

Selection of genetically diverse *Trichoderma* spp. isolates for suppression of *Phytophthora capsici* on bell pepper

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Abstract: Environmentally compatible control measures are needed for suppression of *Phytophthora capsici* on pepper. Twenty-three isolates of *Trichoderma* were screened for suppression of a mixture of 4 genetically distinct isolates of this pathogen on bell pepper (*Capsicum annuum*) in greenhouse pot assays. Of these 23 isolates, GL12, GL13, and Th23 provided significant suppression of *P. capsici* in at least 2 assays. These isolates were then compared with *Trichoderma virens* isolates GL3 and GL21 for suppression of this disease in the presence and absence of the harpin-based natural product Messenger. Isolates GL3 and Th23 provided significant disease suppression ($P \leq 0.05$) in 3 of 4 assays, while GL12, GL13, and GL21 provided significant suppression in 2 of 4 assays. There was no apparent benefit from the application of Messenger. Phylogenetic analysis of these 5 isolates (based on the ITS1 region of the nuclear rDNA cluster and *tef1*), and an additional 9 isolates that suppressed *P. capsici* in at least 1 assay, separated isolates into 2 clades, with 1 clade containing GL3, GL12, GL13, and GL21. There were also 2 more distantly related isolates, one of which was Th23. We report here the identification of genetically distinct *Trichoderma* isolates for potential use in disease management strategies employing isolate combinations directed at suppression of *P. capsici* on pepper.

Key words: biological control, methyl bromide replacement, pepper, *Phytophthora capsici*, *Trichoderma*, *Trichoderma virens*.

Résumé : Des mesures de contrôle compatibles avec l'environnement sont requises pour enrayer la contamination du poivron par *Phytophthora capsici*. Vingt-trois isolats de *Trichoderma* ont été criblés quant à leur capacité à enrayer un mélange de 4 isolats génétiquement distincts du pathogène sur le poivron (*Capsicum annuum*), lors de tests en serre. De ces 23 isolats, GL12, GL13 et Th23 enrayaient significativement *P. capsici* dans au moins 2 tests. Ces isolats ont alors été comparés aux isolats GL3 et GL21 de *Trichoderma virens* quant à leur potentiel de suppression de cette maladie en présence ou en absence de Messenger, un produit naturel à base d'harpine. Les isolats GL3 et Th23 inhibaient significativement ($P \leq 0.05$) la maladie dans 3 des 4 analyses alors que GL12, GL13, et GL21 l'inhibaient significativement dans 2 des 4 analyses. Il n'y avait pas de bénéfice apparent à l'application de Messenger. L'analyse phylogénique de ces 5 isolats (basée sur la région ITS1 de la grappe d'ADNr nucléaire et de *tef1*) et de 9 isolats supplémentaires qui supprimaient la croissance de *P. capsici* dans au moins 1 analyse, a permis de séparer les isolats en 2 clades, 1 clade contenant GL3, GL12, GL13 et GL21. Il y avait aussi 2 isolats reliés mais plus distants, un de ceux-ci étant Th23. Nous rapportons ici l'identification d'isolats génétiquement distincts de *Trichoderma* potentiellement utilisables dans des stratégies de gestion de la maladie faisant appel à des combinaisons d'isolats visant à enrayer *P. capsici* sur le poivron.

Mots-clés : contrôle biologique, remplacement du bromure de méthyle, poivron, *Phytophthora capsici*, *Trichoderma*, *Trichoderma virens*.

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Introduction

Phytophthora blight, caused by the soil-borne oomycete

pathogen *Phytophthora capsici* Leonian, is an important disease of pepper worldwide, with the polycyclic nature of the disease cycle resulting in massive crop losses in some grow-

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ing seasons (Ristaino and Johnston 1999; Roszkopf et al. 2005). For the past 4 decades, management of soil-borne pathogens of vegetable crops, including *P. capsici* on pepper, has relied heavily on preplant injection of soil with the fumigant methyl bromide (Martin 2003; Roszkopf et al. 2005; Ruzo 2006; Lodovica Gullino et al. 2007). However, methyl bromide usage in vegetable production in the United States has been largely phased out in accordance with the Montreal Protocol. Currently registered chemicals have been reevaluated as replacements for methyl bromide for preplant soil fumigation, but none are considered equivalent substitutes (Martin 2003; Roszkopf et al. 2005; Ruzo 2006). There is no guarantee that currently registered chemicals will remain available for commercial growers or that nonregistered chemical compounds currently in development will be registered for commercial use (Duniway 2002; Noling 2002). This is due to an increased awareness regarding hazards associated with the use of chemical fumigants, which has resulted in a more stringent regulatory environment (Ruzo 2006). Additionally, oomycete pathogens such as *P. capsici* are genetically and biochemically distinct from fungi and are not susceptible to most broad-spectrum fungicides. For these reasons growers are heavily reliant on a limited number of chemical controls, such as the phenylamide fungicide metalaxyl, for *P. capsici* (Lamour and Hausbeck 2000). Insensitivity to metalaxyl has been reported in field populations of *P. capsici* on bell pepper (Lamour and Hausbeck 2000). Further complicating the development of control measures for *P. capsici* is the genetic complexity of this species (Oudemans and Coffey 1991; Mchau and Coffey 1995; Bowers et al. 2007). Considerable potential exists for sexual recombination and additional genetic variation among and within *P. capsici* populations in the United States, as both compatibility types (A1, A2) have been detected in the same field (Ristaino 1990; Lamour and Hausbeck 2000, 2001).

'Paladin', a cultivar of pepper with resistance to Phytophthora blight, is available, but plants are only resistant to the soil-borne phase of this disease (Chellemi 2002; Hausbeck and Lamour 2004). Continued research has identified several quantitative trait loci in pepper that confer resistance to the root-rot and foliar blight diseases of pepper (Ogundiwin et al. 2005). However, no pepper accessions showing complete resistance to *P. capsici* have been found (Bonnet et al. 2007). Other strategies to control Phytophthora blight are ecologically based and rely on the prevention of a build-up of inoculum through crop rotation and on controlling water movement in production fields, as *P. capsici* is dispersed by rain splash and surface water (Bowers et al. 1990; Ristaino et al. 1992; Ristaino and Johnston 1999). Safe, environmentally compatible, and economically feasible replacements for methyl bromide are clearly needed for controlling soil-borne plant pathogens of pepper such as *P. capsici* (Roszkopf et al. 2005).

Biological control of soil-borne plant pathogens has attracted significant recent attention as an alternative disease management strategy because of its ability to provide environmentally friendly disease control, particularly when included in an integrated pest management strategy. Unfortunately, inconsistent performance has hampered the commercial development and application of these microbes

(Meyer and Roberts 2002; Alabouvette et al. 2006). Inconsistent performance has been attributed to genetic diversity of the target pathogen, varying initial population levels of the target pathogen, interactions with nontarget organisms, varying rhizosphere or soil colonization by the biological control agent, host plant species and cultivar effects, and varying physical and chemical composition of the soil or rhizosphere (Stirling 1991; Ownley et al. 1992; Boeger et al. 1993; Sikora and Hoffman-Hergarten 1993; Pierson and Weller 1994; Kerry and Bourne 1996; Raupach and Kloepper 1998; Schisler et al. 2000; Bowers et al. 2007). An approach to overcome inconsistent performance is to combine 2 or more genetically distinct microbes in a biological control preparation (Pierson and Weller 1994; Raupach and Kloepper 1998). A combination of biological control agents is more likely to have a greater variety of traits responsible for suppression of a genetically diverse population of a single pathogen or multiple pathogens. Combinations of biological control agents are also more likely to have these traits expressed over a wide range of soil environmental conditions (Lemanceau and Alabouvette 1991; Lemanceau et al. 1993; Pierson and Weller 1994; Compant et al. 2005). The objective of this study was to identify genetically diverse isolates of *Trichoderma* with disease suppressive capabilities against a collection of isolates of *P. capsici* on bell pepper. These *Trichoderma* isolates will be used in future studies where combinations of isolates are analyzed for suppression of Phytophthora blight on pepper in a number of different soil environments.

Materials and methods

Microbial isolates

See Table 1 for *Trichoderma* isolates used in this study and Table 2 for *P. capsici* isolates used in this study. All *Trichoderma* and *P. capsici* isolates were maintained on potato dextrose agar at room temperature and are from the culture collection at the Sustainable Agricultural Systems Laboratory, Beltsville, Maryland.

Preparation of *P. capsici* inoculum and biological control agents

Petri dishes containing corn meal agar (Becton Dickinson, Sparks, Md.) were inoculated with *P. capsici* isolates R1198, RA4, 223, and R899 (2 plates per isolate) and the plates incubated at room temperature for 5 days under light. Four 1 cm² pieces of clarified V8 Juice (CV8) agar containing hyphae of an individual *P. capsici* isolate were added to a separate mycobag (Unicorn Imp. and Mfg. Corp., Commerce, Tex.) for each isolate and incubated at room temperature for 2–4 weeks prior to use in disease suppression assays. Mycobags were filled with 100 g Biodac (20/50; Kadant Gran Tek, Inc., Green Bay, Wis.); amended with CV8 (5.25 g CaCO₃ was added to 340 mL CV8 Juice, the volume brought to 350 mL with sterile distilled water, the mixture centrifuged at 8000g for 20 min, and agar added to the supernatant to a 1.5% final concentration; Bowers and Mitchell 1990) at a ratio of 100 g Biodac to 100 mL CV8; and autoclaved for 60 min on 2 consecutive days prior to inoculation. Mycobags containing Biodac, prepared as above,

Table 1. *Trichoderma* isolates used in this study.

Isolate	Prior designation ^a	Source ^b	Features ^c	DNA accession No. ^d
<i>T. hamatum</i> Tri-4		China		
<i>Trichoderma</i> sp. Th1				
<i>T. harzianum</i> Th23	ATCC MYA-647			HM991711
<i>Trichoderma</i> sp. Th24				
<i>T. harzianum</i> Th32				
<i>Trichoderma</i> sp. Th38				
<i>T. virens</i> Th66	<i>T. harzianum</i> Th66			GU046488/HM991714
<i>T. virens</i> Th89	<i>T. harzianum</i> Th89			GU046486/HM991713
<i>T. virens</i> Th91	<i>T. harzianum</i> Th91			GU046489/HM991712
<i>T. harzianum</i> 104				GU046490/HM991710
<i>T. virens</i> GL1	G1, GVR J-1	C. Howell, College Station, Tex.		GU046497/HM991721
<i>T. virens</i> GL2	G2	Soil from Beltsville, Md.		GU046496/HM991723
<i>T. virens</i> GL3	G3	Isolated from <i>Sclerotium rolfsii</i> in Beltsville, Md. soil	Viridin, glioviren	AF099006/HM991718
<i>T. virens</i> GL4	G4, GL4A2			GU046493
<i>T. virens</i> GL5	G5, MTD356-14	Soil from Beltsville, Md.		
<i>T. virens</i> GL7	G7, MTD29-7	Soil from Beltsville, Md.		
<i>T. virens</i> GL8	G8, GV8	Isolated from <i>Sclerotinia minor</i> in soil from Beltsville, Md.		GU046495
<i>T. virens</i> GL9	G9, GVP	C. Howell, isolated from <i>Rhizoctonia solani</i> in College Station, TX soil	Viridin, glioviren	GU046494/HM991715
<i>T. virens</i> GL10	G10, GVMT			
<i>T. virens</i> GL12	G12, MTD356-11	Soil from Beltsville, Md.		GU046492/HM991716
<i>T. virens</i> GL13	G13, MTD290-18	Soil from Beltsville, Md.		GU046491/HM991722
<i>T. virens</i> GL14	G14, MTD138-10	Soil from Beltsville, Md.		HM991720
<i>T. virens</i> GL15	G15, MTD189-10	Soil from Beltsville, Md.		
<i>T. virens</i> GL16	G16, MTD31-10	Soil from Beltsville, Md.		
<i>T. virens</i> GL21	G20		Viridin, gliotoxin	AF099008/HM991717

^aPrior isolate names occurring in the literature or at the American Type Culture Collection.

^bAll isolates are from the culture collection at the Sustainable Agricultural Systems Laboratory. Additional information regarding the source for each isolate is provided as known.

^cAntibiotics produced. Information is from Lumsden et al. (1992).

^dDNA accessions used in strain identification and phylogenetic analysis.

Table 2. Isolates of *Phytophthora capsici* used in this study.

Isolate	Host	Origin	AFLP clade ^a	ITS subclade ^a
R899	Bell pepper	New Jersey	A	nd
R1198	Bell pepper	New Jersey	A	A3
223 ^b	Bell pepper	New Jersey	A	nd
RA4	Bell pepper	New Jersey	A	A1

Note: nd, not determined.

^aAFLP and sequence analysis of the internal transcribed spacer (ITS) region of rDNA (Bowers et al. 2007).

^bEvidence existed to place isolate 223 in a separate clade (Bowers et al. 2007).

were inoculated with individual biological control agents and incubated at room temperature 2–4 weeks prior to use.

Disease suppression assay

Pepper seed (*Capsicum annuum* L., Enterprise hybrid) treated with Thiram was sown in starter cells (2 × 2 × 2.25 in. (1 in. = 25.4 mm); T.O. Plastics, Inc., Clearwater, Minn.) containing potting mix (PGX ProMix, Premier Horticulture, Inc., Quakertown, Pa.) amended with nothing, Biodac alone (0.1%, v/v), or with Biodac colonized with biological control agents (0.1%, v/v) and grown in the greenhouse for 8 weeks prior to transplant. Water-soluble 20–20–20 (N₂–P₂O₅–K)

fertilizer was applied weekly. Natural soil (compost:sand mixture, 1:7) was moistened with tap water (1 L per 10 L soil) and equilibrated for 1 week prior to transplant. *Phytophthora capsici* in Biodac was added to moistened soil 2 days prior to transplant at a rate of 0.15 g/L soil. Individual pepper plants, 8–10 weeks old, in potting mix (non-amended or amended with Biodac or with Biodac plus biological control agents) formed by starter cells were transplanted into empty 3 in. pots, and the remaining volume of the pot was filled with soil infested with *P. capsici* or non-infested soil as a control. Biological control agents were also applied to the appropriate treatment as a 10 mL drench

($\sim 10^6$ colony-forming units/mL in sterile distilled water) to the soil 1 week prior to transplant and at transplant. The number of colony-forming units of biological control agents in the drench was determined by dilution-plating onto TME (Papavizas and Lumsden 1982). The natural product Messenger (Eden Bioscience, Woodenville, Wash.) was applied individually or in combination with *Trichoderma* isolates. When used, Messenger was applied according to the manufacturer's instructions as a seed treatment and as a foliar application 1 week prior to transplant. Messenger was included to determine whether the use of an additional control tactic would improve disease suppression. Each experiment consisted of 20 replicate pots per treatment, with one plant per pot, arranged in a completely randomized design. Days to symptom expression was determined for each plant for the 14 day period after transplant. Kaplan and Meier (1958) logistic disease progression curves were fitted to the data using SAS version 9.2 LIFETEST (SAS Institute Inc. 2007), which also provided estimates and 95% confidence intervals for median disease progression (i.e., days to 50% symptomatic plants) using the product-limit method (Kalbfleisch and Prentice 1980). Treatments were compared by comparing days to 50% symptomatic plants and considered significant at $P < 0.05$. Experiments were analyzed independently.

Cloning and sequencing the ITS1 region and *tefl* from *Trichoderma* spp.

Genomic DNA isolation from *Trichoderma* isolates GL1, GL2, GL3, GL4, GL8, GL9, GL12, GL13, GL21, Th23, Th66, Th89, Th91, and Th104 was by standard protocols (Sambrook and Russell 2001). The ITS1 region of the nuclear rDNA cluster was amplified from these genomic preparations using primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTA-A-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990; Gardes and Bruns 1993). A 258 bp fragment of *tefl* was amplified using primers EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') (Druzhinina et al. 2005; Jaklitsch et al. 2006). Two microlitres of genomic DNA (0.384 to 1.319 $\mu\text{g DNA}/\mu\text{L}$) was added to 23 μL PCR mixture containing 0.4 $\mu\text{mol/L}$ of each primer and 1 \times AmpliTaq Gold PCR Master Mix (6.25 U AmpliTaq Gold DNA polymerase, 2.5 mmol/L MgCl_2 , and 200 $\mu\text{mol/L}$ of each dNTP). PCR amplification was performed in an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, Westbury, N.Y.). The amplification program consisted of an initial 5 min denaturation step at 94 °C; 35 cycles at 94 °C for 30 s, 44 °C (for the ITS1 region) or 48 °C (for the *tefl* fragment) for 30 s, and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. Following amplification, PCR products were electrophoresed in 1% agarose gels buffered with 1 \times TAE containing 0.01 mg/mL ethidium bromide. ITS1 and *tefl* amplicons were purified from agarose gels using the UltraClean15 DNA purification kit (MoBio Laboratories Inc., Solana Beach, Calif.) and cloned using the pGEM-T Easy Vector System (Promega Corporation, Madison, Wis.). *Escherichia coli* DH5 α transformants containing the cloned amplicons were selected on Luria-Bertani agar plates with 100 $\mu\text{g/mL}$ ampicillin, 0.5 mmol/L IPTG (isopropyl β -D-1-thiogalactopyranoside), and 80 $\mu\text{g/mL}$ X-gal

(5-bromo-4-chloro-3-indolyl- β -galactopyranoside). Recombinant plasmid DNA was isolated from overnight cultures using the UltraClean 6 Minute Mini Plasmid Prep Kit (MoBio Laboratories Inc.). DNA from these plasmid inserts was amplified using M13 forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa) and sequenced using BigDye Terminator v1.1 or v 3.1 Cycle Sequencing Kits (Applied Biosystems Inc., Foster City, Calif.). Sequencing reactions were run for the respective forward and reverse primers for the ITS1 region and *tefl*. Sequencing reactions were purified using the Performa DTR Ultra 96-Well Plate Kit (EdgeBio, Gaithersburg, Md.) and analyzed on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, Calif.). Sequences were trimmed of vector sequence, quality checked, and aligned using the DNASTar suite of software (DNASTar, Madison, Wis.). Contigs were created using paired forward and reverse sequencing reads, and the consensus sequence was used for phylogenetic analysis.

Phylogenetic analysis

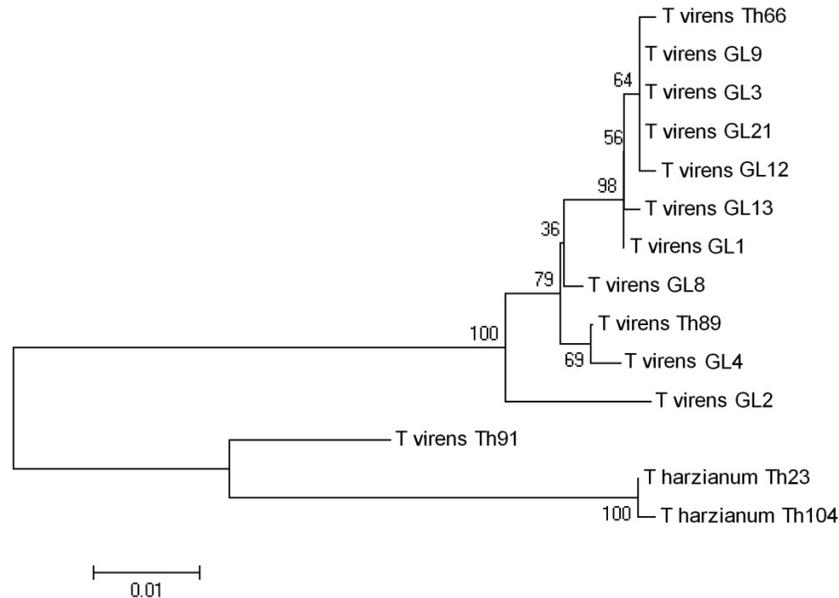
For species identification a batch of FASTA files containing an ITS sequence from the 14 isolates (listed above) were submitted to GenBank using trichOKEY (<http://www.isth.info/tools/molkey>). These sequences were automatically scanned against the 104 vouchered *Hypocreales/Trichoderma* sequences. For phylogenetic analysis consensus sequences for the ITS1 region and *tefl* for each *Trichoderma* strain were individually queried against the NCBI nonredundant database using BLAST. The 15 accessions with the highest bit scores were cataloged, and the FASTA output from each was used to create a local database. This database was later used to populate an input file for subsequent multiple sequence alignments and phylogenetic tree building using the DNASTar suite of software and MEGA4 (Tamura et al. 2004). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987; Tamura et al. 2004). The evolutionary distances were computed using the maximum composite likelihood method and are measured as the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). Phylogenetic analyses were conducted in MEGA4. There were a total of 400 positions in the final data set shown in Fig. 1. The ITS1 region and *tefl* sequences from each organism were concatenated into a single test sequence, as shown in Fig. 2. There were a total of 681 positions in the final data set.

Results

Screening of *Trichoderma* isolates for suppression of *P. capsici* on pepper

Twenty-three isolates from 3 species of *Trichoderma* (*Trichoderma hamatum*, *Trichoderma harzianum*, *Trichoderma virens*) and a variety of host – geographic location combinations were screened for suppression of *P. capsici* on bell pepper. Certain biochemically characterized isolates were included that also varied in antibiotic production, some isolates producing gliotoxin and others producing glioviren (Table 1). These fungi were applied in the planting medium

Fig. 2. Evolutionary relationships of 14 of the *Trichoderma* isolates tested using concatenated ITS1 and *tef1* sequence data. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree, inferred from 500 replicates, was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to each branch. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.



respective second screen runs and were selected for further testing. Isolate Th89 was not run in a second screen despite providing significant disease suppression in the first screen run. There was no evidence of phytotoxicity with any of the 23 isolates tested.

Isolates Th23, GL12, and GL13 were compared with *T. virens* isolates GL3 and GL21 for suppression of *P. capsici* on bell pepper in four greenhouse pot assays (Table 4). These isolates were applied in the planting medium as a drench 1 week prior to transplant and as a drench at transplant in these experiments. GL3 was shown to suppress *P. capsici* on bell pepper in previous work (Mao et al. 1998), while GL21 is the strain in the commercial biological control product SoilGard. Isolates GL3 and Th23 provided significant suppression of *P. capsici* in 3 of 4 runs (Runs 10, 11, and 13), while isolates GL12, GL13, and GL21 provided significant suppression in 2 of 4 runs (Runs 10 and 11). Application of the harpin-based product Messenger, alone or in combination with these isolates, failed to improve disease suppression (Table 4). Messenger was included to determine whether the use of an additional control tactic would improve disease suppression. There was no evidence of phytotoxicity with any of the treatments in any experiment.

Phylogenetic analysis of promising *Trichoderma* isolates

All isolates that performed well in their first screen as well as GL3 and GL21 were identified with high reliability as being from the genus *Hypocrea/Trichoderma*. Each individual analysis identified a minimum of the first 4 of 5 barcodes used for species discrimination (<http://www.isth.info/tools/molkey>). Two isolates, Th104 and Th23, were identified as *Hypocrea lixii/Trichoderma harzianum*, while the remaining isolates (Th66, Th89, Th91, GL1, GL2, GL3, GL4,

GL8, GL9, GL12, GL13, GL21) were identified as *Hypocrea virens/Trichoderma virens*. These results were verified by the phylogenetic relationship trees reported in Fig. 1, in which isolates Th66, Th89, Th91, GL1, GL2, GL3, GL4, GL8, GL9, GL12, GL13, and GL21 clustered with other previously reported *Hypocrea virens/Trichoderma virens* species. Isolates Th23 and Th104 clustered with isolates previously reported to be *Hypocrea lixii/Trichoderma harzianum* species.

Phylogenetic analysis was conducted with these isolates using sequence from the ITS1 region of the nuclear rDNA cluster (Fig. 1). *Trichoderma virens* isolates separated into 2 major clades of closely related isolates consisting of GL1, GL3, GL9, GL12, GL13, GL21, Th66, and Th91 and of GL2, GL4, GL8, and Th89. The *T. harzianum* isolates, Th23 and Th104, were more distantly related. The concatenation of phylogenetically informative genes (the ITS1 region and *tef1*) yielded higher resolution discrimination between the closely related *Trichoderma* isolates. The inferred tree (Fig. 2) was generally consistent with the groupings identified in Fig. 1, with the exception of Th91. The shift in positioning of Th91 with the inclusion of the *tef1* sequence suggests that there may be additional genetic diversity contained within the genome of this strain, which makes it a potentially promising *Trichoderma* isolate.

Discussion

The long-term goal of this project is to develop technically and economically feasible disease management strategies for use as alternatives to methyl bromide for the high-value pepper crop. Microbial biological control shows promise for use in integrated disease management strategies, where these microbes are combined with other microbial biological con-

Table 3. Days to 50% symptomatic plants in a greenhouse pot assay of *Trichoderma* spp. isolates for suppression of *Phytophthora capsici* on bell pepper.

Isolate	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9
HCK	—, —, —	—, —, —	—, —, —	—, —, —	—, —, —	—, —, —	—, —, —	—, —, —	—, —, —
PathCK	8.0, 8.0, 15.0	3.0, 3.0, 6.0	0.0, 0.0, 6.0	0.0, 6.5, 7.0	5.0, 11.0, 12.0	0.0, 5.0, 6.0	4.0, 5.0, 6.0	4.0, 6.0, 7.0	4.0, 4.0, 7.0
Tri-4	8.0, 13.0, 15.0								
Th1	8.0, 15.0, 15.0								
Th23	11.0, 15.0, 15.0				12.0, 17.0, 17.0*	5.0, 6.0, 7.0			
Th24	8.0, 9.5, 14.0								
Th32	8.0, 9.5, 15.0								
Th38	8.0, 12.5, 15.0								
Th66		6.0, 7.0, 7.0*			10.0, 12.5, 14.0				4.0, 4.0, 7.0
Th89		6.0, 7.0, 7.0*							
Th91		6.0, 7.0, 7.0*			10.0, 11.0, 13.0				
Th104		6.0, 7.0, 7.0*			10.0, 11.0, 13.0				
GL1							6.0, 8.0, 9.0*	6.0, 7.0, 8.0	
GL2							6.0, 8.0, 9.0*	5.0, 7.0, 8.0	
GL4							6.0, 6.0, 8.0*	4.0, 7.0, 8.0	
GL5							5.0, 6.0, 6.0		
GL7							5.0, 6.0, 7.0		
GL8			6.0, 8.0, 13.0*						4.0, 4.0, 6.0
GL9			6.0, 7.0, 8.0*						4.0, 5.0, 7.0
GL10			5.0, 12.0, 15.0				5.0, 6.0, 7.0		
GL12			—, 18.0, —*				6.0, 6.0, 7.0*		
GL13			8.0, 18.0, 18.0*				7.0, 7.0, 8.0*		
GL14			5.0, 8.0, 8.0						
GL15				6.0, 9.5, 10.0					
GL16				0.0, 9.0, 10.0					

Note: Treatments consisted of 20 replicate pots each containing a single bell pepper plant. Time to symptom expression was determined for each plant. Symptom expression on 50% of plants was used to determine differences among treatments. HCK, healthy check, no *P. capsici* or biological control agent; PathCK, *P. capsici* was added but no biological control agent. 95% confidence limits: lower 95% limit, median, upper 95% limit. “—” for upper and lower 95% limits indicates that there was no variability; all plants within this treatment behaved the same. “—” for the median indicates that the percentage of disease-free plants never dropped below 50% during the experiment. *, significant disease suppression ($P \leq 0.05$) by this treatment in this run relative to the pathogen check (PathCK).

Table 4. Days to 50% symptomatic plants for treatments containing *Trichoderma* spp. isolates with and without Messenger for suppression of *Phytophthora capsici* on bell pepper in greenhouse pot assays.

Isolate	<i>P. capsici</i>	Run 10	Run 11	Run 12	Run 13
No treatment control	No	—, —, —	—, —, —	—, —, —	—, —, —
Biodac only control	No	—, —, —	—, —, —	—, —, —	—, —, —
Biodac + Messenger control	No	nd	nd	—, —, —	—, 12.0, —
No treatment control	Yes	0.0, 2.5, 5.0	—, 8.0, —	nd	nd
Biodac only control	Yes	—, 0.0, —	6.0, 6.5, 8.0	3.0, 6.0, 8.0	0.0, 6.5, 8.0
<i>Trichoderma</i> sp. Th23	Yes	4.0, 5.0, 6.0*	8.0, 10.5, 15.0*	3.0, 5.5, 9.0	11.0, 12.0, 12.0*
<i>T. virens</i> GL3	Yes	5.0, 6.0, 8.0*	—, 15.0, —*	6.0, 7.0, 8.0	8.0, 12.0, 12.0*
<i>T. virens</i> GL12	Yes	7.0, 8.0, 12.0*	8.0, 9.0, 13.0*	5.0, 7.0, 8.0	0.0, 4.0, 8.0
<i>T. virens</i> GL13	Yes	5.0, 6.0, 8.0*	13.0, 15.0, 15.0*	6.0, 7.0, 8.0	6.0, 7.0, 8.0
<i>T. virens</i> GL21	Yes	4.0, 5.0, 5.0*	8.0, 11.0, 15.0*	3.0, 8.0, 9.0	6.0, 7.0, 8.0
Messenger	Yes	nd	nd	3.0, 3.0, 8.0	8.0, 12.0, 12.0*
<i>Trichoderma</i> sp. Th23 + Messenger	Yes	nd	nd	7.0, 8.0, 9.0	8.0, 12.0, 12.0*
<i>T. virens</i> GL3 + Messenger	Yes	nd	nd	5.0, 6.5, 7.0	6.0, 7.0, 8.0
<i>T. virens</i> GL12 + Messenger	Yes	nd	nd	6.0, 7.5, 8.0	4.0, 7.0, 8.0
<i>T. virens</i> GL13 + Messenger	Yes	nd	nd	5.0, 6.5, 8.0	7.0, 8.0, 8.0
<i>T. virens</i> GL21 + Messenger	Yes	nd	nd	5.0, 7.0, 9.0	nd

Note: Treatments consisted of 20 replicate pots each containing a single bell pepper plant. Time to symptom expression was determined for each plant. Symptom expression on 50% of plants was used to determine differences among treatments. 95% confidence limits: lower 95% limit, median, upper 95% limit. “—” for upper and lower 95% limits indicates that there was no variability; all plants within this treatment behaved the same. “—” for the median indicates that the percentage of disease-free plants never dropped below 50% during the 14 day experiment. nd, not determined. *, significant disease suppression ($P \leq 0.05$) by this treatment in this run relative to the Biodac only plus *P. capsici* control.

control agents, cover crops, resistant pepper varieties, or reduced levels of chemical pesticides (Chellemi 2002; Jacobsen et al. 2004; Roskopf et al. 2005). Key to development of this multitactic disease control strategy is the establishment of a collection of genetically distinct microbial biological control agents that show promise for suppression of the target disease. We report here the identification of several *Trichoderma* isolates with capacity for suppression of a genetically diverse population of *P. capsici* on bell pepper. Isolates GL3 and Th23 provided the most consistent experiment to experiment disease suppression, while isolates GL12, GL13, and GL21 showed some promise (Tables 3 and 4). Isolate GL3 was previously reported to provide suppression of *P. capsici* on bell pepper (Mao et al. 1998) and has the additional benefit of suppression of *Meloidogyne incognita* on bell pepper (Meyer et al. 2001). Isolates GL3, GL12, GL13, and GL21 are genetically distinct but separated into a single clade, with GL13 showing the greatest difference within the clade. Additionally, isolates GL3 and GL21 differ in production of antibiotic compounds (Lumsden et al. 1992). Isolate GL3 produced viridin, gliovirin, and viridiol, while GL21 produced viridin, gliotoxin, dimethylgliotoxin, and viridiol in a soilless planting medium. Th23 was more distantly related to these other 4 isolates based on ITS1 and *tefl* sequence analysis (Figs. 1 and 2).

Future work will involve PCR, targeting genes for production of antibiotic compounds, and biochemically based experiments to determine potential mechanisms (Chung et al. 2008) used by these 5 isolates for suppression of *P. capsici*. Experiments to determine persistence and disease suppression in soils with differing edaphic properties will also be conducted. Isolates GL1, GL2, GL4, GL8, GL9, Th66, Th89, Th91, and Th104 will be included to increase germplasm diversity, making it more likely to detect isolates

with differing disease suppression mechanisms and capabilities in broadly different soils. This new information will be used to make strategic combinations of these isolates for more consistent biological control over broad soil physical-chemical conditions. These isolates will also be used in combination with resistant pepper varieties in future studies. Integration of microbial biological control agents into strategies utilizing resistant varieties has resulted in enhanced disease suppression in previous studies when the resistant varieties did not provide high levels of disease resistance (Jacobsen et al. 2004). Other potential uses for these isolates are their integration into disease management strategies employing cover crops with disease suppression capabilities or reduced levels of chemical pesticides. Cover crops with activity against *P. capsici* have been reported (Kim et al. 1997; Ristaino et al. 1997).

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References

- Alabouvette, C., Olivain, C., and Steinberg, C. 2006. Biological control of plant diseases: the European situation. *Eur. J. Plant Pathol.* **114**(3): 329–341. doi:10.1007/s10658-005-0233-0.
- Boeger, J.M., Chen, R.S., and McDonald, B. 1993. Gene flow between geographic populations of *Mycosphaerella graminicola* (anamorpha *Septoria tritici*) detected with restriction fragment length polymorphism markers. *Phytopathology*, **83**(11): 1148–1154. doi:10.1094/Phyto-83-1148.

- Bonnet, J., Danan, S., Boudet, C., Barchi, L., Sage-Palloix, A.M., Caromel, B., et al. 2007. Are the polygenic architectures of resistance to *Phytophthora capsici* and *P. parasitica* independent in pepper? *Theor. Appl. Genet.* **115**(2): 253–264. doi:10.1007/s00122-007-0561-x. PMID:17497121.
- Bowers, J.H., and Mitchell, D.J. 1990. Effect of soil-water matric potential and periodic flooding on mortality of pepper caused by *Phytophthora capsici*. *Phytopathology*, **80**(12): 1447–1450. doi:10.1094/Phyto-80-1447.
- Bowers, J.H., Sonoda, R.M., and Mitchell, D.M. 1990. Path coefficient analysis of the effect of rainfall variables on the epidemiology of *Phytophthora* blight of pepper caused by *Phytophthora capsici*. *Phytopathology*, **80**(12): 1439–1446. doi:10.1094/Phyto-80-1439.
- Bowers, J.H., Martin, F.N., Tooley, P.W., and Luz, E.D.M.N. 2007. Genetic and morphological diversity of temperate and tropical isolates of *Phytophthora capsici*. *Phytopathology*, **97**(4): 492–503. doi:10.1094/PHYTO-97-4-0492. PMID:18943290.
- Chellemi, D.O. 2002. Nonchemical management of soilborne pests in fresh market vegetable production systems. *Phytopathology*, **92**(12): 1367–1372. doi:10.1094/PHYTO.2002.92.12.1367. PMID:18943895.
- Chung, S., Kong, H., Buyer, J.S., Lakshman, D.K., Lydon, J., Kim, S.-D., and Roberts, D.P. 2008. Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. *Appl. Microbiol. Biotechnol.* **80**(1): 115–123. doi:10.1007/s00253-008-1520-4. PMID:18542950.
- Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E.A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* **71**(9): 4951–4959. doi:10.1128/AEM.71.9.4951-4959.2005. PMID:16151072.
- Druzhinina, I.S., Kopchinskiy, A.G., Komoń, M., Bissett, J., Szakacs, G., and Kubicek, C.P. 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol.* **42**(10): 813–828. doi:10.1016/j.fgb.2005.06.007. PMID:16154784.
- Duniway, J.M. 2002. Status of chemical alternatives to methyl bromide for pre-plant fumigation of soil. *Phytopathology*, **92**(12): 1337–1343. doi:10.1094/PHYTO.2002.92.12.1337. PMID:18943890.
- Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes — application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**(2): 113–118. doi:10.1111/j.1365-294X.1993.tb00005.x. PMID:8180733.
- Hausbeck, M.K., and Lamour, K.H. 2004. *Phytophthora capsici* on vegetable crops: research progress and management challenges. *Plant Dis.* **88**(12): 1292–1303. doi:10.1094/PDIS.2004.88.12.1292.
- Jacobsen, B.J., Zidack, N.K., and Larson, B.J. 2004. The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. *Phytopathology*, **94**(11): 1272–1275. doi:10.1094/PHYTO.2004.94.11.1272. PMID:18944466.
- Jaklitsch, W.M., Komoń, M., Kubicek, C.P., and Druzhinina, I.S. 2006. *Hypocrea crystalligena* sp. nov., a common European species with a white-spored *Trichoderma* anamorph. *Mycologia*, **98**(3): 499–513. doi:10.3852/mycologia.98.3.499. PMID:17040079.
- Kalbfleish, J.D., and Prentice, R.L. 1980. The statistical analysis of failure time data. John Wiley and Sons, New York, N.Y.
- Kaplan, E.L., and Meier, P. 1958. Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**(282): 457–481. doi:10.2307/2281868.
- Kerry, B.R., and Bourne, J.M. 1996. The importance of rhizosphere interactions in the biological control of plant-parasitic nematodes—case study using *Verticillium chlamydosporium*. *Pestic. Sci.* **47**(1): 69–75. doi:10.1002/(SICI)1096-9063(199605)47:1<69::AID-PS386>3.0.CO;2-6.
- Kim, K.D., Nemeč, S., and Musson, G. 1997. Control of *Phytophthora* root and crown rot of bell pepper with composts and soil amendments in the greenhouse. *Appl. Soil Ecol.* **5**(2): 169–179. doi:10.1016/S0929-1393(96)00138-2.
- Lamour, K.H., and Hausbeck, M.K. 2000. Mefenoxam insensitivity and the sexual stage of *Phytophthora capsici* in Michigan cucurbit fields. *Phytopathology*, **90**(4): 396–400. doi:10.1094/PHYTO.2000.90.4.396.
- Lamour, K.H., and Hausbeck, M.K. 2001. Investigating the spatio-temporal genetic structure of *Phytophthora capsici* in Michigan. *Phytopathology*, **91**(10): 973–980. doi:10.1094/PHYTO.2001.91.10.973. PMID:18944124.
- Lemanceau, P., and Alabouvette, C. 1991. Biological control of Fusarium diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. *Crop Prot.* **10**(4): 279–286. doi:10.1016/0261-2194(91)90006-D.
- Lemanceau, P., Bakker, P.A.H.M., de Kogel, W.J., Alabouvette, C., and Schippers, B. 1993. Effect of pseudobactin 358 production by *Pseudomonas putida* on suppression of Fusarium wilt of carnations by nonpathogenic *Fusarium oxysporum* Fo47. *Appl. Environ. Microbiol.* **58**: 2978–2982.
- Lodovica Gullino, M., Savigliano, R., Gasparrini, G., and Clini, C. 2007. Critical Use Exemption for methyl bromide for soil disinfection: Italy's experience with the European Union process. *Phytoparasitica*, **35**(4): 321–329. doi:10.1007/BF02980693.
- Lumsden, R.D., Ridout, C.J., Vendemia, M.E., Harrison, D.J., Waters, R.M., and Walter, J.F. 1992. Characterization of major secondary metabolites produced in soilless mix by a formulated strain of the biocontrol fungus *Gliocladium virens*. *Can. J. Microbiol.* **38**(12): 1274–1280. doi:10.1139/m92-210.
- Mao, W., Lewis, J.A., Lumsden, R.D., and Hebbbar, K.P. 1998. Biocontrol of selected soilborne diseases of tomato and pepper plants. *Crop Prot.* **17**(6): 535–542. doi:10.1016/S0261-2194(98)00055-6.
- Martin, F.N. 2003. Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annu. Rev. Phytopathol.* **41**(1): 325–350. doi:10.1146/annurev.phyto.41.052002.095514. PMID:14527332.
- Mchaur, G.R.A., and Coffey, M.D. 1995. Evidence for the existence of two subpopulations in *Phytophthora capsici* and a redescription of the species. *Mycol. Res.* **99**(1): 89–102. doi:10.1016/S0953-7562(09)80321-3.
- Meyer, S.L.F., and Roberts, D.P. 2002. Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. *J. Nematol.* **34**(1): 1–8. PMID:19265899.
- Meyer, S.L.F., Roberts, D.P., Chitwood, D.J., Carta, L.K., Lumsden, R.D., and Mao, W. 2001. Application of *Burkholderia cepacia* and *Trichoderma virens*, alone and in combinations, against *Meloidogyne incognita* on bell pepper. *Nematropica*, **31**: 75–86.
- Noling, J.W. 2002. The practical realities of alternatives to methyl bromide: concluding remarks. *Phytopathology*, **92**(12): 1373–1375. doi:10.1094/PHYTO.2002.92.12.1373. PMID:18943896.
- Ogundiwin, E.A., Berke, T.F., Massoudi, M., Black, L.L., Huestis, G., Choi, D., et al. 2005. Construction of 2 intraspecific linkage maps and identification of resistance QTLs for *Phytophthora capsici* root-rot and foliar-blight diseases of pepper (*Capsicum*

- annuum* L.). Genome, **48**(4): 698–711. doi:10.1139/g05-028. PMID:16094437.
- Oudemans, P., and Coffey, M.D. 1991. A revised systematics of twelve papillate *Phytophthora* species based on isozyme analysis. Mycol. Res. **95**(9): 1025–1046. doi:10.1016/S0953-7562(09)80543-1.
- Ownley, B.H., Weller, D.M., and Aldredge, J.R. 1992. Relation of soil chemical and physical factors with suppression of take-all by *Pseudomonas fluorescens* 2-79. In Plant growth-promoting rhizobacteria: progress and prospects. Edited by C. Keel, B. Koller, and G. Defago. IOBC/WPRS Bull. 14. pp. 299–301.
- Papavizas, G.C., and Lumsden, R.D. 1982. Improved medium for isolation of *Trichoderma* spp. from soil. Plant Dis. **66**: 1019–1020.
- Pierson, E.A., and Weller, D.M. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology, **84**(9): 940–947. doi:10.1094/Phyto-84-940.
- Raupach, G.S., and Kloepper, J.W. 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology, **88**(11): 1158–1164. doi:10.1094/PHYTO.1998.88.11.1158. PMID:18944848.
- Ristaino, J.B. 1990. Intraspecific variation among isolates of *Phytophthora capsici* from pepper and cucurbit fields in North Carolina. Phytopathology, **80**(11): 1253–1259. doi:10.1094/Phyto-80-1253.
- Ristaino, J.B., and Johnston, S.A. 1999. Ecologically based approaches to management of Phytophthora blight on bell pepper. Plant Dis. **83**(12): 1080–1089. doi:10.1094/PDIS.1999.83.12.1080.
- Ristaino, J.B., Hord, M.J., and Gumpertz, M.L. 1992. Population densities of *Phytophthora capsici* in field soils in relation to drip irrigation, rainfall, and disease incidence. Plant Dis. **76**: 1017–1024.
- Ristaino, J.B., Parra, G., and Campbell, C.L. 1997. Suppression of Phytophthora blight in bell pepper by a no-till wheat cover crop. Phytopathology, **87**(3): 242–249. doi:10.1094/PHYTO.1997.87.3.242. PMID:18945166.
- Roskopf, E.N., Chellemi, D.O., Kokalis-Burelle, N., and Church, G.T. 2005. Alternatives to methyl bromide: a Florida perspective [online]. USDA Horticultural Research Laboratory, Fort Pierce, Fla. Available from <http://www.apsnet.org/publications/apsnetfeatures/Documents/2005/MethylBromideAlternatives.pdf> [accessed 25 August 2010].
- Ruzo, L.O. 2006. Physical, chemical and environmental properties of selected chemical alternatives for the pre-plant use of methyl bromide as soil fumigant. Pest Manag. Sci. **62**(2): 99–113. doi:10.1002/ps.1135. PMID:16308867.
- Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**(4): 406–425. PMID:3447015.
- Sambrook, J., and Russell, D.W. 2001. Molecular cloning. A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory, Woodbury, N.Y.
- SAS Institute Inc. 2007. SAS version 9.2 [computer program]. SAS Institute Inc., Cary, N.C.
- Schisler, D.A., Slininger, P.J., Hanson, L.E., and Loria, R. 2000. Potato cultivar, pathogen isolate and antagonist cultivation medium influence the efficacy and ranking of bacterial antagonists of Fusarium dry rot. Biocontrol Sci. Technol. **10**(3): 267–279. doi:10.1080/09583150050044547.
- Sikora, R.A., and Hoffman-Hergarten, S. 1993. Biological control of plant-parasitic nematodes with plant-health promoting rhizobacteria. In Pest management: biologically based technologies. Proceedings of Beltsville Symposium XVIII. Edited by R.D. Lumsden and J.L. Vaughn. American Chemical Society, Washington D.C. pp. 166–172.
- Stirling, G.R. 1991. Biological control of plant-parasitic nematodes. CAB International, Wallingford, UK.
- Tamura, K., Nei, M., and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U.S.A. **101**(30): 11030–11035. doi:10.1073/pnas.0404206101. PMID:15258291.
- White, T.J., Bruns, T.D., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: a guide to methods and applications. Edited by M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White. Academic Press, San Diego, Calif. pp. 315–322.