Techniques

Use of Polymerase Chain Reaction-Amplified Ribosomal Intergenic Sequences for the Diagnosis of *Verticillium tricorpus*

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ABSTRACT

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Verticillium dahliae, V. albo-atrum, and *V. tricorpus* are common pathogens of potato. Currently, polymerase chain reaction (PCR) assays based on sequence differences in their ribosomal RNA genes are available for the specific diagnosis of the first two species. Using the same principles, we developed an analogous assay to detect *V. tricorpus*. The 18-28S rDNA intragenic region of *V. tricorpus* was obtained by PCR amplification of genomic DNA from the fungus. Sequence analyses indicated that the 5.8S rDNA sequences were conserved among all three *Verticillium* species but the internal transcribed spacer regions of *V. tricorpus* clearly were divergent. These sequence differences were used to synthesize a specific primer set for the diagnosis of *V. tricorpus*. The same primers (VT primers) also were used to prepare a heterologous internal control DNA template, which can be added to assays to standardize the quantification of fungal biomass. VT primers amplified a specific 337-bp fragment from DNA extracted from *V. tricorpus* cultures isolated from various host species, including potato, or from infected potato stems, but no amplification occurred with DNA from *V. dahliae, V. albo-atrum,* or from uninfected potato stems. The addition of *V. tricorpus* internal control DNA template allowed the quantification of the pathogen in diseased field plants. The development of an assay that is specific for *V. tricorpus* completes the diagnostic set necessary for the investigation and monitoring of the *Verticillium*-potato pathosystem.

Additional keywords: nucleic acid hybridization, wilt.

The development of polymerase chain reaction (PCR) technology (7) has resulted in new opportunities in plant pathogen diagnostics (1). In view of the economic importance of *Verticillium*-induced wilt diseases, PCR-based DNA assays have been developed for the identification (9) and quantification (3) of *Verticillium dahliae* (Kleb.) and *V. albo-atrum* (Reinke & Berthold). Both of these species of *Verticillium* are destructive disease-causing agents in potato, which also can be infected with the less pathogenic species, *V. tricorpus* (I. Isaac), especially as a component of the potato early-dying complex (15). As well as losses in yield and quality of the commercial crop, producers suffer losses in seed sales, since infected potato plants produce tubers that also may be infected or infested with any of the three *Verticillium* species. Potentially, PCR-based assays are convenient, sensitive tools that could be used for the monitoring of wilt disease, testing of farm soils, and certification of seed tuber; however, the effective diagnostic evaluation of the *Verticillium*-potato pathosystem also requires a comparable assay for *V. tricorpus*.

The two existing assays are based on small differences in the genomic sequences that were identified in the internal transcribed spacer (ITS) regions of the ribosomal genes from *V. albo-atrum* and *V. dahliae*. Clusters of three and two nonhomologous nucleotides in ITS1 and ITS2, respectively, permitted the synthesis of oligonucleotide primers that annealed differentially with the rDNA of the two species and allowed for an efficient, species-specific amplification of either DNA sequence by PCR (9). Testing of more than two-hundred *Verticillium* isolates from many plant species has shown this approach to be a reliable method for taxonomic identification.

In this paper, we sequenced the homologous region in a ribosomal gene from *V. tricorpus*. Based on the same principles (9), we designed a primer set that specifically identifies *V. tricorpus*. In addition, we developed a suitable internal standard DNA (3), which can be added to each PCR sample to allow quantitative evaluation of this *Verticillium* species.

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MATERIALS AND METHODS

Growth of fungal and plant materials. Isolates of *Verticillium tricorpus* were stored axenically as a conidial suspension in 25% aqueous glycerol at −70 C. Throughout the text, individual isolates are referred to as *V.t.* with a subscript designating the culture number listed in Table 1. To revive stock cultures, single droplets of thawed suspension were pipetted directly onto potato-dextrose agar. To obtain conidia, the resulting mycelial colonies were maintained at 22 C in the dark and spores were harvested 4 wk later in sterile distilled water.

For field experiments, potato seed (*Solanum tuberosum* L. 'Kennebec') was inoculated at the time of second hilling. The roots of plants were damaged with a soil probe and inoculated with a soil drench containing a suspension of 2×10^6 conidia/ml of sterile distilled water. For controlled environment experiments, potato plants were grown in a growth chamber with a 14-h-photoperiod and day and night temperatures of 24 and 19 C. When the plants were 1-mo old, stem cuttings were infused for 1 h with a spore suspension (1×10^7 conidia/ml of sterile distilled water), using enhanced transpiration (10). The infused cuttings were returned to chambers with the same growth conditions.

Preparation of genomic or plasmid DNA. Genomic DNA from *V. tricorpus* or from infected or healthy potato stems was extracted by the hexadecyl trimethylammonium bromide (CTAB) method of Rogers and Bendich (13) as modified by Nazar and co-workers (9). *Verticillium* spores or finely cut plant tissues (i.e., leaves, petioles, or stems) were ground to a coarse powder in the presence of liquid nitrogen; 1–3 g of ground tissue was suspended in extraction buffer (1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl, pH 8.0, containing 1% polyvinylpyrrolidone-40 and 2% CTAB). The suspension was extracted twice with chloroform/isoamyl alcohol (24:1, v/v); the DNA was recovered by precipitation with two volumes of ethanol containing 2% potassium acetate, washed once with 95% ethanol, and dissolved in 20 μl of Tris-EDTA buffer.

Plasmid recombinant DNAs containing *V. tricorpus* genomic fragments or artificially constructed internal control template sequences were prepared using the method of Holmes and Quigley (2), and all DNAs were further purified by CsCl density-gradient centrifugation (11). In each case, the amount of DNA was determined from the absorbency at 260 nm.

PCR. PCR amplification was conducted in 50 μl of 1× PCR buffer (10× solution: 500 mM KCl, 100 mM Tris-HCl pH 9.0 at 25 C, 15 mM $MgCl_2$, 0.1% gelatin [w/v], and 1% Triton X-100) containing 0.2 mM bovine serum albumin, 0.2 mM of each deoxyribonucleotide triphosphate, 12.5 pmol of each oligonucleotide primer, 0.05 μg of DNA, and two units of Taq DNA polymerase (Promega Corp., Madison, WI). For quantitative analyses 0.5 μCi [α^{32}P]ATP and 0.1 pg control DNA were added to each reaction (3). *V. tricorpus*-specific primers (VT) as well as the previously described (3) primer sets for *V. dahliae* (VD primers) and *V. albo-atrum* (VA primers) were synthesized using a Cyclone Plus automated oligonucleotide synthesizer (Milligen/Biosearch, Milford, MA). The target 18–25S rDNA intragenic region (e.g., Fig. 1, 337 bp fragment) was amplified in a programmable heating block (Ericomp Co., San Diego, CA) using 30 reaction cycles, each consisting of a 1-min denaturation step at 95 C, a 1-min annealing step at 60 C, and a 2-min elongation step at 72 C. For qualitative analyses, a 5-μl aliquot of the PCR reaction mixture was fractionated on a 1% agarose gel. The gel was stained with ethidium bromide (14) and viewed over ultraviolet light. For quantitative analyses, a 5 μl aliquot of the reaction mixture was fractionated on an 8% polyacrylamide gel. The products were detected by autoradiography and quantified by scintillation counting (3). The ratio of the intensity of the fungal signal to the internal control signal (i.e., PCR product ratio) was calculated and used to determine the amount of fungal DNA present from the standard curve (Fig. 3B). For DNA cloning or sequence analysis, the PCR product was extracted with phenol saturated with Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM NaEDTA), then further extracted with an equal volume of chloroform/isoamyl alcohol, and the DNA was precipitated from the aqueous phase with two volumes of ethanol containing 1% potassium acetate.

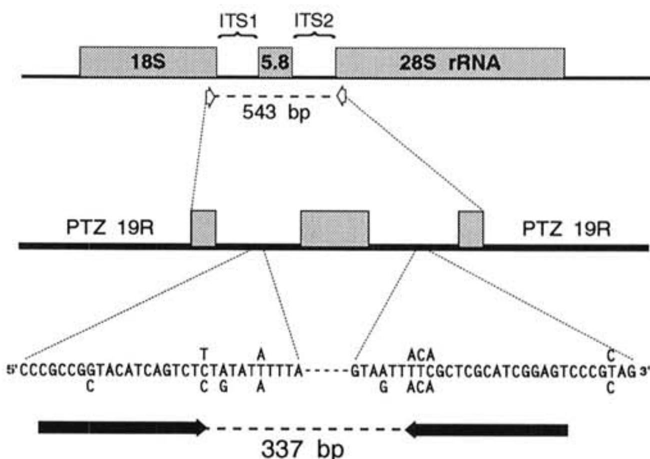

Fig. 1. Identification of exploitable sequence differences in the intervening transcribed sequences of rDNA from *Verticillium* species. Genomic DNA was prepared from two *V. tricorpus* isolates ($V.t._{25}$, $V.t._{117}$), and ribosomal subunit RNA-specific primers (open arrows) were used to amplify the 18–25S rRNA intragenic regions using polymerase chain reaction assays (9). The sequences of the small and large subunit primers were 5′ CAAGGTTTCCGTAGGTG 3′ and 5′ CGCTTATTGATATGCTT 3′, respectively. The fragments (543 bp) were subcloned into pTZ 19R and sequenced by direct plasmid sequencing (17). The sequences were found to be identical. Shaded regions indicate DNA corresponding to the mature rRNA sequences. Regions of sequence difference that were used in the development of *V. tricorpus*-specific primers (solid arrows) are specific variations from *V. albo-atrum* and *V. dahliae* (9) as indicated above and below, respectively. The respective primer sequences within the ITS1 and ITS2 regions are 5′ GCGGCCATGTAGTCAGAG 3′ and 5′ AAGCGAGCGTAGCCTCA 3′. The complete *V. tricorpus* sequence, including the conserved 5.8S rRNA sequence, is available from the EMBL Data Library, accession number L28679.

TABLE 1. Polymerase chain reaction assays of *Verticillium tricorpus* isolates using *V. albo-atrum* (VA), *V. dahliae* (VD) and *V. tricorpus* (VT) primers

V. tricorpus culture no.[a]	Original host	VA primers	VD primers	VT primers	Origin: Country, Researcher	Origin: Tissue
25	cotton	−	−	+	USA, California, J. DeVay	stem
114	potato	−	−	+	Netherlands,[b] H.W. Platt HPPT4 (216060),[c] 1989	tuber
115	potato	−	−	+	Netherlands, H.W. Platt HPP55 (216061), 1989	stem
116	potato	−	−	+	Netherlands, H.W. Platt HPP58 (216070), 1989	stem
117	potato	−	−	+	Canada,[d] PEI, H.W. Platt HP1901, 1990	stem
133	potato	−	−	+	Canada, Manitoba, C. Richard 186←[e], J. A. Hoes 1724, 1969	tuber
162	potato	−	−	+	Netherlands, H.W. Platt HPP55, 1989	stem
163	potato	−	−	+	Netherlands, H.W. Platt HPPT4, 1989	tuber
164	potato	−	−	+	Netherlands, H.W. Platt HPPT10 (216062), 1989	tuber
197	potato	−	−	+	Canada, PEI, H. W. Platt 2901	stem
198	potato	−	−	+	Canada, Quebec, H.W. Platt 2932	stem
200	potato	−	−	+	Canada, PEI, G. Lazarovits, 1990	stem
204	potato	−	−	+	Canada, PEI, G. Lazarovits, 1990	stem
205	potato	−	−	+	Canada, PEI, G. Lazarovits, 1990	stem
206	tomato	−	−	+	Israel, G. Lazarovits←, J. Krikun	stem

[a] J.R. private culture. These numbers are used as subscripts to designate individual isolates throughout the text.
[b] Isolates from the Netherlands were obtained from different plants in adjacent fields in one locality.
[c] Numbers given in parentheses are the Canadian National Identification Service DAOM numbers, where available.
[d] Isolates obtained from Prince Edward Island, Canada, were obtained from different plants in different fields either at the Agriculture Canada Research Station in Charlottetown or commercial farms at various localities throughout the island.
[e] Where more than one individual is listed as a source, the final name is the person who originally isolated the culture and arrows indicate the direction of transfer.

Cloning and sequencing of rDNA. The entire 18–28S rDNA intergenic region (Fig. 1, 543-bp fragment) from two isolates of *V. tricorpus* ($V.t._{25}$, $V.t._{117}$) was amplified by PCR using the above protocol with fungal genomic DNA as a template and primers that were specific for highly conserved regions in the large and small ribosomal subunit RNAs (8; Fig. 1). This fragment subsequently was subcloned into pTZ 19R (6) and sequenced by the direct plasmid dideoxy sequencing methods of Zhang et al (17).

Preparation of an internal control template. Based on previous experience (3), a control DNA template was prepared with *V. tricorpus*-specific primer sequence borders (Fig. 1) and a heterologous internal sequence. The standard VT primers (Fig. 1) were used to amplify genomic DNA from an unrelated fungus, *Fusarium oxysporum* f. sp. *vasinfectum*, under nonspecific annealing conditions (37 C for 2 min). Except for the annealing temperature, the PCR protocol was the same as previously described. The resulting DNA fragment (Fig. 1, 543-bp fragment) was cloned into pTZ R19 and verified using dideoxy sequencing methods as described above.

Quantification of ***V. tricorpus*** **in infected plant material.** Genomic DNA from *Verticillium*-infected or from healthy potato stems was extracted using the CTAB method. Plant tissues (leaf, petiole, and stem) were surface-sterilized in 70% ethanol for 30 s and washed twice in sterile water with gentle agitation. Samples (1.0 g) were homogenized in 4 ml of extraction buffer using a mortar and pestle. A 2-ml aliquot of homogenate was used for DNA isolation, PCR analysis, and quantification as previously described.

RESULTS AND DISCUSSION

As indicated in Figure 1, eukaryotic genes encoding ribosomal RNA contain two intervening transcribed spacers (ITS1 and ITS2), which separate the three mature ribosomal RNA sequences (18S, 5.8S, and 25S rRNAs). Numerous sequence analyses of rRNA genes of diverse origins have indicated the existence of highly conserved rRNAs but striking variability in the ITS regions (8). These differences which commonly have been used for phylogenetic studies (e.g., 16) are the basis of our approach to PCR-based assays for *Verticillium* (3,9).

As a first step in the adaptation of PCR probe technology to the detection of *V. tricorpus*, in this study we sequenced the 18–25S rDNA intragenic region, which was obtained by PCR amplification of genomic DNA from a *V. tricorpus* isolate ($V.t._{25}$) originally isolated from cotton. This isolate had been classified as *V. tricorpus* (4) by its general growth habit as well as by the presence of chlamydospores, large scattered microsclerotia, and dark mycelium, as specified in the Commonwealth Mycological Institute Descriptions of Pathogenic Fungi and Bacteria.

The exploitable nucleotide sequence differences for the two ITS regions in rDNA of *V. dahliae, V. albo-atrum,* and *V. tricorpus* are compared in Figure 1. While earlier sequencing studies of *V. dahliae* and *V. albo-atrum* (9) indicated that there were only three nucleotide differences in ITS1 and two in ITS2 between these two species, *V. tricorpus* (12; EMBL Data Library, accession number L28679) was found to be more divergent. Seventeen base differences were identified when *V. dahliae* was compared with *V. tricorpus*. There were also 12 base changes between *V. albo-atrum* and *V. tricorpus*, five in ITS1 and seven in ITS2. *V. albo-atrum* and *V. tricorpus* share five nucleotide differences with *V. dahliae*. Hence, this comparison suggests that *V. tricorpus* is more divergent than the other two species but more closely related to *V. albo-atrum*.

Using this sequence information, we synthesized a set of *V. tricorpus*-specific oligonucleotide primers that could differentiate *V. tricorpus* genomic DNA from that of the other two *Verticillium* species. As shown in Figure 1, two or three nucleotide differences were incorporated into each primer including, where possible, a mismatch at the 3′-end where elongation is initiated. In subsequent PCR amplification reactions, the new primers failed to amplify a fragment when DNA from either *V. albo-atrum* or *V. dahliae* was used as a template (Fig. 2A), but, as anticipated, an amplified band indicating a positive result was obtained with *V. tricorpus* genomic DNA extracted from the original isolate ($V.t._{25}$). As predicted by the nucleotide sequence (Fig. 1), this gave a distinct band, which corresponded to a fragment length

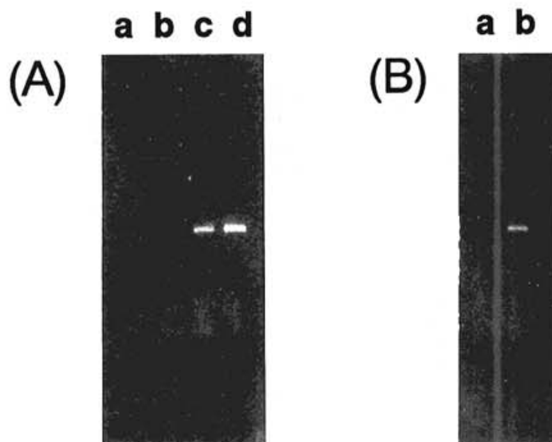

Fig. 2. Detection and differentiation of *Verticillium tricorpus* by a standardized polymerase chain reaction (PCR) assay. **A,** DNA extracted from spores of cultures of previously characterized isolates (9) of *V. albo-atrum* (lane a, J.R. private collection $V.a.a._{11}$) parasitic on alfalfa and *V. dahliae* (lane b, J.R. private collection $V.d._{14}$) parasitic on sunflower as well as of *V. tricorpus* from cotton (lane c, $V.t._{25}$) or potato (lane d, $V.t._{117}$) were subjected to PCR amplification in 1 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM BSA, and 0.2 mM dNTP, with 12.5 pmol of *V. tricorpus*-specific primers and two units of Taq DNA polymerase. The polymerase chain reaction consisted of 30 cycles of denaturation at 95 C, annealing at 60 C, and polymerization at 72 C. **B,** DNA extracted from uninfected control (lane a) or *V. tricorpus*-infused (lane b) potato tissue were tested using the same PCR reaction described in A.

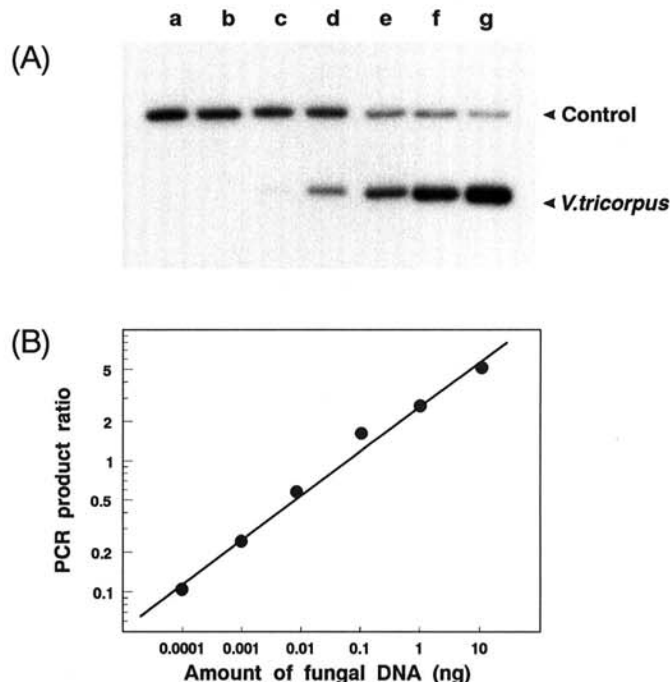

Fig. 3. Relationship between the amount of fungal DNA and the resulting polymerase chain reaction (PCR) product ratio. A constant amount (1.0 pg) of heterologous internal control template was amplified in the presence of varying amounts (picograms) of $V.t._{25}$ genomic DNA: lane a 0.0, lane b 0.0001, lane c 0.001, lane d 0.01, lane e 0.1, lane f 1.0, lane g 10.0. The resulting products were fractionated on an 8% polyacrylamide gel, **A,** detected by autoradiography, and **B,** quantified by liquid scintillation counting. In the autoradiograph, lane a contained only internal template DNA; lanes b–g correspond to the six points in B. The regression line in B was determined from the PCR product ratios (*V. tricorpus* product/internal control product).

of 337 bp when fractionated on agarose gels. PCR testing of genomic DNA obtained from other potato *V. tricorpus* isolates also yielded positive results when the reaction was primed with the VT primer set (Fig. 2A, Table 1), but none of the *V. tricorpus* isolates responded positively when DNA was assayed using either *V. dahliae-* or *V. albo-atrum*-specific primers (Table 1). Further studies of *V. tricorpus*-infused potato tissue indicated that the pathogen could be detected in the host by PCR assay with the *V. tricorpus*-specific primers, but no band appeared when extracts of healthy potatoes were tested similarly (Fig. 2B).

In order to develop a quantitative assay, a heterologous control template DNA was prepared from an unrelated fungal DNA (*Fusarium oxysporum* f. sp. *vasinfectum*) using the *V. tricorpus*-specific primers under less specific annealling conditions (3). The fragment size (about 543 bp) was chosen to be distinct from the *V. tricorpus*-specific product (337 bp) as well as from the control templates, which were developed previously for *V. albo-atrum* and *V. dahliae* (3). To prepare a reliable source of this control template, the fragment was further cloned in pTZ 19R (6), and the resulting recombinant plasmid DNA was used subsequently in all quantitative assays. When control DNA was added to the fungal DNA in a PCR assay, two distinct bands of predicted sizes were visible (Fig. 3A).

The fungal biomass in a sample of plant tissue or soil can be determined as the amount of fungal DNA present by using a standard aliquot of internal control template DNA per sample and a suitable calibration curve (3). An example of such a calibration curve used in the present studies is illustrated in Figure 3B; the sample curve spans the range of DNA concentrations that are observed in biological samples (3). Extrapolating from this curve, the PCR product ratio for each PCR reaction (*V. tricorpus*-amplified DNA/internal control template-amplified DNA) can be used to determine the actual amount of fungus as picograms of fungal DNA per gram of plant tissue, a value that can be compared readily from tissue to tissue between different laboratories. Previous time-course studies (3) of the development of *V. albo-atrum* in alfalfa showed that quantification of the pathogen by such a PCR-based assay yielded results that were very similar to those obtained by more traditional maceration and plating techniques; however, the PCR-based assays were faster (1–2 days vs. 1–2 wk) and more sensitive.

In order to demonstrate the quantitative use of PCR-based assays in the study of the *V. tricorpus*-potato pathosystem, plants (cv. Kennebec) showing early symptom development were sampled from an experimental field about 2.5 mo after planting in *V. tricorpus*-infested soil. The varying amounts of fungal DNA

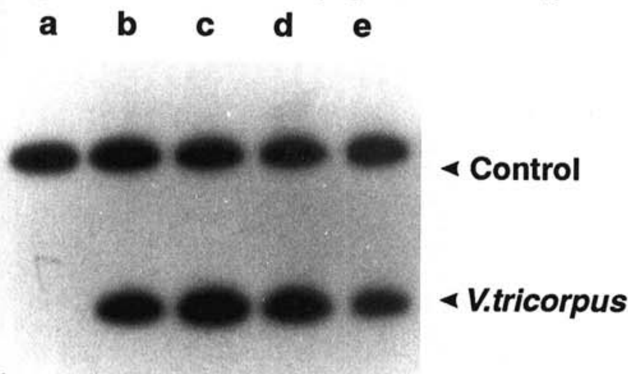

Fig. 4. Quantification of *Verticillium tricorpus* in field plants. DNA was extracted from plants sampled from a *V. tricorpus*-infested experimental field about 2.5 mo after planting. Because of the presence of natural inhibitors of the polymerase chain reaction (PCR) in infected potato tissues, the DNA extracts were diluted 10-fold before testing as previously described (3). An example is given from a plant that showed early symptoms including chlorosis of the lower leaves and reversible wilting of the apical leaves and stem to show the level of colonization in the a, leaves; b, petioles; c, upper stem; and d, lower stem; and for comparison e, 50 pg of *V. tricorpus* genomic DNA. A constant amount (1 pg) of heterologous internal control template was amplified in each sample. The corresponding PCR product ratios were: a 0.0, b 0.8, c 1.5, and d 1.3.

present in different plant tissues are illustrated by the example in Figure 4. Extrapolating from the calibration curve (Fig. 3B) the amount of fungal DNA present in the leaves, petioles, upper stem, and lower stem was calculated as 0, 65, 300, and 200 pg, respectively, per 0.005 g of plant tissues. While the quantitative approach illustrated here relies on the use of autoradiography, ethidium bromide staining (14) also can be used as effectively, providing suitable gel documentation and analysis equipment is available to quantify band intensities. Alternatively, quantification methods based on colorimetry, bioluminescence, or chemiluminescence could be applied (5).

Worldwide governmental requirements that potatoes shipped for produce and, more particularly, for seed, be disease-free, have made the development of rapid, accurate diagnostic technologies imperative. *Verticillium* species are important pathogens of potato in many parts of the world. The development of a PCR-based assay for the specific identification of *V. tricorpus* completes the diagnostic set (i.e., for *V. dahliae*, *V. albo-atrum*, and *V. tricorpus*) necessary for the analysis of pathogenic *Verticillium* species in potato. In addition, we have developed an internal control DNA that is suitable for the quantitative analysis of fungal biomass. A quantitative test is potentially useful to seed inspection branches and government extension programs as well as being an important tool for fundamental studies of the *V. tricorpus*-potato pathosystem.

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