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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

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A validated, multigene-based method using real-time quantitative PCR (qPCR) and the Razor Ex BioDetection system was developed for detection of *Phymatotrichopsis omnivora*. This soilborne fungus causes Phymatotrichopsis root rot of cotton, alfalfa, and other dicot crops in the southwestern United States and northern Mexico, leading to significant crop losses and limiting the range of crops that can be grown in soils where the fungus is established. It is on multiple lists of regulated organisms. Because *P. omnivora* is difficult to isolate, accurate and sensitive culture-independent diagnostic tools are needed to confirm infections by this fungus. Specific PCR primers and probes were designed based on *P. omnivora* nucleotide sequences of the genes encoding rRNA internal transcribed spacers, beta-tubulin, and the second-largest subunit of RNA polymerase II (RPB2). PCR products were cloned and sequenced to confirm their identity. All primer sets allowed early detection of *P. omnivora* in infected but asymptomatic plants. A modified rapid DNA purification method, which facilitates a quick (~30-min) on-site assay capability for *P. omnivora* detection, was developed. Combined use of three target genes increased the assay accuracy and broadened the range of detection. To our knowledge, this is the first report of a multigene-based, field-deployable, rapid, and reliable identification method for a fungal plant pathogen and should serve as a model for the development of field-deployable assays of other phytopathogens.

Phymatotrichopsis root rot, also known as cotton root rot, Texas root rot, or Ozonium or Phymatotrichum root rot, is an important soilborne disease of over 2,000 dicotyledonous species, including cotton, alfalfa, vegetable crops, and fruit and nut trees (1). The causal fungus, *Phymatotrichopsis omnivora*, is a member of the family Rhizinaceae (Ascomycota: Pezizomycetes) and allied to the genera *Psilopezia* and *Rhizina* (2). The disease occurs in most of Texas (excluding the panhandle), southern Oklahoma, and New Mexico, southern and western Arizona, and northern Mexico (1, 2). Because of its extensive host range, *P. omnivora* represents a significant threat to agricultural productivity throughout its geographic range. Phymatotrichopsis root rot is a considerable economic concern, causing up to $100 million in annual losses to the U.S. cotton crop alone (2). Due to its high economic impact and broad host range, *P. omnivora* has been included in lists of regulated organisms by the European and Mediterranean Plant Protection Organization (EPPO A1 list no. 21 [http://www.eppo.org/QUARANTINE/listA1.htm]), the California Department of Food & Agriculture (no. 3261 in the *CDFA Plant Quarantine Manual*, 1989 [http://pi.cdfa.ca.gov/pqm/manual/pdf/309.pdf]), and the United Nations Security Council's monitoring program of Iraq (S/1995/208 [http://www.fas.org/news/un/iraq/s/s1995-0208.htm]). Isolation of *P. omnivora* from infected plants and infested soils is difficult and often not possible. Hence, culture-independent (molecular) approaches to pathogen identification are desirable. Accurate and sensitive detection and discrimination assays for early diagnosis would facilitate disease management. Methods developed for plant pathogen detection include enzyme-linked immunosorbent assay (ELISA) (3), loop-mediated isothermal amplification (LAMP) (4), oligonucleotide array (5), endpoint PCR (6), and real-time quantitative PCR (qPCR) (7). PCR-based techniques are generally more sensitive than immunological methods and have high specificity and discriminatory capabilities. Real-time qPCR offers greater sensitivity and speed than endpoint PCR in the detection of target DNA (7, 8). TaqMan and SYBR green qPCR are the two most popular qPCR formats and chemistries (9). SYBR green qPCR detects all amplified double-stranded DNA, including nonspecific reaction products, while TaqMan qPCR detects only specific amplification products because its amplification-dependent cleavage of probes, incorporating reporter and quencher dyes, results in increased fluorescence (10).

Currently, methods for on-site, rapid, reliable, and sensitive detection of *P. omnivora* are not available. On-site accurate and sensitive detection of fungi is a challenge. Available PCR assays require PCR inhibitor-free DNA purification from fungus-infected plants and access to laboratory facilities equipped with PCR and qPCR machines. Simple on-site DNA purification and PCR protocols for accurate pathogen identification would facilitate diagnosis and regulation, as well as disease monitoring and management. Ideally, these protocols would use a portable, battery-operated real-time qPCR platform designed for on-site molecular testing, which allows plant pathogen detection by minimally trained operators in the absence of laboratory facilities and conditions, including electricity, centrifuges, liquid nitrogen, water

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TABLE 1 qPCR detection of *Phymatotrichopsis omnivora* from infected alfalfa/cotton/unknown weed plant root and soil samples collected in 2010 from fields of the Noble Foundation and about 100 miles west of Ardmore, OK[a]

Sample no.	Sample code	Host	Status of sample	Mo. of collection	C_T value obtained using primer/probe set:		
					PO4	Pobt1	PoRPB2-2
1	OA	Alfalfa	Symptomatic	July	17.54	26.62	28.86
2	OB	Alfalfa	Symptomatic	July	18.46	27.52	29.8
3	O1A	Alfalfa	Dead	October	10.51	19.51	21.48
4	O1B	Alfalfa	Dead	October	15.66	25.07	26.25
5	O2A	Alfalfa	Asymptomatic	October	23.81	33.06	33.49
6	O2B	Alfalfa	Asymptomatic	October	25.73	36.7	38.14
7	O3A	Alfalfa	Dead	October	18.04	26.88	30.23
8	O3B	Alfalfa	Dead	October	18.43	27.81	29.63
9	O6A	Alfalfa	Asymptomatic	October	26.35	35.08	38.32
10	O6B	Alfalfa	Asymptomatic	October	26.49	34.86	37.36
11	O7A	Alfalfa	Dead	October	20.76	30.02	33.15
12	O9A	Alfalfa	Dead	October	20.48	29.6	31.6
13	O9B	Alfalfa	Asymptomatic	October	27.45	35.8	39.21
14	O10A	Alfalfa	Dead	October	26.67	35.56	41.56[b]
15	O22C	Alfalfa	Dead	November	13.43	X	X
16	O27A	Alfalfa	Asymptomatic	November	27.60	X	X
17	O28A	Alfalfa	Asymptomatic	November	32.50	X	X
18	O30A	Alfalfa	Asymptomatic	November	29.36	X	X
19	O22A	Unknown weed	Asymptomatic	November	29.24	X	X
20	O23A	Unknown weed	Dead	November	27.66	X	X
21	O24A	Unknown weed	Asymptomatic	November	29.53	X	X
22	O24B	Unknown weed	Dead	November	31.77	X	X
23	O33A	Alfalfa	Asymptomatic	December	32.90	X	X
24	O34	Alfalfa	Asymptomatic	December	33.69	X	X
25	O37	Alfalfa	Dead	December	35.15	X	X
26	O38A	Alfalfa	Dead	December	35.34	X	X
27	O39	Alfalfa	Dead	December	—	X	X
28	O40	Alfalfa	Dead	December	24.42	X	X
29	O41	Alfalfa	Dead	December	—	X	X
30	O42	Alfalfa	Symptomatic	December	30.46	X	X
31	O43A	Alfalfa	Symptomatic	December	34.90	X	X
32	O45A	Alfalfa	Dead	December	32.90	X	X
33	O46A	Alfalfa	Dead	December	34.65	X	X
34	CO1	Cotton	Symptomatic	December	27.18	X	X
35	CO2	Cotton	Symptomatic	December	24.62	X	X
36	O3C	Soil (A)	NA	October	32.0	X	X
37	O4A	Soil (A)	NA	October	—	X	X
38	O5A	Soil (A)	NA	October	—	X	X
39	O7C	Soil (A)	NA	October	—	X	X
40	O10C	Soil (A)	NA	October	—	X	X
41	O11C	Soil (A)	NA	October	—	X	X
42	O13A	Soil (A)	NA	October	—	X	X
43	O28B	Soil (A)	NA	November	—	X	X
44	O29B	Soil (A)	NA	November	32.0	X	X
45	O30B	Soil (A)	NA	November	32.2	X	X
46	O38B	Soil (A)	NA	December	—	X	X
47	O44B	Soil (A)	NA	December	—	X	X
48	O45B	Soil (A)	NA	December	—	X	X
49	O47	Soil (A)	NA	December	37.0	X	X
50	O48	Soil (A)	NA	December	—	X	X
51	O51	Soil (A)	NA	December	30.54	X	X
52	O52	Soil (A)	NA	December	31.26	X	X
53	O53	Soil (A)	NA	December	34.52	X	X

(Continued on following page)

TABLE 1 (Continued)

Sample no.	Sample code	Host	Status of sample	Mo. of collection	C_T value obtained using primer/probe set: PO4	Pobt1	PoRPB2-2
54	O54	Soil (A)	NA	December	34.99	X	X
55	O55	Soil (A)	NA	December	—	X	X
56	O56	Soil (A)	NA	December	34.26	X	X
57	O57	Soil (A)	NA	December	—	X	X
58	O58	Soil (A)	NA	December	33.17	X	X
59	O59	Soil (A)	NA	December	28.95	X	X
60	O60	Soil (A)	NA	December	—	X	X
61	O61	Soil (A)	NA	December	—	X	X
62	CO3	Soil (C)	NA	December	—	X	X
63	CO4	Soil (C)	NA	December	33.59	X	X
64	CO5	Soil (C)	NA	December	—	X	X
65	CO6	Soil (C)	NA	December	—	X	X
66	CO7	Soil (C)	NA	December	—	X	X

[a] —, no amplification (pathogen not detected); Soil (A), soil from alfalfa fields; Soil (C), soil from cotton fields; X, not tested; NA, not applicable (only a single replicate was used for sample numbers 23 to 64).
[b] The number of PCR cycles was 45.

baths, incubators, and hazardous chemicals. Portable instruments developed previously for on-site pathogen detection include the SmartCycler (Cepheid, Sunnyvale, CA), the R.A.P.I.D. system (Idaho Technologies, Salt Lake City, UT), the LightCycler (Roche Applied Science, Indianapolis, IN), and the Bio-Seeq instrument (Smiths Detection, Edgewood, MD). Recently, Tomlinson and coworkers (10, 11) demonstrated the on-site detection of *Phytophthora ramorum* using qPCR (Cepheid SmartCycler II) and loop-mediated isothermal amplification. Use of the SmartCycler has been successful for on-site detection of the bacterium *Xylella fastidiosa* in grapevines (12) and for diagnosis of foot-and-mouth disease (13). These methods take about 2 h and detect a single target gene. The Razor Ex BioDetection system (Idaho Technology, Inc., Salt Lake City, UT) was designed originally for military use to identify biological-threat organisms on-site. The Razor Ex BioDetection system offers ready-to-use, freeze-dried reagent pouches and bar code-based PCR cycling program upload. These features make the Razor Ex BioDetection system user-friendly. Matero and coworkers (14) developed 40-min on-site detection methods based on the Razor Ex for anthrax, brucellosis, tularemia, and plague.

Nucleic acid-based detection of fungi in plant material requires the purification of genomic DNA (15), which can often be contaminated with phenolics and other PCR inhibitors. Therefore, on-site molecular detection requires both a battery-operated real-time qPCR thermocycler and a rapid and PCR inhibitor-free genomic DNA extraction method.

In this paper, we describe the development of TaqMan qPCR assays that may be performed rapidly, on-site, in about 30 min using the field-deployable Razor Ex BioDetection system protocols for reliable, sensitive, and accurate detection of *P. omnivora* using three target fungal genes. We also describe a modified magnetic-bead-based method for on-site DNA extraction (~10 min) from fungus-infected plant roots. These tools provide enhanced investigative capability for applications in plant disease diagnostics and management, pathogen population monitoring, agricultural biosecurity, and microbial forensics.

MATERIALS AND METHODS

Plant materials and *P. omnivora* genomic DNA. Asymptomatic (apparently healthy), symptomatic (wilted), and dead plants as well as soil samples were collected from affected alfalfa fields at the Samuel Roberts Noble Foundation in Ardmore, OK, and cotton/alfalfa fields from other areas of southwest Oklahoma (Table 1). All healthy plants included in the exclusivity panel (Table 2) were grown in a biosafety level two (BSL-2) greenhouse at the Noble Research Center, Oklahoma State University (OSU), Stillwater, except orange, rose, peach, pecan, grape, and Boston fern, which were collected directly as plant tissue from different sources (Table 2). The genomic DNA of *P. omnivora* isolates (Table 3) used in the inclusivity panel were received from C. Garzon, Department of Entomology and Plant Pathology, Oklahoma State University. The members of the microbial exclusivity panel are listed in Table 2.

DNA isolation from plant, microbes, and soil. Genomic DNA from microbes and plants (Tables 2 and 3) was purified using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Soil DNA was isolated using a MoBio soil extraction kit (MoBio, Carlsbad, CA). DNA concentrations were determined using a NanoDrop v.2000 spectrophotometer (Thermo Fisher Scientific Inc., Worcester, MA). Control *P. omnivora*-free soil was collected from Stillwater, OK, an area in which Phymatotrichopsis root rot has not been reported.

DNA isolation from infected plant roots for Razor Ex BioDetection system application. A Dynabeads DNA Direct Universal kit (Invitrogen, Carlsbad, CA) was used with modifications to the manufacturer's instructions. The target *P. omnivora* templates were prepared using 10 to 30 mg infected plant root tissues and 100 to 150 μl Tris-EDTA (TE) buffer (Promega, Madison, WI). Samples were macerated using an Eppendorf tube and pestle without liquid nitrogen. The 40 μl of macerated supernatant and 200 μl of Dynabeads were mixed and incubated for 5 min. Tubes containing the macerated supernatant and Dynabead mix were placed in a magnetic rack until the beads formed a tight pellet, following which the supernatant was carefully removed and discarded, followed by two rinses with wash buffer. Finally, 40 μl of kit suspension buffer was added to the washed beads to release and suspend isolated DNA. These modifications permitted DNA isolation in the field.

Primer and probe design. Sequences of the three target genes, encoding rRNA internal transcribed spacers (ITS-ribosomal DNA [rDNA]), beta-tubulin, and the second-largest subunit of RNA polymerase II

TABLE 2 Plant and microbial exclusivity panel used for validation of specific primers and probes for *Phymatotrichopsis omnivora* qPCR assays[a]

Plant, microbe, or control	Amplification with specific primer/probe:			Source
	PO4	Pobt1	PoRPB2-2	
Plants				
Medicago sativa (alfalfa)	−	−	−	S. M. Marek, OSU
Arabidopsis thaliana (arabidopsis)	−	−	−	Lehle Seeds, Round Rock, TX
Hordeum vulgare (barley)	−	−	−	R. Hunger, OSU
Secale cereale (rye)	−	−	−	R. Hunger, OSU
Sorghum bicolor (sorghum)	−	−	−	R. Hunger, OSU
Avena sativa (oat)	−	−	−	R. Hunger, OSU
Citrus sinensis (orange)	−	−	−	F. M. Ochoa-Corona, OSU
Petroselinum crispum (parsley)	−	−	−	F. M. Ochoa-Corona, OSU
Solanum tuberosum (potato)	−	−	−	F. M. Ochoa-Corona, OSU
Rosa sp. (rose)	−	−	−	F. M. Ochoa-Corona, OSU
Prunus persica (peach)	−	−	−	A. Payne, OSU
Carya illinoinensis (pecan)	−	−	−	A. Payne, OSU
Arachis hypogaea (peanut)	−	−	−	H. Melouk, OSU
Vitis aestivalis (grape)	−	−	−	L. Overall, OSU
Gossypium hirsutum (cotton)	−	−	−	C. Bender, OSU
Nicotiana tabacum (tobacco)	−	−	−	J. Verchot, OSU
Lens culinaris (lentil)	−	−	−	M. Arif, OSU
Triticum aestivum (wheat)	−	−	−	S. Rogers, OSU
Glycine max (soybean)	−	−	−	Payco Seeds, Dassel, MN
Helianthus annuus (sunflower)				L. L. Olds Seed Co., Madison, WI
Lycopersicon esculentum (tomato)	−	−	−	L. L. Olds Seed Co., Madison, WI
Zea mays (corn)	−	−	−	Ferry-Morse Seed Co., Fulton, KY
Nephrolepis exaltata (Boston fern)	−	−	−	Noble Research Center, OSU
Soil mix	−	−	−	A. Wayadande, OSU
Microbes				
Rhizina undulata M1	−	−	−	R. Vasaitis, SLU
R. undulata M2	−	−	−	R. Vasaitis, SLU
R. undulata M3	−	−	−	R. Vasaitis, SLU
R. undulata M4	−	−	−	R. Vasaitis, SLU
R. undulata M5	−	−	−	R. Vasaitis, SLU
Pythium aphanidermatum	−	−	−	C. Garzon, OSU
Sclerotinia trifoliorum	−	−	−	H. Melouk, OSU
Sclerotinia minor	−	−	−	H. Melouk, OSU
Rhizoctonia solani	−	−	−	J. Olson, OSU
Phytophthora capsici	−	−	−	S. M. Marek, OSU
Plasmid DNA (positive controls)	+	+	+	Generated in the NIMFFAB laboratory through cloning of target sequences for each gene

[a] −, no amplification (negative); +, amplification (positive); OSU, Oklahoma State University, Stillwater, OK; SLU, Swedish University of Agricultural Sciences, Uppsala, Sweden.

(RPB2), were used to design three primer and probe sets (Table 4). Multiple sequences of the *P. omnivora* genes were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). Sequences from *P. omnivora* ITS-rDNA GenBank accession numbers FJ914885, EF494043, EF494042, EF494041, EF494040, EF494039, EF494038, EF494037, EF442000, EF441999, EF441998, EF441995, EF441994, EF441991, AY549457, AY549456, AY549455, and AY549454 were aligned and used to design primers PO4 F (forward) and PO4 R (reverse) and probe PO4. Sequences from accession numbers EF494066, EF494065, EF494064, EF494063, EF494062, and EF494061 were used to design the primer/probe set Pobt1, and those of EF494070, EF494069, EF494068, and EF494067 were used to design the primer/probe set PORPB2-2. Sequences of each gene were aligned using Clustal X v.2 (16) and examined for conserved regions. Percent identity matrices and nucleotide sequence alignments were constructed using GeneDoc (17). Each primer and probe set, targeting a different gene, was designed using Primer3 (18) from a consensus sequence within the target gene of *P. omnivora*. Primer thermodynamics, internal structures, and self-dimer formation were examined *in silico* with mFold (19). The specificity was confirmed *in silico* by screening the primer and probe sequences with BLASTn on the NCBI GenBank database (20) (Table 4). Primers and double-quencher probes 5′ 6-carboxyfluorescein (FAM)/ZEN/3′ Iowa Black FQ (5′ 6-FAM/ZEN/3′ IBFQ) were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, IA).

Positive controls. For each primer set, positive controls carrying target gene segments of *P. omnivora* were generated. Endpoint PCR amplicons were eluted from the agarose gel using Quantum Prep Freeze 'N Squeeze Spin Columns (Bio-Rad, Hercules, CA) and cloned using a TOPO-TA cloning kit (Invitrogen). Plasmid DNA carrying the target sequence for each primer set was purified from overnight bacterial cultures using a QIAprep Spin Miniprep kit (Qiagen). The concentrations of total plasmid DNA were determined using a NanoDrop v.2000 spectrophotometer. Inserts from isolated plasmids were sequenced at the OSU Recombinant DNA/Protein Core Facility using M13F and M13R primers. Amplicon sequences were compared against sequences available on the GenBank nucleotide database using BLASTn.

TABLE 3 *Phymatotrichopsis omnivora* isolates used in the inclusivity panel for validation of *P. omnivora*-specific primers and probes

Host plant and *P. omnivora* isolate accession no.	Region and state of origin[a]	C_T values with specific primer/probe[b]:		
		PO4	Pobt1	PoRPB2-2
Alfalfa				
NFAlf-7	SC Oklahoma	10.67	17.25	18.11
PECOS TXAlf 07-2	W Texas	10.12	17.89	19.07
CCAZ Alf07-2	C Arizona	12.39	19.65	20.32
FS TXAlf07-1	W Texas	8.92	16.37	16.29
Cotton				
SANE TXCO7-10	W Texas	+	+	+
Maudlowe CO5-1	SE Texas	10.98	20.60	20.34
Braden TXCO7-6	W Texas	13.04	20.30	22.70
TAMD CO5-3	NC Texas	+	+	+
EC59 CO5	SE Texas	+	+	+
Marana AZC07-4	SE Arizona	10.60	17.98	19.38
Rick CO5-5	SE Texas	+	+	+
MS AZC07-4	SE Arizona	12.68	18.79	18.76
Yuma2 AZC07-7	SW Arizona	11.8	18.67	17.65

[a] C, central; E, east; N, north; S, south; W, west.
[b] +, positive by using only endpoint PCR (qPCR was not performed).

Endpoint PCR amplification. Preliminary PCR assays to assess the three primer sets were carried out in 20-μl reaction mixtures containing 10 μl GoTaq Green Master Mix (Promega), 1 μl of each forward and reverse primer (5 μM), 1 μl of DNA template, and 7 μl nuclease-free water (Ambion, Austin, TX) in an Eppendorf thermal cycler (Eppendorf, Hauppauge, NY). The cycling parameters were as follows: initial denaturation for 3 min at 94°C followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s, followed by a final extension at 72°C for 3 min. Positive (plasmid DNA; carrying the target gene) and negative (nontemplate [water]) controls were included in each PCR amplification. A volume of 20 μl of amplified PCR product was electrophoresed in a 1.5% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer, and amplicon sizes were estimated using 1Kb Plus ladders (Invitrogen).

qPCR amplification. Amplifications with each primer set were carried out in 20-μl reaction mixtures containing 10 μl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.8 μl (5 μM) of each forward and reverse primer, 0.8 μl (5 μM) probe, 0.12 μl bovine serum albumin (BSA) (50 mg/ml), 1 μl of template genomic DNA, and 6.48 μl of nuclease-free water (Ambion). Positive (plasmid DNA, carrying the target gene) and negative (nontemplate [water]) controls were included in each round of qPCR amplification, and each reaction was performed in three replicates. Cycling parameters included two initial holds, each for 2 min at 50°C and 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The assays were performed in a Rotor-Gene 6000 thermocycler, and data analysis was done using the Rotor-Gene 6000 series software 1.7 (Built 87) (Corbett Research, Sydney, Australia) with a manual cycle threshold (C_T) of 0.2.

FIG 1 Bar codes generated to operate the Razor Ex BioDetection system. (A) Pouch protocol bar code; (B) PCR cycling program bar code.

qPCR sensitivity and spiked assays. To determine the detection limits of all three primer and probe sets, four sensitivity assays were performed with each set in the Rotor-Gene 6000 thermocycler. Plasmid or genomic DNA was serially diluted in 10-fold increments and used at 1 fg to 10 ng per reaction mixture. Each mixed assay was done by adding 1 μl (per reaction mixture) of soil or cotton leaf extract (uninfected soil or a healthy cotton leaf was macerated in 1 ml of TE buffer and clarified by a 2-min centrifugation at 14,000 rpm; the supernatant was used to spike the DNA preparation) into serially diluted *P. omnivora* genomic DNA. Each reaction was performed in three replicates.

Razor Ex BioDetection system amplification. Empty pouches with regular TaqMan reagent were used in place of freeze-dried reagent pouches. Amplification with each primer set was carried out in 150-μl reaction mixtures containing 75 μl of Platinum Quantitative PCR SuperMix-UDG, 6.0 μl (5 μM) of each forward (biotinylated) and reverse primer, 6.0 μl (5 μM) probe, 4 μl of template DNA, and 53 μl of nuclease-free water. Positive and negative controls were included in each round of Razor Ex BioDetection system amplification. Each reaction was performed in duplicate for infected plant samples due to the limited number (12) of well slots. Cycling parameters included one initial hold for 2 min at 50°C and a first cycle at 94°C for 4 min and 60°C for 15 s, followed by 54 cycles at 91°C for 3 s and 60°C for 15 s. The program was uploaded using a bar code (Fig. 1). The assays were performed with a Razor Ex BioDetection system.

PCR sensitivity comparison with and without primer modification.
(i) Effect of primer biotinylation. The forward primer for each target gene was labeled with biotin. Two concentrations (100 pg and 10 pg) of plasmid DNA carrying the target gene sequences were used for each set of primers in TaqMan qPCR.

(ii) Effect of the addition of two different 5′ AT-rich flaps to primers. Three qPCR assays were performed to compare the sensitivity obtained

TABLE 4 *Phymatotrichopsis omnivora*-specific primers and probe sets used for qPCR and Razor Ex BioDetection system amplification

Primer name	Primer sequence (5′–3′)	Primer and probe location	Target gene region	Amplicon size (bp)	GC%	ΔG[a]	NCBI BLASTn result		
							E value	Query coverage (%)	Identity (%)
PO4 F	GTTCGAGCGTCAGCATAACA	425–444	ITS2-5.8S rDNA	116	50	0.0	0.064	100	100
PO4 R	AAGACACCACCCATACATTTCAG	519–541	ITS2		43	0.8	0.002	100	100
PO4 probe	TGGCTTGGTCATTGGCGGTG	473–492	ITS2		60	0.8	0.064	100	100
PObt1 F	GGTCTTGATGGTTCTGGTGTGT	104–125	Beta-tubulin	126	50	0.9	0.006	100	100
PObt1 R	TCCAACTGGAGGTCAGAGGTA	208–228	Beta-tubulin		52	0.8	0.024	100	100
PObt1 probe	TCCCCATAGAGCCGATAGAGTGCTG	132–156	Beta-tubulin		56	0.8	2e−04	100	100
PORPB2-2F	GTGTCTGCCGTCCACTTTTC	1265–1284	RPB2	135	55	0.8	0.064	100	100
PORPB2-2R	CCATCCGAACCTCTCCTCTT	1379–1398	RPB2		55	0.5	0.064	100	100
PORPB2 probe 2	ATAACGACCCGACGAGCGAGAGG	1292–1314	RPB2		61	0.7	0.002	100	100

[a] Plot ΔG value calculated by mFOLD.

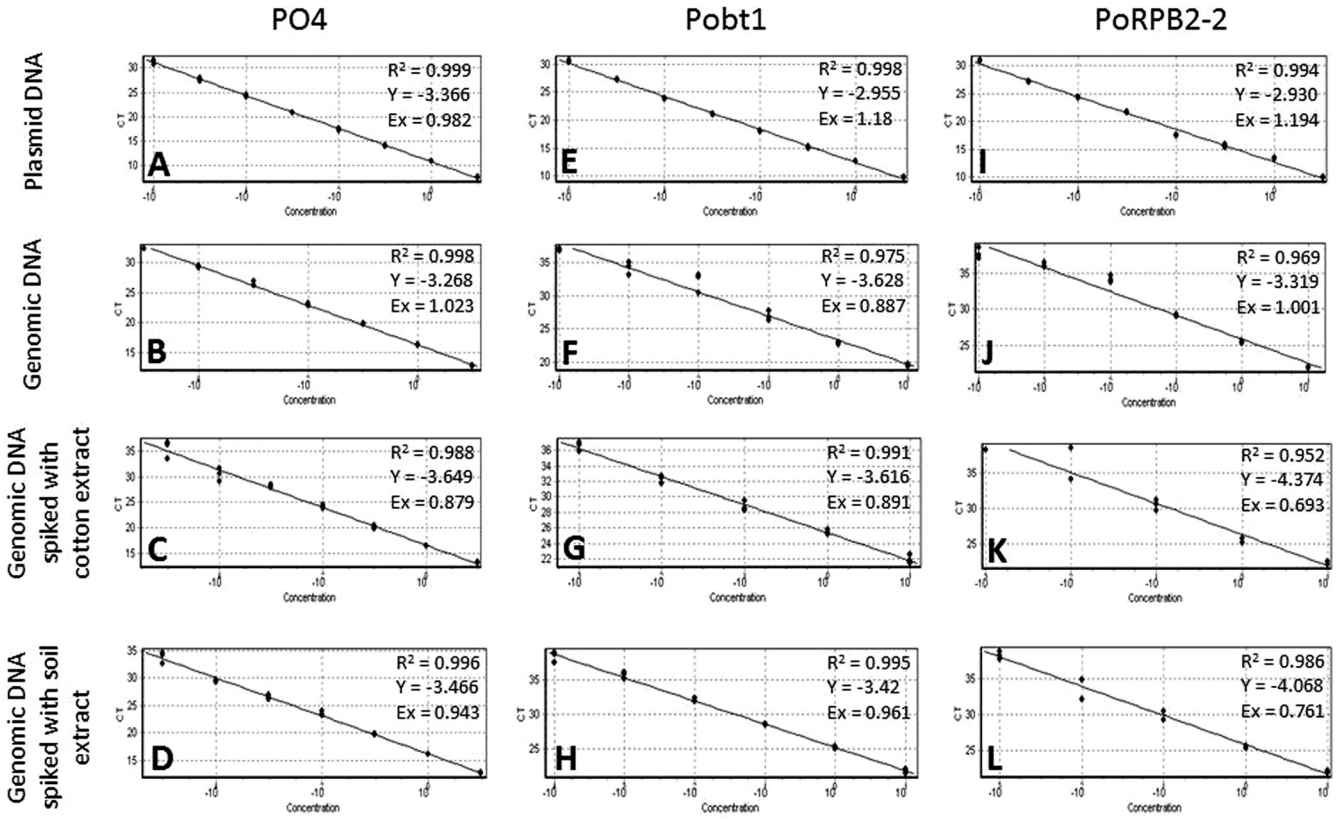

FIG 2 Standard curves were obtained for qPCR amplification of 10-fold serially diluted plasmid DNA and genomic DNA using probes PO4, Pobt1, and PoRPB2-2. Probe PO4 amplified with plasmid DNA (A), genomic DNA (B), genomic DNA mixed with cotton extract (C), and genomic DNA mixed with soil extract (D). Probe Pobt1 amplified with plasmid DNA (E), genomic DNA (F), genomic DNA mixed with cotton extract (G), and genomic DNA mixed with soil extract (H). PoRPB2-2 amplified with plasmid DNA (I), genomic DNA (J), genomic DNA mixed with cotton extract (K), and genomic DNA mixed with soil extract (L). The unit of concentration is ng/reaction mixture; CT, cycle threshold; R^2, linear correlation; Ex, reaction efficiency; Y, slope.

using primers designed (i) with a 5′ AT-rich flap or (ii) without flaps. Two different flaps (5′-AATAAATCATAA-3′ and 5′-AAAATTATTTT-3′) were used at the 5′ position of the primers (21, 22).

RESULTS

DNA isolation. The yield of *in vitro* DNA isolated using the commercially available kits was good and appropriate for qPCR and endpoint PCR. For on-site *P. omnivora* detection, the DNA extracted from four different infected alfalfa root samples using the modified Dynabeads-based protocol was free from PCR inhibitors. The method was reproducible, took only about 10 min to complete, and can be done without centrifuges, incubators, or liquid nitrogen.

Primer and probe design. Three sets of primers and probes were designed to target the three *P. omnivora* genes encoding the ITS-rDNA, beta-tubulin, and RPB2. All primers and probes met the desired 100% query coverage and 100% identity after an alignment using BLASTn in the NCBI GenBank nucleotide database (Table 4). Predictions for internal structures and delta G (ΔG) plot values of each primer and probe were calculated using mFold (Table 4). All primers and probes had plot ΔG of <1.0 at 60°C. The double-quenched probes contain a 5′ FAM fluorophore, a 3′ IBFQ quencher, and an internal ZEN quencher to maximize signal and minimize background.

Primer and probe specificity. Each primer and probe set (Table 4) was designed for use in endpoint PCR, qPCR, and the Razor Ex BioDetection system. Primer and probe specificity in qPCR was tested against a fungal and oomycete panel that included *Rhizina undulata*, which is closely related (confamilial) to *P. omnivora*, and against a plant exclusivity panel (Table 2). Broad-range detection was tested against an inclusivity panel (Table 3) of *P. omnivora* genomic DNA. The primer and probe sets PO4, Pobt1, and PoRPB2-2 showed no cross-reactivity with any species in the exclusivity panel, and the expected 116-, 126-, and 135-bp PCR products, respectively, were amplified only from *P. omnivora*. To further confirm the specificity, the amplified products were cloned, sequenced, and assessed using BLASTn against the available NCBI nucleotide database. Sequences of PCR amplicons generated using primer sets PO4, Pobt1, and PoRPB2 showed maximum similarity only with the target *P. omnivora* sequence with E values of 6e−53 to 3e−40, 6e−58, and 7e−63, respectively. The plasmid DNA isolated from the cloned fragment was used to make the standard curve and as a positive control in each reaction mixture. A total of 14 dead and nonsymptomatic infected alfalfa plant roots tested positive (Table 1) with C_T values of 10.5 to 27.5, 19.5 to 35.8, and 21.5 to 41.6 with primer and probe sets PO4, Pobt1, and PoRPB2-2, respectively. The other symptomatic, nonsymptomatic, and dead alfalfa, cotton, and unknown weeds and soil samples collected between October and December 2010 from alfalfa and cotton fields were screened with the PO4 primers and probe set. Of 52 samples, 31 were positive (5/5 asymptomatic al-

falfa and 8/10 dead/symptomatic alfalfa; 2/2 asymptomatic unknown weeds and 2/2 dead unknown weeds; 2/2 symptomatic cotton; and 11/26 soil from alfalfa fields and 1/5 soil from cotton fields) (Table 1). Alfalfa samples O39 and O41, collected in December 2010, were negative for *P. omnivora*, perhaps because at the time of collection the samples were deteriorated, dry, and asymptomatic. The fact that the soil samples were collected randomly between October and December, when the crop was already harvested, may possibly account for the observation that the pathogen was detected in only 12 of 31 soil samples.

Sensitivity assays. Each primer and probe set detected as little as 1 fg of plasmid DNA (positive controls) in qPCR. Almost identical C_T values (between 7.64 and 9.93) were obtained when each primer set was used to amplify 10 ng of positive control and their C_T values were compared, suggesting high efficiency for these primer sets (Fig. 2A, E, and I). The detection limit of the PO4 primer and probe set was higher (down to 10 fg; Fig. 2B) than those of the Pobt1 and PoRPB2-2 primer and probe sets (down to 100 fg; Fig. 2F and J) with *P. omnivora* genomic DNA. Using 10 ng of fungal genomic DNA, primer set PO4 had a C_T value (12.78) lower than those of Pobt1 (19.4) and PoRPB2-2 (21.95). A small difference in sensitivity was observed when a *P. omnivora* genomic DNA sample was mixed with crude extracts of cotton leaf or field soil (Fig. 2). The primer and probe set PO4 retained its sensitivity when mixed with plant and soil extracts. However, set Pobt1 was 10 times less sensitive when mixed with the plant extract. Set PoRPB2-2 was 10 times less sensitive when mixed with either plant or soil extracts and showed lower reaction efficiency (Ex), 0.693 or 0.761, when mixed with plant or soil extracts, respectively (Fig. 2K and L). All other sensitivity assays, including those samples that were mixed with plant or soil extracts, showed acceptable reaction efficiencies of between 0.88 and 1.19. A normalized fluorescence value of 0.2 was chosen for comparing all standard graphs generated for all three primer and probe sets. The mixed and nonmixed sensitivity assays generated standard curves (Fig. 2), suggesting that the plant and soil extract had little or no inhibitory effect on qPCR sensitivity.

The sensitivity of each primer set was compared also after the addition of biotin to each forward primer, but almost no change of C_T values was noted (Table 5). In other modifications, two different 5′ AT-rich flaps, wf and wf3, were added to each primer in an effort to enhance sensitivity (21, 22). These values indicate that there was no enhancement in qPCR fluorescence (Table 5) after addition of either flap, which suggested that all of the primers and probes developed in this study have suitable thermodynamic properties.

Razor Ex BioDetection system. Primer and probe sets and PCR conditions were validated for the battery-operated, field-deployable Razor Ex BioDetection system, and the results were reproducible. The entire protocol took only ~30 min, with no need for laboratory equipment. The PO4 primer/probe set showed the lowest C_T value, i.e., 34, with infected plant sample (highest sensitivity) (Fig. 3).

TABLE 5 C_T values obtained with primer modifications using biotin and 5′ AT-rich flaps

Primer/probe set name and description	C_T values with plasmid DNA amt of:		
	10 ng	100 pg	10 pg
PO4			
No biotin	NA[a]	14.06	16.62
Biotin labeled	NA	14.35	17.0
Pobt1			
No biotin	NA	14.1	18.8
Biotin labeled	NA	14.2	18.9
PoRPB2-2			
No biotin	NA	16.3	18.4
Biotin labeled	NA	16.2	18.5
PO4			
No flap	8.84	NA	NA
With wf flap	9.39	NA	NA
With wf3 flap	9.84	NA	NA
Pobt1			
No flap	9.97	NA	NA
With wf flap	9.93	NA	NA
With wf3 flap	9.85	NA	NA
PoRPB2-2			
No flap	10.67	NA	NA
With wf flap	10.33	NA	NA
With wf3 flap	10.78	NA	NA

[a] NA, not available; primer and probe set were not tested.

DISCUSSION

We report the development of a reliable, accurate, and sensitive qPCR assay for detection of the plant-pathogenic fungus *P. omnivora*, using three pairs of primers and probes targeting three different genes, ITS-rDNA, beta-tubulin, and RPB2 genes. These primer and probe sets appear to be ready for use in the field-deployable Razor Ex BioDetection system. ZEN double-quenched probes having the internal ZEN quencher with the traditional 3′ end quencher shortened the distance between the reporter and quencher, leading to highly efficient quenching with significantly less background signal and greater precision (GEN, New Rochelle, NY).

High assay specificity, accuracy, and reliability are critical in biosecurity, quarantine, and microbial forensics applications. The use of a multigene format maximizes reliability, specificity, and broad-range detection within *P. omnivora* variants by minimizing the risk of false positives and false negatives because each gene acts as an internal control for the two other genes, which enhances the assay's applicability in microbial forensics. No nonspecific cross-reactivity (false positives) was observed for the three primer and probe sets when tested against plant and microbial exclusivity panels (Table 2). Positive PCR results with genomic DNA of 13 diverse *P. omnivora* isolates collected from different locations confirmed broad-range detection of the target pathogen (Table 3). All 14 symptomatic, asymptomatic, and dead plant roots were PCR positive when tested by all three primer and probe sets. Thirty-one samples of 52 symptomatic and asymptomatic plant and soil samples from alfalfa and cotton fields were qPCR positive for *P. omnivora* using the PO4 primers and probe. A few of the positive samples included asymptomatic or infected/dead weed plants collected from the alfalfa field.

The developed assays are sensitive, detecting as little as 1 fg of plasmid DNA (positive control) and 10 fg (PO4) to 100 fg (Pobt1 and PoRPB2-2) of *P. omnivora* genomic DNA. The higher sensitivity achieved with primer set PO4 may be due to the greater

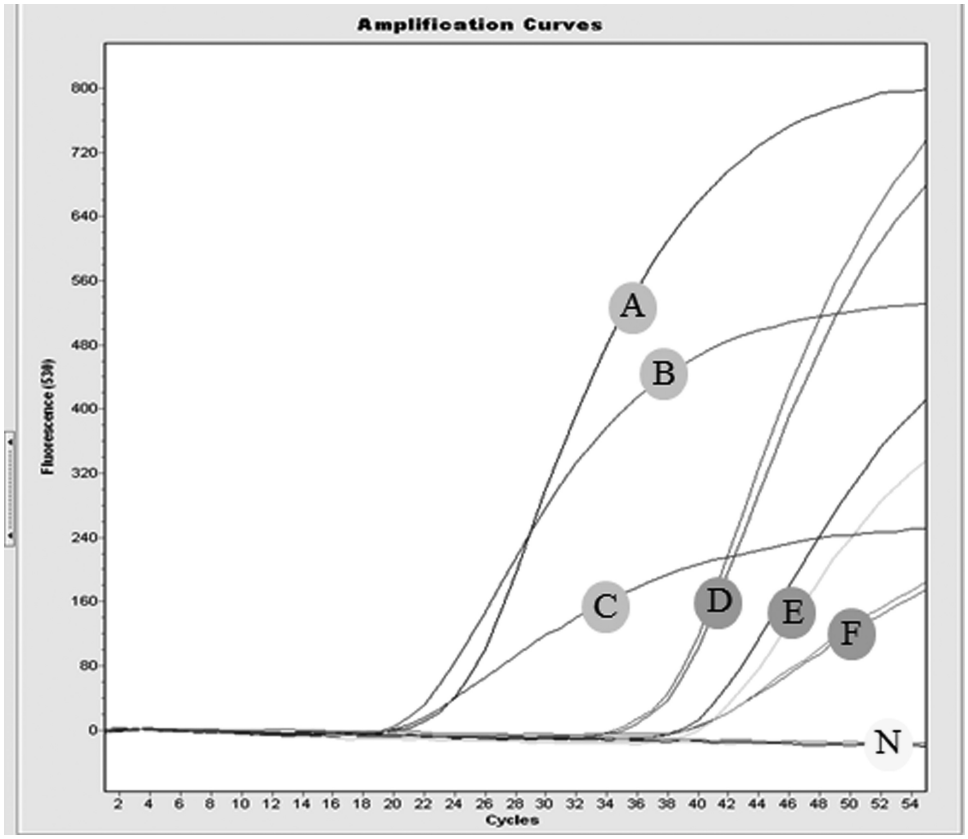

FIG 3 Razor Ex BioDetection system graph obtained with primer and probe set PO4, Pobt1, and PoRPB2-2 amplification of plasmid DNA (positive control) and *P. omnivora*-infected symptomatic alfalfa samples POM1 (positive by real-time qPCR). The results were reproduced using a second symptomatic alfalfa sample POM2 (data not shown). Curves A, B, and C are for positive controls and show estimated C_T values of 21, 23, and 23 for primer/probe sets PO4, Pobt1, and PoRPB2-2, respectively; curves D, E, and F are for *P. omnivora*-infected sample POM1 tested in two replicates with primer/probe sets PO4, Pobt1, and PoRPB2-2 and show estimated C_T values of 36, 40, and 40, respectively; curve N is that of nontemplate controls (water) for each primer/probe set. Both POM1 and POM2 samples were collected from fields at the Samuel Roberts Noble Foundation, Ardmore, OK, in July 2010.

number of rDNA copies per genome (50 to 200 copies per fungal genome [23]) relative to the single or low-copy-number protein-encoding beta-tubulin and RPB2 genes. The high copy number of rDNA allowed detection even from fragmented *P. omnivora* DNA. The genome size of *P. omnivora* is estimated to be ~115 Mb (24). Using a previously published formula (25), 1 pg = 978 Mb (or 1 Mb = 1.022×10^{-3} pg), the detection limit of a single genome of *P. omnivora* should be around 117.5 fg. However, the PO4 primers and probe detect down to 10 fg. Using SYBR green qPCR, Arif and coworkers (26) detected purified *P. omnivora* genomic DNA at amounts as low as 1 fg. However, SYBR green qPCR can result in false positives due to the ability of SYBR green dye to bind to any double-stranded DNA, including primer dimers, limiting the application of this protocol for forensic applications and quarantine inspections.

Previous work had shown that the addition of an AT-rich sequence at the 5′ terminus of primer sequences could lead to increases in PCR yields, sensitivity, and fluorescence (21, 22, 27). But in this study, the addition of 5′ AT-rich flaps made no difference in C_T values, indicating that the designed primers and probes already had optimal PCR thermodynamics.

An important capability for biosecurity, quarantine, and microbial forensics applications is the ability to use the procedure in the field. Tomlinson and coworkers (4, 10) developed on-site, loop-mediated isothermal amplification for phytoplasmas and SmartCycler protocols for *Phytophthora ramorum*. The SmartCycler has been used also for *Xylella fastidiosa* detection (12). Razor Ex BioDetection system-based methods are more rapid than those employing the SmartCycler (10, 12, 14). Matero and coworkers (14) found no difference in sensitivity or accuracy between the Razor Ex and Applied Biosystem 7300/7500 formats. Recently, Idaho Technology, Inc., announced the development of the Razor CRP BioThreat-X kit, which represents the first U.S. Department of Defense system capable of testing for 10 biothreat agents at one time in the field within 30 min. In our investigation, the Razor Ex BioDetection system protocol took about 30 min from sample preparation (DNA extraction, ~10 min) to final detection (~20 to 25 min). The rapid DNA isolation method, unlike many laboratory DNA extraction methods, does not require centrifugation, incubation, organic solvents, or the use of liquid nitrogen for sample homogenization. Commercially available Razor Ex BioDetection system pouches generally contain lyophilized PCR reagents to minimize contamination and eliminate the need for cold storage, but in our experiment we injected regular TaqMan qPCR components into the pouch using a disposable syringe. The primers for the Razor Ex BioDetection system were biotinylated, so as to capture the amplified product using streptavidin magnetic beads for fur-

ther confirmation, if required. No assay inhibition was observed with biotinylated primers.

Multigene TaqMan qPCR and field-deployable Razor Ex BioDetection system-based detection assays for *P. omnivora* are rapid, reliable, sensitive, and efficient, and their use can speed phytosanitary diagnostics and pathogen detection. Early detection can help to prevent pathogen dissemination through plant materials during interstate or international commerce. The assays also have potential applications for farm management and monitoring of resistance in plant breeding programs. The modified Dynabeads-based rapid DNA isolation method, validated in this work for *P. omnivora*, can be applied to other plant pathogens, as well as for field and routine diagnostics.

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