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Trichoderma metabolites as biological control agents against *Phytophthora* pathogens

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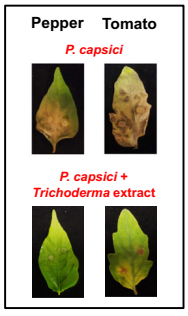
HIGHLIGHTS

- *Trichoderma* spp. were screened for anti-*Phytophthora* activity.
- *Trichoderma atroviride*/*petersenii* (KACC 40557) showed the best anti-*Phytophthora* activity.
- *Trichoderma* metabolite treatment caused plant hormonal and transcriptional changes.

GRAPHICAL ABSTRACT



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ABSTRACT

Trichoderma species are well-known biological control agents. In this study, metabolites from 128 *Trichoderma* isolates were extracted from liquid cultures using ethyl acetate and tested for their activities against seven *Phytophthora* isolates. Following preliminary analysis, eight *Trichoderma* isolates were selected for further tests. Among them, the metabolites from *Trichoderma atroviride*/*petersenii* (KACC, Korea Agricultural Culture Collection, 40557) and *Trichoderma virens* (KACC 40929) showed the strongest inhibitory activities against *Phytophthora* isolates. Treatment with KACC 40557 extract inhibited *Phytophthora* growth, induced defense-related genes, and caused plant hormonal changes during *Phytophthora* infection in the detached leaves of pepper and tomato plants. Our results showed the potential for use of *Trichoderma* metabolites as biological control agents against *Phytophthora* pathogens.

1. Introduction

The genus *Trichoderma* belongs to ascomycetic fungi found in the soil (Samuels, 2006). *Trichoderma* spp. are well-known biocontrol agents against phytopathogens (Romão-Dumaresq et al., 2012). For example, *Trichoderma harzianum*, *Trichoderma virens*

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and *Trichoderma viride* are currently marketed as biocontrol agents. The mechanisms for anti-phytopathogen activities include antibiosis, mycoparasitism, induced resistance and niche exclusion (Bae, 2011). Antibiosis involves the production of various antimicrobial compounds that function as inhibitors of phytopathogen growth (Vinale et al., 2008). More than 100 antimicrobial compounds have been identified from *Trichoderma* spp. (Vinale et al., 2008). During the mycoparasitism process, phytopathogen cell walls are degraded by cell wall-degrading enzymes produced from *Trichoderma* (Reithner et al., 2011). There is competition between *Trichoderma* spp. and phytopathogens for infection sites and nutrients, which is known as niche exclusion.

The genus *Phytophthora* is a devastating plant pathogen that infects almost all plant species (Hansen et al., 2011). In 1861, deBary identified *Phytophthora infestans* as the causal agent of late potato blight, which was responsible for the Irish potato famine (Raffaele et al., 2010). *Phytophthora* species are oomycetes, which are fungi-like eukaryotic microorganisms also known as water molds. More than 100 species of *Phytophthora* are potential plant pathogens. *Phytophthora* mycelia contain non-partitioned hyphae with several diploid nuclei. While chitin is the primary component of fungal cell walls, β-glucan and cellulose are the major components of *Phytophthora* cell walls. *Phytophthora* species produce different types of spores (oospores, chlamydospores and zoospores), but cannot synthesize sterols, which are the target of many fungicides (Gaulin et al., 2010). As a result, *Phytophthora* pathogens are difficult to control using the majority of currently available fungicides. In addition, *Phytophthora* species can overcome chemical control agents and resistance to plant hosts via genetic flexibility.

In this study, the possibility for use of *Trichoderma* metabolites as biocontrol agents against *Phytophthora* was investigated. A total of 128 *Trichoderma* isolates were screened for their anti-*Phytophthora* activities using ethyl acetate extracts isolated from liquid cultures. Further confirmation of eight selected *Trichoderma* isolates was conducted by minimum inhibitory concentration, disk diffusion and antibiosis tests. In addition, we investigated the molecular and biochemical responses of *Phytophthora* and plants (pepper and tomato) after application of the ethyl acetate extract of KACC 40557.

2. Materials and methods

2.1. Trichoderma and Phytophthora isolates

A total of 128 *Trichoderma* isolates were obtained from the Rural Development Administration (RDA) Genebank Information Center (GIC) (Suwon, Republic of Korea) (data not shown). *Trichoderma* isolates were grown on potato dextrose agar (PDA) (Difco, Sparks, MD, USA) at 25 °C for 14 days under dark conditions. Seven *Phytophthora* isolates were maintained on 20% clarified V8 (cV8) juice agar (20% V8 juice, 8% $CaCO_3$, 1.5% Bacto agar). The following seven species were used in this study: *Phytophthora cactorum* (KACC, Korea Agricultural Culture Collection, 40166), *Phytophthora capsici* (KACC 40157), *Phytophthora drechsleri* (KACC 40463), *Phytophthora infestans* (KACC 43071), *Phytophthora melonis* (KACC 40197), *Phytophthora nicotianae* (KACC 44717), and *Phytophthora sojae* (KACC 40412). These isolates were grown at the following temperatures to optimize their growth: *P. infestans*, 18 °C; *P. cactorum* and *P. sojae*, 25 °C; *P. capsici, P. drechsleri*, *P. melonis* and *P. nicotianae*, 30 °C. The isolates were cultivated for 5–13 days under dark conditions.

2.2. Extraction of Trichoderma metabolites using ethyl acetate

After 14-days of growth, sterile water (8 mL) was poured into the plates, and mycelia were harvested by scratching using a glass stick. The amount of harvested mycelia was dependent on isolates; however, the same concentration of extracts was used for the treatment. The harvested mycelia were poured into 500 mL of minimal salts broth (MIN) media in 1000 mL Erlenmeyer flasks and grown for 14 days at 25 °C at 150 rpm (Bae et al., 2011). Half of the volume of ethyl acetate (EtOAc, 250 mL) was added into the liquid culture and shaken at 150 rpm for 10 min. After 1 h of incubation without shaking, the top layer was transferred into a flask, concentrated, and dried using a rotary vacuum evaporator at 36 °C (Rouini et al., 2006). The dried extracts were weighed and dissolved in acetone–water (1:9, v/v).

2.3. Preliminary screening for anti-Phytophthora assay

The EtOAc extracts from 128 *Trichoderma* isolates were screened for anti-*Phytophthora* activity. *Phytophthora* plugs (0.6 cm diameter) were cut from actively growing edges of cV8 plates using a sterile cork borer and then placed mycelial side up in the middle of a plate with a 6 cm diameter containing 10 mL PDA. cV8 plates were used for *P. infestans* and *P. sojae* instead of PDA because of slow growth in the PDA media. Two layers of sterile Whatman filter paper discs with a 0.6 cm diameter that contained either *Trichoderma* EtOAc extracts (100 μg/10 μL) as treatment, acetone–water (1:9, v/v) as a negative control, or propamocarb Pestanal® (1 μg/10 μL; Sigma, St. Louis, MO, USA) as a positive control were placed on top of the *Phytophthora* plug. The plates were then incubated at 18–30 °C for 5–13 days until *Phytophthora* growth in the negative control plates reached the edge of the plates, during which time the colony diameter was measured daily. The optimal temperature of *Phytophthora* growth is dependent on the species mentioned above. Each experiment was repeated twice, with three biological replications per experiment. The percent inhibition of mycelial growth was calculated as follows: growth inhibition (%) = [(control mycelia diameter–treated mycelia diameter)/control mycelia diameter] × 100 (Satish et al., 2007). *Trichoderma* isolates selected through preliminary screening that showed 100% inhibitory activity against at least three *Phytophthora* species via EtOAc extract with a concentration of 100 μg/10 μL were further tested for anti-*Phytophthora* activity using: (i) minimum inhibitory concentration (MIC) test using the EtOAc extract, (ii) disk diffusion test using the EtOAc extract, and (iii) antibiosis test using the liquid culture filtrate of potato dextrose broth (PDB).

2.4. Additional tests

The MIC of the EtOAc extract against *Phytophthora* was determined by the modified agar diffusion method (Espinel-Ingroff et al., 2007). The MIC was determined using the same method used for preliminary screening with three different concentrations of EtOAc extracts, 100, 10 and 1 μg, in 10 μL of acetone–water (1:9, v/v). Seven *Phytophthora* species were treated with the extracts from eight *Trichoderma* isolates, which were selected through preliminary screening. Each experiment was repeated two times, with three biological replications per experiment.

For the disk diffusion test, a small plate (6 cm diameter) containing 10 mL PDA or cV8 was used for the disk diffusion test. cV8 plates were used for *P. infestans* and *P. sojae* instead of PDA plates owing to slow growth in the PDA plate. Two layers of sterile filter paper discs (0.6 cm diameter) that contained EtOAc extracts [100 μg/10 μL of water (1:9, v/v)] as treatment and acetone–water (1:9, v/v) as a control were placed on the surface of PDA or cV8 plates at 3 cm from a *Phytophthora* plug (0.6 cm diameter) that had been cut from the actively growing edge of and placed on the plate mycelial side down. The plate was then incubated for 5–13 days at 18–30 °C until *Phytophthora* growth in the control reached the edge of the plate. The colony diameters of each