Isolation and evaluation of salt tolerant strains induced by ethyl methanesulfonate in ectomycorrhizal fungus *Rhizopogon roseolus*

Ethyl methanesulfonate によって誘導された外生菌根菌ショウロの耐塩性菌株系統の分離と評価に関する研究

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Chapter 1

General introduction

Pinus thunbergii which also has been called Japanese black pine or black pine is the northeast Asian deciduous forest. The pine is native to coastal areas of Japan, northeast China, North and South Korea and widely distributed in the along the shoreline of Kyushu from Honshu. This tree is remarkably resistant to harsh conditions whether cold winter winds, salt spray, drought, and or low nutrient soils. The coastal forests this tree occupies have been long recognized as an important aid in keeping beach erosion in check as well as acting as a buffer to strong winds and tidal surge from the sea including typhoons that haunt these shorelines in summer and early fall (Taniguchi et al., 2007 a b).

A magnitude-9.3 massive earthquake named the 2011 Tohoku-oki earthquake struck off the coast of Japan on March 11, 2011.The ensuing tsunami smashed into the country's northeastern coastal area (Roy et al., 2014). In the disaster, 23,600 hectares of farmland were inundated by the tsunami. These include four prefectures in the Tohoku region (Aomori, Fukushima, Iwate and Miyagi) and two adjacent prefectures (Ibaraki and Chiba) in Kanto region (Roy et al., 2014). The forests of *P. thunbergii* and *Pinus densiflora* trees along the coast of the Sendai Plain and other disaster areas were almost completely destroyed by the tsunami (Miyawaki, 2014). After the tsunami, the water-soluble ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Br^- and Cl^-) carried by the tsunami seawater affect soil physiochemical properties (Rhoades, 1990), and the salinity level of

topsoil mixed with the tsunami deposits reached a maximum electrical conductivity (EC) value of 37.8 dS m^{-1}, whereas the pre-tsunami average EC value of the soil in most of the agricultural lands was below 1 dS m^{-1} (Haraguchi et al., 2012). In order to repair the coastal pine forests, planting of black pine seedlings have been implemented of Japan. However, the retention rate of the seedlings is low, because of salt accumulated in the soil of the coastal area.

1.1 Effects of salt stress on plant growth

Properties of saline soils including unfavorable pH, imbalance of essential cations and anions reduce plant survival, inhibit plant development, and alter soil structure and texture, resulting in a decrease in aeration and water holding capacity (Abrol and Sandhu, 1985; Bettenay, 1986). The direct effects of salt on plant growth may involve reduction in the osmotic potential of the soil solution that reduces the amount of water available to the plant causing physiological drought. To counteract this problem plants must maintain lower internal osmotic potentials in order to prevent water movement from roots into the plant soil (Feng et al., 2002; Jahromi et al., 2008). The toxicity of excessive Na^+ and Cl^- ions effects towards cell that include disruption to the structure of enzymes and other macromolecules, damage to cell organelles and plasma membrane, disruption of photosynthesis, respiration and protein synthesis (Juniper and Abbott, 1993; Feng et al., 2002). And the high salt concentrations also result in cellular ion imbalance. High Na^+ uptake from saline substrates competes with the

uptake of other nutrient ions, especially K^+, resulting in K^+ deficiencies (Parida and Das, 2005). Another study has shown that increased salt stress induces increases in Na^+ and decreases in Ca^{2+}, K^+ and Mg^{2+} levels in *Halopyrum mocoronatum* (Khan et al., 1999).

Plants, in their natural environment are colonized both by external and internal microorganisms (Evelin et al., 2009). Some microorganisms, particularly beneficial bacteria and fungi can improve plant performance under stress environments (Brown, 1974; Levy et al., 1983; Creus et al., 1998). As important terrestrial mutualistic fungal groups, ectomycorrhizal fungi have been intensively studied regarding their effects on plant growth, plant communities and ecosystem processes (Zheng et al., 2014).

1.2 Physiological characters and benefits of ectomycorrhizal fungi

Ectomycorrhizal fungi form a symbiotic relationship with the roots of various plant species. The structure of ectomycorrhizal fungi is composed three parts. The first is the intraradical hyphae that penetrate into the root and form a network between the outer cells of the root axis called Hartig net (Blasius et al., 1986). This region of juxtaposition is where nutrient and carbon exchange occurs. Enveloping the root tip, and often containing more biomass than the Hartig net interface, is a hyphal sheath known as the mantle. There exists considerable variation in the structure of the mantle, ranging from a loose network of hyphae to a structured and stratified arrangement of tissue. And the last is the extraradical hyphae and extend outward from the mantle into the soil, fulfilling the role of the suppressed root hairs by increasing the surface area of the colonized root.

The extramatrical mycelia radiating into the soil and rhizomorph organization can vary considerably (Agerer, 2001) and can spread in the soil over striking distances. Due to the amount, range, and differentiation of the mycelial structures emanating from the extramatrical mycelium into the soil, several exploration types can be distinguished (Agerer, 2001; 2007). The contact exploration type covers ectomycorrhizas with almost smooth mantles and lack rhizomorphs with ectomycorrhizas in close contact with the surrounding substrate. The short-distance exploration type has distinct emanating hyphae and rather limited growth into the surrounding soil. The medium-distance exploration type has rather far reaching rhizomorphs that are internally either undifferentiated or can in some fungal relationships possess internal hyphae of enlarged diameter. The long-distance exploration types are highly differentiated, features very long rhizomorphs with internal vessellike transport hypha. Long distance types are associated with increased levels of organic nitrogen uptake, and rhizomorphs of exploration types differ in their capacities to transport phosphate (Kammerbauer et al., 1989).

Ectomycorrhizas improved water relations as one of the benefits of ectomycorrhizal symbiosis has become customary (Lehto and Zwiazek, 2011). The external mycelium of ectomycorrhizal fungi transports water to the host plant (Duddridge et al., 1980), and extensions water, phosphorus and nitrogen uptake (Smith and Read, 2008). The benefits of ectomycorrhizal fungi colonization are likely to arise particularly under stress conditions, and it could be of benefit through their capacity to provide the minimum requirements for survival of the plant during

environment stress conditions (Read and Boyd, 1986). The aquaporin expression can be enhanced in ectomycorrhizal compared to non-mycorrhizal seedlings, and this is suggested to be particularly important in water stress conditions (Marjanović et al., 2005). Salt stress-tolerant ectomycorrhizal fungi enhanced plant growth and alleviated the negative effects of excess NaCl, such as decrease in Na^+ and Cl^- uptake together (Bandou et al., 2006), improving plant nutrient uptake and ion balance, protecting enzyme activity (Tang et al., 2009). *P. thunbergii* usually lives with various ectomycorrhizal fungi such as *Suillus bovinus*, *Suillus luteus* and *Rhizopogon roseolus* to resist the salt in the coastal areas.

1.3 Characteristics of *Rhizopogon roseolus* and objectives

Rhizopogon roseolus (Corda) Th. M. Fr. (=*R. rubescens* Tul. & Tul.), known as “shoro” in Japanese, is a hypogeous basidiomycete (Morina and Trappe, 1994). This fungus is an important ectomycorrhizal symbiont of Pinaceae. The fruiting bodies of this mushroom are found in the sandy soils of *Pinus thunbergii* Parl. forest in seashore habitats, and are prized as an edible mushroom, and traded at high prices in local markets (Kawai et al., 2008). Recently, aspects of the life cycle of *R. roseolus* have been revealed. Shimomura et al. (2008) showed that eight basidiospores are usually produced on the apex of each sterigma on the basidium, that the basidiospores are discharged by sterigma collapse and subsequently develop accompanying changes in the spore wall architecture. Depend on the maturity of fruiting bodies and the layers of spore walls the

germination rate of *R. roseolus* was significant variation (Nakano et al., 2016). The germinated spores subsequently become homokaryotic hyphae, equivalent to a basidiospore isolate (Sawada et al., 2014). Kawai et al. (2008) reported that *R. roseolus* exhibits a bipolar incompatibility system. After mating, the hybrid strains could become heterokaryotic hyphae with a clamp connection.

Dunabeitia et al. (2004) reported that *R. roseolus* was adaptable and showed a great range of tolerance to adverse environmental conditions in pure culture. In previous studies, the salt-tolerant strains were also obtained by crossbreeding technology (Nakano et al., 2015). Because of in the high salt concentration conditions the spore germination rate of *R. roseolus* is very low and the large number of bacteria associated with fruiting bodies, it is difficult to isolate a large number of salt-tolerant and halophilic strains by the traditional method. Moreover, the salt tolerant assessment system of the isolates, especially in soil, need to be established. In addition to the single tolerance to the salt, the non-specific stress tolerance of the breeding strains is worthy to detect. Therefore, in this study, sensitive, tolerance and halophilic strains of *R. roseolus* were induced by chemical mutation and hybridization. And the stability of the selected strains was confirmed by long-term subculture. The soil salinity tolerance was determined by culturing in sandy soil, and finally the composite tolerance of each strain was determined under the environmental factors of drying and temperatures. Through the above series breeding method, the complex tolerant strains of *R. roseolus* that is more suitable for various natural environments could be selected.

Chapter 2

Usefulness of ethyl methanesulfonate in isolation of halophilic of *Rhizopogon roseolus*

2.1 Introduction

Rhizopogon roseolus (Corda) Th.M. Fr. (=*R.rubescens* Tul. & C. Tul.), is an ectomycorrhizal basidiomycete with a worldwide distribution (Molina and Tranppe, 1994). The fruiting bodies of this fungus are found in the sandy soils of *Pinus thunbergii* Parl. forests in seashore habitat and are prized as an edible mushroom, called "shoro" in Japanese (Kawai et al., 2008; Shimomura et al., 2010). Saline soils pose a serious environmental and agricultural problem (Yeo, 1983; Jain et al., 1989; Munns and Tester, 2008). The main effects of salt stress which inhibit plant development or reduce survival include unfavorable pH, altered soil structure, ion toxicity and osmotic stress (Dixon et al., 1993; Dashtebani et al., 2014). Recent studies have suggested that ectomycorrhizal fungi can help the host plant uptake phosphorus and water more efficiently (Duddridge et al., 1980), indirectly increase plant growth, and reduce the toxicity of ion effects (Pond et al., 1984; Azcón and El-Atrash, 1997; Tang et al., 2009).

Soils having high levels of contaminants elicited toxic effects in fungi (Colpaert and van Assche, 1987), such as in saline-alkaline environment, where high levels of salts can inhibit fungal growth (Tresner and Hayes,

1971; Dixon et al., 1993). Tresner and Hayes (1971) surveyed the NaCl tolerance of a wide range of terrestrial fungi. The *Penicillia* and *Aspergilli* were the most resistant, with the majority of species able to grow in the presence of 20% or more NaCl. Basidiomycetes were the least tolerant, with over half the species unable to withstand more than 2% NaCl (Tresner and Hayes, 1971). Dixon et al. (1993) found various levels of inhibition of growth, protein production, and colonization potential in selected species at 200 mM concentrations of various sodium salts. Kernaghan et al. (2002) showed that the growth of *R. roseolus* was completely suppressed by 100 mM and 200 mM NaCl. On the other hand, a salt-tolerant strain was produced by crossbreeding in *R. roseolus* (Nakano et al., 2015). However, no halophilic strains have been produced in this mushroom species.

Breeding based on physical and chemical mutagenesis has been more effective than traditional breeding in producing cultivars with high resistance to biotic and abiotic stresses (Zhao et al., 2013). Ethyl methanesulfonate (EMS) has been found to be mutagenic in a wide variety of genetic test systems from viruses to mammals (Sega, 1984). EMS alkylates guanine bases and leads to mispairing; alkylated G pairs with T instead of C, resulting primarily in G/C- to-A/T transitions. However, no mutagenesis studies on the selection of ectomycorrhizal fungi of *R. roseolus* strains have been performed. Here, to induce mutations, we treated basidiospores of *R. roseolus* with EMS, isolated homokaryotic strains and evaluated mycelial growth activity on agar plates containing NaCl. Furthermore, we obtained hybrid strains by

crossing the homokaryotic strains, examined them for salt sensitivity, and then proposed useful strains that should be adaptive for salt-containing environments.

2.2Materials and methods

2.2.1 Fungal strains

A fresh wild fruiting body of *R. roseolus* MCL2014Rhz41 was collected from the *P. thunbergii* forest of the Arid Land Research Center, Tottori University, in March 2014. The collected fruiting body was cut with a scalpel and the spores extruded in sterilized water. The basidiospore suspension was passed through a cell strainer with a 100 μm filter and the spore concentration was determined using a Thoma hemocytometer. In general, the concentration of basidiospores was 10^6-10^7 spores/mL; the suspension was stored in a refrigerator at 4°C for later use.

Homokaryotic strains used in this study were obtained by monosporous isolation after EMS treatments. Hybrid strains were obtained by crossing homokaryotic strains. For crossing, each of the two homokaryotic strains was inoculated on the same agar plate and incubated for nearly one week. Thereafter, the crossing region was cut and inoculated on a clamp detection medium (0.1% malt extract, 0.2% Tween 80, 2% agar medium pH 7.0, Shimomura et al., 2012a) for 5 days at 25°C. Production of a hybrid strain was confirmed by observation of hyphae

bearing clamp connections.

2.2.2 Treatment of basidiospores with EMS mutagen

A stock solution of EMS (100 mg/mL; 10% v/v) was prepared using 1 mL EMS dissolved in 9 mL sterilized distilled water. Different volumes of EMS stock solution were put in microtubes and mixed with the basidiospore suspension to 2 mL. The concentration of EMS in each treatment was 5, 10, 20, 30, 40 and 50 mg/mL (0.5~5% v/v). The basidiospore suspension without EMS was used as a control. After 5 h, the microtubes were centrifuged at 5,500 rpm for 10 min and rinsed thrice with sterilized distilled water. Subsequently, a 0.1 mL EMS-treated basidiospore suspension was spread on a 5-fold diluted Modified Melin-Norkrans (Marx, 1969) agar (MMNA) plate. Following a 20 days culture at 25°C, the number of colonies derived from the EMS-treated basidiospore suspension was counted. Each experiment was repeated five times.

2.2.3 Preparation of mycelial fragments and treatment with EMS mutagen

Mycelial fragment of basidiospore isolates MCL2014Rhz41·Sp1 (Sp1) and MCL2014Rhz41·Sp2 (Sp2) were used for the mutation experiment. These strains were incubated on 1/5 MMNA medium for 2 weeks at 25°C in the dark for preparing mycelial fragments. The mycelial fragments were prepared as per the method reported by Shimomura et al. (2012b).

Two week-old mycelial colonies were cut into 5 mm^2 mycelial agar plugs and then inoculated into 50 mL of malt extract medium (20 g/L malt extract and 0.2% Tween 80) in an Erlenmeyer flask and incubated at 25°C in the dark. After 20 days, the mycelia were washed thrice with sterilized DW. The washed mycelia were suspended at 3.5 g wet weight in 50 mL sterilized DW and blended with an AHG-160A homogenizer (AS ONE Corp., Osaka, Japan) at 14,000 rpm for 1 min.

The resulting mycelial fragments were treated with EMS (20 mg/mL; 2% v/v) for 5 h. Subsequent processing was the same as that for mutated basidiospores. The mycelial fragments were collected by centrifugation and washed. Debris of mycelial fragments were removed by 100 μm-filtering through a Cell Strainer, and the filtrate was spread on 1/5 MMNA medium. Following the culture for 30 days, the numbers of colonies were recorded.

2.2.4 Evaluation of salt-tolerances

Colonies formed by germinated basidiospores and mycelial fragments were isolated on 1/5 MMNA plates. Homokaryotic strains were observed for clamp connection. The isolate lacking clamp connection was regarded as a homokaryotic strain. Circular agar blocks (6.0 mm diameter) of recovered homokaryotic isolates were cut and inoculated on 1/5 MMNA medium containing 0 and 300 mM NaCl. After culturing for 25 days, the diameter of the colonies was measured, and the relative growth rate of each isolate in 300 mM NaCl was calculated to select halophilic and

sensitive strains. Three replicate experiments were performed for each strain.

Selected EMS-free and EMS-treated homokaryotic strains were continuously sub-cultured for one year on1/5 MMNA medium containing 0 and 300 mM NaCl for observing the variation in mycelial growth ability and examining the salt stability. For comparison with homokaryotic strains, the hybrid strains were also used to evaluate the salt stability after sub-culturing for six months.

2.2.5 Data analysis

Data were analyzed by ANOVA. The significant differences between means were determined by Duncan's multiple-range test. Unless otherwise stated, the differences were considered statistically significant when $p < 0.05$.

2.3 Results

2.3.1 Basidiospore mutation

When the basidiospores were treated with EMS, germination and subsequent colony formation were delayed with increasing concentrations of EMS. For the EMS-free basidiospores, colony formation could be observed within 10-20 days of incubation. In the case of EMS-treated basidiospores, colony formation was observed after incubation for 20-30

days. The number of colonies of isolates derived from the EMS-treated basidiospore suspension of fruiting body MCL2014Rhz41 decreased in a concentration-dependent manner (Fig. 2.1). When EMS concentration was over 40 mg/mL, no colony formed.

In total, 88 homokaryotic strains derived from the basidiospore suspension treated with different concentration of EMS were isolated. The average colony diameter of all strains for each treatment is shown in Table 2.1. Without the salt stress, there was no significant difference in the colony diameter between EMS-treated and EMS-free isolates. However, at a salt stress of 300 mM NaCl, the colony diameter of homokaryotic strains increased with increasing EMS concentration and peaked at 20 mg/mL EMS treatment. At a concentration of 20-30 mg/mL of EMS, both the colony diameter and relative growth rate on an agar plate containing 300 mM NaCl were significantly higher than at 0-10 mg/mL EMS treatment of the isolates.

There was a significant variation in mycelial growth ability of each isolate. At low-EMS concentrations or in the absence of EMS treatment, there were no halophilic homokaryotic strains with a relative growth rate over 100% on 300 mM NaCl medium. At high-EMS concentrations (20-30 mg/mL), five halophilic homokaryotic strains MCL2014Rhz41-ESp1 to MCL2014Rhz41·ESp5 (ESp1 to ESp5) that exhibited relative growth rates of over 100% on 300 mM NaCl medium could be isolated (Fig. 2.2). In addition to halophilic mutant strains, a salt-sensitive strain MCL2014Rhz41·ESp6 (ESp6) was isolated from basidiospores treated with low concentration (5 mg/mL) of EMS. The relative growth rate of

Esp6 was only 10% and showed retarded mycelia growth in medium containing 300 mM NaCl, compared to that of isolates derived from EMS-free basidiospores. These results indicate that EMS treatment of *R. roseolus* basidiospores at a concentration of 20-30 mg/mL seems to be optimal for the selection of halophilic mutants, while the EMS at a low concentration (5 mg/mL) might tend to induce salt-sensitive mutants.

2.3.2 Salt stability of selected strains and hybridization

After one-year of subculture, selected EMS-free and EMS-treated homokaryotic strains were inoculated on 1/5 MMNA medium containing 0 and 300 mM NaCl for observing the variation in mycelial growth ability and examining the salt stability (Table 2.2). After subculture for one year, mycelial growth ability of all of homokaryotic strains, except for Sp2 and ESp4, increased significantly, when compared to that prior to subculture on NaCl-free medium. On 300 mM NaCl medium, mycelial growth ability was significantly decreased in homokaryotic strains ESp2, ESp3 and ESp5. Furthermore, the halophilic ability of Sp1, ESp1，ESp2, ESp3 and ESp5 decreased after one-year continuous subculture, since the relative growth rate of these strains was significantly lower than one year ago (Table 2.2). However, the relative growth rate was significantly increased in Sp2 and ESp6. These results indicated that after one-year of subculture, the salt-tolerant ability increased in Sp2 and ESp6. From these results, it is evident that except for ESp4, all the homokaryotic strains showed a significant salt instability after subculture.

In the F1 generation, ten hybrid strains were obtained by mating between EMS-free homokaryotic strain and EMS-treated homokaryotic strain (Table 2.3). The halophilic was evident from the vigorous growth of the following six hybrid strains on 300 mM NaCl medium than in salt-free medium: ESp2 × ESp4, ESp2 × ESp3, ESp5 × ESp3, ESp6 × ESp3, ESp6 × ESp4, and Sp1 × ESp4. On the other hand, the hybrid strain, ESp1 × Sp2, exhibited a salt-sensitive trait, with a relative growth rate of 59% on 300 mM NaCl medium. The relative growth rate of the hybrid strains obtained by crossing between two strains from EMS-free original was 85.5%.

After six months of continuous subculture, except for ESp5 × Sp3 and ESp6 × Sp4, the relative growth rate of hybrid strains had no significant difference with a half year ago (Table 2.3). The result suggests that heterokaryotic strains tend to be more stable than homokaryotic strains after subculture in salt-free conditions.

2.3.3 Mycelia fragment mutation

Homokaryotic mycelial fragments filtered through a Cell Strainer revealed micro-fragments formed by a few mycelial cells. The length of some of the micro-fragments was 4.95 μm, which was similar to the diameter of a spore. Unfiltered mycelial fragments mostly formed a large aggregation of colonies. After coating on a plate, the unfiltered fragments could also be observed by the naked eye. Compared with mycelium and unfiltered fragments, the micro-fragments were easily accessible to the

chemical mutagen EMS, which increased the susceptibility to mutation.

After one week, micro-fragments of homokaryotic mycelium formed colonies. The colony numbers of homokaryotic strains showed significant variation. The number of colonies derived from mycelial fragments of basidiospore isolate Sp1 was significantly higher than that of Sp2 (Fig. 2.3). Without EMS, the recovery number of colonies was 143.9/plate. With EMS treatment at 20 mg/mL, the number was 30.2/plate. Moreover, the recovery colony formation was also delayed by EMS in both of the mycelial fragments.

The numbers of colonies recovered from mycelial fragments in the basidiospore isolate Sp2 decreased with increasing EMS concentrations. Importantly, no colonies formed in excess of 40 mg/mL EMS treatment. The recovery numbers of Sp2 were very low, at 13.0/plate (Fig. 2.3). Mycelial fragments treated with EMS at a concentration of 20 mg/mL yielded 3 colonies on 27 incubated plates. Treatment with EMS at 30mg/mL produced only one colony on 30 plates.

Eleven EMS-treated (20 mg/mL) and five EMS-free recovery strains were isolated from mycelial fragments of homokaryotic strain Sp1. These strains were incubated on agar plate containing different concentration of salt to evaluate their salt tolerance ability. On the non-NaCl medium, no significant difference was observed on mycelia growth ability between recovery isolates and original strain (Table 2.4). Under salt stress, mycelial growth ability and relative growth rate of EMS-treated recovery strains were significantly lower than the original strain. However, all of the strains exhibited impaired mycelial growth in the medium containing

300 mM NaCl, compared to that of the original strain (Fig. 2.4a). Among the recovery strains, there was no strain showing relative growth rate over 100% on 300 mM NaCl medium.

In contrast, mycelial growth of EMS-treated and EMS-free recovery strains derived from homokaryotic strain Sp2 was significantly vigor than the original strain on both salt and non-salt medium (Table 2.4). Under a salt stress of 300 mM NaCl, the relative growth rate of EMS-treated recovery strains was significant higher than that of original strain (Table 2.2, Fig. 2.4b). A halophilic EMS-treated strain showing a relative growth rate of 111% on 300 mM NaCl medium was selected, which exhibited an increased colony diameter in a salt concentration-dependent manner.

2.4 Discussion

The mature spore wall of the *R. roseolus* complex is comprised of at least four distinct layers: outer electron-lucent thin double layers, a mottled electron-dense third layer, and an electron-lucent fourth layer in which electron-lucent granular substances are dispersed (Shimomura et al., 2008). Shimomura et al. showed that the spore wall of *R. roseolus* may be physically reinforced by four distinct layers and heavily pigmented by deposition of electron-dense materials. Therefore, it seems that these specific spore wall structures may play a role in physical tolerance against the harsh environmental conditions such as in sandy soil, where survival depends on resistance to dryness and UV light. Another study also reported similar findings with reference to *Bacillus subtilis*

(Riesenman and Nicholson, 2000). In the present study, in order to exclude the possibility of a spore wall barrier against UV light, we used EMS, which is biologically reactive due to its ethyl group, which can be transferred to a variety of cellular nucleophilic sites (Sega, 1984), to induce mutations.

The optimal concentration of EMS in different fungi varied significantly. In the case of *Mucor mucedo*, 1, 10 and 50 mg/mL of EMS prevented either spore swelling or germ tube emergence, whereas an increased EMS concentration of 100 and 150 mg/mL, it inhibited the elongation of germ tube and hyphae, as well as spore germination (Moturi and Charya, 2010). In the case of *Aspergillus niger*, spore suspension treated with 2 mg/mL EMS did not show any change in morphology, whereas significant morphological change and a 0.75% survival rate were observed upon exposure to EMS for 1 h at 6 mg/mL (Radha et al., 2012). Pradeep and Narasimha (2011) reported that the spore survival rate of *A. niger* was less than 1% upon exposure to EMS at a concentration of 4 mg/mL for 60 and 90 min. In the case of the ectomycorrhizal fungus, *Tricholoma lascivum*, mutation occurred upon EMS treatment at 200 and 300 μg/mL and the fungus survived at a temperature of 36°C (Kamble and Mulani, 2012). In the present study, following EMS treatment for 5 h, no colonies derived from the basidiospore suspension were observed at a concentration over 40 mg/mL of EMS. Therefore, it may be inferred that the optimum concentration of EMS mutagen for the treatment of *R. roseolus* basidiospores for the selection of halophilic mutants was 20-30 mg/mL.

Most of the genetic mutations are either neutral or deleterious, while beneficial mutations are relatively rare. In order to increase the number of halophilic mutants, the culture media are usually supplemented with NaCl for directed selection of mutants, such as the method of screening for salt tolerance of sweet potato (Luan et al., 2007). Since basidiospore germination rate of *R. roseolus* was very low during salt stress, in this study, colony formation was not observed on salt medium (150-450mM NaCl) derived from EMS-treated spore suspensions (data not show). Without salt culture, the salt-sensitive mutant MCL2014Rhz41·ESp6 derived from low concentration (5 mg/mL) EMS-treated spore suspension, exhibited a relative growth rates of only 10% in medium containing 300 mM NaCl, and was also selected. The salt-sensitive strain has been used in research to analyze the resistance mechanism of salt stress in plants and fungi (Gossett et al., 1994; Amtmann et al., 2001; Sannazzaro et al., 2007).

Intraspecific variability of fungal isolates has to be considered in selection processes aimed at obtaining superior isolates for controlled inoculation (Trapper, 1977; Parladé et al., 2011). In the present study, the recovery numbers of colonies derived from different homokaryotic mycelial fragments showed significant variance. The difference between the two original homokaryotic strains could be associated with their salt-tolerance ability. The original homokaryotic strain Sp1, which exhibited a relative growth rate of 83.4%, showed a relatively higher salt-tolerance. Compared with Sp1, the original strain Sp2 seems to be a salt sensitive homokaryotic strain. In suboptimal conditions, the gene

products that are commonly induced include enzymes involved in the synthesis of putative osmoprotectants, proteases, and cyclophilins (Marivet et al., 1992). Hare et al. (1997) reported that the elevated internal cytokinins levels resulting from mycorrhizal symbiosis could be a primary factor causing improved plant growth, particularly under unfavourable conditions. Slankis (1973) has emphasized the possible involvement of cytokinins in mycorrhizal associations. Several reports have indicated that cytokinins have been successfully extracted from the culture filtrate of *R. roseolus* (Miller, 1967, 1971; Carol and Miller, 1974). In order to associate with the salt tolerance, the tolerant strain Sp1 could secrete more cytokinins, resulting in increased cytokinesis and vigorous increase in colony diameter at a salt stress of 300 mM NaCl. In addition, differential cytokinins secretion might affect asexual reproduction of micro homokaryotic mycelial fragments to form new colonies. Accordingly, it led to a significant difference in the number of recovery colonies derived from different salt-tolerant homokaryotic strains. However, this inference requires to be proven by a series of experiments, such as analysis of cytokinins activity in mycelia exposed to differential salt stress.

Another interesting finding was that during salt stress the regulatory ability of recovery strains from mycelial fragments seem to mutate directly opposite to their original strains. This phenomenon has also been observed after the EMS-free original strains Sp1 and Sp2 were subjected to continuous subculture for one year. In addition to EMS-free homokaryotic strains, the salt stability of almost all homokaryotic strains

exhibited a significant difference after subculture. After continuous subculture or recovery form micro-fragments, the salt tolerant ability of halophilic homokaryotic strains decreased. In contrast, when we used salt sensitive strains, their salt tolerant ability increased. These results may be explained by the fact that subculture or recovery of salt sensitive homokaryotic strains still exhibit vigorous cytokinesis, causing increased ability of reproduction, leading to a vigorous mycelial growth in 300 mM NaCl stress than their original strains. This inference can be proved by the observation that after one-year subculture, the mycelial growth ability of most homokaryotic strains on the salt-free medium was significantly higher than one year ago. In addition to cytokinesis ability, the main survival strategies under salt pressure for fungi require additional adjustment capability. Exposure to high salinity includes two different environmental stimuli for the cell: osmotic stress, and ionic stress (Plemenitaš et al., 2014). The morphological and physiological mechanism(s) of salt tolerance in soil-borne fungi have not been elucidated. An excess of ions may alter membrane integrity, enzymatic activity, as well as protein and nucleic acid metabolism (Yeo, 1983; Gadd, 1993; Hasegawa et al., 2000; Posas et al., 2000; Bois et al., 2006). Subculture in a salt-free environment led to fungal cell reproduction and adaptive ability to decrease osmotic stress and ionic stress. These serious reactions led to the subculture and recovery salt-tolerant strains exhibited a significant negative mycelial growth and relative growth rate on 300 mM NaCl medium, compared to that of original strains.

Along with physiological stress, which is implicated in chromosome

rearrangement of fungal genomes, several potentially destabilizing genetic elements are known to occur in fungi (Smith, 2008). In the present study, after several rounds of mitosis, EMS-free recovery strains showed variability in salt stress compared to original strains. However, the related growth rate between EMS-free recovery strains and original strains had no significant differences in the statistical analysis. These results suggest that natural variation appeared through vegetative propagation, but the magnitude of variation was small and had no significant differences between the recovery strains. EMS can cause a high frequency of gene mutations and low frequency of chromosome aberrations (Van Harten, 1998). With EMS-treatment, the variability was more significant between mutant strains and original strains. After EMS treatment, the magnitude of variation increased, and which exhibited significant differences between EMS-treated recovery strains.

Recently, some parts of the life cycle of *R. roseolus* were revealed by Shimomura et al. (2008). After meiosis, generally eight monokaryotic spores per basidium are produced (Shimomura et al., 2008). The germlings from the spores subsequently become homokaryotic hyphae, equivalent to a basidiospore isolate (Sawada et al., 2014). Kawai et al. (2008) reported that *R. roseolus* exhibits a bipolar incompatibility system. After mating, the hybrid strains could become heterokaryotic hyphae and form clamp connection. In the present study, hybrid strains, which were mated with all halophilic mutant parents, uniformly showed a higher relative growth in 300 mM NaCl stress. The relative growth of the hybrid strains that were mated with one halophilic and one salt-sensitive parent

was variable. These results indicate that the phenotype of salt tolerance in *R. roseolus* may be controlled by multiple recessive genes (Nakano et al., 2015). Individuals with two copies of each gene should have a higher phenotypic stability than those with one copy (Barbara and Sarah, 1998). In the present study, compared with homokaryotic strains, most hybrid strains showed no significant change in the relative growth rate after six months of subculture. This result suggests that subculture of heterokaryotic strains appears to be more stable than subculture of homokaryotic strains under salt stress.

Ectomycorrhizal fungi are basically propagated in soil conditions, not on agar. Furthermore, their propagation is considered to depend on host pine activity. If halophilic characteristics are also found in soil, this will be applicable to reforestation in salt-containing environments. Therefore, evaluation of halophilic characteristics is now ongoing not only in soil but also in symbiosis with host pine roots.

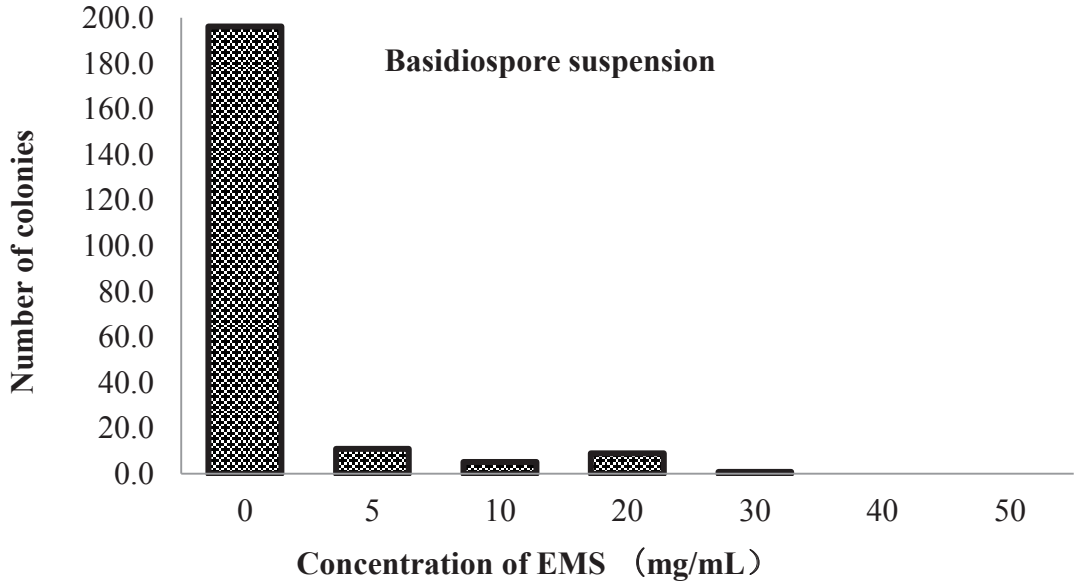

Fig. 2.1. The numbers of colonies derived from basidiospore suspensions of *Rhizopogon roseolus* with EMS treatment at different concentration.

Table 2.1. Colony diameter of recovered strains from different EMS treatment of *Rhizopogon roseolus* basidiospore suspensions on agar medium containing 0 and 300 mM NaCl

Concentration of EMS (mg/mL)	Number of strains	Colony diameter in different NaCl concentration (mm)		Relative growth rate (%)
		0 mM NaCl	300 mM NaCl	
0	15	52.8 ± 5.7 a	37.8 ± 7.9 b	71.5 ± 13.4 b
5	17	55.7 ± 7.2 a	37.0 ± 10.4 b	66.3 ± 17.4 b
10	26	52.2 ± 9.3 a	39.6 ± 8.7 ab	74.5 ± 10.6 b
20	26	53.8 ± 8.7 a	45.1 ± 9.1 a	84.4 ± 14.9 a
30	4	50.1 ± 12.9 a	43.5 ± 5.0 a	91.9 ± 26.5 a

The values are the average values of colony diameter of all strains in each treatment, and ± SD of different strains. The data were analyzed by one-way ANOVA and compared by LSD. The value with the same letter within a column was not significantly different at $p < 0.05$. Each strain had at least three repeats.

Relative growth rate (%) = (colony diameter on 300 mM NaCl medium / colony diameter on 0 mM NaCl medium) ×

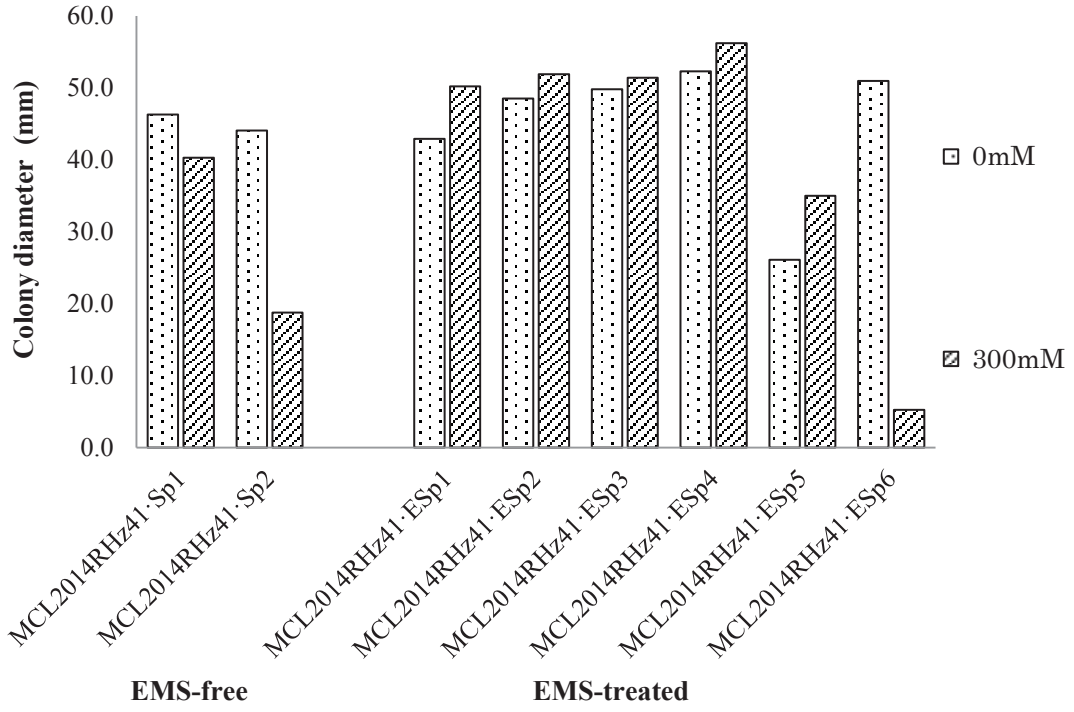

Fig. 2.2. Colony diameter of selected EMS-free and EMS-treated homokaryotic strains of *Rhizopogon roseolus* on agar medium containing 0 and 300 mM NaCl.

Table 2.2. The variety of salt stability in homokaryotic strains of *Rhizopogon roseolus* after one-year continuous subculture.

Homokaryotic strains	Colony diameter in different NaCl concentrations (mm)				Relative growth rate (%)	
	0 mM NaCl		300 mM NaCl			
	One year ago	After one year	One year ago	After one year	One year ago	After one year
EMS-free						
MCL2014RHz41·Sp1	51.3	56.6 **	45.3	44.1	88.4	78.0**
MCL2014RHz41·Sp2	49.1	43.3**	23.7	30.7*	48.4	71.0*
EMS-treated						
MCL2014RHz41·ESp1	47.9	53.5**	55.2	53.5	115.4	100.3**
MCL2014RHz41·ESp2	53.5	64.2 **	56.9	53.0*	106.4	83.1**
MCL2014RHz41·ESp3	54.8	58.7**	56.4	53.9 *	102.9	91.9**
MCL2014RHz41·ESp4	57.3	57.3	61.2	59.6	106.9	104.1
MCL2014RHz41·ESp5	31.1	33.8**	40.0	27.5 **	128.7	81.5**
MCL2014RHz41·ESp6	56.0	64.2 **	10.2	41.7 **	18.6	65.0**

The values are the average values of colony diameter (before or after one year subculture) of each strain. The data were analyzed by one-way ANOVA and compared by LSD. Each strain had at least three repeats.

Relative growth rate (%) = (colony diameter on 300 mM NaCl medium / colony diameter on 0 mM NaCl medium) × 100

* means the value had a significant difference at $p < 0.05$ after subculture.

** means the value had a significant difference at $p < 0.01$ after subculture.

Table 2.3. The variety of salt stability in *Rhizopogon roseolus* hybrids F1 strains after half-year continuous subculture.

Hybrid strains	Colony diameter in different NaCl concentrations (mm)				Relative growth rate (%)	
	0 mM NaCl		300 mM NaCl			
	6 months ago	After 6 months	6 months ago	After 6 months	6 months ago	After 6 months
MCL2014RHz41·ESp2× MCL2014RHz41·ESp4	54.4	56.4	54.6	58.6*	100.8	103.7
MCL2014RHz41·ESp2× MCL2014RHz41·ESp3	51.7	50.6	54.5	52.4	105.5	103.2
MCL2014RHz41·ESp5× MCL2014RHz41·ESp3	39.2	42.2	44.5	38.1**	114.7	90.3%**
MCL2014RHz41·ESp6× MCL2014RHz41·ESp3	70.5	70.5	72.4	71.5	102.2	101.4
MCL2014RHz41·ESp6× MCL2014RHz41·ESp4	67.6	61.2 **	70.0	58.3 **	105.2	96.6*
MCL2014RHz41·Sp1 × MCL2014RHz41·ESp3	44.9	45.8	42.5	39.4 *	95.0	86.3
MCL2014RHz41·Sp1 × MCL2014RHz41·ESp4	46.3	48.6*	47.9	50.0	103.4	102.9
MCL2014RHz41·ESp1× MCL2014RHz41·Sp2	60.9	65.5**	35.8	37.0	58.7	56.4
MCL2014RHz41·ESp6× MCL2014RHz41·Sp2	64.5	56.3 **	55.9	47.6 **	86.8	84.6
MCL2014RHz41·Sp1 × MCL2014RHz41·Sp2	59.8	55.7 **	51.5	46.1**	85.5	83.6

The values are the average values of colony diameter (before or after six months subculture) of each strain. The data were analyzed by one-way ANOVA and compared by LSD. Each strain had at least three repeats.

Relative growth rate (%) = (colony diameter on 300 mM NaCl medium / colony diameter on 0 mM NaCl medium) × 100

* means the value had a significant difference at $p < 0.05$ after subculture.

** means the value had a significant difference at $p < 0.01$ after subculture.

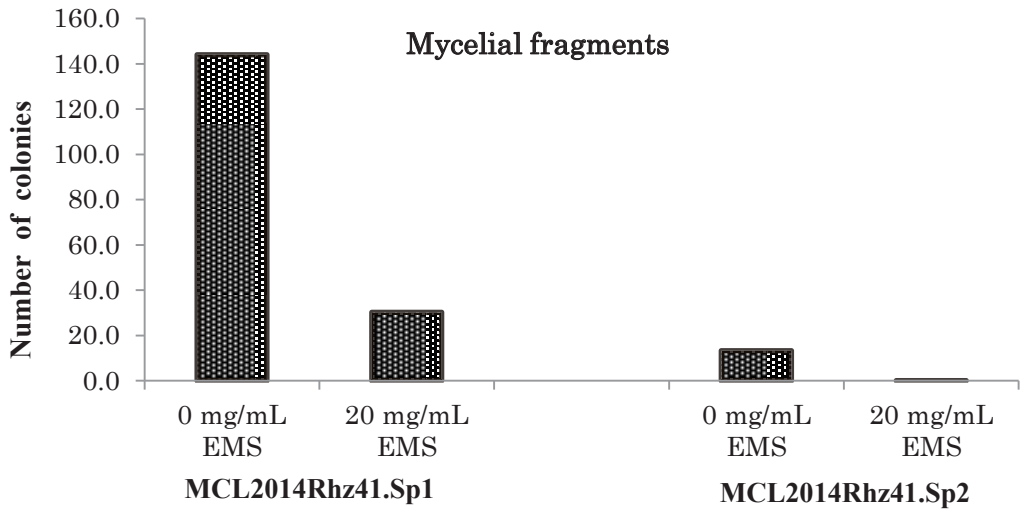

Fig.2.3. The numbers of colonies derived from EMS-treated and EMS-free mycelial fragments of *Rhizopogon roseolus* homokaryotic strains MCL2014Rhz41·Sp1 and MCL2014Rhz41·Sp2. The concentration of EMS was 20 mg/mL.

Table 2.4. Colony diameter of recovered strains from *Rhizopogon roseolus* homokaryotic mycelial fragments on agar medium containing 0-300 mM NaCl.

Recovered strains from homokaryotic mycelial fragments	Number of strains	Colony diameter in different NaCl concentrations (mm)		Relative growth rate (%)
		0 mM NaCl	300 mM NaCl	
MCL2014Rhz41·Sp1	1	48.7	40.4	83.4
0 mg/mL EMS	5	48.6 ± 9.1	36.6 ± 9.2	75.0 ± 5.8
20 mg/mL EMS	11	43.7 ± 8.4	30.5 ± 10.1*	69.0 ± 14.2*
MCL2014Rhz41·Sp2	1	45.6	28.9	63.3
0 mg/mL EMS	5	51.9 ± 5.3*	39.8 ± 5.6*	76.9 ± 3.6
20 mg/mL EMS	4	54.4 ± 3.7*	48.0 ± 10.3*	87.8 ± 14.9*

The values are the average values of colony diameter of all strains in each treatment, and ± SD of different strains. The data were analyzed by one-way ANOVA and compared by LSD. Each Strain had at least three repeats.

Relative growth rate (%) = (colony diameter on 300 mM NaCl medium / colony diameter on 0 mM NaCl medium) × 100

* means the value had a significant difference at $p < 0.05$ to compare with original strain.

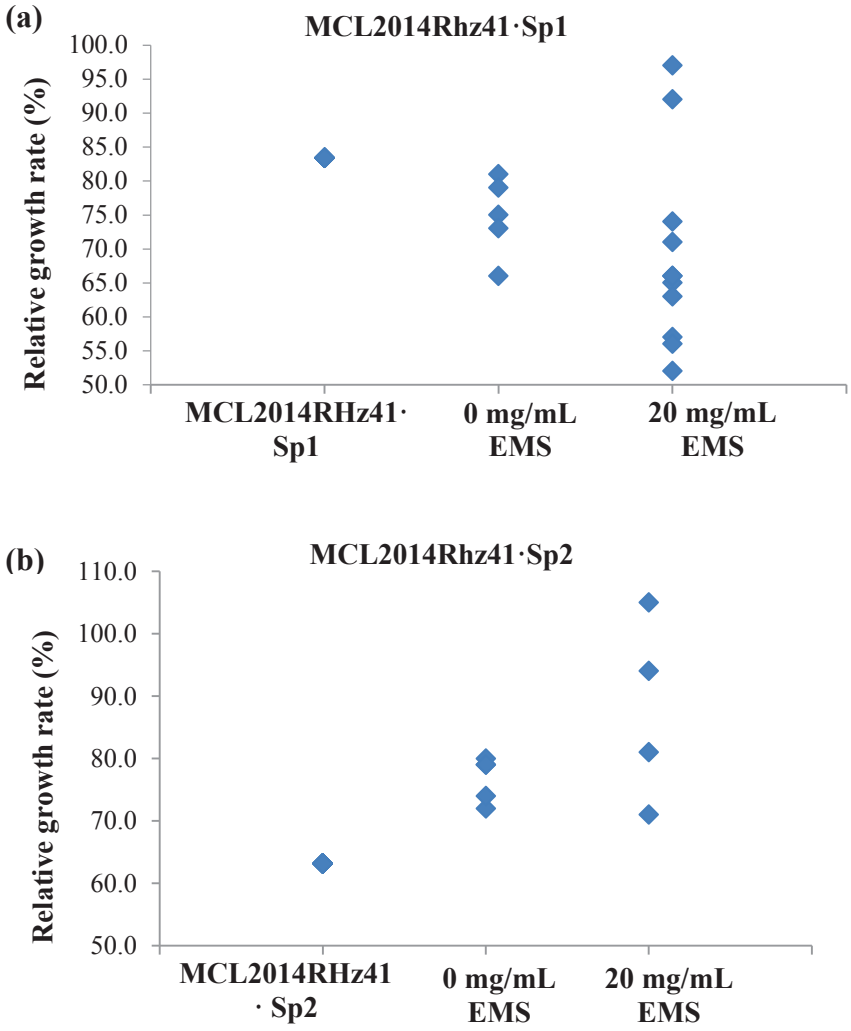

Fig.2.4. Comparison of the salt tolerance ability of original homokaryotic strains and recovered strains from *Rhizopogon roseolus* mycelial fragments by calculating their relative growth rate on agar medium containing 300 mM NaCl.

a: The relative growth rate (%) of original homokaryotic strains MCL2014Rhz41·Sp1 and its recovered strains

b: The relative growth rate (%) of original homokaryotic strains MCL2014Rhz41·Sp2 and its recovered strains

Chapter 3

Comparison of salt-tolerance of ectomycorrhizal fungi *Rhizopogon roseolus* in soil and on agar

3.1 Introduction

Saline soils occupy approximately 7% of the earth's land surface (Yeo, 1983; Jain et al., 1989), and cause serious environmental and agricultural problems in many areas of the world, particularly in arid and semi-arid regions (Dashtebani et al., 2014). Salt-affected soils have physiochemical properties that could in turn reduce their suitability for plant growth and thus reduce their value for agriculture (Rhoades, 1990).

Recent studies have suggested that ectomycorrhizal fungi can help the host plant take up phosphorus and water more efficiently (Duddridge et al., 1980), indirectly increase plant growth, and reduce the toxicity due to effects of ions (Pond et al., 1984; Azcón and El-Atrash, 1997; Tang et al., 2009). Plants relying on symbiotic relationships for adequate mineral nutrition and water uptake may differ in salt tolerance, depending on the tolerance of host and symbiotic fungi to soil salinity (Reddell et al., 1986; Perry et al., 1987; Marcar et al., 1991). Extreme soil salinity can have adverse effects on mycorrhizal propagules in the rhizosphere, fungal colonization of roots, and ectomycorrhizal structure and function (Reddell, 1986; Hennessey et al., 1989). Soils having high levels of salts also can inhibit fungal growth (Tresner and Hayes, 1971; Dixon et al.,

1993).

Rhizopogon roseolus (Corda) Th.M. Fr. (=*R.rubescens* Tul. & C. Tul.) is an ectomycorrhizal basidiomycete with a worldwide distribution (Molina and Trappe, 1994) that is called “shoro” in Japanese. The fruiting bodies of this fungus are found in the sandy soils of *Pinus thunbergii* Parl. forests in seashore habitats and are prized as an edible mushroom (Kawai et al., 2008; Shimomura et al., 2010). This fungus is also salt tolerant (Nakanoet al., 2015). In previous studies, salt-tolerant strains were obtained by cross-breeding (Nakano et al., 2015) and mutation (Gao et al., 2016). However, there are no studies characterizing the salt tolerance of *R. roseolus* strains in soil. The ability of ectomycorrhizal fungus to grow in soil without host plants is still being studied. Here, I used sandy soil collected in the wild to investigate the growth of *R. roseolus* strains in soil, and analyzed the correlation of mycelial growth when cultured on soil and in agar.

3.2 Material and methods

3.2.1 Fungal isolates

Fresh wild fruiting bodies of *R. roseolus* were collected from the *P. thunbergii* forest of the Arid Land Research Center, Tottori University. A tissue culture isolate (MCL2015Rhz77) was obtained from the fruiting body (Table 3.1). The homokaryotic strains used in this study were obtained by basidiospore isolation from fruiting body MCL2014Rhz41 (Table 3.1). Hybrid strains were obtained by crossing between selected

untreated and EMS-treated homokaryotic strains (Table 3.1). All of the strains were cultured on 1/5 Modified Melin-Norkrans agar (Marx, 1969) (MMNA) plates in preparation for the experiments.

3.2.2 Mycelial growth on agar medium

Daigo's Artificial Seawater SP was used to adjust the level of salinity in agar medium. Circular agar blocks (6.0 mm diameter) of each strain were cut and inoculated on 1/5 MMNA medium containing 0, 50, and 100% seawater. After culturing for 25 days, the diameter of the colonies was measured. Five replicate experiments were performed for each strain.

3.2.3 Mycelial growth in soil substrate

The sandy soil used in this study was collected at the *P. thunbergii* forest of Arid Land Research Center, Tottori University. The sandy soil was air-dried for one month at room temperature. Dried soil was sieved through an iron mesh (pore size 25 mm) to remove dry mycorrhizal roots and external hyphae. Then, 1/5 MMN liquid or distilled water (DW) were injected into the soil until the moisture content was saturated. The wet soil was put in a plastic bag, following sterilization at 121°C for 60 minutes. The strains were inoculated in two ways, viz., upper side inoculation and bottom side inoculation. For upper side inoculation, a Petri dish was filled with about 35 g sterilized soil and the surface was

Petri dish was filled with about 35 g sterilized soil and the surface was inoculated with a circular agar block (6.0 mm diameter) bearing mycelia of each strain. For bottom side inoculation, a 1/5 MMNA agar plate was inoculated with mycelia, incubated at 25°C for one week, and then filled with sterilized soil. After 60 days of culture, colony formation and mycelial growth in the soil substrate were observed by stereomicroscope and light microscope.

The sandy soil was injected with 1/5 MMN liquid containing 0, 50 and 100% the salinity of seawater for use in these experiments. Individual glass tubes were filled with the wet soil. The filled glass tubes were sterilized for 60 min at 120°C. Two-week-old mycelial colonies were cut into 3 mm^2 mycelial agar plugs and then inoculated into the soil substrate. After incubation at 25°C for 60 days, the mycelial growth in soil was observed through a stereomicroscope and recorded daily.

3.2.4 Data analysis

Microsoft Office Excel 2010 and SPSS 7.0 software were used for data calculation and statistical treatments. One-way ANOVA and Fisher's least significant difference (LSD) test were conducted to identify significant differences. Correlation analysis used the Pearson correlation function provided by SPSS, with $p < 0.05$ or $p < 0.01$ determined to be a statistically significant correlation.

3.3 Results

3.3.1 Screen observation

In this study, all strains could grow well and formed a mycelial colony on the surface of sandy soil containing 1/5 MMN liquid or DW (Fig. 3.1a-f). While thin mycelial colonies were observed in sandy soil saturated with DW, more visible mycelial colonies were observed in sandy soil saturated with 1/5 MMN (Fig. 3.1b, d). On soil containing either 1/5 MMN or DW, colonies of the homokaryotic strain MCL2014Rhz41 • Sp2 were significantly smaller and thinner than colonies of heterokaryotic strains (Fig. 3.1e, f).

3.3.2 Quantitative determination of mycelial growth

3.3.2.1 Mycelial growth on agar medium

NaCl is usually used for evaluation of salt tolerance and in breeding studies (Tresner and Hayes, 1971; Dixon et al., 1993; Tang et al., 2009; Nakano et al., 2015). However, in nature, soil salt is composed not only of sodium chloride, but also magnesium, calcium, and sulfate salts, and secondarily potassium, bicarbonate, carbonate, nitrate, and boron salts (Rhoades, 1990). In this study, the salt tolerance of *R. roseolus* strains was studied using artificial seawater to simulate the natural seashore soil environment in order to evaluate the salt tolerance of *R. roseolus* strains under various ionic salt conditions.

The salt tolerance of each strain varied significantly on agar medium

(Fig. 3.2a). Mycelial growth of hybrid strain H1 on agar medium increased with increasing salt concentration, demonstrating that this strain is halophilic. For hybrid strain H7, mycelial growth on agar medium containing 50% of the salinity of seawater was more vigorous than on salt-free agar medium; however, mycelial growth was slightly decreased on agar medium containing 100% seawater. Even so, hybrid strain H7 still exhibited salt tolerance on agar medium. For hybrid strain H9, mycelial growth was significantly inhibited by salt stress on agar media with both 50% and 100% of the salinity of seawater; consequently, it was recognized as a salt-sensitive strain. On 50% and 100% seawater agar medium, mycelial growth of wild strain MCL2015Rhz77 was decreased and its relative growth rate was between that of a salt-tolerant strain and a salt-sensitive strain. Among the homokaryotic strains, mutant strain MCL2014Rhz41·ESp3 exhibited salt tolerance as high as that of hybrid strain H7. Mycelial growth of single basidiospore isolate MCL2014Rhz41·Sp2 was significantly decreased on agar medium containing 100% seawater, and its relative growth rate was 86.7% of that on agar medium containing 50% seawater.

3.3.2.2 Mycelial growth in soil substrate

The mycelial growth rate in glass tubes containing soil was determined quantitatively (Fig. 3.3). All strains grew well in sandy soil without host plants. The mycelia of *R. roseolus* seemed more sensitive to salt in soil than on agar, because the mycelial growth of almost all strains

was inhibited by 50% and 100% of the salinity of seawater in soil. However, hybrid strains H1 and H7 consistently exhibited a higher mycelial growth rate than other strains in soil containing 50% and 100% of the salinity of seawater (Fig. 3.2b). In addition, salt-sensitive hybrid strain H9 was completely inhibited at 100%, showing no apparent mycelial growth. Compared to wild and homokaryotic strains, hybrid strains exhibited vigorous growth in soil (Figs. 3.2b, 3.3). A significant positive correlation ($p < 0.05$) was observed between the mycelial growth of *R. roseolus* in non-saline soil and on non-saline agar medium (Fig. 3.4a). Mycelial growth in soil containing 50% of the salinity of seawater tended to correlate with that in 50% seawater on agar. Furthermore, mycelial growth both 50% and 100% salinity soil positively correlated ($p < 0.05$) with mycelial growth on 100% seawater agar (Fig. 3.4c). These results indicated that the mycelial growth of *R. roseolus* shows a positive correlation in soil substrate and on agar medium, but that the mycelia are more sensitive to salt in soil than on agar, so sandy soil is useful for characterizing the salt tolerance of strains.

3.4 Discussion

Growth of extraradical mycelia in artificial soil (peat moss and vermiculite) with host plants has been widely reported (Wallander et al., 2001; Hortal et al., 2008, 2009; Weigt et al., 2012). However, there are few reports on the mycelial growth of ectomycorrhizal fungi in natural soils without a host. In the present study, both homokaryotic and

heterokaryotic strains of *R. roseolus* grew well in natural sandy soil without a host. The sandy soil used in this study was a natural soil collected from a *P. thunbergii* forest. Therefore, the soil may have contained host root secretions that promote mycelial growth.

Ectomycorrhizal fungi can easily colonize substrates with low organic matter content, since they have a carbon source provided from the tree, and they are good at transporting substances over long distances (Wallander et al., 2001). The density of the extraradical mycelium of ectomycorrhizal fungi decreases gradually with increasing distance from the ectomycorrhizal mantle (Weigt et al., 2012). Without a host plant, the distribution of water within soils plays a crucial role in governing fungal development and activity, as does the spatial distribution of nutrients (Ritz and Young, 2004). In the present study, the mycelium was also more likely to proliferate in soil with added nutrient solution. Hyphae of *R. roseolus* might absorb the carbon sources in moist sand, network in gravel gaps and aggregate to form homogeneous mats on the surface of sandy soil. A similar phenomenon was reported by Harris et al. (2003), which demonstrated that hyphae of *Rhizoctonia solani* can bridge air gaps in pore networks, though there may be a metabolic cost associated with such growth, and that ultimately surface-associated growth is more efficient

Mycelial biomass has also been quantified using mesh bags (Wallander et al., 2001, 2010; Hendricks et al., 2006; Hedh et al., 2008; Majdi et al., 2008). The use of biochemical markers such as ergosterol and phospholipid fatty acids (PLFAs) has become a popular method for

biomass quantification (Nilsson et al., 2005; Clemmensen et al., 2006; Wallander et al., 2010). However, much less attention has been directed at mycelial growth. In the present study, I evaluated the correlation of mycelial growth in soil substrate and on agar medium. I observed significant positive correlation of the final growth rate in both media. The mycelia of *R. roseolus* seemed more sensitive to salt in soil than on agar.

Research on salt tolerance of mycorrhizal fungi is mostly focused on the growth and dry weight of their symbiotic plants in a saline environment, on the effects of absorption of nutrients such as P and N (Harley, 1989; Juniper and Abbott, 1993; Turjaman et al., 2006), and on the absorption of water-soluble ions such as K^+ and Ca^{2+} in plant cells (Bandou et al., 2006). Even in vitro studies of mycorrhizal fungi mostly concentrate on pure cultures on plates and in liquid environments to evaluate their potential salt tolerance (Dixon et al., 1993; Chen et al., 2001; Bois et al., 2006; Mulligan, 2007). However, few studies have characterized the salt tolerance of mycorrhizal fungi in soil or analyzed the correlation of mycelial growth in soil and on agar. With this method, I could clearly observe the behavior of mycelia and track their growth trajectory in a soil substrate. Colpaert and van Tichelen (1996) pointed out that one of the best ways of studying the effect of environmental stress factors on mycorrhizas may be to focus on the growth of the external mycelium. The salt-tolerance mechanism induced by *R. roseolus* ectomycorrhizal fungi is not well understood. In further study, I will employ this assay to reveal the interactions between the extraradical mycelium from ectomycorrhizas and their host plants under various environmental conditions.

Table 3.1. The strains used in this study

Strains	Description	Original
H1	MCL2014RHz41·ESp2× MCL2014RHz41·ESp4	Chapter 2
H7	MCL2014RHz41·ESp6× MCL2014RHz41·ESp3	Chapter 2
H9	MCL2014RHz41·ESp1× MCL2014RHz41·Sp2	Chapter 2
MCL2015Rhz77	Tissue culture isolate	Fruiting body collected in the *P. thunbergii* forest of the Arid Land Research Center, Tottori University in March 2015.
MCL2014Rhz41·ESp3	Basidiospore isolate recovered from spore suspension treated with EMS	Chapter 2
MCL2014Rhz41·Sp2	Basidiospore isolate	Chapter 2

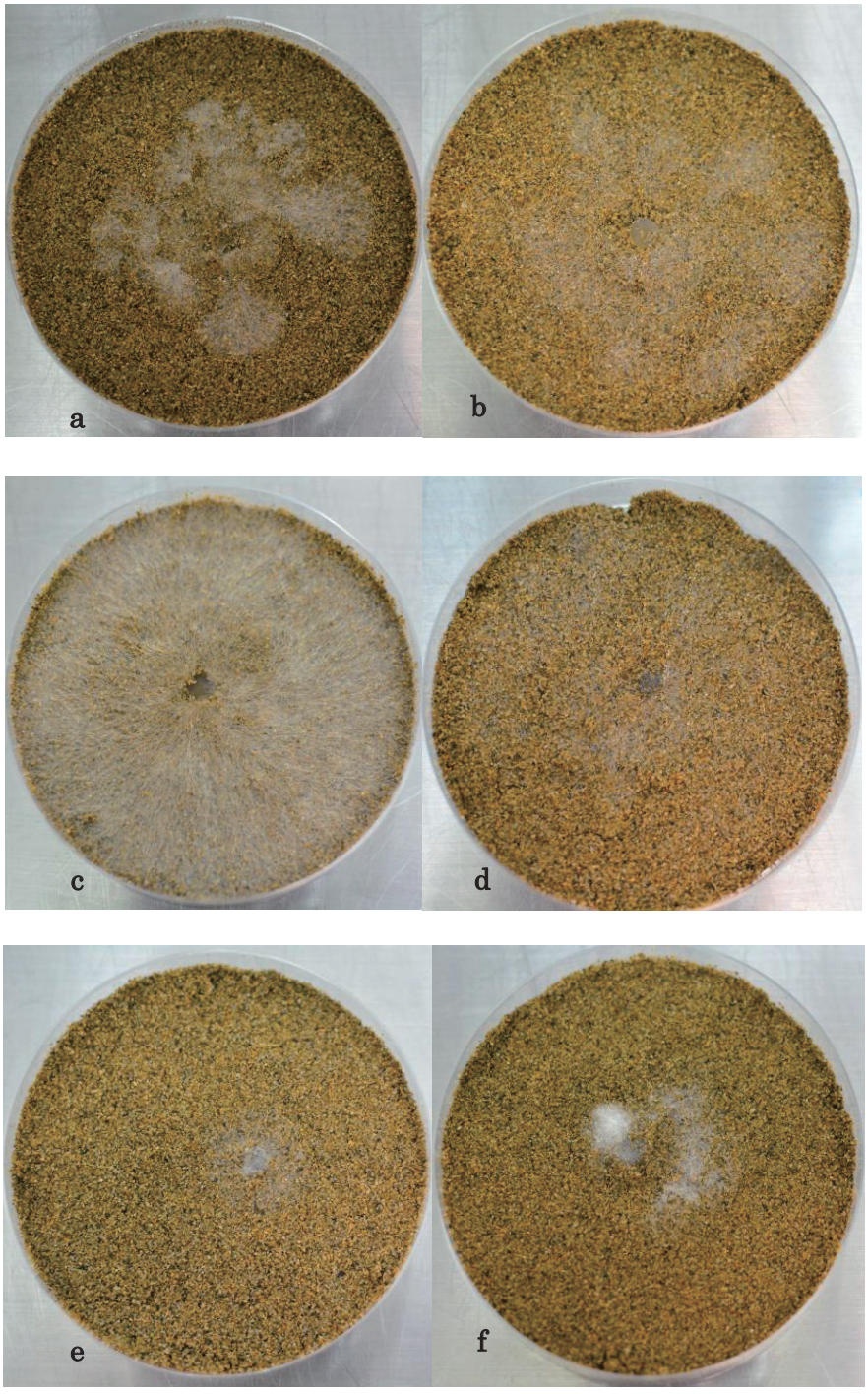

Fig 3.1. Colony formation of *R. roseolus* on soil medium containing 1/5 MMN or DW.

a: Wild strain MCL2015Rhz77 with 1/5 MMN

b: Wild strain MCL2015Rhz77 with DW

c: Hybrid strain H1 with 1/5 MMN

d: Hybrid strain H1 with DW

e: Homokaryotic strain MCL2014Rhz41·Sp2 with 1/5 MMN

f: Homokaryotic strain MCL2014Rhz41·Sp2 with DW

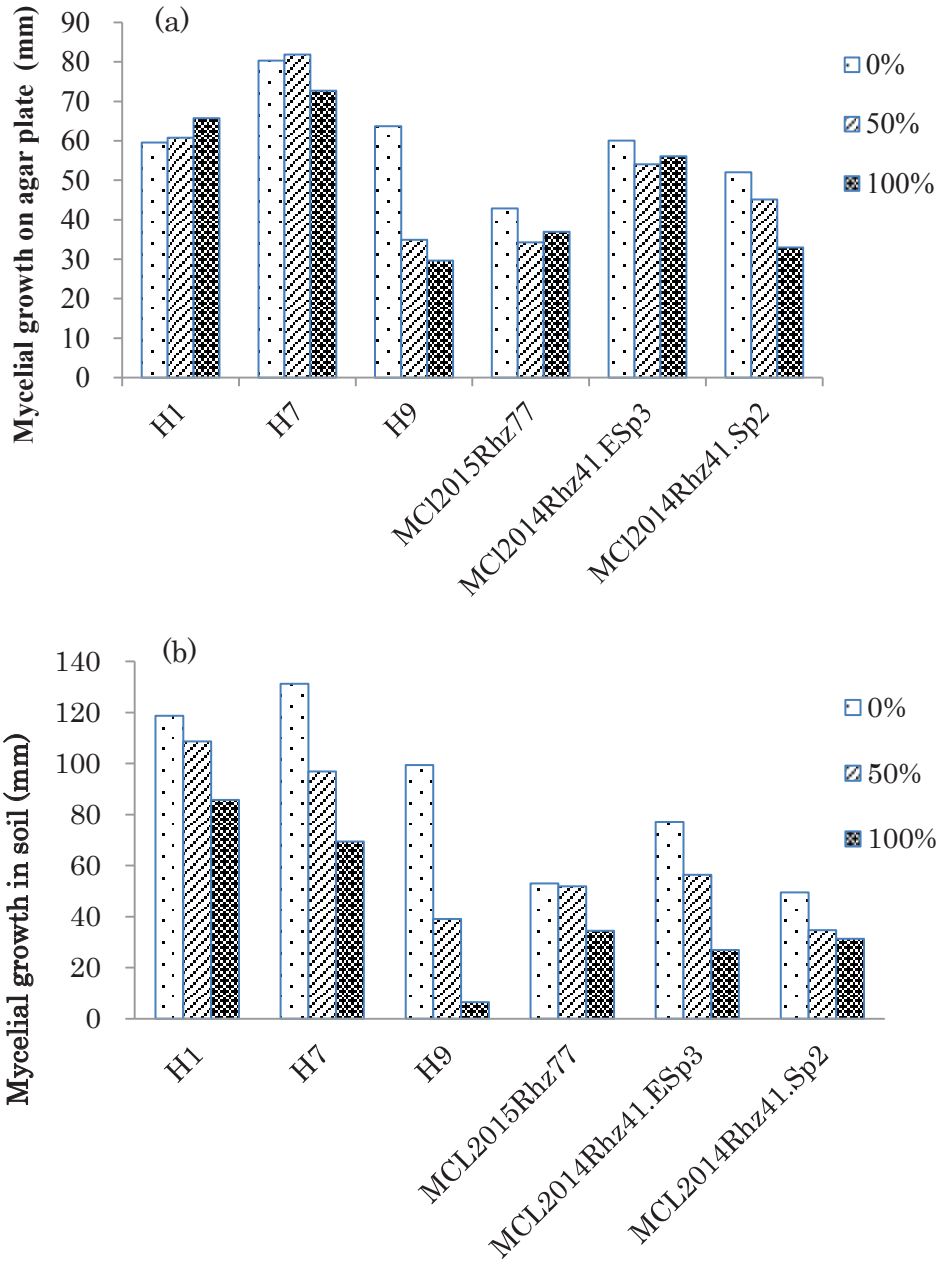

Fig 3.2. Mycelial growth of *Rhizopogon roseolus* in agar medium (a) or in soil (b) containing seawater. % means the percentage salinity of seawater.

Fig 3.3. The mycelial growth of *R. roseolus* in a glass tube.

Arrows indicate the position of the front edge of mycelial growth.

a-1: Hybrid strain H1 in soil with salinity of seawater

a-2: Hybrid strain H1 in non-saline soil

b-1: Hybrid strain H7 in soil with salinity of seawater

b-2: Hybrid strain H7 in non-saline soil

c-1: Hybrid strain H9 in soil with salinity of seawater

c-2: Hybrid strain H9 in non-saline soil

d-1: Wild strain MCL2015Rhz77 in soil with salinity of seawater

d-2: Wild strain MCL2015Rhz77 in non-saline soil

e-1: Homokaryotic strain MCL2014Rhz41·ESp3 in soil with salinity of seawater

e-2 Homokaryotic strain MCL2014Rhz41·ESp3 in non-saline soil

f-1: Homokaryotic strain n MCL2014Rhz41·Sp2 in soil with salinity of seawater

f-2: Homokaryotic strain MCL2014Rhz41·Sp2 in non-saline soil

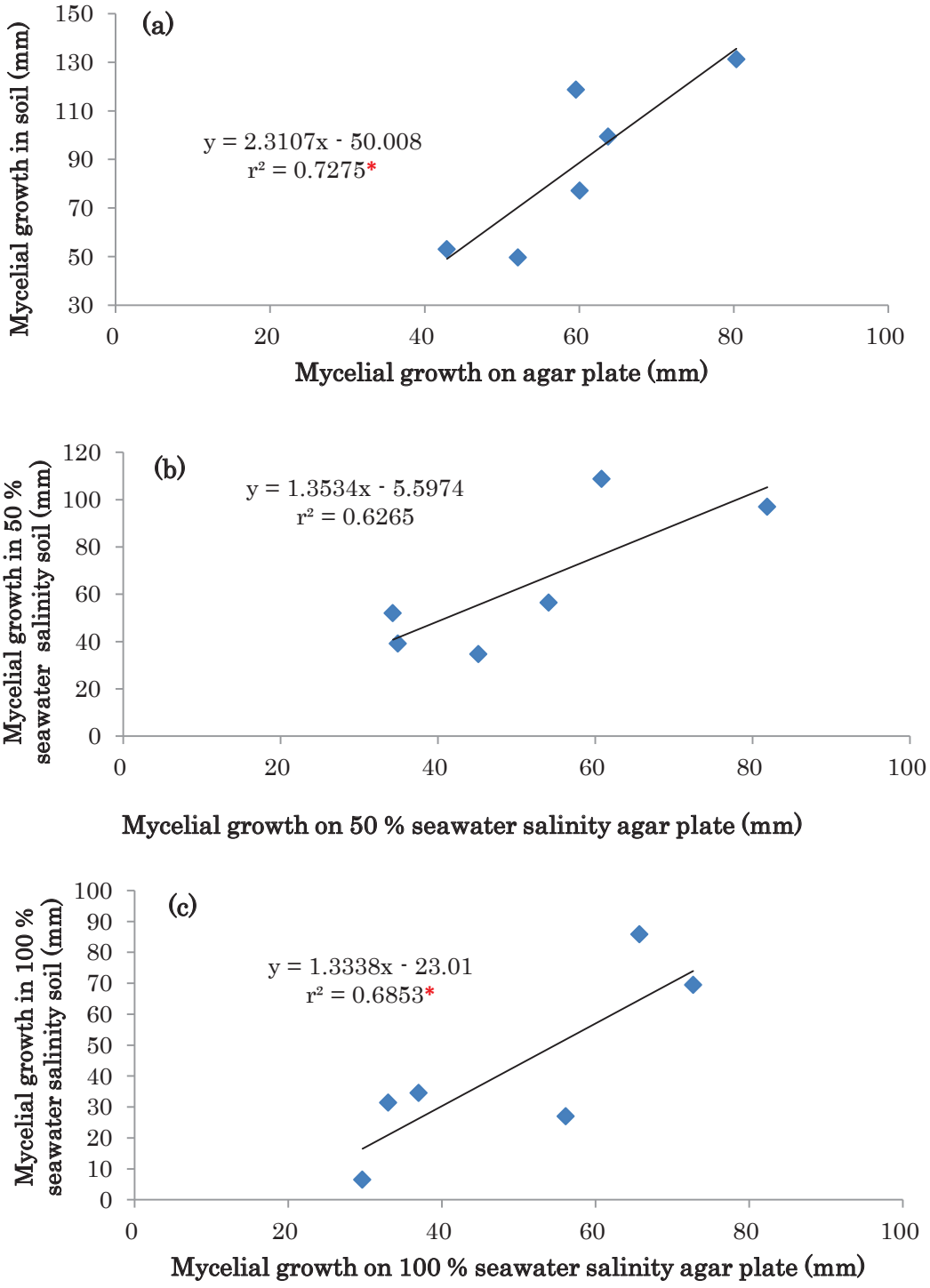

Fig. 3.4. The correlation of mycelial growth in soil and on agar at different levels of salinity.

a: Growth in non-saline conditions

b: Growth at 50% salinity of seawater

c: Growth at 100% salinity of seawater

* means positive correlation of mycelial growth between in soil and on agar has been obtained by Pearson correlation using SPSS ($p < 0.05$).

Chapter 4

Detection of non-specific stress tolerance of the ectomycorrhizal fungi *Rhizopogon roseolus*

4.1 Introduction

Drought is considered one of the most impactful and stressful environmental conditions, as water stress leads to limited crop productivity in arid and semi-arid regions (Maggio et al., 2000; Chaves et al., 2003). In general, ectomycorrhizal symbiosis is ubiquitous on the roots of many plant families, such as *Pinaceae*, *Fagaceae,* and *Betulaceae* (Rincón et al., 2007). Ectomycorrhizal fungi play an important role in the development of forest ecosystems, because the dominant tree species in most of the world's temperate and boreal forests are ectomycorrhizal (Zhou et al., 1997). The external mycelia extend the root systems for water, phosphorus, and nitrogen uptake (Smith and Read, 2008; Lehto and Zwiazek, 2011). Read and Boyd (1986) suggested that the benefits of colonization are likely to arise particularly under stress conditions, and mycorrhizas could beneficial through their capacity to provide the minimum requirements for survival of the plant during episodes of drought.

Many mycorrhizal fungi can tolerate drought and high temperature stress (Han et al., 2006). Many ectomycorrhizal fungi have a maximal underwater growth potential of -0.5 to -1.5 MPa, while some species

grow well under a water potential of -2.0 MPa (Zhao and Guo, 1989). Other fungi that are believed to be more drought-tolerant include species with strong external mycelia, such as *Rhizopogon* species (Parke et al., 1983; Boyle and Hellenbrand, 1991; Dunabeitia et al., 2004). In addition to between-species variation, within-species variation may also make a difference in different conditions (Newsham et al., 1995; Lehto and Zwiazek, 2011).

Rhizopogon roseolus (Corda) Th. M. Fr. (=*R. rubescens* Tul. & Tul.), known as "shoro" in Japanese, is a hypogeous basidiomycete that is an important ectomycorrhizal symbiont of *Pinaceae* (Morina and Trappe, 1994). The fruiting bodies of this mushroom are found in the sandy soils of *Pinus thunbergii* Parl. forests in seashore habitats, and are prized as an edible mushroom and traded at high prices in local markets (Kawai et al., 2008). Dunabeitia et al. (2004) reported that *R. roseolus* was more adaptable and showed a greater range of tolerance to adverse environmental conditions than *Rhizopogon luteolus* and *Scleroderma citrinum* in pure culture did. The fungi that performed best in pure culture generally improved the performance of conifer seedlings during water stress (Boyle and Hellenbrand, 1991; Dunabeitia et al., 2004). Newsham et al. (1995) pointed out that the same mycorrhizal fungus can produce different effects on the same host under different water and temperature stress conditions. Osmotic solutions are used to induce water stress reproducibly in *in vitro* conditions (Pandey and Agarwal, 1998). In the present study, I used three solutions to adjust water stress: seawater, glycerol, and polyethylene glycol (PEG) 6000, in order to evaluate the

tolerance of *R. roseolus* strains in different water stress and temperature stress conditions.

4.2 Materials and methods

4.2.1 Determination of water potential

The strains used in this experiment were the same as those described in Chapter 3. All strains were cultured on 1/5 Modified Melin-Norkrans agar (Marx, 1969) (MMNA) plates in preparation for the experiments. I used three solutions to adjust the water potential in the medium: seawater, glycerol, and polyethylene glycol (PEG) 6000. Daigo's Artificial Seawater SP was used to adjust the salinity of seawater in agar. A plurality of groups of different concentration values were taken for measurement, and the curves of the concentration of the solution versus the water potential were plotted. The osmotic pressure was measured using a vapor pressure osmometer (Wescor Vapro 5520), and then converted to water potential by using the following formula:

$$\psi_{TT}=-nRT$$

where, n (mmol/kg) is osmotic pressure in different liquid medium, R [L·kPa/ (K·mol)] is the Avogadro constant, and T is the temperature in kelvin.

4.2.2 Mycelial growth in different water potentials

The different seawater salinities (0%, 25%, 50%, and 100%) in 1/5

MMNA medium were produced with corresponding water potentials of -0.03, -0.54, -1.13, and -1.92 MPa, respectively. The medium containing glycerol and PEG were produced with the same water potential as seawater salinity (0%, 25%, 50%, and 100%), and the concentrations of these were calculated using the curve formula shown in Figure 4.1. Because increasing PEG concentrations can reduce solidification of agar, 1/5 MMN medium without PEG-6000 was poured into 9 cm diameter Petri dishes at first. As it cooled, I poured 20 mL solution (sterilized at 121°C for 30 min) with different concentrations of PEG-6000 into the dishes, and placed them in a laminar flow super clean bench for 48 h. Then, I spilled the PEG-6000 solution to obtained different concentrations of stress culture medium (Li et al., 2008). The 1/5 MMNA media containing different concentration of seawater and glycerol were autoclaved at 121°C for 30 min.

Circular agar blocks (6.0 mm diameter) of each strain were cut and inoculated on 1/5 MMNA medium with different water potentials. After culturing for 25 days, the diameter of the colonies was measured. The edge of the colony was cut with a scalpel into a 1 × 1 cm-sized block. The mycelial block was transferred to a slide, and the penetration rate of mycelium into different media was observed with an optical microscope. Ten replicate experiments were performed for each strain.

4.2.3 Mycelial growth in different temperatures

The temperature stress tolerance of each strain was assayed via

treatment with low (15°C) and high temperatures (35°C and 38°C). Circular agar blocks (6.0 mm diameter) of each strain were cut and inoculated on 1/5 MMNA medium. After 5 days of culture, the plates were incubated at different temperatures. For high temperature stress, the plates were incubated for either 3 or 5 days, after which they were removed from the incubator and cultured at room temperature (25°C). For the low temperature treatment, the plates were incubated at 15°C for 20 days. Strains cultured at 25°C were used as the control. All strains were cultured for 25 days. The diameter of the colonies was measured at 5 and 25 days of culture. The mycelial growth rate of each strain was calculated as follows:

Mycelial growth rate (mm/day) = (25 day colony diameter - 5 day colony diameter) / 20 days

4.2.4 **P. thunbergii** *association in drought and salt stress*

The cryopreserved seeds of *P. thunbergii* were secured in a net and washed for ~10 min with 1–2% surfactant solution, and then washed in running water for >24 h. The seeds were surface-sterilized via immersion in 30% hydrogen peroxide for 20 min, and then soaked and washed with sterile water twice. The net was cut with dissecting scissors to place the moist pine seeds onto sterile filter paper. Approximately 10 seeds were placed on a petri dish containing 1% agar medium, and cultured in the dark at 25°C. When the seeds formed ~5 mm buds, they were transplanted to a 15 mL test tube filled with sandy soil. Autoclaved

modified MMN liquid (diammonium phosphate, 0.25 g/L; potassium dihydrogen phosphate, 0.5 g/L; heptahydrate magnesium sulfate, 0.15 g/L; calcium chloride, 0.05 g/L; sodium chloride, 0.025 g/L; iron chloride solution, 1%; thiamine hydrochloride, 100 μg/L) was used as the nutrient solution and supplied to the seedlings once a week.

The strains were incubated on 1/5 MMNA medium for 2 weeks at 25°C in the dark. Mycelial fragments were prepared as per the method reported by Shimomura et al. (2012b). Two-week-old mycelial colonies were cut into 5 mm^2 mycelial agar plugs and then inoculated into 50 ml malt extract medium (20 g/L malt extract and 0.2% Tween 80) in an Erlenmeyer flask and incubated at 25°C in the dark. After 20 days, the mycelia were washed thrice with sterilized dH_2O. The washed mycelia were suspended at 3.5 g wet weight in 50 mL sterilized modified MMN liquid containing 0% or 100% seawater salinity, and blended with an AHG-160A homogenizer (AS ONE Corp., Osaka, Japan) at 14,000 rpm for 10 s.

Two months of culture, the resulting mycelial fragments were inoculated on the surface of soil. The inoculation liquid containing 100% seawater salinity was used as salt stress treatment, and the water supplement used was modified MMN liquid with 100% seawater salinity. For the drought stress, no water supplement was provided to the seedlings. After 45 days association, the growth status of each seedling was recorded and analyzed. Seedlings without inoculation were grouped as the control, and each treatment was repeated nine times.

4.2.5 Data analysis

Microsoft Office Excel 2010 and SPSS 7.0 were used for calculations and statistical analysis. One-way ANOVA and Fisher's least significant difference (LSD) test were used to evaluate significant differences.

4.3 Results

4.3.1 Solution water potential

Solution water potential increased with increasing salt, glycerol, and PEG concentrations. The water potential of salt and glycerol increased linearly, and that of PEG increased in a polynomial manner (Fig. 4.1). The PEG regression equation generated was:

$Y= 0.0034X^2 - 0.0525X + 0.1628$ ($r^2 = 0.9849$),

concentration of PEG and Y is water potential(-MPa). This equation was used to calculate the PEG concentrationsfor each of the desired water potentials at 25°C. The water potential values obtained were similar to those previously published (Michel and Kaufmann, 1972; Michel et al., 1983).

4.3.2 Mycelial growth in different water potentials

The mycelial growth rate varied for different strains in different treatments. For the PEG treatment, except for the homokaryotic strain

MCL2014Rhz41·Sp2, the relative growth rates of all strains were significantly higher than that of the control, and the mycelial growth rates increased with increasing PEG water potential (Fig. 4.2). At the same water potential, the strains in salt treatment and glycerol treatment showed a completely opposite pattern of growth. For example, the hybrid strain H1 showed the halophilic characteristic of increasing mycelial growth rate with increasing water potential by seawater. In glycerol stress, the mycelial growth rate of H1 significantly decreased with increasing water potential by glycerol (Fig. 4.2). The hybrid strain H9 showed the lowest mycelial growth in seawater stress, and had the highest mycelial growth rate of all strains in glycerol stress (Fig. 4.2). Similar phenomenon was also observed in the salt-tolerant hybrid strain H7, and the homokaryotic strains MCL2014Rhz41·Sp2 and MCL2014Rhz41·ESp3. The mycelial growth rate of wild strain MCL2015Rhz77 was the lowest in most treatments.

The infiltration rate of hyphae varied with different treatment. The anterior hyphae of the control infiltrated ~80% of the medium (Fig. 4.3a), and the anterior hyphae under seawater and glycerol treatment completely infiltrated the entire medium (Fig. 4.3b c). However, in the PEG treatment, the hyphae of *R. roseolus* grew only on the surface, and had difficulty infiltrating the medium (Fig. 4.3d).

4.3.3 Mycelial growth in different temperatures

Temperature had a significant impact on the mycelial growth of *R.*

roseolus, andthere were obvious differences between strains. In the low temperature (15°C) treatment, after 25 days the mycelial growth rate of each strain was significantly inhibited (Fig. 4.4), and the value of relative growth rate was 45% to 55%. However, after low temperature treatment, the strains still had the ability to recover. After recovering at 25°C for 25 days, all the low temperature-treated strains, except hybrid strain H7, had almost identical colony diameters (Fig. 4.5). The relative growth rate of hybrid strain H7 was 88% after low temperature treatment and recovery. In the 35°C high temperature test, after 3 days of treatment the mycelial growth rate of most strains had no significant inhibition, and homokaryotic strain MCL2014Rhz41·Esp3 showed a higher mycelial growth rate compared to the control (Fig. 4.5). After 5 days of treatment at 35°C, the mycelial growth rate of all strains, except homokaryotic strain MCL2014Rhz41·Esp3, had significant inhibition (Fig. 4.5), and the relative growth rate of each strain was between 81% - 89%. However, homokaryotic strain MCL2014Rhz41·Esp3 still showed a vigorous mycelial growth rate in the 35°C high temperature test. In the 38°C test, the mycelial growth of H1, wild strain MCL2015Rhz77, and homokaryotic strain MCL2014Rhz41·Esp3 were observed after 3 days of treatment. H1 showed the highest relative growth rate (77%) among the strains after 3 days of treatment at 35°C. After 5 days of 35°C treatment, all strains were dead, and no mycelial growth was observed (Fig. 4.5).

4.3.4 P. thunbergii *association*

All strains of *R. roseolus* had the ability to increase the drought tolerance of *P. thunbergii*. Without inoculation, the leaves of 66.7% of seedlings became yellow (Table 4.1). In contrast, all the leaves of inoculated seedlings remained green and healthy (Table 4.1, Fig. 4.6). In salt stress, leaf yellowing was common in the seedlings inoculated with the salt-sensitive strain H9. Leaf yellowing was also found in the seedlings without inoculation. When the seedlings were inoculated with H1 or MCL2014Rhz41·ESp3, half of the seedlings retained green leaves. However, when the seedlings were inoculated with H7, only one seedling retained green leaves, indicating that this strain did not contribute to salt tolerance.

4.4 Discussion

The relative effects of non-ionic (glycerol), ionic (NaCl), and inert (PEG 6000) solute stress which simulates the way water is held in soil and food systems (Medina et al., 2015). When seawater was used to modify water potential, the cells of *R. roseolus* strains encountered both osmotic and ion stress. In osmotic stress, the fungus must take up high concentrations of solutes to ensure that water will enter rather than leave the cell by osmosis (Clipson and Hooley, 1995). In many fungal species, the cells adjust osmotically by synthesizing substantial quantities of mannitol, glycerol, and trehalose, and other metabolites such as proline (Lewis and Smith, 1967; Jennings and Burke, 1990; Shen et al., 1999). These substances, if present at sufficiently high concentrations, will allow

the flow of water into the cell. In addition, high salt concentrations result in cellular ion imbalance, which can lead to ion toxicity (Ashraf, 1994, Mittler, 2002). As a primary stress factor, high Na^+ uptake from saline substrates competes with the uptake of other nutrient ions, especially K^+, resulting in K^+ deficiencies (Parida and Das, 2005). K^+ is taken up via high- and low-affinity K^+ transporters (Ko and Gaber, 1991). The high-affinity K^+ transporter shows a higher K^+ to Na^+ discrimination than the low-affinity transporter. Under salt stress, high-affinity K^+ uptake allows cells to accumulate more K^+ than Na^+, and thus they maintain a low Na^+ to K^+ ratio (Haro et al., 1993). Another method is the use of excess ions absorbed by vacuolization to decrease damage to the membrane (Clipson and Jennings, 1992; Clipson and Hooley, 1995).

In this study, the hybrid strain H9 showed a high tolerance to glycerol stress, but was sensitive to salt. It is possible that when glycerol was used to modify osmotic potential, the content of glycerol increased in the hyphae cell for osmotic adjustment, perhaps via passive diffusion and/or endogenous synthesis. This phenomenon has been observed in *Fusarium graminearum* (Ramirez et al., 2004). However, strain H9 may be susceptible to ionic toxicity, so that the mycelial growth of this strain was completely inhibited. In contrast, hybrid strains H1 and H7 could carry out ion balance, and absorb excess ions to enhance intracellular osmotic pressure for osmotic regulation. Therefore, these two strains showed strong halophilic characteristics and sensitivity to glycerol stress.

In PEG stress, almost all strains of *R. roseolus* showed vigorous growth on agar plates for all water potentials observed in this study. This

result is significantly different from those reported by Zhang et al. (2011) for ectomycorrhizal fungi *Suillus tomentosus*, *Suillus laricinus,* and *Aminita vaginata*. In liquid culture, the colony diameter and dry weight of *R. roseolus* significantly decreased with increasing water potential by PEG 6000 (Dunabeitia et al., 2004). However, despite the vigorous mycelial growth of *R. roseolus* strains observed in PEG stress, hyphae grew only on the surface of agar medium, and few hyphae penetrating the medium were observed. Moreover, the mycelia showed obvious drought stress symptoms, including leaf yellowing and sparse growth. In the absence of water-stress, the anterior mycelia could invade ~80% of the agar medium. While under salt and glycerol stress, the anterior mycelia completely invaded to the bottom of the culture medium. This may be because the stress-free hyphae did not need the entire medium to obtain sufficient nutrients and water for growth and metabolism. Under salt and glycerol stress, the nutrient and water uptake of hyphae was inhibited, and the hyphae had to penetrate deeper into the medium to obtain sufficient nutrients. The water stress of agar medium after PEG treatment inhibited mycelial invasion and growth, thus the mycelium could only get sufficient water and nutrition by increasing the amount of surface spreading.

Ecotomycorrhizal associations significantly alter water relationships of host plants, and this enhancement has been attributed in part to rhizomorph production and their function in water transport (Duddridge et al., 1980). A positive relationship in a parallel assay developed under field conditions was observed by Ortega et al. (2004). Nursery

inoculations with *R. roseolus* and *S. citrinum* improved *Pinus radiata* growth during the first 2 years after field planting, particularly at a drier site (Ortega et al., 2004). In the present study, all *P. thunbergii* seedlings that were inoculated with *R. roseolus* showed higher drought tolerance than the control seedlings did. However, the reaction of inoculated *P. thunbergii* seedlings to salt stress was strain-dependent. Seedlings inoculated with the halophilic strain H1 showed vigorous mycelial growth in 100% seawater salinity in pure culture, and showed high tolerance in continuous 100% seawater treatment. In addition, the salt-sensitive strain H9 could not help the host against salt stress. For the halophilic hybrid strain H7, even though it increased the host's salt tolerance, it was significantly lower than that of strain H1. Similarly, H1 also had vigorous mycelial growth compared to that of H7 in 100% seawater salinity soils.

Temperature and water availability have been identified as two of the main abiotic factors modulating fungal growth (Medina et al., 2015). The effective water content of sand dune soil that contained the fruiting bodies of *R. roseolus* was only 7% (Honna, 2000). The diurnal temperature of the surface of sand dune soil was more than 40°C, and ~5–10 cm below the surface was the dry sand layer (Honna, 2000). Therefore, in addition to water stress, the study of the effect of temperature stress on mycelial growth is required. In the present study, the strains of *R. roseolus* showed strain-specific variability of temperature tolerance *in vitro*. The halophilic hybrid strain H1 showed the highest tolerance to high temperature stress in this study.

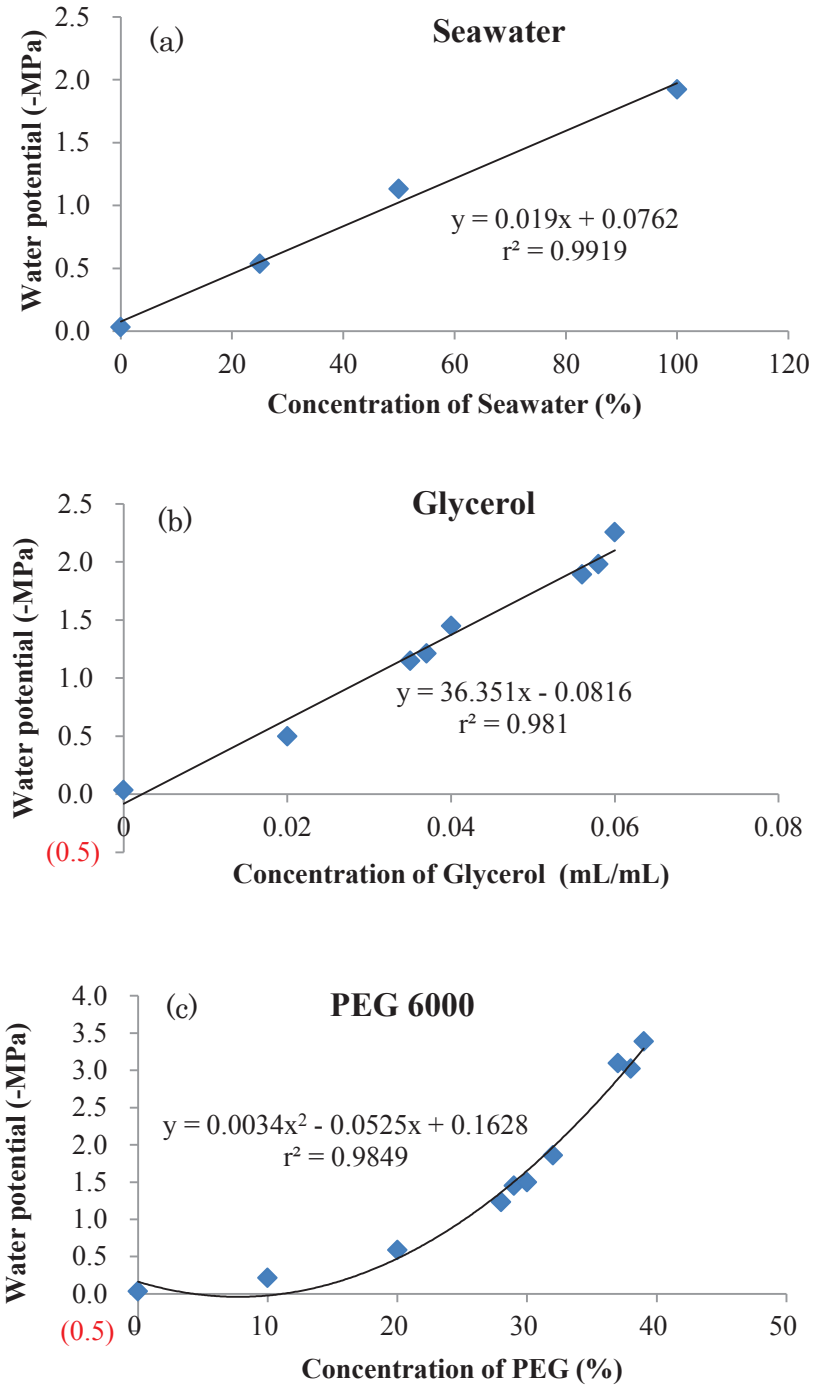

Fig. 4.1. Curves showing the effect on water potential by increasing concentrations of a: seawater; b: glycerol; c: PEG 6000

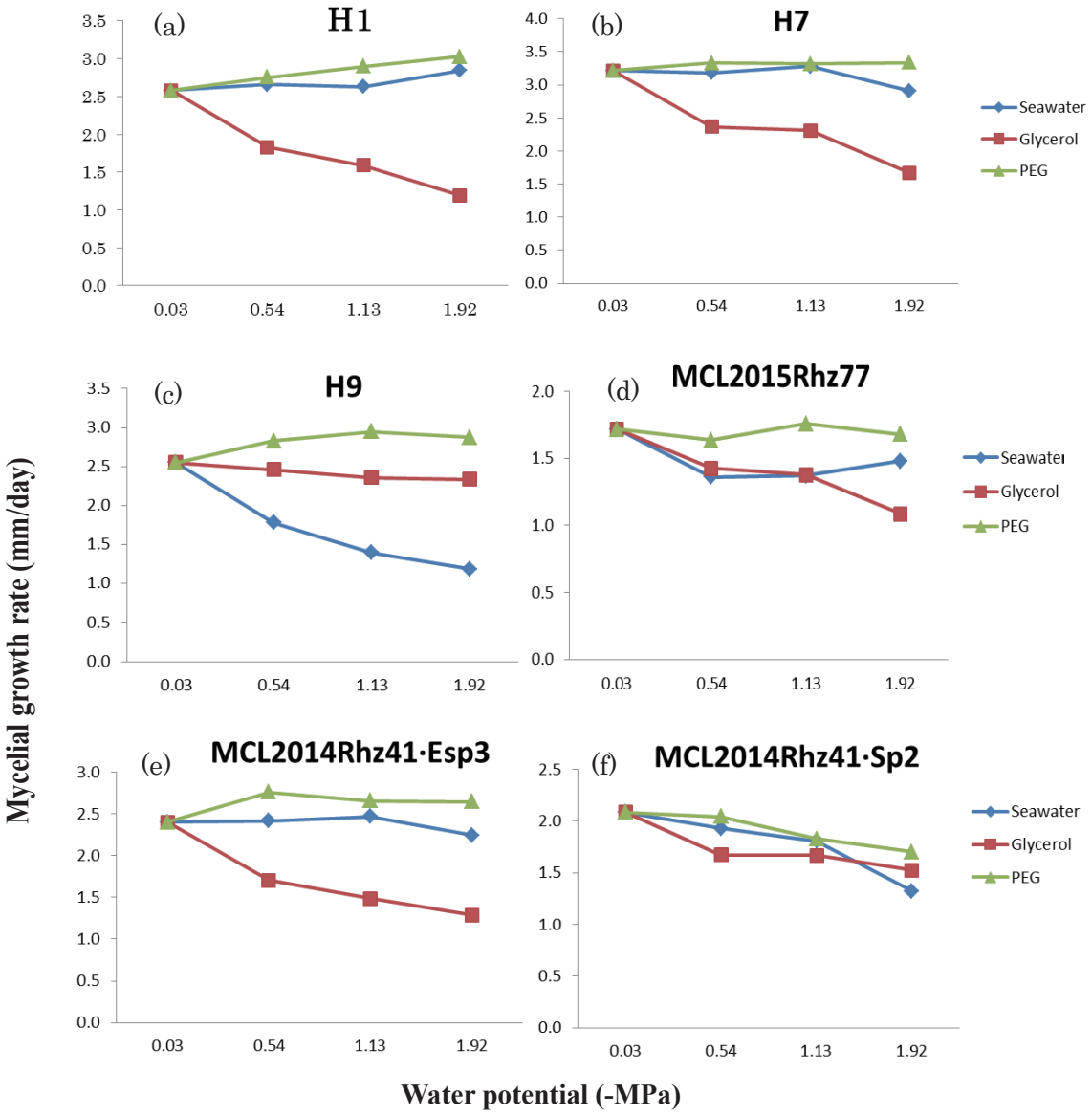

Fig. 4.2. Mycelial growth rate of *Rhizopogon roseolus* strains in different water potential treatments.

a: H1; b: H7; c: H9; d: MCL2015Rhz77; e: MCL2014Rhz41·Esp3

f: MCL2014Rhz41·Sp2

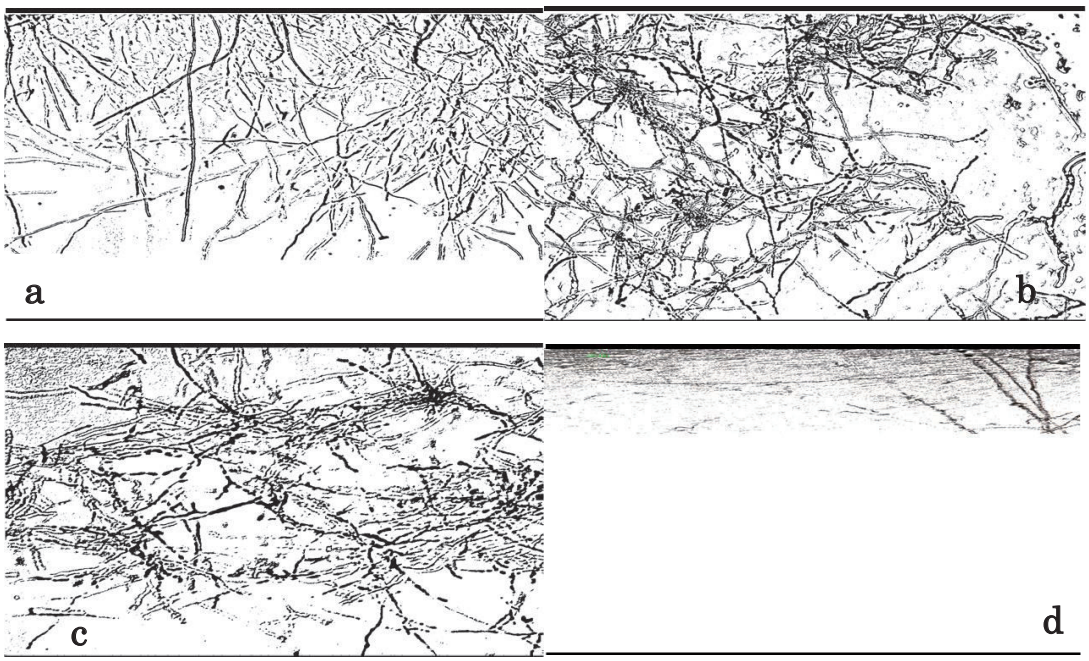

Fig. 4.3. The infiltration rate of *Rhizopogon roseolus* hybrid strain H1 into the medium in different treatments. a: control; b: seawater; c: glycerol; d: PEG

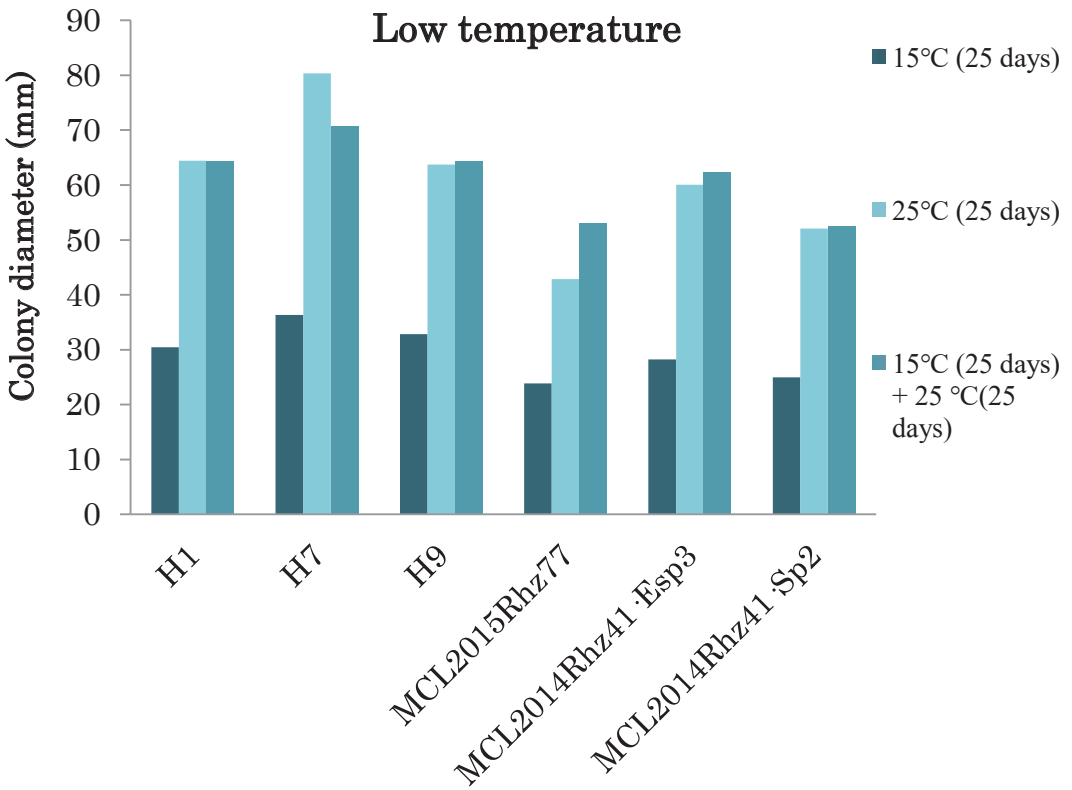

Fig. 4.4. Colony diameters of *Rhizopogon roseolus* strains treated to low temperature stress.

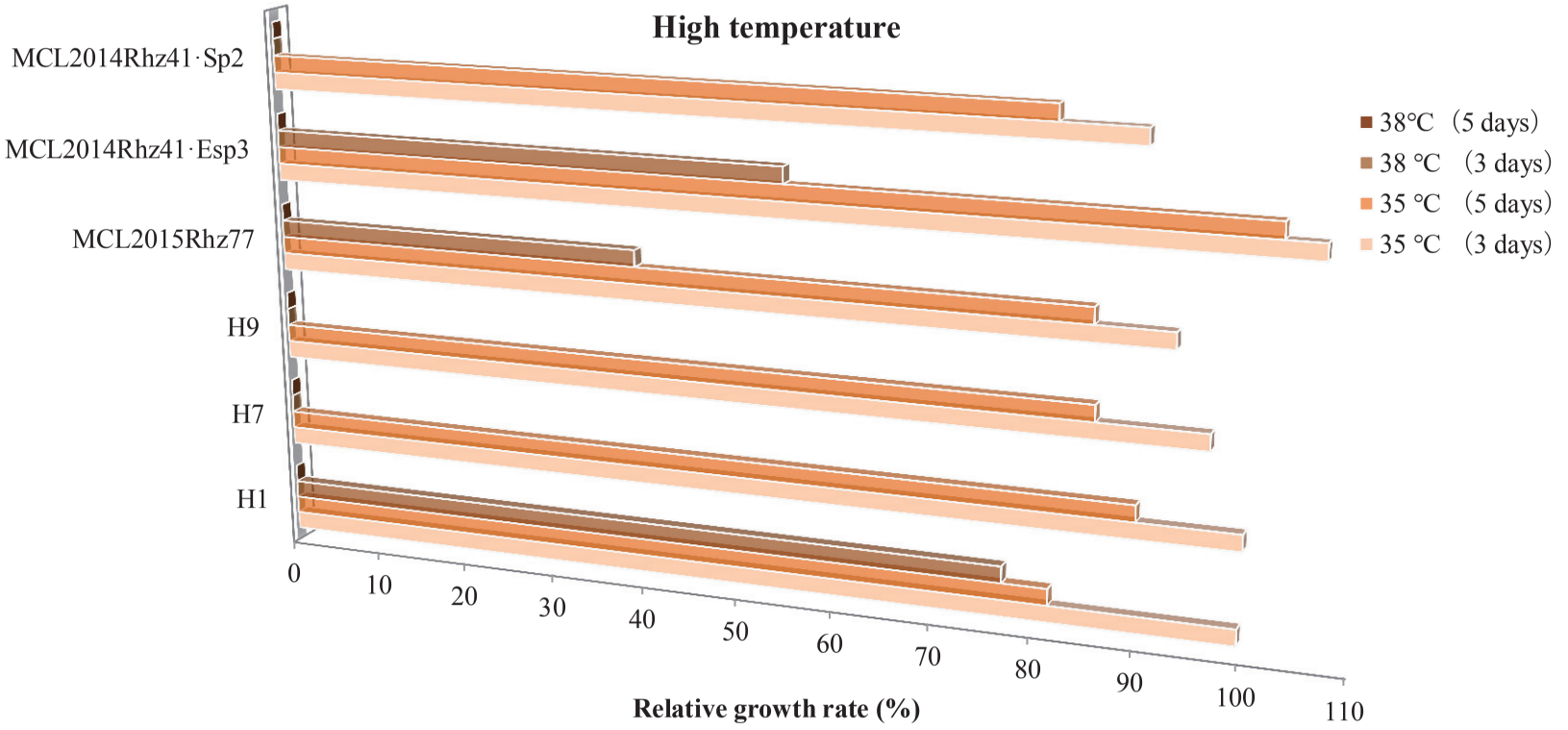

Fig. 4.5. Relative growth rates of *Rhizopogon roseolus* strains treated to high temperature stress.

Relative growth rate (%) = (mycelial growth rate of each temperature treatment / mycelial growth rate at 25°C) × 100

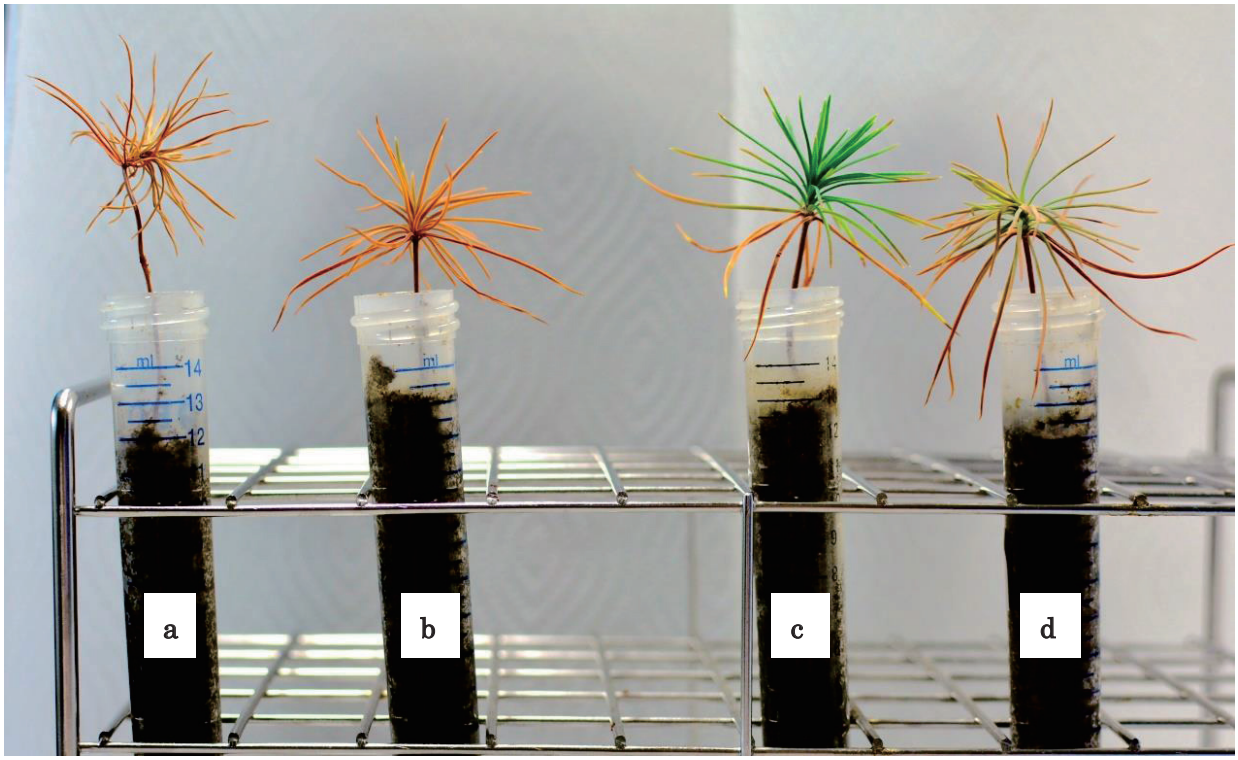

Fig. 4.6. Drought and salt stress for *Pinus thunbergii* inoculated with *Rhizopogon roseolus*

a: Drought stress without inoculation

b: Salt stress without inoculation

c: Drought stress with inoculation of hybrid strain H1

d: Salt stress with inoculation of hybrid strain H1

Table 4.1. Yellowing death rate of *Pinus thunbergii* inoculated with different *Rhizopogon roseolus* strains in drought and salt stress.

Inoculation strains	Yellowing death rate (%)	
	Drought	Salt
Non-inoculation	66.7	100.0
H1	0.0	55.6
H7	0.0	88.9
H9	0.0	100.0
MCL2015Rhz77	0.0	77.8
MCL2014Rhz41·Esp3	0.0	66.7
MCL2014Rhz41·Sp2	0.0	44.4

Chapter 5

General discussion and conclusion

Classical breeding is an important technique in plant breeding, and involves the selective propagation of plants with desirable characteristics and the elimination of those with less desirable characteristics (Carol, 2000). Another technique used is cross-breeding, which is the deliberate interbreeding of closely or distantly related but sexually compatible parental lines to produce new varieties or lines with desirable properties. Since the 1930s, mutation breeding uses a plant's own genetic make-up, mimicking the natural process of spontaneous mutation (Schouten and Jacobsen, 2007). The mutation process generates random genetic variations, resulting in mutant plants with new and useful traits. Breeding based on mutagenesis has been more effective than traditional breeding in producing cultivars with high resistance to biotic and abiotic stresses (Zhao et al., 2013).

Ethyl methanesulfonate (EMS) can cause a high frequency of gene mutations and low frequency of chromosome aberrations (Van Harten, 1998). This mutation may expand the salt tolerance variation of isolates from *R. roseolus*. In Chapter 2, I described the observation of halophilic and salt-sensitive strains derived from both basidiospores and homokaryotic mycelial fragments of *R. roseolus*. By cross-breeding, the stable halophilic and salt-sensitive hybrids strains were successfully selected (Fig. 5.1).

However, the mutation experiments in basidiospores and mycelial fragments had their advantages and disadvantages. Basidiospore of *R. roseolus* as a sexual reproduction, with the characteristics of genetic diversity, based on the mutation breeding that showed a wide range of variation. Due to effects of fruiting body maturity (Nakano et al., 2016), culture environment (Kikuchi et al., 2007), and spore preservation method and storage time (Quintanilla et al., 2002), the reproducibility of spore mutation breeding is low. In the present study, I used fresh, moderately mature, and bacteria-free fruiting bodies in each experiment to obtain spore suspensions. In recent years, due to environmental factors, a large number of *R. roseolus* fruiting bodies have reduced production (Nagasawa, 2000), and basidiospore mutation breeding of *R.roseolus* has had a role in restricting production. The mycelial fragments have the advantages of a high recycling rate and easy preservation compared with that of the basidiospores. However, since intraspecific variability of fungal isolates has to be considered in selection processes (Trapper, 1977; Parladé et al., 2011), the selection of mutant halophilic strains of mycelia fragments is largely dependent on their original strains. I examined whether the basidiospore or mycelia fragment mutation method could successfully obtain the halophilic mutation isolate with EMS treatment in this study.

The sandy soil in the seashore ecosystem where *P. thunbergii* and *R. roseolus* inhabit is mostly (90%) composed of sand particles sized 0.02-2 mm, and the silt and clay component is less than a few percent. Humus content is also very low, and total carbon content is lower than 4%

(Honna, 2000). In general, research on salt-tolerant mycorrhizal fungi has mostly focused on the growth and dry weight of their symbiotic plants in salt environments, including the effects of nutrient absorption such as phosphorus and nitrogen (Harley, 1989; Juniper and Abbott, 1993; Turjaman et al., 2006), and the absorption of water-soluble ions in plant cells such as K^+ and Ca^+ (Bandou et al., 2006). Even *in vitro* studies of mycorrhizal fungi mostly used plate and liquid pure culture environments to evaluate salt tolerance potential (Tresner and Hayes, 1971; Dixon et al., 1993; Bois et al., 2006; Mulligan, 2007; Tang et al., 2009; Nakano et al., 2015). Because it is difficult to quantitate the mycelium of ectomycorrhizal fungi in soil, few studies have characterized the salt-tolerant ability of mycorrhizal fungi in soil. In addition to salt stress in seashore ecosystems, fungi that are present in sand dunes are subjected to drought and temperature stress. Therefore, non-specific tolerance that allows adaptation to various environmental conditions is worth examining in the selected strains. In present study, the salt-tolerance abilities of the selected strains in soil were examined (Chapter 3), moreover, their water stress and temperature tolerance abilities were also assessed (Chapter 4).

In Chapter 3, I described the observation that all *R. roseolus* strains grew well in sandy soil without a host plant. Moreover, a significant positive correlation was observed between the mycelial growth of *R. roseolus* in soil and on agar. These results reveal that sandy soil is useful for characterization of the salt tolerance of *R. roseolus* strains in soil substrates. Donnelly et al. (2004) pointed out that the measurement of the surface area parameters of mycelial morphology and quantitation of

hyphal lengths are desirable. In this method, a flat plate was used to observe the morphology of mycelium colonization on the soil surface, and the ability of mycelia to infiltrate the soil was measured with a glass tube. From this method, the three-dimensional network formed by the extrametrical mycelium of *R. roseolus* in a natural soil environment could be simulated. Furthermore, fungal interaction also could be observed on the soil surface (Donnelly and Boddy, 2001). In addition, strain H1 showed vigorous mycelial growth both in non-saline and high salinity soil, which suggests that extensive external mycelia of H1 is better at taking up water for the host plant in salt stress. With H1 inoculation, half of the *P. thunbergii* seedlings retained green leaves in salt stress. In contrast, all the non- and salt-sensitive strain H9-inoculated seedlings experienced leaf yellowing (Chapter 4).

Water stress plays a major role in limiting the success of conifer seedling regeneration (Ortega et al., 2004). In Chapter 4, I used three solutions to adjust the water stress, including seawater, glycerol, and polyethylene glycol (PEG) 6000, to evaluate the composite tolerance of *R. roseolus* strains to different environmental factors, including drought, salt, and temperature. The mycelial growth rate of different strains of *R. roseolus* was variable in the same water potential with seawater and glycerol treatment. The strain that was tolerant to salt stress became sensitive with glycerol treatment, and the salt-sensitive strain of *R. roseolus* was tolerant to glycerol. These results suggest that the different strains of *R. roseolus* have different mechanisms to modify osmotic potential. This research provides a basis for further elucidating the salt

tolerance mechanism of *R. roseolus*. Moreover, studies on compatible organic solutes, ion transportation, and cell membrane stability need to be carried out to further understand the mechanism of salt tolerance.

In addition, most strains of *R. roseolus* showed vigorous mycelial growth on PEG-treated plates as compared to the control. Moreover, 66.7% of seedlings without inoculation had yellow leaves, while 100% of the seedlings inoculated with *R. roseolus* strains retained green leaves. These results are similar to those reported in the study by Dunabeutia et al. (2004), wherein *R. roseolus* appeared drought-tolerant, showing the widest range of water stress tolerance. In addition to drought tolerance ability, hybrid strain H1 showed the highest relative growth rate (77%) among the strains after 3 days' treatment at 38°C.These results suggest thatH1 is a non-specific stress-tolerant strain of *R. roseolus*. Inoculation with H1 may help *P. thunbergii* adapt to a seashore environment, and enhance its salt and drought resistance. In future studies, H1 will be used for field inoculation, and its adaptive and tolerance properties will be observed in the natural environment.

In conclusion (Fig. 5.1), the basidiospores and mycelial fragments of *R. roseolus* induced by EMS can produce a high number of halophilic homokaryotic strains, and through cross-breeding and continuous subculture, stable halophilic heterokaryotic strains can be selected. The strains showing non-specific stress tolerance can be obtained by evaluating their potential in conditions of sandy soil, drought, high and low temperatures, and inoculation into host plants. In this study, the hybrid strain H1 had a stable halophilic ability, vigorous mycelial growth

both in non-saline and high salinity soil, drought- and high temperature-tolerant characteristics, and was determined to be a non-specific stress-tolerant strain of *R. roseolus* that could help *P. thunbergii* adapt to various natural environments.

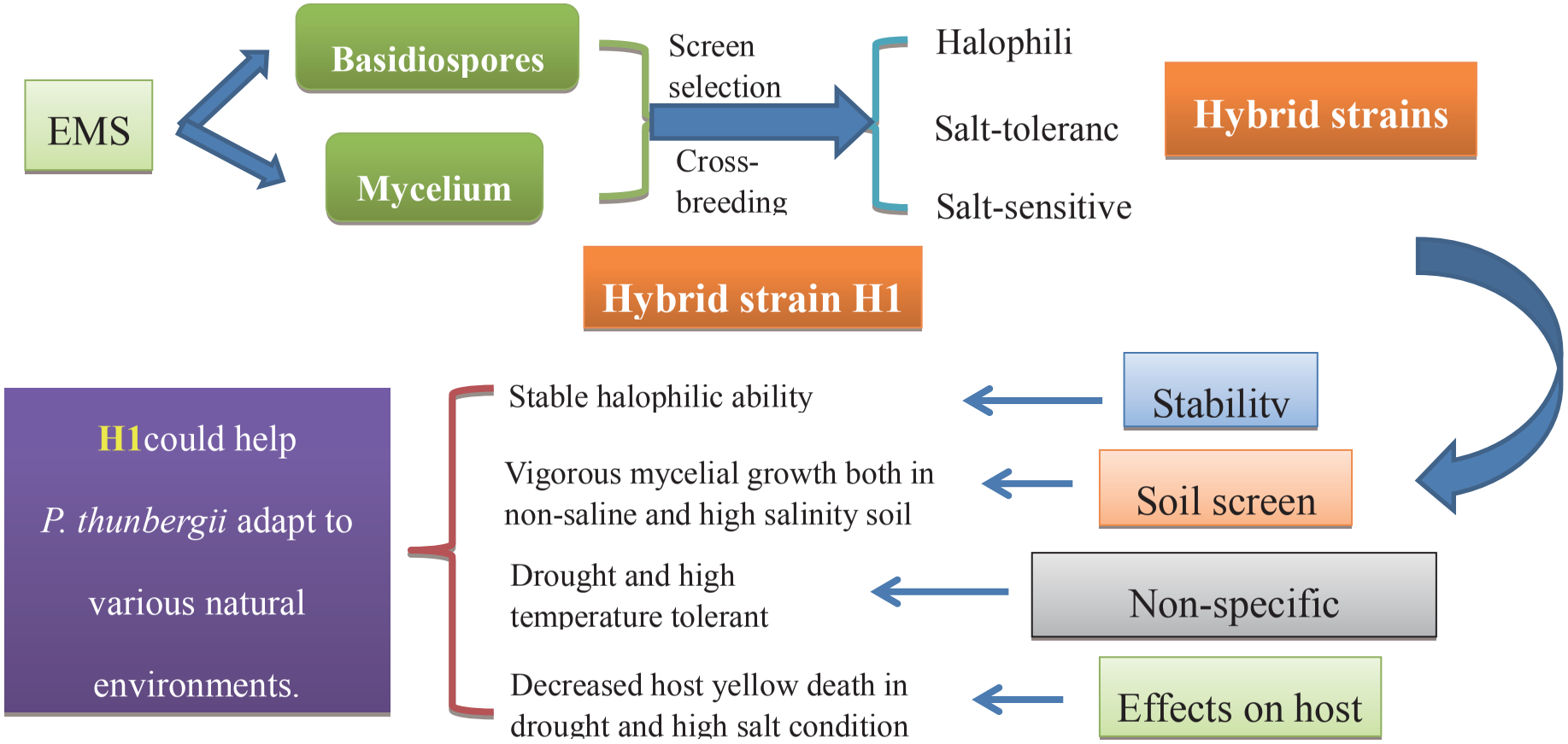

Fig. 5.1. Outline of the methods used in this study to isolate and evaluate salt-tolerant strains induced by ethyl methanesulfonate in the ectomycorrhizal fungus *Rhizopogon roseolus*.

Abstract

Rhizopogon roseolus (Corda) Th. M. Fr. (=*R. rubescens* Tul. & Tul.), a hypogeous basidiomycete also known as “shoro” in Japan, is an important ectomycorrhizal symbiont of Pinaceae. Recent studies suggest that ectomycorrhizal fungi play an important role in the protection of host roots from environmental stressors such as drought, heavy metals and excessive salt. However, no mutagenesis study on the selection of halophilic ectomycorrhizal strains of *R. roseolus* has been performed. Moreover, there is no study on characterizing their salt tolerance in soil and on their specificity against stress responses such as drought and high temperature.

Here, to induce mutations, I treated two types of specimen, basidiospores, and homokaryotic mycelial fragments of *R. roseolus*, with ethyl methanesulfonate (EMS), which is a widely used chemical mutagen, and subsequently evaluated salt-tolerance on 300 mM salt agar plates. Among the strains recovered from EMS-treated basidiospores, I isolated halophilic strains that showed a more vigorous mycelial growth in medium containing 300 mM NaCl, compared to that of the strains derived from untreated basidiospores. The salt-sensitive strains could also provide useful characteristics for subsequent research in which the resistant mechanism against salt stress will be revealed. EMS treatment expanded the variations in salt tolerance ability of these strains derived from homokaryotic mycelial fragments of *R. roseolus*. By crossbreeding system, the heterokaryotic hybrid strains showed a higher tolerance and

stability in NaCl stress than homokaryotic ones. Hybrids strains which were crossed with halophilic mutants showed a higher relative growth in medium containing 300 mM NaCl.

I used wild sandy soil to investigate the growth of *R. roseolus* strains in soil, and comparatively analyzed the correlation of mycelial growth between in soil substrate and one agar medium. All the strains grew well in sandy soil without a host plant and on the surface of the soil, several hyphae aggregated to form a mycelial colony. The quantitative assay revealed that, a significant positive correlation ($p < 0.05$) had been observed between the mycelial growth of *R. roseolus* in soil substrate and on agar medium. In saline culture, the mycelial growth in 100% seawater salinity soil were positive correlation ($p < 0.05$) with the mycelial growth on 100% seawater salinity agar medium. These results suggest that mycelium of *R. roseolus* seems more sensitive with salt in soil than on agar, and sandy soil is useful to characterize salt tolerant ability of *R. roseolus* in soil substrate.

Finally, to investigate specificity against stress response, I have examined effect of drought stress and heat stress on salt halophilic strain of *R. roseoluse*. I used three types of solutions to adjust the water stress, seawater, glycerol containing water or polyethylene glycol (PEG) 6000 containing water to evaluate the stress tolerant of *R. roseolus* against three environments factors, viz., ion stress, non-ion osmotic stress and drought stress. As the results, in same water potential, the strains in salt and glycerol exhibited a completely opposite pattern of growth. While the strain that was tolerant salt stress was sensitive to glycerol, the salt

sensitive strain H9 exhibited a vigorous mycelial growth in glycerol treatment than other strains. Almost strains of *R. roseolus* exhibited a vigorous mycelial growth on PEG-treated plates than control. Moreover, all *R. roseolus* strains contributed to promote drought tolerance ability of seedlings of *Pinus thunbergii*. While 66.7% of seedlings without inoculation became yellow, 100% of the seedlings inoculated with hybrid strains H1, H7 and H9 still kept in green. In salt stress, 50% of the seedlings inoculated with halophilic strain H1 still kept the green leaves. In contrast, 100% of the seedlings inoculated with salt-sensitive strain H9 have changed to yellowing. Yellowing was also observed in all of the seedlings without inoculation. When mycelium of *R. roseolus* were treated with high temperature at 38°C for 3 days, the highest relative growth rate (77%) was observed in the strain H1, indicating that strain H1 is tolerant against not only salt stress but also high temperature stress.

These results in this study suggest that the combination of EMS mutant and the crossbreeding system could be an effective method to obtain halophilic strains of *R. roseolus*. The strains showing non-specific stress tolerant would be obtained by evaluating its potential in sandy soil, drought, temperature, and inoculation into host plants. Hybrid strain H1 recovered in this study considered to possess a stable halophilic ability, vigorous mycelia growth, and non-specific tolerant characteristics against drought and high temperature. Furthermore, the strain H1 will be expected as useful bio-fertilizer that could support *P. thunbergii* adapting to various natural environments.

和文摘要

ショウロ *Rhizopogon roseolus* (Corda) Th. M. Fr.は，海岸砂地という特殊な環境でマツ科の樹木の根に外生菌根を作り共生する典型的な外生菌根菌である．近年，外生菌根には，宿主樹木根において水，重金属および塩ストレスを緩和する役割があることが報告されている．また，ショウロの担子胞子由来一次菌糸同士を交配することで得られた耐塩性の交雑 F1 菌株を作出できることが報告された．しかし，突然変異誘発剤を用いて耐塩性や好塩性ショウロ菌株を育成した報告例はなく，さらに，土壌基質における菌株の耐塩性評価や耐塩性に加えて高温や水ストレスなど複数の環境ストレスに対する反応について多面的に評価した研究例についてもない．そこで，本研究では，突然変異誘発剤として広く用いられている ethyl methanesulfonate (EMS) を利用して好塩性を具備したショウロ菌株の作出を試みた．また，作出した菌株のストレス耐性について多面的に評価した．

EMS を用いてショウロの担子胞子または一次菌糸断片を処理して突然変異を誘発した．先ず，担子胞子に処理する EMS の影響についてコロニー形成を指標にして調べた結果，20 mg/mL の濃度で処理すること適正であると思われた．EMS を処理したショウロ担子胞子から 300 mM NaCl 含有培地で旺盛に成長した好塩性一次菌株を分離した．また，NaCl に対して感受性の菌株も見出された．一方，ショウロの一次菌糸断片に EMS を処理すると耐塩性に関する変異が拡大したが，その拡大範囲は用いる一次菌株の特性に依存した．分離した好塩性菌株を交配し交雑 F1 菌株を作出し，300 mM NaCl 含有寒天平板培地に接種した所，旺盛に成長した．また，6 ヶ月間連続して継代培養してもその好塩性の特性は持続した．

次に，野外から採取した砂土壌を用いてショウロ菌株の耐塩性を評価した．

その結果, 砂土壌基質がショウロ菌株の耐塩性を評価するに有効であることが判明した. 砂土壌基質にショウロ菌糸体を接種した結果, すべてのショウロ菌株は宿主植物が存在しない砂土壌基質で旺盛に成長した. 砂土壌基質と寒天培地における菌糸体成長速度を比較した結果, 好塩性菌株 H1 や耐塩性菌株 H7 は人工海水を含む砂土壌基質で旺盛に成長し, 塩感受性菌株 H9 は成長が完全に抑制された. また, 砂土壌基質の菌糸成長と寒天培地との間で有意な正相関が認められた.

次に, 様々なストレス耐性について調べるために, 人工海水, グリセロールおよびポリエチレングリコール（PEG）6000 を添加した寒天平板培地を用いて, イオンストレス, 非イオン浸透圧ストレスおよび水ストレスのショウロ菌糸成長に及ぼす影響について調査した. その結果, 好塩性菌株 H1 はグリセロールによる非イオン浸透圧に感受性を示し成長が低下した. 一方, 塩感受性菌株 H9 は, 他の菌株よりグリセロールによる非イオン浸透圧に耐性を示し旺盛に成長した. PEG による水ストレス処理をしたところ場合, ほとんどのショウロ菌株において, 未処理より旺盛に菌糸が成長した. ショウロの菌糸成長に及ぼす高温処理の影響について調査した. その結果, 好塩性菌株 H1 は 38℃で 3 日間処理した後においても最も高い相対成長率(77 %)を示した.

最後に, 宿主クロマツ実生 *Pinus thunbergii* にそれぞれのショウロ菌株を人工的に接種して, 感染苗木を育成し, 塩ストレスおよび水ストレスを与えて, 生育状況を調査した. その結果, 未接種実生に水ストレスを 45 日間与えると 66.7%の実生が枯死したが, ショウロ菌を接種した実生での枯死率は 0%であった. 塩ストレス処理においては, ショウロ菌を接種しなかった実生と塩感受性菌株 H9 を接種したクロマツは全て黄化し, 緑色葉を保持した実生は 0%であった. 一方, 好塩性株菌株 H1 を接種したクロマツ実生で約 50%が緑色葉を保持しており, 本菌株は宿主クロマツにストレス耐性を付与する有望菌株であ

ると推定された.

本研究は，突然変異誘発剤を用いてショウロの好塩性菌株を作出に成功し，その菌株の好塩性の持続性を確認するとともに，菌糸体の土壌基質における増殖性，水ストレスや高温ストレスなどへの耐性，さらには，宿主クロマツ実生への水ストレスや塩ストレス耐性付与効果についても，多面的に評価した内容である．得られた一連の結果はショウロの有望菌株の育成と活用に向けた新規性のある基礎的知見を提供するものであり，今後の外生菌根菌の利活用に大きく貢献しうる内容である．

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References

Abrol IP, Sandhu SS (1985). Growth responses of *Eucalyptus tereticornis* and *Acacianilotica* to selected methods of site preparation in a highly sodic soil. IntlTree Crops J. 3: 171-183.

Agerer R (2001). Exploration types of ectomycorrhizal mycelial systems. A proposal to classify mycorrhizal mycelial systems with respect to their ecologically important contact area with the substrate. Mycorrhiza. 11: 107-114.

Agerer R (2007). Diversity of ectomycorrhizae as seen from below and above ground: the exploration types. Z Mykol 73(1): 61-88.

Amtmann A, Fischer M, Marsh EL, Stefanovic A, Sanders D, Schachtman DP (2001). The wheat cDNA LCT1 generates hypersensitivity to sodium in a salt-sensitive yeast strain. Plant Physiol. 126: 1061-1071.

Ashraf M (1994). Breeding for salinity tolerance in plants. Crit Rev Plant Sci. 13: 17-42.

Azcón, R, El-Atrash F (1997). Influence of arbuscular mycorrhizae and phosphorus fertilization on growth, nodulation and N_2 fixation (15N) in *Medicago sativa* at four salinity levels. Biol Fert Soils. 24: 81-86.

Bandou E, Lebailly F, Muller F, Dulormne M, Toribio A, Chabrol J, Courtecuisse R, Plenchette C, Prin Y, Duponnois R, Thiao M, Sylla S, Dreyfus B, Ba AM (2006). The ectomycorrhizal fungus *Scleroderma bermudense* alleviates salt stress in seagrape (*Coccoloba uvifera* L.) seedlings. Mycorrhiza. 16: 559-565.

Barbara KM, Sarah PO (1998). The evolution of life cycles with haploid and diploid phases. Bio Essays. 20: 453-462.

Bettenay E (1986). Salt affected soils in Australia. Rec Revegetation Res. 5: 167-179.

Blasius D, Feil W, Kottke I, Oberwinkler F (1986). Hartig net structure and formation in fully ensheathed ectomycorrhizas. Nord J bot. 6: 837-842.

Bois G, Bertrand A, Piché Y, Fung M, Khasa DP (2006). Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations. Mycorrhiza. 16: 99-109.

Bois G, Bigras FJ, Bertrand A, Piche Y, Fung MYP, Khasa DP (2006). Ectomycorrhizal fungi affect the physiological responses of *Picea glauca* and *Pinus banksiana* seedlings exposed to an NaCl gradient. Tree Physiol. 26: 1185-1196.

Boyle CD, Hellenbrand KE (1991). Assessment of the effect of mycorrhizal fungi on drought tolerance of conifer seedlings. Can J Bot 69: 1764-1771.

Brown ME (1974). Seed and root bacterization. Annual Rev Phytopathol 12: 181-197.

Carol BC, Miller CO (1974). Detection and identification of cytokinins produced by mycorrhizal fungi. Plant Physiol. 54: 586-588.

Carol D (2000). Breed Your Own Vegetable Varieties. Chelsea Green Press. pp: 237-244.

Chaves MM, Maroco JP, Pereira JS (2003). Understanding plant

responses to drought-from genes to the whole plant. Funct Plant Biol. 30: 239-264.

Chen DM, Ellul S, Herdman K, Cairney JWG (2001). Influence of salinity on biomass production by Australian *Pisolithus* spp. isolates. Mycorrhiza. 11: 231-236.

Clemmensen KE, Michelsen A, Jonasson S, Shaver GR (2006). Increased ectomycorrhizal fungal abundance after long-term fertilization and warming of two arctic tundra ecosystems. New Phytol. 171: 391-404.

Clipson N, Hooley P (1995). Salt tolerance strategies in marine fungi. Mycologist. 9: 3-5.

Clipson NJW, Jennings DH (1992). *Dendryphiella salina* and *Debaryomyces hensenii*: models for ecophysical adaptation to salinity by fungi that grow in the sea. Can J Bot. 70: 2097-2105.

Colpaert JV and van Assche JA (1987). Heavy metal tolerance in some ectomycorrhizal fungi. Funct Ecol. 1: 415-421.

Colpaert JV, van Tichelen KK (1996). Mycorrhizas and environmental stress. In: Fungi and environmental change (Frankland JC, Magan N, Gadd GM, eds.). Symposium of the British mycological society. Cambridge University Press, Cambridge. pp: 109-128.

Creus CM, Sueldo RJ, Barassi CA (1998). Water relations in *Azospirillum* inoculated wheat seedlings under osmotic stress. Can J Bot. 76: 238-244.

Dashtebani F, Hajiboland R, Aliasgharzad N (2014). Characterization of salt-tolerance mechanisms in mycorrhizal (*Claroideoglomus etunicatum*) halophytic grass, *Puccinellia distans*. Acta Physiol Plant.

36: 1713-1726.

Dixon RK, Rao MV, Garg VK (1993). Salt stress affects in vitro growth and in situ symbioses of ectomycorrhizal fungi. Mycorrhiza. 3: 63-68.

Donnelly DP,·Boddy L, Leake JR (2004). Development, persistence and regeneration of foraging ectomycorrhizal mycelial systems in soil microcosms. Mycorrhiza. 14: 37-45.

Donnelly DP,·Boddy L (2001). Mycelial dynamics during interactions between*Stropharia caerulea*and other cord-forming,saprotrophic basidiomycetes. New Phytol. 151: 691-704.

Duddridge JA, Malbari A, Read DJ (1980). Structure and function of ectomycorrhizal rhizomorphs with especial reference to their role in water transport. Nature. 287: 834-836.

Dunabeitia MK,Hormilla S, Garcia-Plazaola JI, TxarterinaK, Arteche U, Becerril JM (2004). Differential response of three fungal species to environmental factors and their role in the mycorrhization of *Pinus radiata* D. Don. Mycorrhiza. 14: 11-18.

Evelin H, Kapoor R, Giri B (2009). Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. Ann Bot. 104: 1263-1280.

Feng G, Zhang FS, Li XL, Tian CY, Tang C, Rengel Z (2002). Improved tolerance of maize plants to salt stress by arbuscular mycorrhiza is related to higher accumulation of soluble sugars in roots. Mycorrhiza. 12: 185-190.

Gadd GM (1993). Interaction of fungi with toxic metals. New Phytol. 124: 25-60.

Gao Q, Nakano S, Aimi T and Shimomur N (2016). Usefulness of ethyl

methanesulfonate in isolation of halophilic strains of *Rhizopogon roseolus*, Mushroom Sci Biotechnol (in press).

Gossett DR, Milhollon EP, Lucas MC (1994). Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivar of cotton. Crop Sci. 34: 706-714.

Han XL, Jia GX, Niu Y (2006). Research of the mechanism of ectomycorrhizae to drought resistance. Res Soil Water Conserv. 5: 42-44.

Haraguchi N, Kitagawa I, Tomosho T, Mineta T, Zukemura C, Wakasugi K (2012). Paddy fields damaged along the coast part of Miyagi Prefecture by the Tsunami originated from the 2011 off the Pacific Coast of Tohoku Earthquake. J Jpn Soc Soil Phys. 121: 11-17 (in Japanese).

Hare PD, Cress WA, van Staden J (1997). The involvement of cytokinins in plant responses to environmental stress. Plant Growth Regul. 23: 79-103.

Harley JL (1989). The significance of mycorrhiza. Mycol Res. 92: 129-139.

Haro R, Banuelos M, Quintero FJ, Rubio F, Rodriguez-Navarro A (1993). Genetic basis of sodium exclusion and sodium tolerance in yeast. Physiol Plant. 89: 868-874.

Harris K, Young IM, Gilligan CA, Otten W, Ritz K (2003). Effect of bulk density on the spatial organisation of the fungus *Rhizoctonia solani* in soil. FEMS Microbiol Ecol. 44: 45-56.

Hasegawa PM, Bressan RA. Zhu JK, Bohnert HJ (2000). Plant cellular

and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51: 463-499.

Hedh J, Wallander H, Erland S (2008). Ectomycorrhizal mycelial species composition in apatite amended and non-amended mesh bags buried in a phosphorus poor spruce forest. Mycol Res. 112: 681-688.

Hendricks JJ, Mitchell RJ, Kuehn KA, Pecot SD, Simms SE (2006). Measuring external mycelia production of ectomycorrhizal fungi in the field: the soil matrix matters. New Phytol. 171: 179-186.

Hennessey JC, Vishniac HS, Lorenzi EM, Williams JC (1989). Dinitrogen fixation in a water stressed *Alnus* clone is limited by host xerotolerance. Plant and Soil. 118: 89-96.

Honna S (2000). Sandy soil. In: Dunes research to open the century - from sand dunes to the world desert (Japan Society of Sand Dunes Research, eds.). Association of Agriculture and Forestry Statistics, Japan. pp: 55-61 (in Japanese).

Hortal S, Pera J, Parladé J (2008). Tracking mycorrhizas and extraradical mycelium of the edible fungus *Lactarius deliciosus* under field competition with *Rhizopogon* spp.Mycorrhiza. 18: 69-77.

Hortal S, Pera J, Parladé J (2009). Field persistence of the edible ectomycorrhizal fungus *Lactarius deliciosus*: effects of inoculation strain, initial colonization level, and site characteristics. Mycorrhiza. 19: 167-177.

Jahromi F, Aroca R, Porcel R, Ruiz-Lozano JM (2008). Influence of salinity on the in vitro development of *Glomus intraradices* and on the in vivo physiological and molecular responses of mycorrhizal lettuce

plants. Microbial Ecol. 55: 45-53.

Jain RK, Paliwal K, Dixon RK, Gjerstad DH (1989). Improving productivity of multipurpose trees on substandard soils in India. Indian J For. 87: 38-42.

Jennings DH, Burke RM (1990). Compatible solute-the mycological dimension and their role as physiological buffering agents. New Phytol. 116: 277-283.

Juniper S, Abbott LK (1993). Vesicular-arbuscular mycorrhizas and soil salinity. Mycorrhiza. 4: 45-57.

Kamble VR, Mulani RM (2012). Mutation Studies in ECM Fungus *Tricholoma lascivum* (Fr.) Gillet From Maharashtra. Biosci Intl. 1: 66-73.

Kammerbauer H, Agerer R, Sandermann H (1989). Studies on ectomycorrhiza XXII. Mycorrhizal rhizomorphs of *Thelephora terrestris* and *Pisolithus tinctorius* in association with Norway spruce (*Picea abies*): formation in vitro and translocation of phosphate. Trees 3: 78-84.

Kawai M, Yamahara M, Ohta A (2008). Bipolar incompatibility system of an ectomycorrhizal basidiomycete, *Rhizopogon rubescens*. Mycorrhiza. 18: 205-210.

Kernaghan G, Hambling B, Fung M, Khasa D (2002). In vitro selection of Boreal ectomycorrhizal fungi for use in reclamation of saline–alkaline habitats. Restor Ecol. 10: 1-9.

Khan MA, Ungar IA, Showalter AM (1999). Effects of salinity on growth, ion content, and osmotic relations in *Halopyrum mocoronatum* (L.).

Stapf J Plant Nutr. 22: 191-204.

Kikuchi K, Matsushita N, Suzuki K, Hogetsu T (2007). Flavonoids induce germination of basidiospores of the ectomycorrhizal fungus *Suillus bovinus*. Mycorrhiza. 17: 563-570.

Ko CH, Gaber RF (1991). TRK1 and TRK2 encode structurally related K1 transporters in Saccharomyces cerevisiae. Mol Cell Biol. 11: 4266-4271.

Lehto T, Zwiazek JJ (2011). Ectomycorrhizas and water relations of trees: a review. Mycorrhiza. 21: 71-90.

Levy Y, Dodd J, Krikun J (1983). Effect of irrigation water salinity and rootstock on the vertical distribution of vesicular-arbuscular mycorrhiza in citrus roots. New Phytol. 95: 397-403.

Lewis DH, Smith DC (1967). Sugar alcohols (Polyols) in fungi and green plants. I. Distribution, physiology and metabolism. New Phytol 66: 143-184.

Li B, Sun FF, Hu HZ, Jia HZ, Zhang WS, Li X (2008). Comparison of imported and the domestic PEG on seed germination and root growth in Arabidopsis. Chin J Eco-Agric. 4: 1070-1072.

Luan YS, Zhang J, Gao XR, An LJ (2007). Mutation induced by ethylmethanesulphonate (EMS), in vitro screening for salt tolerance and plant regeneration of sweet potato (*Ipomoea batatas* L.). Plant Cell Tiss Organ Cult. 88:77-81.

Maggio A, Reddy MP, Joly RJ (2000). Leaf gas exchange and solute accumulation in the halophyte *Salvadora persica* grown at moderate salinity. Environ Exp Bot. 44: 31-38.

Majdi H, Truus L, Johansson U (2008). Effects of slash retention and wood ash addition on fine root biomass and production and fungal mycelium in a Norway spruce stand in SW Sweden. For Ecol Manage. 255: 2109-2117.

Marcar NE, Dart P, Sweeney C (1991). Effect of root-zone salinity on growth and chemical composition of *Acacia ampticeps* BR Maslin, *A. auriculiformis* A. Cunn. ex Benth, and *A. mangium* Willd. at two nitrogen levels. New Phytol. 119: 567-573.

Marivet J, Frendo P and Burkard G (1992). Effects of abiotic stresses on cyclophilin gene expression in maize and bean and sequence analysis of bean cyclophilin cDNA. Plant Sci. 84: 171-178.

Marjanović Z, Nehls U, Hampp R (2005). Mycorrhiza formation enhances adaptive response of hybrid poplar to drought. Ann NY Acad Sci 1048: 496-499.

Marx DH (1969). The influence of ectotrophic ectomycorrhizal fungi on the resistance of pine roots to pathogenic colonizations. I. Antagonism of mycorrhizal fungi to pathogenic fungi and soil bacteria. Phytopathol. 59:153-163.

Medina A, Schmidt-Heydt M, RodríguezA, Parra R, Geisen R, Magan N (2015). Impacts of environmental stress on growth, secondary metabolite biosynthetic gene clusters and metabolite production of xerotolerant/xerophilic fungi. Curr Genet. 61: 325-334.

Michel BE, Kaurmann MR (1973). The osmotic potential of polyethylene glycol 6000. Plant Physiol. 51: 914-916.

Michel BE, Wiggins OK, Outlaw WH (1983). A guide to establishing

water potential of aqueous two-phase solutions (polethylene glycol plus dextran) by amendment with mannitol. Plant Physiol. 72: 60-65.

Miller CO (1971). Cytokinin production by mycorrhizal fungi. Proceedings of the 1st North American Conference on Mycorrhizae. US Department of Agriculture, Washington. pp: 168-174.

Miller CO (1967). Zeatin and zeatin riboside from a mycorrhizal fungus. Science. 157: 1055-1057.

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405-410.

Miyawaki A (2014). The Japanese and Chinju-no-mori, Tsunami-protecting forest after the Great East Japan Earthquake 2011. Phytocoenologia. DOI: 10.1127/0340-269X/2014/0044-0571.

Molina R, Trappe JM (1994). Biology of the ectomycorrhizal genus, *Rhizopogon*. I. Host associations, host-specificity and pure culture syntheses. New Phytol. 126: 653-675.

Moturi B, Charya MAS (2010). Influence of physical and chemical mutagens on dye decolourising *Mucor mucedo*. Afr J Microbiol Res. 4: 1808-1813.

Mulligan D (2007). Effect of salt on the growth of ectomycorrhizal isolates and implications for the coal mining industry in central Queensland. Ecol Manag Restor. 8: 63-65.

Munns R, Tester M (2008). Mechanisms of salinity tolerance. Annu Rev Plant Biol .59: 651-681.

Nagasawa E (2000). Cultivation of *Rhizopogon rubescens* in the seashore pine forest (in Japanese). Sand Dune Res. 47:140-143.

Nakano S, Gao Q, Aimi T, Shimomura N (2016). *Rhizopogon roseolus* basidiospore germination decreases as the fruiting bodies mature and the spore wall becomes more complex. Bot. 94: 311-320.

Nakano S, Sawada K, Gao Q, Aimi T, Shimomura N (2015). Production of a salt tolerant strain by cross-breeding in the ectomycorrhizal mushroom *Rhizopogon roseolus* (=*R. rubescens*). Mushroom Sci Biotechnol. 23: 75-79.

Newsham KK, Fitter AH, Watkinson AR (1995). Multifunctionality and biodiversity in arbuscular mycorrhizas. Trends Ecol Evol. 10:407-411.

Nilsson LO, Giesler R, Bååth E, Wallander H (2005). Growth and biomass of mycorrhizal mycelia in coniferous forests along short natural nutrient gradients. New Phytol. 165: 613-622.

Ortega U, Duñabeitia M, Menendez S, Gonzalez-Murua C, Majada J (2004). Effectiveness of mycorrhizal inoculation in the nursery on growth and water relations of *Pinus radiata* in different water regimes. Tree Physiol. 24: 65-73.

Pandey R, Agarwal RM (1998). Water stress-induced changes in praline contents and nitrate reductase activity in rice under light and dark conditions. Physiol Mol Biol Plants. 4: 53-57.

Parida AK, Das AB (2005). Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Saf. 60: 324-349.

Parke JL, Linderman RG, Black CH (1983). The role of ectomycorrhizas in drought tolerance of Douglas-fir seedlings. New Phytol. 95: 83–95.

Parladé J, Hortal S, de la Varga H, Pera J (2011). Intranspecific variability of *Lactarius deliciosus* isolates: colonization ability and survival after

cold storage. Mycorrhiza. 21: 393-401.

Perry DA, Molina R, Amaranthus MP (1987). Mycorrhizae, mycorrhizosphere and reforestation: current knowledge and research needs. Can J For Res. 17: 929-940.

Plemenitaš A, Lenassi M, Konte T, Kejžar A, Zajc J, Gostinčar C, Gunde-Cimerman N (2014). Adaptation to high salt concentrations in halotolerant / halophilic fungi: a molecular perspective. Front Microbiol. 5: 199.

Pond EC, Menge JA, Jarrell WM (1984). Improved growth of tomato in salinized soil by vesicular-arbuscular mycorrhizal fungi collected from saline soils. Mycologia. 76: 74-84.

Posas F, Chambers JR, Heyman JA, Hoeffler JP, Nadal E, Arino J (2000). The transcriptional response of yeast to saline stress. J Biol Chem. 275: 17249-17255.

Pradeep MR, Narasimha G (2011). Utilization of pea seed husk as subsrate fro cellulose production by mutant *Asperdillus niger*. Insight Biotechnol. 1: 17-22.

Quintanilla LG, Amigo J, Pangua E, Pajarón S (2002). Effect of storage method on spore viability in five globally threatened fern species. Annals of Botany. 90: 461-467.

Radha S, Babu RH, Sridevi A, Prasad NBL, Narasimha G (2012). Development of mutant fungal strains of *Aspergillus niger* for enhanced production of acid protease in submerged and solid state fermentation. European J Exp Biol. 2: 1517-1528.

Ramirez ML, Chulze SN, Magan N (2004). Impact of osmotic and matric

water stress on germination, growth, mycelial water potentials and endogenous accumulation of sugars and sugar alcohols by *Fusarium graminearum*. Mycologia. 96: 470-478.

Read DJ, Boyd R (1986). Water relations of mycorrhizal fungi and their host plants. In: Water, fungi and plants (Ayres PG, Boddy L, eds.). Cambridge University Press, Cambridge. pp: 215-240.

Reddell P, Foster RC, Bowen GD (1986). The effects of sodium chloride on growth and nitrogen fixation in *Casuarina obesa* Miq. New Phytol. 102: 397-408.

Rhoades JD (1990). Soil salinity – cause and control. In: Techniques for desert reclamation (eds., Goudie AS). John Wiley & Sons Press, England. pp: 109-134.

Riesenman PJ, Nicholson WL (2000). Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. Appl Environ Microb. 66: 620-626.

Rincón A, Parladé J, Perab J (2007). Influence of the fertilization method in controlled ectomycorrhizal inoculation of two Mediterraean pines. Ann For Sci. 64: 577-583.

Ritz K, Young I (2004). Interactions between soil structure and fungi. Mycologist. 18: 52-59.

Rousseau JV, Sylvia DM, Fox AJ (1994). Contribution of ectomycorrhizal to the potential nutrient absorbing surface of pine. New Phytol. 128: 639-644.

Roy K, Sasada K, Kohno E (2014). Salinity status of the 2011 Tohoku-oki tsunami affected agricultural lands in northeast Japan. Intl

Soil Water Conserv Res. 2: 40-50.

Sannazzaro AI, Echeverria M, Alberto´ EO, Ruiz OA, Mene´ndez AB (2007). Modulation of polyamine balance in Lotus glaber by salinity and arbuscular mycorrhiza. Plant Physiol Bioch. 45: 39-46.

Sawada K, Wan J, Oda K, Nakano S, Aimi T, Shimomura N (2014). Variability in nucleus number in basidiospore isolates of *Rhizopogon roseolus* and their ability to form ectomycorrhizas with host pine roots. Mycol Progress.13: 745-751.

Schouten HJ, Jacobsen E (2007). Are Mutations in Genetically Modified Plants Dangerous? J Biomed Biotechnol. doi: 10.1155/2007/82612

Sega GA (1984). A review of the genetic effects of ethyl methanesulfonate. Mutat Res. 134: 113-142.

Shen B, Hohmann S, Jensen RG, Bohnert HJ (1999). Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol. 121: 45-52.

Shimomura N, Aimi T, Matsumoto T, Maekawa N, Otani H (2008). Ultrastructure of developing basidiospores in *Rhizopogon roseolus* (=*R. rubescens*). Mycoscience. 49: 35-41.

Shimomura N, Aimi T, Otani H, Park P (2010). Cytological features of ectomycorrhizae aseptically synthesized between *Rhizopogon roseolus* (shoro) and *Pinus thunbergii*. Mushroom Sci Biotechnol. 18:103-106.

Shimomura N, Egi T, Sawada K, Aimi T (2012a). Development of detecting medium for the clamp formation of *Rhizopogon roseolus* mycelium. Mushroom Sci Biotechnol. 20: 85-88

Shimomura N, Matsuda M, Ariyoshi K, Aimi T (2012b). Development of

mycelial slurries containing surfactant for cultivation of the edible ectomycorrhizal mushroom *Rhizopogon roseolus* (syn. *Rhizopogon rubescens*). Botany. 90: 839-844.

Slanki V (1973). Hormonal relationships in mycorrhizal development. In, Ectomycorrhizae: their Ecology and Physiology (Marks GC, Kozlowski TT, eds.). Academic Press, New York. pp: 231-298.

Smith ML (2008). Genetic stability in fungal mycelia. In: The fungal colony (Gow NAR, Robson GD, Gadd GM, eds.). Cambridge University Press, New York. pp: 283-301.

Smith SE, Read DJ (2008). Mycorrhizal symbiosis, 3 rd ed. Academic Press. pp: 787.

Tang M, Sheng M, Chen H, Zhang FF (2009). In vitro salinity resistance of three ectomycorrhizal fungi. Soil Biol Biochem. 41: 948-953.

Taniguchi T, Kanzaki N, Tamai S, Yamanaka N, Futai K (2007a). Does ectomycorrhizal fungal community structure vary along a Japanese black pine (*Pinus thunbergii*) to black locust (*Robinia pseudoacacia*) gradient? New Phytol. 173: 322-334.

Taniguchi T, Tamai S, Yamanaka N, Futai K (2007b). Inhibition of the regeneration of Japanese black pine (*Pinus thunbergii*) by black locust (*Robinia pseudoacacia*) in coastal sand dunes. J For Res. 12: 350-357.

Trappe JM (1977). Selection of fungi for ectomycorrhizal inoculation in nurseries. Annu Rev Phytopathol. 15: 203-222.

Tresner HD, Hayes JA (1971). Sodium chloride tolerance of terrestrial fungi. Appl Microbiol. 22: 210-213

Turjaman M, Tamai Y, Segah H, Limin SH, Osaki M, Tawaray K (2006).

Increase in early growth and nutrient uptake of *Shorea seminis* seedlings inoculated with two ectomycorrhizal fungi. J Trop Forest Sci. 18: 243-249.

Van Harten AM (1998). Mutation Breeding: Theory and Practical Applications. Cambridge University Press, London. pp: 127-140.

Wallander H, Johansson U, Sterkenburg E, Brandström Durling M, Lindahl BD (2010). Production of ectomycorrhizal mycelium peaks during canopy closure in Norway spruce forests. New Phytol. 187: 1124-1134.

Wallander H, Nilsson LO, Hagerberg D, Bååth E (2001). Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. New Phytol. 151: 753-760.

Weigt RB, Raidl S, Verma R, Agerer R (2012). Exploration type-specific standard values of extrametrical mycelium – a step towards quantifying ectomycorrhizal space occupation and biomass in natural soil. Mycol Progress. 11: 287-297.

Yeo AR (1983). Salinity resistance: physiologies and prices. Physiol Plant. 58: 214-222.

Zhang HH, Tang M, Yang Y (2011). The response of ectomycorrhizal (ECM) fungi under water stress induced by polyethylene glycol (PEG) 6000. Arf J Microbiol Res. 5: 365-373.

Zhao MX, Sun HY, Ji RR, Hu XH, Sui JM, Qiao LX, Chen J, Wang JS (2013). In vitro mutagenesis and directed screening for salt-tolerant mutants in peanut. Euphytica. 193: 89-99.

Zhao ZP, Guo XZ (1989). Ecological studies on ectomycorrhizal fungi in

pure cultures. For Res. 2: 136-141.

Zheng W, Morris EK, Rillig MC (2014). Ectomycorrhizal fungi in association with *Pinus sylvestris* seedlings promote soil aggregation and soil water repellency. Soil Biol Bioch.78: 326-331

Zhou MY, Sharik TL, Jurgensen MF, Richter DL (1997). Ectomycorrhizal colonization of *Quercus rubra* seedlings in response to vegetation removals in oak and pine stands, For Ecol Manage. 93: 91-99.

List of related publication

1. Qi Gao, Shota Nakano, Tadanori Aimi and Norihiro Shimomura(2016). Isolation of halophilic strains induced by ethyl methanesulfonate in *Rhizopogon roseolus*. Mushroom Science and Biotechnology 24 (4): in press. (The corresponding content is in Chapter 2).

2. Qi Gao, Shota Nakano, Tadanori Aimi and Norihiro Shimomura(2016). Comparison of salt tolerance of ectomycorrhizal mushroom *Rhizopogon roseolus* in soil and on agar.Mushroom Science and Biotechnology25 (1): in press. (The corresponding content is in Chapter 3).