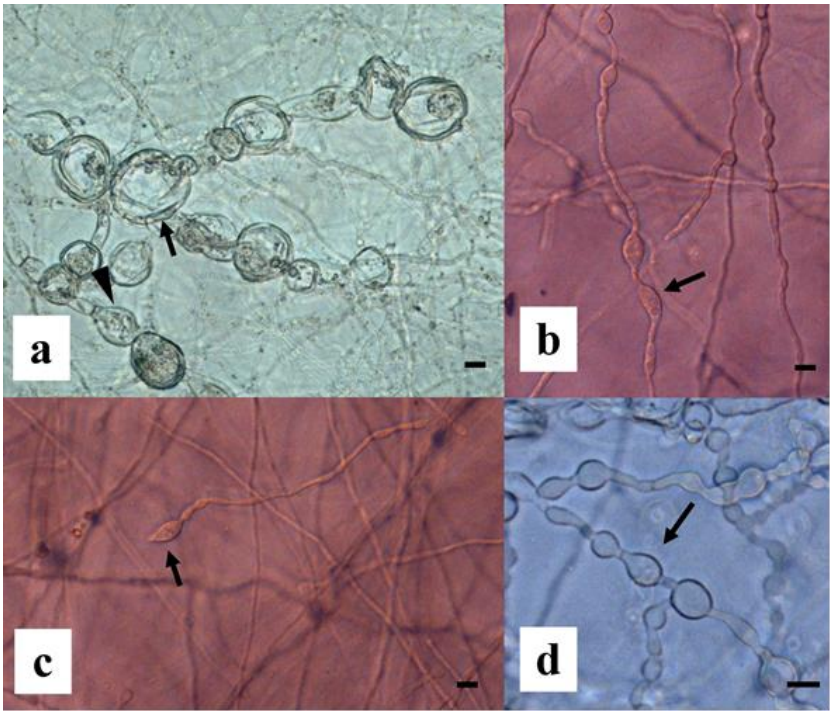

Figure 3.6 Chlamydospore-like structures of *Rhizopogon roseolus*. Round (arrow) and ovoid (arrowhead) shape of the structures (a). Pyriform shape at the middle (b, arow) and terminal hyphae (c, arrow). Moniliform (arrow) growth of hyphae. Bars =10 µm.

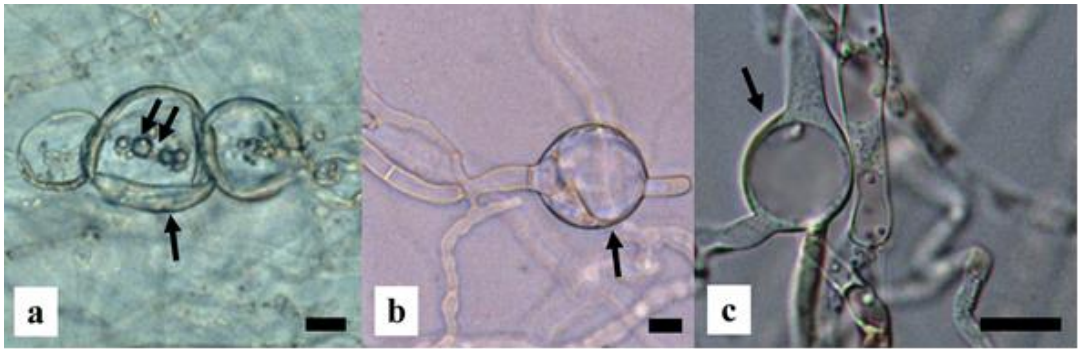

Figure 3.7 The characteristics of chlamydospore-like structures of *Rhizopogon roseolus*. The structures with thick layers of cell wall (arrow), uneven thickness, and some interior granules (double arrows) (a). The structure with a thin layer (arrows) of cell wall (b-c). Bars =10 µm.

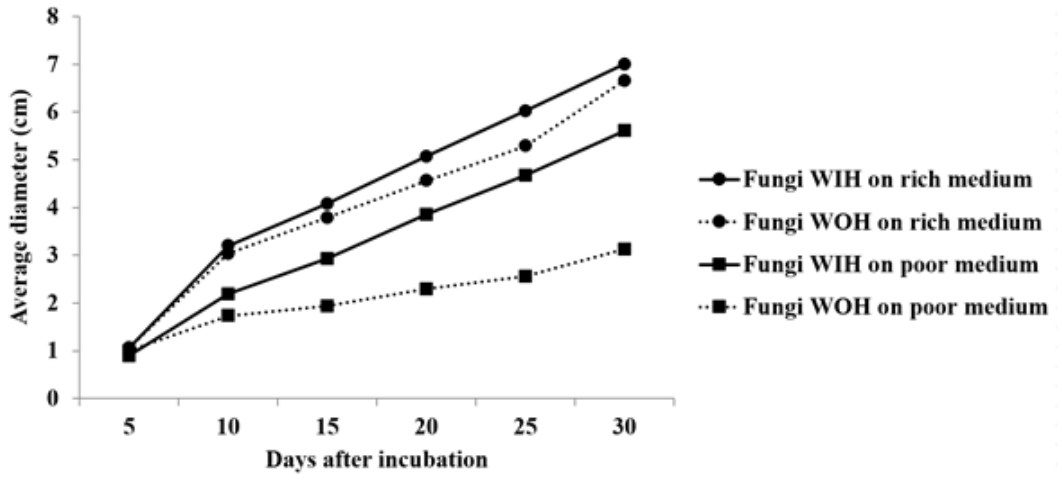

Figure 3.8 Colony diameter of *Rhizopogon roseolus* WIH and WOH on rich and poor 1/5 dilution of MMN medium.

Table 3.1 Comparison of the hyphal coils diameter and width of *Rhizopogon roseolus* with host (WIH) and without host (WOH) on rich and poor 1/5 dilution of Modified Melin and Norkrans medium. Values in a column followed by different letter differ significantly at $P < 0.05$ (Student-Newman-keuls) WIH and WOH on the same medium.

Combination	Colony diameter after 1 month of incubation (cm)	Colony diameter growth (cm/day)	Hyphal length (mm)	Anastomosis number (mm^{-1})	Branch number (μm^{-1})	Chlamydospore- like structure diameter (µm)
Fungi WIH on rich medium	7.00 ± 2.02^{a}	0.23 ± 0.04	76.44 ± 30.71^{a}	1.05 ± 0.45^{a}	$7.82 \times 10^{-3} \pm 0.004^{a}$	19.51 ± 7.57^{a}
Fungi WOH on rich medium	6.66 ± 1.86^{a}	0.22 ± 0.04	64.85 ± 19.03^{b}	1.25 ± 0.53^{a}	$8.54 \times 10^{-3} \pm 0.004^{a}$	11.67 ± 5.01^{b}
Fungi WIH on poor medium	5.61 ± 1.84^{a}	0.19 ± 0.01	51.31 ± 22.42^{a}	1.13 ± 0.32^{a}	$8.98 \times 10^{-3} \pm 0.005^{a}$	22.08 ± 7.57^{a}
Fungi WOH on poor medium	3.22 ± 0.87^{b}	0.10 ± 0.04	35.96 ± 12.62^{b}	1.26 ± 0.72^{a}	$11.87 \times 10^{-3} \pm 0.004^{b}$	9.85 ± 5.01^{b}

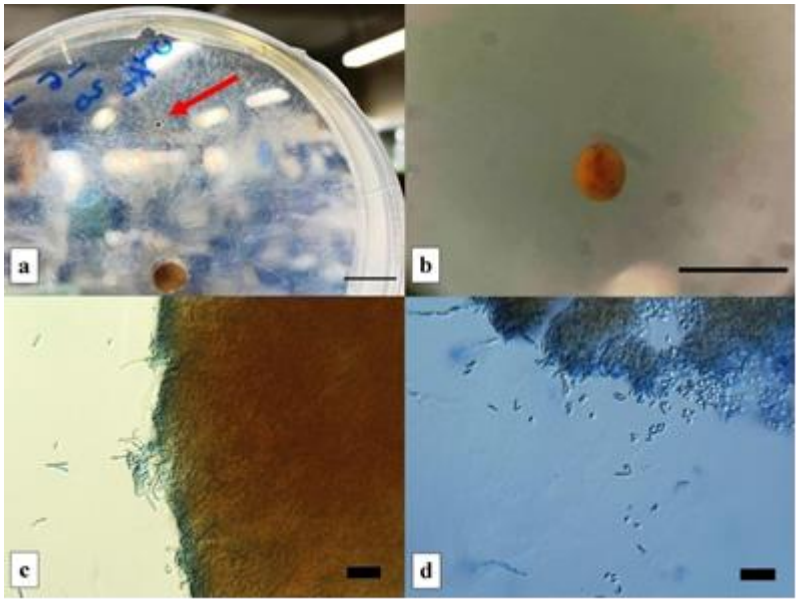

Figure 3.9 The sclerotium of *R. roseolus* in this study. a) Sclerotium production on extraradical mycelia in laboratory cultures (arrow). b) Sclerotium colour and shape. c) Tissue layer of sclerotium. d) The suspected spores inside the sclerotium. Bars= a: 1 cm, b: 0.5 mm, c-d: 10 µm.

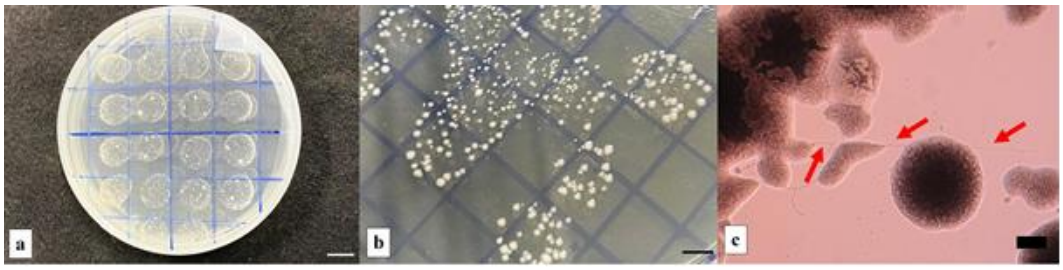

Figure 3.10 No germination of sclerotium of *R. roseolus* on TM7 medium. a-b) Bacteria colonies appeared after 4 days of incubation. c) Sclerotium hyphae (arrows) connected to bacteria colonies. Bars= a-b: 1 cm, c:10 µm.

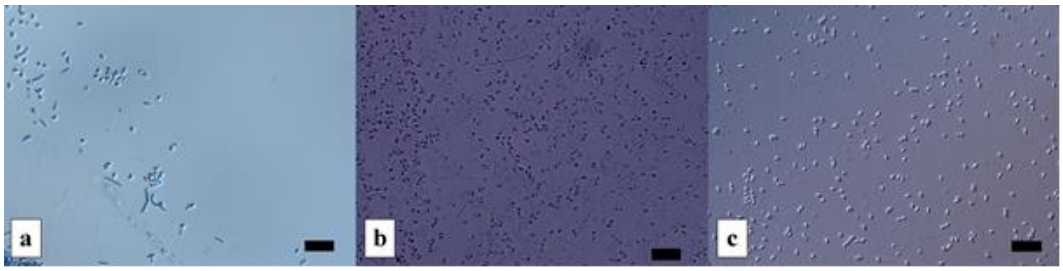

Figure 3.11 The comparison of shape and size of bacterial cells from sclerotium of *R. roseolus*. a) The observation of bacteria from the inside of sclerotium. b) The observation of pure cultures of bacteria on TM7 medium. c) The observation of pure cultures of bacteria on LA medium. Bars: 10 µm.

Chapter 4
Hyphal coil morphogenesis and its role as a source of thromboplerous hyphae in *Rhizopogon roseolus*

4.1 Introduction

The hyphal coil of mycorrhiza is the hypha that forms a loop within a plant root cell (Peterson et al. 2004). In some mycorrhizal associations (ectendomycorrhizas, arbuscular mycorrhizas, orchid mycorrhizas), intracellular penetration occurs, and hyphal coils are mainly produced in autotrophic cells (Peterson et al. 2004; Deguchi et al. 2017; Smith and Read 2008; Sathiyadash et al. 2012). However, information on the coiled hyphae of ectomycorrhizal (ECM) fungi is scarce. Villarreal-Ruiz et al. (2012) reported the ability of ECM fungi to form intracellular hyphal coils with ericaceous plants. In addition, Luoma et al. (2011) described extracellular coiled, dark-walled hyphae on the sporocarps and rhizomorphs of *Rhizopogon vesiculosus* with important taxonomic value. I recently observed that the hyphae of *R. roseolus* also produce coiled hyphae, which have a potential role in the formation of thromboplerous hyphae.

Thromboplerous hyphae are commonly known as oleiferous hyphae, and their development and function are unclear. This oily structure is found in the basidiocarp tissue of many agarics (Lentz 1954; Smith and Zeller 1966; Moreau et al. 2012; Vizzini and Ercole 2012; Gelardi et al. 2019). Previously, the only comprehensive report of the possible development and function of thromboplerous hyphae was from the rhizomorph of *Ossicaulis lignatilis* (Clémençon 2005). These thromboplerous hyphae are derived from the tubular hyphae of the rhizomorph (Clémençon 2005).

To date, there have been no studies reporting the correlation between thromboplerous hyphae and hyphal coils in ECM fungi, the morphogenesis of hyphal coils in ECM fungi, and the role of coiled hyphae in thromboplerous hyphae morphogenesis. In this study, I indicated another way to develop thromboplerous hyphae through the coiled hyphae of *R. roseolus.* Hence, this chapter aimed to characterize the coiled hyphae of *R. roseolus* from laboratory cultures and to document the formation of thromboplerous hyphae via hyphal coils.

4.2 Materials and methods

4.2.1 Biological materials

The ECM fungi *R. roseolus* (Corda) Th.M. Fr. (= *R. rubescens* Tul. & C.

Tul.) TUFC10010 was obtained from the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Tottori, Japan. The fungi were prepared on malt extract agar containing 20 gr malt extract, 20 gr agar, and 1 L tap water with a final medium pH of 5.5. Following incubation for 21 days at 25 °C, a fungal colony that had developed was used to inoculate the Black Japanese pine (*Pinus thunbergii)* as the plant host. Surface-sterilized seeds of *P. thunbergii* were germinated on water agar at 25 °C for 1–2 weeks. The pine seedlings with fine lateral roots were transferred to a five-fold dilution of Modified Melin and Norkrans (1/5 MMN) medium with (rich medium) or without (poor medium) the addition of glucose and malt in a 90 × 10 mm Petri dish (Figure 4.1). The combinations used in this study were fungi with the host in rich medium, fungi without a host in rich medium, fungi with the host in poor medium, and fungi without a host in the poor medium. Each Petri dish contained a 7 mm diameter inoculum of fungi with or without one seedling on 30 mL of solid 1/5 MMN medium. The Petri dish was sealed with parafilm and incubated in a controlled chamber at 25 °C, 50% relative humidity, and 16-h illumination at 5000 lx, for 3 months.

4.2.2 Morphological characterization of hyphal coils

Sixty plates of *R. roseolus* with and without the host were observed. The hyphal coils were observed at low magnification using a light microscope, Eclipse 80i microscope (Nikon, Tokyo, Japan). Detailed coil structures were confirmed in slide cultures at higher magnification under the same microscope (Nugent et al. 2006) with modification of the medium, fungal plug position, and plant host occurrence (Figure 4.2). The samples were then mounted with distilled water. Images were captured using a DS-L2 digital camera (Nikon). The assessed morphological features of coiled hyphae included hyphae septum, coil shape, coil position, coil formation, coil layer(s), coiled hyphae width, hyphal coil loop, thromboplerous hyphae formation, and comparison of melanizing coiled hyphae. The coiled hyphae were also morphologically compared with thromboplerous hyphae from the same mycelia and mycelial cords of *R. roseolus*. The hyphal coil loop, hyphal coil width, and melanized hyphal coil width were subjected to an analysis of variance (one-way ANOVA). Mean values were ranked by the Student-Newman-Keuls test at $P < 0.05$.

4.3 Results

The ECM structures (white to cream, Figure 4.3) were formed between *P. thunbergii* and *R. roseolus* both in rich and poor agar media. The hyphal coils in all experimental treatments showed the same characteristics. Coils were formed in both rich and poor media conditions, with host (WIH) or without the host (WOH). Hyphal coils were originally formed from margin of fungal colony. The initiation of hyphal coils occurred from the tip of the hyphae, with hook-shaped hyphal indentations (Figure 4.4a and 4.4d). The hook then coiled further (Figure. 4.4b, 4.4c, and 4.4e) to form fused rope-like hyphal strands (Figure 4.5a) followed by an extensive complex of coil layers (Figures. 4.6c and 4.6d). The coiled hyphae had the same width as the adjacent vegetative hyphae. The hyphal coils were round (Figure 4.5b), oval (Figure 4.5c), and ellipsoid (Figure 4.5d) in shape. Some of the coils decayed as their cytoplasm leaked, but most coils retained their structure as the colony continued to grow and formed multiple loops (Figure 4.5a). The hyphal coils were aseptate and composed of one (Figure 4.6a) to several layers (2–5) of hyphae (Figures 4.6b-d). The coils can be observed on the hyphal tip (Figure 3.5c) or in the middle section (Figure 4.5b) of hyphae when the hyphal tip continued growing outside the loop.

Some anastomosis and hyphal fusion were evident in the coiled hyphae, between coils and the adjacent hyphae through an anastomosis bridge (Figures 4.7a-b), and fusion between the hyphal coil and the adjacent hyphae (Figure 4.7c). The terminal part of the coiled and melanized hyphae was separated from vegetative hyphae by a simple septum (Figures 4.8a–d) or sometimes by a clamp connection. The melanized part of the mature coil broke off and became thick condensed hyphae that were scattered throughout the colony (Figure 4.8e). The melanized hyphae were thick-walled, pale to dark brown, extremely melanized, smooth with some cracks on the surface, and were reminiscent of oil. These characteristics confirmed that the coiled hyphae, produced by *R. roseolus*, were thromboplerous hyphae. In addition, I also noted the larger diameter of the thromboplerous hyphae derived from the mycelial cords of *R. roseolus* (FIG. 8f), not from the coils.

The coiled hyphae loop diameter of *R. roseolus* WOH was consistently larger than WIH in both rich and poor media (Table 4.1). The occurrence of the host had a significant impact on the coiled hyphae loop diameter in both the rich and poor media ($P<0.05$). Conversely, the host had no impact on the width of the hyphal in coils in both media ($P>0.05$), and the width of melanized hyphal in coil on the rich medium (Table 1). The host had an impact on the width of the melanized hyphal

in coils only on the poor medium. The hyphal coils had a similar width to the adjacent vegetative hyphae (Table 4.1). In addition, the average hyphal width of melanized hyphae in coils was slightly shorter than that of the thromboplerous hyphae from the mycelia and mycelial cords of *R. roseolus* (data not shown).

3.4 Discussion

In this study, hyphal coils were observed WIH and WOH in both rich and poor MMN media. In some fungi, the hyphal coil is affected by several factors, such as medium nutritional content (Persson et al. 1985), the role of bacteria (Hildebrandt et al. 2002), and hyphal penetration process in the plant host tissues (Peterson et al. 2004; Smith and Read 2008). I found that the coil loop WIH had a higher diameter than WOH in both media. The reason for this is unclear. Furthermore, the hyphae of coils had the same width as the adjacent vegetative hyphae, in contrast to the coil of *Arthrobotrys oligospora*, which is twice the diameter of normal hyphae (Persson et al. 1985). In this study, hyphal coil width was not affected by the occurrence of the host in both media. The initial formation of hyphal coils showed that the hook shape of the hyphae was, to some extent, similar to the hook-shaped pathogenic fungal hyphae reported by Brasch and Gr ser (2005) but differed in the consistency of the hyphal width. The initial formation of hyphal coils was always on the terminal portion of the hyphae, and hyphal branching sometimes appeared inside the coil loop.

The singly-layered fused rope-like hyphal strands were similar to the coiled hyphae, of *R. vesiculosus,* from the sporocarp and rhizomorph structure (Luoma et al. 2011), human pathogenic fungi *Cladophialophora humicola* (Crous et al. 2007), and the endophytic fungi *Muscodor coffeanum* (Hongsanan et al. 2015). Moreover, I also found extensive coil hyphae with 2–5 hyphal layers, which, to some degree, resemble the hyphal coil in arbuscular mycorrhiza, ericoid mycorrhiza, ectendomycorrhizas, and orchid mycorrhizas (Cavagnaro et al. 2001; Peterson et al. 2004), but differed in the order of the layers. Most of the coils were round, while some were oval or ellipsoid in shape. I also observed a novel anastomosis and hyphal fusion between the coil and adjacent vegetative hyphae. The initiation of anastomosis was the tip-to-side type (from the hyphal coil direction) based on the description by Hickey et al. (2002).

Thromboplerous hyphae are modified fungal hyphae with obscure details of development and function (Lentz 1954; Clémençon 2012). The present observations demonstrate that melanized hyphal coils and thromboplerous hyphae

shared similar morphological properties. Statistical analyses indicated a host effect on the melanized hyphal coil width only in the poor medium. The melanized, refractive, and solid mass of homogenous dense material of mature coiled hyphae met the characteristics of the thromboplerous hyphae described by Clémençon (2005, 2012). In addition, the present data are the first description of the development of thromboplerous hyphae via hyphal coils of *R. roseolus.* In mature and melanized coils, a septum appears to separate this structure from the unmelanized hyphae. The detached melanized coils generally only occurred in the terminal position and did not form thromboplerous hyphae from the middle of the hyphae.

Clémençon (2005) proposed a possible explanation for the formation of thromboplerous hyphae from the melanized tubular hyphae of the rhizomorph of *Ossicaulis lignatilis*. The present findings provide alternative information regarding the formation of thromboplerous hyphae via hyphal coils. However, consistent with Clémençon (2005), I also found several thromboplerous hyphae from the mycelial cords of *R. roseolus* with an exceptionally large hyphal width compared to the hyphal coil. I suggest that their formation is not through the hyphal coil. The hyphal coils and thromboplerous hyphae formed WIH and WOH, in rich and poor media. These hyphae were formed from the beginning of hyphae development and were produced consistently until the medium became dry. This result is in line with Lentz (1954), who stated that these hyphae pose an essential physiological role, which is still unclear. My data showed that they were continuously produced under a variety of environmental conditions, which might have an essential role in *R. roseolus* growth.

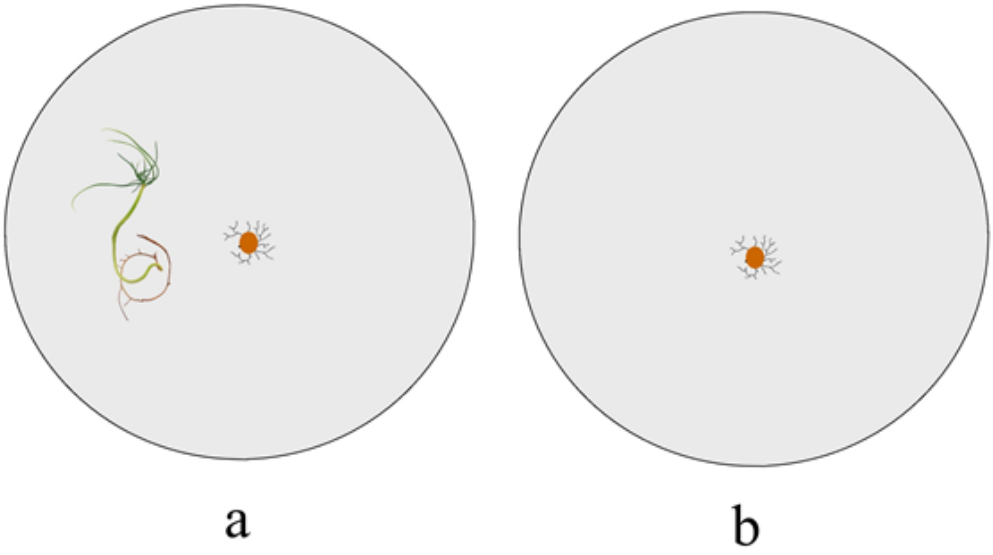

Figure 4.1 Preparation for coiled hyphae observation from plates. a. Fungi were grown with host in a 1/5 dilution of rich and poor Modified Melin and Norkrans medium. b. Fungi were grown on the same medium without host.

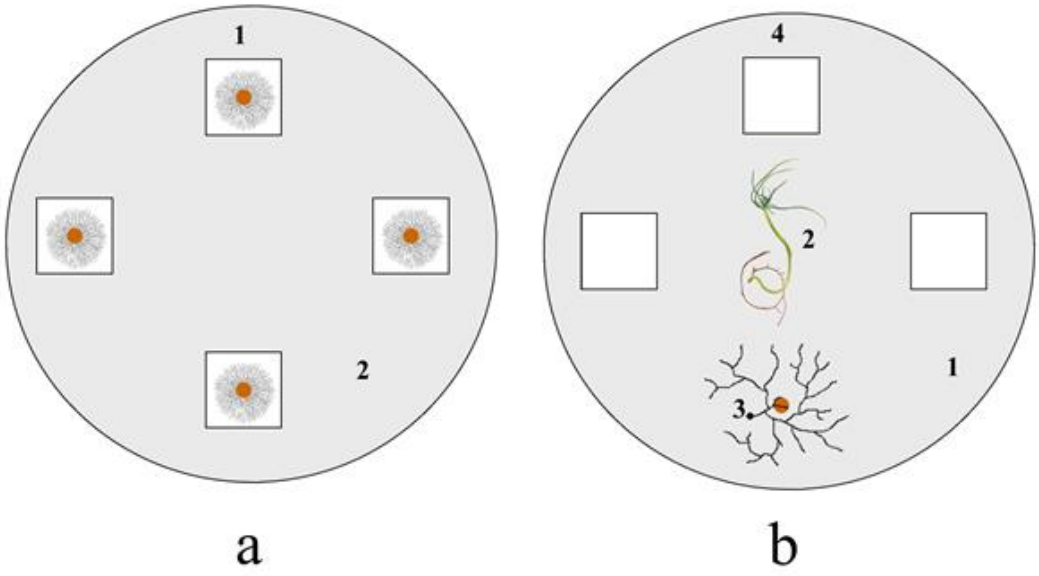

Figure 4.2 Preparation for detailed coiled hyphae observation. a. Fungi were grown without host on sterile glass cover (1) inside a Petri dish containing a 1/5 dilution of Modified Melin and Norkrans medium (2). b. Fungi were grown on the same medium (1) with host (2). The hyphae (3) grew towards host and filled the space inside the Petri dish. The hyphae on the surface of sterile cover glass (4) were used for investigation.

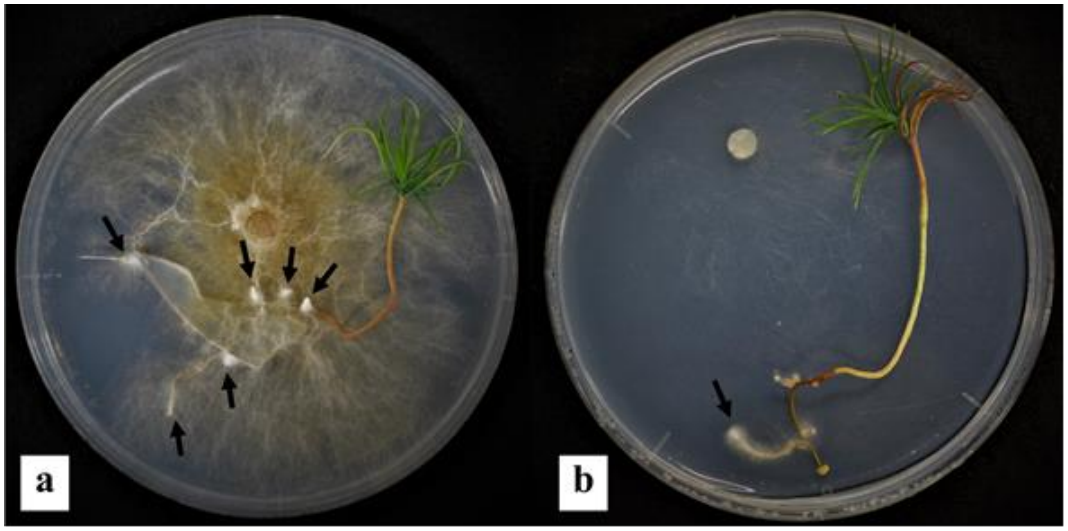

Figure 4.3 The plant- fungi system on 1/5 dilution of Modified Melin and Norkrans medium in 90x10 mm Petri dish. Fungi with host on rich medium (a) and poor medium (b) showing white to cream ectomycorrhizal structures (arrow).

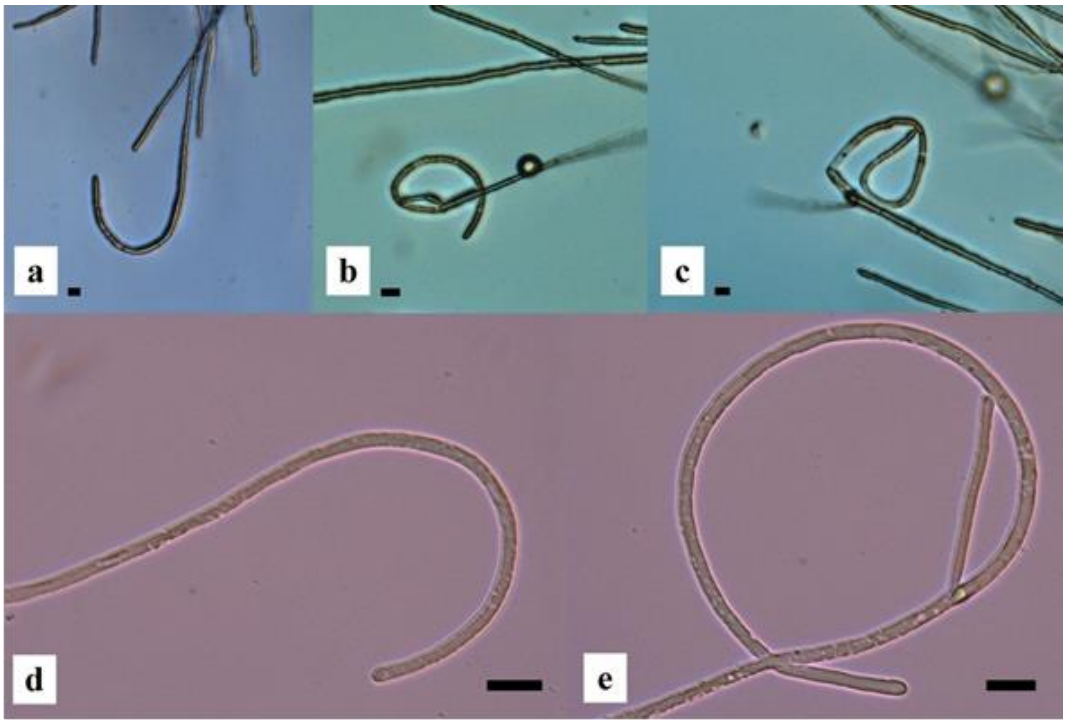

Figure 4.4 Initial formation of coiled hyphae of the ectomycorrhizal fungus, *Rhizopogon roseolus* at low and high magnification. A hook shape appeared on the hyphal tips (a, d). The subsequent extensive coiling of the hyphae (b,c,e). Bars = 10 µm.

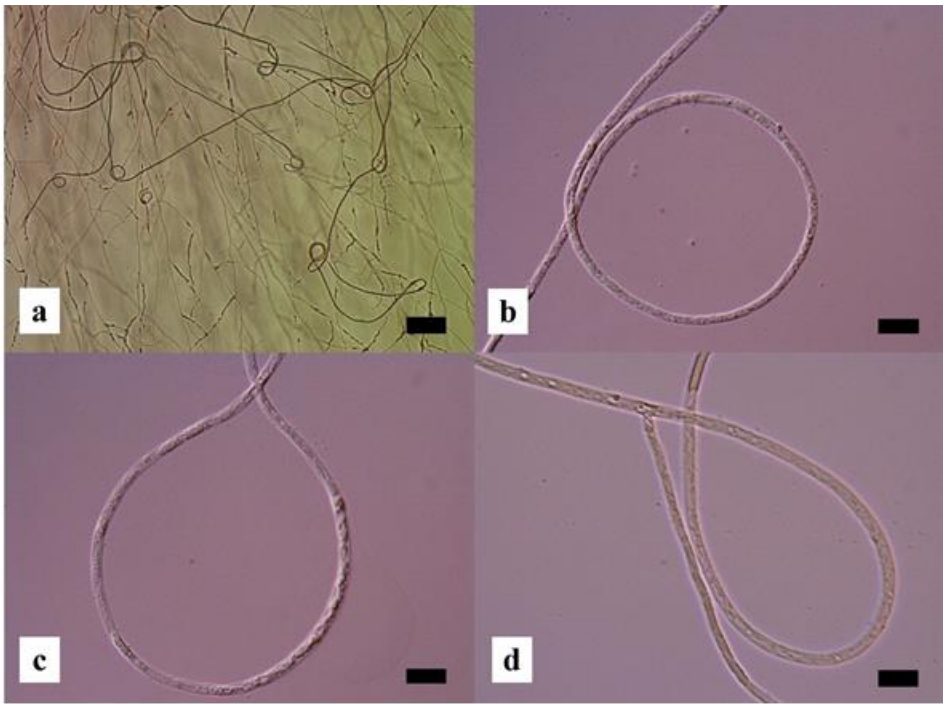

Figure 4.5 The shape and position of hyphal coils of the ectomycorrhizal fungus, *Rhizopogon roseolus*. Hyphal coils with fused rope-like hyphal strands were dispersed on the mycelia of *R. roseolus* in all experiments (a). Round (b), oval (c), and ellipsoid (d) shape of coils formed both on the middle (b) and terminal (c, d) of the hyphae. Bars: a = 100 µm, b–d = 10 µm.

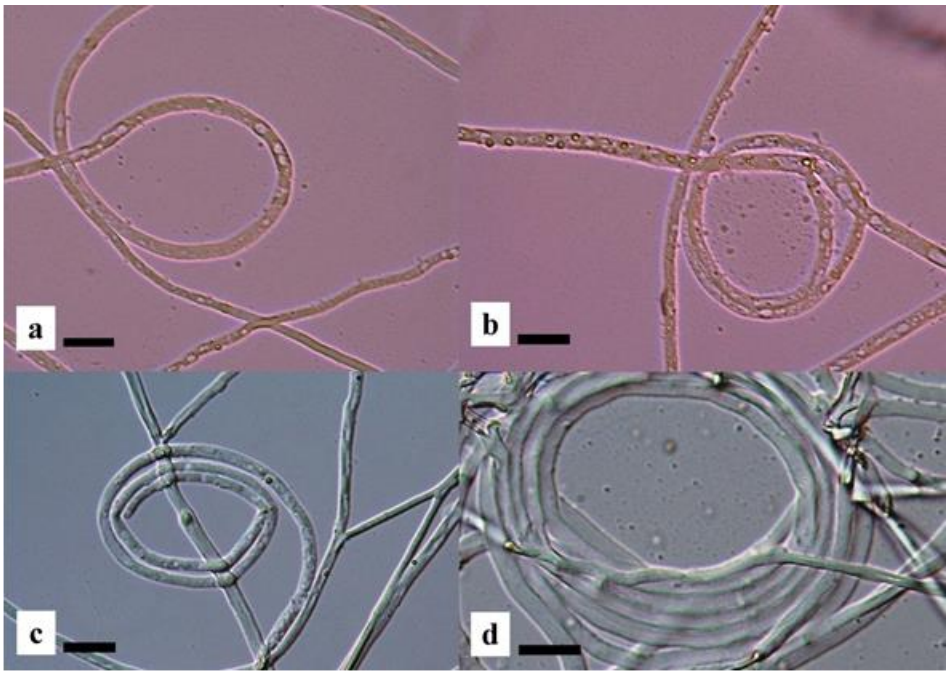

Figure 4.6. Variation in the number of layers of hyphal coils of *Rhizopogon roseolus*. Hyphal coil with one (a), two (b), three (c), and five layers (d) of hyphae. Bars = 10 µm.

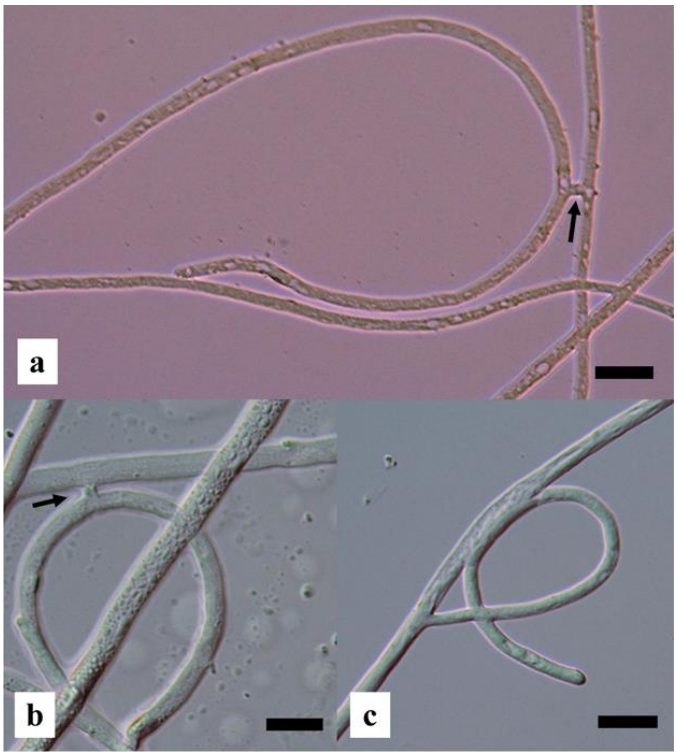

Figure 4.7 Anastomosis formation on hyphal coils of the ectomycorrhizal fungus, *Rhizopogon roseolus*. Anastomosis bridge between hyphal coils and adjacent hyphae (a,b, arrows). Hyphal fusion between hyphal coils and adjacent hyphae (c). Bars = 10 µm.

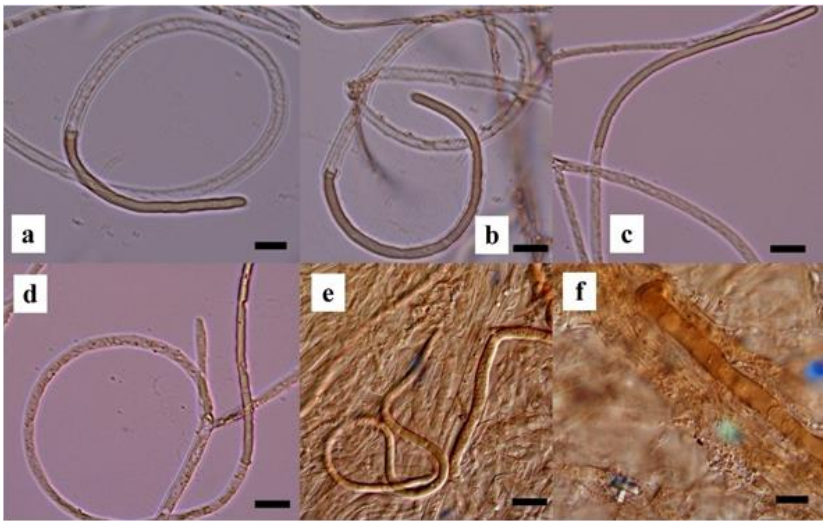

Figure 4.8. Thromboplerous hyphae formation from hyphal coils in the ectomycorrhizal fungus, *Rhizopogon roseolus.* The melanized hyphal coils (a,b,c) with clear septae. The melanized hyphal coils with some cracks on its surface (d) resemble the thromboplerous hyphae (e) on the mycelia. The Thromboplerous hyphae at mycelial cords with the exceptional large size of diameter (f). Bars = 10 µm.

Table 4.1. Comparison of the *Rhizopogon roseolus* hyphal coils diameter and width with and without host on rich and poor Modified Melin and Norkrans medium of 1/5 dilution. Values in a column followed by different letters differ significantly at $P < 0.05$ (Student-Newman-Keuls) with the host (WIH) and without the host (WOH) on the same medium.

Combination	Hyphal coils loop diameter (µm)	Width of the hyphae in coils (µm)	Width of the vegetative hyphae near coils (µm)
Fungi WIH on rich medium	32.06 ± 10.41[a]	2.93 ± 0.68 [a]	2.68 ± 0.37 [a]
Fungi WOH on rich medium	49.82 ± 15.96[b]	3.15 ± 0.45 [a]	2.45 ± 0.42 [a]
Fungi WIH on poor medium	41.41 ± 6.92[a]	2.94 ± 0.32 [a]	2.64 ± 0.35 [a]
Fungi WOH on poor medium	50.02 ± 16.01[b]	2.68 ± 0.83 [a]	2.49 ± 0.48 [a]

Chapter 5
Basidium formation of *Rhizopogon roseolus* with *Pinus thunbergii* in agar medium

5.1 Introduction

Rhizopogon roseolus, known as 'shoro' in Japanese is an important edible ectomycorrhizal (ECM) mushroom in Japan. This species is known as the common symbiont of *Pinus thunbergii* (Japanese black pine) and is usually distributed in sandy soils or coastal areas in Japan. The basidiomata are appraised as a delicacy and are expensive in local markets (Kawai et al. 2008). However, the market demand is still dependent on the collection of basidiomata from the field, as this mushroom has not been fully successfully cultivated. Hence, basic biological research on *R. roseolus* cultivation is still in progress (Visnovsky et al. 2010; Shimomura et al. 2012a).

Previous reports have shown that *R. roseolus* employs a bipolar incompatible mating system (Kawai et al. 2008; Wang et al. 2012). Therefore, selection and breeding trials could contribute to cultivation efforts. Because monokaryotic mycelia develop from basidiospores, obtaining basidiospores from diverse parental sources is crucial. However, a limited number of *R. roseolus* basidiomata can be collected from the field, as it follows the fruiting body formation season. In addition, field propagation of *R. roseolus* requires at least one year of limited fruiting body formation (Yamada et al. 2001; Shimomura et al. 2012a), and information on *in vivo* sporulation is still lacking.

Previous reports have shown that environmental factors play an essential role in the production of basidia and basidiospore (Adam and Butler 1983; Fu et al. 2013; Chen et al. 2021). Furthermore, Wickes et al. (1996) suggested that the conditions required for sexual reproduction and monokaryotic fruiting are similar, including low temperature (25 °C) culture, low moisture, and nutrient limitation. However, information regarding basidiospore formation in *R. roseolus* and other ECM fungi in agar media is not available. During my observation of a mantle of ECM between pure synthetic cultures of *R. roseolus* and *P. thunbergii* under temperature shock treatment, notable basidia formation was evident. Therefore, this study aimed to provide initial information regarding the mycelial basidia characteristics of *R. roseolus* in laboratory culture. This information might contribute to the development of a sporulation method for *R. roseolus* in agar medium.

5.2 Materials and methods

5.2.1 Biological materials

Rhizopogon roseolus (Corda) strain TUFC10010 was used in this experiment. ECM fungi were obtained from the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Japan. The fungi were propagated on 2% malt extract agar (MEA) containing 20 g malt extract (Oriental Yeast Co., Ltd., Tokyo, Japan), 20 g agar, and 1 L tap water at a final pH of 5.5, and incubated at 25 °C for three weeks prior to use. *Pinus thunbergii*, also known as the Japanese black pine, was used as the host. Surface-sterilized seeds were germinated on water agar for 1–2 weeks. Pine seedlings with fine lateral roots were transferred to a five-fold dilution of modified Melin and Norkrans (1/5 MMN) medium (Marx 1969) in a Petri dish (90 × 20 mm). Each Petri dish had three 7 mm rounds with one seedling on 30 mL of half-size solid MMN medium (Figure 5.1). The Petri dish was sealed with 3M™ transpore surgical tape and incubated in a controlled chamber at 25 °C, 50% relative humidity, for 16-h d photoperiod with 5000 lx using cool-white fluorescent lamps for six months.

5.2.2 Morphological observation of ECM mantle

After six months of incubation, 40 plates of *R. roseolus* and *P. thunbergii* were observed. Twenty plates of ECM with fine mantle structures were then incubated at low shock temperature (4°C) for one month, while the other 20 plates were kept at 25 °C. Three ECM roots were observed from each plate. Roots with thick mantle were observed using a stereo microscope. The selected samples were cut and fixed in a fixative solution (3:1 99.5% ethanol:acetic acid), and the air was subsequently removed. Samples were then stained with lactophenol cotton blue, squashed on a slide culture glass, and observed under the Eclipse 80i light microscope (Nikon, Tokyo, Japan). Images were captured with the DS-L2 digital camera (Nikon).

5.3 Results

In the plant-host ectomycorrhizal system, the mantle was observed after three months of incubation (Figures 5.2a–b). However, the basidia were evident on the outer layer of the old mantle structure only after six months of incubation in 1/5 dilution of MMN medium with shock-temperature treatment. Two out of 20 plates had basidia investigated from three ECM structures (root's mantle) from each plate. The agar medium was almost dry (Figure 5.2c), the ECM colour became cream, and the plant was dry.

Furthermore, the outer mantle layer was composed of a net prosenchymatous tissue with loosely interwoven aseptate hyphae (Figures 5.3a–b). The basidia were 21.61 µm– 22.67 µm × 5.98 µm–6.61 µm, cylindrical to clavate, thin-walled, with 2–4 sterigmata and no basidiospores (Figure 5.4a). Immature sterigmata were also observed (Figure 5.4a). Basidioles were 11.65 µm–13.98 µm × 2.79 µm–4.74 µm, ellipsoid to clavate, and sometimes with prominent lipid body (Figures 5.4b–c). In addition, the cystidia were less abundant (Figures 5.3c–d) in the mantle and were unbranched (Figures 5.5a–b) or with two or three branches (Figure 5.5c). Cystidia clavate to ovoid, sometimes with granules, 7.46 µm–25.36 µm in length, proximal end diameter 3.28 µm–9.51 µm, diameter of distal end 2.58 µm–8.93 µm, formed on the terminal hyphae, mostly without granules, thick-walled, smooth at surface, with short stalk and thick septa, and sometimes chained with two cells. ECM mantle without temperature shock showed no evidence of basidia and cystidia formation (Figure 5.6).

4.1 Discussion

I found a maximum of four sterigmata without basidiospores on the mycelial basidiome, whereas *R. roseolus* usually produces eight sterigmata on basidia from the fresh fruiting body (Shimomura et al. 2008; 2012b). Sterigma formation is an indication of spore formation in many Basidiomycota species (Clémençon 2012). In *R. roseolus,* the initial sterigmata formed when the first meiotic division occurred (Shimomura et al. 2012b). However, this process could not be completed in the present study for unknown reasons. In addition, I showed the novel formation of branched cystidia of *R. roseolus* on agar. To some extent, the cystidia were identical to russuloid-type cystidia (Agerer 2006). He described that cystidia in the ECM structures are not commonly known and that *Rhizopogon* species generally lack cystidia. Massicotte et al. (1999) discussed the development of peg-like appendages in the outer mantle of the ECM roots of *R. subcaerulescens* and *P. ponderosa* in greenhouse. Peterson et al. (2004) considered this structure as peg-shaped cystidia. Massicotte et al. (1999) suggested that this structure appears to be unique to *R. subcaerulescens*. Here, I report the formation of cystidia in the ECM of *R. roseolus* and *P. thunbergii* in agar cultures.

Mycelial basidiome formation might have been induced by environmental stress conditions, such as dry medium and cold shock temperatures. In contrast, the identical set of ECM synthesis between *R. roseolus* and *P. thunbergii* incubated in the same medium at a constant temperature (25 °C) did not produce mycelial basidome and cystidia. This indicated that environmental conditions could be a

limiting factor for mycelial basidiome formation in the current study. Previous studies have revealed that temperature and light are critical factors in inducing basidia in pure cultures and inhibiting basidiospore formation (Uchida et al. 1986; Bastos and Andebrhan 1987). These studies reported that basidia were formed at a minimum temperature of 24°C, contrary to the present study (4°C). Therefore, the present study reminds us of the formation of fruiting bodies of *R. roseolus* in the autumn season in Japan. The basidiomata appeared after the decrease in temperature, similar to the mycelial basidiome production in this study. In addition, prior works reported that *R. roseolus* is one of the most resistant species to the heating treatment (up to 70°C) (Kipfer et al. 2010) and might be related to basidiospore production for a survival strategy.

Furthermore, I suggest that basidia formation may be related to the survival mechanism of *R. roseolus* as the medium becomes dry. Environmental factors play important roles in fungal spore production. Some fungi produce spores that can withstand harsh conditions (Wang et al. 2017). In the dry medium used in the present study, basidia could not be observed from mycelia and mycelial cords but could be found on the mantle structure; this observation may be correlated with the function of the mantle as a nutrient sink in the ECM system. Smith and Read (2008) reported that trehalose, mannitol, and glycogen constitute sinks in the ECM mantle. In addition, mantle hyphae can accumulate lipids, proteins, phenolics, and polyphosphates, which can be preserved for short or long periods of time (Peterson et al. 2004). Furthermore, these compounds are important during basidiosporogenesis, as a high demand for energy and precursor compounds must prevail during basidiospore formation (Bago et al. 1999; Bago et al. 2002; Campos et al. 2008; Campos and Costa 2010). In the present study, I observed lipid bodies in some mycelial basidia, indicating a partial basidiosporogenesis process. Shimomura et al. (2008) reported that *R. roseolus* spores contained prominent lipid bodies.

Crossbreeding using spores of homokaryons is considered a promising tool for obtaining superior strains and phenotypes of ECM fungi (Kropp 2005). Previously, attempts have been made to select superior strains of *R. roseolus* for cultivation (Shimomura et al. 2012a; Nakano et al. 2015). However, researchers still rely on basidiomata from the field to obtain spores. In this study, basidiospores were not found, which might indicate partial development of basidium structure, fungal response to survival mechanism, and incomplete life cycle of *R. roseolus*. The basidiosporogenesis of *R. roseolus* is substantial under field conditions, but not in agar. Notably, my results showed evidence of mycelial

basidiome formation *in vitro* and might also stimulate the basidiosporogenesis of *R. roseolus*. The development of the mycelial basidiome might reveal the feasibility of sexual production of *R. roseolus* under certain circumstances. The possibility of obtaining spores in laboratory cultures will contribute significantly to improved breeding of *R. roseolus*.

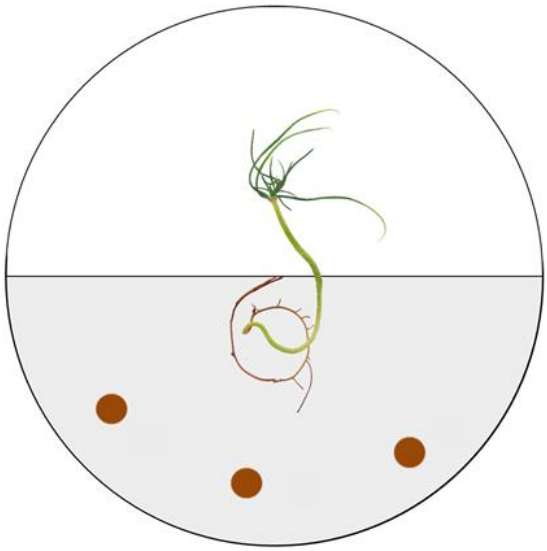

Figure 5.1 Experimental set-up. *Pinus thunbergii* inoculated on modified Melin and Norkrans medium with three plugs of *Rhizopogon roseolus*.

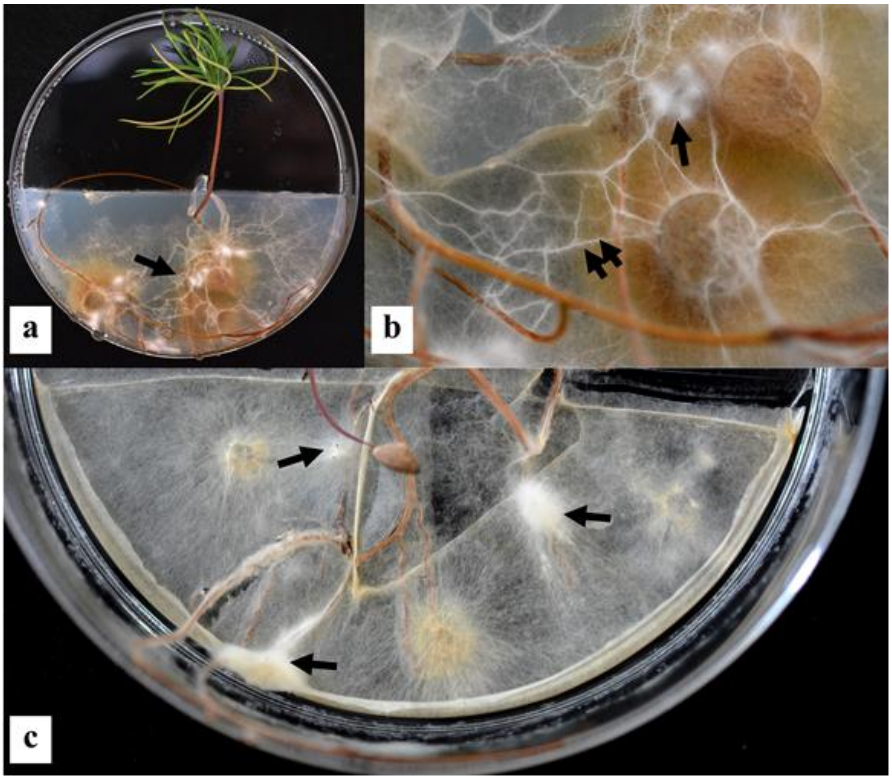

Figure 5.2 The plant–host ectomycorrhizal system in 1/5 dilution of Modified Melin and Norkrans agar medium in 90x20 mm Petri dish. Some mantle structures (**a**,**b** arrow) and mycelial cords (**a**,**b**, double arrow) can be observed near the 7 mm of plug of inoculum after three months of incubation. After six months (**c**), the agar was dried, and some mantles remained (arrow) and subjected to a low shock temperature.

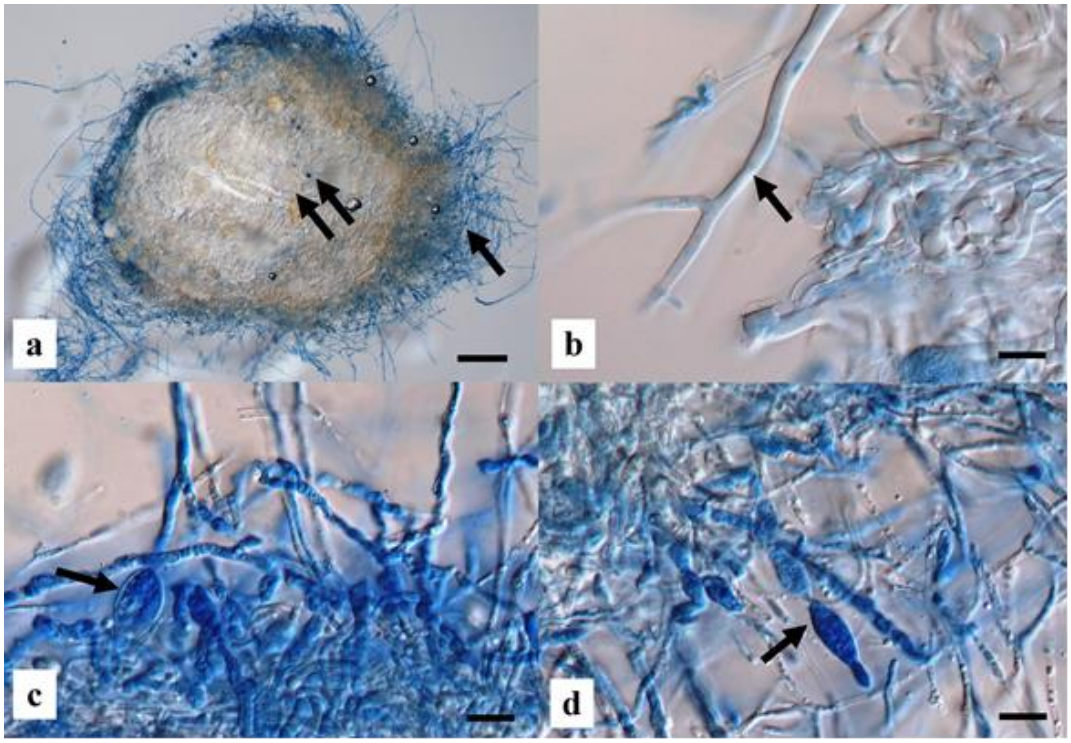

Figure 5.3 Cross section of ectomycorrhizal (ECM) root after temperature shock showing mantle hyphae with cystidia. Plant tissue (**a**, double arrow) with mantle of ECM (arrow). Aseptate hyphae of outer layer of mantle (**b**, arrow). Cystidia with prominent granules (**c**,**d**, arrows) were evident. Bars: a = 100 µm, b-d = 10 µm.

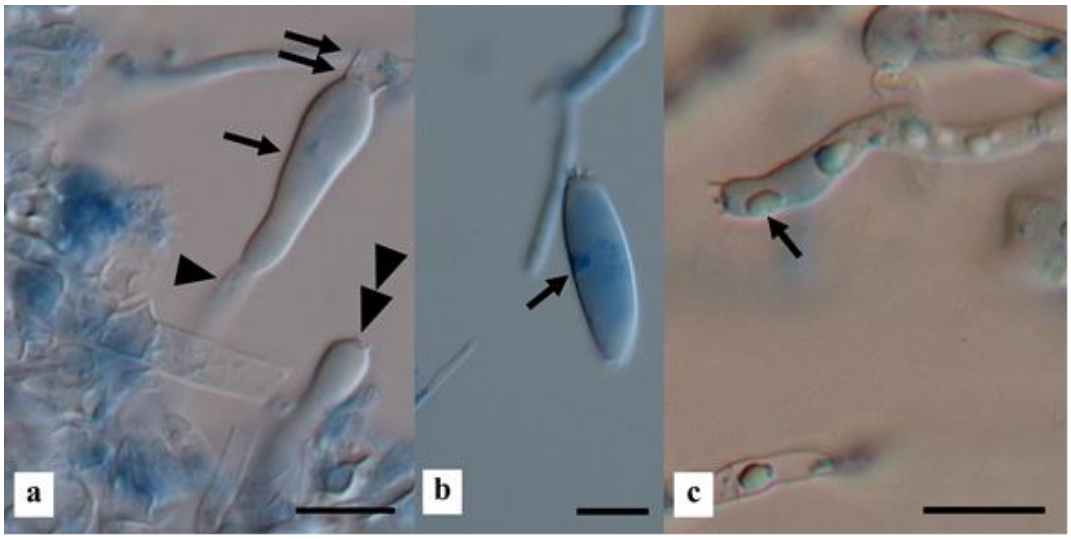

Figure 5.4 Basidia and basidiole formation in the mantle of ECM in Modified Melin and Norkrans medium. Basidia (**a**, arrow) with four sterigma (double arrow) emerged from terminal hyphae (arrowhead) of outer mantle structure. Immature sterigma (**a**, double arrowhead). Basidiole (**b**, arrow) with lipid body (**c**, arrow) were evident. Bars: 10 µm.

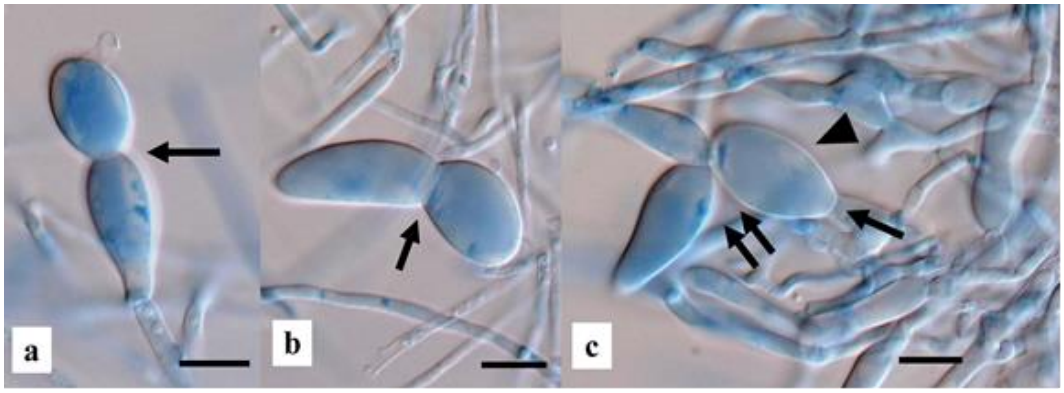

Figure 5.5 Clavate to ovoid cystidia cells in the mantle of ectomycorrhizal roots in Modified Melin and Norkrans medium. Two chained cystidia (**a**,**b**, arrows). Three branched (double arrow) of cystidia (**c**) with thick wall (arrowhead) and septate (arrow). Bars: 10 µm.

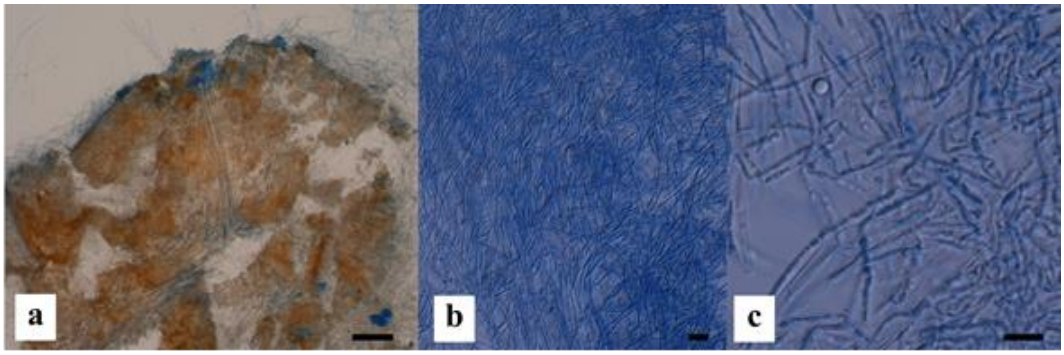

Figure 5.6 Cross section of the root of the ectomycorrhizal plant (**a**) without temperature shock, showing no evidence of basidia formation on the mantle (**b,c**). Bars: a = 100 µm, b–c = 10 µm.

Chapter 6
General discussion and conclusion

In chapter 2, the mycelial cords of *R. roseolus* with and without host are rather similar, with some cytological differentiations. The most notable features are the complexity of the aggregates, the tubular hyphae features (including size, partially and completely dissolved septa, the conjunction septa shape), and the thromboplerous hyphae. These findings showed the morphological response of ECM fungi to the plant host. The information contributes to deepening the cytological understanding of ECM fungal hyphae in plant-fungi interactions. Further investigation is needed to reveal how the occurrence or absence of host impacted the morphological characters of the mycelial cords features of ECM fungi .

Previously, assessments of the mycelia of ECM fungi are scarce compared to those of arbuscular mycorrhizae. In fact, the observations in laboratory conditions have rarely been reported. To my knowledge, the study in chapter 3 is the first attempt to assess and quantify the mycelial features of ECM fungi with or without host occurrence. I revealed a significant impact of the host on some of the mycelial features of *R. roseolus*. Statistical analyses of colony diameter, hyphal length, hyphal branch, and formation of the chlamydospores-like structure revealed a host effect, in contrast to the anastomosis number per hyphal length. Chapter 3 provides some new information regarding host impact on the cytological aspects of the ECM fungus, *R. roseolus*. Further research is needed to reveal whether the effect is case-dependent species or general in ECM symbiosis.

During the assessment of mycelial cords of *Rhizopogon roseolus* on poor nutrient of Modified Melin-Norkrans (MMN) medium in chapter 3, I found some sclerotia produced on the surface of extraradical mycelia. The sclerotia were 0.27 mm in average of diameter and produced after 2 months of incubation. The current knowledge defined the sclerotium as mass of hyphae and normally having no spores in or on it. However, I found and suspected the small structures like spores (1-1.5 um) inside the sclerotium. These structures were ellipsoid, hyaline, with the smooth surface. I then incubated the sclerotium and these small structures on TM7 detecting medium whether they can produce the secondary mycelia of *R. Roseolus*, but no germination was observed. Interestingly, the bacterial colonies which connected to hyphae of sclerotium were appeared. The colonies were transferred to Luria agar (LA) medium. The morphological observation of bacterial cells from TM7 and LA confirmed that they were the same as small structures inside the sclerotium. This is the first report on production of unusual sclerotium of *R. roseolus* in pure cultures.

Further study is required to reveal the role of bacteria on production of sclerotium of *R. Roseolus.*

In chapter 4, I provide for the first time, the formation of hyphal coils in the ECM fungus *R. roseolus*, which are involved in the development of thromboplerous hyphae. Generally, hyphal coils are formed inside the plant tissue of many mycorrhizal symbioses (Peterson et al. 2004; Smith and Read 2008). Coils are modified fungal hyphae that increase the surface area for resource exchange between the plant tissues and fungi (Johnson and Gehring 2007; Smith and Read 2008). In contrast, this study documented hyphal coil formation of ECM fungi from laboratory cultures and revealed a different role than in the prior reports of the coils in mycorrhiza. Finally, my findings indicate the occurrence and formation of hyphal coils in the ECM fungus *R. roseolus.* This data fills the gap in the morphogenesis details of thromboplerous hyphae. Further investigation of hyphal coils and thromboplerous hyphae is needed to completely understand their specific roles in fungi.

In the study of chapter 5, I reported a novel basidia formation of *R. roseolus* in pure culture synthesis of ECM fungi inoculated with *P. thunbergii*. Several researchers have previously described the production of basidia by saprophytic and parasitic fungi in agar (Lam et al. 1988; Yamanaka and Sagara 1990; Ainsworth and Rayner 1991; Tu et al. 1992). However, information on basidia formation on agar by ECM-forming fungi remains unavailable. Clémençon (2012) defined basidia produced on mycelia in pure cultures as mycelial basidiomes; I adopted this terminology in this study. Whether the other strains of *R. roseolus* inoculated with *P. thunbergii* will exhibit the same response to environmental changes as that reported in the present study remains unknown. Further research (Figure 6.1) with manipulation of the temperature condition of ECM cultures might solve the remaining challenge of the low frequency of mycelial basidia. In addition, it might offer the opportunity to induce *R. roseolus* spore formation in agar medium.

In conclusion, the present work emphasizes the fungal response to ECM formation and provides the first information on host plant impact (quantification and statistical analyses) to ECM fungus. In chapter 2, further investigation using electron microscope and 3D analyses should be done to visualize the tubular hyphae diameter and septa length of *R. roseolus* with and without host. In chapter 3, I suggest the use of cellophane membrane to obtain the optimum imaging of ECM hyphae observation. In addition, the significant impact of host plant to mycelial features of ECM fungus should be on other fungi and or plants. I also, show the first

information of sclerotium formation of *R. roseolus* in agar medium, contain the bacteria. The origin and specific role of the bacteria in *R. roseolus* should be investigated in further research. Following the result from chapter 3, I provide another information on how the thromboplerous hyphae were formed in chapter 4. I report for the first time the morphogenesis of hyphal coils in ECM fungi and its relation to transform the thromboplerous hyphae. However, the role of the two structures is remain unclear and warrant a future work. Those basic biological findings from chapter 1-3 will contribute to the development strategy of basidiospores production in laboratory. In chapter 5, I successfully induce the basidium formation of ECM fungus in agar medium. Nevertheless, the environmental modification to promote basidiospores production is need in the following research.

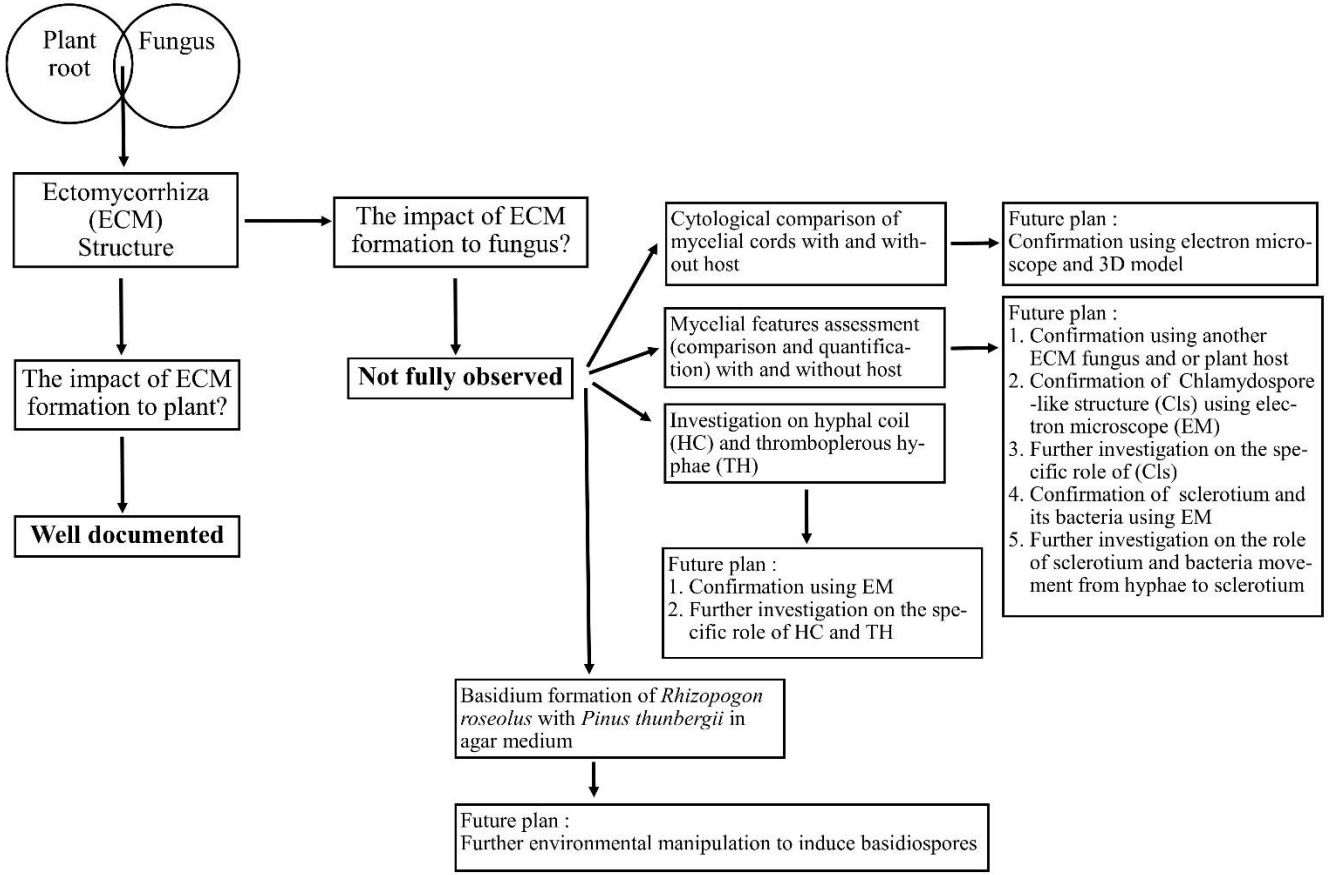

Figure 6.1 Research outline and future plan toward host impact to fungal structures (*Rhizopogon roseolus*) in ectomycorrhiza (ECM) symbiosis and the establishment of primary mycelium production of ECM fungi in agar medium.

Summary

Most cytology reports on ectomycorrhiza (ECM) have been less focused on the fungal structures, especially on the exploratory organ. Chapter 2 in this this study aimed to evaluate the morphological response of explorative mycelia of ECM forming fungi with and without the occurrence of the ECM host. I assessed the mycelial aggregates of *Rhizopogon roseolus* which was inoculated with and without *Pinus thunbergii* under controlled laboratory conditions. The mycelial aggregates with the host produced strikingly complex hyphal strands relative to those without host. Light microscopy revealed that the cytology and plectology of both mycelial aggregates had the approximately similar architecture. The tubular hyphae diameter without the host was consistently larger than that with the host. This study confirmed that the septa diameter of the tubular hyphae conjunction of mycelial aggregates with the host were shorter than those without the host. In addition, partially and completely dissolved septa of the tubular hyphae were evident with and without host. I also described the thromboplerous hyphae, which have rarely been reported in vitro. These hyphae were produced in higher numbers near the fungal inoculum with and without the host. However, further investigation needs to be done regarding the morphogenesis and specific role of the thromboplerous hyphae.

The impact of host plant on the mycelial features of mycorrhiza symbiont and its characteristics has been poorly investigated. Chapter 3 in this study aimed to compare and quantify (statistically tested) some of the mycelial features of an ectomycorrhiza (ECM) forming fungus with and without the ECM host. The ECM forming fungus, *R. roseolus,* inoculated with or without *P. thunbergii* on both rich and poor nutrient media, was observed under laboratory conditions. On rich medium, fungi with the host had the highest colony diameter and consistently produced the highest hyphal length relative to fungi on other media. Thus, the host had a significant impact on the mycelia production of *R. roseolus* in both rich and poor media. Further, fungi without the host had higher hyphae anastomosis numbers per hyphal length on both poor and rich media than fungi with the host in the same medium. Anastomosis, which refers to the fusion of two parallel hyphae, was evident in all experiments. However, there was no significant impact of the host on the number of hyphal anastomoses. In addition, fungi without the host had more frequent hyphal branching both on rich and poor media than fungi with the host. The occurrence of a host only had a significant impact on the formation of the hyphal branch on poor medium. Further, a chlamydospores-like structure was

identified, which had a higher diameter when formed with the host both on rich and poor media.

During the assessment of mycelial cords in chapter 3, I noted the occurrence of sclerotium on extraradical mycelia. The sclerotia were 0.27 mm and can be observed after 2 months of incubation. I found and suspected the small structures like spores inside the sclerotium. These structures were ellipsoid, hyaline, with the smooth surface. I then incubated the sclerotium and these small structures on TM7 detecting medium whether they can produce the secondary mycelia of *R. Roseolus*, but no germination was observed. Interestingly, the bacterial colonies which connected to the hyphae of sclerotium were appeared. The colonies were transferred to Luria agar (LA) medium. The morphological observation of bacterial cells from TM7 and LA confirmed that they were the same as the small structures inside the sclerotium. This data is the first report on the occurence of sclerotium of *R. roseolus* on pure cultures. Further study is required to reveal the role of bacteria on the production of sclerotium of *R. Roseolus.* In addition, the investigation regarding the origin of the bacteria and mechanism of bacterial transfer to the sclerotium should be done.

While observing the mycelial features of the ectomycorrhizal (ECM) fungus, *R. roseolus* in chapter 4, I noted the formation of hyphal coils in laboratory cultures. The coiled hyphae initially formed at the hyphal tip of the ECM fungi with or without the plant host (*P. thunbergii*), both in rich and poor Modified Melin-Norkrans (MMN) media. Hyphal coils formed from the hyphal tips to the center of the hyphae, with rope-like hyphal strands fused to the extensive circles. Hyphal coils were generally round, oval, and ellipsoid in shape. They were composed of 1–5 layers of hyphae. The hyphal coil loop was consistently larger without the host than with the host in both media. Host occurrence had a significant impact on the diameter of the coil loops. In addition, the terminal part of the mature coils was melanized and separated from the unmelanized coil by a septum. The melanized coils resembled thick condensed hyphae and were detached and scattered throughout the fungal colony. The observation of morphological characteristics confirmed that the mature coil released into the mycelia is the thromboplerous hyphae. I also observed the formation of thromboplerous hyphae from hyphal coils. This is the first report on hyphal coil morphogenesis and its role in the initial development of thromboplerous hyphae.

The edible ectomycorrhizal (ECM) mushroom *R. roseolus* usually develops basidium and basidiospores in the gleba of its basidiomata. In chapter 5, I report a novel production of basidia in laboratory cultures of the edible ECM

mushroom. The basidium with sterigma was observed on the old mantle structure (> six months) of the ECM between *R. roseolus* and *P. thunbergii* in a modified Melin and Norkrans (MMN) medium that was subjected to a temperature shock from 25 °C to 4 °C. The basidia were cylindrical to clavate, with prominent sterigmata and no basidiospores. In addition, branched cystidia were evident in two or three clavate-to-ovoid cells. The absence of basidiospores might indicate partial development of the basidium structure as a response to environmental stress, and incomplete life cycle of *R. roseolus.* This study suggests the possibility of obtaining the primary mycelium of *R. roseolus* from pure cultures and may be an alternative genetic source for cultivation purposes. Further observations are required to induce basidiosporogenesis of *R. roseolus* basidia in an agar medium focusing on temperature. The production of *R. roseolus* spores in laboratory cultures will contribute significantly to improve the breeding of *R. roseolus*.

The findings of the current study will add to the current knowledge regarding host impact to ECM fungi mycelial features. Furthermore, this study reveals some aspects of the fungal response in regard to fungi-plant interaction strategy on the occurrence and absence of the host. This study also provides new information on the origin and morphogenesis of some ECM fungal structures. In addition, this study showed the importance of basic mycology work which essential to clarify the life cycle of an edible ECM mushroom. The better understanding of life cycle of *R. roseolus* will greatly increase the possibility of obtaining spores in laboratory cultures and significantly improved the future works of breeding of this edible mushroom.

和文摘要

外生菌根菌におけるこれまでの細胞学的研究において, 宿主根に形成された外生菌根を観察対象にした研究が多く，人工培地で培養した菌糸形状について焦点を当てた研究例は少ない．そこで，先ずは外生菌根菌ショウロ *Rhizopogon roseolus* の菌糸体形状に及ぼす宿主クロマツ *Pinus thunbergii* の影響について in vitro 条件下で細胞学的に評価した．

ショウロ菌糸の菌糸塊形成は宿主クロマツが有無に関わらず外見上は類似した形状を呈していた．しかし，宿主クロマツが存在する条件下での管状構造菌糸体の直径は，宿主が存在しない条件下で形成されたそれと比較して小さかった．また，宿主存在下での隔壁直径も，宿主が存在しない条件下での直径と比較して小さかった．管状構造菌糸の隔壁の崩壊や部分崩壊は，宿主の有無に関わらず観察された．一方，thromboplerous（凝血状）菌糸が接種源近くで見受けられた．

次に, 宿主存在下と宿主非存在下での外生菌根菌ショウロの菌糸の形状を定量的に解析した．宿主が存在することでショウロ菌糸体の形成が著しく促進された．菌糸の融合頻度は，宿主非存在下において高頻度で認められる傾向があったが，宿主の顕著な影響は認めらなかった．菌糸の分岐頻度は，宿主の存在下によって低下し，貧栄養の培地条件下ではその影

響は拡大し，宿主の存在によって分岐頻度が有意に低下した．また厚膜胞子状菌糸が認められ，本構造の直径は宿主が存在することで顕著に大きくなることが判明した．

次に，ショウロ菌糸の菌核形成について調査した．本菌核は大きさが約 0.27 mm 以下であり，2 か月培養した菌糸において認められた．本菌核の中に小さな胞子状構造体が認められた．本構造体を分離培養したところ，菌糸体は分離できなかったが細菌が分離できた．分離できた細菌の形状は，菌核内で認められた胞子状構造体と酷似していた．本結果は純粋培養ショウロ菌糸体において細菌を包含した菌核形成の初めての報告である．次に，菌糸コイル形成について調査した．菌糸コイルは菌糸の先端部で認められた．菌糸コイルは先端からループを形成し，次いでループ状の束となった．本菌糸コイルは，円形，卵形，楕円形を呈し，1〜5 層の菌糸で構成されていた．本菌糸コイルの大きさは宿主存在下の方が宿主非存在下のものより小さかった．さらに，成熟した菌糸コイルは着色し，厚く密集した菌糸体と類似してきた．これらの観察結果から，成熟した菌糸コイルが thromboplerous （凝血状）菌糸に分化しているものと推察された．

最後に, 寒天培地において子実体形成を経由しない担子器形成について調査した．MMN 寒天培地において宿主クロマツにショウロ菌を接種

した後２か月を経過した感染苗木実生を寒天培地と一緒に低温処理（4℃）したところ，外生菌根の菌鞘において担子器がまれに形成した．形成された担子器は円筒～棍棒状であり，担子胞子の形成は認めらなかった．本観察結果は，ショウロの有性生殖の生活環を外形菌根の菌鞘において実現する可能性があることを示唆している．今後，本手法を改良することで菌鞘において担子胞子を形成させることができれば，子実体を形成することなく効率よく一次菌糸体を取得することが可能性になると考えられた．

本研究は，宿主クロマツが及ぼす菌糸体形状に関する基礎的知見，さらには，外生菌根菌の育種技術向上に繋がる可能性がある有益な情報を提供した．得られた一連の知見は，今後の菌学，微生物学，きのこ学，さらには，きのこ育種学に貢献するものと期待される．

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List of related publications

1. Putra IP, Aimi T, Shimomura, N. 2022. Cytological comparison of mycelial aggregates of *Rhizopogon roseolus* with and without the ectomycorrhiza host. Mycologia 114(3):533-543. https://doi.org/10.1080/00275514.2022.2035156. (The corresponding content is in chapter 2)
2. Putra IP, Aimi T, Shimomura N. 2022. Thromboplerous hyphae of the ectomycorrhizal mushroom *Rhizopogon roseolus* with and without a host tree. Nusantara Bioscience 4(1): 47–52. (The corresponding content is in chapter 2)
3. Putra IP, Aimi T, Shimomura N. 2022. The impact of host plant (*Pinus thunbergii*) on the mycelial features of the ectomycorrhiza fungus *Rhizopogon roseolus*. Mycologia https://doi.org/10.1080/00275514.2022.2071119. (The corresponding content is in chapter 3)
4. Putra IP, Hermawan R, Aimi T, Shimomura N. 2021. The unusual sclerotium of *Rhizopogon roseolus* reported from pure cultures. *IOP Conference Series: Earth and Environmental Science*, 948. (The corresponding content is in chapter 3)