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In vitro Biological Control Against *Trichoderma harzianum* Using Antifungal Bacteria

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Abstract - *Trichoderma harzianum* is an aggressive causal agent of green mold disease on mushroom cultivation. Some bacterial strains isolated, from oyster mushroom compost in Wonju, were found to have *in vitro* antifungal activity against *Trichoderma harzianum* ATCC 6385, 6504, and our isolates *Trichoderma* spp. Y and G. Further *in vitro* antifungal studies on several strains of phytopathogenic fungi showed that all of 12 phytopathogenic fungal strains were significantly inhibited by the isolated antifungal bacteria in Petri dishes. Of these, KATB 99121 showed the broadest inhibiting effect and displayed as negative coagulase, negative sulfide production and rod shape. KATB 99121 was resistant to ampicillin, chlorampenicol, and kanamycin. Identification of isolates was determined by Biolog GN system, and KATB 99121 was identified as *Photobacterium logei* because of 96 probability, 0.65 similarity, and 4.97 disturbance. With electron microscopy, thin section of KATB 99121 strain revealed typical rod-like shaped cell (0.6~0.8 μm × 1.5~2.0 μm) with prokaryotic structure and organization.

Key words : Antifungal agent, Biological control, *Photobacterium logei, Trichoderma harzianum*

Introduction

Sinden and Hauser in 1953 formally recognized the importance of *Trichoderma* spp. in limiting commercial production of the button mushroom, *Agaricus bisporus*. *Trichoderma* disease, commonly referred to as green mold, was previously considered a minor problem in mushroom production, because it typically occurred episodically in association with low-quality compost or poor hygiene (Sinden and Hauser 1953; Chen *et al.* 1999). Therefore, the disease could be effectively managed by modifying the composting process, improving sanitation, or chemical intervention. Severe outbreaks of green mold occurred in Northern Ireland in 1985 and, in the ensuing years, in England, Scotland, Canada, the United States, and Korea. In the early stages of the disease, *Trichoderma* flourishes in the composted mushroom substrate as white mycelia and eventually develops a dark green color after sporulation (Ospina-Giraldo *et al.* 1999). The mechanism of pathogenesis is not understood, but a cessation in the formation of mushrooms occurs in areas of the substrate colonized by *Trichoderma*. Because green mold mycelium resembles the mushroom mycelium, the green mold disease is not recognized in the early states. Historically, *Trichoderma* viride, *T. harzianum, T. hamatum*, and *T. koningii* caused green mold in mushrooms (Sinden and Hauser, 1953).

In the past few years, *Trichoderma* spp., the most common saprophytic fungi in the rhizosphere, has received considerable attention as potential bio-control agents for a number of soil-borne pathogens. Recent progress in the purification and identification of *Tricho-*

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derma metabolites has led to the notion that, in most cases, the antagonistic process relies on the production of antibiotics and /or hydrolytic enzymes associated with possible competition for nutrients in the rhizosphere.

As we know, *Trichoderma* spp. is a valuable asset to crop plants as a bio-control agent. At the same time it is a significant pathogen on mushroom (Yedida *et al.* 1999).

The purpose of our study is to isolate and identify the anti-*Trichoderma* agents from bacteria. The anti-fungal activity of some compounds is due to their ability to affect the function or the structure of the plasmalemma and other membranes of the fungal cell.

These compounds, often indicated as antibiotics, may include small molecules and peptides, proteins, enzymes, which act as a fungicide. Cell wall degrading enzymes produced by plants, bacteria, and fungi are also powerful anti-fungal agents *in vitro*, especially if mixtures of these different enzymes were applied. The final goal of this work is to find commercially useful chemicals or proteins which act as antagonists for *Trichoderma* spp. on mushroom compost.

Materials and Methods

Fungal strains and growth conditions

In this study, the strains for green mold disease *T. harzianum* ATCC 6043, 6385, 6426,6504, *T. viride* (KACC 40519), *T. hamatum* (KACC 40508) and *Pleurotus ostreatus* (wonhyung, 2209, chunchu2) were obtained from the Korean Agricultural Culture Collection. *Trichoderma* spp. Y and G were isolated in this study. Phytopathogenic strains *Alternaria alternata* (KACC 40020), *A. porri* (KACC 40568), A. solani (KACC 40570), *Botrytis cinerea* (KACC 40574), *Collectorichum gloesporioodes* (KACC4005), *Fusarium oxysporum* (KACC 40236), *Gliocladium roseum* (KACC 40320), *Phytophthora capsici* (KACC 40158), *P. cryptogea* (KACC 40189), *Pyricularia grisea* (KACC 40415), *Rhizoctonia solani* (KACC 40111), *Sclerotinia sclerotiorum* (KACC 40457) were obtained from the Korean Agricultural Culture Collection. All strains were grown at 25°C in potato dextrose agar medium.

Isolation of bacteria

Bacteria were isolated from an oyster mushroom farm site in Wonju, South Korea. Sixty grams (dry weight) of compost was suspended in 200 ml of 0.015 M phosphate saline buffer (pH 7.2) and blended three times for 1-minute intervals by stirrer, at low speed. Aliquots (100 μl) in direct and 10^{-1} dilutions were spread on nutrient agar plate (NA). The bacteria spray plates were incubated at 30°C for 24 hours. Colonies that were transferred to *Trichoderma* plates were cultured at 25°C for 2 days. The plates were incubated at 25°C and screened for bacteria that produced clearing zones in the opaque *Trichoderma* layer. Screening was done several times during a period of 5 days. Subsequently, bacteria were estimated as positive, moderate, and negative bio-control agents by the size of clearing zone (2,6). All bacterial suspensions were stored in 50% (vol/vol) glycerol at −80°C. All bacteria in this work, unless stated otherwise, were grown on Luria-Bertani medium supplemented with 0.10% (wt/vol) glucose and 1.5% (wt/vol) BiTek Agar (Difco).

Characterization by classical tests

Gram and spore stains by classical staining method, plus catalase, coagulase, SIM (Sulfur Indole Motility), and antibiotic resistance tests were used to examine the bacteria. Transmission electron microscopy was conducted for shape and size.

Identification and characterization by Biolog GN2

One or two days before the inoculation of Biolog GN2 plates (Biolog, Inc., USA), the isolates were streaked on tryptic soy agar (Difco, USA) plates from frozen cultures. On the day of inoculation, a prewetted sterile cotton swab was used to transfer bacteria to a 30 ml muffled glass tube with 19 ml of 0.85% NaCl solution and adjusted to a bacterial concentration of optical density at 600 nm (OD_{600}) of 0.12 ($\sim 3 \times 10^8$ cells/ml). Within 10 minutes, the Biolog GN2 plates were inoculated with 150 μl of the adjusted bacterial suspension in each well and incubated at 30°C for 24 h., leaving out the 4-h measurement as recommended by Holmes *et al.* 1994. The development of color was read the following day by using a 590 nm wavelength filter in a microplate reader,

as well as read manually. Reactions were recorded as positive (+), negative (−), or borderline (/). Manual readings from the first and second runs were entered in the Biolog GN2 database, release 3.50, to provide identification. Identification was acknowledged when the similarity index used by Biolog was 0.5 or more (Harris-Baldwin and Gudmestad, 1996).

Results

Isolation of bacteria

The three strains of bacteria with anti-fungal activities were isolated on the *Trichoderma* plates. Of these, KATB 99121 produced clearing zones at a higher rate than others.

Identification by classical tests and Biolog GN2

All three isolates were shown to be gram-positive, spore negative, catalase positive, indole positive and motility negative. Of these, KATB 99121 was rod shape and displayed coagulase activity negative, sulfide production negative. KATB 99122 was detected to be coagulase activity positive, sulfide production negative and cocci shape. KATB 99123 appeared to be coagulase activity positive, sulfide production positive and rod shape (Table 1). After antibiotic tests, all three isolates showed resistance to ampicillin, bacitracin, gentamycin, kanamycin, oxacillin, and tetracycline (Table 2).

Table 1. Identifications and characteristics of Anti-*Trichoderma* isolates

Test	Strains		
	KATB 99121	KATB 99122	KATB 99123
Classical			
Gram stain	+	+	+
Spore stain	−	−	+
Catalase	+	+	+
Coagulase	−	+	+
Morphology	rod	cocci	rod
Sulfide production	−	−	+
Indole formation	+	+	+
Motility	−	−	+

Identification of isolates was carried by Biolog GN system. KATB 99121 had 96 probability, 0.65 similarity, and 4.97 disturbance to *Photobacterium logei*. KATB 99122 had 0.0 probability, 0.40 similarity, and 8.93 disturbance to *Vibrio harveyi*. And KATB 99123 had 100 probability, 0.60 similarity, and 6.26 disturbance to *Bacillus licheniformis*.

By thin section studies with electron microscopy, KATB 99121 strain revealed typical rod shaped cell (0.6~0.8 μm × 1.5~2.0 μm) with prokaryotic structure and organization (Fig. 1). The very dark electron-dense area was nuclear region with fibrillar DNA-like materials. The low-density bodies observed around plasma membrane were assumed to be secreting granules.

Anti-fungal effects of KATB 99121

The three isolates were selected by anti-fungal activities. A lytic zone assay was used to determine the anti-fungal activity. Eight types of *Trichoderma* spp., three types of *Pleurotus* spp., and twelve types of phytopathogenic fungi were examined. *T. harzianum* ATCC 6385, 6504, *T. hamatum* KACC 40508, *Trichoderma* spp. Y and G displayed growth inhibition zone by KATB 99121 and its culture supernatant. *T. harzianum* ATCC 6043, 6426, *T. viride* KACC 40519 showed slightly inhibited by KATB 99121, but growth inhibition was not detected by the culture supernatant (Fig. 2).

Table 2. Interpretive chart of antibiotics tests by BBL Sensi-Disc zone diameter (mm)

Antibiotics	Strains								
	KATB 99121			KATB 99122			KATB 99123		
	Resistant	Intermediate	Susceptible	Resistant	Intermediate	Susceptible	Resistant	Intermediate	Susceptible
Ampicillin^{K} 10 μg	9			15			11		
Bacitracin^{f} 10U	5			0			0		
Chlorampenicol 30 μg	12				15		12		
Gentamycin^{A} 10 μg	7			8			10		
Kanamycin 30 μg	10			10			10		
Oxacillin 1 μg	7			10			8		
Tetracycline 30 μg	6			10			10		

KATB 99121 and its culture supernatant showed growth inhibition ability against all types of phytopathogenic fungi. The mycelial growth of some of these phytopathogenic fungi, *Botrytis cinerea* KACC 40574 and *Sclerotinia sclerotiorum* KACC 40457, were restricted by culture supernatant of KATB 99121 but not affected by KATB 99121 itself. On the other hand, *Phytophthora cryptogea* KACC 40189 and *Rhizoctonia solani* KACC 40111 were inhibited by KATB 99121 but not by supernatant (Fig. 3).

In oyster mushrooms, the growth of *Pleurotus ostreatus* types, Wonhyung, 2209, and Chunchu 2 were slightly affected by KATB 99121 and its culture supernatant on early culture time (Fig. 4), but the growth inhibition effect disappeared with time (data not shown).

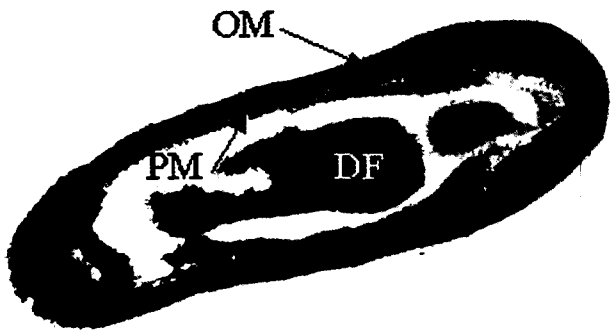

Fig. 1. Transmission electron microscopy, thin section of KATB 99121 (×50,000) DF; DNA fibril, OM; outer membrane, PM; plasma membrane.

Discussion

The anti-fungal activity of some compounds is due to their ability to affect the function or the structure of the plasmalemma and other membranes of the fungal cell. These compounds, often indicated as antibiotics, may include small molecules and peptides, proteins, enzymes, and chemical fungicides. Cell wall degrading enzymes produced by plants, bacteria, and fungi are also powerful anti-fungal agents *in vitro*, especially, if mixtures of different enzymes are applied. Some membrane affecting compounds and cell wall degrading enzymes are able to interact synergistically in the inhibition of pathogenic fungi. It has been suggested that this synergism is involved in both plant defense and microbial biocontrol mechanisms (Harris-Baldwin and Gudmestad 1996). In this study, we isolated three strains that produced anti-fungal substances. One of these isolates was identified as *Photobacterium logei*. *P. logei* (KATB

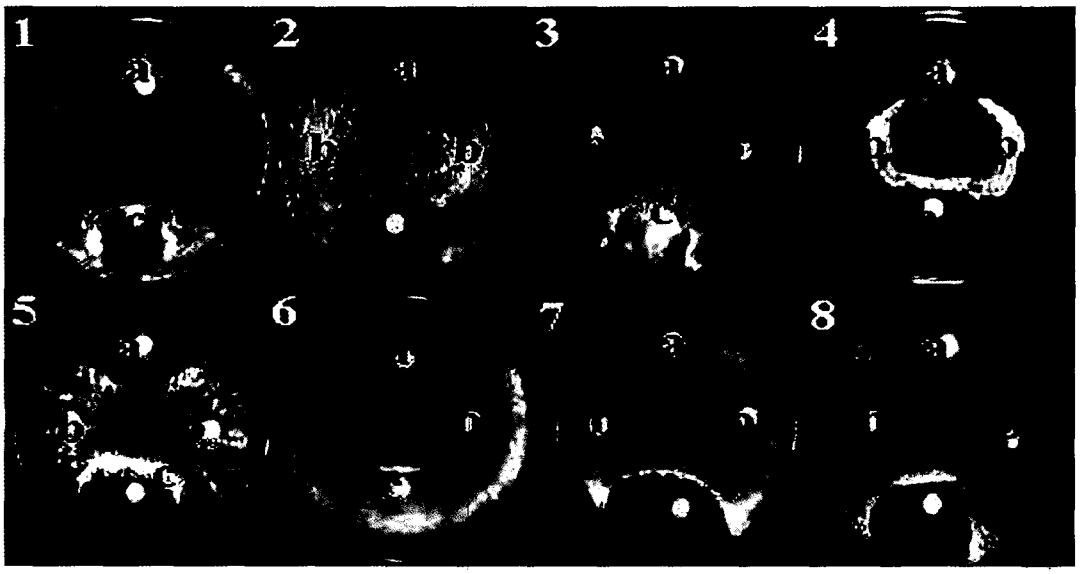

Fig. 2. Anti-*Trichoderma* effects of *Photobacterium logei* (KATB99121) and its culture supernatant.
1; *Trichoderma harzianum* ATCC 6043, 2; *Trichoderma harzianum* ATCC 6385,
3; *Trichoderma harzianum* ATCC 6426, 4; *Trichoderma harzianum* ATCC 6504,
5. *Trichoderma hamatum* KACC 40508, 6; *Trichoderma viride* KACC 40519,
7; *Trichoderma* spp. Y, 8; G were isolated from the authors
a; *Photobacterium logei* KATB 99121, b; distilled water, c; culture supernatant

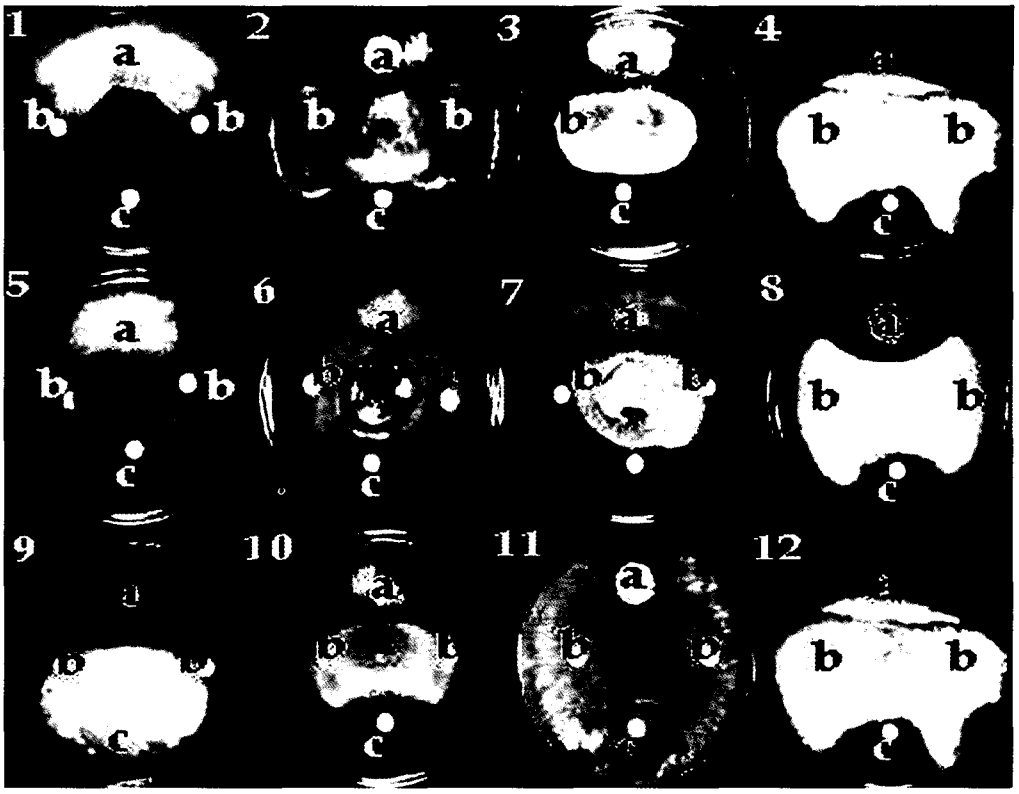

Fig. 3. Anti-fungal effects of *Photobacterium logei* (KATB99121) and its culture supernatant on phytopathogenic fungi.
1; *Alterinaria porri* KACC 40568, 2; *Alternaria alternata* KACC 40020,
3; *Alternaria solani* KACC 40570, 4; *Botrytis cinerea* KACC 40574,
5; *Collectorichum gloesporioodes* KACC 4005, 6; *Fusarium oxysporum* KACC 40236,
7; *Gliocladium roseum* KACC 40320, 8; *Phytophthora capsici* KACC 40158,
9; *Phytophthora cryptogea* KACC 40189, 10; *Pyricularia grisea* KACC 40415,
11; *Rhizoctonia solani* KACC 40111, 12; *Sclerotinia sclerotiorum* KACC 40457.
a; *Photobacterium logei* KATB 99121, b; distilled water, c; culture supernatant

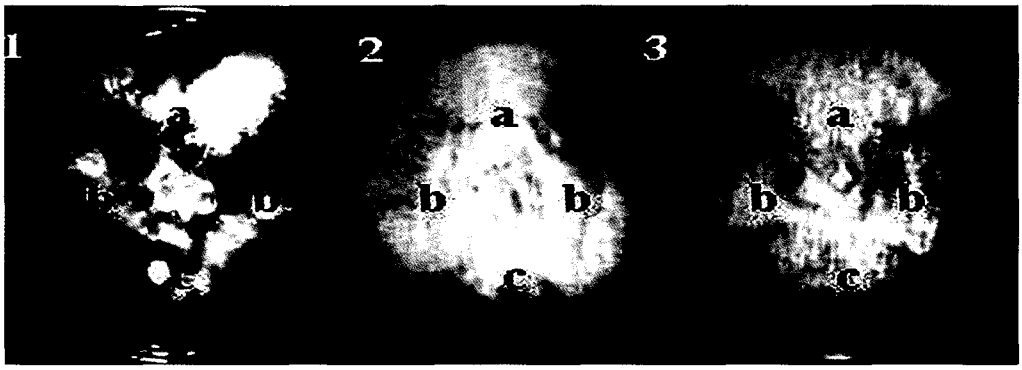

Fig. 4. Anti-*Pleurotus ostreatus* (oyster mushroom) effects of *Photobacterium logei* (KATB99121) and its culture supernatant.
1; 2209, 2; Chunchu 2, 3; Wonhyung
a; *Photobacterium logei* KATB 99121, b; distilled water, c; culture supernatant

99121) produced clearing zones at a higher rate than others. KATB 99121 was shown to be coagulase negative, sulfide production negative and rod shape. KATB 99121 was exhibited resistance against ampicillin,

chlorampenicol, and kanamycin. With electron microscopy, thin sections of KATB 99121 revealed that this strain is typical rod shaped cells with prokaryotic structure and organization. *T. harzianum* ATCC 6385, 6504, *T. hamatum* KACC 40508, *Trichoderma* spp. Y and G were displayed growth inhibition zone by KATB 99121 and its culture supernatant. KATB 99121 and its culture supernatant had growth inhibiting ability against all types phytopathogenic fungi. The growth of *Pleurotus ostreatus* type Wonhyung, 2209, and Chunchu 2 were slightly affected by KATB 99121 and culture supernatant during the early culture time, but the growth inhibition effect disappeared with time. Like previously studies on anti-fungal substances; gramicidin, valinomycin, and phospholipase B, the anti-fungal substances of KATB 99121 were not specific to any phytopathogenic fungal, but had a broad spectrum (Holmes *et al.* 1994; Espinel-Ingroff *et al.* 1995). There were differences between KATB 99121 itself and the culture supernatant in the antifungal characteristics on each phytopathogenic fungus. From these results, KATB 99121 was estimated to produce one or more anti-fungal substances. According to our data, we think that KATB 99121 is a new model for new bio-control agents in green mold disease as bacteria itself and mixed treatments of antifungal substances.

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