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Molecular Identification, Enzyme Assay, and Metabolic Profiling of *Trichoderma* spp.

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The goal of this study was to identify and characterize selected *Trichoderma* isolates by metabolic profiling and enzyme assay for evaluation of their potential as biocontrol agents against plant pathogens. *Trichoderma* isolates were obtained from the Rural Development Administration Genebank Information Center (Wanju, Republic of Korea). Eleven *Trichoderma* isolates were re-identified using ribosomal DNA internal transcribed spacer (ITS) regions. ITS sequence results showed new identification of *Trichoderma* isolates. In addition, metabolic profiling of the ethyl acetate extracts of the liquid cultures of five *Trichoderma* isolates that showed the best anti-*Phytophthora* activities was conducted using gas chromatography-mass spectrometry. Metabolic profiling revealed that *Trichoderma* isolates shared common metabolites with well-known antifungal activities. Enzyme assays indicated strong cell wall-degrading enzyme activities of *Trichoderma* isolates. Overall, our results indicated that the selected *Trichoderma* isolates have great potential for use as biocontrol agents against plant pathogens.

Keywords: *Trichoderma*, molecular identification, metabolic profiling, cell wall degradation enzyme activity

Introduction

Trichoderma (teleopmorph *Hypocrea*) is a genus of ascomycetic fungi found in soil that contains over 100 phylogenetically characterized species [1]. Many members of *Trichoderma* are well-known biocontrol agents of plant pathogens [2–5]. *Trichoderma* spp. reproduce asexually through conidia spore production on conidiophores that have branched structures and develop on hyphal tips [6]. The conidia spores are green, yellow, or white, which is one of the characteristics of the fungi. *Trichoderma* species are opportunistic, avirulent plant symbionts with known potential to control plant diseases [7]. There are other beneficial functions of *Trichoderma*, such as plant growth promotion and tolerance induction to abiotic/biotic stresses [8–11]. Some *Trichoderma* species such as *T. harzianum*, *T. virens*, and *T. viride* are commercially marketed as biopesticides [12]. There are several mechanisms through which *Trichoderma* spp. control plant pathogens, including mycoparasitism, antibiosis, induced resistance, and niche exclusion [10]. Mycoparasitism involves direct feeding of pathogens using cell wall-degrading enzymes (CWDEs) [12]. *Trichoderma* spp. degrade the host fungal cell wall using CWDEs, resulting in the release of oligomers [13]. *Trichoderma* spp. recognize pathogens through the sequential expression of CWDEs such as chitinases, glucanases, and proteases [14]. The released oligomers then induce expression of the genes involved in exochitinase. *Trichoderma* spp. attach and coil around the pathogen hyphae and produce appressorium-like structures that penetrate pathogen cells [15]. Following appressorium formation, CWDEs and antibiotics are secreted to facilitate penetration into the pathogen lumen. During antibiosis, *Trichoderma* spp. produce various antimicrobial compounds that can inhibit pathogen growth [6, 14]. Antibiotics can be classified into three groups: volatile (*e.g.*, 6-pentyl-α-pyrone and several isocyanide derivatives), water-soluble (*e.g.*, heptelidic acid and koningic acid), and peptide antibiotics (*e.g.*, peptaibols)

[17–19]. *Trichoderma* spp. protect plants via resistance induced through a hypersensitive response (HR), systemic acquired resistance (SAR), and induced systemic resistance [14, 20]. During the process, plants accumulate metabolites and enzymes that are involved in defensive mechanisms, such as phenyl-alanine ammonio-lyase and chalcone synthase for HR, pathogenesis-related proteins for SAR, superoxide dismutase and amino acid oxidase for oxidative stress response, as well as chitinases and glucanases for cell wall modification. Niche exclusion includes competition between *Trichoderma* and pathogens for infection sites and nutrients.

In this study, we identified and characterized selected *Trichoderma* isolates by metabolic profiling and enzyme assay to evaluate their potential for use as biocontrol agents against plant pathogens.

Materials and Methods

Analysis of Internal Transcribed Spacer Sequences

All *Trichoderma* isolates were obtained from the Rural Development Administration (RDA) Genebank Information Center (GIC) (Wanju, Republic of Korea). Isolates were maintained on potato dextrose agar (PDA) (Difco, USA) at 25°C for 14 days under dark conditions. Overall, there were 11 *Trichoderma* isolates consisting of three known and eight unknown species (Table 1).

Table 1. Molecular identification of selected *Trichoderma* isolates using internal transcribed spacer sequences.

KACC[a] No.	GIC[b]	NCBI[c]	Identity (%)	Homolog sequences
40552	*T.* sp.	*T. atroviride*	100	AF414324
		T. petersenii	100	DQ323441
40553	*T.* sp.	*T. gamsii*	100	JQ398842
		T. koningiopsis	100	JQ398842
40556	*T.* sp.	*T. harzianum*	100	AJ224009
40557	*T.* sp.	*T. atroviride*	100	AF414324
		T. petersenii	100	DQ323441
40776	*T. atroviride*	*T. atroviride*	100	JQ712578
40871	*T. harzianum*	*T. harzianum*	100	AJ224009
40929	*T. virens*	*T. virens*	100	AF099007
40931	*T.* sp.	*T. brevicompactum*	99	JQ040334
41707	*T.* sp.	*T. brevicompactum*	100	JQ040334
41715	*T.* sp.	*T. virens*	99	AF099007
41717	*T.* sp.	*T. brevicompactum*	100	JQ040334

[a]Korean Agricultural Culture Collection; [b]Genebank Information Center, Rural Development Administration, Republic of Korea; [c]National Center for Biotechnology Information.

Total genomic DNA was extracted from *Trichoderma* mycelia that had been scraped from PDA plates using a DNeasy plant mini kit (Qiagen, USA). The following universal eukaryotic primers were used to amplify *Trichoderma* ribosomal DNA internal transcribed spacer (ITS) regions 1 and 2: ITS 1 (5′- TCCGTAGGTGAACCT GCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) [21, 22]. Amplification and sequence analyses were conducted as previously described [23].

Analysis by Gas Chromatography-Mass Spectrometry

The mycelia of five *Trichoderma* isolates (KACC 40552, 40557, 40929, 40931, and 41717) were harvested from 14-day-old *Trichoderma* PDA cultures with 8 ml of sterile water using a glass stick, and then poured into 500 ml of minimal salts broth medium (glucose (5 g/l), L-asparagine (anhydrous, 0.013 g/l), KH_2PO_4 (1.0 g/l), $MgSO_4{\cdot}7H_2O$ (0.6 g/l), KCl (0.5 g/l), $FeSO_4{\cdot}H_2O$ (0.01 g/l), $C_4H_6MnO_4{\cdot}4H_2O$ (0.008 g/l), $Zn(NO_3)_2{\cdot}6H_2O$ (0.002 g/l), $Ca(NO_3)_2{\cdot}4H_2O$ (0.05 g/l), $CuSO_4{\cdot}5H_2O$ (0.003 g/l), and NH_4NO_3 (0.008 g/l)) in 1,000 ml Erlenmeyer flasks [10]. After 14 days of growth at 25°C, 150 rpm, ethyl acetate (EtOAc, 250 ml) was added to the fungal liquid culture. The mixture was then mixed by shaking at 150 rpm for 10 min, after which it was incubated for 1 h without shaking. The top phase was subsequently transferred into a round flask and concentrated using a rotary vacuum evaporator at 36°C [24]. Concentrated metabolites were transferred into a 10 ml vial, and then completely dried in a fume hood. After complete drying, the extract powder was weighed and dissolved in acetone:water (1:9 (v/v)). Metabolic profiling of the EtOAc extracts of five *Trichoderma* isolates (KACC 40552, 40557, 40929, 40931, and 41717) was conducted using an Agilent 6890N GC (Agilent, USA) fitted with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) linked to a JMS 700 mass spectrometer (Jeol, Japan) at the Korea Basic Science Institute (Korea). Helium was applied as the carrier gas at a flow rate of 1 ml/min and a 1:50 split ratio. Relative amounts of individual components were calculated as the percentage of the peak area relative to the total peak area.

Analysis of Cell Wall-Degrading Enzymes

Five *Trichoderma* isolates (KACC 40552, 40557, 40929, 40931, and 41717) were grown on PDB media at 25°C with continuous shaking at 200 rpm for 5 days. Aliquots (1 ml) of PDB culture were inoculated into 100 ml of Czapek-Dox medium containing 0.3% Avicel, 0.3% carboxymethyl cellulose (CMC), 0.3 beechwood xylan, 0.3% pectin, 0.3% chitin, 0.42% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.02% urea, 0.03% $CaCl_2$, 0.03% $MgSO_4{\cdot}7H_2O$, 0.1% protease peptone, 0.2% Tween 80, and 0.2% trace element solution (0.5% $FeSO_4{\cdot}7H_2O$, 0.16% $MnSO_4{\cdot}H_2O$, 0.14% $ZnSO_4{\cdot}7H_2O$, and 0.2% $CoCl_2$) in a 500 ml baffle flask for 5 days at 25°C with shaking at 200 rpm. Cell-free supernatants were then harvested by centrifugation at 6,300 ×*g* for 20 min at 4°C, after which the protein concentrations of the *Trichoderma* liquid cultures were measured by the Bradford method. Six different carbon sources were used as substrates: 1% Avicel, 1% CMC, 1% β-glucan, 1% xylan, and 0.1% pectin. Enzyme

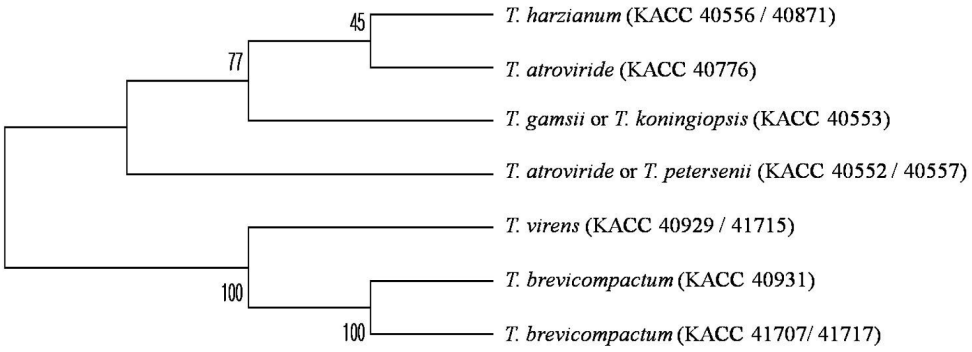

Fig. 1. Phylogenetic tree based on internal transcribed spacer (ITS) sequences of the selected *Trichoderma* isolates.
The tree was generated from the ITS sequences of *Trichoderma* isolates by the neighbor-joining method using MEGA5 (http://www/megasoftware.net/). The numbers at the nodes indicate bootstrap values from 1,000 replications.

activities were estimated by measuring the reducing sugars released after hydrolysis under optimal conditions with 3,5-dinitrosalicylic acid reagent and a glucose standard curve. The following assay conditions were used: citrate buffer at 1 M (pH 5.0) for 20 min at 50°C in a 200 µl volume (100 µl of 1% Avicel, 1% CMC, 1% β-glucan, 1% xylan, or 0.1% pectin plus 5 µl of 1 M sodium acetate buffer (pH 5.0), and 95 µl of enzyme and water). Duncan's multiple-range test was applied to identify differences among groups at a significance of $p \leq 0.05$ using the SAS software (SAS Inc., USA).

Results and Discussion

In previous reports, we screened 128 *Trichoderma* isolates for anti-*Phytophthora* activity [25]. We used the EtOAc extracts of *Trichoderma* isolates, and disk diffusion and antibiosis tests to select the best isolates for anti-*Phytophthora* activity. Five *Trichoderma* isolates (KACC 40552, 40557, 40929, 40931, and 41717) showed strong inhibitory activity against seven *Phytophthora* species. In this study, we further characterized the selected isolates through analyses of the ITS region, metabolic profiling, and CWDE activities.

DNA barcoding employs standard 500–800 bp sequences to identify species of all eukaryotic kingdoms using a broad range of primers. Among different barcoding methods, those targeting the ITS region have the greatest probability of successful identification of the broadest range of fungi [20, 26–28]. In this study, molecular identification of 11 *Trichoderma* isolates was conducted using the ITS sequence (Table 1). The results revealed that the Korea Agricultural Culture Collection (KACC) strains 40552 and 40557 shared 99% sequence identity with each other and were 100% homologous with both *T. atroviride* and *T. petersenii*. KACC 40553 was 100% homologous with *T. gamsii* and *T. koningiopsis*, whereas KACC 40556 and 40871 had identical ITS sequences that matched *T. harzianum*, and KACC 40776 matched that of *T. atroviride*. The results of the present study matched the identification determined by the RDA GIC for KACC 40776 (*T. atroviride*), 40871 (*T. harzianum*), and 40929 (*T. virens*). Three isolates (KACC 40931, 41707, and 41717) were homologous with *T. brevicompactum*, which is known to produce trichothecene, a potential mycotoxin agent against plant pathogenic fungi. Trichothecene activity increases in response to overexpression of the *tri5* gene [6, 29]. *T. brevicompactum* also produces polypeptide antibiotics (peptaibiotics) that act as potential antagonistic agents against pathogenic microbes [30]. The ITS sequences of the 11 *Trichoderma* isolates were also used to construct a phylogenetic tree with the MEGA5 software. The tree revealed the presence of two clades: (i) KACC 40552/40557, KACC 40553, 40776, and KACC 40556/40871; and (ii) KACC 40929/41715, KACC 40931, and KACC 41701/41717 (Fig. 1). However, some isolates had more than one match; therefore, further identification is necessary.

The detected metabolites are shown in Table 2. Dioctyl phthalate (DOP) and dilaurylthiodipropionate (DLTDP) were the major metabolites detected, but DOP was not detected in KACC 40931. On average, DOP accounted for 0.75% to 8.75% of the total area for the remaining *Trichoderma* isolates. There are several reports of the occurrence of DOP in plants and fungi such as *Limonium bicolor*, *Dracaena cohinensis*, *Caesalpinia sappan*, *Penicillium lividum*, and *Trichoderma lignorum* [31]. DOP exerts antimicrobial activity against bacteria, including *Bacillus subtilis*, *Escherchia coli*, *Sarcina lutea*, *Shigella dysenteriae*, *Shigella shiga*, *Shigella sonnei*, and *Staphylococcus aureus*. DLTDP is a secondary metabolite with the ability to degrade hydroperoxides of fish oils and an antifungal agent [32]. DLTDP was detected in five *Trichoderma* isolates at levels ranging from 0.75% to

Table 2. List of major compounds of five *Trichoderma* isolates detected by GC-MS analysis (>1% area; > 50% quality).

Trichoderma spp.	Library result	Contents (%)
KACC 40552	3-Nitropyrrole	16.44
	Erucamide	6.26
	Dilaurylthiodipropionate	5.25
	Dioctyl phthalate	2.4
	Hexadecanoic acid	1.67
	Valeric acid	1.34
KACC 40557	Erucamide	11.3
	Dilaurylthiodipropionate	10.95
	Dioctyl phthalate	8.75
	3-Nitropyrrole	7.62
	Valeric acid	1.5
KACC 40929	Benzoic acid, 3-(3-hydroxy-3-methyl-1-butyn-1-yl)-, ethyl ester	22.3
	1,4-*Trans*-6-methoxyisocalamenene	5.3
	Methyl palmitate	1.79
	Dilaurylthiodipropionate	1.54
	t-Muurolol	2.76
	Dioctyl phthalate	1.47
	α-Calacorene	1.44
	Valerenal	1.09
	α-Muurolene	1.05
KACC 40931	1,10-Phenanthroline, 3,8-*bis*(1,1-dimethylethyl),	71.95
	3-Furancarboxamide, 2,5-dihydro-4-hydroxy-5-oxo-*n*-phenyl-2-(phenylimino)	3.11
	1-Cyclohexene-1-propanol, α-ethynyl-α,2,6,6-tetramethyl	1.54
	Dilaurylthiodipropionate	0.75
KACC 41717	1,10-Phenanthroline, 3,8-*bis*(1,1-dimethylethyl)	79.24
	3-Furancarboxamide, 2,5-dihydro-4-hydroxy-5-oxo-*n*-phenyl-2-(phenylimino)	2.77
	Dioctyl phthalate	1.94
	1-Cyclohexene-1-propanol, α-ethynyl-α,2,6,6-tetramethyl	1.48
	Dilaurylthiodipropionate	1.47

KACC, Korean Agricultural Culture Collection.

10.95%. The major compounds of the five isolates were found to differ. In KACC 40552, 3-nitropyrrole (16.44%), which has been reported to have antimicrobial activity, was the major compound [33]. KACC 40557 exhibited a similar metabolic profile pattern to that of KACC 40552. Moreover, *cis*-13-docosenoamide (erucamide), which has been reported to have potential antimicrobial activity, was a major metabolite (11.30%) in KACC 40557 [34]. As shown in Table 1, the ITS sequences of these two isolates (KACC 40552 and 40557) also showed significant homology (99%). Erucamide acts as a receptor in cyclic AMP accumulation and a receptor of G-protein coupled fatty acid [34]. The major metabolic compounds of KACC 40929 were benzoic acid and 3-(3-hydroxy-3-methyl-1-butyn-1-yl)-ethyl ester, which accounted for 22.3% of the total compounds (Table 2). The metabolites detected in this isolate were found to differ from those of other isolates, and included several terpenoids, such as α-muurolene [35], α-calacorene [35], *t*-muurolol [37, 38], valerenal, methyl palmitate, and 1,4-*trans*-6-methoxy isocalamenene [39]. ITS sequence analysis revealed that KACC 40931 and 41717 matched *T. brevicompactum* and showed significant sequence identity to each other (Table 1). Similar metabolites were also detected in both isolates, including 1,10-phenanthroline, 3,8-*bis*(1,1-dimethylethyl), 3-furancarboxamide, 2,5-dihydro-4-hydroxy-5-oxo-*n*-phenyl-2-(phenylimino), 1-cyclohexene-1-propanol, α-ethynyl-α,2,6,6-

Table 3. Enzyme assays for five *Trichoderma* isolates on six different carbon sources.

Trichoderma isolates (KACC)	Protein conc. (μg/μl)	Specific activities (unit/mg)					
		Avicel	CMC	Xylan	β-Glucan	Pectin	Chitin
40552	0.244	$0.952^{d} \pm 0.003$	$11.548^{c} \pm 0.026$	$94.197^{d} \pm 1.564$	$47.785^{a} \pm 0.272$	$1.667^{c} \pm 0.008$	$6.915^{c} \pm 0.296$
40557	0.232	$1.007^{c} \pm 0.005$	$11.837^{b} \pm 0.136$	$96.350^{d} \pm 0.239$	$47.405^{b} \pm 0.204$	$1.664^{c} \pm 0.006$	$7.353^{c} \pm 0.131$
40929	0.175	$0.984^{cd} \pm 0.009$	$16.331^{a} \pm 0.163$	$113.534^{c} \pm 1.125$	$42.847^{c} \pm 0.235$	$1.685^{c} \pm 0.041$	$5.551^{c} \pm 0.065$
40931	0.093	$1.376^{b} \pm 0.029$	$4.538^{e} \pm 0.097$	$152.112^{b} \pm 9.595$	$15.957^{e} \pm 0.177$	$3.075^{b} \pm 0.001$	$14.264^{b} \pm 0.123$
41717	0.041	$3.930^{a} \pm 0.073$	$9.616^{d} \pm 0.209$	$226.591^{a} \pm 5.854$	$31.049^{d} \pm 0.031$	$12.992^{a} \pm 0.335$	$78.953^{a} \pm 5.105$

Three replicate cultures of each *Trichoderma* isolate were prepared from each medium. The numbers indicate the mean ± standard error of three biological replications. The letters on top of each number indicate significant differences between the mean values at a significance of $p \leq 0.05$.
KACC, Korean Agricultural Culture Collection

tetramethyl, and DLTDP. Among these compounds, 1,10-phenanthronline and 3,8-*bis* (1,1-dimethylethyl) were the most abundant (more than 70%). These compounds have been reported as metalloprotease inhibitors and strong growth inhibitors against a number of fungal species [40].

The cell walls of fungal pathogens are excellent targets for development of antimicrobial agents. The cell walls of most fungi are chitin-based, and disruption of the cell walls has profound effects on cell growth and morphology. In this study, we measured the activities of various CWDEs from five *Trichoderma* isolates (KACC 40552, 40557, 40929, 40931, and 41717) to determine which isolates had the potential for use as biocontrol agents with the ability to degrade cell walls of fungal pathogens (Table 3). High enzyme activities for Avicel, CMC, xylan, β-glucan, pectin, and chitin degradation were found in KACC 41717, 40929, 41717, 40552/40557, 41717, and 41717, respectively. It is well known that *Trichoderma* species such as *T. harzianum*, *T. atroviride*, and *T. virens* produce active CWDEs such as chitinase, protease, and β-1,3-glucanases, which function against fungal pathogens [41, 42].

Trichoderma isolates were characterized through analyses of the ITS region, metabolic profiling, and CWDE activities. ITS sequence analysis showed new classification of the isolates. Metabolic profiling showed that isolates produced common metabolites with antifungal activities. Enzyme assay revealed strong CWDE activities. Overall, the results of this study indicate that the selected *Trichoderma* isolates have good potential for use as biocontrol agents against plant pathogens.

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