Morphological, pathogenic and molecular characterization of *Phoma* spp. isolated from onion field soils in Puerto Rico[1,2]

Ismael E. Badillo-Vargas[3], Lydia I. Rivera-Vargas[4] and Juan Calle-Bellido[5]

J. Agric. Univ. P.R. 92(1-2):73-86 (2008)

ABSTRACT

Thirteen *Phoma* spp. isolates collected during a survey conducted in onion field soils in Santa Isabel, Puerto Rico, were examined on the basis of morphology, pathogenicity and molecular characteristics. Twelve isolates were identified as *Phoma putaminum* Speg. and one isolate as an atypical *Phoma macrostoma* var. *incolorata* (section *Phyllostictoides*). This is the first report of *P. putaminum* and *P. macrostoma* var. *incolorata* for Puerto Rico and the Caribbean. In vitro, *Phoma putaminum* isolates were pathogenic to onion cvs. Mercedes and Excalibur, resulting in necrosis of young bulbs and roots seven days after inoculation. Disease incidence caused by *P. putaminum* was higher in cultivar Mercedes, ranging from 75 to 100%, than in *P. macrostoma* var. *incolorata* (0 to 25%). No symptoms were observed on cultivar Excalibur inoculated with *P. macrostoma* var. *incolorata* or on control plants. In vitro inoculations of commercial onion bulbs and field inoculations of roots failed to reproduce symptoms, thus showing that *P. putaminum* and *P. macrostoma* var. *incolorata* are weak pathogens. Sequence size of the nuclear internal transcribed spacer (ITS) of ribosomal DNA (rDNA) gene ranged from 458 to 610 base pairs (bp) for *P. putaminum* and was 456 bp for *P. macrostoma* var. *incolorata* isolate. Analysis of rDNA ITS region by PCR-RFLP showed that restriction enzyme, HindIII, among other restriction enzymes evaluated (AluI, EcoRI, ClaI and ScaI), differentiate between *P. putaminum* and *P. macrostoma* var. *incolorata* isolates.

Key words: onions, *Phoma putaminum*, *P. macrostoma* var. *incolorata*, pathogenicity tests, ITS region of rDNA, restriction enzymes, RFLP

RESUMEN

Caracterización morfológica, patogénica y molecular de *Phoma* spp. aislados del suelo de predios de cebolla en Puerto Rico

Se examinaron 13 aislados de *Phoma* spp. recolectados durante un catastro de hongos de suelo llevado a cabo en predios de cebolla en Santa Isabel, Puerto Rico, a base de sus características morfológicas, patogénicas y

[1]Manuscript submitted to Editorial Board 20 June 2007.

[2]This research was supported by an USDA TSTAR-97 grant and the Arturo Roque Fellowship of the Agricultural Experiment Station, University of Puerto Rico.

[3]Former Undergraduate Student, Crop Protection Department, University of Puerto Rico, Mayagüez Campus, P.O. Box 9030, Mayagüez, PR 00681-9030.

[4]Professor, Crop Protection Department.

[5]Former Graduate Student, Agronomy and Soils Department.

moleculares. Doce aislados fueron identificados como *P. putaminum* y un aislado como *P. macrostoma* var. *incolorata* atípico (sección *Phyllostictoides*). Este es el primer informe de *P. putaminum* y *P. macrostoma* var. *incolorata* para Puerto Rico y el Caribe. In vitro, *P. putaminum* fue patogénico a los cvs. de cebolla Mercedes y Excalibur, resultando en necrosis de los bulbos y raíces jóvenes siete días después de la inoculación. La incidencia de la enfermedad causada por *P. putaminum* fue mayor en el cultivar Mercedes (75 a 100%) que en *P. macrostoma* var. *incolorata* (0 a 25%). No se observaron síntomas en el cultivar Excalibur inoculado con *P. macrostoma* var. *incolorata* o en las plantas control. Ni las inoculaciones in vitro de bulbos comerciales de cebolla, ni de raíces en condiciones de campo reprodujeron síntomas, demostrando así que *P. putaminum* y *P. macrostoma* var. *incolorata* son patógenos débiles. El tamaño de la región espaciadora interna (ITS) del gen del ADN ribosómico fluctuó entre 458 a 610 bp para *P. putaminum* y 456 bp para el aislado de *P. macrostoma*. El análisis de la región ITS del rADN mediante PCR-RFLP demostró que la enzima de restricción, HindIII, entre otras enzimas de restricción evaluadas (AluI, EcoRI, ClaI y ScaI) diferenció entre *P. putaminum* y *P. macrostoma* var. *incolorata*.

Palabras clave: cebolla, *Phoma putaminum*, *P. macrostoma* var. *incolorata*, pruebas de patogenicidad, región ITS del rADN, enzimas de restricción, RFLP

INTRODUCTION

Worldwide onion (*Allium cepa* L.) production reaches 48 billion kilograms (FAO, 2006). In the Caribbean, onion production was estimated at 164,557 mt for 2004 (FAO, 2006). According to the Department of Agriculture of Puerto Rico (2006), the estimated value of the onion crop was $2.3 million for fiscal year 2005-2006. Over the past ten years an increase in onion production and consumption on the island has been observed (Martínez, 1999; Department of Agriculture of Puerto Rico, 2006). The increase in onion production under tropical conditions has renewed the importance of studying pathogens limiting yield.

Several diseases limit onion production by affecting plant development and quality of the product. In general, bulbs stimulate bacteria and fungal development, some of which can invade the root cortex and act as pathogens or saprophytes (Schwartz and Mohan, 1999). Fungal diseases occurring in onion crops have been reported worldwide. Many of these diseases also affect other *Allium* species (Schwartz and Mohan, 1999). Among these diseases, pink root disease caused by *Phoma terrestris* (Hansen) has been reported as a severe onion pathogen occurring in fields in tropical and subtropical climates (Boerema et al., 2004). Both pink root and basal plate rot (caused by *Fusarium oxysporum* f. sp. *cepae*) occurring together appear to cause more losses in yield and quality than either disease occurring alone (Thornton and Mohan, 1996).

Phoma (Sacc. emend. Boerema & G.J. Bollen), a fungal genus of soil-inhabitant fungi, has been commonly isolated from soils of onion fields in the southern region of Puerto Rico (Vélez-Rodríguez, 2001; Calle-Bellido, 2005). Most species were saprobes, but have plant pathogenic

potential causing numerous diseases of vegetables and other annual plants (Schwartz and Mohan, 1999; Boerema et al., 2004). *Phoma* spp. infects subterranean organs directly and aerial parts of the plants indirectly (Boerema et al., 2004). In Puerto Rico, *Phoma sorghina* and *Phoma* sp. were reported causing gray lesions in young onion bulbs and roots in vitro, and a pink coloration at root inoculation sites with both species (Vélez-Rodriguez and Rivera-Vargas, 2007). In addition, *Phoma* spp., anamorph of *Didymella bryoniae* Auersw (syn. *Mycosphaerella melonis*), has been isolated and identified on the island as the causal agent of the gummy stem blight of watermelons [*Citrullus lanatus* (Thunb.) Matsum & Nak.] and tropical pumpkins [*Cucurbita moschata* (Duchesne ex Lam.)] (Rosa-Márquez and Fornaris-Rullán, 2003).

Traditional fungal plant pathogen taxonomy is based on morphological and pathogenic characterization. A generally accepted complementary approach for fungal plant pathogen characterization has been the use of molecular markers. Sequence comparisons of the internal transcriber spacer (ITS) region of the nuclear encoded ribosomal DNA (rDNA) gene have frequently been used in fungal identification, because of its great variability among organisms (White et al., 1990). Restriction fragment length polymorphism (RFLP) is another commonly used molecular technique for fungal plant pathogen characterization. This technique is based on the use of restriction enzymes that recognize a particular sequence in double-stranded DNA, cutting it at a specific region and creating multiple fragments of the genetic material. This technique has been shown to differentiate between isolates of the same genus and species. Restriction of the rDNA ITS region creates polymorphic fingerprint patterns that can be used for phylogenetic inferences of the pathogen under study (Weiland and Sundsback, 2000). The main objective of this research was to characterize thirteen *Phoma* spp. isolates from onion field soils collected from the southern region of Puerto Rico. Isolate characterization was based on morphology, pathogenicity and molecular composition of the ITS region of the 5.8S rDNA gene.

MATERIALS AND METHODS

Fungal isolates: *Phoma* spp. isolates were obtained during a survey conducted in onion field soils in Santa Isabel, Puerto Rico. Composite soil samples were taken at 0- to 10-cm and 10- to 20-cm depth at 0, 59 and 107 days after planting onion cultivars Mercedes and Excalibur. Ten grams of soil for each composite sample was diluted in 90 ml sterile di-ionized distilled water and shaken for 30 min. Ten microliters of a serial dilution (10^{-2} and 10^{-3}) of a soil suspension were placed on Ohio Agar and incubated at 28° C (Dhingra and Sinclair, 1995). After 72 h,

fungal colonies were selected on the basis of morphological characteristics such as dematiaceous mycelia and transferred to acidified potato dextrose agar (PDA). Fungal isolates were kept on PDA at 4° C. Field lot location and sampling methodology were as described in detail by Calle-Bellido (2005).

Morphological characterization: Thirteen *Phoma* spp. isolates were grown on PDA at 28° C for further characterization and identification. Semi-permanent slides were prepared to make detailed observations of the morphological features of fungal structures (i.e., mycelium, pycnidia, conidia, chlamydospores and swollen cells) with a light microscope (Olympus Optical CO, LTD. Model BX40F4, Tokyo, Japan).[6] Each fungal isolate was grown in oatmeal (OA) and malt agar (MA). Oatmeal agar was used to stimulate pycnidia production whereas MA was used to stimulate pigment production and dendritic crystal formation, both of which have been reported as important criteria used in *Phoma* spp. identification (Boerema et al., 2004). Colony growth and development was evaluated every two days for two weeks. Furthermore, the isolates were grown on OA and MA to determine colony margin and color. For each isolate, a NaOH spot test was used to observe the production of a diffusible antibiotic metabolite 'E' (Boerema et al., 2004). Four *Phoma* spp. representative isolates were identified and certified by Dr. R.A. Samson from the Fungal Biodiversity Centre, The Netherlands.[7]

Pathogenicity Tests: Three representative isolates of *P. putaminum* (P1, P2 and P4) and one *P. macrostoma* var. *incolorata* (P3) isolate were selected for the pathogenicity tests conducted at the Plant Pathology Laboratory, University of Puerto Rico-Mayagüez Campus, and in fields of the Agricultural Experiment Station (AES) at Juana Díaz, Puerto Rico. Onion cultivars Mercedes (Seminis®) and Excalibur (Sunseeds®) were used in both laboratory and field tests. Certified onion seeds were planted directly into an experimental plot located at the AES, following the recommended practices for onion production (Rivera, 1999).

In vitro pathogenicity tests: Mycelial disks from each *Phoma* isolate (P1 to P4) were used to inoculate 55-day-old field-grown onion bulbs with roots. Plant tissues were superficially disinfected with 70% ethanol, 0.5% sodium hypochlorite and rinsed with sterile distilled

[6]Trade names in this publication are used only to provide specific information. Mention of a trade name does not constitute a warranty of equipment or materials by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

[7]Fungal identification service was mentioned to provide specific information and does not constitute a warranty by the University of Puerto Rico, nor is this mention a statement of preference over other identification services.

water, prior to inoculation. Bulbs and roots were placed inside a glass petri plate (150 × 15 mm) acting as a moisture chamber. Bulbs were wounded at the center with a sterile needle whereas roots were not wounded. Bulbs and roots were inoculated with 0.4-cm mycelial disks of the evaluated fungal isolates. Four replicates were done per onion cultivar for each fungal isolate. Controls were inoculated with acidified PDA disks. After inoculation, young bulbs and roots were placed in humid chambers that consisted of petri plates (100 × 15 mm) placed inside plastic boxes (91 × 41 × 15 cm) under high humidity (i.e., 90 to 100%) at 25° C. Plastic boxes were randomly placed on laboratory benches. Disease incidence was determined by the number of onion plants with symptoms out of the total of inoculated plants.

Commercial onion bulbs (post-harvested with no roots) were superficially disinfected, as mentioned above, and inoculated with isolates previously selected (P1 to P4). The experimental design was the same as described for laboratory studies. For a month, periodic observations were made to examine symptom development on onion bulbs.

Field pathogenicity tests: We prepared fungal inoculum using 400 g of rice soaked in water for 24 h. Rice was placed in a one-liter Erlenmeyer and sterilized in an autoclave three consecutive times at intervals of 24 h between sterilizations. Rice was inoculated with a conidial suspension (10^6 to 10^7 conidia /ml) and kept for 30 days at room temperature (26° C). *Phoma* spp. inoculum was examined under the microscope to guarantee fungal purity.

Four plants each of onion cultivars Excalibur and Mercedes were used during field experiments. Seeds were sown in 60-cm wide rows with 15 cm of separation between plants. Each treatment was separated by 30 cm from each other. In the field, ten grams of rice colonized by each *Phoma* isolate (10^9 conidia per gram) were applied at sunset around the soil line of four onion plants (60 days post-planting) per cultivar and then covered with wet soil (Vélez-Rodríguez and Rivera-Vargas, 2007). Ten grams of sterile non-inoculated rice was applied to control plants. Periodic observations were made for four weeks to detect any symptom development. Four weeks after inoculation plants were harvested and evaluated for root development.

In all studies, symptomatic signs, if any, were used to re-isolate the pathogen. Plant tissues were superficially disinfected and transferred to PDA. Semi-permanent slides were prepared from developed colonies to observe *Phoma* spp. reproductive structures under the light microscope.

Molecular characterization: Fungal mycelium from thirteen *Phoma* spp. isolates was grown in potato dextrose broth (PDB, Difco, Becton Dickinson and Company, USA) for 8 h at 25° C. Mycelium (0.8

g) was collected on filter paper and washed with sterile double-distilled water (Calle-Bellido, 2005). Genomic DNA was extracted from mycelium by using the FastDNA®Kit (Q-Biogene, Irvine, CA) and was used to amplify the ITS region including the 5.8s unit of rDNA gene by polymerase chain reaction (PCR) with a thermocycler (Perkin Elmer, Model 2400, Wellesley, MA). Polymerase chain reaction was performed in 25µl of a reaction mix that included 20 pM of each primer (ITS1 and ITS4), 2.5 µl 10× PCR buffer (New England Biolabs, Beverly, MA), 1.5 mM of $MgCl_2$, 50 µM of each dNTP, 1U/µl of DNA *Taq* polymerase (Promega Corp., Madison, WI), and 25 ng/µl of fungal DNA (Calle-Bellido, 2005). Primers set ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (3'TCCTCCGCTTATTGATATGC5') were used for PCR at 94° C for 4 min followed by 25 denaturalization cycles at 94° C for 1 min, 30 sec at 55° C for hybridization and 1 min at 72° C for elongation (White et al., 1990). Polymerase chain reaction products were visualized in 1.3% agarose gel by using ethidium bromide and a Biorad UV transilluminator model 416703 (Biorad, Hercules, CA). Polymerase chain reaction products were purified with a MinELute PCR Purification Kit (Qiagen®, Maryland) following manufacturer specifications.

For RFLP's analysis, restriction enzymes *Alu*I, *Eco*RI, *Cla*I, *Hind*III and *Sca*I were used to digest the ITS region of the rDNA in order to create polymorphic patterns. Digestion products were visualized as previously described in 3% agarose gels. An electrophoresis documentation system, Quantity One® 4.5, 2003 (Biorad, Hercules, CA) was used to differentiate band weight and number of fragments. Molecular markers of 100 bp (Promega, Madison, WI) and 1 kb (New England Biolabs, Ipswich, MA) were included in gel images, and DNA was sequenced by the Molecular Resources Facilities of the New Jersey Medical School (http://www. umdnj.edu/mrfweb/).

Sequences of the ITS rDNA region from 38 other *Phoma* species and related genera, such as *Didymella applanata* and *Leptosphaerulina trifolii*, were downloaded from the GenBank at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) was used for sequence comparison with GenBank data bases. Computer program Clustal X (version 1.83) was used to perform DNA sequence alignment. A phylogenetic tree was created by using Phylo_win a program developed at the Genome Populations, Interactions, Adaptations Laboratory from the Montpellier University in Paris, France (GPI Laboratory; http://www.genetix.univ-montp2.fr/labo.htm). Pair-wise distance, or the evolutionary distance, measure was performed by using Kimura's two-parameter model for superimposed mutation. Bootstrap values were above 50% (100 replicates).

RESULTS AND DISCUSSION

Phoma isolates collection: Fifty percent of *Phoma* spp. isolates were collected from soil samples previous to onion sowing. Twenty-two percent and 28% of the isolates were collected 59 and 107 days after planting, respectively; findings showed that as onion plants developed, *Phoma* population dropped. The majority of the isolates were collected from 10- to 20-cm soil depth (60%) as compared to those collected from 0- to 10-cm depth (39%).

Morphological characterization: On the basis of morphology, 92% (12 of 13) of *Phoma* isolates were identified as *P. putaminum* Speg., and only one isolate as *P. macrostoma* var. *incolorata* (A.S. Horne) Boerema & Dorenb. This last isolate formed two-celled conidia in addition to the one-celled, thus indicating that this species belongs to the section *Phyllostictoides*, and its cultural characteristics resembled an atypical isolate. It is sometimes confused with *Phoma exigua* Desm. var. *exigua* and *Phoma pomorum* Thum. var. *pomorum* (Boerema et al., 2004). This is the first report of *P. putaminum* and *P. macrostoma* var. *incolorata* for Puerto Rico and the Caribbean. Fungal isolates were certified as *P. putaminum* (P1, P2 and P4) and one as *Phoma* sp. (P3) (atypical *P. macrostoma* var. *incolorata*) by Dr. R.A. Samson from the Fungal Biodiversity Centre, The Netherlands.

Colony color ranged from olivaceous green (three isolates) to dark brown or black (10 isolates) and colony growth rate was similar for all *Phoma* isolates in both culture media (i.e., OA and MA) (Figure 1A, B). Colony margins for 66% of *P. putaminum* (eight out of 12 isolates) and *P. macrostoma* var. *incolorata* isolates were regular in both culture media used. Mycelia of both *Phoma* species were septated hyaline ranging from 4 to 7.5 µm. *Phoma putaminum* conidia were elliptical 3 to 4 µm long and 2 to 2.5 µm wide. *Phoma macrostoma* var. *incolorata* conidia were globose 8.5 to 14 µm (length) and 2.5 to 4 µm wide. Both *P. putaminum* and *P. macrostoma* var. *incolorata* produced abundant reproductive structures such as pycnidia and chlamydospores in PDA, and were used as discriminating characters. *Phoma macrostoma* var. *incolorata* pycnidia were pear-shaped (11.70 × 10.55 µm) whereas *P. putaminum* were globose (13.65 × 12.40 µm) (Figure 1C, D). Solitary chlamydospores (1.68 × 1.55 µm) were observed in *P. macrostoma* var. *incolorata* whereas in *P. putaminum* they occurred in series (1.55 × 1.08 µm) (Figure 1E). *Phoma putaminum* produced swollen cells (2.10 × 2.50 µm) (Figure 1F); these swollen cells are a very common species character (Boerema et al., 2004), but swollen cells were not observed in *P. macrostoma* var. *incolorata* included in section *Phyllostictoides*.

None of the isolates under study produced pigmentation or dendritic crystal formation in MA after the NaOH spot test (Boerema et al., 2004). A negative test for NaOH indicated that neither of the *Phoma* species studied produced a diffusible antibiotic metabolite 'E' (Figure 1A, B).

Pathogenic Characterization

In vitro pathogenicity test: Pathogenicity tests conducted under laboratory conditions showed that all *P. putaminum* isolates (P1, P2 and P4) evaluated were pathogenic to young onion bulbs and roots of cultivars Mercedes and Excalibur (Table 1). Necrosis of young roots and bulb was observed seven to 12 days after inoculation. Disease incidence was higher in cultivar Mercedes. *Phoma putaminum* isolate P2 was the most virulent to onion tissue, with an incidence of 100%. *Phoma macrostoma* var. *incolorata* isolate P3 caused 25% of incidence only in cultivar Mercedes and did not cause any symptoms in cultivar Excalibur (Table 1). Control plants did not show symptom development after four weeks of periodical observations. *Fusarium* spp. was frequently observed as a contaminant of onion tissues.

Pathogenicity tests on commercial onion bulbs showed that none of the *Phoma* spp. isolates caused symptoms. Eventually, other fungi, such as *Aspergillus niger, Penicillium* sp. and *Rhizopus* sp. colonized mature bulbs. These species have been reported as causing the black and blue mold, and the mushy rot of onion, respectively; they commonly occurred in onion during storage conditions and were re-isolated from bulbs showing symptoms (Schwartz and Mohan, 1999; Vélez-Rodríguez and Rivera-Vargas, 2007). *Fusarium* spp. and *Alternaria* spp. were isolated from onion bulbs and roots showing symptoms. Both have been reported as occurring in onion fields in southern Puerto Rico (Vélez, 2001; Calle-Bellido, 2005; Fernández-García, 2005; Vélez-Rodríguez and Rivera-Vargas, 2007).

Field pathogenicity tests: None of the onion plants inoculated under field conditions developed symptoms during a four-week period after inoculation. *Phoma putaminum* is generally regarded as a saprophyte or a weak pathogen and may act as an opportunistic parasite on roots of some plants. *Phoma macrostoma* var. *incolorata* is considered a cosmopolitan fungus, ubiquitous on woody plants and incidental on herbaceous substrates (Boerema et al., 2004).

Molecular characterization: Sequence size of the ITS region of the nuclear encoded rDNA gene ranged from 458 to 614 base pairs (bp) for *P. putaminum* and 458 bp for *P. macrostoma* var. *incolorata* isolate (Table 2). Our findings differed from those of rDNA ITS region reported for *Leptosphaeria maculans* (anamorph *Phoma lingam*) species com-

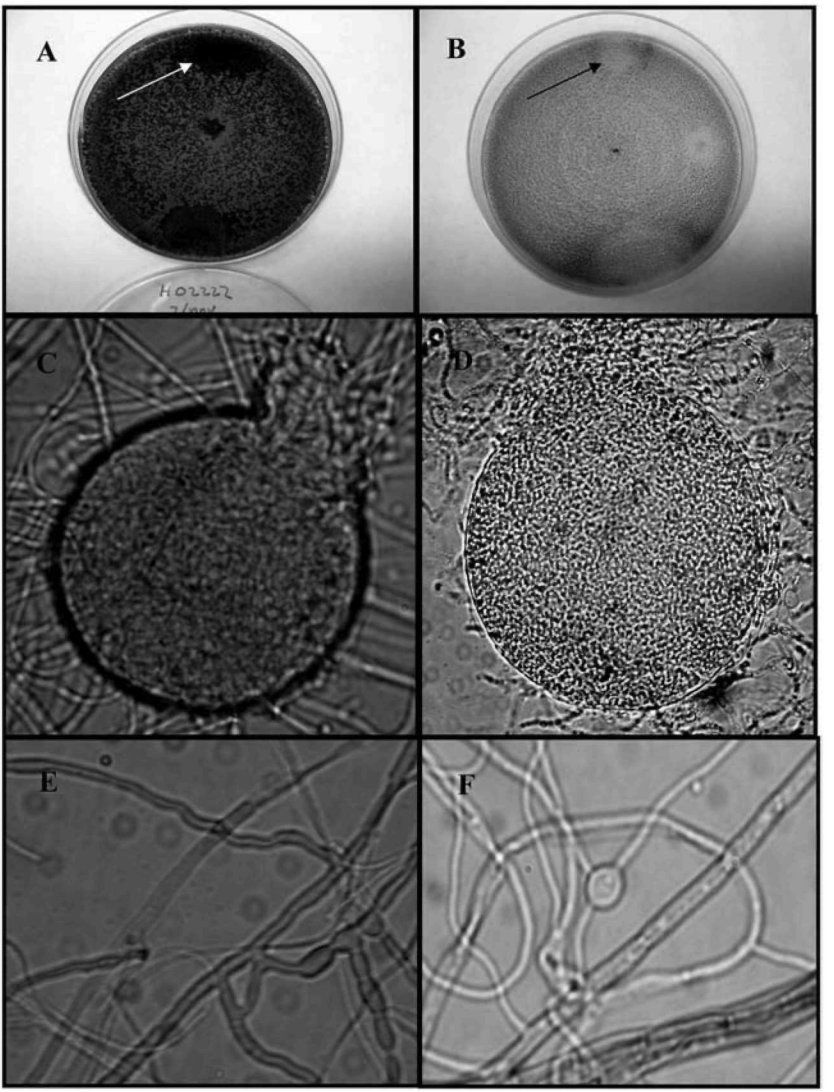

FIGURE 1. *Phoma putaminum* (A) and *P. macrostoma* (B) colonies on Oatmeal Agar. Arrows indicate negative NaOH spot test for both species. Morphological structures: pycnidia of *P. macrostoma* var. *incolorata* (C) and *P. putaminum* (D); chlamydospores (E) and swollen cells (F) of *P. putaminum*.

TABLE 1.—*Disease incidence caused by* Phoma *spp. young onion bulbs and roots of cultivars Mercedes and Excalibur inoculated in vitro.*

Source of inoculum[1]	Incidence (%)[2]	
	Mercedes	Excalibur
Phoma putaminum (P1)	75	50
Phoma putaminum (P2)	100	25
Phoma putaminum (P4)	75	50
Phoma macrostoma var. *incolorata* (P3)	25	0
Control	0	0

[1]Onion bulbs and roots from 55-day-old plants cultivars Mercedes and Excalibur were inoculated in vitro with mycelial disks (4 mm) of the different *Phoma* sp. isolates. Controls were inoculated with 4 mm PDA disks.

[2]Disease incidence was calculated by the number of onion plants infected from the total of plants multiplied by 100.

plex, whose sizes ranged from 470 to 500 bp (Balesdent et al., 1998). Reports of rDNA ITS region size for *Dydimella bryoniae*, the telomorph of *Phoma cucurbitaceatum*, ranged from 500 to 527 bp (Somai et al., 2002).

Digestion of the ITS region of the nuclear encoded rDNA with restriction enzyme *Hind*III distinguished *Phoma* isolates at species level. All other restriction enzymes (*Alu*I, *Eco*RI, *Cla*I and *Sca*I) evaluated were unable to differentiate between species producing identical DNA fragments patterns (Figure 2). Of the 13 *Phoma* isolates evaluated, 12 showed two DNA fragments with *Hind*III, all belonging to *P. putaminum*, and a one-fragment or uncut DNA for the isolate of *P. macrostoma*

TABLE 2.—*Phoma isolate's code, accession number, nucleotide sequence length and BLAST® results using the ITS region of rDNA gene from the GenBank.*

Isolate	Code	GenBank Accession no.	Nucleotide sequence length (bp)	BLAST results
P. putaminum	P1	AM689929	467	*Leptosphaerulina trifolii*
P. putaminum	P2	AM689930	529	*Leptosphaerulina trifolii*
P. macrostoma	P3	AM691012	458	*Didymella ligulicola*
P. putaminum	P4	AM691000	528	*Phoma sp.* P073
P. putaminum	P5	AM691001	458	*Leptosphaerulina trifolii*
P. putaminum	P6	AM691002	535	*Phoma sp.* P073
P. putaminum	P7	AM691003	592	*Leptosphaerulina trifolii*
P. putaminum	P8	AM691004	576	*Leptosphaerulina trifolii*
P. putaminum	P9	AM691005	610	*Leptosphaerulina trifolii*
P. putaminum	P10	AM691006	606	*Phoma multirostrata*
P. putaminum	P11	AM601007	468	*Leptosphaerulina trifolii*
P. putaminum	P12	AM691008	614	*Phoma sp.* P075
P. putaminum	P14	AM691009	557	*Leptosphaerulina trifolii*

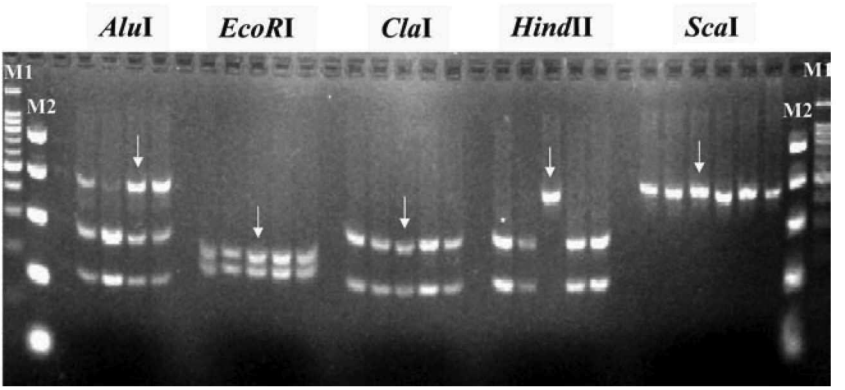

FIGURE 2. Polymorphic patterns of the ITS region of the rDNA of *Phoma* isolates using five restriction enzymes (from left to right: *Alu*I, *Eco*RI, *Cla*I, *Hind*III and *Sca*I). Legend: Molecular markers 1 kb (M1) and 100 bp (M2); arrows indicate *Phoma macrostoma* var. *incolorata* isolate, all other isolates are *P. putaminum*.

var. *incolorata* (Figure 3). The unique band observed had a molecular weight of 552 bp, whereas the two *P. putaminum* bands were 347 and 200 bp, respectively. This technique has proven to be a useful molecular tool for differentiating *Phoma* species occurring in the soil studied.

Table 2 shows the different *Phoma* isolates studied and the BLAST® (NCBI) results by using the nucleotide sequence of the ITS region of

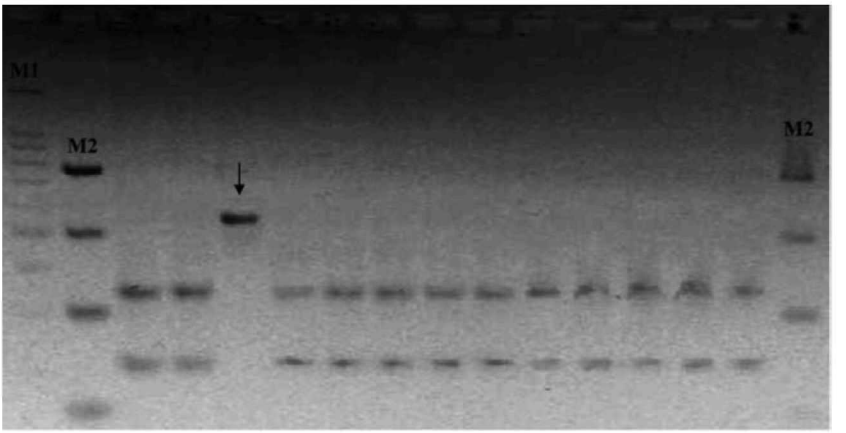

FIGURE 3. Polymorphic patterns of the ITS region from the rDNA obtained with the restriction enzyme *Hind*III for thirteen *Phoma* isolates. Legend: Molecular markers 1 kb (M1) and 100 bp (M2); arrow shows *Phoma macrostoma* var. *incolorata* isolate, all other isolates are *P. putaminum*.

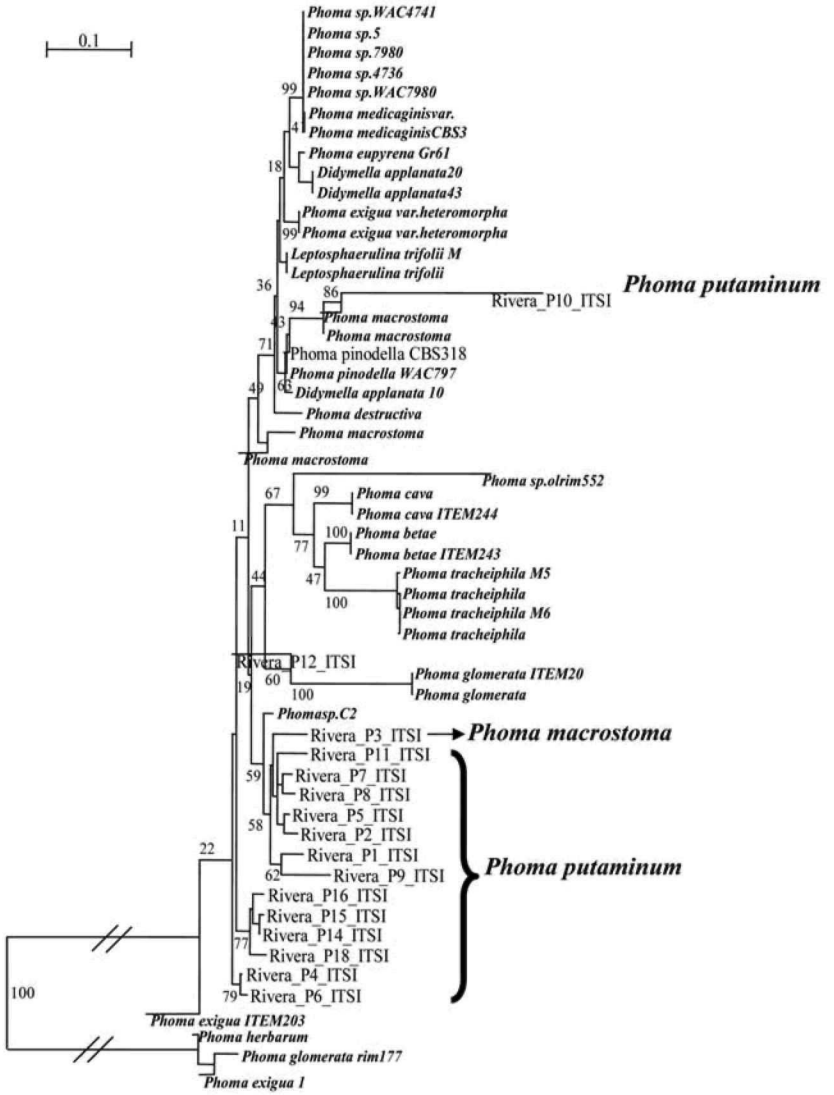

FIGURE 4. Phylogenetic tree created by neighbor-joining method using DNA sequences of the ITS region of the rDNA of *Phoma* spp. isolates. Sequences of closely related genera retrieved by BLAST searches available in the database were used for comparison. Pairwise distance measure was performed with the Kimura two-parameter model for superimposed mutation. Bootstrap values above 50% are indicated (100 replicates). Bar = Kimura distance.

rDNA. The sequence alignment of *Phoma* spp. isolates was matched with sequences of closely related genera such as *Leptosphaerulina trifolii*, *Didymella ligulicola*, *Phoma multirostrata* and *Phoma* sp. (isolates P073 and P075), available sequence databases retrieved by a BLAST search.

A phylogenetic tree created with the ITS region of the rDNA sequences using the neighbor-joining method grouped 12 *P. putaminum* isolates characterized in this study in one big cluster sustaining our morphological characterization, including *P. macrostoma* var. *incolorata* isolate (P3) (Figure 4). One *P. putaminum* isolate (P10) was clustered together with two *Phoma macrostoma* isolates from the GenBank. The difficulty encountered during this study was mainly due to the lack of nucleotide sequences of *P. putaminum* or *P. macrostoma* var. *incolorata* in the GenBank for comparison and the lack of diversity of sequences from *Phoma* spp.

On the evidence presented using morphology, pathogenicity and molecular techniques, we isolated two *Phoma* species from onion soils, *P. putaminum* being predominant over *P. macrostoma*. We observed that their populations declined as onion plants developed, both acting as weak pathogens or opportunistic parasites on roots as shown by the in vitro pathogenicity tests. Even though the use of molecular tools such as RFLP allowed us to differentiate among *Phoma* isolates, future studies using DNA analysis of other genetic regions will be important in order to resolve relationships among these taxa.

LITERATURE CITED

Agrios, G. N., 2005. Plant Pathology, Fifth Edition. Elsevier Academic Press Publications. San Diego, CA. 922 pp.

Balesdent M. H., M. Jedryczka, L. Jain, E. Mendes-Pereira, J. Bertrandy and T. Rouxel, 1998. Conidia as a substrate for Internal Transcribed Spacer-Based PCR identification of members of the *Lepthosphaeria maculans* species complex. *Phytopathology* 88:1210-1217.

Boerema, G. H., J. de Gruyter, M. E. Noordeloos and M. E. C. Hamers, 2004. *Phoma* Identification Manual: Differentiation of Specific and Infra-specific Taxa in Culture. CABI Publishing. Wallingford, Oxfordshire, UK.

Calle-Bellido, J., 2005. Caracterización morfológica y molecular de hongos fitopatógenos de suelo e identificación de bacterias foliares en el cultivo de cebolla. M.S. Thesis, Department of Agronomy and Soils, University of Puerto Rico-Mayagüez Campus. 115 pp.

Department of Agriculture of Puerto Rico, 2006. Agricultural Annual Gross Income, revised data 2003-2004 and preliminary data 2004-2005. Commonwealth of Puerto Rico, San Juan, PR.

Dhingra O. D. and J. B. Sinclair, 1995. Basic Plant Pathology Methods. Second edition. Ed Lewis Publishers. CRC Press, Boca Ratón, FL.

FAO, 2006. FAO Statistical Databases. http://www.faostat.fao.org.

Fernández-García, J., 2005. Incidencia y caracterización: morfológica, patogénica y genética de *Alternaria* spp. en cultivos de cebolla del sur de Puerto Rico. M.S. Thesis, Department of Crop Protection, University of Puerto Rico-Mayagüez Campus. 185 pp.

Martínez, S., 1999. Introduction. *In*: Technological package of onion production. Agricultural Experiment Station. Publication 156. Río Piedras, PR.

Minter, D. W., M. Rodríguez-Hernández and J. Mena Portales, 2001. Fungi of the Caribbean, An Annotated Checklist. PDMS Publishing. Isleworth, London, UK. 946 pp.

Rivera, L. E., 1999. Preparación de terreno y siembra. *In:* Technological package of onion production. Agricultural Experiment Station. Publication 156. Río Piedras, PR.

Rosa-Márquez, E. and G. J. Fornaris-Rullán, 2003. Survey of fungal diseases affecting vegetable crops in southern Puerto Rico. *J. Agric. Univ. P.R.* 87(3-4):155-160.

Schwartz, H. F. and S. K. Mohan, 1999. Compendium of Onion and Garlic Diseases. The American Phytopathological Society Press, St. Paul, MN.

Somai, B. M., R. A. Dean, M. W. Farnham, T. A. Zitter, and A. P. Keinath, 2002. Internal transcribed spacer regions 1 and 2 and Random Amplified Polymorphic DNA analysis of *Didymella bryoniae* and related *Phoma* species isolated from cucurbits. *Phytopathology* 92:997-1004.

Thornton, M. K. and S. K. Mohan, 1996. Response of sweet Spanish onion cultivars and numbered hybrids to basal rot and pink root. *Plant Dis.* 80:660-663.

Vélez-Rodríguez, L., 2001. Catastro de hongos asociados al cultivo de la cebolla (*Allium cepa* L.) en la zona sur de Puerto Rico. M.S. Thesis, Department of Crop Protection, University of Puerto Rico-Mayagüez Campus. 99 pp.

Vélez-Rodríguez, L. and L. I. Rivera-Vargas, 2007. Recent studies of fungal pathogens of onion in Puerto Rico. *J. Agric. Univ. P.R.* 91(1-2):31-45.

Weiland, J. J., and J. L. Sundsback, 2000. Differentiation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS region of the rRNA Gene. *Plant Dis.* 84:475-482.

White, T. J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. Academic Press, Inc. pp. 315-322.