Development of an Assay for Rapid Detection and Quantification of *Verticillium dahliae* in Soil

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ABSTRACT

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Verticillium dahliae is responsible for Verticillium wilt on a wide range of hosts, including strawberry, on which low soil inoculum densities can cause significant crop loss. Determination of inoculum density is currently done by soil plating but this can take 6 to 8 weeks to complete and delay the grower's ability to make planting decisions. To provide a faster means for estimating pathogen populations in the soil, a multiplexed TaqMan real-time polymerase chain reaction (PCR) assay based on the ribosomal DNA (rDNA) intergenic spacer (IGS) was developed for *V. dahliae*. The assay was specific for *V. dahliae* and included an internal control for evaluation of inhibition due to the presence of PCR inhibitors in DNA extracted from soil samples. An excellent correlation

was observed in regression analysis ($R^2 = 0.96$) between real-time PCR results and inoculum densities determined by soil plating in a range of field soils with pathogen densities as low as 1 to 2 microsclerotia/g of soil. Variation in copy number of the rDNA was also evaluated among isolates by SYBR Green real-time PCR amplification of the *V. dahliae*-specific amplicon compared with amplification of several single-copy genes and was estimated to range from ≈ 24 to 73 copies per haploid genome, which translated into possible differences in results among isolates of ≈ 1.8 cycle thresholds. Analysis of the variation in results of *V. dahliae* quantification among extractions of the same soil sample indicated that assaying four replicate DNA extractions for each field sample would provide accurate results. A TaqMan assay also was developed to help identify colonies of *V. tricornutus* on soil plates.

Additional keywords: rDNA copy number, *V. tricornutus*.

Strawberry (*Fragaria* $\times$ *ananassa*) is an important crop in California, with production representing $\approx 89\%$ of the fresh and frozen market strawberries produced in the United States for 2009, with a market value of \$1.7 billion (7). Estimated yearly production costs are $> \$82,000/\text{ha}$ ($\$33,000/\text{acre}$) (33); therefore, disease losses due to soilborne pathogens could have an economic impact for the strawberry producer and are a major reason that nonorganic commercial production fields are fumigated prior to planting. Historically, one of the primary reasons for soil fumigation was for control of the vascular wilt pathogen *Verticillium dahliae* Kleb. (44). This pathogen has a wide host range and can cause significant losses in highly susceptible crops, such as strawberry, that lack genetic resistance. Soil inoculum densities of as little as 1 to 2 microsclerotia (MS) g^{-1} of soil can result in plant losses (20) and 7 to 8 MS g^{-1} of soil can result in 90% losses in some cases (F. Martin, *unpublished*). With the phase-out of methyl bromide for soil fumigation (43,56), the longer intervals needed between soil fumigation and planting when using alternative fumigants, and the increase in organic production, there is a need for having a method for rapid and accurate determination of soil inoculum densities of *V. dahliae*.

Verticillium spp. survive in the soil as MS, which consist of a mass of fungal mycelium surrounded by a rind of tissue high in melanin that protects it from UV light; these structures can survive in the soil for many years in the absence of susceptible hosts (40,49). The current method for determining soil inoculum density involves soil plating onto semiselective media and counting colonies of *V. dahliae* after several weeks of incubation. Both wet and dry sieving techniques have been developed but an inter-laboratory ring trial showed inconsistency in results between the techniques (18,63). Furthermore, it is not possible to differentiate colonies of *V. dahliae* from the crucifer pathogen *V. longisporum* (*V. dahliae* var. *longisporum* C. Stark [61]; comb. nov. Karapapa, Bainbr. & Heale [32]) and, in some cases, from colonies produced by *Verticillium tricornutus* I. Isaac, that are nonpathogenic on strawberry. These other species can be encountered in areas of California where strawberry is grown and, therefore, may potentially influence the accuracy of the plate counts (S. Koike, *unpublished*). The dry sieving technique is commonly used in California but this technique can take 6 to 8 weeks to get results (29). This timeline can be too long when strawberry growers have planting decisions to make; therefore, techniques that could provide accurate results more quickly would be a benefit to the industry.

One approach to provide a faster means of pathogen detection and quantification in the soil is the use of polymerase chain reaction (PCR) techniques. Conventional PCR assays using single (30,45) or nested amplifications (53) have been described for detection of *V. dahliae*. Techniques for quantifying this pathogen in plant tissue (11,26,37) and soil (40) have also been reported, using competitive PCR with an internal control (IC) for relative quantification of *V. dahliae*. However, techniques using real-time PCR will provide a more accurate quantification of the pathogen with a higher level of sensitivity. Schena et al. (58,59) reported a real-time PCR technique using scorpion primers designed against

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species-specific sequences of the intergenic region (IGS) of the ribosomal DNA (rDNA) repeat as a target to detect *V. dahliae* in olive trees; however, an IC to evaluate whether PCR inhibitors in the extracted DNA were affecting the results was not included. Lievens et al. (38) reported a SYBR Green assay targeting the internal transcribed spacer (ITS) region of the rDNA for quantification of a *Verticillium* sp. infecting tomato that included an exogenous IC DNA amplified in a separate well to allow for relative quantification. More recently, Atallah et al. (2) developed a quantification assay for *V. dahliae* in potato using Plexor (Promega Corp., Madison, WI) real-time PCR targeting the single-copy β -tubulin gene. A plant DNA target was also multiplexed in the amplification and the ratio between the results of the pathogen/potato plant target was used to quantify the pathogen. Given how important it is for the California strawberry industry to have a soil quantification assay for *V. dahliae* sensitive enough to be able to detect pathogen inoculum densities as low as 1 to 2 MS/g of soil, it was believed that a new technique needed to be developed focusing on a high-copy-number target sequence that would have the added specificity of a TaqMan probe in addition to primer sequence specificity to reduce potential problems with nonspecific amplification. Recently, several potential real-time PCR soil quantification methods were presented at the 10th International *Verticillium* symposium but were not available for evaluation in California (12,51,65).

There are several challenges for developing a molecular quantification assay for soilborne fungal plant pathogens, including the ability to efficiently disrupt the resting structures present in the soil in order to extract the DNA and provide intact DNA template of sufficient purity that PCR inhibitors do not affect the amplification efficiency of the assay. DNA extraction procedures for soilborne pathogens (including *Verticillium* spp.) have been evaluated with variable success. Methods using liquid nitrogen and phenol-chloroform DNA extraction combined with adding powdered skim milk to reduce PCR inhibition were used for *Fusarium oxysporum* f. sp. *ciceris* Matuo & K. Sato (16), *V. tricornutum* (24), and *V. dahliae* (50,62). Lees et al. (36) also used phenol-chloroform in conjunction with glass beads and a bead beater for assaying *Rhizoctonia solani* Kühn in potato tissue and soil. Commercial kits were used with a FastPrep homogenization instrument (MP Biomedical, Solon, OH) for detection of *Rhizoctonia* spp. from soil (47). Rather than using mechanical disruption, pressure cycling technology was evaluated for improving DNA extraction from soil for detection of *Rhizoctonia* and *Pythium* spp. (46). For some of these methods, reagents such as powdered skim milk, polyvinylpyrrolidone, and polyvinylpolypyrrolidone were added to remove PCR inhibitors during DNA extraction; however, these additions were not always effective in doing this.

The most common approach used to address problems with PCR inhibitors has been to dilute the DNA extract and retry amplification. Although this may eventually allow for amplification, it will reduce the sensitivity of the assay due to dilution of target sequences. Because of this, DNA dilution may not be useful when trying to quantify low levels of pathogen inoculum. This approach can also lead to inaccurate results unless there is some mechanism to detect whether PCR inhibitors are affecting the amplification efficiency of the target DNA; just because there is amplification does not mean that PCR inhibitors are not reducing amplification efficiency and shifting the cycle threshold (C_t) higher, leading to incorrect conclusions. One technique used to assess the impact of inhibitors is to include an IC in the amplification mixture, an approach that has been used in detection of hepatitis virus (39,62), *Enterovirus* spp. (19), or *Enterococcus* spp. (22) in water samples. Perhaps the optimal method is to add a standardized amount of target DNA to the amplification master mix that is designed to be amplified with the same primers used to amplify pathogen template but has a different TaqMan probe and, thus, can be multiplexed in the same amplification. If there is a change

in amplification of this IC multiplexed with the pathogen DNA compared with a control amplification with the IC alone, this is an indication that PCR inhibitors are present and altering the accuracy of the assay. It is essential that an IC be included in soil quantification molecular assays where differences in soil type or cropping histories of the field may result in differing levels of PCR inhibitors making it through the DNA extraction procedures.

Having an accurate real-time PCR procedure for soil quantification of *V. dahliae* will reduce the time needed for soil assays from 6 to 8 weeks to a matter of days, thereby providing growers more lead time to make planting decisions. It would also eliminate questions about the correct identification of *V. dahliae*, *V. longisporum*, and sometimes *V. tricornutum* colonies in soil plating assays, thereby improving the accuracy of results. The objectives and approach of this research were to (i) develop a real-time PCR diagnosis and quantification assay for *V. dahliae*; (ii) develop an IC to determine whether PCR inhibitors in DNA extracted from soil were influencing the amplification of *V. dahliae* target sequences and, thus, the accuracy of results; and (iii) validate the method by comparing the results of the molecular assay with a soil plating assay using different types of field soil.

MATERIALS AND METHODS

***Verticillium* isolates.** All isolates of *Verticillium* spp. used in this study are listed in Table 1 and represent most of the recognized *Verticillium* spp. (56). Mycelia were grown and the DNA extracted following the procedures described by Qin et al. (56) and Collado-Romero et al. (9). Isolate Ls331a from lettuce (*Lactuca sativa* L.) was used as the positive control and for the serial dilution for the standard curves. Assays for *V. tricornutum* used isolate Ls443 from lettuce for positive controls and standard curves.

Isolation of soil fungi. To evaluate the potential of common soil fungi to interfere with the *Verticillium* detection assay, 1 g of field soil from a commercial California strawberry production field (Elder sandy loam) was diluted in 9 ml of sterile water, and seven 1:10 serial dilutions were done. A total of 100 to 500 μ l of each dilution was plated on 10 plates of potato dextrose agar (PDA)-rose-Bengal medium (PDA, rose Bengal at 40 μ g/ μ l, rifampicin at 10 μ g/ml, and ampicillin at 250 μ g/ml), which is a modification of the medium reported by Dhingra and Sinclair (14). Five plates were incubated at 24°C and five at 32°C for 2 days. Morphologically different colonies were transferred to new plates and incubated at their respective temperatures. Pure cultures were grown in potato dextrose broth (Difco, BD, Franklin Lakes, NJ) at 32°C for several days and DNA was extracted using the Fast DNA Spin Kit and the lysis buffer (CLS-Y) for fungi provided by the manufacturer (MP Biomedical). The ITS region was amplified using primers ITS 1 and 4 using the procedures of White et al. (68), and the amplicon was sequenced at the Nucleic Acid Sequencing Facility at the Penn State University (University Park, PA). Taxonomic identification of the isolates was done by BLAST analysis against GenBank (1). DNA from a total of 27 fungal isolates representing a range of *Aspergillus*, *Chaetosartorya*, *Eupenicillium*, *Fusarium*, *Geomyces*, *Mucor*, *Penicillium*, and *Ulocladium* spp. (Supplemental Material S1) were included in evaluations of marker specificity.

***Verticillium* sp.-specific probe and primers design.** To help facilitate detection of low inoculum levels of the pathogen, the high-copy-number IGS from rDNA was used to design primers and probe. An alignment of 65 IGS sequences of different *Verticillium* spp. (*V. dahliae*, *V. albo-atrum*, *V. tricornutum*, and *V. longisporum*) from Qin et al. (56) (PopSet alignment GI: 76781573) was used to design species-specific PCR primers and probes for *V. dahliae* and *V. tricornutum* (Table 2). The software IDT SciTools OligoAnalyzer 3.1 (Integrated DNA Technologies Inc., Coralville, IA) was used for primer design. Specific primers were designed

so that the nucleotides unique to the target were at the 3' end position of the primer, the amplicons were <200 bp, and a TaqMan probe (23) could be designed in the middle of the amplicon. For the TaqMan probe design, the following parameters were used: annealing temperature 10°C higher than the amplification primers, 15 to 30 bp in length, and the total number of Gs or Cs in the last five nucleotides at the 3' end of the probe not exceeding two. Mismatching nucleotides responsible for species specificity were positioned as close as possible to the middle of the probe rather than at the ends while avoiding positions with secondary structure (15). The 5' end of the TaqMan probe for *V. dahliae* was labeled with FAM (fluorescein) and for *V. tricornutus* with CAL FluorRed 610 (Table 2). The 3' end of all probes was labeled with the Black Hole Quencher-1 (BHQ1; Biosearch Technologies, Inc., Novato, CA). The amplification master mix contained 1.0 µM each of the species-specific primer pair, 0.4 µM TaqMan probe, 1.5 mM MgCl₂, and Real Master Mix without Rox (5 Prime; Fisher Scientific, Waltham, MA) with PCR cycling conditions set at 95°C for 2 min and 55 cycles at 95°C for 15 s and 62°C for 30 s, in a reaction volume of 25 µl on the ICycler instrument (IQ4; Bio-Rad Laboratories, Hercules, CA). The amplification conditions were the same for *V. tricornutus*, with the exception that 0.5 µM amplification primers (IGS_Vtric_1F and IGS_Vtric_1R) (Table 2), 0.2 µM Vtrico_probe, and 6 mM MgCl₂ were used with cycling conditions set at 95°C for 1.5 min and 55 cycles at 95°C for 15 s and 57°C for 30 s.

Copy number of rDNA in *V. dahliae*. To estimate the number of rDNA repeat units present in the nuclear genome and evaluate whether there was intraspecific variation that could compromise the accuracy of pathogen quantification, three single-copy genes (endochitinase, glyceraldehyde-3-phosphate dehydrogenase [G3PD], and β-tubulin) were selected based on analysis of the genome sequences of *V. dahliae* and *V. albo-atrum* (http://www.broadinstitute.org/annotation/genome/Verticillium_dahliae/MultiHome.html) and primers were designed to amplify a fragment of ≈160 bp for each of these three genes (Table 3). BLAST analysis with the genome of *V. dahliae* Ls17 was used to confirm that these represented single-copy genes. Amplifications of the *V. dahliae* IGS species-specific target sequence and the single-copy genes were done individually using SYBR Green with the master mix containing 0.4 µM forward and reverse primer, 1.5 mM MgCl₂ and 1× SYBR Green Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA) in a reaction volume of 25 µl with each template run in duplicate. DNA templates from 11 *V. dahliae* isolates from different hosts and geographic locations (Table 1) were used with an approximate concentration of 1 ng/µl determined on a ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). PCR cycling conditions were set at 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 55 cycles at 95°C for 15 s and 60°C for 30 s, and a melting curve with 60 cycles from 55°C increasing at each cycle by 0.5°C. The difference of the mean C_t value between the IGS region and each single-copy locus was calculated for individual *V. dahliae* isolates to estimate the copy number using Excel 2003 (Microsoft, Redmond, WA) (Table 4).

Design of the IC. To serve as an internal reference for each amplification to identify whether there was any alteration in amplification efficiency due to the presence of PCR inhibitors, an IC was developed using a cloned mitochondrial plasmid from *Pythium aphanidermatum* isolate 96-4 (42). The plasmid was initially cloned into the HindIII site of the multiple cloning site in pBluescript (Stratagene, La Jolla, CA) and had been subsequently subcloned into pUC119 (pUC96-4) (Fig. 1). The forward and reverse amplification primers were designed based on plasmid sequences and the multiple cloning sites for pUC119, respectively, with binding sites for the forward and reverse amplification primers for *V. dahliae* tailed on so that the IC would amplify in *V. dahliae* assays without the need for a second set of amplification

primers. The TaqMan probe (PPF_Probe_543) was designed following the procedure mentioned in the previous section with the sequence including regions of the *Pythium* plasmid and portions of the multiple cloning sites of pBluescript and pUC119 (Fig. 1). The targeted amplicon was 121 bp and the amplification conditions to generate this amplicon were 0.4 µM each hybrid primer (Vdf929-PPF1F, CGTTTCCCGTTACTCTTCTTAGA ATAATATATATAACGCGT and Vdr1076-PPF1R, GGATTTCG CCCCAGAAACTCCAAGCTTGCATGCCT) (underlined sequences represent the *V. dahliae* primer sequences), 0.2 mM dNTPs, 2 mM MgCl₂, 1× reaction buffer, and 1.25 units of AmpliTaq DNA Polymerase (Applied Biosystems Inc.), and template DNA (pUC96-4 purified plasmid). PCR cycling conditions to generate the IC amplicon were 95°C for 3 min and 40 cycles at 95°C for 1 min, 60°C for 45 s, and 72°C for 1 min 30 s in a reaction volume of 25 µl. After evaluation of this amplicon on a 2% Seakem agarose electrophoresis gel (Cambrex, Charles City, IA) and staining with ethidium bromide to confirm amplification, the PCR amplification mixture was treated with ExoSap-IT (USB Corporation, Cleveland) following the manufacturer's instructions to remove residual primers and dNTPs. To determine the concentration of the IC that should be added to the amplification master mix of the *V. dahliae* assay, a dilution series from 10⁻² to 10⁻¹² of this amplicon was tested. The TaqMan probe was labeled with Cy5 at the 5' end and with the quencher Iowa Black RQ at the 3' end (Integrated DNA Technologies Inc.). Preliminary evaluations determined that 1 µl of the 10⁻⁹ dilution (0.2 fg/µl) of the IC amplicon had a C_t of ≈30 to 31 while the 10⁻¹⁰ dilution (0.02 fg/µl) was ≈34 to 36 (data not shown). In multiplexed amplifications with the *V. dahliae* marker, the IC probe (PPF_Probe_543) was added to the amplification master mix at a concentration of 0.2 µM.

DNA extraction from soil. Soil samples (500 mg of air-dried soil) were placed in the 2-ml DNA extraction tubes for the FastDNA spin kit for soil (MP Biomedical), frozen in liquid nitrogen for 2 min, and then put in a water bath (70°C) for 2 min. These two steps were repeated for a total of three cycles before addition of lysing matrix E, 978 µl of sodium phosphate buffer, and 122 µl of MT buffer. The tubes were placed in a FastPrep homogenization instrument (MP Biomedical) and homogenized at a setting of 6.5 for 45 s. The manufacturer's recommendations were followed for subsequent steps of DNA extraction, with a final elution volume of 50 µl. The DNA was further purified using components from the Quick Pick kit (BioNobile, Parainen, Finland). DNA extract (20 µl) was put in a 1.5-ml tube, and 10 µl of plant DNA MagaZorb magnetic particles and 250 µl of plant DNA binding buffer was added. This was mixed continuously during incubation at room temperature for 10 min. The tube was placed in the Dynal MPC Magnet rack (Invitrogen, Carlsbad, CA) and, once the paramagnetic beads were attached to the side of the tube, the liquid was removed. A volume of 400 µl of plant DNA wash buffer was added and the tubes were mixed with quick finger flicks prior to replacing in the magnet rack for 1 min before removing the liquid. This washing was repeated. The DNA was eluted with 20 µl of plant DNA elution buffer by gentle mixing at room temperature for 10 min. The magnetic particles were collected on the side of the tube with the magnetic rack and the eluted DNA transferred into a new tube.

Quantification of *V. dahliae* in field soil. Soil samples were collected from commercial strawberry and lettuce fields in different production areas in California (Table 5). Inoculum densities of *V. dahliae* in these soils were assayed using NP-10 semi-selective medium and standard plate count methods following the procedure of Kabir et al. (29), with some modifications. Soils were air-dried at room temperature (23 ± 2°C), mixed thoroughly, and pulverized with a mortar and pestle. The samples were then passed through a 20-mesh sieve to remove hard particles and stones. For each sample, 10 g of the sieved soil was placed in

vials along with 3 ml of a methionine solution (7.5 mg/ml) (31). Vials were sealed and incubated in the dark for 1 week at 30°C. After this incubation, vials were opened and allowed to air dry at room temperature for an additional week. The dry samples were repulverized and stored until tested for *Verticillium* spp. Prepared soils were evenly distributed onto the agar surfaces of 100-mm petri plates containing NP-10 semiselective medium for *V. dahliae* (29,60) using the modified Anderson air sampler (6,35). For each sample, 0.5 g of soil was distributed onto six petri plates. For six soils that had very low inoculum densities (soils 05, 07, 25, 29, 30, and 31) (Table 5), 1.0 g of soil was distributed on a total of 12 plates to improve the accuracy of the results obtained. Plates were subsequently stored in plastic sleeves and incubated in the dark at 23 ± 2°C for 3 weeks. After incubation, agar surfaces were gently rubbed by hand under running water to remove soil particles. Washed plates were drained and then examined for the presence of microsclerotial colonies of *V. dahliae* by viewing plates with a dissecting microscope having a transmitted light source. Some colonies were observed that were difficult to classify as *V. dahliae* or *V. tricornis*. In these instances, a transfer needle was used to remove part of the colony from the agar surface (taking care to

limit the amount of agar transferred) and DNA was extracted using the same procedures as for DNA extraction from soil, with the exception that one colony was used instead of 500 mg of soil. Samples were assayed individually with the *V. dahliae* and *V. tricornis* TaqMan assay to conclusively determine the correct classification of the colony.

For soil quantification of the pathogen using the *V. dahliae* quantification molecular assay, DNA was extracted from 500 mg of air-dried field soil using the method described above. The DNA samples were assayed in multiplexed amplifications for *V. dahliae* with the IC added at 10⁻¹⁰ as described above; all samples were run in duplicate on the same 96-well plate. To determine the relationship between soil plate counts of inoculum density and results from the TaqMan real-time PCR quantification assay, regression analysis was conducted using Microsoft Excel 2003.

Evaluation of DNA extraction procedure with different soil types. Soils were collected from commercial strawberry fields in different production districts in California to evaluate the effectiveness of the DNA extraction procedure for removing contaminants that will reduce the efficiency of PCR amplification, thereby providing DNA of sufficient quality for conducting quantification

TABLE 1. Isolates of *Verticillium* spp. used in this investigation and evaluation of species specificity of the TaqMan assay for *Verticillium dahliae* and *V. tricornis* evaluated with fungal DNA present at 1 ng/μl^a

Isolate	Species	Host	Origin	VCG ^b	<i>V. dahliae</i> C _t	<i>V. tricornis</i> C _t
V48I ^c	<i>V. albo-atrum</i>	Hop	United Kingdom	–	N/A	N/A
Ms102 ^d	<i>V. albo-atrum</i>	Alfalfa	Pennsylvania, United States	–	N/A	N/A
CHILE1 ^e	<i>V. albo-atrum</i>	Kiwi	Chile	–	N/A	N/A
PSU140 ^e	<i>V. albo-atrum</i>	Alfalfa	Pennsylvania, United States	–	N/A ^f	N/A
VAA 383 ^e	<i>V. albo-atrum</i>	Potato	Ontario, Canada	–	N/A ^f	N/A
462 ^e	<i>V. albo-atrum</i>	Potato	Minnesota, United States	–	N/A	N/A
48557 ^e	<i>V. albo-atrum</i>	Potato	England	–	N/A ^f	N/A
Vaa4ATC ^e	<i>V. albo-atrum</i>	Potato	Ontario, Canada	–	N/A ^f	N/A
1852 ^{g,h}	<i>V. albo-atrum</i>	Potato soil	Cuba	–	N/A ^f	N/A
1856 ^{g,h}	<i>V. albo-atrum</i>	Potato	Manitoba, Canada	–	N/A	N/A
5009 ^{g,h}	<i>V. albo-atrum</i>	Potato	P.E.I., Canada	–	N/A	N/A
4901 ^{g,i}	<i>V. albo-atrum</i>	Potato soil	P.E.I., Canada	–	N/A ^f	N/A
V104 ^{g,i}	<i>V. albo-atrum</i>	Potato soil	P.E.I., Canada	–	N/A ^f	N/A
V112 ^{g,i}	<i>V. albo-atrum</i>	Potato soil	P.E.I., Canada	–	N/A	N/A
CBS451.88 ^j	<i>V. albo-atrum</i>	Unknown	Belgium	–	N/A	N/A
CBS321.91 ^j	<i>V. albo-atrum</i>	Tomato	The Netherlands	–	N/A	N/A
CBS385.91 ^j	<i>V. albo-atrum</i>	Tomato	The Netherlands	–	N/A	N/A
Ca63 ^d	<i>V. dahliae</i>	Bell pepper	California, United States	–	22.1	N/A
Cf40 ^d	<i>V. dahliae</i>	Chili pepper	California, United States	–	23.9	N/A
Cf45 ^{d,k}	<i>V. dahliae</i>	Chili pepper	California, United States	–	19.9	N/A
Cs80 ^{d,k}	<i>V. dahliae</i>	Artichoke	California, United States	2	19.6	N/A
Cv79 ^d	<i>V. dahliae</i>	Watermelon	California, United States	–	20.9	N/A
Cv85 ^d	<i>V. dahliae</i>	Watermelon	California, United States	2	22.3	N/A
Fca21 ^{d,k}	<i>V. dahliae</i>	Strawberry	California, United States	2	20.8	N/A
Fca29 ^{d,k}	<i>V. dahliae</i>	Strawberry	California, United States	–	19.9	N/A
Gh101 ^d	<i>V. dahliae</i>	Cotton	California, United States	1	22.2	N/A
Le88 ^{d,k}	<i>V. dahliae</i>	Tomato	California, United States	4	21.7	N/A
Ls17 ^{d,k}	<i>V. dahliae</i>	Lettuce	California, United States	–	19.8	N/A
Ls7 ^d	<i>V. dahliae</i>	Lettuce	California, United States	2	21.2	N/A
Mp89 ^d	<i>V. dahliae</i>	Mint	Oregon, United States	2	20.3	N/A

(continued on next page)

^a Baseline of 5 to 15 and threshold of 350 was used for *V. dahliae* and auto baseline with threshold at 25 for *V. tricornis*. Baseline of 13 to 28 and threshold of 50 was used for the internal control (IC). The IC was also run with the *V. dahliae* assay to evaluate competition. IC amplicon of 10⁻¹⁰ dilution was used to test polymerase chain reaction inhibition. No inhibition was observed with a cycle threshold (C_t) of 33 to 35. N/A = no amplification and nt = not tested.

^b VCG = vegetative compatibility group, HSI = heterokaryon self-incompatible and – = means not determined.

^c DNA provided by Rafael M. Jiménez-Díaz, Universidad de Córdoba, Spain; Collado-Romero et al. (8,9).

^d Isolates used by Qin et al. (56).

^e DNA provided by Katherine Dobinson, Agriculture and Agrifood Canada, London, ON, Canada.

^f C_t value > 40 cut-off, background amplification.

^g DNA provided by Bud Platt, Agriculture and Agrifood Canada, Charlottetown, Prince Edward Island (P.E.I.), Canada.

^h Determined as a *V. albo-atrum* group 1.

ⁱ Determined as a *V. albo-atrum* group 2.

^j DNA provided by Bart Lievens, Scientia Terra Research Institute, Sint-Katelijne-Waver, Belgium; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MUCL = Mycologie de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

^k Isolates used for evaluating intraspecific intergenic spacer copy number variation.

^l *V. dahliae* Ls331a DNA used for standard curve.

^m Cultures provided by Lindsey du Toit, Washington State University Mount Vernon Northwestern Washington Research and Extension Center.

ⁿ No DNA template control.

assays for *V. dahliae*. The percent sand, silt, and clay, as well as soil pH, were determined at the University of California–Davis Analytical Laboratory using standard techniques (<http://anlab.ucdavis.edu>). Because *V. dahliae* was not present in all of these samples, the amplification of the IC was used to assess the presence of PCR inhibitors. The real-time PCR amplification mixture was spiked with the IC at a concentration of 10^{-10} and the IC amplification was compared with control wells containing the IC in water.

RESULTS

Verticillium markers and IC development. Based on the alignment of 65 IGS sequences from different *Verticillium* spp., PCR primers and a TaqMan probe were designed for detection of *V. dahliae* and *V. tricornis* (Table 2). All 40 isolates of *V. dahliae* were amplified with the *V. dahliae* species-specific primers and probe whereas no amplification occurred with any of the other *Verticillium* spp. (Table 1). Background amplification was not observed for the 28 isolates representing a range of soil fungi commonly encountered in strawberry production fields (data not shown). Likewise, the primer pair and probe for *V. tricornis* was species specific and amplified all isolates of this species evalu-

ated. There was a linear relationship between log of DNA concentration and C_t for both the *V. dahliae* and *V. tricornis* species-specific markers (Fig. 2A and B).

The IC designed from the cloned mitochondrial plasmid of *P. aphanidermatum* isolate 96-4 amplified with the *V. dahliae*-specific primer pair and gave a positive result in TaqMan real-time PCR with the IC probe but was negative when tested with the *V. dahliae*-specific TaqMan probe (data not shown). Conversely, the IC probe did not anneal to amplified *V. dahliae* target DNA. Under the amplification conditions used in these experiments, a dilution of the amplified IC to 10^{-9} (amplification product at 0.2 fg/ μ l) had a C_t of ≈ 31 while a 10^{-10} dilution (amplification product at 0.02 fg/ μ l) had a C_t of ≈ 34 in singleplex amplifications.

Estimation of rDNA copy number in *V. dahliae*. The copy number of the rDNA region of *V. dahliae* was estimated by real-time PCR amplification by comparing the mean C_t of the single-copy genes endochitinase, G3PD, and β -tubulin with the result obtained for the *V. dahliae* species-specific amplification based on the IGS region of the rDNA. At a total DNA concentration of ≈ 1 ng/amplification, the single-copy genes had a C_t value of 21 to 24, depending on the isolate used, with variation in the C_t between replicates generally <0.8 (not shown). In general, mean C_t differences between the IGS region and individual single-copy

TABLE 1. (continued from preceding page)

Isolate	Species	Host	Origin	VCG ^b	<i>V. dahliae</i> C_t	<i>V. tricornis</i> C_t
Sm113 ^d	<i>V. dahliae</i>	Eggplant	Maryland, United States	4	22.3	N/A
St91 ^{d,k}	<i>V. dahliae</i>	Potato	Oregon, United States	4	21.9	N/A
V1371 ^c	<i>V. dahliae</i>	Olive	Spain	1A	19.4	N/A
V2921 ^c	<i>V. dahliae</i>	Cotton	Israel	4B	19.5	N/A
V3101 ^{d,k}	<i>V. dahliae</i>	Israel	Cotton	4B	19.5	N/A
V3141 ^c	<i>V. dahliae</i>	Olive	Spain	1A	18.7	N/A
V3641 ^c	<i>V. dahliae</i>	Olive	Syria	2A	16.8	N/A ^f
V3961 ^c	<i>V. dahliae</i>	Artichoke	Spain	2B	18.7	N/A ^f
V4031 ^c	<i>V. dahliae</i>	Artichoke	Spain	1A	16.4	N/A ^f
V5321 ^c	<i>V. dahliae</i>	Artichoke	Spain	2B	18.9	N/A
V5371 ^c	<i>V. dahliae</i>	Artichoke	Spain	HSI	17.7	N/A
V5391 ^c	<i>V. dahliae</i>	Artichoke	Spain	2B	20.1	N/A
V5481 ^c	<i>V. dahliae</i>	Artichoke	Spain	2A	18.7	N/A
V6611 ^c	<i>V. dahliae</i>	Cotton	Greece	1B	16.1	N/A ^f
V7071 ^c	<i>V. dahliae</i>	Artichoke	Spain	2A	19.4	N/A ^f
V7141 ^c	<i>V. dahliae</i>	Olive	Italy	2A	19.1	N/A ^f
V7171 ^c	<i>V. dahliae</i>	Olive	Italy	2A	20	N/A
V7921 ^{c,k}	<i>V. dahliae</i>	Olive	Spain	4B	19.7	N/A
V8001 ^c	<i>V. dahliae</i>	Olive	Spain	2A	24.7	N/A
V8541 ^{c,k}	<i>V. dahliae</i>	Cotton	Spain	1A	18.6	N/A
V8701 ^c	<i>V. dahliae</i>	Cotton	Spain	1A	19	N/A
69212A ^g	<i>V. dahliae</i>	Potato	P.E.I., Canada	—	22.7	N/A ^f
C026-3 ^g	<i>V. dahliae</i>	Potato	P.E.I., Canada	—	26.6	N/A
10010 ^g	<i>V. dahliae</i>	Potato	P.E.I., Canada	—	24.9	N/A
CBS179.66 ^j	<i>V. dahliae</i>	Tomato	The Netherlands	—	22.3	N/A
CBS381.66 ^j	<i>V. dahliae</i>	Tomato	Canada	—	22	N/A
Ls331a ^{k,l}	<i>V. dahliae</i> 0.3 ng/ μ l	Lettuce	California, United States	—	20.4	N/A
Ls331a ^l	<i>V. dahliae</i> 0.03 ng/ μ l	Lettuce	California, United States	...	25	nt
Ls331a ^l	<i>V. dahliae</i> 0.003 ng/ μ l	Lettuce	California, United States	...	31.3	nt
Ls331a ^l	<i>V. dahliae</i> 0.0003 ng/ μ l	Lettuce	California, United States	...	N/A	nt
Ls331a ^l	<i>V. dahliae</i> 0.00003 ng/ μ l	Lettuce	California, United States	...	N/A	nt
Bob70 ^d	<i>V. longisporum</i>	Cauliflower	California, United States	—	N/A	N/A
Bob71 ^d	<i>V. longisporum</i>	Cauliflower	California, United States	—	N/A	N/A
Ar136 ^d	<i>V. longisporum</i>	Horseradish	Illinois, United States	—	N/A	N/A
V511 ^c	<i>V. nigrescens</i>	Potato	United Kingdom	—	N/A	N/A
VN-10 ^m	<i>V. nigrescens</i>	Spinach	Washington, United States	—	N/A ^f	N/A
MUCL8266 ^j	<i>V. nubilum</i>	Soil	Germany	—	N/A ^f	N/A
V531 ^c	<i>V. tricornis</i>	Soil	United States	—	N/A	nt
Ls442 ^d	<i>V. tricornis</i>	Lettuce	California, United States	—	N/A ^f	nt
Ls443 ^d	<i>V. tricornis</i>	Lettuce	California, United States	—	nt	16.8
Cs234 ^d	<i>V. tricornis</i>	Artichoke	California, United States	—	N/A	19.6
2931 ^g	<i>V. tricornis</i>	Soil	Quebec, Canada	—	N/A	19
2933 ^g	<i>V. tricornis</i>	Soil	Quebec, Canada	—	N/A	22.7
1901 ^g	<i>V. tricornis</i>	Potato leave	P.E.I., Canada	—	N/A	33
VT-10 ^m	<i>V. tricornis</i>	Spinach	Washington, United States	—	N/A	21.8
Control, rep 1 ⁿ	N/A	...	...	...	N/A	...
Control, rep 2 ⁿ	N/A	...	...	...	N/A	...

genes were 5.8 to 6.3 (Table 4), with a mean of 5.9 and standard deviation of 0.4. Variation of <13% was observed for the C_t differences between genes. Based on an amplification efficiency of 90.6% for the IGS assay with a standard dilution of Ls331a DNA, the mean IGS copy number could be estimated using the equation copy number = $1.906^{C_t \text{ difference}}$ (52). With a mean C_t difference of 5.9, this would represent ≈ 45 copies of the rDNA per haploid nucleus for *V. dahliae*. The amplification efficiency of

the single-copy genes was 97.7, 81.9, 92.1, and 81.5% for the endochitinase, G3PD, and two β -tubulin regions, respectively. Because these values were different from the 90.6% amplification efficiency of the IGS marker, these copy number estimates should be considered approximations. To determine whether there were rDNA copy number differences among isolates of *V. dahliae*, the amplifications were repeated with 11 different isolates with estimated relative copy number differences of ≈ 24 to 73 per haploid genome.

TABLE 2. Primers and probe used for TaqMan real-time polymerase chain reaction diagnostic assay for detection and quantification of *Verticillium dahliae* and *V. tricornutum*

Primer name	Primer sequence ^a	Target region
<i>V. dahliae</i> assay primers		
Vd-F929-947	CGTTTCCCGTTACTCTTCT ^b	Intergenic spacer
Vd-R1076-1094	GGATTTCGGCCCAGAACT ^b	
<i>V. dahliae</i> probe		
VdhrC FAM	[5' 6-FAM] CACCGCAAGCAGACTCTTGAAAGCCA [3' BHQ1] ^c	
<i>V. tricornutus</i> assay primers		
IGS_Vtric_1F	TAGTAGAATACTAGATARCTAG ^b	Intergenic spacer
IGS_Vtric_1R	AGCCTAGGTCTTTATAGCTAG ^b	
<i>V. tricornutus</i> probe		
Vtrico_probe	[CAL FluorRed 610] TCCTACTAATACCTTACTATAACTCTTAAGGTA [BHQ2] ^c	
Internal control probe		
PPF_Probe_543	[5' CY5] AAAGTAAGCTTATCGATACCGTCGACCT [3' IOWA BLACK RQ] ^b	<i>Pythium</i> ^d

^a For dye and quencher used for each probe, dye on 5' end and quencher on 3' end. 6-FAM = 6-carboxyfluorescein, BHQ1 = Black Hole Quencher 1, BHQ2 = Black Hole Quencher 2, and IOWA BLACK RQ = Iowa Black quencher.

^b Synthesized at Integrated DNA Technologies, Inc., Coralville, IA.

^c Synthesized at Biosearch Technologies, Inc., Novato, CA.

^d Mitochondrial plasmid clone.

TABLE 3. Primers for amplification of single-copy genes used for evaluating intraspecific variation in the copy number of the ribosomal DNA repeat unit in isolates of *Verticillium dahliae*

Gene region	Primer name	Primer sequence
Endochitinase	Vd_endoch_1F	CTCGGAGGTGCCATGTACTG
Endochitinase	Vd_endoch_1R	ACTGCCTGGCCAGGTTCT
β -Tubulin	Vd_Btub_1F	GCGACCTTAACCACCTCGTT
β -Tubulin	Vd_Btub_1R	CGCGGCTGGTCAGAGGA
β -Tubulin	VertBt-F ^a	AACAACAGTCCGATGGATAATTC
β -Tubulin	VertBt-R ^a	GTACCGGGCTCGAGATCG
G3PD ^b	Vd_G3PD_2F	CACGGCGTCTTCAAGGGT
G3PD	Vd_G3PD_1R	CAGTGGACTCGACGACGTAC

^a β -Tubulin primers from Atallah et al. (3).

^b G3PD = glyceraldehyde-3-phosphate dehydrogenase.

TABLE 4. Evaluation of ribosomal DNA (rDNA) copy number differences in isolates of *Verticillium dahliae* by comparing real-time polymerase chain reaction (PCR) results for the rDNA with results for single-copy genes

Isolate	Mean C_t difference between IGS and single-copy gene region ^a				Mean C_t diff. ^b	SD	Variation (%) ^c	Copy number ^d
	Endo-IGS	G3PD-IGS	Btub1-IGS	Btub2-IGS				
Ls331a	6.3	5.1	5.5	5.4	5.6	0.5	9.2	36.4
Cs80	6.1	5.4	6.0	5.5	5.8	0.4	6.1	40.8
Ls17	6.9	5.7	6.6	6.6	6.4	0.5	8.1	62.1
Fca29	7.1	6.6	5.9	6.3	6.4	0.5	7.9	63.1
Fca21	7.4	7.0	5.8	6.4	6.7	0.7	10.5	72.9
CF45	6.9	6.8	6.4	6.4	6.6	0.3	4.0	71.7
St91	6.6	5.6	5.5	6.1	5.9	0.5	8.6	44.9
Le88	5.5	4.6	4.1	5.4	4.9	0.7	13.6	23.6
V310 I	5.8	6.3	5.7	5.8	5.9	0.3	4.6	44.9
V792 I	5.4	5.4	5.8	5.2	5.5	0.3	4.6	33.6
V854 I	5.6	5.4	5.8	5.9	5.7	0.2	3.9	38.9
Between isolates								
Mean C_t difference	6.3	5.8	5.7	5.9	5.9	0.4	7.4	45.9
SD	0.7	0.8	0.6	0.5	0.5	...	...	...
Variation (%)	10.9	13.1	11.1	8.0	9.2	...	...	...

^a Standard deviations (SDs) of cycle thresholds (C_t s) between PCRs are 0.1–1.0. PCR efficiency of 90.6% for the intergenic spacer (IGS) region based on standard dilution curve of isolate Ls331a: $1.906^{C_t \text{ difference}} = \text{copy number}$ (52). PCR efficiency for the four regions tested was 97.7, 81.9, 92.1, and 81.5%, respectively. Gene designations: Endo = endochitinase, G3PD = glyceraldehyde-3-phosphate dehydrogenase, Btub1 = β -tubulin (primers Vd_Btub_1F and Vd_Btub_1R), and Btub2 = β -tubulin (primers VertBt-F and VertBt-R).

^b Mean C_t difference for each single gene.

^c Percent C_t difference variation between regions.

^d Copy number estimate based on mean C_t difference.

Validation of the molecular detection and quantification assay using field soil. Soil plate counts of *V. dahliae* were 0 to 622 MS/g of soil, with counts >100 MS/g of soil obtained from lettuce fields naturally infested with *V. dahliae*. In some soils, colonies on culture medium were observed that were difficult to identify as *V. dahliae* or *V. tricornutus*; the patterns of micro-sclerotial production in the colony were similar to *V. tricornutus* and less dense than those normally observed for *V. dahliae*, yet the typical yellow pigment associated with colonies of *V. tricornutus* (17) was not always observed. These colonies were removed from the agar plate and their identification confirmed in individual assays using the *V. dahliae* and *V. tricornutus* TaqMan real-time PCR assay before they were counted. There was an excellent correlation ($R^2 = 0.96$) between C_t value of the PCR assay and the number of MS per gram of soil based on the plate counts (Table 5; Fig. 3). The correlation was a little lower ($R^2 = 0.85$) when looking only at the data for soils with a plate count of ≤ 14 MS/g (Supplementary Figure S1B). A count of ≤ 4 MS/g of soil was found to have a C_t of ≈ 32 in these soils.

The effect of the IC concentration on the results of field soil assays. The concentration of the added IC can have an influence on the accuracy of the amplification results obtained (Fig. 2A; Table 6). As the concentration of *V. dahliae* target DNA increases, there can be competition for primers between the pathogen template and IC, resulting in an increase in the C_t for the IC. Likewise, increasing the concentration of the IC can cause competition for amplification primers, resulting in an increase in the C_t of *V. dahliae* when the pathogen DNA is present in low amounts (Fig. 2A). For example, in a soil with a low pathogen inoculum density (soil 06 or 11; 2 MS/g of soil) there was an increase in the C_t for the soil assay for *V. dahliae* when the IC was added (soil 06, $C_t = 35.6$ when the IC was not added versus 36.9 and 37.3 for the 10^{-10} and 10^{-9} dilution, respectively). Similar results were observed for soil 01 (6 MS/g of soil), although addition of a 10^{-10} dilution of the IC did not alter amplification of the *V. dahliae* marker (Table 6). Conversely, high concentrations of *V. dahliae* template can also influence the amplification of the IC as exemplified by soil 24, where the inoculum density of *V. dahliae* was 156 MS/g of soil. The presence of the IC in this sample had a limited effect on pathogen amplification (at 10^{-9} dilution of the IC, there was a slight increase in C_t for pathogen detection), yet there was an increase in the C_t for the IC from 30.8 for the water control to 33.2 with the 10^{-9} IC dilution (but no amplification for the 10^{-10} IC dilution). There also was a lack of

amplification of the IC when present at 10^{-10} dilution in other field soils that had a high inoculum density (soils 26 and 28) (Table 5). Similar increases in the C_t for the IC at these dilution levels was also observed in amplifications with increasing concentrations of culture-purified *V. dahliae* DNA added (data not shown).

TABLE 5. Assay of field soils for *Verticillium dahliae* by standard direct plating methods for determining inoculum densities and by TaqMan real-time polymerase chain reaction (PCR)^a

Soil samples (undiluted)	<i>V. dahliae</i> mean C_t ^b	SD	IC C_t mean (10^{-10} dilution)	<i>V. dahliae</i> (MS/g) ^c
Soil 01	33.4	0.4	35.8	6
Soil 02	33.9	0.1	35.7	6
Soil 03	34.5	0.5	35.0	4
Soil 04	32.4	0.1	35.4	8
Soil 05 ^d	N/A ^e	...	34.5	2
Soil 06	36.9	3.3	36.3	2
Soil 07 ^d	32.3	0.2	36.5	11
Soil 08	33.4	1.1	36.7	6
Soil 09	33.4	0.4	35.8 ^f	4
Soil 10	34.8	0.4	35.3	2
Soil 11	35.0	0.7	34.8	2
Soil 12	35.4	0.4	34.3	2
Soil 13	30.5	0.1	37.5	40
Soil 19	30.5	1.5	36.9	12
Soil 20	33.7	1.1	37.2	6
Soil 22	N/A ^e	...	34.1	0
Soil 23	N/A	...	34.9	0
Soil 24	26.1	0.2	N/A	156
Soil 25 ^f	36.4	1.1	36.2	1
Soil 26	22.4	0.1	N/A	622
Soil 27	31.0	0.1	36.7	14
Soil 28	25.2	0.3	N/A	126
Soil 29 ^d	31.5	0.8	39.4 ^f	12
Soil 30 ^d	32.9	0.7	37.1 ^f	4
Soil 31 ^d	N/A	...	34.4	0
H ₂ O	N/A	...	34.2	...

^a SD = standard deviation, IC = internal control, and N/A = no amplification.

^b Mean cycle threshold (C_t) of two PCR replicates.

^c Plate count, microsclerotia per gram of soil.

^d For those soils, 12 plates were used for the plate count instead of 6 to provide greater accuracy with the low inoculum density.

^e Only one of two replicates had a value and, with this high $C_t > 40$, was considered negative.

^f Only one C_t value of the two was counted for this soil; the other value was >40.

Fig. 1. Diagram of the amplicon used to design the internal control (IC). Primers and probe for the IC assay were based on the clone of mitochondrial *Pythium* plasmid 96-4 (43). The sequence consisted of a construction of a *Pythium* plasmid sequence (white arrow) with multiple cloning sites (MCS) of the cloning vectors pBluescript and pUC119. Amplification primers for *Verticillium dahliae* that were tailed on the end of the plasmid amplification primers are indicated by the black bars.

To minimize the competition between amplification of the IC and *V. dahliae* target sequences, the following approach for running field soil samples was selected. All samples are first run with the IC at a 10^{-10} concentration (amplification product at 0.02 fg/ μ l). If there was a limited difference in IC amplification compared with the IC control and the *V. dahliae* assay result had a $C_t > 34$, then it was necessary to rerun the assay without the IC added to get an accurate determination of *V. dahliae*. When the *V. dahliae* C_t is < 29 , the competition with the pathogen template will cause the C_t of the IC to increase; therefore, the sample should be diluted and rerun to ensure an accurate evaluation of IC amplification and *V. dahliae* quantification.

Consistency of results among DNA extractions. To evaluate the consistency of results among different soil DNA extractions, four soils were extracted five times and the results of the assay for *V. dahliae* compared. Mean C_t and the level of variation were calculated for all possible combinations of data from a combination of two, three, and four separate extractions (Table 7). The standard deviation in C_t within each soil replicate was 0.1 to 0.7, with the lowest values observed for the mean of four replicates. When these data were converted to MS counts, this translated into an $\approx 8\%$ variation in results when looking at the means of all combinations of four DNA extractions. The means of three replicates gave nearly identical MS counts but with an increase in variation to $\approx 11\%$. There was one replicate for soil 20 that had a much lower C_t than the rest of the replicates and was not included in the analysis. In view of this, it was concluded that four DNA extractions should be done for each soil sample because this provides a balance between accuracy of results and soil processing as well as providing a buffer in the event that one DNA extraction gives results far different from the other replicates.

Fig. 2. A, Standard curve of *Verticillium dahliae* DNA dilution series with cycle threshold (C_t) value plotted against the log of the DNA concentration. Circles and regression analysis represent amplification without the internal control (IC) added. Squares represent the data for amplification with the IC; regression analysis for this data was not done because of polymerase chain reaction competition between the IC and *V. dahliae* target sequences when the pathogen DNA concentration is low, causing the data to be nonlinear above a C_t of 29. B, Standard curve of *V. tricornis* DNA dilution series with C_t value for the *V. tricornis*-specific TaqMan assay plotted against the log of the DNA concentration.

Evaluation of soil DNA extraction procedure using different soil types. When the DNA extraction procedure was tested with a range of soil types encountered in California strawberry production, there was no effect on the amplification of the IC; the C_t was the same as was observed in the water control (Table 8). Soil texture types included the following: sand, sandy loam, loam, clay loam, and silty clay loam.

DISCUSSION

The described TaqMan molecular assay for *V. dahliae* provided an accurate means for quantification of the pathogen in the soil. It was species specific when tested against a range of other *Verticillium* spp. as well as other common soil fungi and detected all 40 isolates of *V. dahliae* that were collected from a range of hosts and geographic areas and represented different vegetative compatibility groups. The results from this molecular assay had an excellent correlation ($R^2 = 0.96$), with pathogen inoculum density ranging from 1 to 2 up to 622 MS/g of soil in 25 naturally infested field soils, as determined by a standard soil plate assay. Of particular interest for the California strawberry industry is the ability to accurately quantify the lower inoculum densities of the pathogen, with 1 to 4 MS/g of soil being detected with a C_t of 34 to 36, which is in the linear range of the standard curve regression analysis of DNA concentration and C_t . Using a procedure for DNA extraction from the soil that removed many of the PCR inhibitors that could reduce amplification efficiency and targeting the multiple-copy IGS region of the rDNA contributed to this level of sensitivity. Although the three isolates of *V. longisporum* that were tested did not amplify, in view of the recent work by Inderbitzin et al. (28) identifying three lineages of this species that share sequence homology with *V. dahliae* (representatives of lineage A1/D1 and A1/D2 were included in the evaluation), it would be prudent to conduct additional evaluations with lineage A1/D3 of *V. longisporum* to confirm species specificity of the *V. dahliae* diagnostic markers.

It has been reported that variation in the rDNA IGS sequences is present among *Verticillium* spp. (54,58,59) and even among different isolates of *V. dahliae* (54); however, based on sequence alignments and the fact that multiple isolates from different hosts and geographic regions were detected, the primer and probe annealing sites used in this assay appear to be highly conserved in *V. dahliae*. In addition to having sequence differences that can be

Fig. 3. Linear regression of mean cycle threshold (C_t) value from TaqMan real-time polymerase chain reaction quantitative assay for *Verticillium dahliae* versus log of microsclerotia of *V. dahliae* per gram of soil from soil plating of the same soil samples.

used to design a species-specific TaqMan assay, the IGS region is also present in multiple copies, which increases the sensitivity of the assay compared with single-copy target sequences. Standard curves using DNA isolated from pure cultures indicated that as little as 3 fg of pathogen DNA could be accurately quantified using the assay, and analysis of field soils indicates a lower limit of detection of 1 MS/g of soil. The copy number of the rDNA in *V. dahliae* is not known; however, using real-time PCR amplification of single-copy genes compared with amplification of the diagnostic amplicon from the IGS region, it was estimated to be

≈24 to 73 copies per haploid genome (average of ≈46) depending on the isolate. A similar approach was used for determination of gene copy number variation in Rhesus macaques (13). Given that the amplification efficiency for each of the single-copy genes and the IGS region were different, these values should be considered an estimate only but useful for relative comparison of copy number among isolates. This difference in copy number among isolates could contribute to variability in quantification assays if they were present in the same field; however, based on the raw data obtained during the course of this experimentation, this

TABLE 6. Evaluation of the influence of *Verticillium dahliae* inoculum density in different field soils and internal control (IC) dilution on the results of the TaqMan real-time polymerase chain reaction (PCR) molecular assay for quantification of *V. dahliae*

Soil sample isolates ^a	C _t mean per IC concentration used ^b					<i>V. dahliae</i> (MS/g) ^c
	<i>V. dahliae</i> amplification			IC amplification		
	No IC	IC 10 ⁻¹⁰	IC 10 ⁻⁹	IC 10 ⁻¹⁰	IC 10 ⁻⁹	
Soil 01	33.6	33.4	35.0	35.8	31.0	6
Soil 02	32.9	33.9	34.7	35.7	31.3	6
Soil 06	35.6	36.9	37.3	36.3	31.2	2
Soil 11	35.0	41.2	40.2	34.8	30.9	2
Soil 24	25.9	26.1	26.6	N/A	33.2	156
Soil 25	34.3	36.4	37.1	36.2	31.3	1
Soil 26	21.6	22.4	22.3	N/A	42.5	622
Soil 28	24.5	25.2	25.0	N/A	35.9	126
Soil 29	31.4	31.5	31.4	44.6	31.2	12
Soil 30	32.1	32.9	31.8	42.4	30.8	4
Soil 31	N/A	N/A	N/A	34.4	30.9	0
H ₂ O	N/A	N/A	N/A	34.2	30.8	...

^a All soil DNA were purified with paramagnetic particles and undiluted.

^b Mean cycle thresholds (C_ts) are based on two PCR replicates. N/A = no amplification.

^c Plate count, microsclerotia per gram of soil.

TABLE 7. Evaluation of the variation in TaqMan real-time polymerase chain reaction results for *Verticillium dahliae* quantification in soil when replicated DNA extractions are compared^a

Soil	C _t value, mean of					Calculated microsclerotial counts, mean of ^c				
	Two replicates	Three replicates	Four replicates	All replicates	CV (%) ^b	Two replicates	Three replicates	Four replicates	All replicates	CV (%) ^b
A	31.8 (0.3)	31.8 (0.2)	31.8 (0.1)	31.8 (0.6)	1.9	11.1 (1.7)	11.4 (0.9)	11.1 (0.8)	11.1 (3.0)	27
B	32.3 (0.4)	32.3 (0.3)	32.3 (0.2)	32.3 (0.7)	2.1	9.2 (1.7)	9.1 (1.2)	9.2 (0.7)	9.2 (3.0)	33
C	31.6 (0.5)	31.6 (0.3)	31.6 (0.2)	...	2.2	12.4 (2.2)	12.6 (1.4)	12.4 (0.9)	...	24
D	30.8 (0.4)	30.8 (0.2)	30.8 (0.2)	30.8 (0.6)	1.9	17.8 (3.0)	17.9 (1.9)	17.8 (1.3)	17.8 (5.2)	29

^a Numbers in parentheses indicate standard deviation between the means obtained for each combination; ... indicates only four replicates used for this soil; one replicate had an inconsistent reading with a cycle threshold (C_t) of 28 that was not included in the analysis.

^b Coefficient of variation.

^c Calculation equation: $y = -2.224\ln(x) + 37.063$, $R^2 = 0.9565$.

TABLE 8. Evaluation of the DNA extraction procedure on the ability to obtain DNA from different soil types collected from commercial strawberry production fields in California that can be amplified in the *Verticillium dahliae* TaqMan real-time polymerase chain reaction diagnostic assay

Soil ^a	Soil type	Mean C _t IC ^b	SD ^c	pH	OM (%) ^d	Sand (%)	Silt (%)	Clay (%)
1-A	Pittville sandy loam	34.5	0.31	6.6	0.63	73	14	13
1-B	Pittville sandy loam	34.9	0.64	...	...	...	...	...
2-1	Anacapa sandy loam	34.7	1.27	...	...	...	...	...
2-2	Hueneme sandy loam	34.6	0.32	...	...	...	...	...
2-3	Salinas clay loam	33.9	0.15	7.3	1.20	16	53	31
2-4	Sorrento silty clay loam	34.5	1.00	6.7	1.48	22	45	33
3-1	Suey silt loam	34.4	0.29	...	...	...	...	...
3-2	Bayshore silty clay loam	34.3	0.32	7.3	2.79	37	32	31
3-3	Oceano sand	34.2	0.38	...	...	...	...	...
4-1	Chular loam	34.2	0.26	...	...	...	...	...
4-2	San Emygdio variant sandy loam	34.8	1.21	...	...	...	...	...
4-3	Elder sandy loam	34.3	0.60	7.0	1.08	78	13	9
4-4	Pacheco clay loam	34.6	0.55	6.9	1.78	28	45	27
H ₂ O	Not applicable	34.5	0.17	...	...	...	...	...

^a Soil numbers indicate soil used from 1, strawberry transplant region, nursery, Fall River Valley; 2-1, Ventura County strawberry fields; 3, Santa Maria strawberry fields; and 4, Salinas, Watsonville strawberry fields.

^b Internal control (IC) concentration used 10⁻¹⁰ dilution.

^c Standard deviation between the different cycle thresholds (C_ts) used for the mean C_t based on three replicates.

^d Organic matter.

should account for a difference in the final C_t of ≈ 1.8 . With the high correlation ($R^2 = 0.96$) observed for comparison between the molecular quantification and microsclerotial plate counts for the field soils collected from a range of locations in California, it does not appear that this possibility of copy number variation among isolates appreciably affected the accuracy of our results. Variation in rDNA copy number has been reported in a number of eumycotan fungal genera (5,41,48,55,57).

The sensitivity of this assay allowed accurate detection of 1 to 2 MS/g and 3 fg of pure *V. dahliae* DNA, which is a lower level relative to other assays for this pathogen. With conventional competitive PCR, Mahuku and Platt (40) had a maximum sensitivity of 5 MS/g of soil. Using the single-copy gene β -tubulin for detection of *V. dahliae* in potato, Atallah et al. (2) reported a limit of detection of 148 fg of pathogen DNA. Likewise, using an ITS target sequence and SYBR Green real-time PCR, Lievens et al. (38) reported a detection limit of 8 to 13 MS and purified pathogen DNA at 1 pg/ μ l. Compared with these techniques, the addition of a TaqMan probe in the assay reported herein could potentially improve the specificity of detection by adding an additional annealing site in order to get a positive amplification.

The IC was an important part of this *V. dahliae* assay because it provided a means to evaluate the presence of PCR inhibitors in the sample. This not only prevented false negative results (if only the pathogen probe was present there would be no way to determine whether the lack of amplification was due to the absence of the pathogen or the inability of amplification to take place due to PCR inhibitors) but also allowed for normalization of results when inhibitors were present in low amounts, which led to more accurate quantification of the pathogen. The cloned mitochondrial plasmid of *P. aphanidermatum* was chosen as a template for the IC because of its limited distribution in *Pythium* spp. (42) making it uncommon in the soil. The uniqueness of the IC was further enhanced by targeting a combination of sequences from the mitochondrial plasmid and multiple cloning sites of the plasmid cloning vectors for the TaqMan probe (Fig. 1). Designing the IC so that it used the same amplification primers as *V. dahliae* target sequences further simplified running the assay. Depending on the concentration of the IC added to the master mix and the inoculum density of *V. dahliae* that was present in the soil, some competition in the amplification of the IC or pathogen target was observed. However, the impact of this competition on the accuracy of the results could be moderated by rerunning the assays without adding the IC (if the $C_t > 34$) or by diluting the soil DNA extract ($C_t < 29$).

An IC also has been used in real-time PCR quantification assays of other microorganisms. Plant sequences were targeted as an IC for assays designed to determine the amount of the pathogen in potato tissue by using a ratio of the amplification results of plant and pathogen to quantify colonization levels (2,3,10). Although this approach may be useful for quantification in plant tissue, other target sequences are needed for assays focusing on the soil, and the IC template should be added to the master mix to ensure that an equal amount is present for each amplification. For quantification of a bacterial biocontrol agent (67) and *Ralstonia solanacearum* (27), an exogenous plasmid and commercially available IC template, respectively, was added to the master mix. Exogenous plasmids were also used as an IC for assays quantifying pathogens responsible for Fusarium crown rot (25) and *Synchytrium endobioticum* (66), while purified DNA from another fungal species was added as the IC template for quantification of soilborne fungal potato pathogens (4). In these examples, the amplification primers for the IC were different from the target organism; however, designing an IC that uses the same amplification primers as the target pathogen will simplify setting up the amplification reactions. This was the approach used for the assay for *V. dahliae* described herein and was patterned after the techniques used for quantification of *Enterovirus* spp. (19) from water

samples and hepatitis B virus (39). Recently, Haudenschild et al. (21) described a similar approach with several ICs for quantification of *Phakopsora pachyrhizi*.

Before a molecular assay can be used to quantify the amount of a pathogen in the soil, basic information on the consistency of results observed between replicate samples is needed so that a sampling strategy can be devised that will provide accurate results without unnecessary sample processing. In an effort to assess this for the *V. dahliae* assay, five replicate samples of several soils were processed and the means calculated for all possible combinations of three and four replicate samples (Table 7). It was concluded that running four replicate samples was advisable when processing field samples because this would provide some buffering to account for "outliers" that should be discarded. This higher level of variation in the result that was observed for one replicate of soil 20 could be due to nonuniform distribution of inoculum in the soil or, perhaps, be caused by organic matter containing a cluster of MS that was not completely disrupted during grinding of the dry soil prior to DNA extraction.

One approach that could improve the sensitivity and accuracy of this assay when low inoculum densities are present is to increase the amount of soil that is used for DNA extraction. Due to the size of the tubes currently used for DNA extraction and the volume of extraction buffer they can hold, the current upper limit is 0.5 g of soil per extraction; increasing the amount of soil will result in a lower yield of DNA, with more PCR inhibitors persisting through the extraction procedure. With this small amount of soil, more variation in the results of the real-time assay may be encountered for low inoculum density soils (< 4 MS/g) due strictly to the likelihood that pathogen inoculum distribution is not completely uniform. Although extraction tubes capable of holding a total of 15 ml are available for the FastPrep instrument and would allow greater amounts of soil to be processed, this would also significantly increase the cost of running the assay due to the need for much larger volumes of extraction buffer. Running three to four replicate DNA extractions with sample tubes capable of holding 2 ml of total volume (0.5 g of soil each) gives adequate consistency in results with minimal increase in processing cost. Another approach described by Debode et al. (12) used sucrose flotation of soil suspensions for concentrating MS from soil. This procedure could significantly increase the accuracy of quantification of soils with a low inoculum density by allowing much larger amounts of soil to be processed in each DNA extraction.

One advantage of using a molecular approach for quantification of *V. dahliae* in the soil is that the specificity of the assay eliminates possible interference from other *Verticillium* spp. The recent report by Inderbitzin et al. (28) confirms that *V. longisporum* is a hybrid species with *V. dahliae* as one parent; however, the colony morphology of this hybrid species on NP-10 medium used for soil plating assays cannot be differentiated from *V. dahliae* (morphological differentiation of these species is based on more elongated conidia for *V. longisporum*) (34). This could lead to incorrect soil inoculum density estimates of *V. dahliae* using soil plating when *V. longisporum* is present. Likewise, it can sometimes be difficult to differentiate colonies of *V. dahliae* from *V. tricorpus*. As noted by Good et al. (17), formation of MS in *V. dahliae* colonies has a more radial pattern, consisting of profuse numbers of MS with larger globose to irregular-shaped MS, compared with a scattered MS pattern for *V. tricorpus*, with fewer MS produced having more of an oval to blocky shape. The colonies of *V. tricorpus* have been reported to produce a yellow pigment as well but, in our experience, this is not always the case. Having an accurate molecular assay to confirm the identity of ambiguous colonies improved the accuracy of our *V. dahliae* plate counts.

The assay developed for *V. tricorpus* was also specific for this species and was a useful tool to confirm the identity of *Verticillium* spp. on NP-10 soil assay plates. Studies are currently in

progress evaluating the use of this marker for quantification of *V. tricorpus* in soil samples using the same approach as described for *V. dahliae*; however, evaluation of additional isolates of *V. tricorpus* is needed to confirm marker specificity. Preliminary analysis of one isolate from tomato in the United Kingdom (MUCL9792) that was provided as *V. tricorpus* was not detected by this marker; sequence analysis of the IGS region for this isolate revealed polymorphisms in primer and probe annealing sites, and the sequence was different from all other *Verticillium* spp. in GenBank (G. Bilodeau and F. Martin, *unpublished*). It was recently reported that *V. tricorpus* causing lettuce wilt in Japan was genetically distinct from California lettuce isolates of this species (64), indicating that additional taxonomic work is needed to clarify species boundaries and the correct classification of these isolates.

One possible shortcoming when using molecular techniques for quantifying a soilborne pathogen that may influence the accuracy of results is that nonviable propagules have the potential to contribute DNA to the assay, thereby causing incorrect quantification. The length of time DNA may remain intact in nonviable propagules of *V. dahliae* in the soil is unknown and may be influenced by a range of factors such as soil moisture or the degree to which MS resist decay. Some insight into this for *V. dahliae* may be gleaned from observations for field soil 26, which had an inoculum density of 622 MS/g of soil in plate counts when initially sampled but, when resampled 5 months after the field was commercially fumigated with methyl bromide + chloropicrin, the plate counts and results of the molecular assay indicated negligible levels of *V. dahliae* (data not shown). The excellent correlation between molecular results and plate counts of viable MS ($R^2 = 0.96$) (Fig. 3) obtained for a range of soils in this experimentation suggests that nonviable propagules have not compromised the final results.

The described TaqMan soil assay should provide an accurate and rapid means for quantification of *V. dahliae* over a wide range of inoculum densities in agricultural soils. The ability to use commercial DNA extraction kits without the use of organic solvents, the potential for using robotic techniques for DNA extraction, and a 96-well format for processing samples will facilitate high-throughput analysis. The ability to accurately quantify low inoculum levels will be particularly useful for the California strawberry industry because this host is highly susceptible to *V. dahliae*. Although this technique was effective for quantification of *V. dahliae* in the soil samples we processed, before it is implemented on a larger scale in regions with different soils a full validation comparing the results with plate counts should be completed to ensure the accuracy of results. Whereas the soil DNA extraction procedure used for purifying template DNA was found to be effective on a range of soil types from California strawberry fields, there is no guarantee that it will be as effective in providing clean template DNA from all soil types. This can have an influence not only on the accuracy of the assay but also on the relationship between C_i and microsclerotia counts. It should be noted that the brand of the real-time PCR master mix can have an influence on the sensitivity of this assay. Initial validation trials of the *V. dahliae* diagnostic assay were run with TaqMan Universal Master Mix (Applied Biosystems); however, switching to the Real Master Mix without Rox (5 Prime) increased the sensitivity of the assay by reducing the C_i values by 4 to 6 depending on the concentration of the target sequences. The species-specific marker for *V. dahliae* described herein should also be useful for pathogen identification and quantification in plant tissue although, for the latter purpose, another IC that will allow comparison of the quantity of pathogen relative to the plant DNA will need to be used. Likewise, due to the unique sequence of the IC and the ease with which it can be modified to be amplified with other primers, the IC from the *V. dahliae* assay could also be used in other molecular quantification assays.

NOTE ADDED IN PROOF

Debode et al. recently published their work using sucrose flotation to recover microsclerotia as part of their molecular soil quantification assay in addition to a new SYBR green detection assay for *V. dahliae*, *V. longisporum*, and *V. tricorpus* (Plant Dis. 95:1571-1580). Inderbitzin et al. recently reported a multilocus phylogenetic analysis of the genus *Verticillium* and *V. tricorpus* isolates Ls442 and Ls443 used in this study have been reclassified as *V. isaacii* and the *V. albo-atrum* isolate Ms102 has been reclassified as *V. alfalfae* (PLOS ONE 6:e28341).

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