Highly Sensitive End-Point PCR and SYBR Green qPCR Detection of *Phymatotrichopsis omnivora*, Causal Fungus of Cotton Root Rot

M. Arif, S. Dobhal, P. A. Garrido, G. K. Orquera, and A. S. Espíndola, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, USA; C. A. Young, Samuel Roberts Noble Foundation, Ardmore, OK, USA; F. M. Ochoa-Corona, S. M. Marek, and C. D. Garzón, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, USA

Abstract

Arif, M., Dobhal, S., Garrido, P. A., Orquera, G. K., Espíndola, A. S., Young, C. A., Ochoa-Corona, F. M., Marek, S. M., and Garzón, C. D. 2014. Highly sensitive end-point PCR and SYBR green qPCR detection of *Phymatotrichopsis omnivora*, causal fungus of cotton root rot. Plant Dis. 98:1205-1212.

Phymatotrichopsis omnivora, the causal pathogen of cotton root rot, is a devastating ascomycete that affects numerous important dicotyledonous plants grown in the southwestern United States and northern Mexico. *P. omnivora* is notoriously difficult to isolate from infected plants; therefore methods for accurate and sensitive detection directly from symptomatic and asymptomatic plant samples are needed for disease diagnostics and pathogen identification. Primers were designed for *P. omnivora* based on consensus sequences of the nuclear ribosomal internal transcribed spacer (ITS) region of geographically representative isolates. Primers were compared against published *P. omnivora* sequences and validated against DNA from *P. omnivora* isolates and infected plant samples. The primer combinations amplified products from a range of *P. omnivora* isolates representative of known ITS haplotypes using standard end-point polymerase chain reaction (PCR)

methodology. The assays detected *P. omnivora* from infected root samples of cotton (*Gossypium hirsutum*) and alfalfa (*Medicago sativa*). Healthy plants and other relevant root pathogens did not produce PCR products with the *P. omnivora*-specific primers. Primer pair PO2F/PO2R was the most sensitive in end-point PCR assays and is recommended for use for pathogen identification from mycelial tissue and infected plant materials when quantitative PCR (qPCR) is not available. Primer pair PO3F/PO2R was highly sensitive (1 fg) when used in SYBR Green qPCR assays and is recommended for screening of plant materials potentially infected by *P. omnivora* or samples with suboptimal DNA quality. The described PCR-based detection methods will be useful for rapid and sensitive screening of infected plants in diagnostic laboratories, plant health inspections, and plant breeding programs.

The soilborne fungus *Phymatotrichopsis omnivora* (Duggar) Hennebert causes root rots of important crops, such as cotton (*Gossypium hirsutum* L.), alfalfa (*Medicago sativa* L.), soybean (*Glycine max* (L.) Merr.), vegetable crops, and fruit and nut orchards, in the southwestern United States and northern Mexico. The common name of the disease is Phymatotrichopsis root rot, but it is also known as cotton root rot, Texas root rot, Ozonium root rot, and Phymatotrichum root rot. The disease was first reported in the late 1880s (26,27) and has since remained of considerable economic concern, causing up to \$100 million in annual losses to the U.S. cotton crop alone. The average loss of raw cotton fiber yield has been estimated to be 3.5% in Texas and 2.2% in Arizona, with losses ranging from 8 to 13% in severely infested areas (15,22,33). *P. omnivora* has a large host range that includes around 2,000 species of dicotyledonous plants (31). Initial root infection occurs when soil temperatures and moisture rise in late spring to early summer and is usually symptomless. However, after the roots are girdled by the resulting rot and soil moisture decreases later in the summer, plants quickly wilt and almost inevitably die, usually forming circular patterns of wilting and dead plants in the field that progressively expand to form large patches (Fig. 1). Due to its potentially high economic impact, broad host range, and limited geographic range, *P. omnivora* is included on the quarantined/

regulated pest lists of many organizations, including the European and Mediterranean Plant Protection Organization (11,25), the California Department of Food and Agriculture (8), the Australian Cotton Research Institute (3), and the United Nations Security Council's monitoring program of Iraq (32).

Rapid and accurate detection of plant pathogens is important for timely implementation of disease management. Nucleic acid based techniques, especially those that rely on the polymerase chain reaction (PCR), are among the most widely used techniques for plant pathogen detection (17,34). PCR allows reliable and sensitive microbial detection in surveillance programs. Real-time quantitative PCR (qPCR) can be used for both detection and quantification of plant pathogens (17,23) and, due to its use of fluorescence, is up to 100 times more sensitive than end-point PCR and can detect down to a single fungal spore (*Glomus intraradices*) (2). Among qPCR formats, TaqMan and SYBR Green qPCRs are the two most popular (7). SYBR Green qPCR is relatively inexpensive and more suitable for routine and large-scale monitoring and surveillance (6,14,19,20,28).

This research addresses the development of end-point and SYBR Green qPCR assays for isolate identification and for detection and/or monitoring of the root rot pathogen *P. omnivora* directly from infected plant tissues.

Materials and Methods

Samples. Information regarding fungal strains and plants used in this study are summarized in Table 1. Sixteen *Phymatotrichopsis omnivora* isolates from cotton and alfalfa, isolated as previously described (22), were used in validation tests. These isolates were collected from locations in Arizona, Texas, and Oklahoma. Isolates of fungal soilborne pathogens isolated from *P. omnivora* known hosts, *Rhizina undulata* Fr., *P. omnivora*'s closest known relative, healthy tissues from host and common rotation crops, commercial Jiffy Seed Starting Mix (Ferry Morse Seed Co., Fulton, KY), and loam soil from a location outside of the geographic distribution of

Corresponding authors: C. D. Garzón, E-mail: carla.garzon@okstate.edu and S. M. Marek, E-mail: stephen.marek@okstate.edu

*The e-Xtra logo stands for "electronic extra" and indicates that Figure 1 appears in color in the online edition.

Accepted for publication 17 January 2014.

P. omnivora (OSU Agronomy Research Station, Stillwater, OK) were included in specificity tests (Table 1). Isolation and culture of *P. omnivora*, *Pythium aphanidermatum*, and *Sclerotinia minor* were performed as previously reported (1,22,24). Pure cultures of *Fusarium solani*, *Neocosmospora vasinfecta*, *Macrophomina phaseolina*, *Colletotrichum truncatum*, and *Acrostalagmus luteoalbus* were obtained from the fungal collection maintained at the Oklahoma State University, grown on potato dextrose agar (PDA)

for 5 days at room temperature, then transferred to potato dextrose broth (PDB) and grown for 5 to 7 days at room temperature before harvesting. Dried *R. undulata* apothecia and cultures of *Rhizoctonia solani* and *Sclerotinia minor* were provided by other researchers.

Samples of 37 symptomatic and apparently healthy alfalfa (Ardmore, OK) and cotton (Altus, OK) were obtained for protocol validation using field samples of the standard GoTaq end-point

Fig. 1. Cotton root rot symptoms and signs. **A**, Disease focus visible in otherwise healthy field. **B**, Wilt and necrotic plants due to root rot. **C**, Mycelial growth on the surface of plant crown. **D**, Vascular discoloration. **E**, Cortical lesion under scraped epidermis near plant crown. **F**, Hyphae and sclerotia may be present on crown surface.

PCR assay and the SYBR Green qPCR assay (Table 2). Pure culture isolation of *P. omnivora* from these samples was performed as reported previously (22). Also, samples of symptomatic or apparently healthy alfalfa, cotton, and soybean collected in Texas, Arizona, and Oklahoma ($n = 148$; Table 3) were used for validation with field samples of the modified GoTaq end-point PCR assay using primer set PO3F/PO2R. Alfalfa and cotton seedlings used as

noninoculated negative controls were grown from seed for 7 days in growth chambers at 28°C, 80% relative humidity, and under 12 h of light.

DNA isolation. DNA from *P. omnivora*, *F. solani*, *A. luteoalbus*, *M. phaseolina*, *C. truncatum*, *N. vasinfecta*, *R. solani*, *P. aphanidermatum*, and *R. undulata* mycelia, healthy plant tissues (Table 1), 37 cotton and alfalfa field samples (Table 2), and from Jiffy

Table 1. Fungal and healthy plant samples used in this study

Species	Sample ID	Host plant	Origin/source
<i>Phymatotrichopsis omnivora</i>	NFAIf-7	Alfalfa	Carter Co., OK
<i>P. omnivora</i>	OKAIf-8	Alfalfa	Love Co., OK
<i>P. omnivora</i>	PECOS TXAIf 07-2 ^a	Alfalfa	Reeves Co., TX
<i>P. omnivora</i>	SANE TXC07-10 ^a	Cotton	Tom Green Co., TX
<i>P. omnivora</i>	Braden TXC07-6 ^a	Cotton	Pecos Co., TX
<i>P. omnivora</i>	FS TXAIf07-1	Alfalfa	Pecos Co., TX
<i>P. omnivora</i>	TAMD C05-3 ^{a,b}	Cotton	Collin Co., TX
<i>P. omnivora</i>	EC 59 C05 ^a	Cotton	Wharton Co., TX
<i>P. omnivora</i>	EC 358 C05-4	Cotton	Wharton Co., TX
<i>P. omnivora</i>	Rick C05-5 ^a	Cotton	Refugio Co., TX
<i>P. omnivora</i>	Trumbull C05-4 ^a	Cotton	Ellis Co., TX
<i>P. omnivora</i>	Maud Lowe C05-1 ^a	Cotton	Refugio Co., TX
<i>P. omnivora</i>	Yuma 2 AZC07-7 ^a	Cotton	Yuma Co., AZ
<i>P. omnivora</i>	CCAZAIf07-2	Alfalfa	Maricopa Co., AZ
<i>P. omnivora</i>	Marana AZC07-4	Cotton	Pima Co., AZ
<i>P. omnivora</i>	MS AZC07-4	Cotton	Pima Co., AZ
<i>Rhizina undulata</i>	Rhizina-1	...	Sweden
<i>Sclerotinia minor</i>	Sminor-9	Peanut	Stillwater, OK
<i>Fusarium solani</i>	NFAIf	Alfalfa	Ardmore, OK
<i>Macrophomina phaseolina</i>	AlfMC-11	Alfalfa	Belleville, OK
<i>Colletotrichum truncatum</i>	OSUAIf	Alfalfa	Stillwater, OK
<i>Acrostalagmus luteoalbus</i>	Vt.2 cherry leaf	Cherry	Davis, CA
<i>Neocosmospora vasinfecta</i>	AlfMC-3	Alfalfa	Belleville, OK
<i>Rhizoctonia solani</i>	RS7	Unknown	Jennifer Olson, OSU, OK
<i>Pythium aphanidermatum</i>	P18	Poinsettia	Gary Moorman, PSU, PA
<i>Medicago sativa</i>	...	Alfalfa	Stillwater, OK
<i>Gossypium hirsutum</i>	...	Cotton	Stillwater, OK
<i>Sorghum bicolor</i>	...	Sorghum	Stillwater, OK
<i>Triticum aestivum</i>	...	Wheat	Stillwater, OK
<i>Arachis hypogaea</i>	...	Peanut	Stillwater, OK
<i>Glycine max</i>	...	Soybean	Stillwater, OK
<i>Zea mays</i> subsp. <i>mays</i>	...	Corn	Stillwater, OK
Potting mix	...	...	Stillwater, OK
Loam soil	...	...	Stillwater, OK

^a Isolates used to compare standard and modified end-point polymerase chain reaction (PCR) protocols.

^b Isolate used to assess standard end-point PCR assay sensitivity.

Table 2. Comparison of GoTaq Green (GTG) end-point polymerase chain reaction (PCR) and quantitative PCR (qPCR) protocols using field samples collected at two Oklahoma locations in 2012^a

Sample name ^b	Field site	Host plant	No. samples	PCR positives PO/ITS ^c	qPCR positives (estimated concentrations)
ALF-P1	Field 1, Ardmore, OK	Alfalfa	3	0/1	3 (4.7 ng; 29 pg; 3.6 ng)
ALF-P2	Field 2, Ardmore, OK	Alfalfa	3	0/1	3 (6.5 pg; 63 fg; 3 fg)
ALF-P3	Field 3, Ardmore, OK	Alfalfa	3	0/3	3 (245 fg; 4.4 pg; 92 fg)
ALF-R1	Field 1, Ardmore, OK	Alfalfa	3	0/2	3 (193 fg; 2.5 ng; 499 pg)
ALF-R2	Field 2, Ardmore, OK	Alfalfa	3	0/3	3 (1.8 pg; 198 fg; 88 fg)
ALF-R3	Field 3, Ardmore, OK	Alfalfa	3	3/3	3 (58 pg; 44 pg; 8 pg)
ALF-H1	Field 1, Ardmore, OK	Alfalfa	3	0/2	3 (23 fg; 27 fg; 2.5 pg)
ALF-H2	Field 2, Ardmore, OK	Alfalfa	3	3/3	3 (39 pg; 3.9 pg; 4.4 pg)
ALF-H3	Field 3, Ardmore, OK	Alfalfa	3	0/3	3 (34 fg; 266 fg; 280 fg)
Cott-H	Altus, OK	Cotton	4	4/4	4 (36 pg; 91 fg; 2.9 fg; 18 pg)
Cott-P	Altus, OK	Cotton	3	1/1	3 (>10 ng; >10 ng; >10 ng)
Cott-R	Altus, OK	Cotton	3	2/1	3 (978 pg; >10 ng; >10 ng)
Cott-N	Growth chamber, Stillwater, OK	Cotton	1	0 ^d	0
ALF-N	Growth chamber, Stillwater, OK	Alfalfa	1	0 ^d	0
Total			39	13/27	37

^a Field samples assayed with GTG end-point PCR using *Phymatotrichopsis omnivora* specific primer set PO2F/PO2R (PO) were also assayed with universal primers ITS4/ITS5 to identify false positives and false negatives. The qPCR protocol used primer set PO3F/PO2R.

^b ALF = alfalfa; Cott = cotton; H = healthy looking root tissue; P = partially rotted root tissues; R = fully collapsed, rotted root tissue; N = noninoculated negative controls.

^c All the samples positive for universal ITS primers but negative for *P. omnivora* specific primers produced sequences of either the host plant or fungi other than *P. omnivora*.

^d Assayed with PO2F/PO2R only.

Seed Starting Mix (Ferry Morse Seed Co.) was isolated using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). DNA from 148 field samples (Table 3) was extracted from approximately 20 mg of lyophilized root samples using the MagAttract 96 DNA Plant Kit (Qiagen). DNA extraction from *Sclerotinia minor* was performed as previously reported (1). DNA from loam soil was isolated using a Soil Extraction Kit (MoBio, Carlsbad, CA).

DNA sequence analysis and primer design. The internal transcribed spacer (ITS) regions of 125 *P. omnivora* isolates, obtained for a population biology study that will be reported elsewhere, were amplified using ITS region specific primer set ITS4/ITS5 (34) and conditions described by Marek et al. (22). These isolates were isolated from diverse locations in the United States (Texas, Oklahoma, and Arizona) and the North of Mexico, representing the genetic diversity of the species across its geographic range (21). PCR products were purified using PureLink PCR Purification Kit (Invitrogen Inc., Carlsbad, CA) and sequenced using an Applied Biosystems DNA 3730 Analyzer (Oklahoma State University Nucleic Acids and Proteins Core Facility). The 125 ITS sequences obtained and 19 additional *P. omnivora* accessions retrieved from the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) were aligned using CLUSTALX v. 2 (18). Sequence alignment errors were corrected manually and conserved regions were identified using percent identity matrices in GeneDoc (Genedoc, a Tool for Editing and Annotating Multiple Sequence Alignments. 1997. K. B. Nicholas, H. B. Nicholas Jr., and D. W. Deerfield II. Distributed by the author.). PCR primers for *P. omnivora* (Table 4) were designed based on the consensus of aligned sequences using Primer3 (29). The presence of internal secondary structures and homo- and self-dimers in the primers was examined using mFold (35).

Preparation of plasmid positive controls and qPCR standards. PCR products amplified from genomic DNA of *P. omnivora* isolate EC 358 C05-4 (Table 1) with each of three *P. omnivora*-specific primer sets were gel purified using Quantum Prep Freeze 'N Squeeze Spin Columns (Bio-Rad Laboratories, Hercules, CA) and cloned into a plasmid vector (pCR2.1-TOPO) using a TOPO-TA Cloning Kit (Invitrogen). Plasmid DNA was purified from

overnight bacterial cultures using a QIAprep Spin Miniprep Kit (Qiagen). Isolated plasmid DNA was quantified using a NanoDrop v. 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and sequenced. Plasmids containing PCR products were used in sensitivity tests, as positive controls, and as standards for qPCR.

End-point PCR assay development. The GoTaq Green (GTG) Master Mix (Promega, Madison, WI) was used in end-point PCR assays using primer set PO1F/PO1R with the reaction conditions and temperature cycling parameters recommended by the manufacturers, since it consistently produced superior results (*data not shown*) in preliminary comparisons between that kit and the Takara Taq kit (Takara Bio Inc., Shiga, Japan). Additionally, a modification of the standard GoTaq Green Master Mix (MG TG) protocol was evaluated for smaller reaction volumes and shorter temperature cycling times (i.e., total PCR program run for PO2F/PO2R was 38 min with MG TG compared to 78 min with GTG). In the MG TG protocol reactions were 20 µl (10 µl master mix, 10 ng template, and 1 µl of each 5 µM primer), and the thermocycling protocol included an initial denaturation of 3 min at 95°C, followed by 30 (PO1F/PO1R and PO2F/PO2R) or 35 (PO3F/PO2R) cycles of 20 s denaturation at 94°C, 15 s annealing (annealing temperatures reported in Table 4), and 20 s extension at 72°C, and final extension at 72°C for 3 min.

SYBR Green qPCR assay development. Reactions (20 µl) contained 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Grand Island, NY), 0.8 µl (5 µM) of each forward and reverse primer, 1 µl of template genomic DNA (various DNA concentrations), and 7.4 µl of nuclease-free water (Ambion, Austin, TX). Positive (plasmid carrying the target gene sequence or *P. omnivora* genomic DNA) and negative (nontarget template or water) controls were included in each set of qPCR assays. Each reaction was performed in triplicate. Cycling parameters included two initial holds at 50°C and 95°C, each for 2 min, and 40 cycles of 95°C for 15 s, and 60°C for 60 s. The assays were performed in a Rotor-Gene 6000 thermocycler (Corbett Research, Sydney, Australia).

End-point PCR sensitivity test. The detection limits of each of the three primer sets when used with the GTG and MG TG proto-

Table 3. Efficiency of the modified GoTaq Green (MG TG) end-point polymerase chain reaction (PCR) protocol to detect *Phymatotrichopsis omnivora* DNA in cotton, alfalfa, and soybean, root samples collected from *Phymatotrichopsis* root rot affected sites in 2009 and research plots with primer set PO3F/PO2R

Sample name	Field site	Host plant/substrate	Total samples	<i>P. omnivora</i> positive
Liberty 1	Field 1, Bryan Co., OK	Alfalfa	3	3
Liberty 2	Field 1, Bryan Co., OK	Alfalfa	10	10
Liberty 3	Field 1, Bryan Co., OK	Alfalfa	10	10
Liberty 4	Field 1, Bryan Co., OK	Alfalfa	8	8
Liberty 5	Field 1, Bryan Co., OK	Alfalfa	10	9
Liberty 1 Soy	Field 1, Bryan Co., OK	Soybean	10	9
ELRD 1	Field 2, Bryan Co., OK	Alfalfa	10	10
Deport 1	Field 3, Red River Co., TX	Cotton	10	6
Deport 2G ^a	Field 4, Red River Co., TX	Cotton	10	0
Deport 2N ^a	Field 4, Red River Co., TX	Cotton	5	0
Deport 2	Field 4, Red River Co., TX	Cotton	10	1
Deport 2 W	Field 4, Red River Co., TX	Cotton	10	4
Plant RSYD4 LLA ^b	Greenhouse, Carter Co., OK	Alfalfa	20	19
Plant 19 and 21 CCOMT ^b	Greenhouse, Carter Co., OK	Alfalfa	15	13
OKAlf8 ^c	Laboratory strain	Sterile wheat grain	7	7
Total			148	109

^a Healthy looking plants.

^b Plants inoculated with *P. omnivora* OKAlf8.

^c Pure culture used as positive control.

Table 4. Details of the primers designed for this study

Primer name	Primer sequence (5'-3')	Primer location	Annealing temp. (°C)
PO1F	CAAATGGATGCCCGTAGT	ITS1	58.5
PO1R	CCAATGACCAAGCCAACC	ITS2	58.5
PO2F	AAACCCCCAAATGGATGC	ITS1	60.0
PO2R	CACCACCATACTACGTCAAAGG	ITS2	60.0
PO3F	CGAGCGTCAGCATAACAAA	5.8S rDNA, ITS2	60.0

cols were determined by conducting PCR reactions using 10-fold dilutions (10 ng to 1 ag) of genomic DNA of *P. omnivora* isolate TAMDC05-3 (Table 1).

SYBR Green qPCR sensitivity test. Sensitivity of the qPCR assay was evaluated using 10-fold dilutions of plasmid DNA containing cloned PO3F/PO2R PCR product, genomic DNA, or genomic DNA mixed with host plant DNA. To simulate interference from host plant DNA, 1 ng genomic DNA from healthy alfalfa or healthy cotton was added to each reaction in addition to 10-fold dilutions of genomic DNA from *P. omnivora* isolate TAMDC05-3 at concentrations from 1 ng to 1 ag per reaction. Each qPCR sensitivity test was replicated three times.

Primer specificity test. The specificity of the three primer sets was challenged using genomic DNA of fungal plant pathogens, the nonpathogenic fungus *Rhizina undulata*, healthy plants, or obtained from soil and potting mix (Table 1) using the MGTG protocol. To confirm that targeted gene sequences were amplified in the PCR assays, amplicons obtained with the three primer sets from genomic DNA (10 ng) of *P. omnivora* isolate EC 358 C05-4 (Table 1) were directly sequenced using the same forward and reverse primers. BLASTn (4) was used to compare the amplicon sequences against the GenBank database.

End-point PCR protocol validation. Three end-point PCR validation experiments were conducted. In the first experiment, the three primer sets were tested on 16 *P. omnivora* isolates representing the genetic diversity of the fungus in Texas, Oklahoma, and Arizona (Table 1), using the MGTG protocol. In the second experiment, the utility of the GTG protocol using primer set PO2F/PO2R for detection of *P. omnivora* during routine tests was assessed using DNA samples of 37 symptomatic (alfalfa = 18; cotton = 6) (Table 2), and apparently healthy plants (alfalfa = 9; cotton = 4) that had the risk of having latent infections due to their proximity to symptomatic plants in the field. The DNA samples used in this experiment varied in quality (DNA 260/280 ratio 0.9 to 2.2) and were not further purified to test the primers in reactions with sub-optimal DNA quality. Positive (TAMDC05-3 genomic DNA; Table 1) and negative (noninoculated cotton and alfalfa) controls were included. In order to identify false negatives (infected but not amplified), the same samples were assayed with ITS-region-specific universal primers ITS4 and ITS5 (34). The PCR products obtained with primer sets PO2F/PO2R and ITS4/ITS5 (Table 2) were directly sequenced, and their sequences were blasted against the GenBank database.

Finally, the MGTG protocol was validated using primer set PO3F/PO2R and DNA isolated from 148 samples (DNA 260/280 ratio >1.7) comprised of symptomatic alfalfa ($n = 86$), cotton ($n = 30$), and soybean ($n = 10$), and apparently healthy cotton ($n = 15$) collected from Texas, Arizona, and Oklahoma, and one laboratory isolate originally collected from an infested alfalfa field in Oklahoma (Table 3).

SYBR Green qPCR protocol validation. The SYBR Green qPCR protocol was validated using the 37 plant DNA samples and controls used in the validation of GTG end-point PCR assay (Table 2). DNA was diluted to a final concentration of 2 ng/μl, but was not further purified. Each qPCR reaction was carried out in a total volume of 15 μl (7.5 μl of FastStart SYBR Green Master [Roche Applied Sciences, Indianapolis, IN], 0.45 μl of PO3F primer at 5 μM, 0.45 μl of PO2R primer at 5 μM, and 6.6 μl (13.2 ng) DNA) in a MyiQ real-time PCR detection system (Bio-Rad). Amplification conditions were: one cycle of 20 s at 95°C, followed by 55 cycles of 15 s at 94°C, 15 s at 60°C, and 20 s at 72°C. Positive (isolate TAMDC05-3 genomic DNA) and negative controls (healthy alfalfa and cotton seedlings; PCR grade water) were included. All the reactions were performed with three replicates. The standard curve was calculated using 10-fold serial dilutions of *P. omnivora* isolate TAMDC05-3 genomic DNA, between 10 ng/μl and 100 ag/μl.

Results

Primer design and PCR assay development. *Primer design.* Three sets of PCR primers (PO1F/PO1R, PO2F/PO2R, and

PO3F/PO2R) were designed (Table 4). All primers annealed to conserved regions of the consensus sequence (Fig. 2). Primer sequences, positions, and annealing temperatures are shown in Table 4. Primer sets PO1F/PO1R, PO2F/PO2R, and PO3F/PO2R produced PCR products approximately 451 bp, 564 bp, and 168 bp long, respectively (Fig. 2). Because of its small PCR product size, primer set PO3F/PO2R was compatible with SYBR Green qPCR.

Determination of primer specificity. Primers PO1F, PO1R, PO2F, PO2R, and PO3F were blasted against NCBI's GenBank nucleotide database. All the primers showed 100% query coverage and 100% identity with *P. omnivora* accessions exclusively, and no other 100% coverage and high-identity (>90%) hits were observed. The three primer sets did not produce PCR products with DNA from healthy plants, noninoculated potting mix, or soil samples, or from oomycete and fungi other than *P. omnivora* (Table 1).

Sensitivity of end-point PCR assays. The GTG protocol was 10 times more sensitive than the MGTG protocol with primer set PO2F/PO2R, but similar in sensitivity with primer sets PO1F/PO1R and PO3F/PO2R (Fig. 3). The detection limits of primer sets PO1F/PO1R, PO2F/PO2R, and PO3F/PO2R with the GTG protocol were 100 pg, 100 fg, and 10 pg *P. omnivora* genomic DNA per reaction, respectively (Fig. 3). The detection limits of primer sets PO1F/PO1R, PO2F/PO2R, and PO3F/PO2R with the MGTG protocol were 100 pg, 1 pg, and 10 pg *P. omnivora* genomic DNA per reaction, respectively (Fig. 3). Primer set PO2F/PO2R was consistently more sensitive than the other two primer sets, followed by primer set PO3F/PO2R (Figs. 3 and 4). Since PO1F/PO1R was the least sensitive primer set, field validation test results for this primer set were not included in this report.

Sensitivity of SYBR Green qPCR. The qPCR assay with the PO3F/PO2R primer set was 10,000-fold more sensitive than the end-point PCR assay with the same primer set when genomic DNA was tested (Fig. 4, Table 5). The generated standard curve showed R^2 values between 0.985 and 0.999, and reaction efficiency values of 1.01 and 1.23 (Table 5). A threshold (statistical significant point above the calculated baseline fluorescence) of 0.2 was used to compare the standard curves generated for assays using plasmid, genomic, and mixed genomic DNA as templates. The PO3F/PO2R

Fig. 2. Primer binding sites within the internal transcribed spacer (ITS) region (A) and specific sequences within the consensus ITS sequence from 144 isolates of *Phymatotrichopsis omnivora* (B). Primer binding site sequences are in bold and underlined (overlapping sites are double underlined) and primer names and directions appear below sequences. Variable positions in the consensus sequence are shaded.

primer set showed high sensitivity with genomic DNA (1 fg) and plasmid DNA (100 ag). When 1 ng of either cotton or alfalfa genomic DNA was added to each reaction, the observed cycle threshold (Ct) values in the presence of host plant DNA were similar to the *P. omnivora* genomic sensitivity assays' Ct values, indicating that host genomic DNA did not interfere with real-time PCR assays (Table 5).

Validation of PCR assays. *P. omnivora* isolation. Eleven pure cultures of *P. omnivora* were isolated from 210 root pieces obtained from the 37 symptomatic samples of cotton and alfalfa obtained from the field.

P. omnivora end-point PCR primer validation. The three primer sets designed amplified the targeted sequences from genomic DNA of 16 *P. omnivora* isolates representing the genetic diversity of *P. omnivora* in Texas, Oklahoma, and Arizona. Four sequenced amplicons showed 99% identity among them and 95 to 99% identity to BLASTn results, only closely matching *P. omnivora* accessions (query coverage *P. omnivora* = 100%; next nearest blast hit *Rhizina* = 39 to 51%).

GTG protocol validation. Out of 37 symptomatic plant samples from the field tested using DNA of suboptimal quality, 13 tested positive with the *P. omnivora*-specific primer set PO2F/PO2R (Table 2), while 27 produced amplicons with the universal primer set ITS4/ITS5. The DNA sequences of all the PCR products were obtained and blasted against the GenBank nucleotide database. No false positives or false negatives were produced by primer set PO2F/PO2R. All the sequences obtained with the *P. omnivora*-specific primers matched *P. omnivora* accessions in GenBank; while DNA sequences obtained with the universal ITS primers either confirmed *P. omnivora* infection or matched the host plant (*Medicago sativa*) or other fungi (*Alternaria arborescens*, *A. alternata*, *Aspergillus terreus*, or *Paecilomyces* sp.).

MG TG protocol validation. Out of 148 field plant samples that were tentatively identified as infected, a total of 109 were positive for *P. omnivora* using primer set PO3F/PO2R (Table 3). Fifteen asymptomatic plants (Deport 2G and 2N) collected next to wilted plants from Field 4, Red River Co., Texas, were negative for *P. omnivora*.

Fig. 3. End-point polymerase chain reaction (PCR) sensitivity test using the three primer sets with the standard Promega GoTaq Green Master Mix protocol (GTG) or the modified Promega GoTaq Green Master Mix protocol (MG TG) and 10-fold serial dilution of *Phymatotrichopsis omnivora* genomic DNA at the indicated concentrations per reaction. Lane ML: 1 kb Plus DNA ladder (Invitrogen).

SYBR Green qPCR protocol validation. The 37 samples of cotton and alfalfa that were symptomatic for Phymatotrichopsis root rot or were at risk for asymptomatic infections were positive for the presence of *P. omnivora* (Table 2). The detection limit of the assay was 2.9 fg. DNA samples obtained from healthy alfalfa and cotton plants grown in growth chambers were negative for the presence of *P. omnivora*.

Discussion

This study describes the development of sensitive and highly specific end-point PCR and SYBR Green real-time qPCR assays for detection of *P. omnivora*. *P. omnivora* is a destructive soilborne pathogen occurring only in the alkaline calcareous soils of the southwestern United States and northern Mexico. Thus, plants and soils from *P. omnivora*'s endemic regions are subject to regulations and quarantines by U.S. and international agencies. Although the symptoms and signs of Phymatotrichopsis root rot are usually conspicuous on cotton, such diagnostic manifestations may be absent or subtle on other hosts, especially perennial hosts such as alfalfa. Also, confirmatory cultures of the pathogen from infested plant tissue are not always possible because *P. omnivora* is difficult to isolate from infected plants (5). Therefore, sensitive diagnostic methods are needed to rapidly and reproducibly detect the *P. omnivora* within roots from field samples.

The multicopy ITS region was selected as the target gene due to its unique sequence, specificity to *P. omnivora* (5), and high copy number per genome (~1,000 copies per haploid genome, based on draft genome sequences, S. M. Marek, *unpublished results*). All primers developed for this study were designed and optimized with regard to compatibility with one another and low tendency to form hairpins. Moreover, all of the primer sets for this study were designed based on the consensus sequence of ITS sequences from diverse isolates of *P. omnivora* isolated from several hosts collected in southern Arizona and Oklahoma, northern Mexico, and much of Texas over 30 years (21). While the aligned ITS sequences used were diverse, all primers bound within regions conserved across all sequences, as confirmed by successful amplification of PCR products from genomic DNA of 16 diverse *P. omnivora* isolates from Texas, Oklahoma, and Arizona. Primer sets were specific for *P. omnivora*, with no PCR products amplified from genomic DNA isolated from any of the other soilborne fungi and oomycetes tested, including the closely related fungus, *Rhizina undulata*.

Fig. 4. End-point polymerase chain reaction (PCR) sensitivity of the three primer sets with a 10-fold serial dilution at the indicated concentration per reaction of plasmid DNA carrying the corresponding PCR products amplified from *Phymatotrichopsis omnivora* with primer sets **A**, PO1F/PO1R, **B**, PO2F/PO2R, and **C**, PO3F/PO2R. Lane M: 1 kb Plus DNA ladder (Invitrogen). The band produced by PO2F/PO2R using 10 µg template DNA looks faint due to a pipetting error while loading the PCR product on the agarose gel.

(confamilial with *P. omnivora* in Rhiziniaceae). The primers also did not amplify products from DNA isolated from healthy plant samples, or soil collected in Stillwater, OK, which is outside of *P. omnivora*'s endemic range.

The described primer sets can be used in end-point PCR assays, and one set, PO3F/PO2R, can also be used in SYBR Green qPCR assays. Primer set PO2F/PO2R was the most sensitive for end-point PCR, since it detected the target sequence in 100 fg genomic DNA from *P. omnivora*, and 1 fg of plasmid DNA carrying the respective target sequence, and as little as 29 fg *P. omnivora* genomic DNA from infected roots. The sizes of amplicons produced by primer pair PO2F/PO2R (~564 bp) make these suitable for direct sequencing for assay confirmation. The SYBR Green qPCR assay using primers PO3F/PO2R detected the *P. omnivora* target sequences at concentrations down to 1 fg genomic DNA from cultured mycelium, 2.9 fg DNA from infected plant roots, and 100 ag plasmid DNA carrying the cloned amplicon. Thus, the SYBR Green qPCR assay was 100-fold more sensitive than the end-point PCR assay using primer set PO2F/PO2R in the detection of *P. omnivora* genomic DNA alone, and at least 10-fold more sensitive in the detection of *P. omnivora* using DNA from infected plants, in spite of the presence of PCR inhibitors in the samples analyzed.

Remarkably, the 1 fg detection limit for *P. omnivora* genomic DNA using SYBR Green qPCR equates with detecting six to seven haploid nuclei, assuming the 150 bp ITS target occurs as 1,000 copies per genome equivalent and 1 fg $\approx$ 978 kbp (10). Such sensitive detection could be used to quantify in planta growth of *P. omnivora* during the asymptomatic phases of infection in susceptible and resistant hosts, as demonstrated in other pathosystems, such as late blight of potato and apple scab (9,19). The described PCR and qPCR assays were tested on numerous infected and healthy plant samples collected from agricultural fields. The assays successfully detected *P. omnivora* in roots from cotton and alfalfa plants displaying symptoms and signs of Phymatotrichopsis root rot and occasionally detected the fungus in healthy-appearing roots from infested fields. These asymptomatic roots could have been in the initial or latent stages of disease development. The modified end-point PCR protocol detected *P. omnivora* in 73.6% of infected field samples using primer sets PO3F/PO2R. By comparison, cultural confirmation of *P. omnivora* usually requires numerous attempts to isolate a pure culture from surface-sterilized root pieces with approximately 0.05% of pieces producing axenic cultures.

The standard GoTaq Master mix protocol was 10 times more sensitive than the modified protocol; however, the modified protocol allowed much shorter runs, which may be convenient for routine diagnostics from mycelial strands or mycelium, or from high-quality infected plant DNA. In order to assess the robustness of the described protocols for routine plant sample processing, where samples of variable DNA concentrations and containing PCR in-

hibitors must be analyzed, we compared the efficiency of primer set PO2F/PO2R in standard end-point PCR reactions and primer set PO3F/PO2R in SYBR Green qPCR to detect *P. omnivora* on symptomatic alfalfa and cotton plant samples showing varying levels of deterioration, using DNA samples obtained with DNA isolation kits without further purification or dilution. The end-point PCR protocol detected *P. omnivora* in only 13 of 37 (35%) samples, while the SYBR Green qPCR protocol using diluted DNA detected *P. omnivora* in all 37 infected samples. The presence of alfalfa or cotton genomic DNA or PCR inhibitors derived from host plant tissues did not appear to reduce the sensitivity of the SYBR green qPCR assay; while the end-point PCR assay's sensitivity was severely affected. Good quality DNA should be used in the end-point PCR assay, particularly when working with known recalcitrant host samples. Because of these limitations, PCR primer set PO2F/PO2R is recommended for sensitive detection of *P. omnivora* using the GTG end-point PCR protocol with high-quality DNA samples obtained from infected plants when qPCR is not available, and for identity confirmation of pure cultures of *P. omnivora* or mycelial strands obtained from infected plants using either the GTG or the MGTG protocols. Primer set PO3F/PO2R is recommended for highly sensitive detection of *P. omnivora* using SYBR green qPCR, regardless of the quality of the DNA samples, but can also be used for end-point PCR, with either the GTG or the MGTG protocols, using high-quality fungal or infected plant DNA samples.

Phymatotrichopsis root rot can occur as well-defined rings of dead plants in fields of perennial hosts, such as alfalfa, or late in the season in fields of annual hosts, such as cotton and soybean. However, earlier in the season (summer) the disease begins as several wilted and dead plants adjacent in a row (13). As above-ground disease symptoms are not apparent until the main taproot and/or crown have been girdled, early diagnosis is not likely. In addition, low soil moisture and cool temperatures can inhibit fungal growth and disease expression, even though the fungus persists in the soil and on roots (16,30). The reported SYBR green qPCR assay could make early field diagnosis possible and facilitate pre-emptive application of the recently described fungicide, flutriafol, with activity against *P. omnivora* (12).

The reported PCR-based detection assays are sensitive, cost effective, quick, and efficient tools that can be used in the routine diagnosis of Phymatotrichopsis root rot and may permit diagnosis of plants lacking characteristic symptoms and signs. This study reports the first fully validated end-point PCR assays for detection of *P. omnivora* and a SYBR Green qPCR protocol with a primer set 10 times more sensitive than those previously described (5). In addition to routine diagnostics, potential applications of these assays include assessment of *P. omnivora* soil inoculum levels to predict a field's disease potential, monitoring of the pathogen's survival during crop rotations with

Table 5. Comparison of quantitative polymerase chain reaction (qPCR) sensitivity with different templates^a

Template conc. per reaction	Target DNA in plasmid	Average Ct values (three replicates)		
		<i>Phymatotrichopsis omnivora</i> genomic DNA	<i>P. omnivora</i> genomic DNA with cotton genomic DNA	<i>P. omnivora</i> genomic DNA with alfalfa genomic DNA
1 ng	10.62	15.09	15.42	15.40
100 pg	13.33	18.48	18.59	18.62
10 pg	14.93	21.94	21.58	21.77
1 pg	17.40	25.67	24.84	25.38
100 fg	21.06	28.97	28.03	27.82
10 fg	24.27	32.11	30.80	29.29
1 fg	27.56	34.43	N/A	N/A
100 ag	30.38	Not detected	N/A	N/A
R²	0.991	0.996	0.999	0.985
Ex	1.23	1.01	1.10	1.23
Y	-2.871	-3.297	-3.098	-2.876

^a Ct (cycle threshold), R² (linear correlation), Ex (reaction efficiency), and Y (slope) values obtained in sensitivity assays with primer pair PO3F/PO2R using dilutions of plasmid DNA containing PCR product, *P. omnivora* isolate EC358-C05-4 genomic DNA alone or with 1 ng alfalfa or cotton genomic DNA. N/A = data not available, since those concentrations were not tested.

nonhosts, and evaluation of fungal growth on resistant germplasm used in breeding programs. The described assays could also be used in regulatory agricultural biosecurity and in microbial forensic applications.

Acknowledgments

This work was supported in part by grants from the Oklahoma State Regents for Higher Education, NSF-EPSCoR, the Oklahoma Department of Agriculture, Food and Forestry, and by the Oklahoma Agricultural Experiment Station Projects OKL02536 to Stephen Marek, OKL02698 to Carla Garzón, and OKL02773 to Francisco Ochoa-Corona. We thank Jacque Fletcher and John Damicone (OSU), for reviewing this manuscript; Madhavi Dhulipala, James N. Enis, Sandrine Casanova, Tim Samuels, Ian Moncrief, Carrie Smith, Kihyuck Choi, Francisco Flores, Chelsea Shafley, Christen Shelton, Johanna Takach, Stormy Posey, Andrea Medina, Gabriela Garcés, Pablo Espinosa, Fernanda Proaño, and Omar Arias for technical assistance; and Nathan Walker, Terry Pitts, Shane Osborne, Clay Jones, Thomas Thiltgen, Michael Morrow, Tom Isakeit, Kevin Ong, Mark Black, James E. Marek, Barry Pryor, and Randy Norton for help sampling. We extend our appreciation to Rimvydas Vasaitis (Swedish University of Agricultural Sciences) for providing *Rhizina undulata* apothecia used for validation of the techniques developed in this study, and to Jennifer Olson and Hassan Melouk (Oklahoma State University), and Gary Moorman (The Pennsylvania State University) for providing *Rhizoctonia*, *Sclerotinia*, and *Pythium* isolates, respectively. The mention of trade names or commercial products in this publication does not imply recommendation or endorsement by Oklahoma State University.

Literature Cited

1. Abd-Elmagid, A., Garrido, P. A., Hunger, R., Lyles, J. L., Mansfield, M. A., Gugino, B. K., Smith, D. L., Melouk, H., and Garzon, C. D. 2013. Discriminatory simplex and multiplex PCR for four species of the genus *Sclerotinia*. *J. Microbiol. Methods* 92:293-300.
2. Alkan, N., Gadkar, V., Coburn, J., Yarden, O., and Kapulnic, Y. 2004. Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. *New Phytol.* 161:877-885.
3. Allen, S. J. 1995. Plant quarantine and diseases of cotton. *Australas. Plant Pathol.* 24:70-73.
4. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
5. Arif, M., Fletcher, J., Marek, S. M., Melcher, U., and Ochoa-Corona, F. M. 2013. Development of a rapid, sensitive, and field-deployable razor ex biodetection system and quantitative PCR assay for detection of *Phymatotrichopsis omnivora* using multiple gene targets. *Appl. Environ. Microbiol.* 79:2312-2320.
6. Arikawa, E., Sun, Y., Wang, J., Zhou, Q., Ning, B., Dial, S. L., Guo, L., and Yang, J. 2008. Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. *BMC Genomics* 9:1-12.
7. Bustin, S. A. 2005. Real-time, fluorescence-based quantitative PCR: A snapshot of current procedures and preferences. *Expert Rev. Mol. Diagnostics* (5):493-498.
8. California Department of Food and Agriculture. 2012. *Ozonium Root Rot*. Page 3261 in: *Plant Quarantine Manual*. CDFA.
9. Daniels, B., De lantsheer, A., Dreesen, R., Davey, M. W., and Keulmans, J. 2012. Real-time PCR as a promising tool to monitor growth of *Venturia* spp. in scab-susceptible and -resistant apple leaves. *Eur. J. Plant Pathol.* 134:821-833.
10. Dolezel, J., Bartos, J., Voglmayr, H., and Greilhuber, J. 2003. Nuclear DNA content and genome size of trout and human. *Cytometry* 51:127-128.
11. EPPO, C. a. *Phymatotrichopsis omnivora*. Pages 1-5 in: *Data Sheets on Quarantine Pests*. EPPO.
12. Isakeit, T., Minzenmayer, R. R., and Abameit, A. 2010. Flutriafof for control of cotton root rot, caused by *Phymatotrichopsis omnivora*. (Abstr.) *Phytopathology* 100:S54.
13. Jeger, M. J., Kenerley, C. M., and Gerik, T. J. 1987. Spatial dynamics of *Phymatotrichum* root rot in row crops in the Blackland region of north central Texas. *Phytopathology* 77:1647-1656.
14. Karsai, A., Muller, S., Platz, S., and Hauser, M. 2002. Evaluation of a homemade SYBR Green I reaction mixture for real-time PCR quantification of gene expression. *BioTechniques* 32:790-796.
15. Kenerley, C. M., and Jeger, M. J. 1992. Fungal diseases of the root and stem. Pages 161-190 in: *Cotton Diseases*. R. J. Hillocks, ed. CAB International, UK.
16. Kenerley, C. M., and Jeger, M. J. 1990. Root colonization by *Phymatotrichum omnivorum* and symptom expression of *Phymatotrichum* root rot in cotton in relation to planting date, soil temperature and soil water potential. *Plant Pathol.* 39:489-500.
17. Kim, J. S., Lee, G. G., Kim, J., Kwon, J. Y., and Kwon, S. T. 2008. The development of rapid real-time PCR detection system for *Vibrio parahaemolyticus* in raw oyster. *Lett. Appl. Microbiol.* 46:649-654.
18. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. 2007. ClustalW2 and ClustalX version 2. *Bioinformatics* 23:2947-2948.
19. Llorente, B., Bravo-Almonacid, F., Cvitanich, C., Orlowska, E., Torres, H. N., Flawia, M. M., and Alonso, G. D. 2010. A quantitative real-time PCR method for in planta monitoring of *Phytophthora infestans* growth. *Lett. Appl. Microbiol.* 51:603-610.
20. Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Koikeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F., and Takashiba, S. 2003. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. *FEMS Immunol. Med. Mic.* 39:81-86.
21. Marek, S. M. 2009. Phylogeography of the cotton root rot fungus *Phymatotrichopsis omnivora*. (Abstr.) *Phytopathology* 99:S79.
22. Marek, S. M., Hansen, K., Romanish, M., and Thorn, R. G. 2009. Molecular systematics of the cotton root rot pathogen, *Phymatotrichopsis omnivora*. *Persoonia* 22:63-74.
23. Martin, R. R., James, D., and Lévesque, C. A. 2000. Impacts of molecular diagnostic technologies on plant disease management. *Annu. Rev. Phytopathol.* 38:207-239.
24. Moorman, G. W., Kang, S., Geiser, D. M., and Kim, S. H. 2002. Identification and characterization of *Pythium* species associated with greenhouse floral crops in Pennsylvania. *Plant Dis.* 86:1227-1231.
25. OEPP/EPPO. 2011. EPPO A1 and A2 Lists of Pests Recommended for Regulation as Quarantine Pests. Pages 16. European and Mediterranean Plant Protection Organization, Paris, France.
26. Pammel, L. H. 1888. Root rot of cotton, or cotton blight. *Texas Agric. Exp. Stn. Bull.* 4:3-18.
27. Pammel, L. H. 1889. Cotton root-rot. *Texas Agric. Exp. Stn. Bull.* 7:5-30.
28. Pellissier, F., Glogowski, C. M., Heinemann, S. F., Ballivet, M., and Ossipow, V. 2006. Lab assembly of a low-cost, robust SYBR Green buffer system for quantitative real-time polymerase chain reaction. *Anal. Biochem.* 350:310-312.
29. Rozen, S., and Skaletsky, H. J. 2000. Primer3 on the WWW for general users and for biologist programmers. Pages 365-386 in: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. S. A. M. S. Kraetz, ed. Humana Press, Totowa, NJ.
30. Rush, C. M., Gerik, T. J., and Lyda, S. D. 1984. Factors affecting symptom appearance and development of *Phymatotrichum* root rot of cotton. *Phytopathology* 74:1466-1469.
31. Streets, R. B., and Bloss, H. E. 1973. *Phymatotrichum* root rot. *Monogr. American Phytopathological Society* 8:38.
32. United Nations. 1995. Plan for Future Ongoing Monitoring and Verification of Iraq's Compliance with Relevant Parts of Section C of Security Council Resolution 687 (1991). Report of the Secretary-General (s/22871/rev.1). Revised Annexes II, III and IV.
33. Uppalapati, S. R., Young, C. A., Marek, S. M., and Mysore, K. S. 2010. *Phymatotrichum* (cotton) root rot caused by *Phymatotrichopsis omnivora*: Retrospects and prospects. *Mol. Plant Pathol.* 11:325-334.
34. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
35. Zuker, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31:3406-3415.