Detection and Identification of *Monosporascus* spp. with Genus-Specific PCR Primers and Nonradioactive Hybridization Probes

B. R. Lovic, Former Graduate Research Assistant, **V. A. Valadez,** Former Research Associate, **R. D. Martyn,** Professor, Department of Plant Pathology and Microbiology, Texas A&M University, College Station 77843-2132; and **M. E. Miller,** Associate Professor, Texas A&M University, Agricultural Research and Education Center, Weslaco 78596

ABSTRACT

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Methodology was developed and evaluated for purposes of polymerase chain reaction (PCR)–mediated detection of *Monosporascus cannonballus*, a soilborne ascomycete causing root rot/vine decline on Cucurbitaceae. In previous studies the sequence of the internal transcribed spacer (ITS) region of the ribosomal DNA unit was shown to be conserved within the genus *Monosporascus* yet different from that of fungi taxonomically and ecologically most closely related. Among five PCR primers derived from the ITS region a primer pair was selected that amplified the DNA from infected roots most efficiently and most consistently. The method developed for DNA extraction and included as part of the PCR-detection protocol uses 10-mg samples, and requires neither incubation nor organic solvents. The use and applicability of the method is illustrated for fresh or dry roots, individual ascospores, and processed soil samples. The detection method is based on amplifying the DNA by PCR with *Monosporascus*-specific PCR primers (B. R. Lovic et al., 1995, Phytopathology 85:655-661), performing agarose gel electrophoresis followed by dot blot hybridization with digoxigenin-labeled portions of the *Monosporascus* ITS region. The duration of the procedure and amounts and hazardous nature of the chemicals have been minimized for each step. The average duration of the procedure for 30 root samples, including DNA extraction, PCR amplification, and gel electrophoresis, is less than 7 h. This detection method proved especially valuable for identifying a relatively large (20%) portion of *Monosporascus* population that does not produce perithecia.

Additional keywords: *M. eutypoides*, muskmelon, rDNA, watermelon

Monosporascus cannonballus Pollack & Uecker is a soilborne fungus that causes root rot/vine decline of muskmelon (*Cucumis melo* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) (11, 17,33). This species was first described and placed into the new genus *Monosporascus* in 1974 (22) based on the report of its isolation from necrotic cantaloupe roots in Arizona (32). It was first documented as a pathogen of muskmelon and other cucurbits in Japan in 1985 (33) and from the United States in 1990 (17,18). Within the United States it is now known to occur in several western states: Texas (17), Arizona (28,32), and California (M. Stanghellini, Tom Gordon, *personal communication*). It also has been reported from Spain (11,12) and Tunisia (14). *Monosporascus cannonballus* colonizes developing muskmelon roots early in the season, causing root rot and necrosis. Damage to the root system leads to progressive defoliation and partial or complete vine decline that occurs toward the end of the growing season as the fruit matures. In the melon-growing areas of the Lower Rio Grande Valley of south Texas, Monosporascus root rot/vine decline has accounted for an average 15% annual loss in yield each year since 1986, when the disease first appeared (16).

The only other putative species in this genus is *M. eutypoides* (Petrak) Von Arx. *Monosporascus eutypoides* has been reported from Israel (23) and is similar to *M. cannonballus* in most of its characteristics (26) including pathogenicity to muskmelon (23); an increasing volume of evidence suggests that they are conspecific (12,15). We have recently reported that the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) repeat unit are identical in DNA sequence in a series of representative isolates of the two "species" (12). In the same study we also demonstrated that the polymerase chain reaction (PCR) primers derived from the ITS regions did not amplify the DNA of any other soilborne fungi while consistently amplifying predicted-size fragments from all of the *Monosporascus* isolates tested. These findings demonstrated suitability of the primers and probes derived from the ITS regions of the rDNA for purposes of PCR-mediated identification and detection.

Soilborne pathogens are notoriously difficult to identify but certain characteristics of *M. cannonballus* make this problem even more pronounced. This species is frequently associated with other potentially pathogenic fungi, lacks a conidial stage, perithecia form only after 3 to 4 weeks in culture, and the ascospores do not germinate under standard laboratory conditions. Efforts to develop a selective medium also have proved unsuccessful to date (16). A particular difficulty is presented by phenotypic variability (excessive pigmentation, slow and uneven growth) observed in a large proportion of field isolates of *Monosporascus* spp. (13). These isolates often fail to produce perithecia in culture, making their identification as *Monosporascus* spp. virtually impossible.

Monosporascus cannonballus has a wide host range and can colonize roots of plants belonging to several different families (18). It also appears to survive and proliferate saprophytically in the soil, which, in addition to a broad host range, implies that its presence could be expected in agricultural soils regardless of the previous cropping history. It would, therefore, be especially useful to be able to detect the spores and mycelia in the soil samples before planting crops known to be susceptible. Soil, however, is a very complex environment and it has proved to be very difficult to develop convenient and reliable procedures for extracting PCR-amplifiable DNA from soil (21,27,29,35). However, unique characteristics of the ascospores of *M. cannonballus*, i.e., their size, shape, and specific gravity, permitted the development of a relatively simple method for their direct extraction from the soil (19, 28). Following sieving through two different-mesh sieves and a short centrifugation in sucrose solution, a relatively clean suspension of ascospores can be obtained and the ascospores can be enumerated using a dissecting microscope. However, since these spores germinate rarely or not at all (33), positive identification of the ascospores relies solely on recognizing their physical characteristics. A DNA extraction method that uses partially processed soil samples followed by PCR amplification with *Monosporascus*-specific primers

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would provide a convenient way to identify *Monosporascus* ascospores.

Even though often praised for its sensitivity and applicability in a range of environments (1,5,29), PCR detection has not been developed into a method for routine testing of root and soil samples. This is due partly to limitations of the method itself but also to the lack of simple alternatives to the currently used, often cumbersome methodology that makes the detection procedure itself time-, money-, and labor-consuming. This is not necessarily a major inconvenience when doing research but it may severely hamper the applicability of a method on a larger scale, e.g., in plant disease diagnostic clinics.. Some of the previously described protocols (10) lend themselves to modifications that can reduce the amount of time and chemicals without compromising either the sensitivity or the reliability of the method. In this report we describe methods for DNA extraction, PCR amplification, and dot blot hybridization that were developed and evaluated for a rapid and convenient detection of *Monosporascus* spp. in muskmelon roots and in soil. Taken into consideration were the cost and hazardous nature of the chemicals, and procedures that are least time- and labor-consuming, without compromising either reliability or sensitivity of detection.

MATERIALS AND METHODS

Origin and condition of root and soil samples. Fresh root material was from a greenhouse pathogenicity study in which muskmelon (cv. Magnum 45) was grown in soil infested with a mixture of *M. cannonballus* spores and mycelia, as described previously (16). After 60 days, roots were harvested and kept at –80°C for up to 4 months.

Dry root material from the watermelon cultivar Giza from Tunisia was obtained from Daryl Maddox (Seed Testing of America, Longmont, CO). The plants from which the roots originated were exhibiting severe symptoms of root rot/vine decline and the roots bore perithecia resembling *M. cannonballus.* Several small pieces of roots were in transit for 6 weeks before they were received. In an attempt to isolate *M. cannonballus* from these roots, a few pieces of tissue were surface-disinfected in 0.5% NaOCl and plated on 2% water agar with 200 ppm of streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) as described previously (16). Remaining pieces of root tissue were used for DNA extractions and PCR amplifications.

Soil samples, containing putative ascospores of *Monosporascus,* were obtained from the Brazos river bottom soil previously used for simulated field trials testing the host range of *M. cannonballus* (19). Several samples were collected from the control plots and several originated from *M. cannonballus*–infested plots.

DNA extraction from fresh roots. Large-scale DNA extractions were performed on greenhouse-grown muskmelon roots by a standard cetyltrimethylammonium bromide protocol (12). A small-scale DNA extraction procedure was a modification of the original method developed by Edwards et al. (4) and modified by Cenis et al. (3). Five- to 10-mg samples of root tissue were placed into 1.5-ml Eppendorf Safe-Lock (Brinkmann Instruments, Inc., Westbury, NY) tubes and homogenized using Eppendorf micropestles (Brinkmann) for 30 s in 300 µl of the extraction buffer (1% SDS [sodium dodecyl sulfate], 250 mM NaCl, 150 mM Tris-HCl, pH 7.5). The samples were vortexed briefly and centrifuged for 5 min at 10,000 × *g* in a microcentrifuge. The supernatant was added to another 1.5-ml tube and the DNA precipitated by adding an equal volume of isopropanol, mixing briefly, and incubating for 10 min at –20°C. The samples were centrifuged for 5 min at 10,000 × *g*, the DNA pellets thoroughly washed with 1 ml of 70% ethanol, dried under vacuum, and resuspended in 25 µl of sterile distilled water. Two-microliter aliquots were used for PCR amplifications.

Extraction of ascospores from the soil. The procedure used was originally developed by Stanghellini and Rasmussen (28), and modified by Mertely et al. (19). Soil was initially sieved through a 2.4-mm sieve and large aggregates discarded. Twenty-gram samples of the dry, screened soil were placed in 600-ml beakers with 200 ml of water and stirred for 20 min. Contents of the beaker were transferred onto a 75-µm (200-mesh) sieve nested over a 38-µm (400-mesh) sieve and the contents of the top sieve washed for 3 min

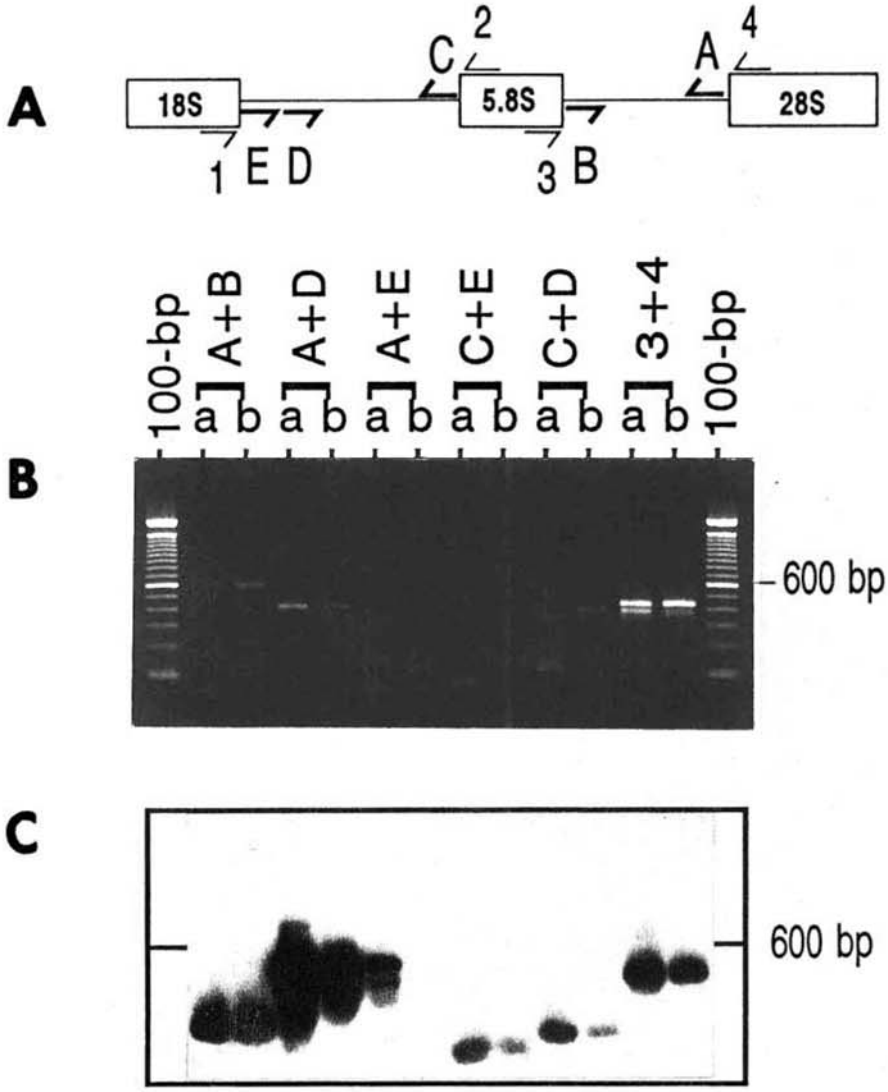

Fig. 1. Analysis of the polymerase chain reaction amplification (PCR) product using the DNA extracted from two muskmelon roots infected with *Monosporascus cannonballus* and different combinations of *Monosporascus*-specific primers. **(A)** Schematic representation of the internal transcribed spacer regions of ribosomal DNA showing approximate locations and orientation of the primers. **(B)** Gel electrophoresis of the PCR amplification products using all possible primer combinations and the DNA from two *Monosporascus*-infected roots (a and b). **(C)** Southern hybridization using digoxigenin-labeled A+D (panel A) amplification product as a probe.

with running tap water. Contents of the top sieve were discarded and contents of the bottom sieve were washed for another 2 min, transferred into a 50-ml centrifuge tube, and centrifuged for 4 min at maximum speed in a clinical centrifuge. The supernatant was discarded, the pellet resuspended in 40 ml of 50% sucrose, and the suspension centrifuged for 2 min as above. The supernatant was transferred onto a small (3 inch diameter) 38-µm sieve, the spores and/or soil particles washed briefly, and stored at 4°C until used for either PCR amplification or spore enumeration.

DNA extraction from ascospores, dry roots, or processed soil samples. Either individual perithecia or small pieces (5 to 10 mg) of root tissue were ground in 1.5-ml Eppendorf Safe-Lock tubes and the DNA extracted using the same small-scale procedure described for fresh root samples. Processed soil samples containing ascospores were resuspended in 50 ml of sterile, distilled water, 300 ml of the extraction buffer was added, and the procedure carried out as described above.

PCR amplification. PCR primers used in this study were described previously (12) and their approximate location along the rDNA repeat unit is illustrated in Figure 1. The nonspecific PCR primers 1, 2, 3, and 4 from the conserved regions of rDNA were used as positive controls to ensure that the amplification conditions were optimal. PCR amplifications were performed in 15- to 30-µl volumes containing 1 µM each of the primers; 500 µM of each of the four deoxyribonucleotides; 2.5 mM $MgCl_2$; 0.6 and 2.0 (for 30- and 100-ml reactions, respectively) units of *Taq* polymerase (Promega, Madison, WI) in 1× PCR buffer (Promega). Temperature parameters were 94°C for DNA denaturation, 5 min for the first cycle, and 1 min for the remaining cycles, 45°C for 1 min for primer annealing, and 72°C for 1 min for primer extension. The total number of cycles was 25 plus a final extension of 7 min. The reactions were run using the Perkin-Elmer Cetus 480 (Norwalk, CT) thermocycler. The efficacy of amplification was analyzed by electrophoresing 5-µl aliquots through a mini 1.2% agarose gel, staining with ethidium bromide, and visualizing under UV light. The number of amplification cycles was 45.

Southern transfer and dot blot hybridization. Three DNA hybridization probes were constructed by digoxigenin labeling of the PCR-amplified portions of the ITS region(s) and the 5.8S gene with the Genius labeling kit (Boehringer Mannheim, Indianapolis, IN). Probe A+E included most of the ITS 1 sequence, the entire 5.8S gene and most of the ITS 2 sequence, probe A+B was homologous to the ITS 2, and probe C+E contained the DNA sequence of the ITS 1. Processes of gel electrophoresis, Southern transfer, hybridization, and autoradiography were as suggested by the manufacturer and described previously (12). A dot blot method was developed and tested by spotting aliquots of the PCR amplification reactions (primers A+D) onto a nylon membrane (Boehringer Mannheim, Indianapolis, IN) and hybridizing with one of the three probes. A 1:10 dilution of the PCR-amplified product in manufacturer-suggested buffer (herring sperm DNA in 10 mM Tris-Cl, 1 mM EDTA, pH = 7.4) was boiled for 10 min and chilled on ice, and 5-ml aliquots spotted directly onto the membrane. The DNA was UV cross-linked and allowed to air-dry for 15 min. The prehybridization and hybridization conditions were as suggested by the manufacturer (Boehringer Mannheim, Indianapolis, IN). The membranes were washed twice in 2× SSC (0.3 M NaCl, 30 mM $Na_3C_6H_5$, pH = 7.5, 0.1%) (1× SSC is 0.15 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% SDS for 15 min at 65 C, and twice with 0.5× SSC, 0.1% SDS for 20 min at 65°C. The nonspecific sites were blocked by treating the membrane for 45 min with a blocking reagent (Boehringer Mannheim, Indianapolis, IN) followed by 30-min treatment with antidigoxigenin-alkaline phosphatase diluted 1:10,000 in the blocking reagent. The membranes were washed twice in 150 mM NaCl, 100 mM Tris for 10 min at room temperature, rinsed with TE (10 mM Tris-Cl, 1 mM EDTA, pH = 7.4) for 2 min, placed between two plastic sheets, treated with Lumiphos (Boehringer Mannheim, Indianapolis, IN) and exposed to X-ray film (Jersey Lab Supply, Livingston, NJ). The initial exposure time was 1 h at room temperature. Alternatively, a chromogenic assay based on histochemical substrate (nitroblue tetrazolium salt) to locate alkaline phosphatase-conjugated antidigoxigenin was used according to the manufacturer's

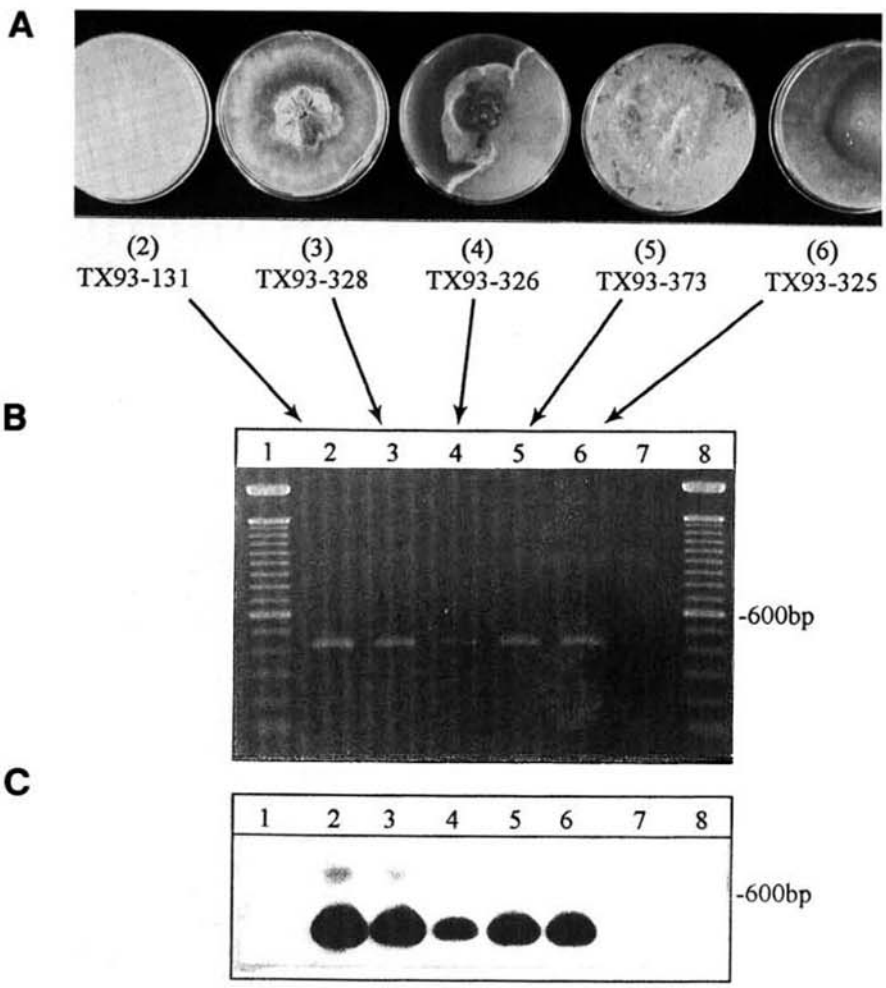

Fig. 2. Analysis of the polymerase chain reaction (PCR) amplification products using primers A+D (see Fig. 1A) and the DNA from *Monosporascus cannonballus* isolates characterized by wild-type and atypical colony morphologies. **(A)** Colony morphologies of five fungal isolates initially characterized as *M. cannonballus* based on microscopic observation of hyphae. **(B)** Gel electrophoresis of the PCR amplification products. Lanes 1 and 8, 100-bp ladder; lane 2, isolate TX93-131 exhibiting *M. cannonballus* wild-type morphology (panel A); lanes 3 to 6, four isolates (TX93-328,-326,-373, and -325) that failed to produce perithecia and exhibited atypical culture characteristics, not resembling wild-type culture morphology illustrated with isolate TX93-131 (panel A); lane 7, negative control (no DNA). **(C)** Southern transfer and hybridization with A+B (see Fig. 1A) digoxigenin-labeled probe.

instructions (Boehringer Mannheim, Indianapolis IN).

RESULTS

PCR amplification from the DNA of *M. cannonballus*–infected muskmelon roots using different primer combinations. Primer pair A and D (Fig. 1A) consistently yielded the most abundant product that could be observed after gel electrophoresis and ethidium-bromide staining only (Fig. 1B). After Southern transfer and hybridization with digoxigenin-labeled probe, products of predicted sizes could be observed using all five possible combinations of *Monosporascus*-specific primers (Fig. 1C). The apparently complex band pattern observed for primers A+D is a result of overexposure necessary to visualize less abundant fragments (e.g., C+E and C+D products) on autoradiographies. The abundant 420-bp-long amplification products using primers 3+4 observed on the gel (Fig. 1B) are presumably of plant origin. The hybridization bands observed in lanes showing 3+4 amplification products on autoradiography (Fig. 1C) correspond to slightly smaller (approximately 380 bp) fragments, which correspond to the size of the PCR product amplified using primers 3+4 and the DNA of *Monosporascus* spp. (12).

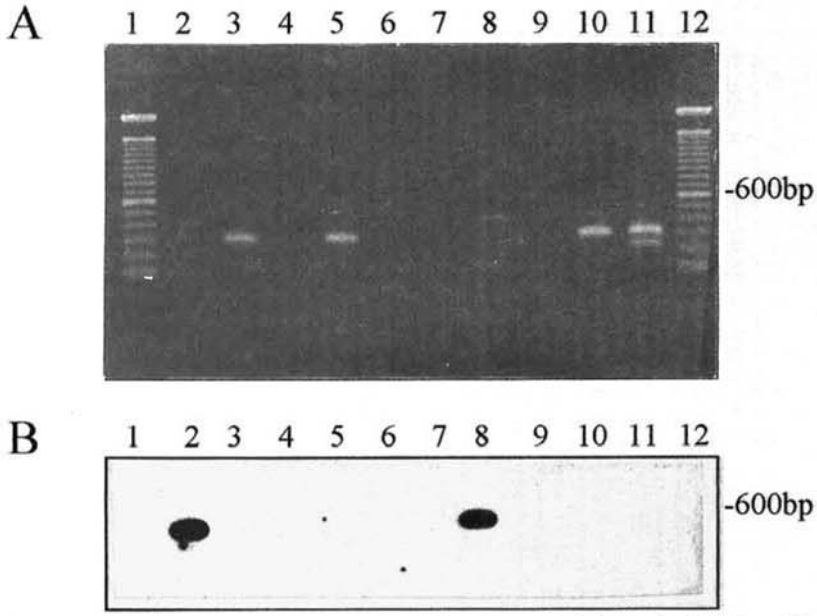

Fig 3. Gel electrophoresis and Southern analysis of the polymerase chain reaction (PCR) amplification products with primers A+D and the DNA from *Monosporascus*-infected roots extracted by small-scale or large-scale DNA extraction procedure. **(A)** Lanes 1 and 12, 100-bp DNA ladder; lanes 2, 4, and 6, small-scale DNA extraction and primers A+D; lanes 3, 5, and 7, small-scale DNA extraction and primers 3+4 from the conserved portions of ribosomal DNA (see Fig. 1A); lane 8, large-scale DNA extraction and primers A+D (see Fig. 1A); lane 9, negative control (no DNA); lane 10, primers 1+2 (see Fig. 1A) and DNA from healthy muskmelon roots; lane 11, primers 1+2 and DNA from infected muskmelon roots. **(B)** Southern transfer and hybridization with A+B (see Fig. 1A) digoxigenin-labeled probe.

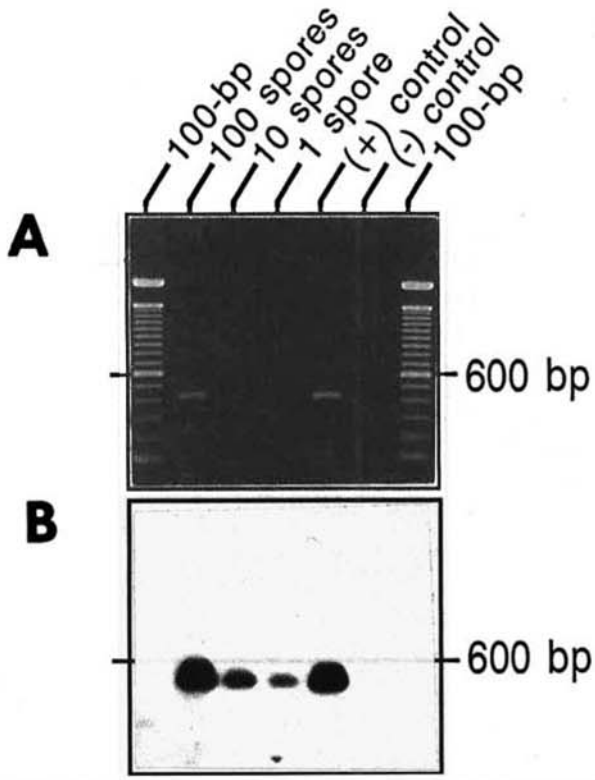

Fig. 4. Gel electrophoresis and Southern analysis of the polymerase chain reaction (PCR) amplification product using the DNA extracted from the ascospores of *M. cannonballus* and primers A+D (see Fig. 1A). **(A)** Gel electrophoresis of the PCR amplification products. **(B)** Southern hybridization with digoxinenin-labeled PCR amplification product using primers A+B (see Fig. 1A).

Identification of fungal cultures as *Monosporascus* spp. Primer pair A and D was further evaluated on a number of isolates that had been characterized as *M. cannonballus* based on their hyphal characteristics but that could not be positively identified since they failed to produce perithecia in culture (Fig. 2). Isolates TX93-328, -326, -373, and -325 are examples of the isolates with odd culture characteristics and no identifiable structures (Fig. 2A). PCR amplification followed by gel electrophoresis (Fig. 2B) and Southern hybridization (Fig. 2C) conclusively established their identity as *Monosporascus* spp.

Comparison of the small- and large-scale DNA extraction procedures from muskmelon roots. The small-scale DNA extraction procedure was developed by varying the parameters of two similar extraction methods described previously (3, 4). Addition of sodium acetate to a final concentration of 1 M as described by Cenis (3) did not convey any identifiable advantage (data not shown) and was not included in the protocol. Extraction procedures using two different pH values of the extraction buffer, 7.5 (4) and 8.5 (3), yielded equivalent amounts of equally amplifiable DNA (data not shown).

The small-scale DNA extraction procedure gave a sufficient amount of DNA for at least 10 PCR amplification reactions using 45 amplification cycles (Fig. 3, lane 2). However, as illustrated in Figure 3, not all subsamples from the same root system resulted in amplification of the diagnostic fragment. Only one (lane 2) of three subsamples (lanes 2, 4, and 6) from the *M. cannonballus*–infected root gave a positive signal, which is representative of the results of several DNA extractions and PCR amplifications from other *M. cannonballus*–infected roots (data not shown).

DNA extraction and amplification from ascospores. When DNA was extracted from a single ascospore, 45 reaction cycles using primers A+D consistently amplified a sufficient amount of product to be detected by Southern hybridization but not by gel electrophoresis in

ethidium bromide–stained gels only (Fig. 4). Starting from a larger number of spores (e.g., 10 or 100) the amplification products could consistently be visualized in ethidium bromide–stained gels following electrophoresis and were confirmed by Southern transfer and hybridization (Fig. 4).

PCR "amplifiability" of the substrate at different stages of the spore extraction protocol. Several attempts were made to extract DNA directly from the soil fraction retained on the 38-µm sieve with both large- and small-scale DNA extraction procedures. Even though DNA was extracted in considerable quantity, as judged by fluorimetry, no PCR-amplified products could be detected when using up to 50 cycles and any of the *Monosporascus*-specific or PCR primers originating from the conserved regions of ribosomal DNA (12) (data not shown). A second round of amplifications with "nested" primer pair (e.g., primers C and D following amplification with primers A and E, Fig. 1) also failed to yield any detectable product (data not shown). The soil fraction that remained in the supernatant after sucrose centrifugation, however, was readily amplifiable. Figure 5 illustrates products of PCR amplification from five soil samples that contained anywhere from 0 to 15 spores as determined by microscopic observation of the ascospores prior to DNA extraction and PCR amplification.

Comparison of gel electrophoresis, Southern blot, and dot blot hybridization methods for detecting *M. cannonballus*. Figure 5 illustrates the amplification products from DNA extracted from five different soil samples (lanes 1 to 5), two predetermined numbers of ascospores (lanes 6 to 8), and from the DNA extracted from *M. cannonballus* mycelium (lane 9). While the predicted-size products could be detected in only two samples following ethidium bromide staining, hybridization with the digoxigenin-labeled probe following either a Southern transfer or dot blotting gave a very strong signal for four of the five soil samples (lanes 2 to 5). Hybridization of the Southern blotted (Fig. 5B) and dot blotted (Fig. 5C) DNA produced signals of similar intensity.

PCR detection of *M. cannonballus* in dry root tissue. The DNA extracted from either individual perithecia or from pieces of root tissue could be amplified with primers A+D and 45 cycles, and resulted in an abundance of the 430-bp product that could be detected in ethidium bromide–stained gels. The amplification products from perithecia and root tissue from Tunisia were identical in size to the amplification products from the DNA of the representative isolates from Texas (Fig. 6). The sequence was confirmed as *M. cannonballus* by Southern hybridization (Fig. 6B).

DISCUSSION

The methods described have established that PCR detection with primers derived from the DNA sequence of the ITSs of rDNA is a convenient tool for detection of *M. cannonballus* in root tissue and processed soil samples. The procedures have been shortened considerably and avoid the use of hazardous chemicals (e.g., phenol, chloroform, P^{32}, etc.). In addition, the protocols developed can be followed easily by anyone with a working knowledge of several standard molecular biology techniques.

Even though our main goal was to develop a detection method for this fungus, the tools described in this report also are useful for its identification. At least 20% of *Monosporascus* spp. isolates obtained from muskmelon roots fail to produce ascospores (unpublished data) and are currently identifiable only by the methods described in this study.

The small-scale DNA extraction procedure followed by PCR amplification allows detection from either root tissue or soil samples, reduces the processing time to 5 min per sample, and requires no special qualifications and/or experience to perform. However, presumably due to a very small sample size, when several

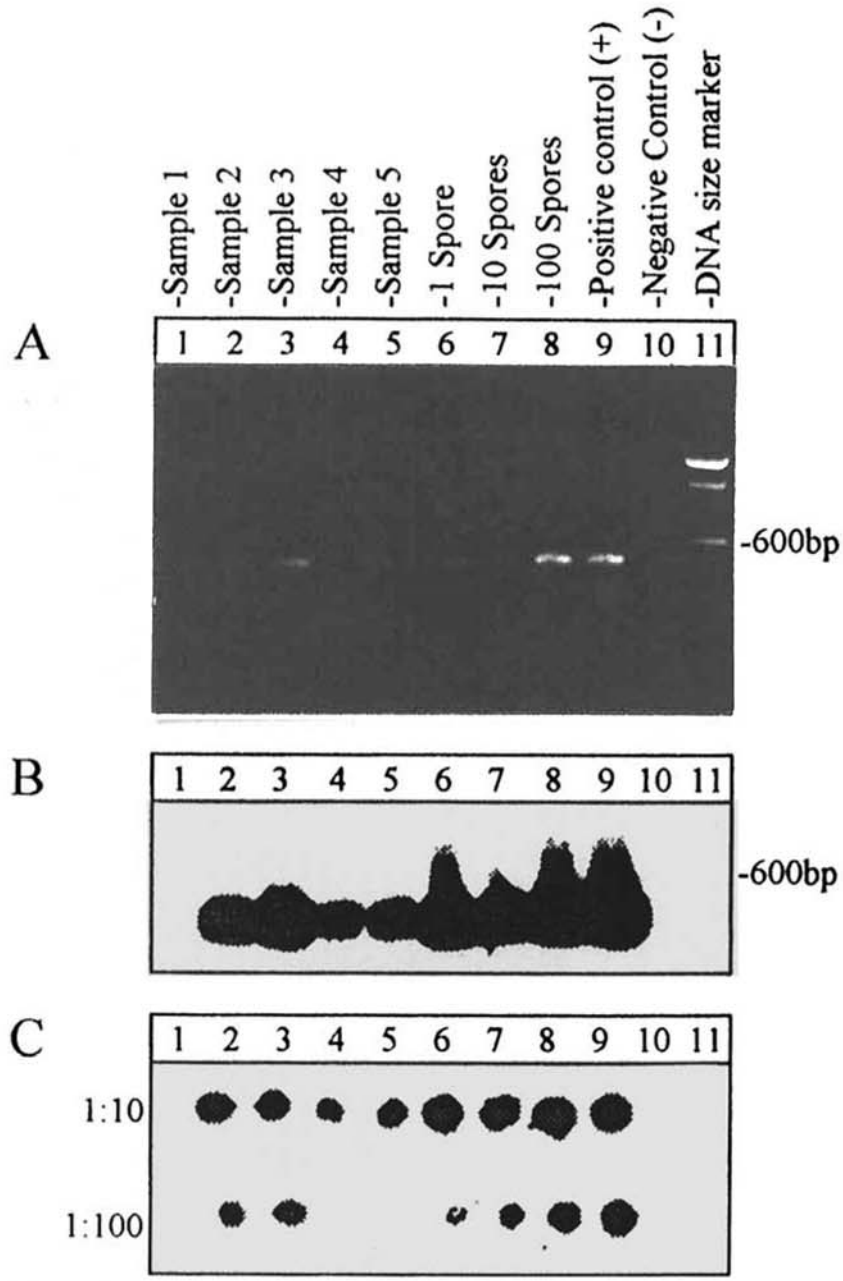

Fig. 5. Comparison of gel electrophoresis, Southern hybridization, and dot blot hybridization for detection of polymerase chain reaction (PCR) amplification products from processed soil samples. **(A)** Gel electrophoresis of the PCR amplification products using the DNA extracted from processed soil samples (samples 1 to 5) and a known number of spores (1, 10, 100). Ten microliters of PCR amplification product was loaded in each lane. Positive control contained 10 ng of *M. cannonballus* DNA. **(B)** Southern hybridization using digoxinenin-labeled A+B (see Fig. 1A) probe. **(C)** Dot blot analysis using 0.5 µl of the amplification products (1:10) or 0.05 µl of the amplification product (1:100).

samples were taken from the same *Monosporascus*-infected root, the fungus could not be detected in every sample (Fig. 3 and unpublished data). Even though histological studies of root colonization by *M. cannonballus* have not been done it is not thought to be a systemic or vascular pathogen. It is therefore likely that portions of the root are not colonized. Consequently, if the assay of the entire root system is based on a single 10-mg sample, it is likely that false negatives will result. False negatives are a disadvantage of using small samples for purposes of determining infection. This problem can be reduced, however, by taking multiple samples from the root, combining them, and then assaying a 10-mg aliquot of the mixture. Examination of different root sampling strategies is an objective of a current study.

Using the same small-scale DNA extraction procedure described for roots, *Monosporascus* could be detected via PCR from DNA extracted from ascospores (Fig. 4). As presented in Figure 4, however, the amount of the A+D–amplified product from a single spore could not be detected in ethidium bromide-stained agarose gels. Failure to amplify enough product from a single ascospore may be because only 10% of the total volume of the DNA extracted from a spore was used for PCR amplification. If the DNA is resuspended in a smaller volume and all the DNA extracted from a spore is used, the product is much more abundant and can easily be visualized by ethidium bromide staining (data not shown). This is not surprising, since a single spore of *M. cannonballus* has an average of eight nuclei (22), each of which may have up to 200 copies of the rDNA repeat unit (2). This would theoretically give 1,600 initial targets for the primers.

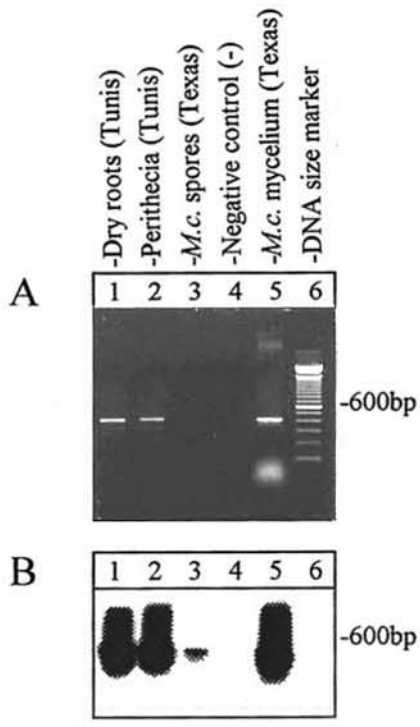

Fig 6. Gel electrophoresis and Southern hybridization of the polymerase chain reaction (PCR) amplification product using the DNA extracted from dry watermelon roots from Tunisia and primers A+D (see Fig. 1A). **(A)** Gel electrophoresis of the PCR amplification using the DNA from root tissue and individual perithecia. Ten spores or 10 ng of DNA from standard *M. cannonballus* isolate were used as positive controls. **(B)** Southern transfer and hybridization with digoxigenin-labeled A+B probe.

Monosporascus could not be detected in the soil fraction retained on the 38-µm sieve (data not shown). Since individual ascospores of *M. cannonballus* could be detected easily by this method (Fig. 4) the sensitivity of the assay per se is not a limiting factor. Most probably, failure to detect *M. cannonballus* at this stage of the spore extraction procedure is due to the presence of components that interfere with the process of PCR amplification. Based on visual detection of the spores before performing the DNA extraction, as little as two spores per sample could be detected after the final stage of spore extraction protocol.

Even though it is very unlikely that *Monosporascus*-specific primers amplify a predicted-size product from any other organism, a DNA-DNA hybridization of the amplification product with the homologous probe is the only way to conclusively prove the identity of the product. Our studies suggest that performing hybridizations on a dotted aliquot of the amplification product (dot blot) is similar in reliability and sensitivity to performing a Southern transfer followed by hybridization. The savings in time (no overnight transfer, 70-min vs. 180-min posthybridization washes) and resources (approximately 20% of the membrane and antibody per reaction) make a dot blot approach particularly attractive. It has been shown that Lumiphos treatment followed by autoradiography may not be necessary since it can be replaced by a colorimetric assay that is less time consuming and less costly (no need for costly Lumiphos and X-ray film), with a minimal reduction in sensitivity.

PCR detection is not particularly dependent on the condition of the sample, which is probably the single most important reason for the increasing popularity of this approach in detection of plant, animal, and human pathogens (6,9,20,24,25). With the PCR-based method, *M. cannonballus* was detected in dry roots that originated from Tunisia (Fig. 6) while we failed in numerous attempts to isolate the fungus from the same roots. Since we were not able to recover an active culture of the fungus, Koch's postulates could not be fulfilled to confirm that the *M. cannonballus* from Tunisia is actually pathogenic. However, PCR amplification of the predicted-size product using primers A+D conclusively proved its presence on melons from Tunisia (14). This fully illustrates the advantages of PCR detection and the methodology developed in this study; it took approximately 6 h (30 min to extract the DNA, 30 min to set up a PCR reaction, 4 h for 45 cycles of amplifications and a 1-h electrophoresis run) to confirm visual observations of the symptoms and signs on a 2-mm-long piece of dry root.

The experiments and methodology described explore only a part of the PCR parameters and "molecular" tools that can be modified or used to enhance sensitivity limits and to make PCR detection of *M. cannonballus* more applicable. Nested primers were accounted for while designing the method but they were not used, mostly because it was felt that the actual sensitivity of the assay was not a limiting factor in being able to detect this fungus in either roots or soil. However, they remain as a possible tool in developing a method for detection of *M. cannonballus* mycelium in the soil.

Still, the greatest benefit to applicability of this method would be gained by making it quantitative. We performed a number of PCR amplifications using the DNA obtained from a mixture of known amounts of *Monosporascus* mycelia and cantaloupe root tissue. The results suggested that the detection limit of our procedure was anywhere from 10^{-5} to 10^{-8} (e.g., 10 to 0.1 µg of *Monosporascus* mycelium in 10 g of root tissue) (data not shown) but we could not obtain these results consistently. Even though a number of methods have been suggested for quantifying the number of starting DNA templates in PCR (8,30,31, 34) quantification of the initial amount of the target still remains one of the biggest challenges in the advancement of PCR detection methodology. Once technology such as "kinetic PCR" (7) becomes more easily accessible it should add a needed level of confidence to interpreting results of PCR amplifications and allow reliable quantification of the initial amount of the target.

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