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Biocontrol Potential of Streptomyces Strain FY4 Against Heterobasidion Root Rot Pathogen In Vitro

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Abstract: Root and butt rot, caused by *Heterobasidion* species, poses a significant threat to coniferous forests in the Northern Hemisphere. Innovative and effective strategies are crucial to enhance the control of this disease. This study aimed at identifying a *Streptomyces* strain, FY4, and evaluating its biocontrol potential against *H. annosum* and *H. parviporum*. Strain FY4 was identified as *Streptomyces blastmyceticus* based on morphological, physiological, and biochemical characteristics, supported by a multigene phylogenetic analysis using the 16S rRNA, *atpD*, *rpoB*, and *trpB* genes. In vitro dual-culture experiments showed that *S. blastmyceticus* exhibited antagonistic activity against both *H. annosum* and *H. parviporum*, with an inhibition zone diameter exceeding 15 mm. Moreover, the fermentation broth of *S. blastmyceticus* FY4 displayed significant inhibitory effects on the mycelial growth and spore germination of both *Heterobasidion* species. At a 10% concentration, the fermentation broth inhibited the mycelial growth by over 90% and reduced the spore germination rate by more than 60%. Additionally, the fermentation broth exhibited significant inhibitory effects on the mycelial growth of four common pathogenic fungi—*Phytophthora cinnamomi*, *P. sojae*, *Rhizoctonia solani*, and *Verticillium dahlia*, with an inhibition rate over 50%. These findings suggest that *S. blastmyceticus* FY4 produces antifungal substances capable of effectively suppressing infection of *Heterobasidion* species in conifers. Consequently, strain FY4 holds great promise as a biological control agent for managing root and butt rot caused by these pathogens, as well as potential for controlling other fungal diseases.

Keywords: *Heterobasidion* spp.; antifungal activity; *Streptomyces blastmyceticus*; strain identification

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1. Introduction

Root and butt rot conifers, caused by *Heterobasidion* spp., is one of the most destructive diseases, with a wide global distribution [1,2]. The disease has led to a significant economic loss of more than 800 million euros annually in Europe [3]. Detecting above-ground symptoms is particularly challenging, even during prolonged pathogen infection [4], which complicates disease control efforts and highlights the urgent need for effective prevention strategies.

Biocontrol is recognized as an environmentally friendly and sustainable long-term solution for managing *Heterobasidion* root rot [3,5]. Among biocontrol agents, *Phlebiopsis gigantea* has been widely adopted due to its effectiveness against *Heterobasidion* spp. [6,7]. However, its efficacy decreases for pathogens capable of rapid growth at temperatures above 25 °C [8]. Similarly, Proradix®, a biocontrol agent prepared from *Pseudomonas protegens*, has been proven effective against *Heterobasidion* spp. [9]. Despite these advancements, the search for alternative biocontrol agents remains critical to mitigate the risk of pathogen

resistance and improve control strategies. Varrio et al. [10] have reported that some *Streptomyces* belonging to actinomycetes exhibited certain control efficacy against *Heterobasidion* spp. using a dual-culture testing method.

Recent studies have highlighted the potential of *Streptomyces* species, a genus within the Actinomycetes group, as promising candidates for biological control. *Streptomyces* spp. are Gram-positive, saprophytic bacteria abundant in soil and known for producing diverse secondary metabolites, including cell wall-degrading enzymes that inhibit pathogen growth and reproduction [11]. Notably, *Streptomyces* account for over 60% of known antibiotics and have shown efficacy against various fungal pathogens [12–14]. Actinomycetes interact with mycorrhizal fungi, contributing to a collaborative defense against root-infecting fungal pathogens [15]. For instance, *Streptomyces endophytica* sp. nov., isolated from yam root, displayed strong antifungal activity against *Colletotrichum gloeosporioides*, the causative agent of yam leaf infection [16]. Similarly, *S. kasugaensis* was reported as a control agent against pine rot caused by *Fusarium* and *Armillaria* in Brazil [17]. Additionally, a study involving Norway spruce (*Picea abies*) seedlings co-inoculated with *Streptomyces* and *Heterobasidion* showed that *Streptomyces* not only initially promoted pathogen growth but also triggered host defense mechanisms, enhancing disease resistance [18].

Despite the promising potential of *Streptomyces* spp. in biological control, limited reports have focused on their effectiveness against *Heterobasidion* species. In addition, relying solely on a single biocontrol agent poses a high risk of resistance development in pathogens [19]. In the present study, *Streptomyces* strain FY4, previously isolated from a 6-month-old *P. densiflora* seedlings cultivated in shiro soil [10], was identified as *Streptomyces blastmyceticus* based on morphological, physiological, and biochemical characteristics, as well as molecular identification. We further investigated the inhibitory effects of strain FY4 against two major *Heterobasidion* species, *H. annosum* and *H. parviporum*, which mainly attack Scots pine and Norway spruce, respectively [20]. The aim of this study was to further validate the potential of strain FY4 as a potential biocontrol agent against forest pathogens.

2. Materials and Methods

2.1. Streptomyces Strain FY4 and Fungal Pathogens

The *Streptomyces* strain FY4 was isolated from matsutake mycorrhizal root tips of a 6-month-old *P. densiflora* seedling cultivated in shiro soil [10]. The shiro soil was collected beneath matsutake sporocarps in a forest site recognized for matsutake production, dominated by *P. densiflora*, in Wakayama Prefecture, Japan (33°57′22″ N 135°22′11″ E). The pathogens used in this study included *H. annosum* (strain Ha03007, Ha05045, Ha02034, Ha03010) and *H. parviporum* (strain Hp96026, Hp96017, Hp92150, Hp94174), which were obtained from the Department of Forest Sciences, University of Helsinki (Table S1). Additionally, to validate the broad-spectrum potential of the FY4 strain, we also conducted inhibition tests against four other plant pathogens. Four designated plant pathogens—*Phytophthora cinnamomi* (Accession number: OR074127), *P. sojae* (Accession number: HQ261676), *Rhizoctonia solani* (Accession number: ON138616), and *Verticillium dahliae* (Accession number: MF149108)—obtained from the Forest Pathology Laboratory at Nanjing Forestry University were included in the experiment.

2.2. Cultivation and Characterization of Strain FY4

Strain FY4 was streaked on various media, including Gauze's Medium No. 1, ISP1-ISP5, Glucose aspartate medium, and Czapek's Medium, as described previously [21–23]. The plates were then inverted and incubated at 28 °C for 7 to 14 days. Aerial mycelium coloration, substrate mycelium (underside of the plate), and diffusible pigments were observed microscopically.

The ability of strain FY4 to utilize various carbon sources (fructose, glucose, rhamnose, lactose, galactose, arabinose, sucrose, maltose, mannose) as the only carbon and energy sources was assessed using the method recommended by the International *Streptomyces* Project (ISP) and Bacterial Taxonomy [21,24]. Additionally, the ability of the FY4 to produce

enzymes (gelatinase, starch hydrolase, cellulase, protease, rennet, and hydrogen-sulfide-producing enzyme) was evaluated using the media described by Yokota et al. [25]. Gelatin liquefaction, starch hydrolysis, cellulose degradation, milk proteolysis and coagulation, and H_2S production were assessed accordingly [25].

2.3. Phylogenetic Analyses of Strain FY4

DNA extraction was carried out using a Bacterial DNA Kit (TIANGEN Biotech Cooperation, Beijing, China) following the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop-1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA) and was detected via 1% agarose gel electrophoresis. Four pairs of primers (Table S2) were used to amplify housekeeping genes 16s rRNA, *atpD*, *rpoB*, and *trpB*, as described by Guo et al. [26]. PCR reaction (20 µL) contained of 10 µL 2 × Hieff Robust PCR Master Mix, 1 µL each PCR primer (100 µmol·L^{-1}), 10 ng template DNA, and 8 µL ddH_2O. The PCR reaction conditions comprised an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at the primer-pair-specific temperature for 30 s, and extension at 72 °C for 90 s. A final extension was performed at 72 °C for 10 min. A negative PCR control using sterilized water as template was included to assess potential contamination. The PCR products were quality-checked on a 1% agarose gel electrophoresis, purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), and sequenced by SPRINGEN Biotechnology Cooperation (Nanjing, China).

The sequences were classified through sequence homology searches using the BLAST algorithm and subsequently downloaded. The sequences were aligned using BioEdit version 7.0.9.0. Multiple sequence alignments were performed in PhyloSuite version 1.2.2, using MAFFT, followed by concatenation of the aligned sequences with the Concatenate Sequence function. A phylogenetic tree was constructed using the MrBayes method, and the resulting tree was visualized with FigTree version 1.4.4.

2.4. Inhibitory Effect of Strain FY4 Mycelium on Heterobasidion spp.

To assess the inhibitory effect of strain FY4 on *Heterobasidion*, four strains of *H. annosum* and *H. parviporum* were used, respectively. The pathogens were cultured on potato dextrose agar (PDA) medium and incubated at 25 °C for 7 days. Strain FY4 was cultured on ISP2 medium [21] and incubated at 28 °C for 8 days. In a dual-culture experiment assessing the inhibitory effect of strain FY4 on *Heterobasidion* spp. growth, an 8 mm agar plug of strain FY4 was excised using a sterile punch and placed on one side of the plate surface. An 8 mm diameter plug of *Heterbasidion* spp. was placed on the other side of the plates, 3 cm away from the strain FY4 inoculum. Three replicates were examined for each strain of *Heterobasidion*. In the control group, the strain FY4 inoculum was replaced by an ISP2 sterile plug. Seven days after incubation at 25 °C, the antagonistic activity of strain FY4 against *Heterobasidion* spp. was determined by measuring the distance of the inhibition zone between the FY4 colony and the *Heterobasidion* colony margin.

2.5. Inhibitory Effect of Strain FY4 Fermentation Broth on Fungal Pathogen

2.5.1. Preparation of FY4 Fermentation Broth and LC-MS/MS Analysis

Strain FY4 was grown on ISP2 agar medium at 28 °C for 7 days. Subsequently, ten FY4 disks with a diameter of 8 mm were inserted into 100 mL of ISP2 liquid medium in a conical flask. The flasks were placed on a shaker and cultivated at 200 rpm, 28 °C, for 7 days. The fermentation broth was transferred into 50 mL centrifuge tubes and centrifuged twice at 5000× *g* rpm and 20 °C for 20 min, and the supernatant was filtered through 0.22 µm filter membranes. The resulting fermentation broth was stored at −20 °C. The active substances in the fermentation broth were analyzed using a liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) by Shanghai Applied Protein Technology (Shanghai, China).

2.5.2. Inhibitory Effect of Strain FY4 Fermentation on Mycelial Growth of Fungal Pathogens

The inhibitory effects of FY4 fermentation broth on *H. annosum* and *H. parviporum* (strains Hp96017 and Ha03010) were assessed by mixing the broth with PDA to prepare media containing 10% fermentation broth. PDA medium with ISP2 liquid equivalent to the fermentation broth served as the blank control. Additional tests were conducted on four other pathogenic fungi (*P. cinnamomi*, *P. sojae*, *R. solani*, and *V. dahliae*) using fermentation broth concentrations ranging from 0.625% to 10.00% (0.625%, 1.25%, 2.50%, 5.00%, and 10.00%).

Briefly, an 8 mm mycelia inoculum of the pathogen was placed on the surface of the medium, with the side covering the hyphae facing the medium. Each treatment was replicated three times. All plates were incubated at 25 °C until the mycelium in the control group extended to the edge of the plate. The inhibition rate was calculated after measuring the colony diameter by the crossing method, using the following formula:

$$\text{Inhibition rate of mycelium growth (\%)} = (\text{Colony diameter of control group} - \text{Colony diameter of experiment group})/(\text{Colony diameter of control group} - 8) \times 100\%$$

2.5.3. Inhibition of Strain FY4 on Spore Germination of *Heterobasidion* spp.

The effect of FY4 fermentation broth on spore germination of *Heterobasidion* spp. was determined by the spore germination method [27]. The spores of *Heterobasidion* spp. were gently dislodged from the mycelium with sterile water using a glass coater after 10 days of culture on malt extract agar medium. After filtration, mycelium and impurities were removed, and spore precipitate was obtained using a centrifuge. The concentration of the spore suspension was adjusted with sterile water to contain 30–40 spores in one field of view under an objective 40× light microscope. The FY4 fermentation broth was diluted with sterile water and mixed with spore precipitation of the two *Heterobasidion* species in a 1:1 ratio, with the final concentrations of the FY4 fermentation broth being 50%, 25%, 15%, 10%, 5%, and 1%. In total, 10 µL of the suspension mixture was added dropwise to a concave slide and placed in a glass Petri dish with shallow sterile water. The Petri dish was then sealed and incubated in the dark at 25 °C for 24 h. The control group had a 0% concentration of FY4 fermentation broth. Once the spore germination rate of the control group reached 90% or more, the germination of spores under each treatment was checked (the beginning of germination was considered when the length of the spore germ tube was greater than the short radius of the spore). The total number of spores and the number of germinated spores were observed under a light microscope to calculate the inhibition rate of spore germination. Each treatment was repeated three times, and at least 100 spores were randomly observed in three field of view.

2.6. Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted to calculate the significant difference ($p < 0.05$) in antagonistic ability in mycelium growth and spore germination followed by a Tukey's HSD post hoc test using SPSS version 22.0 (IBM, Armonk, NY, USA). Normal distribution was verified, and data were transformed using natural logarithms where necessary. Data visualization and nonlinear regression fitting model were conducted using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Characterization of Strain FY4

Strain FY4 exhibited distinct characteristics on different media, with varying growth rates and mycelium appearance (Figure 1). Notably, colonies of strain FY4 exhibited rapid expansion on glucose aspartate agar and ISP1-ISP4 medium, but no pigmentation was observed on any medium. FY4 demonstrated the ability to utilize a wide range of carbon sources, including fructose, glucose, lactose, galactose, arabinose, maltose, sucrose,

mannose, and rhamnose (Table S3). Physiological and biochemical measurements showed that FY4 produced gelatinase and starch hydrolase but did not produce rennet, cellulase, melanin, protease, or hydrogen-sulfide-producing enzyme (Table S3).

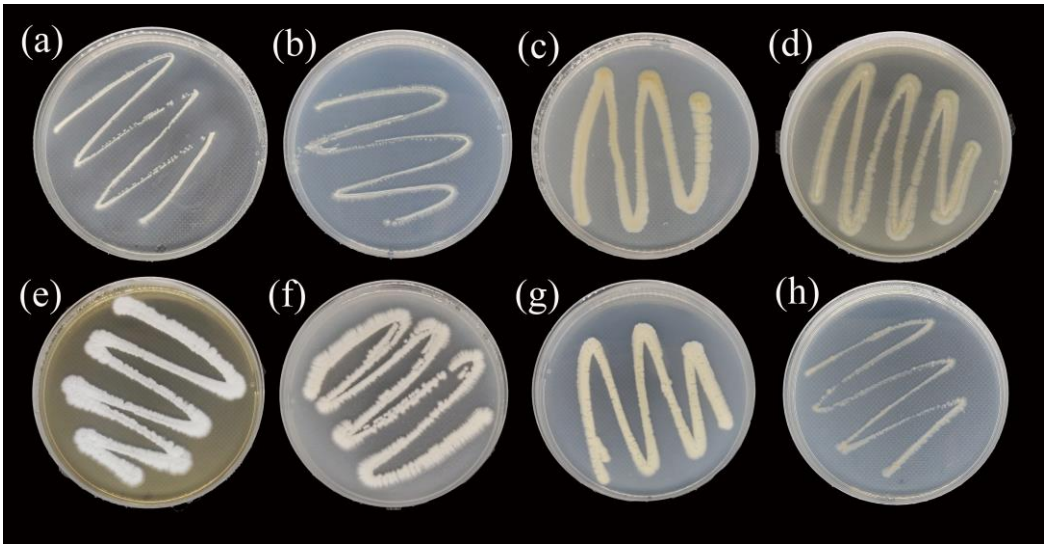

Figure 1. Colony morphological characteristics of strain FY4 on different media. (**a**) Gauze's Medium No. 1; (**b**) Czapek's Medium; (**c**) glucose aspartate agar medium; (**d**) ISP1 medium; (**e**) ISP2 medium; (**f**) ISP3 medium; (**g**) ISP4 medium; (**h**) ISP5 medium.

3.2. Molecular Identification of Strain FY4

Molecular identification of strain FY4 was conducted through PCR amplification of the 16S rRNA, *atpD*, *rpoB*, and *trpB* genes, resulting in fragments of 1420 bp, 706 bp, 735 bp, 726 bp, respectively. These sequences were deposited in GenBank under the accession numbers PP434658, PP230168, PP230166, and PP230167. Comparative sequence analysis in the NCBI database revealed a 99.9% similarity between FY4 and *S. blastmyceticus* NBRC 12747 (NR_112576.1). The phylogenetic analysis using the MrBayes method demonstrated that FY4 and *S. blastmyceticus* NRRL B-5480 clustered together on the same branch with high confidence (99.8%) (Figure 2).

3.3. Inhibitory Effect of S. blastmyceticus FY4 Mycelium on Heterobasidion spp. Mycelial Growth

The inhibitory effect of strain FY4 against *Heterobasidion* spp. was evaluated on a PDA medium in Petri dishes after 7 days. FY4 showed strong inhibition on the growth of *Heterobasidion* spp., resulting in an inhibition zone exceeding 15.00 mm in diameter (Figure 3a). The largest inhibition zone (19.50 ± 1.32 mm) was observed for strain Ha05045, while the smallest inhibition zone (15.00 mm) was observed for strain Hp94174. Moreover, there was no significant difference ($p > 0.05$) in the inhibition zone diameter between *H. parviporum* and *H. annosum* caused by FY4 strain (Figure 3b).

3.4. Inhibition of Strain FY4 Fermentation Broth on Mycelial Growth and Spore Germination of Heterobasidion spp.

The fermentation broth of FY4 significantly inhibited the mycelial growth of *Heterobasidion* spp. At a 10% broth concentration, the inhibition rates reached 92.54% ± 0.93% and 91.01% ± 2.32% for strains Hp96017 and Ha03010, respectively, compared to the control group (Figure 3c,d).

Similarly, the inhibition rate of spore germination increased with increased fermentation broth concentration (Figure 4a). At a 50% concentration, the spore germination inhibition rates reached 87.09% ± 1.72% and 89.84% ± 3.40% for *Heterobasidion* strains Ha03010 and Hp96017, respectively.

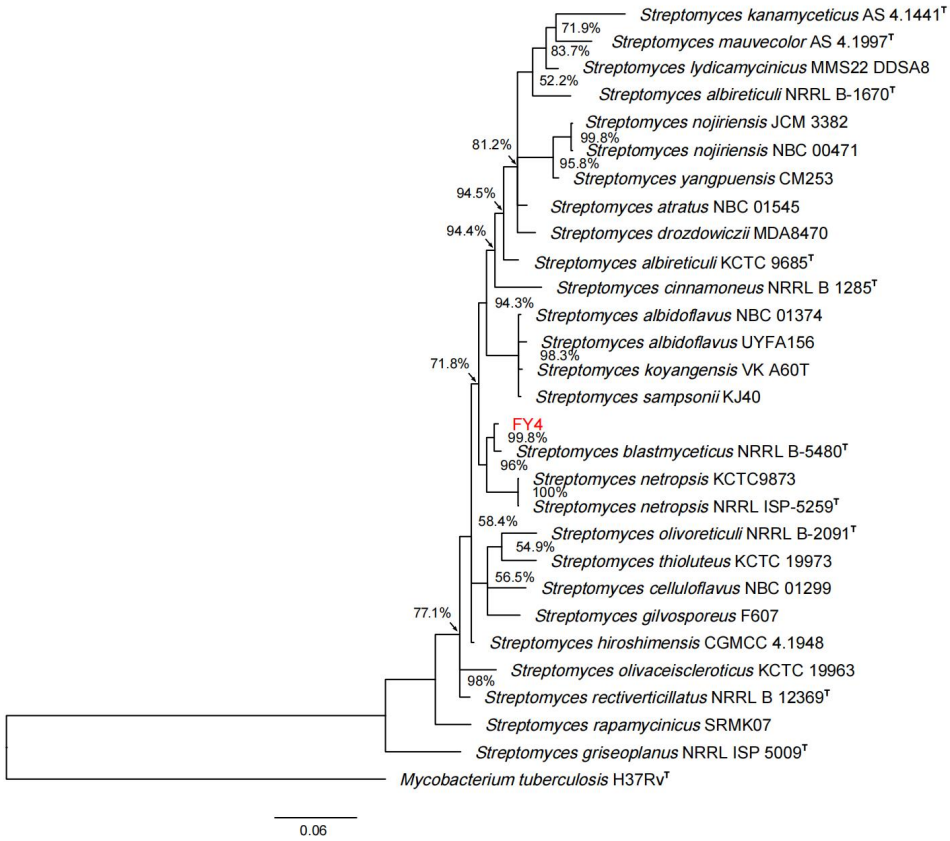

Figure 2. Maximum-likelihood tree obtained from the combined 16s rRNA, *atpD*, *rpoB*, and *trpB* genes of strain FY4. *Mycobacterium tuberculosis H37Rv* was used as the outgroup. Numbers at the branches indicate the percentage of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates).

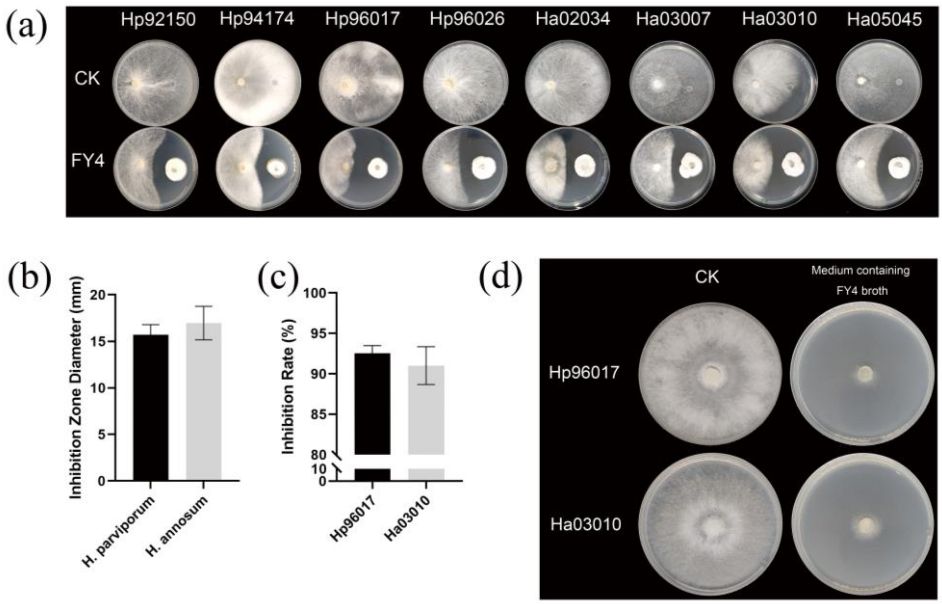

Figure 3. Inhibitory effect of strain FY4 mycelium and fermentation broth against *Heterobasidion* spp. (**a**) Strain FY4 mycelium against *Heterobasidion* spp. in Petri dishes after 7 days (n = 3). (**b**) Inhibition zone diameter of strain FY4 mycelium on mycelium growth of *H. annosum* and *H. parviporum* (n = 3). (**c**) Inhibition rate of strain FY4 fermentation broth on mycelial growth of *Heterobasidion* spp. over 10 days (n = 3). (**d**) Growth of *Heterobasidion* spp. on medium containing strain FY4 broth. CK: Control.

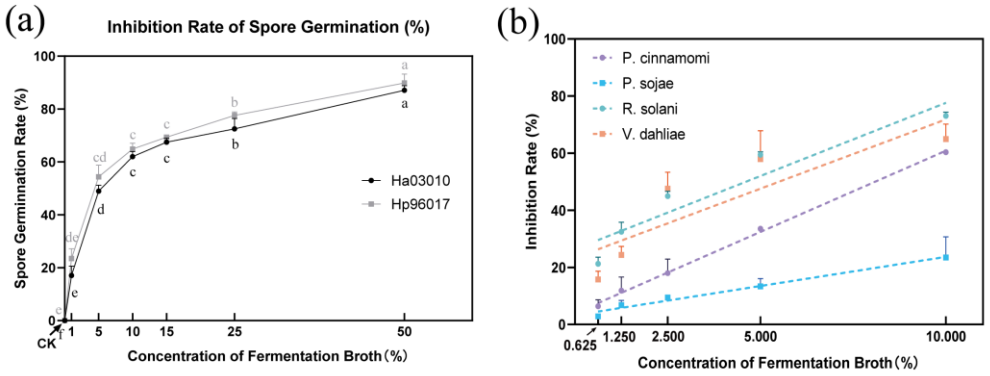

Figure 4. Inhibition rate of strain FY4 fermentation broth on *Heterobasidion* spore germination and on mycelial growth of *Phytophthora cinnamomi*, *P. sojae*, *Rhizoctonia solani*, and *Verticillium dahlia*. (**a**) Inhibition rate of strain FY4 fermentation broth on *Heterobasidion* spore germination (n = 3). The lowercase letters of the same color indicate significant differences ($p < 0.\ 05$) between different treatments. (**b**) Linear regression fitting of inhibition rate of strain FY4 fermentation broth on the mycelial growth of *P. cinnamomi* ($R^2 = 0.98$), *P. sojae* ($R^2 = 0.844$), *R. solani* ($R^2 = 0.89$), and *V. dahliae* ($R^2 = 0.71$) (n = 3).

The fermentation broth also demonstrated inhibitory effects on four additionally tested plant pathogens. The inhibitory rates increased with the higher fermentation broth concentrations (Figure 4b). The fermentation broth exhibited the highest and lowest inhibition rate on the pathogenic fungi at a 10% concentration and 0.625% concentration. At the 10% concentration, the mycelial growth inhibition rates of *P. cinnamomi*, *R solani*, and *V. dahliae* were all greater than 50%. Additionally, the inhibition on *R. solani* and *V. dahliae* surpassed that of *P. cinnamomi* and *P. sojae* at various concentrations.

LC-MS/MS analysis of FY4 fermentation broth detected a total of 41,460 peaks. Database matching identified 915 compounds classified into 13 superclasses, with the most abundant being organic acids and derivatives (23.5%), lipids and lipid-like molecules (21.7%), and benzenoids (10.4%) (Table S4).

4. Discussion

This study elucidates the current challenges in managing *Heterobasidion* root rot, emphasizing the limitations of existing control methods and the need for alternative approaches [28,29]. It specifically aimed to evaluate the biocontrol potential of *S. blastmyceticus* strain FY4 against these pathogens.

Biocontrol strategies offer promising prospects for sustainable disease management [3,5,6]. Members of *Streptomyces* genus, known for their antibiotic production [30], are particularly attractive candidates. They have the ability to produce plant protection substances, including enzymes, secondary metabolites, and volatile organic compounds, while also stimulating plant immunity and responding rapidly to pathogens [12,17,20]. Furthermore, actinomycetes are advantageous as biological control agents due to their rapid reproduction, secretion of metabolites, and short response times [31].

In this study, we successfully identified *Streptomyces* FY4 as *S. blastmyceticus* using morphological, physiological, biochemical, and molecular techniques. Both phenotypic and phylogenetic characterization validated its taxonomy. These findings provide essential information on its potential as a biocontrol agent and lay the foundation for assessing its efficacy against target pathogens.

Our study demonstrated the effective inhibition of FY4 against *H. annosum* and *H. parviporum* in dual-culture experiments, in accordance with previous research on the antifungal properties of various *Streptomyces* species [10,13,14,16]. The consistent inhibition zones underscore FY4's potential for managing root and butt rot disease. Previous research has shown that *Streptomyces* fermentation broth contains secondary metabolites with antifungal properties, such as cycloheximide and naphthoquinone antibiotics, which effectively

inhibit fungal growth [32–35]. The inhibition of FY4 extends beyond direct antagonism, as evidenced by the suppression of *Heterobasidion* spp. mycelial growth and spore germination by its fermentation broth. These outcomes emphasize FY4's capability to limit the reproductive capacity of these pathogens, potentially mitigating their proliferation. Antibiotic action through the production of antifungal compounds may be the main mechanism by which FY4 inhibits *Heterobasidion* spp. In addition, the ability of *S. blastmyceticus* FY4 to produce gelatinolytic compounds enhances its antifungal arsenal. These compounds, which are known to inhibit spore attachment of *Magnaporthe oryzae* [29], further contribute to its efficacy. This dual capacity—antagonistic action and enzymatic suppression of pathogen reproduction—underscores FY4's robust antifungal potential.

The chosen pathogens in this study are significant soil-borne pathogens threatening economically important crops and forest tree species. FY4 exhibited inhibition effects against all tested pathogens. The broad-spectrum antifungal activity of FY4, including its ability to suppress mycelial growth in these pathogens, suggests its utility as a versatile biocontrol agent. This versatility opens avenues for novel strategies to manage critical fungal diseases.

The biocontrol potential of *Streptomyces* spp. has gained increasing recognition. Studies have demonstrated that *S. blastmyceticus* produces a wide range of metabolites, including the hexose-containing peptidyl-nucleoside antibiotic blastmycin and the glycolipid-like compound teleocidin [36–38]. Blastmycin exhibits antifungal activity against diverse fungi, such as *Candida albicans* and *Monilinia fructicola*, while teleocidin possesses nematicidal activity against pine wood nematodes [38]. This study contributes valuable new evidence to the growing body of research on the efficacy of *S. blastmyceticus* against *Heterobasidion* species.

5. Conclusions

This study addresses challenges of managing *Heterobasidion* root and butt rot, highlighting the limitations of current control methods and the need for alternative solutions. Strain FY4 was identified as *S. blastmyceticus* and evaluated as a biocontrol agent against these pathogens. The results demonstrated that FY4 effectively inhibits *Heterobasidion* spp. in vitro, highlighting its potential for managing root and butt rot diseases and contributing to improved forest health and sustainability. In conclusion, the findings support FY4 as a promising biocontrol agent with strong antifungal capabilities. Further research should focus on field trials to assess the practical application of FY4 in forest management. Additionally, the identification and characterization of specific antifungal compounds produced by FY4 could elucidate its mechanisms of action, paving the way for more targeted biocontrol strategies.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f15122124/s1, Table S1: Information of *Heterobasidion* strains used in the study; Table S2: Primers used for PCR amplification and DNA sequences; Table S3: Carbon compound utilization and enzyme producing test of strain FY4; Table S4: Classification of compounds at superclass-level in FY4 fermentation broth.

Author Contributions: H.S. conceptualized and received funding. H.S., Y.L., X.L., S.L., L.G., Z.G., L.-M.V. and L.H. involved in methodology, data curation, and writing—original draft preparation. H.S., Y.L., X.L., Z.G. and L.H. involved in reviewing and editing the English. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw sequences were submitted to National Center for Biotechnology Information (NCBI). The accession numbers have already been listed in the results.

Conflicts of Interest: The authors declare no conflicts of interest related to this work.

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