



Bioactive properties of streptomyces may affect the dominance of *Tricholoma matsutake* in shiro

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Abstract

Tricholoma matsutake is known to be the dominant fungal species in matsutake fruitbody neighboring (shiro) soil. To understand the mechanisms behind matsutake dominance, we studied the bacterial communities in matsutake dominant shiro soil and non-shiro soil, isolated the strains of *Streptomyces* from matsutake mycorrhizal root tips both from shiro soil and from the *Pinus densiflora* seedlings cultivated in shiro soil. Further, we investigated three *Streptomyces* spp. for their ability to inhibit fungal growth and *Pinus densiflora* seedling root elongation as well as two strains for their antifungal and antioxidative properties.

Our results showed that Actinobacteria was the most abundant phylum in shiro soil. However, the differences in the Actinobacterial community composition (phylum or order level) between shiro and non-shiro soils were not significant, as indicated by PERMANOVA analyses. A genus belonging to Actinobacteria, *Streptomyces*, was present on the matsutake mycorrhizas, although in minority. The two antifungal assays revealed that the broths of three *Streptomyces* spp. had either inhibitory, neutral or promoting effects on the growth of different forest soil fungi as well as on the root elongation of the seedlings. The extracts of two strains, including one isolated from the *P. densiflora* seedlings, inhibited the growth of either pathogenic or ectomycorrhizal fungi. The effect depended on the medium used to cultivate the strains, but not the solvent used for the extraction. Two *Streptomyces* spp. showed antioxidant activity in one out of three assays used, in a ferric reducing antioxidant power assay. The observed properties seem to have several functions in matsutake shiro soil and they may contribute to the protection of the shiro area for *T. matsutake* dominance.

Keywords Antifungal · Antioxidant · *Heterobasidium* · Mycorrhiza-associated · Shiro · *Streptomyces*

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Abbreviations

AS	ascomycetes
BA	basidiomycetes
ECM	ectomycorrhizal fungi
PA	pathogenic fungi

1 Introduction

Mycorrhizal symbiosis has a great effect on plant fitness via nutrient and water uptake (Smith and Read 2010). The ectomycorrhizosphere, a specific interface between plants, soil and symbiotic fungi, harbors a large and diverse community where the organisms can inhibit or stimulate each other's growth (Rudnick et al. 2015). The plant partner selects not only its symbiotic fungi but also bacterial species beneficial for the symbiosis; bacteria have been visualized inside mycorrhizas and as colonies on soil-colonizing fungal hyphae (Frey-Klett et al. 2005). Specifically, the growth of ectomycorrhizal

fungi and the formation of mycorrhizas are promoted by certain mycorrhizosphere bacteria, termed “mycorrhiza helper-bacteria” (Garbaye 1994).

Tricholoma matsutake is an ectomycorrhizal symbiotic fungus of forest trees. It develops mycelial aggregations in association with ectomycorrhizal roots and soil particles, which are called ‘shiro’ (Hosford et al. 1997). In Japan, Korea and in the northeastern China, *Pinus densiflora* is a major host tree of *T. matsutake*, where it produces economically valuable mushrooms (Wang et al. 2012). However, its natural yields have been decreasing for several decades (Wang et al. 2012; Vaario et al. 2017). Attempts to produce more matsutake mushrooms have been challenging for a long time; the successful outplanting of matsutake mycorrhizal seedlings is still limited (Guerin-Laguette et al. 2004; Kobayashi et al. 2015; Yamada et al. 1999; Yamada et al. 2006). The major obstacle for the cultivation of matsutake seems to be that although the seedlings are originally mycorrhizal, the development of mycorrhizal symbiosis and shiro are limited in the field (Kobayashi et al. 2015). The reason for this interruption has been the subject of many studies.

Recently, several studies from different continents have focused on the soil microbial communities in matsutake shiro (Kataoka et al. 2012; Kim et al. 2014; Lian et al. 2006; Oh et al. 2016; Usha et al. 2011). The development of the shiro is the key for matsutake fruiting body formation. In shiro, *T. matsutake* is the dominant mycorrhizal species (Lian et al. 2006; Vaario et al. 2011). This supports an early finding that matsutake mycorrhiza inhibits other soil microbes (Ohara and Hamada 1967). However, some recent observations suggest the opposite. The molecular screening of 16S rDNA libraries (Boer et al. 2005) revealed that a wide repertoire of microbes, including OTUs belonging to the genera *Burkholderia*, *Bacillus*, *Mycobacterium* and *Paenibacillus*, were significantly more abundant when matsutake was more abundant in soil (Oh et al. 2016). The genus *Pseudomonas* was also found to be abundant in the mycorrhizal roots of *T. matsutake* sampled from another region (Li et al. 2018). The observations do not allow us, however, to generalize any common features of the bacterial community in matsutake shiro soil. We are also unable to interpret the laboratory experiments in which only a few bacterial species promoted matsutake mycelium growth on a relatively carbon deficient agar medium (Oh et al. 2018; Oh and Lim 2018b). These pieces of previous information altogether hint that matsutake shiro bacterial communities vary among regions, and that different community members may have different roles.

Recently, the plant-interacting actinobacterial genus *Streptomyces* has received much attention (Schrey et al. 2005; Kurth et al. 2013). These Gram-positive filamentous prokaryotes are ubiquitous in soils and marine sediments, and they are commonly found in the rhizosphere or inside the plant roots. They have been extensively investigated in

medicine for decades due to their rich potential for antifungal and antibacterial properties. Numerous *Streptomyces* strains from a wide range of major clades have been shown to have a helper-function in symbioses (Frey-Klett et al. 2007). *Streptomyces* seems to be important in matsutake ecosystem as well. *Streptomyces* were the most common actinobacterial species in matsutake shiro when assessed using culturable methods (Kataoka et al. 2012). Based on PCR-DGGE fingerprinting method, a positive relation between *Streptomyces* spp. and *T. matsutake* in shiro was reported previously (Vaario et al. 2011). However, information about the matsutake mycorrhiza-associated *Streptomyces* and their potential roles for the surrounding fungal growth is limited. *Streptomyces* are known as the sources of novel bioactive compounds, but they have not received attention in matsutake ecosystems (Seipke et al. 2012). The production of these secondary metabolites depends on the strains as well as on their nutritional and growing conditions (Miao et al. 2006; Manikkam et al. 2015).

In this study, we focused on the shiro ecosystem and *T. matsutake* mycorrhiza, and more specifically, on streptomyces occurrence on matsutake mycorrhizal roots. Our aim was to study whether any *Streptomyces* actinobacteria associate with the mycorrhizal root tips of *T. matsutake* and what functions those actinobacteria have in the matsutake ecosystem, specially, if the presence of streptomyces affects the hyphae growth of *T. matsutake* or other soil fungi. We measured the effect directly on hyphae growth or indirectly through the soil bioactivities. Our hypothesis is that streptomyces are in general common in soil, and that they directly associate with matsutake mycorrhiza and contribute to the superiority of *T. matsutake* in shiro.

This study was divided into four steps; (i) assessment of the bacterial communities in shiro soil; (ii) isolation and identification of *Streptomyces* species from matsutake mycorrhizal root tips; (iii) in vitro investigation of the effects of three *Streptomyces* spp. broths on the growth of fungi originating from Japanese red pine forests, and a subsequent testing of two *Streptomyces* spp. broth extracts, which were prepared using different solvents and cultural media, inhibit the growth of pathogen or ectomycorrhizal fungi (ECM) and (iv) examination of the antioxidant activities of the *Streptomyces* spp. extracts.

2 Material and methods

2.1 Study site and soil sampling

A forest site that produces matsutake sporocarps and is dominated by *Pinus densiflora* in Wakayama prefecture, Japan, was selected for the shiro soil sampling. Each ‘shiro’ sample was collected beneath a matsutake sporocarp and was defined

as soil containing the dense mat of fungal filaments adhered to host plant roots and soil particles. Two to five meters from the shiro sample, where no matsutake sporocarp was observed, a ‘non-shiro’ sample was collected; the sample was further confirmed to have no dense matsutake mycelial mat under a stereomicroscope. Each sample was taken 10 cm below the soil surface. In total, 18 soil samples (nine shiro samples and nine non-shiro samples) were taken at the end of October 2015, immediately after the matsutake sporocarp harvest. The samples were immediately taken to the laboratory for further processing. The pieces of the samples (ca. 0.5 g) were randomly transferred into 2-ml tubes and stored at $-20\text{ }^{\circ}\text{C}$ for the DNA analysis. The mycorrhizal root tips of matsutake were collected from three randomly chosen shiro soil samples for the isolation of actinobacteria as described below.

2.2 Mycorrhizal seedling production

The rest of the soil sample was used to produce mycorrhizal seedlings under controlled green-house conditions ($20\text{--}25\text{ }^{\circ}\text{C}$ with a light cycle 16-h day and 8-h night). The seeds of *P. densiflora* were sown on shiro soil in three replicate 500 ml pots and examined for mycorrhizal occurrence six months after germination. The seedlings were irrigated with tap water at 2- to 3-day intervals during the experimental period.

2.3 Soil DNA extraction, pyrosequencing, and community analysis

Environmental DNA was extracted from 0.2 g homogenized soil per sample (18 samples) using the ISOIL for Beads Beating kit (Nippon Genes, Japan). DNA was amplified using a forward primer (27Fmod: AGRGTTTGATYMTGGCTCAG) and a reverse primer (338R: TGCTGCCTCCCGTAGGAGT) (Kim et al. 2013). The average length of the PCR products was 279 bp. Each reaction mixture contained 2.5 μL 2x Type-it Multiplex PCR Master Mix (Qiagen), 2 μL RNase-free water, 0.5 μL forward and reverse primers (5 μM), and 0.5 μL DNA. The total volume of each mixture was 6.0 μL . The reaction program had the following thermal profile: 5 min initial denaturation at $95\text{ }^{\circ}\text{C}$ followed by 30 cycles of 30 s denaturation at $95\text{ }^{\circ}\text{C}$, annealing for 90 s at $57\text{ }^{\circ}\text{C}$, and extension for 45 s at $72\text{ }^{\circ}\text{C}$, followed by a final extension at $60\text{ }^{\circ}\text{C}$ for 30 min. PCR products were adjusted essentially in accordance with Ion 16S Metagenomics kit (Thermo Fisher Scientific), however, the number of cycles for amplifying the library was 30 in this study. The amplified DNAs were set to an IonPGM sequencer (Thermo Fisher Scientific) according to Kurokochi et al. (2015).

All sequencing data were trimmed by CLC genomics workbench 8.5 (QIAGEN) (<https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/>) according to the web tool at the analyzing date with the following conditions;

removing short sequences with less than 150 bp, Ambiguous limit = 2, and Quality limit = 0.05. The trimmed sequences were identified by RDP classifier ver 2.11 (database: 16S rRNA training set 16, concordance rate: 97%) (Wang et al. 2007).

2.4 Isolation of *Streptomyces* species from ECM root tips

Matsutake ectomycorrhizal root tips were picked from shiro soil and from the seedlings of *P. densiflora* under a stereomicroscope. The root tips, 3–5 mm length, were rinsed five times with sterile water followed by stirring in a beaker first in 0.05% Tween 20 for 2 min and then in 0.001% Tween 20 for 30 min. Twenty mycorrhizal root tips per shiro sample were collected into a 2 ml tube with Zirconia beads, 2.0 mm (TOMY, Japan). Warm HNC-medium (200 μL) containing yeast extract (60 g), CaCl_2 (0.5 g), SDS (5 g) in 1 L water (Nonomura and Hayakawa 1988) was added. The root tissues were disrupted using a Micro Smash™ (TOMY, MS-100, Japan) with 20 s pulses at 2000 rpm, repeated three times, then incubated at $42\text{ }^{\circ}\text{C}$ for 30 min. The tubes were centrifuged at 8000 rpm for 2 min. The supernatant was looped by a glass loop onto ISP2 agar medium (Shirling and Gottlieb 1966) surface, following a distinctive zig-zag pattern to ensure obtaining a single colony. The cultures were incubated at $28\text{ }^{\circ}\text{C}$ in the dark. Later, a single colony was transferred onto a fresh ISP2 medium in the same conditions.

2.5 DNA identification of the *Streptomyces* species and matsutake mycorrhizal roots of 6-month-old seedlings

The actinobacterial cultures were identified according to their nucleotide sequences. Genomic DNA was extracted from 0.1 g culture tissue according to Lian et al. (Lian et al. 2003). The 16S rRNA gene was amplified with S-C-Act-0235-a-S-20 and S-C-Act-0878-a-A-19 primers (Stach et al. 2003), or 27f and 1492r primers (Lane 1991). PCR with KAPA Taq Extra PCR kit (Kapa Biosystems, Wilmington, MA) had the following thermal profile: initial denaturation for 8 min at $95\text{ }^{\circ}\text{C}$; 35 cycles of denaturation for 1 min at $95\text{ }^{\circ}\text{C}$, annealing for 1 min at $58\text{ }^{\circ}\text{C}$, extension for 1 min at $72\text{ }^{\circ}\text{C}$; and a final extension step of 7 min at $72\text{ }^{\circ}\text{C}$. PCR products were sequenced by a commercial sequencing service (Macrogen Inc.) with the same primers used in the amplification. Sequences were aligned with those available in GenBank using the BLAST algorithm. At least 97% similarity was used as the limit for classifying an operational taxonomic unit (OTU). When the closest sequences similarity was under 97%, the highest BLAST score was chosen and noted accordingly. All sequence data generated in this study were deposited in GenBank (LC420152-LC420156, LC475423), the

actinobacterial sequence data were also confirmed at EzTaxon database (<http://eztaxon-e.ezbiocloud.net/>) (Yoon et al. 2017).

In order to confirm the matsutake mycorrhizas from the mycorrhizal seedlings, DNA was extracted from matsutake mycorrhiza-like root tips with the NucleoSpin® plant II kit (Macherey-Nagel) according to the manufacture's instruction, and amplified with specific primers (TmF, CATTATTATATACTCGGT; TmR, GACGATTA GAAGCCGACCTA) annealed at 52 °C (Kikuchi et al. 2000).

2.6 Fungal material

Two basidiomycetes (BA) strains (*Agaricomycetidae* sp., *Amanita rubescens*) and three ascomycetes (AS) strains (*Phialocephala fortinii*, *Archaeorhizomyces borealis*, *Helotiales* sp.), isolated from the matsutake forest soil in our previous study (Lian et al. 2017) (Table S1), and one strain of *T. matsutake*, originated from Nagano, Japan (Genebank accession No. LV420162), were used in Experiment_1, where the effects of *Streptomyces* broths on the growth of fungal and plant root growth were studied.

Four pathogenic fungal strains (*Heterobasidion annosum*, *H. parvipoyum*, *Phellinus noxius*, *Armillaria ostoyae*) and four ectomycorrhizal (ECM) fungal strains (*Suillus luteus*, *Cenococcum geophilum*, *Pisolithus* sp., *Tricholoma matsutake*) were used for Experiment_2, where the effect of *Streptomyces* broth extracts prepared with different procedures (solvents and cultural media) on fungal growth were studied (Table S1). All fungi were maintained on modified MMN agar medium (Marx 1969) containing 2 g L⁻¹ of glucose at room temperature.

Experiment_1 *Streptomyces* broths – effect on fungal and plant root growth.

The supernatants of the broths, called *Streptomyces* broth, were prepared using three *Streptomyces* spp. (B2, B9 and B14) that were isolated and identified from matsutake mycorrhizal samples (described above). The strains were first subcultured on ISP2 agar medium for two weeks. Ten plugs (7 mm in diameter) were cut from the margin and transferred into sterile 200 ml glass flasks containing 50 ml liquid ISP2 medium. After 2 weeks of stationary incubation in the dark at 28 °C, the culture broth was centrifuged at 8000 rpm for 2 min. The supernatant was collected and used in Experiment_1 as a screening test.

The supernatant was mixed with MMN agar medium (ca. 50 °C) at the final concentration of 2.5% (v/v), poured on Petri-dish, and a cellophane sheet was placed on the surface of agar medium. The plug (ca. diameter 7 mm) of the fungal culture (the six fungal species selected for testing, Table S1) was placed in the center. A Petri-dish with MMN agar medium with sterile ISP2

medium (2.5%) was used as a control. Four replicates were prepared. The colony size of the growing fungus was measured using a digital planimeter (X-plan, Ushikata Mfg, Co. Ltd., Japan). The biomass of the colony was weighed after drying it on cellophane at 60 °C overnight. Fungal biomass was calculated as the final minus the initial mycelium (dry weights) and the empty cellophane sheet.

The supernatants of *Streptomyces* broths described above were used to study the effects of streptomyces on the root elongation of *P. densiflora* seedlings. First, seeds of *P. densiflora* were surface sterilized with 30% H₂O₂ for 5 min and placed on an autoclaved filter paper in a laminar flow hood to dry. Then, the seeds were placed on water agar medium containing 2 g L⁻¹ glucose. The Petri-dishes were kept at room temperature in the dark. After germination, 2-cm length seedlings were picked and soaked in the supernatant for 2 min. Subsequently, the seedlings were moved to modified MMN agar medium in rectangular plates (230 × 82 × 18 mm) (Radia Industry Co. Ltd., Japan) and placed into a phytotron (16 h light: 350–500 μmol m⁻² s⁻¹ of photosynthetically active radiation at 25 °C; 8 h darkness at 23 °C), 20 replicates with each *Streptomyces* spp.. The root elongation was measured after one month of incubation.

Experiment_2 *Streptomyces* broth extracts prepared with different procedures – effect on fungal growth.

The broth extracts, called *Streptomyces* extracts, were prepared using two strains (B2 and FY4). B2 strain was isolated directly from matsutake mycorrhizal root, and FY4 strain was isolated from a 6-month-old seedling of *P. densiflora*. The strains were used in the following antifungal assay where two cultural media (A3M and ISP2) and two organic solvents (butanol (BuOH) and ethyl acetate (EtOAc)) were tested to prepare the *Streptomyces* extracts. The effects of the extracts on the growth of four pathogenic fungi and four ECM fungi were studied in vitro (Table 1).

A3M medium (Onaka et al. 2011) contained 20 g of soluble starch (Wako Chemical, Osaka Japan), 5 g of glucose (Wako Chemical, Osaka, Japan), 20 g of glycerol (Wako Chemical, Osaka, Japan), 15 g of pharmedia (Southern Cotton Oil Co., TX, USA), 3 g of yeast extract, and 10 g of diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan) (adjusted to pH 7.0 with 1 M NaOH) in 1 L of tap water.

First, two *Streptomyces* spp. were separately seed cultured using 100 ml of ISP2 media in Erlenmeyer flask at 30 °C, at 200 rpm for 2 days. Then, 3 ml of the seed cultures were transferred into the flasks containing 100 ml of either A3M or ISP2 medium (cultural medium treatments). Further, the strains were cultured at 30 °C at

Table 1 Relative fungal growth (% of control) (mean, $n = 4$) after 2–4 weeks of incubation for each pathogenic and ectomycorrhizal (ECM) fungal species in the treatments where different solvents (BuOH, EtOAc) and cultural media (A3M, ISP2) were used to prepare *Streptomyces* extracts

Tested fungi	<i>Streptomyces sp.</i> B2				<i>Streptomyces sp.</i> FY4			
	BuOH ^a		EtOAc		BuOH		EtOAc	
	A3M ^b	ISP2	A3M	ISP2	A3M	ISP2	A3M	ISP2
<i>Heterobasidion annosum</i>	-54.2	-65.9	-66.6	-38.2	21.1	-68.8	-20.6	-91.4
<i>H. parviporum</i>	-76.6	-54.2	-72.5	-52.0	-15.3	-46.1	-4.8	-80.4
<i>Phellinus noxius</i>	-46.9	-18.4	-36.4	-11.5	-8.3	-38.1	0.0	-16.0
<i>Armillaria ostoyae</i>	-3.3	-15.5	-24.7	13.7	6.9	-77.4	1.3	-53.2
<i>Suillus luteus</i>	-4.3	-28.2	-22.6	-25.7	-4.0	-27.7	-4.9	-32.2
<i>Pisolithus sp</i>	1.1	-11.8	-7.4	-9.2	-0.7	-27.2	2.3	-8.5
<i>Tricholoma matsutake</i>	-44.6	-18.8	-36.3	-8.4	-4.0	-42.2	-0.7	-31.7
<i>Cenococcum geophilum</i>	-22.2	4.7	-17.5	2.6	-24.3	-30.4	-7.1	6.2
* $P_{factor} < 0.05$	Fungi, Media				Fungi, Media			
$P_{interaction} < 0.05$	Fungi * Solvent, Solvent * Media, Fungi * Solvent * Media				Fungi * Solvent, Fungi * Media, Fungi * Solvent * Media			

Bars with gradient red or blue fillings show the negative or positive effects compared to the control (without *Streptomyces* extract) (Microsoft Office 365)

^a BuOH, butanol solvent; EtOAc, ethyl acetate solvent

^b A3M and ISP2 medium

* P values in GLM, three-way ANOVA for the significant fixed factors and their interactions ($p < 0.05$)

200 rpm for 6 days. After the cultivation, 15 ml of both A3M and ISP2 culture broths were extracted (in 50 ml conical tube) with 15 ml of BuOH or EtOAc by shaking at 200 rpm for 2 h (solvent treatments). After the centrifuging (8000 rpm, 15 min, 4 °C), 12 ml of each supernatant was transferred into a glass vial and dried in vacuo using centrifugal evaporator system (Sakuma). The dried supernatant was dissolved in 1.2 ml of dimethylsulfoxide (DMSO) to obtain $\times 10$ concentrated sample and stored at -20 °C for further use as the *Streptomyces* extract.

The effects of the *Streptomyces* extracts on the growth of pathogenic and ECM fungi were studied as follows. Each of the treatment combinations was conducted as four replicates. First, a sterile filter paper disc (7 mm) was impregnated with 20 μ l of the *Streptomyces* extract in DMSO (only DMSO for the control). The disc and an agar plug (7 mm) from one test fungal culture were then placed equidistantly on a modified MMN agar plate. The growth rates of the fungal species varied, and the colony sizes were therefore measured either after a 4-weeks incubation for *Armillaria ostoyae*, *T. matsutake* and *Cenococcum geophilum* or after a 2-weeks incubation for the other fungi. The fungal colony sizes were measured using the digital planimeter mentioned above. The relative growth of the tested fungi was calculated as the following: relative growth (%) = [(treatment/control) - 1] \times 100%. A negative value indicates an inhibiting effect and a positive value indicates a promoting effect.

2.7 Antioxidant assays: H₂O₂ scavenging, oxygen radical absorbance capacity and ferric reducing antioxidant power

Filtered broths of two strains (B2 and FY4) were prepared to be used in the antioxidant assays. Ten plugs of both B2 and FY4 culture colonies (diameter 7 mm) were cut from 2-week agar cultures and added to 200 ml flasks containing 10 ml ISP2 liquid medium. The flasks were placed to an incubator at 30 °C and rotated at 200 rpm for 10 days to prepare the seed cultures. Then, 0.5 ml of each culture broth was pipetted into 25 ml fresh ISP2 liquid medium in 50 ml tubes and incubated again at 30 °C at 128 rpm for two weeks. After the incubation, the culture broth was mixed and 1 ml of the broth was filtered through a membrane filter (Nylon Membrane, 25 mm, 0.2 μ m), and the filtered samples were used for screening of their antioxidative properties. The antioxidant and radical scavenging assays were modified to 96-microplate format and measured with Varioskan Flash, Thermo Scientific multimode reader with fluorescence or UV/VIS detector. The pure culture medium without the *Streptomyces* broth was used as a control sample. Three seed cultures of each strain were prepared. Subsequently, each seed culture is subdivided into three sub-cultures. The results for each strain was the means of the three subcultures.

The hydrogen peroxide (H₂O₂) scavenging activity was determined using a method modified from Hazra et al. (Hazra et al. 2008) and Jiang et al. (Jiang et al. 1990). An aliquot of 60 μ l of 2 mM H₂O₂ (Merck

KGaA) was added to the reaction mixture with 60 μl of the sample and a mixture (190 μl) containing 2.56 mM ammonium ferrous (II) sulphate (VWR International) in 0.25 mM H_2SO_4 (Merck KGaA) and 27.8 μM xylenol orange disodium salt (Sigma-Aldrich) in 4.4 mM sorbitol (D(-)-sorbitol, AppliChem GmbH). After 30 min of incubation, the absorbance of violet-colored ferric-xylenol orange complexes at 560 nm was measured. The assay measures the ability of the sample to scavenge H_2O_2 and prevent the oxidation of Fe(II) to Fe(III), which is indicated by the formation of ferric-xylenol orange complex. The inhibition of the oxidation is expressed as inhibition % of the reaction and the samples with 100% inhibition activity will remain yellowish. Sodium pyruvate (Sigma-Aldrich) was used as a reference compound.

Oxygen Radical Absorbance Capacity (ORAC) assay measures the oxidative dissociation of fluorescein at the presence of peroxy radicals (R-O-O \cdot), which causes reduction in the fluorescence signal. The antioxidant's protective ability is based on the inhibition of the breakdown of fluorescein caused by the peroxy radicals. The assay was carried out according to Huang et al. (Huang et al. 2002) and Prior et al. (Prior et al. 2003) with microplate fluorescence reader in 96-well format. The reaction mixture contained 25 μl of the sample in 0.075 M phosphate buffer pH 7.5 (Merck), 150 μl of 8.16×10^{-5} mM fluorescein (Sigma Aldrich Chemie GmbH) and 25 μl of 2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH) (Sigma Aldrich Chemie GmbH). Trolox (vitamin E analog) ((\pm)-6-Hydroxy-2,5,7,8,-tetramethylchromane-2-carboxylic acid; Sigma Aldrich Chemie GmbH) was used as the standard compound and the results are expressed as Trolox equivalents ($\mu\text{mol TE/L}$).

Ferric reducing antioxidant power (FRAP) assay measures the ability of an antioxidant to reduce ferric (Fe^{III}) to ferrous (Fe^{II}) ion (Benzie and Strain 1996) in the reaction mixture with the sample (25 μl), 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 μl) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) (20 μl) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 300 mM (80 μl), acetate buffer pH 3.6 in 96-microplate format. The formation of blue colored ferrous-tripyridyltriazine complex in the reaction mixture was measured as absorbance at 593 nm, which indicates the ferric reducing antioxidant power. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a standard compound.

2.8 Statistical analysis

The difference in the bacterial community composition at the phylum level and order level (within Actinobacteria) between shiro soil and non-shiro soil samples was investigated by

a permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance using the function "adonis" in the "vegan" package in RStudio 1.1.383 version (RStudio Team 2016). The results were visualized using Bray-Curtis distance based non-metric multidimensional scaling (NMDS).

The differences among the *Streptomyces* spp. B2, B9 and B14 strains on the growth of basidiomycete and ascomycete fungal groups, as well as on the root elongation of *P. densiflora* were analyzed with one-way ANOVA followed by Tukey's test ($p < 0.05$) or with non-parametric Kruskal-Wallis test ($p < 0.05$).

Three-way ANOVA was used to analyze the effect of the solvent (BuOH and EtOAc), culture medium (A3M and ISP2) and fungal species (four pathogenic, four ECM) on fungal growth. The model included fungal species, solvent, cultural medium and their interactions ($n = 4$). One-way ANOVA was used to find the most negative combination (medium/solvent) for the growth of each fungus separately. The differences in relative growth rates between culture media within the two solvents were examined with Mann-Whitney *U* test for solvents and B2 and FY4 strains separately. The differences in the antioxidant activities among the B2 and FY4 strains and the control were studied using one-way ANOVA followed by Tukey's test ($p < 0.05$). All statistical analyses were performed with SPSS (version 22.0; SPSS Inc., Chicago, Illinois) if not mentioned otherwise.

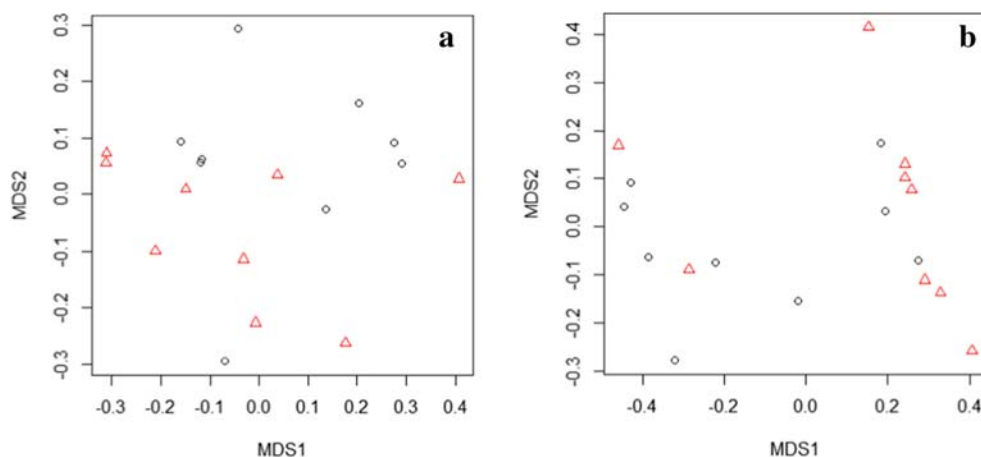
3 Results

3.1 Bacterial community composition in shiro and non-shiro soil and in mycorrhizal root tips

In our study site, the most abundant phylum was Actinobacteria (55%) and the second most abundant phylum was Proteobacteria (34%). However, the difference in bacterial community compositions, either at the phylum or order level (within Actinobacteria) between the two soil types was not significant (Fig. 1a, b), as indicated by PERMANOVA ($p > 0.05$).

Streptomyces, the target genus of this study was detected from six soil samples, including four shiro soils, and two non-shiro soils. However, *Streptomyces* accounted only for 0.12% of the total reads. Six actinobacterial strains were isolated from the matsutake mycorrhizal root tips collected directly from shiro soil. The partial 16S rDNA sequencing data revealed that five out of the seven strains belonged to *Streptomyces* (Table S2). One isolate from a 6-month-old matsutake mycorrhizal seedling growing on shiro soil was closely related to *Kitasatospora* (*Streptomycetaceae*) (Table S2).

Fig. 1 NMDS ordination of bacterial communities at the a) phylum level (stress = 0.06), b) order level within the phylum Actinobacteria (stress = 0.04) in shiro soil (open circles), non-shiro soil (open triangles) samples



3.2 Effect of matsutake-associated *Streptomyces* broth on fungal growth and on *P. densiflora* root elongation

To examine the *Streptomyces* broth's effects on fungal growth, we divided the fungi into ascomycete (AS) and basidiomycete (BA) groups for ANOVA, whereas *T. matsutake* was analyzed alone. The colony size and biomass, indicating fungal growth, did not show consistent differences. B2 strain had a significant inhibiting effect on BA biomass, but not on BS colony size compared to the control (Fig. 2a and b). B2 had a significant

inhibiting effect on AS colony size but not on biomass. B9 and B14 did not have significant effects on AS or BA colony size, but they had significant promoting effects on AS biomass compared to the controls (Fig. 2a and b). *Tricholoma matsutake* colony size was significantly promoted by B9 and B14. However, no significant difference in the respective biomasses were found (Fig. 2c and d). On the other hand, *T. matsutake* was significantly inhibited by B2, as indicated by both colony size and biomass (Fig. 2c and d).

None of the tested *Streptomyces* species had a significant effect on the root elongation of *P. densiflora* seedlings (Fig. 3).

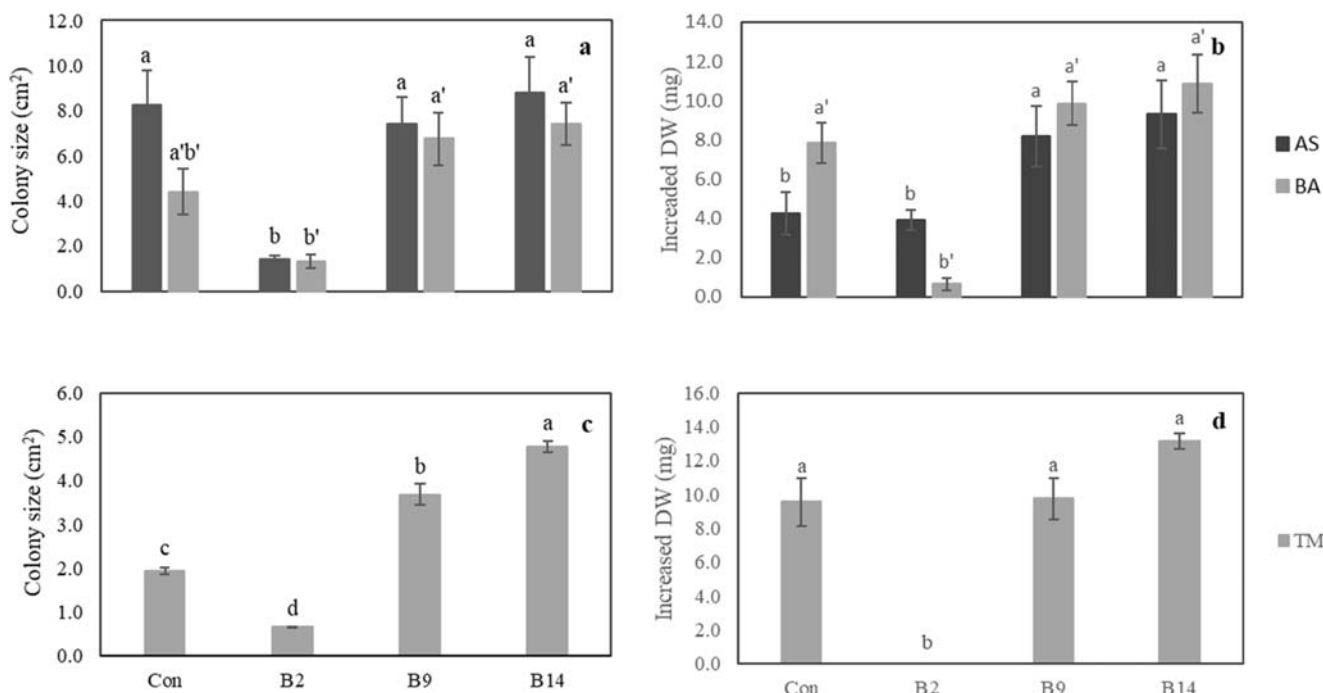
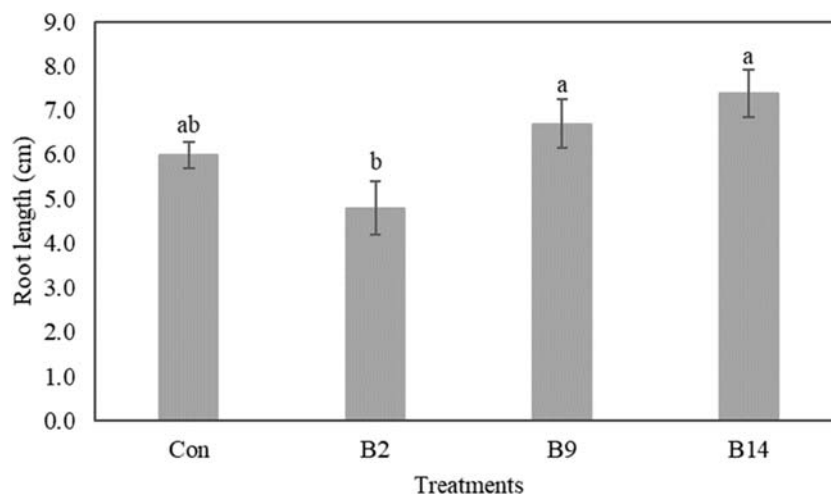


Fig. 2 Fungal growth (colony size (a, c) and increased dry weight (biomass (b, d) after a 4-week incubation on MMN agar media containing no (Con) or different *Streptomyces* spp. (B2, B9, B14) broths. AS, Ascomycota fungi; BA, Basidiomycota fungi, TM, *T. matsutake* fungus.

Error bars refer to SE ($n = 12$, for AS and BA; $n = 4$ for TM). Different letters indicate significant differences between the *Streptomyces* spp. treatments within the same fungi type (Tukey's test, $p < 0.05$)

Fig. 3 Root elongation of *Pinus densiflora* seedlings grown on agar after the treatments with different *Streptomyces* spp. (B2, B9, B14) broths. The cultural medium was used as the control (Con). Error bars refer to SE ($n = 20$). Different letters indicate significant differences between the treatments (Tukey's test, $p < 0.05$)



Based on morphological observations, a slight necrosis on the root tips occurred on those seedlings that were treated with B2 broth.

3.3 Antifungal activities of the *Streptomyces* spp. (B2 and FY4) broth extracts prepared with different procedures (cultural medium, solvent)

Relative fungal growth (pathogenic and ECM) depended on the solvent and cultural medium used to prepare the *Streptomyces* extract. Moreover, the effect of cultural medium varied depending on the *Streptomyces* strains (B2 and FY4). In the case of B2 strain, the extracts from A3M medium showed significantly stronger inhibiting effects on the growth of fungi (all species combined) than the extracts from ISP2 medium, especially with EtOAc solvent (Fig. 4a). For FY4 strain, the result was the opposite (Fig. 4b).

Three-way ANOVA including fungal species, cultural medium and solvent as factors revealed that fungal

species and cultural medium had significant effects, and solvents had non-significant effect on the growth of the tested fungi for both B2 and FY4 strains. Significant interactions between the pairs of factors were also found, indicating that each of the tested fungi reacted differently to different extracts. Among all tested fungi, two strains of *Heterobasidion* sp. were inhibited by both *Streptomyces* (B2, FY4) extracts. The greatest inhibition was observed for FY4 strain that inhibited the growth of *H. annosum* almost totally ($-91.4\% \pm 0.8$; growth compared to control; mean \pm SE) (Table 1). No significant differences were observed for *Suillus luteus*. *Tricholoma matsutake* was mostly inhibited by the extracts from B2 (A3M) and FY4 (ISP2) (Table 1).

3.4 Antioxidant properties

The filtered broths of either of the strains (B2 and FY4) did not show any H_2O_2 -scavenging activity (Fig. 5a).

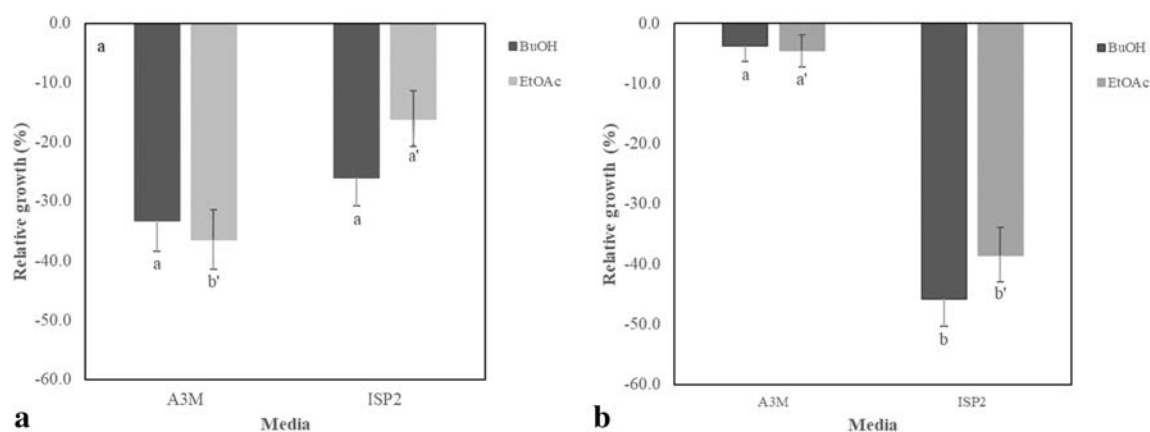


Fig. 4 Relative growth (compared to control) of the fungi (mean of all species) in the treatments where different solvents (BuOH, EtOAc) and cultural media (A3M, ISP2) were used to prepare *Streptomyces* spp. a) B2

and b) FY4 extracts. Different letters indicate significant differences between two media (Mann-Whitney U test, $p < 0.05$). Error bars refer to SE ($n = 32$)

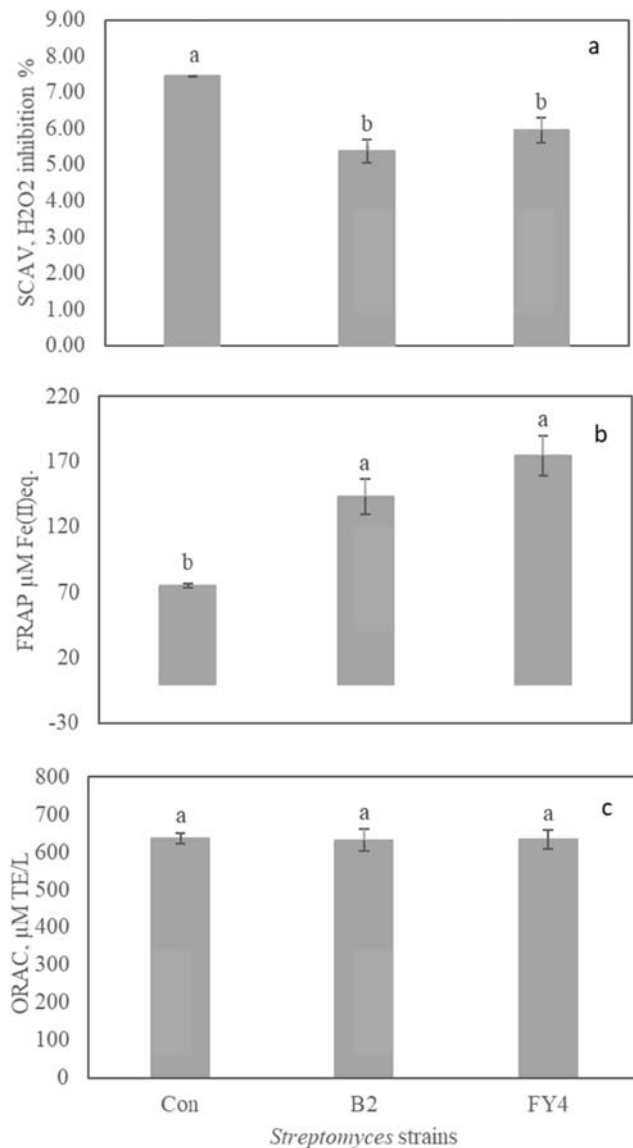


Fig. 5 Antioxidant activity of the filtered cultures of *Streptomyces* spp. B2 and FY4 in the assays of (a) H₂O₂ scavenging, (b) ferric reducing antioxidant power (FRAP), and (c) oxygen radical absorbance capacity (ORAC). The cultural medium was used as the control (Con). Error bars refer to SE (n = 3). Different letters indicate significant differences among B2, FY4 and the control (ANOVA, or Kruskal-Wallis test, $p < 0.05$)

The H₂O₂-scavenging activity was significantly higher in the control without the *Streptomyces* than with them broths. The ferric reducing antioxidant power (FRAP) of B2 (143 μM Fe(II) eq) and FY4 (174 μM Fe(II) eq) were both significantly higher than that of the control (75 μM Fe(II) eq) (Fig. 5b). The oxygen radical absorbance capacity (ORAC) assay showed no difference between B2 and FY4 strains and the control (about 630–640 TE μM/L) (Fig. 5c). The total phenolic concentrations were higher in both broths than in the control, but the difference was not significant (data not shown).

4 Discussion

Bacteria have been considered to represent the third component in mycorrhizal associations. They are loosely or tightly associated with mycorrhizal fungi and most likely, they have functions in mycorrhizal symbiosis. For instance, they seem to promote mycorrhizal formation and root development (Garbaye 1994; Poole et al. 2001; Franco-Correa et al. 2010). Our study focused on understanding if there is any special helper-actinobacteria, particularly *Streptomyces*, associated with the unique ectomycorrhizal fungus *T. matsutake* in nature. We did not find any unique bacterial community in shiro soil (bulk soil) but confirmed the presence of several mycorrhiza-associated *Streptomyces* species. Further, we found that the matsutake mycorrhiza-associated *Streptomyces* actinobacteria had either inhibiting or promoting effects on the in vitro growth of fungi, both root pathogenic and ectomycorrhizal fungi. Moreover, the *Streptomyces* strains showed antioxidant activities. We interpret that *Streptomyces* bacteria contribute to the protection of the shiro area for *T. matsutake* dominance by inhibiting the growth of other fungi. In addition to their functions in shiro soil, *Streptomyces* may enhance plant growth by releasing antioxidants in soil and suppressing root pathogenic fungi. However, the interpretations are not straightforward; our results and previously published results elsewhere are complex and partly contradictory. Therefore, we discuss how we interpreted the results and what we understand of the functional importance of bacteria in matsutake forests.

Streptomyces abundance is well known in the rhizospheres of plants (Barka et al. 2016). To date, *Streptomyces* have been found in association with several ectomycorrhizas (Richter et al. 1989; Schrey et al. 2005) and they, among several other species, have previously been reported to be mycorrhiza helper-bacteria as reviewed by Frey-Klett and colleagues (Frey-Klett et al. 2007). The helper-bacteria have been suggested to facilitate the development of mycorrhizas and fine root formation (Frey-Klett et al. 2005; Frey-Klett et al. 2007; Tarkka et al. 2008) as well as to control plant diseases (reviewed by Schrey and Tarkka 2008). In this study, the *Streptomyces* broth assay (Experiment_1) indicated that functional mycorrhiza-associated *Streptomyces* species exist in matsutake mycorrhiza and that the species have diverse functions. The second assay, the *Streptomyces* extracts assay (Experiment_2) indicated that the case is complex, because the fungal species were inhibited differently depending on the solvent and culture medium combination used to prepare the extract (interaction between species, solvent and cultural medium).

Shiro is one of the most important components of matsutake mushroom formation, and therefore, the microorganisms associated with shiro have been extensively studied. Studies from different continents have suggested that Proteobacteria,

Firmicutes and Actinobacteria are favored in *T. matsutake* shiro soil (Kataoka et al. 2012; Kim et al. 2014; Oh et al. 2016; Vaario et al. 2011). Our results were somewhat different from the previous results, as we did not find any significant differences in Actinobacteria between shiro soil and non-shiro soil. Our results are, however, based on one sampling site. The effect of sites may cause the different characteristics of the bacterial communities in shiro soil from the different regions.

The studies about the functional importance of bacteria originally started from the observations that *T. matsutake* is the dominating mycorrhizal species in matsutake shiro soil (Lian et al. 2006; Vaario et al. 2011). Such a unique ecological feature hints that some ‘guards’ may be existing in matsutake shiro; organisms that protect *T. matsutake* and inhibit other fungal species invading or growing in the shiro. However, some observations suggest the contradictory. To study the role of bacteria in matsutake shiro further, we used a mild disinfection method to isolate the Actinobacteria species directly contacting with matsutake mycorrhiza, either tightly living on mycorrhizal surface or inside the mycorrhiza. Members of the family Streptomycetaceae were isolated from matsutake mycorrhizal root tips, which originated either from shiro soil or from the *P. densiflora* seedlings cultivated in the shiro soil. The strains isolated represent mycorrhiza-associated species. The different mycorrhiza-associated actinobacterial species may have various roles. Our results suggest that some Actinobacteria might live in harmony with matsutake fungus and at the same time, some might suppress surrounding fungal growth including matsutake itself. This phenomenon was studied previously by Oh and his colleagues (Oh and Lim 2018a, 2018b). They showed that most of the bacteria isolated from shiro soil or mycorrhizal root tips suppressed the hyphal growth of *T. matsutake* and thus, the result hinted that bacteria inhabiting shiro soil might not take the major role for conserving or promoting matsutake hyphal growth. Although Oh and Lim (2018b) did not confirm that the roots in question were matsutake mycorrhizal roots, it was found that most bacterial isolates had negative effects on matsutake hyphal growth. In our study, the isolated strains showed positive, neutral or negative effects on matsutake hyphal growth. Oh and Lim (2018b) reported that only a few species promoted matsutake growth, and only under glucose poor conditions (Oh and Lim 2018b). This can be explained by the fact that low carbon nutrition in general decreases the biomass of bacteria, which in turn, may reduce the production of active antifungal compounds produced by bacteria. Reduced bacterial biomass also reduces competition for nutrients, thus, benefiting *T. matsutake*. We observed that matsutake mycorrhiza-associated *Streptomyces* spp. inhibited the growth of several fungal species, either root pathogenic fungi or ECM fungi. This is in agreement with the previous reports of *Streptomyces* that have antifungal activity (Yuan et al. 2012; Seipke et al. 2012). Considering that *Streptomyces* living in

forest soil generally secrete antibiotic or antifungal compounds to compete for resources (e.g., nutrition or space) with other microbes (Boer et al. 2005), we interpret that the organisms having antifungal properties are important for *T. matsutake* and they promote its ability to dominate in shiro.

One fact that has complicated the understanding of the function of mycorrhiza-associated bacteria is the highly variable methods used in previous studies. The media used for the isolation has a great effect on the results (Davis et al. 2005). We used ISP2 medium (Shirling and Gottlieb 1966), which has been commonly used for isolating *Streptomyces* species (Schrey et al. 2012). However, Tryptic soy agar, which is a general medium for the isolation and cultivation of microorganisms, and Reasoner’s 2A agar, which is a medium for slow-growing bacterial species, have been used in previous studies as well (Oh and Lim 2018b). In summarizing the previous studies dealing with microbial communities of *T. matsutake*, we noticed that it was difficult to find any general bacterial species that would regularly inhabit the matsutake rhizosphere and/or hyphosphere within the matsutake shiro. In addition to differences in the methods used, environmental and regional factors, such as soil characteristics (Lindström and Langenheder 2012; Marschner et al. 2001) and climate (Rasche et al. 2011), which are known affect microbial communities, could explain the lack of consistency in the results published. One issue that should be mentioned, is that we did not confirm the *Streptomyces* associating with matsutake mycorrhizas in this study, however, we still have limited information about the composition of the culturable *Streptomyces* on matsutake mycorrhizas because the primers we used in this study were not designed based on the latest sequence data.

It’s worth noting that the extracts of *Streptomyces* strains B2 and FY4 inhibited the growth of root pathogenic fungi, especially *Heterobasidion* species. Both *Heterobasidion* species are the causal agents of the root and butt rot of conifer trees in northern boreal forests in Europe and America. They cause annual economic losses of 800 million euros in Europe alone (Asiegbu et al. 2005; Stenlid and Rayner 1989). Our study shows that certain strains of *Streptomyces* associated with matsutake mycorrhizas might biologically control these root pathogens. To develop practical applications, more studies are needed. For instance, the culturing conditions, such as nutrition, might trigger secondary metabolite production (Miao et al. 2006; Usha et al. 2011).

In addition to antifungal activities, we investigated the antioxidant activities of two *Streptomyces* strains with the idea that antioxidants have two-fold functions. On one hand, many soil microbes enhance plant growth by producing antioxidants. Bacterial inoculation has been shown to increase the antioxidant activity of plants when exposed to drought stress (Kohler et al. 2008). On the other hand, the pathogen infection in plants is controlled by the antioxidant mechanism;

pathogens exploit plant antioxidant mechanisms during an infection. Plants instead use reactive oxygen species as a defense mechanism to block the infection, often by releasing H_2O_2 into the environment as an oxidative burst (Torres et al. 2006). In the current study, the ferric reducing antioxidant power (FRAP) assay indicated the release of compounds with antioxidant activity by both *Streptomyces* strains.

The three antioxidant assays used gave somewhat contradictory results. The FRAP assay indicated positive antioxidant activity. The assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine complex; it is sensitive enough and analytically precise to be used in assessing the antioxidant potential in samples (Benzie and Strain 1996). The other two assays, the oxygen radical absorbance capacity (ORAC) and H_2O_2 scavenging capacity, indicated no antioxidant release. Instead, it seemed that H_2O_2 inhibition was relatively low for the *Streptomyces* strains. The antioxidant potential of microbes usually depends on the growing conditions. In this study, the idea was to study the potential bioactive compounds that had been released into the cultural medium, and therefore, exclude the actinobacterial cells by filtering. This might explain the lack of activities in ORAC and H_2O_2 scavenging assays. In summary, one out of three assays indicated antioxidant activity of the *Streptomyces* strains. The results show that the filtered broths contain compounds that can slightly reduce the oxidative potential or inhibit the destructive action of peroxy radicals.

In conclusion, this study demonstrated that Actinobacteria *Streptomyces* inhabiting matsutake mycorrhizal roots have functional importance. This study increases our understanding of the diverse functions of bacteria associated with matsutake mycorrhiza. Matsutake mycorrhiza-associated *Streptomyces* seem to have several functions in matsutake shiro soil where the bacteria affect fungi, including matsutake itself. Putting the effects together, we conclude that matsutake-associated *Streptomyces* may prevent other fungi from invading and growing in matsutake shiro rather than directly enhance the growth of matsutake. Thus, *Streptomyces* may support the dominance of *T. matsutake* in shiro soil. Moreover, *Streptomyces* seem to have functions with practical relevance. Firstly, they produce antioxidants that theoretically enhance plant growth. Secondly, matsutake-associated *Streptomyces* strongly inhibited the growth of root pathogenic fungi, which gives an idea about possible novel applications for forest management, i.e. the potential of developing a biocontrol agent against fungal pathogens in forests. To explore the value of matsutake mycorrhiza-associated *Streptomyces* is a new opportunity for forests and forestry.

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