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RESEARCH ARTICLE

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Promotion of *Tricholoma matsutake* mycelium growth by *Penicillium citreonigrum*

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ABSTRACT

Tricholoma matsutake has been the most valuable ectomycorrhizal fungi in Asia because of its unique flavor and taste. However, due to the difficulty of artificial cultivation, the cultivation of *T. matsutake* has relied on natural growth in forests. To cultivate the *T. matsutake* artificially, microorganisms in fairy rings were introduced. In this study, we isolated 30 fungal species of microfungi from the soil of fairy rings. Among them, one single fungal strain showed a promoting effect on the growth of *T. matsutake*. The growth effect was confirmed by measuring the growth area of *T. matsutake* and enzyme activities including α-amylase, cellulase, and β-glucosidase. In comparison with control, microfungal metabolite increased the growth area of *T. matsutake* by 213% and the enzyme activity of *T. matsutake* by 110–200%. The isolated fungal strain was identified as *Penicillium citreonigrum* by BLAST on the NCBI database. The Discovery of this microfungal strain is expected to contribute to artificial cultivation of *T. matsutake*.

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Tricholoma matsutake growth; promoting growth; microfungal metabolite; *Penicillium*

1. Introduction

Ectomycorrhizal fungi (EMF) are primarily specialized in mutualistic root symbiosis with host plants [1]. By connecting their own hyphal systems with the host root systems, EMF interchanges the nutrients that they necessary [2]. In the connection, EMF cooperates with microorganisms like bacteria to facilitate mycorrhizal formation [3]. Because of this cooperation that promotes the mycelial growth of EMF, microorganisms are attractive symbiotic partners to EMF [4]. Not only bacteria, but microfungi were also discovered in various structures of EMF including ectomycorrhizae [5], hyphae [6], and fruiting bodies [7]. Despite EMF and microfungi living together in the rhizosphere, EMF has been competing with microfungi for nutrients or space for survival [8]. However, a symbiotic relationship between EMF and microfungi was also reported in a previous study [9].

Tricholoma matsutake, known as pine mushroom, is a member of EMF and has been regarded as a highly prized food for a long time in Asia because of its unique flavor and taste [10]. Like other EMF, *T. matsutake* has a symbiotic relationship with its hosts named *Pinus densiflora*, *Quercus serrata* and *Q. phillyraeoides* [11], by forming a fairy ring named Shiros around the host tree [12]. Since the occurrence of *T. matsutake* fruiting body has been steadily decreased, artificial cultivation of *T. matsutake* has been processed [13]. In recent studies, two cultivations, named field cultivation and bed cultivation, were introduced as artificial cultivation of *T. matsutake*. In field cultivation, pine sapling infected with *T. matsutake* was planted and cultured over 6 years to grow *T. matsutake* without a shiros [14–15]. In bed cultivation, Dr Murata's team reported a stable mutant of *T. matsutake* that degraded the amylose and cellulose, recently [16–17]. In these two studies, however, there is no symbiotic relationship between the *T. matsutake* and microorganisms which inhabited shiros.

In previous studies, promoting effects on *T. matsutake* growth by microorganisms have been reported [18–19]. In addition, metabolites of *Mortierella* species and other species promoted the *T. matsutake* growth about twofold [20]. In this study, *T. matsutake* fruiting bodies and microfungi were collected from shiros in Yangyang. Then, metabolites were obtained from the microfungi and analyzed the growth effect on *T. matsutake* growth. The growth effect was monitored by the growth of colony size and enzyme activity. Based on the growth results, the name of the microfungal strain which promotes the *T. matsutake* growth was identified using molecular markers [21].

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2. Materials and methods

2.1. Preparation of sample and microfungi

Tricholoma matsutake fruiting bodies and soil samples in fairy rings (Shiros) were collected in Yangyang, Gangwon province, Korea in July 2022. From shiros, about 20 g of soil blocks at a depth of 10–20 cm were collected using a sterilized spatula. 5 g of soil blocks were serially diluted and cultured on potato dextrose agar (PDA; Difco, Detroit, MI, USA) with 300 ppm of streptomycin to isolate microfungi. After cultivation at 25 °C for 3–5 days, single colonies of microfungi were sub-cultured on PDA at 25 °C for 5–7 days to obtain pure culture. The colonies were grouped based on the morphologic features such as color of mycelia and the growth size of the colony.

2.2. Isolation of fungal metabolites and growth effect on T. matsutake

15 pieces of microfungi were cultured in Czapek broth medium (Sigma-Aldrich Co., St. Louis, MO, USA) at 25 °C for 15 days. The cultured solutions were filtered using Acrodisc syringe filter with 0.45 µm pore size (Pall Corporation, Ann Arbor, MI, USA) to obtain the metabolite without fungal spores. The metabolites were concentrated 50 times each using a freeze dryer (Ilshin Biobase Co., Ltd, Korea). The 10 µL of concentrated metabolite was injected on the *T. matsutake* mycelium and the mycelium was cultured on *Tricholoma matsutake* media (TMM) (glucose 20 g/L, yeast extract 1.5 g/L, soytone 1.5 g/L, and agar 20 g/L) at 25 °C for a month [22]. After the cultivation, metabolite metabolite-treated *T. matsutake* growth area was compared with that of the control, which was treated with distilled water. In comparison, the growth area of *T. matsutake* was +/measured three times using ImageJ [23].

2.3. Enzyme activity assays

T. matsutake treated by microfungal metabolite was cultured in PDB broth at 25 °C for 4 weeks. Following the filtering of the culture through 110 mm Whatman filter paper (Advantec, Tokyo, Japan), activities of three different enzymes associated with fungal cell wall components (α-amylase, cellulase and β-glucosidase) were measured from the filtered *T. matsutake* solution. To identify the *T. matsutake* growth, each enzyme activity was in comparison with that of the control which was treated with distilled water. Details of the enzyme activity assays are listed below, respectively.

2.3.1. Assay of α-amylase activity

α-amylase activity was assayed based on the study of Bhanja's team [24]. Briefly, 20 mM phosphate buffer with pH 7.0 and soluble starch were mixed with 1.0 mL of the filtered *T. matsutake* solution. Following maintaining the mixture at 37 °C for 5 min, 2.0 mL of 3,5-dinitrosalicylic acid (DNS) was added to identify the release of maltose from the starch. The mixture was boiled for 5 min and cooled down in ice for 10 min. The absorbance of the filtered solution was measured at 540 nm. A standard curve was plotted using maltose. A unit of α-amylase activity was described as the released maltose from hydrolyzed starch. α-amylase activity was recorded as units/L of filtered *T. matsutake* solution.

2.3.2. Assay of cellulase activity

Cellulase activity was assayed based on the study of Ang's team [25]. Briefly, 0.5 mL of 1.0% (w/v) carboxymethyl-cellulose (CMC) in 0.2 M sodium acetate buffer with pH 5.0 was mixed with 1.0 mL of the filtered *T. matsutake* solution. The mixture was kept at 37 °C for 20 min, and 2.0 mL of DNS was added to identify the release of glucose. Then, the mixture was boiled for 5 min and cooled down in ice for 10 min. The absorbance of the filtered solution was measured at 540 nm. A standard curve was plotted using glucose. A unit of cellulase activity was described as the released glucose from hydrolyzed CMC. Cellulase activity was recorded as units/L of filtered *T. matsutake* solution.

2.3.3. Assay of β-glucosidase activity

Based on the reports by Salar's team [26], β-glucosidase was assayed using an assay Kit (Sigma-Aldrich Co., St. Louis, MO, USA) in this study. Briefly, 0.02 mL of the filtered *T. matsutake* solution was mixed with p-nitrophenyl-b-D-glucoside (pNPbG) and assay buffer. With a calibrator and distilled water as a positive and negative control, the initial absorbance of the mixture was measured at 405 nm. After incubation at 37 °C for 20 min, the absorbance of the mixture was measured at 405 nm, again. A unit of β-glucosidase activity was described as the released p-nitrophenol from pNPbG. β-glucosidase activity was recorded as units/L of filtered *T. matsutake* solution.

2.4. Identification of fungal strain

A microfungal strain that promoted the *T. matsutake* growth was cultured on PDA at 25 °C for 3–5 days. From the cultured strain, DNA was extracted using the DNA Extraction Kit (Qiagen, Hilden, Germany). Four DNA regions, including Internal transcribed spacer (ITS), *β*-tubulin (*BenA*), calmodulin (*CaM*),

and RNA polymerase II second largest subunit (*RPB2*), were amplified with the primer pairs ITS4-ITS5, Bt2a-Bt2b, CMD5-CMD6, and 5Feur-7CReur, respectively [27]. The amplified sequence region was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and analyzed using ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) and BigDye® Terminatorv3.1 cycle sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). The analyzed sequence identified its name through the National Center for Biotechnology Information (NCBI) BLASTn search [27].

To prove the result of sequencing in NCBI, a phylogenetic tree was constructed. The obtained strain sequences were aligned using BioEdit v7.2.5. (Clustal W) [28]. In Molecular Evolutionary Genetics Analysis (MEGA) 7 software, a phylogenetic tree was constructed with a maximum likelihood (ML)algorithm using a combination of ITS, *BenA*, *CaM*, and *RPB2* [29]. In addition, an ML tree was drawn based on the previous *Penicillium* identification research [30]. To evaluate the reliability of the phylogenetic tree, number of bootstrap replicates was considered as 1000. The strain sequence was compared with other type strains of *Penicillium* species and the two *Aspergillus* strains, *Asp. fumigatus* and *Asp. clavatus*, were set as the outgroup.

2.5. Statistical analysis

All experiments in this study were conducted three times. The data were shown as the mean ± standard deviation. The data was analyzed using GraphPad Prism and InStat V.3 (GraphPad, San Diego, CA, USA). The means were compared using the student's t-test for two-group comparisons and one-way analysis of variance (ANOVA) for multi-group comparisons.

3. Results

3.1. Growth promotion on T. matsutake by fungal metabolite

From the pure culture of microfungal single colonies, 30 separate microfungal strains were isolated. The spores of microfungi were filtered out to prevent the presence of fungal mycelium in the cultivation of *T. matsutake*. In cultivation, however, *T. matsutake* mycelium treated by metabolites didn't grow except for mycelium treated by metabolites from 5 strains: Y22_F16, F18, F20, F21, and F22. Among the 5 strains, only Y22_F22 significantly promoted the growth effect on *T. matsutake* mycelium in comparison with that of the control ($p < 0.05$) (Figure 1). Compared with the control, Y22_F22 increased *T. matsutake* mycelium growth area by 213%.

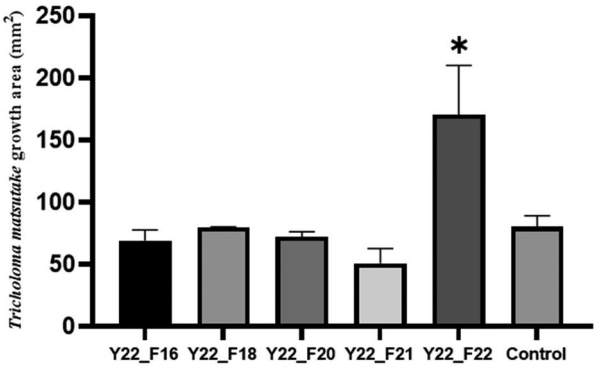

Figure 1. Growth effect on *Tricholoma matsutake* by microfungal metabolite. The mean radial growth area (mm^2) of *T. matsutake* mycelium treated with microfungal metabolites was compared with that of the control treated with nothing. Marker (*) indicates that the mean values differ significantly by the one-way ANOVA test ($p < 0.05$).

3.2. Enzyme activities in fungal metabolite-treated T. matsutake

From *T. matsutake*, the activity of α-amylase, cellulase, and β-glucosidase showed degradation of polysaccharide, associated with fungal cell wall components [31]. As only Y22_F22 metabolite promoted the *T. matsutake* growth, Y22_F22 metabolite-treated *T. matsutake* was assayed for its enzyme activity (Figure 2). In enzyme activity assay, *T. matsutake* treated Y22_F22 metabolite exhibited a significantly higher degree of α-amylase, cellulase, and β-glucosidase activity than that of control ($p < 0.05$). In detail, the activity of α-amylase, cellulase, and β-glucosidase in *T. matsutake*-treated Y22_F22 metabolite was increased by 117%, 110%, and 200%, respectively in comparison with the control. In conclusion, *P. citreonigrum* from the fairy ring in Yangyang has a primarily promoting effect on β-glucosidase activity of *T. matsutake*.

3.3. Identification of fungal species

Using four primers, Y22_F22 strain which promoted the growth area of *T. matsutake* was analyzed and registered in the GenBank database of the NCBI (OQ719837 for ITS, OR412752 for *BenA*, OR412753 for *CaM*, and OR412754 for *RPB2*) (Table 1). As a result of BLAST, ITS region sequence of Y22_F22 strain matched the sequence of *P. citreonigrum* with 100% similarity and other region sequences (*BenA*, *CaM* and *RPB2*) of Y22_F22 strain also showed high similarity (100%, 99%, and 99%, respectively) with a sequence of *P. citreonigrum*. To prove the sequence similarity result of NCBI blast, the relationship between Y22_F22 strain and other strains in *Penicillium* species was identified by the phylogenetic tree with ML (Figure 3). And in the result of phylogenetic tree construction, Y22_F22 strain showed the most closed relationship with *P. citreonigrum*, again.

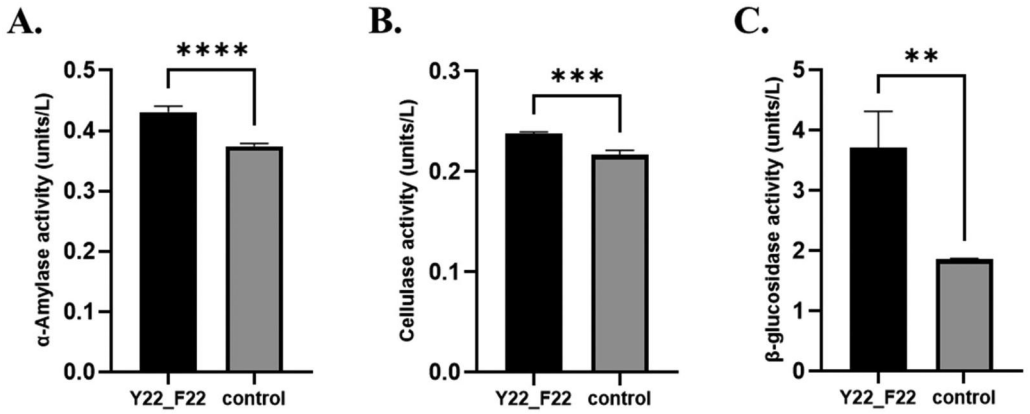

Figure 2. (A) α-amylase activity, (B) cellulose activity and (C) β-glucosidase activity of *Tricholoma matsutake* with or without microfungal metabolite. Marker (*) indicates that the mean values differ significantly by student's t-test ($p < 0.05$).

Table 1. Details of the strains used in phylogenetic analysis.

	Sequence accession numbers			
Species	ITS	*BenA*	*CaM*	*RPB2*
Y22_F22	OQ719837	OR412752	OR412753	OR412754
Penicillium amaliae CBS 134209 T	JX091443	JX091563	JX141557	n.a.
Penicillium arabicum CBS 414.69 T	KC411758	KP016750	KP016770	KP064574
Penicillium chalabudae CBS 219.66 T	KP016811	KP016748	KP016767	KP064572
Penicillium citreonigrum CBS 258.29 T	AF033456	EF198621	EF198628	JN121474
Penicillium citreosulfuratum IMI 92228 T	KP016814	KP016753	KP016777	KP064615
Penicillium fundyense CBS 140980 T	KT887853	KT887814	KT887775	n.a.
Penicillium krskae CCF 6561 T	MW794123	MW774594	MW774595	MW774593
Penicillium restrictum CBS 367.48 T	AF033457	KJ834486	KP016803	JN121662
Penicillium terrenum CBS 313.67 T	AM992111	KJ834496	KP016808	JN406577
Aspergillus fumigatus CBS 133.61 T	EF669931	EF669791	EF669860	EF669719
Aspergillus clavatus CBS 513.65 T	EF669942	EF669802	EF669871	EF669730

The accession numbers of the strains Y22_F22 and others are available in the NCBI database.

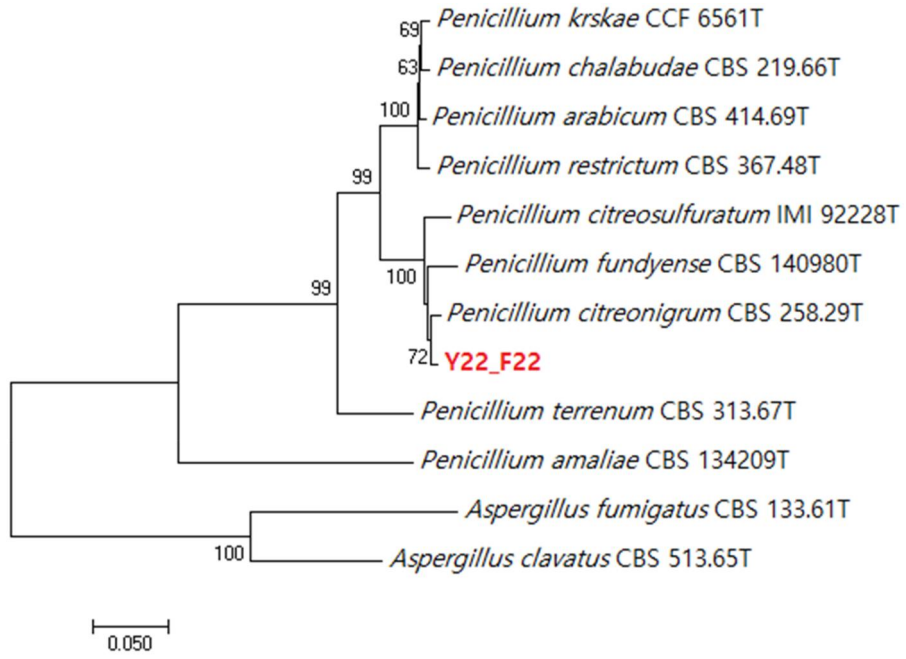

Figure 3. Phylogenetic tree based on maximum likelihood analysis of the combined ITS, *BenA*, *CaM*, and *RPB2* dataset for species classified in the *Penicillium* section. *Aspergillus fumigatus* and *Asp. clavatus* were included as an outgroup. Bootstrap analysis was performed with 1,000 replications. Bootstrap support values of ≥ 60% are indicated at the nodes. The bar indicates the number of substitutions per position. T indicates the type strains of the species. Bar indicates 0.05 substitutions per nucleotide position. The isolate Y22_F22 is marked in red.

4. Discussion

In this study, a total of 30 microfungal strains were isolated from the soil sample of shiros in Yangyang, and microfungal metabolites were isolated, respectively. Among the 30 metabolites, Y22_F22 promoted the growth of *T. matsutake* mycelium. As other

fungal species showed growth-promoting effect on *T. matsutake* mycelium [20], a comparison of growth promotion to *T. matsutake* mycelium was processed. In comparison with *Penicillium ochrochloron* which increased growth area by 172%, Y22_F22, also identified as *P. citreonigrum,* exhibited higher growth promotion to *T. matsutake* mycelium. Also, Y22_F22 showed similar growth promotion with the *Mucor zonatus* which increased growth area by 207%. Such growth result indicates that Y22_F22 not only positive effect on *T. matsutake* growth but also proved its potential as a competitive candidate to promote *T. matsutake* growth.

P. citreonigrum has been known for its metabolites which are reported as cytotoxic compounds [32–33]. On the contrary, the metabolite of *P. citreonigrum* also has been known as a productive element like β-fructofuranosidase which hydrolyzes sucrose and releases fructose from various fructooligosaccharides and fructans [34–35]. However, any reports regarding promoting the growth of other fungi by *P. citreonigrum* have not been published. In this study, fungal isolate Y22_F22 which was identified as *P. citreonigrum*, promoted the growth of *Tricholoma matsutake* mycelium growth.

In addition, the activities of three enzymes including α-amylase, cellulase, and β-glucosidase were measured to analyze the *T. matsutake* growth. Among the activites, β-glucosidase activity which indicates the promotion of cellulose degradation showed the highest degrees of enzyme activity. Cellulose which consist with β-1,4-glycosidic bond of D-glucose is the most abundant organic biomass in plant [36]. As EMF, mushrooms have a closed symbiotic relationship with the host tree by obtaining biological energy from their host [1]. In Y22_F22 metabolite-treated *T. matsutake*, β-glucosidase activity was primarily promoted to consume plant biomass from the *Pinus densiflora*'s cell wall, and based on the result of enzyme activity assay, Y22_F22 metabolite is concern with promoting β-glucosidase activity of *T. matsutake*.

Because of the highly prized value and difficulty in artificial cultivation, studies for *T. matsutake* growth in artificial conditions have been processed [13]. Even though several studies reported artificial cultivation of *T. matsutake* [14–17], improvements to decrease the cultivation time are still necessary. In this study, microfungal isolate Y22_F22, identified as *P. citreonigrum*, promotes *T. matsutake* growth and this promoting effect is primarily concerned with β-glucosidase activity. Because the isolate Y22_F22 shows its potential as a mycorrhizal helper fungus for the growth of *T. matsutake* in artificial cultivation, further studies for ingredients of the Y22_F22 metabolite are recommended.

Disclosure statement

No potential conflict of interest was reported by the authors.

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