Screening and Evaluation of *Streptomyces* Species as a Potential Biocontrol Agent against a Wood Decay Fungus, *Gloeophyllum trabeum*

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### ABSTRACT

Two-hundred and fifty-five strains of actinomycetes isolated from soil samples were screened for their antagonistic activities against four well-known wood decay fungi (WDF), including a brown rot fungus, *Gloeophyllum trabeum* and three white rot fungi *Donkioporia expansa*, *Trametes versicolor*, and *Schizophyllum commune*. A dual culture assay using culture media supplemented with heated or unheated culture filtrates of selected bacterial strains was used for the detection of their antimicrobial activity against four WDF. It was shown that *Streptomyces atratus*, *S. tsukiyonensis*, and *Streptomyces* sp. greatly inhibited the mycelial growth of the WDF tested compared with the control. To evaluate the biocontrol efficacy of *S. atratus*, *S. tsukiyonensis*, and *Streptomyces* sp., wood blocks of *Pinus densiflora* inoculated with three selected *Streptomyces* isolates were tested for weight loss, compression strength (perpendicular or parallel to the grain), bending strength, and chemical component changes.

### 1. Introduction

Increasing efforts have been made to improve disease management for sustainable production systems with a reduced chemical input in agriculture and forestry due to the growing awareness that integrated pest management strategies may provide more environmentally sound and economically feasible alternatives. The uncontrolled use of chemical pesticides has led to side effects, including residual toxicity and environmental pollution [1], demanding immediate replacement of some of the chemical fungicide treatments with biocontrol agents [2].

It is well known that actinomycetes, especially *Streptomyces* species, are saprophytic bacteria that decompose organic matters such as lignocellulose, starch, and chitin in soil [3–5] and are capable of producing a wide range of antibiotics as secondary metabolites [6–8]. Although the role of *Streptomyces* species in mediating soil processes is less studied, the fact that these bacteria stimulate plant growth and protect plant roots against invasion by root pathogenic fungi provides a clear evidence that these are an important part of the rhizosphere microbiome [9,10].

Wood decay fungi (WDF) can be mainly classified into either brown rot or white rot fungi. This is based on the enzymatic capabilities to rapidly depolymerize cellulose through oxidative mechanisms; however, modified lignin remains as a polymeric residue in brown rot infection [11–14]. In contrast, all components of plant cell walls, including cellulose, hemicellulose, and lignin are degraded during white rot infection [15]. Although only a few groups of decay fungi are directly responsible for tree mortality as primary invaders [16], the decay caused by wood rot fungi leads to the structural deterioration of woody tissues and cause significant economic losses [17–19].

A biological control agent should essentially exert sufficient levels of antagonistic activity against a wide variety of pathogens. There are many cases where the goal of sustainable disease control against fungal pathogens is achieved by a wide
range of microorganisms [20]. This includes bacteria belonging to the genus Bacillus [21,22], Xanthomonas, and Serratia [23], as well as fungal species belonging to the genus Trichoderma [24]. Of these, Streptomyces species have received increased attention as biocontrol agents, given their exceptional abilities to produce secondary metabolites such as antibiotics [6–8] and fungal cell wall-degrading enzymes produced, such as cellulases, hemicellulases, chitinases, and glucanases [25,26], as well as their potential to suppress the growth of a wide variety of fungal pathogens [25,27–29]. In this regard, the main aim of this study was to isolate actinomycetes, particularly Streptomyces species, from soil samples and characterize and evaluate the antifungal activities of these isolated species for their application as biocontrol agents against wood rotting fungi.

2. Materials and methods
2.1. Isolation of Streptomyces spp. from soil

Soil samples were obtained from the oak forest in Cheonggye Mountain, Korea, in 2013 to selectively isolate Streptomyces spp. Soil samples collected were placed in a plastic bag and transported to the laboratory for further analyses. A total of 10 g sieved soil sample was placed in a crucible dish and heated in an oven at 45°C for 24 h until dried. The samples were suspended in 100 mL distilled water (DW) and subsequently serially diluted ranging from 10⁻³ up to 10⁻⁷. From each dilution, 0.1 mL suspension was spread evenly onto the surface of humic acid-vitamin (HV) agar media [30] and the plates were incubated at 30°C for 2 weeks. Colonies produced from each serially diluted plate were purified using 2% potato dextrose agar (PDA; Difco, Detroit, MI) and incubated at 30°C for 24 h until dried. The samples were subsequently incubated at 25°C for 2 weeks. Purified isolates presumed to be Streptomyces spp. were selected based on their morphologies [31].

2.2. Isolates of WDF

To test the antifungal activity of Streptomyces spp. against WDF, four WDF reported causing damages to trees, timbers, and wood structures were selected [19]. Of these, Trametes versicolor was isolated from Seokbyung mountain, Gangneung, Korea, and Donkioporia expansa, Gloeophyllum trabeum, and Schizophyllum commune were obtained from Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) (Table 1). All isolates obtained in this study were transferred to 2% PDA and incubated at 25°C for 10 days for further analyses.

| Culture collection No. | Species                          |
|------------------------|---------------------------------|
| TPML 13119 (KACC 46180, CBS 236.91) | Donkioporia expansa            |
| TPML 12104 (KACC 46194, CBS 164.27) | Gloeophyllum trabeum           |
| TPML 13123 (KACC 46294, CBS 103.20) | Schizophyllum commune           |
| TPML 11015             | Trametes versicolor             |

2.3. Screening of Streptomyces spp. with antifungal activity

To evaluate the antifungal activity of 255 Streptomyces spp. isolated from soil against WDF, a dual culture assay was performed as a primary screening method. The fungal inoculum was prepared from the actively growing edge of the plate, excised using a cork borer and transferred onto the center of Petri dishes (90 mm) containing 2% PDA. Four different Streptomyces strains were applied to the edge of Petri dishes in the direction opposite to one another. These were subsequently incubated at 25°C for 10 days and the antagonistic activity of Streptomyces spp. against WDF was inspected.

Based on the result from the primary screening, Streptomyces spp. that exhibited high-antagonistic activity against WDF were selected for the secondary screening of antifungal activity using the dual culture assay (one Streptomyces sp. against one wood decay fungus). The antifungal activity of Streptomyces spp. was scored based on the extent of suppression (inhibition zone) of WDF growth (45–65 mm: +, 25–45 mm: ++, ~25 mm: +++). The fungal inoculum was prepared from the actively growing edge of the plate, excised using a cork borer and transferred onto the center of Petri dishes (90 mm) containing 2% PDA. Four different Streptomyces strains were applied to the edge of Petri dishes in the direction opposite to one another. These were subsequently incubated at 25°C for 10 days and the antagonistic activity of Streptomyces spp. against WDF was inspected.

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2.4. Identification of the selected strains with strong antifungal activity against WDF

To ensure the identity of selected Streptomyces spp. with strong antifungal activity against WDF, these were subjected to DNA sequence comparisons based on the 16S rDNA region using primers, 27 F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1525 R (5′-AAG GAG GTG WTC CAR CC-3′) [32]. Genomic DNAs were extracted using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following manufacturer’s instructions. PCR amplification was performed using the T100™ thermal cycler (Bio-Rad, Hercules, CA). DNA sequence analysis was carried out following the techniques described by Lee et al. [33]. All isolates obtained in this study were deposited at the culture collection of the Tree Pathology and Mycology Laboratory (TPML), Kangwon National University (Table 2).

2.5. Assay using culture filtrates of the selected Streptomyces strains

For preparing culture filtrates for each selected Streptomyces spp., each strain was grown on 2%
potato dextrose broth (PDB; Difco, Detroit, MI) by incubating at 30°C with constant shaking at 140 rpm for 7 days. To decant the supernatant, suspensions of each Streptomyces spp. were centrifuged for 15 min at 9000 rpm. The supernatant was filtered with 0.4 μm nucleopore membranes. The filtered suspension was added to the media. Two types of media, heated and unheated media, were used for the assay to evaluate the antifungal activity against WDF. For the heated media (PHM), filtered suspensions of each strain were directly added to PDA supplemented with 100 mg/L streptomycin sulfate without autoclaving for the unheated media (PUHM).

Agar plugs were excised from the growing edge of WDF isolates grown on PDA at 25°C for 10 days and transferred onto either PHM or PUHM and incubated at 25°C for 7 days to measure the rate of suppression (S) of the fungal growth. Controls were prepared by adding DW to PDA. The rate of suppression was calculated as below:

\[
S (%) = \frac{C - T}{C} \times 100,
\]

where C is the mycelial growth for control (PDA + DW), T is the mycelial growth for treatment (either PHM or PUHM).

### 2.6. Evaluation of Streptomyces spp. as a biocontrol agent using wood blocks

#### 2.6.1. Preparing wood blocks

Sample blocks were prepared from *Pinus densiflora* harvested from the experiment forest in Hongcheon, Korea (belonging to Kangwon National University), as per the Korean Industrial Standards (KS F). Hexahedron wood blocks were prepared as 340 (l) × 20 (w) × 20 (h) mm for the analysis of the bending strength (KS F 2208), 20 (l) × 20 (w) × 30 (h) mm for the analysis of the compression strength (parallel to the grain) (KS F 2206), and 30 (l) × 20 (w) × 20 (h) mm for the analysis of the compression strength (perpendicular to the grain) (KS F 2206). All wood blocks were measured for the weight to evaluate the reduction ratio in the weight owing to WDF-mediated decay before their autoclave sterilization with ethylene oxide at 55°C for 15 h.

#### 2.6.2. Pre-inoculation of Streptomyces spp. on wood blocks

Selected Streptomyces spp. with high-antifungal activity against WDF were transferred to 2% PDB and incubated at 30°C with shaking at 160 rpm for 4 days. To stimulate the colonization of Streptomyces spp. into wood blocks, wood blocks were immersed in the 4-day-old broth cultures of Streptomyces spp. and placed in a stainless-steel container inside plastic bags, which were sealed to prevent contamination. These were incubated at 30°C for 10 days. Controls were prepared using sterile 2% PDB.

#### 2.6.3. Preparation and inoculation of WDF onto Streptomyces-treated wood blocks (STW)

The isolate of WDF that showed maximum growth suppression in the dual culture assay in the presence of selected Streptomyces spp. was subjected to the inoculation test on Streptomyces-treated wood blocks (STW). WDF isolate was grown on 2% PDA at 25°C for 7 days and 7-mm agar disc plugs excised from the growing edge of WDF isolate were inoculated onto STW and incubated in the dark at 25°C for 120 days. All STW inoculated with WDF isolate were evaluated for weight loss, and mechanical and histological changes.

#### 2.6.4. Mechanical test of STW

Changes in the weight of STW caused by WDF were demonstrated in accordance with KS F 2213. To calculate the weight loss, the oven-dry weight of STW (W1) was measured prior to the inoculation. The oven-dry weight of STW inoculated with WDF (W2) that was washed twice with sterilized water was measured 120 days after incubation at 25°C. The weight loss of each wood block was calculated by the equation as follows:

\[
\text{Weight loss} (%) = \frac{W1 - W2}{W1} \times 100.
\]

A universal testing machine (Model: 4482; Instron, Norwood, MA) was used to test changes in mechanical properties of STW in accordance with KS F (compression strength and perpendicular or parallel to the grain: KS F 2206 and bending strength: KS F 2208). Measurements were made at a constant loading speed of 1.5 mm/min and span length of 200 mm. For the bending strength test, the modulus of rupture and modulus of elasticity were determined.
2.6.5. Determination of changes in the chemical components after treatment

To determine the changes in the chemical components of STW treated with WDF isolate, the wood flour (WF) was prepared with a size of 40 mesh from treated STW using a cutter mill (KF-20; KoreaMedi Co. Ltd., Daegu, Korea). The chemical properties were determined following the Soxhlet method (Technical Association of Pulp and Paper Industry, 1994). Four grams of oven-dried WF in a thimble filter was extracted with 210 mL of ethanol/benzene mixture (1:2 v/v) using a Soxhlet extractor fitted with a reflux condenser at 80°C for 6 h. The extracted solution was evaporated under reduced pressure. The extract was subsequently dried at 105°C for 3 h and weighed. The extract-free sample left in the thimble filter was subjected to analyses for lignin and holocellulose contents.

The content of lignin and holocellulose was determined as the delignified residue using sodium chlorate (NaClO₂) [34]. A total of 1.5 g of the extract-free sample in 250 mL flask was treated with 75 mL of DW, 0.6 g of NaClO₂, and 0.09 mL of acetic acid (CH₃COOH) at 70–80°C for 1 h. This procedure was repeated thrice. The solution was filtered using a glass filter (Iwaki Glass Co. Ltd., Tokyo, Japan) and successively washed with 300 mL of cold DW and 30 mL of acetone. The filtrated residue was dried at 105°C for 120 days and weighed. The extract-free sample, and the content of lignin and holocellulose was determined according to the equations below:

\[
L (\%) = \frac{W_2}{W_1} \times 100,
\]

\[
H (\%) = \frac{W_1 - W_2}{W_1} \times 100,
\]

where \( L \) is the content of lignin (%), \( H \) is the content of holocellulose (%), \( W_1 \) is the weight of the extract-free sample, and \( W_2 \) is the weight of the treated sample.

2.6.6. Analysis of histological changes

The treated STWs incubated at 25°C for 120 days were cut on a freezing microtome and subjected to scanning electron microscopy (SEM) analysis to determine histological changes. Wood specimens were mounted on aluminum, sputter-coated with gold–palladium, and observed by a scanning electron microscope (FE-SEM, SUPRA 55VP; Carl Zeiss, Oberkochen, Germany) at 15 kV.

2.7. Statistical analyses and graphs

One-way analysis of variance (ANOVA) and Tukey’s honestly significance difference (Tukey’s HSD) test were used to determine significant differences in the changes in mechanical, chemical, and histological properties of treated STWs based on a p value computed using R v.2.5.1 [http://www.r-project.org/: 35]. All the graphs produced in this study were generated using SigmaPlot v.10.0 (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Isolation of Streptomyces spp. from soil and WDF

In total, 255 Streptomyces spp. were successfully isolated from the soil sample. These were used for screening strains with strong antifungal activities against WDF. In addition to Streptomyces spp. obtained in this study, six WDF isolates were successfully obtained and deposited at the culture collection of TPML, Kangwon National University, Korea (Table 1).

3.2. Screening and identification of Streptomyces spp. with antifungal activity

To screen and identify the strains of Streptomyces spp. with strong antifungal activity against WDF, 255 strains of Streptomyces spp. isolated from the soil were used for the dual culture assay. All Streptomyces spp. with strong antifungal activity against WDF were sequenced and the identities of the strains were confirmed based on the 16S rDNA region. Sequences were subjected to BLASTn analysis against the nucleotide database of NCBI (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). All sequence data produced in this study were deposited at NCBI (MG923820–923824, MG972195) (Table 2). In addition, all strains of Streptomyces spp. obtained in the study were deposited at the culture collection of TPML, Kangwon National University, Korea (Table 2).

On the basis of the primary screening, six strains of Streptomyces spp. were selected for the secondary screening using the dual culture assay. Of the strains used, three strains, including Streptomyces sp. 1 (TPML 13102), Streptomyces sp. 4 (TPML 13106), and Streptomyces sp. 6 (TPML 13094) showing high-antifungal activity against G. trabeum (TPML 12104) were eventually selected. This was followed by testing for their activities against T. versicolor (TPML 11015), D. expansa (TPML 13119), and S. commune (TPML 13123) (Table 3).

3.3. Assay using culture filtrates of the selected strains of Streptomyces spp.

Three strains of Streptomyces spp. (TPML 13102, TPML 13106, and TPML 13094) with strong antifungal activities against four WDF based on the dual
culture assay were used to prepare culture filtrates. Five fungal isolates used as biocontrol agents against WDF \[36,37\] were also used as controls. These included *Trichoderma atroviride* (TPML 13118), *T. viride* (TPML 13117), *T. harzianum* (TPML 13115), *T. virens* (TPML 13116), and *Gliocladium roseum* (TPML 13114).

Of all stains, *Streptomyces* sp. 1 (TPML 13102) showed the highest suppression (\( \% \text{C}_{20} \)) of the growth of WDF. The growth of *G. trabeum* (TPML 12104) was most suppressed in the presence of *Streptomyces* sp. 1 in the culture media, followed by *S. commune* (TPML 13123), *T. versicolor* (TPML 11015), and *D. expansa* (TPML 13119) (Table 4).

Culture filtrate is widely used to determine or screen potential biocontrol agents, especially *Streptomyces* spp., with antagonistic activities against pathogens \[38–41\]. Our results based on the culture filtrate of *Streptomyces* spp. successfully identified the strain of *Streptomyces* sp. 1 that exhibited the potential as a biocontrol agent, given its ability to suppress the mycelial growth of WDF at a higher rate than the controls.

Our results showed that the selected *Streptomyces* sp. 1 strain exhibited higher levels of inhibition against WDF in the culture filtrate assay compared with five fungal isolates used as controls, including *T. atroviride* (TPML 13118), *T. viride* (TPML 13117), *T. harzianum* (TPML 13115), *T. virens* (TPML 13116), and *Gliocladium roseum* (TPML 13114).

3.4. Evaluation of *Streptomyces* spp. as a biocontrol agent using wood blocks

3.4.1. Changes in mechanical properties

The weight loss caused by *G. trabeum* in *P. densiflora* wood blocks treated with the selected *Streptomyces* spp. is presented in Figure 1. The weight loss was least for samples treated with *Streptomyces* sp. 1 (TPML 13102) (\( p = .02 \) at 95% confidence interval) compared with the control (3.5% weight loss).
No significant difference was observed between samples treated with *Streptomyces* sp. 1 (TPML 13102, 2.4% weight loss) and *Streptomyces* sp. 6 (TPML 13094, 2.6% weight loss) ($p = 0.523$ at 95% confidence interval).

Figure 1. Comparison of the weight loss (%) of *Pinus densiflora* wood blocks treated with the selected *Streptomyces* spp. at 120 days after inoculation with *Gloeophyllum trabeum*. Means with the same letter on the bar are not significantly different, as analyzed with Tukey’s honestly significance difference test ($p = 0.017$ at 95% confidence interval).

Figure 2. Comparison of the compression strength (perpendicular or parallel to the grain) of *Pinus densiflora* wood blocks subjected to treatment with selected *Streptomyces* spp. for 120 days after inoculation with *Gloeophyllum trabeum*. Means with the same letter on the bar are not significantly different based on Tukey’s honestly significance difference test ($p = 1.708e^{-16}$ and $2.536e^{-05}$ at 95% confidence interval for compression strength perpendicular and parallel to the grain, respectively).

Figure 3. Comparison of the bending strength of *Pinus densiflora* wood blocks treated with the selected *Streptomyces* spp. for 120 days after inoculation with *Gloeophyllum trabeum*. Means with the same letter on the bar are not significantly different, based on Tukey’s honestly significance difference test ($p = 0.017$ at 95% confidence interval).

Figure 4. Analysis of changes in the chemical components, including lignin and holocellulose, at 120 days after inoculation with *Gloeophyllum trabeum* on the wood block of *Pinus densiflora* treated with the selected *Streptomyces* spp. ($p < 2e^{-16}$ at 95% confidence interval).

No significant difference was observed between samples treated with *Streptomyces* sp. 1 (TPML 13102, 2.4% weight loss) and *Streptomyces* sp. 6 (TPML 13094, 2.6% weight loss) ($p = 0.523$ at 95% confidence interval).
The compression strength (perpendicular or parallel to the grain) and bending strength of *P. densiflora* wood blocks treated with the selected *Streptomyces* spp. are shown in Figures 2 and 3, respectively. In analyses determining the compression strength (perpendicular or parallel to the grain) of *P. densiflora* wood blocks, the smallest decrease was observed for samples treated with *Streptomyces* sp. 1 (TPML 13102; 56.03 Mpa and 2.99 Mpa in compression strength perpendicular to the grain and parallel to the grain, respectively). This tendency was also observed in the analysis of the bending strength of wood blocks, wherein the second lowest decrease was reported for samples treated with *Streptomyces* sp. 1 (TPML 13102; 73.73 Mpa).

### 3.4.2. Changes in chemical properties

Cellulose deconstruction rate was higher in wood blocks treated with the selected *Streptomyces* spp. except for those treated with *Streptomyces* sp. 1 (TPML 13102), as observed in the analyses of changes in chemical properties, including lignin and holocellulose, at 120 days after the inoculation of wood blocks with *G. trabeum* (Figure 4). The average amounts (%) of lignin and holocellulose retained in wood blocks
were 50.1% and 121.2%, respectively, after treatment with Streptomyces sp. 1 (TPML 13102) compared with 30.2% and 116.5% in the control.

3.4.3. Analysis of histological changes

The wood blocks treated with Streptomyces sp. 1 that showed high-antifungal activity against G. trabeum were subjected to histological evaluation using SEM. The cell wall fiber of the wood blocks from the control group became thinner due to fungal attack, indicative of the degradation of the cell wall polymers by the inoculum. In addition, the tracheid collapsed in wood blocks from the control group. However, this tendency was not observed from the wood blocks treated with Streptomyces sp. 1 (TPML 13102) (Figure 5).

G. trabeum is one of the brown rot fungi of ecological importance, as it plays a significantly important role in biomass recycling and soil fertility in forest ecosystems [43,44]. In addition, it is an economic concern, as it causes the most prevalent and destructive type of wood deterioration arising from the rapid structural failure [45]. We observed that the selected strain, Streptomyces sp. 1, successfully protected the wood from decay inflicted by the brown rot fungus, G. trabeum, as observed through mechanical, chemical, and histological properties.

Efforts are underway to explore alternatives for the use of chemical biocides due to increased concern over the environmental side-effects, as well as associated legislative constraints with the use of some chemical treatments. Our results suggest that Streptomyces sp. 1 is a promising biological control agent given the fact that it exhibits strong antagonism toward WDF, especially G. trabeum. Although modes of antagonism of Streptomyces sp. 1 against G. trabeum are remained uncovered, this strain may be a likely source of a large number of bioactive compounds.

Disclosure statement

No potential conflict of interest was reported by the authors.

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