MYCELIAL SLURRIES AS SPAWN FOR CULTIVATION OF THE EDIBLE ECTOECTOMYCORRHIZAL MUSHROOM, *RHIZOPOGON ROSEOLUS* (=*R. RUBESCENS*)

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ABSTRACT

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 the Pinaceae. In order to cultivate this edible ectomycorrhizal mushroom, several researchers have tried to promote ectomycorrhization of this mushroom on roots of its host, *Pinus thunbergii*: Pine seedlings were inoculated with mycelium in vitro, or with crushed fruiting bodies in nature. However, this mushroom has not been successfully cultivated. We have developed useful mycelial slurries that enable production of abundant ectomycorrhizas and promote fruiting body formation under greenhouse nursery. We selected a superior isolate that rapidly colonized and produced a lot of ectomycorrhizas in roots of *P. thunbergii*. The mycelial slurries were composed of saline solution and a homogenate of a submerged culture of the mycelium. Addition of surfactant to the mycelial slurries resulted in stimulation of ectomycorrhizal formation in host roots. When the mycelial slurries were injected into a mother plant system in which the colonized seedling had been planted in the nursery, stimulatory effects were observed not only on ectomycorrhization of the seedlings but also on fruiting body formation. Genotype analysis of the fruiting bodies produced in the nursery showed that they had been produced by the inoculated isolate. These results suggest that the mycelial slurries could serve as mycelial spawn for cultivation of shoro.

Keywords: Ectomycorrhizal mushroom; Fruiting body formation; Mycelial slurry; *Pinus thunbergii*; *Rhizopogon roseolus*; Spawn

INTRODUCTION

Rhizopogon roseolus (Corda) Th.M. Fr. (=*R. rubescens* Tul. & C. Tul.), known as “shoro” in Japanese, is a hypogeous basidiomycete that is an important ectomycorrhizal symbiont of Pinaceae [1]. The fruiting bodies of this fungus are found in the sandy soils of *Pinus thunbergii* Parl. forest in seashore habitats. *R. roseolus* is a popular edible fungus in Japan, but factors such as deforestation, poor forest management and indiscriminate harvesting have led to recent decreases in production [2]. To cultivate this fungus, pine seedlings can be aseptically inoculated with mycelium or spores, then these ectomycorrhizal seedlings can be planted into a natural environment, and the fungus can be encouraged to grow [3-5]. However, cultivation techniques

have not been fully refined, because of the low efficiency with which ectomycorrhizal trees can be obtained, and the low levels of fruiting body production.

To successfully cultivate the fungus, inoculation and propagation methods must be developed, and isolates with superior traits must be selected. Such traits as vigorous mycelial growth, a tendency to form ectomycorrhizas and high levels of fruiting body production are desirable. Recently, we have discovered the superior isolate that rapidly colonized and produced a lot of ectomycorrhizas in root of *P. thunbergii*. Furthermore, we have developed a new inoculation method by using the isolate. In this paper, we demonstrate that the inoculation method enables to produce not only abundant ectomycorrhizas of host root, but also rapid fruiting body formation of this edible mushroom.

MATERIALS AND METHODS

Fungal isolates. Nineteen wild dikaryotic isolates of *R. roseolus* were used in this study (Table 1). The isolates have been deposited in the culture collection of the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Japan. To prepare inoculum, the isolate was inoculated on malt extract agar (20 g malt extract (Oriental Yeast Co., Ltd., Tokyo, Japan), 20 g agar, and 1 L tap water) plates and incubated for 3 wk at 25 °C in the dark. After incubation, 5 mm square mycelial agar plugs were removed by a surgical knife and used for inoculation.

Table 1: Fungal isolates of *Rhizopogon roseolus* used in this study

TUFC number	Collection	
	Location	Year
10003	Tottori	2005
10004	Tottori	2005
10006	Ishikawa	2005
10007	Miyagi	1997
10008	Niigata	2005
10009	Niigata	2005
10010	Niigata	2005
10016	Miyagi	2003
10018	Shimane	2000
10019	Kochi	1998
10026	Tottori	2005
10031	Iwata	2003
10032	Miyagi	2003
10033	Miyagi	1997
10036	Kagoshima	1999
10042	Niigata	2005
10086	Saga	2001
10096	Ibaraki	1998
32018	Tottori	2005

In vitro infection. Roots of *P. thunbergii* were infected in vitro with *R. roseolus* using the Petri dish technique described previously [6]. Sterile *P. thunbergii* seedlings were germinated from seeds collected from a *P. thunbergii* forest at Tottori University in Tottori, Japan. The seeds were rinsed in running water for 24 h and surface-sterilized in 30% H_2O_2 for 30 min. Following three rinses with sterile distilled water, the seeds were aseptically transferred to a water agar medium and germinated at 23 °C under diffused light. At 2 wk following germination, the seedlings were transferred to filter paper overlying modified Melin and Norkrans medium [7] without added glucose and malt extract in Petri dishes (90 × 20 mm). The Petri dishes were sealed with lanolin and Parafilm (American Can Co., Detroit, MI, USA) and incubated in controlled environmental chambers at a constant 23 °C with 85% relative humidity under a 16-h day at 60 µmol m^{-2} s^{-1}. After 4 wk of incubation, 5 mm square *R. roseolus* mycelial agar plugs were transferred to *P. thunbergii* lateral roots and the seedlings were incubated as above for an additional 8 wk.

Preparation of mycelial slurries. Agar plugs of a dikarytotic isolate, *R. roseolus* TUFC10010 were inoculated into 50 ml of malt extract liquid medium (20 g/L malt extract and 0.2% Tween 80) in an Erlenmeyer flask and incubated at 25 °C. After 4 wk, the mycelia were harvested by filtration on 1 mm mesh Nitex cloth followed by washing with tap water. The harvested mycelia were suspended at 10 g wet weight in 100 mL saline solution (30 mM NaCl, 7 mM KCl and 4 mM $MgCl_2{\cdot}6H_2O$, pH 7.0) containing 0.2% Tween 80. About 500 mL of suspension was blended with an AHG-160A homogenizer (AS ONE Corp., Osaka, Japan) at 14,000 rpm for 1 min. The resulting mycelial slurries were used as inocula without dilution.

Inoculation in mother plant system in greenhouse nursery. For container-grown seedlings, seeds of *P. thunbergii* were planted in a plastic container (50 cm × 35 cm × 15 cm) containing horticultural grade vermiculite and bark-compost in equal proportions by volume. After 8 wk planting, an ectomycorrhizal mother seedling that had been inoculated in vitro with *R. roseolus* TUFC10010 was transferred into the center of the container. At 12, 16 and 20 wk after planting, seedlings were inoculated two or three times by injecting 500 mL of mycelial slurries into the root zone, approximately 2 cm underground, using a pipette. Ectomycorrhizal formation was evaluated 32 wk after planting by counting the total number of ectomycorrhizas.

RESULTS AND DISCUSSION

Pinus thunbergii seedlings grew well in plastic Petri dishes and produced root systems with first-order laterals suitable for ectomycorrhizal synthesis over a 5- to 6-wk period after germination. The total number of ectomycorrhizas formed by each isolate at 2, 4, 8 and 12 wk from inoculation is shown in Fig. 1. From 2 wk following inoculation, initial ectomycorrhizal formation could be observed in 4 isolates (TUFC10004, 10008, 10010 and 10042). After 8 wk, 2 isolates (TUFC10004 and 10010) formed abundant ectomycorrhizas. Figure 2 shows typical ectomycorrhizas of the seedling inoculated with TUFC10004.

Intraspecific variability of fungal isolates has to be taken into account in the selection processes aimed at obtaining superior isolates for controlled inoculation [8, 9]. The candidate fungi should exhibit the physiological capacity to form abundant ectomycorrhizas on the desired hosts [10]. Molina & Trappe [1] pointed out differences in colonization ability among *Rhizopogon* spp. fungal isolates. Habitat differences might be reflected in the colonization patterns, indicating some degree of specialization or host preference among isolates. Recent studies [11, 12] showed priority effects in inoculation experiments with *Rhizopogon* species,

demonstrating that more rapid root tip colonization of one species resulted in almost complete exclusion of the slower species. Our results obtained with in vitro experiments also showed intraspecific variation in colonization speed and ability to produce ectomycorrhiza among *R. roseolus* isolates. Rapid colonization ability may be a competitive advantage under natural conditions. Therefore, the fungal isolate used in natural conditions should have an excellent capability for rapid colonization as well as formation of a great number of ectomycorrhizas.

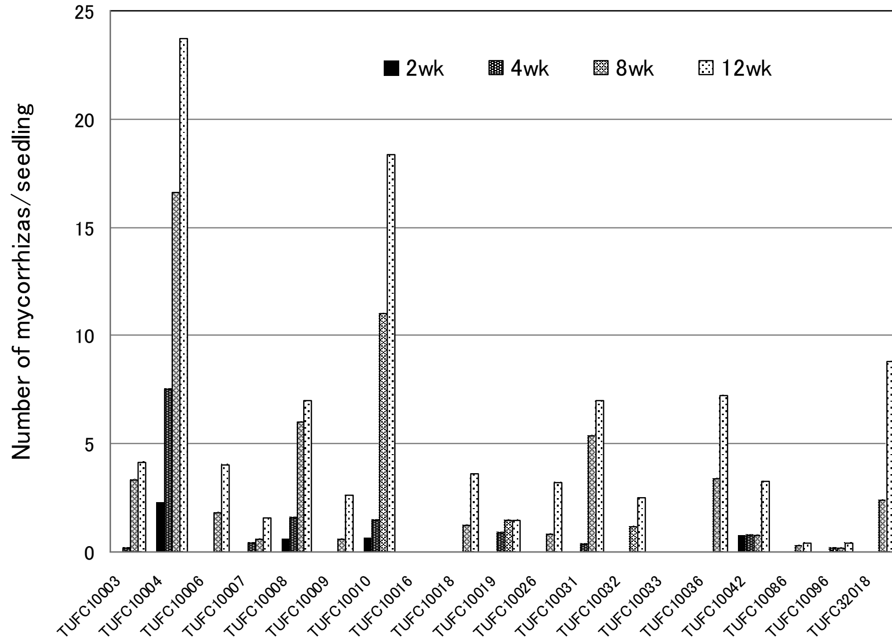

Figure 1: Variation in ectomycorrhizal forming ability among isolates of *Rhizopogon roseolus* on *Pinus thunbergii* seedlings using a Petri dish method.

Figure 2: Ectomycorrhizal root system of a *Pinus thunbergii* seedling inoculated with *Rhizopogon roseolus* TUFC10004.

Effects of injection of mycelial slurries on ectomycorrhizal formation in the mother plant system are shown in Fig. 3. Evaluation of ectomycorrhizal formation was carried out 32 wk after planting by counting the total number of ectomycorrhizas. A stimulatory effect of mycelial slurries on ectomycorrhizal formation by pine seedlings in the mother plant system was observed. At 32 wk after planting, 14 fruiting bodies were found in the nurseries: 1 fruiting body in the uninoculated treatment, 8 fruiting bodies in the treatment with two inoculations, and 5 fruiting bodies in the treatment with three inoculations (Table 2). Figure 4 shows the fruiting body produced in the nursery. Genotype analysis of the fruiting bodies produced in the nursery showed that they had been produced by the inoculated isolate.

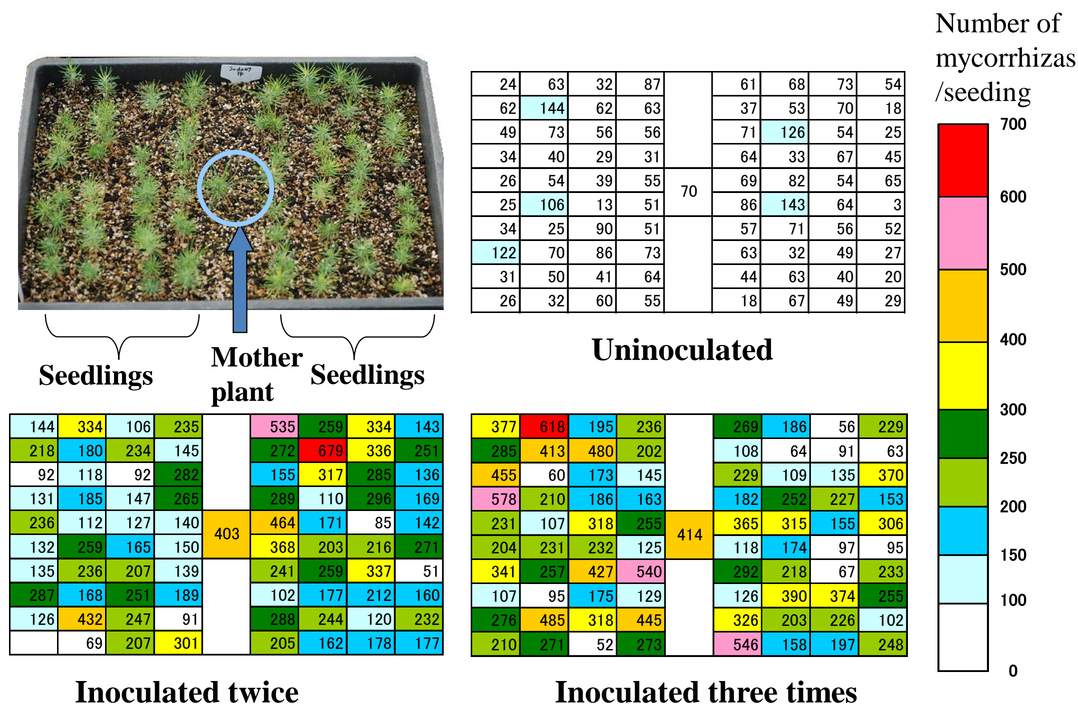

Figure 3: Effect of inoculation of mycelial slurries of *Rhizopogon roseolus* TUFC10010 on ectomycorrhizal formation on *Pinus thunbergii* seedlings in the mother plant system. Values in the figures indicate number of ectomycorrhizas/seedling.

Table 2: Effect of inoculation of mycelial slurries of *Rhizopogon roseolus* TUFC10010 on fruiting body formation in the mother plant system

Inoculation treatment	Number of fruiting bodies in four planters
Uninoculated	1
Inoculated twice	8
Inoculated three times	5

Figure 4: Fruiting body formation (arrow) of *Rhizopogon roseolus* in a *Pinus thunbergii* nursery.

In these experiments, we showed the usefulness of mycelial slurries as inocula. Although the use of solid carriers for mycelium is the standard method used to inoculate seedlings [13], it appears that mycelial slurries may have some advantages over solid for certain practical situations. Blended mycelial slurries of different ectomycorrhizal fungi were assessed for their suitability as inocula for the production of ectomycorrhizal trees, especially for plantations [14, 15]. On the other hand, difficulty obtaining ectomycorrhizal seedlings inoculated with mycelia of *Rhizopogon* spp. has been reported [1]. In the present experiments, we mixed a non-ionic surfactant with the inocula. Addition of surfactant promoted mycelial growth and ectomycorrhizal colonization (data not shown). Previous research [16, 17] showed that incorporation of Tween nonionic surfactants in soil-containing substrate strongly stimulated not only mycelial growth but also formation of shiro-like structure of the edible ectomycorrhizal mushroom *Tricholoma matsutake*. This stimulation is considered to be related to the higher hydrophilicity of treated hyphae, or to enhance lytic excretion and activity of hyphae [17]. Use of a surfactant might help overcome the difficulty in obtaining ectomycorrhizal seedlings.

The mother plant technique has been used extensively in the production of *Tuber magnatum* colonized plants, which are difficult to obtain using spore inoculation [18]. However, this method carries a high risk of spreading contaminating and possibly very competitive ectomycorrhizal fungi. Mycelial slurries developed in this study may reduce the risk of contamination.

CONCLUSIONS

In the present study, we showed that inoculation using mycelial slurries containing surfactant stimulates not only ectomycorrhizal colonization of host pine seedlings but also fruiting body formation, indicating that the slurries could be used as spawn for cultivation of the edible ectomycorrhizal mushroom, shoro.

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