Alternaria spp. implicated in a disease complex of onion leaf blight in the tropics[1,2]

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ABSTRACT

Alternaria **isolates were collected from onion foliage at different stages of the plant life cycle. Incidence of** ***Alternaria*** **species in cultivars 'Mercedes' and 'Excalibur' was determined during two consecutive growing seasons in fields located in southern Puerto Rico. Leaves showing purple to brown sunken elliptical lesions with chlorotic halos were taken at random. Five leaf sections (0.5 cm) from each sample were superficially disinfested, transferred to culture media and incubated, and isolations were documented. Disease incidence ranged from 25 to 52% in 60- to 100-day-old plants. An increase in** ***Alternaria*** **incidence was observed in response to high relative humidity in the fields. A total of 280 isolates were obtained, and 35 were selected for morphological, pathogenic and molecular characterization. A complex of five different** ***Alternaria*** **species is associated with onion leaf blight on the island.** ***Alternaria destruens, A. tenuissima, A. palandui, A. allii*** **and a group of small-spore** ***Alternaria*** **sp., belonging to a taxonomically undescribed group, were identified. Sixty-two percent of selected isolates belong to this group having an** ***A. arborescens*** **intermediate sporulation pattern.** ***Alternaria destruens*** **and** ***A. palandui*** **have not been previously reported as associated with onions in the Caribbean or in the Western Hemisphere.** ***Pathogenicity tests showed that A. allii, A. tenuissima*** **and** ***Alternaria*** **sp. were pathogenic to onion foliage, with** ***A. allii*** **as the most virulent. Molecular characteristics of the isolates were determined by using the ITS of the rDNA gene. Phylogenetic relationships based on rDNA ITS sequences from** ***Alternaria*** **isolates and other Pleosporaceae distinguished three clades. The first clade**

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of large filiform-beaked spores included *A. allii* from this study, as well as isolates from the GenBank (*A. porri, A. solani, A. macrospora, A. zinniae* and *A. sesamicola*). These formed a monophyletic group, discrete from other members of the genus. The second clade included a diverse group of small-spore *Alternaria*: *A. tenuissima, A. alternata, A. palandui, A. destruens* and *Alternaria* sp.; the third clade included *Stemphylium* spp.

Key words: *Alternaria, Allium cepa*, rDNA ITS region, late blight of onion

RESUMEN

Alternaria spp. implicadas en el complejo de la enfermedad del tizón foliar de la cebolla en los trópicos

Se colectaron aislados de *Alternaria* del follaje de cebolla en diferentes etapas del ciclo de vida de la planta. Se determinó la incidencia de las especies en los cultivares Excalibur y Mercedes durante dos épocas consecutivas de crecimiento en predios localizados en el sur de Puerto Rico. Se colectaron hojas al azar que mostraban lesiones elípticas hundidas de coloración púrpura a marrón con halos cloróticos. Se desinfestaron superficialmente cinco secciones (0.5 cm) de las hojas por cada muestra, se transfirieron a medio de cultivo, se incubaron y se documentaron los aislamientos. La incidencia de la enfermedad fluctuó de 25 a 52% en plantas de 60 a 100 días de madurez. Se observó un aumento en la incidencia de *Alternaria* en respuesta al aumento en la humedad relativa en los predios. En total se obtuvieron 280 aislados, de los cuales 35 se seleccionaron para la caracterización morfológica, patogénica y molecular. Un complejo de cinco especies diferentes de *Alternaria* está asociado a la quemazón o tizón de la hoja de cebolla en la isla. Se identificó a *A. destruens, A. tenuissima, A. palandui, A. allii* y un grupo de *Alternaria* sp. de esporas pequeñas perteneciente a un grupo taxonómicamente no descrito. Sesenta y dos por ciento de los aislados seleccionados pertenecen a este grupo, caracterizado por el patrón de esporulación intermedio de *A. arborescens. Alternaria destruens* y *A. palandui* no han sido reportadas anteriormente asociadas a cebolla en el Caribe ni en el Hemisferio Occidental. Las pruebas de patogenicidad mostraron que *A. allii, A. tenuissima* y *Alternaria* sp., fueron patogénicas al follaje de cebolla, siendo *A. allii* la más virulenta. Se utilizó la región ITS del rADN para la caracterización molecular de los aislados. Las relaciones filogenéticas basadas en las secuencias de la región ITS del rADN de los aislados de *Alternaria* y otras Pleosporaceae distinguen tres grupos o clados. El primer clado de aislados con esporas grandes y puntas filiformes incluye a *A. allii*, aislados durante este estudio, así como secuencias de aislados obtenidos del GenBank (*A. porri, A. solani, A. macrospora, A. zinniae* y *A. sesamicola*). Estas especies forman un grupo monofilético separado de otros miembros del género. El segundo clado incluye un grupo diverso de *Alternaria* de esporas pequeñas: *A. tenuissima, A. alternata, A. palandui, A. destruens* y *Alternaria* sp.; y un tercer clado que incluye a *Stemphylium* spp.

Palabras clave: *Alternaria, Allium cepa*, región ITS del rADN, quemazón, tizón tardío de la cebolla

INTRODUCTION

Species of *Alternaria* are important pathogens that cause plant and post-harvest diseases. They can be potential fungal contaminants that

produce toxic metabolites (i.e., mycotoxins), or saprophytes, present in the air and in soil litter (Andersen et al., 2002; Zur et al., 1999). As an important worldwide plant pathogen, *Alternaria* spp. causes purple leaf blotch or late blight of onions under warm and humid conditions (Everts and Lacy, 1990; Miller and Lacy, 1995). Characteristics of the disease are sunken elliptical lesions in older leaves varying in color from purple to brown with chlorotic halos and concentric rings (Nolla, 1927; Schwartz and Mohan, 1995). *Alternaria porri* (J. B. Ellis) Ciferri has been identified as the causal agent of purple blotch in garlic, leek and onion foliage around the world (Koike and Henderson, 1998; Schwartz and Mohan, 1995). Erroneously the name *Alternaria porri* has been used for any large-spore *Alternaria* that was found on members of the Alliaceae (Simmons, 2007). In fact, *A. allii* Nolla was first described in 1927 by J. A. B. Nolla from foliar lesions in onions cv. Bermuda grown in the northern region of Puerto Rico, but the name fell into disuse (Nolla, 1927; Simmons, 2007). Recently, other notable species with large long-beaked conidia affecting onions such as *A. iranica* Simmons & Ghosta from Iran, and *A. vanuatuensis* Simmons & Hill from Vanuatu in Oceania have been described (Simmons, 2007).

In the field, *Alternaria* lesions disrupt photosynthesis and reduce the final output of the onion bulb. In Colorado, *A. porri*, *A. alternata* (Fries) Keissler (synonymous with *A. tenuis*) and *A. tenuissima* (Nees & Nees:Fr.) Wiltshire, have been implicated in a disease complex of onion leaf blight causing purple and brownish foliar lesions (Skiles, 1953). Optimum field conditions for disease development occurred during long wet periods of high relative humidity (RH > 90%) or dew deposition with continuous temperatures ranging from 20 to 25 °C (Everts and Lacy, 1990). During environmental conditions that favor pathogen sporulation and conidia germination, fungicide control is ineffective (Miller, 1983). Yield losses can range from 30 to 50% and up to 100% because of the rapid dissemination of the disease in the field (Schwartz, 1999).

During February and April 2000, field surveys conducted by personnel of the University of Puerto Rico (UPR) in onion fields located at Santa Isabel and Guánica, Puerto Rico, showed that up to 52% of foliar lesions in onions were caused by at least three different *Alternaria* species: *A. tenuissima*, *A. alternata*, and *Alternaria* sp. (Vélez et al., 2004; Vélez-Rodríguez and Rivera-Vargas, 2007). They were easily isolated from foliar lesions of onion and were pathogenic under laboratory and greenhouse conditions. Isolates evaluated caused ellipsoidal sunken brownish to purple lesions that eventually extended to the tip of the leaf (Vélez-Rodríguez and Rivera-Vargas, 2007). Tip blight was well de-

veloped seven days after inoculation. *Alternaria tenuissima* produced ellipsoidal light brown lesions with a dark halo margin measuring 0.6 × 0.3 cm, twelve days after inoculation. Profuse mycelial growth and sporulation were observed at the center of the lesions. Brownish to purple lesions were observed with *A. alternata* and with an *Alternaria* sp. that was not identified. Interestingly, *A. porri* and *A. allii* were not isolated during these studies. The inability to isolate *A. allii* was probably due to the poor or non-sporulation of this species isolates that make them impossible to identify morphologically; they were therefore classified as unknowns by the authors (Vélez et al., 2004).

Alternaria taxonomy is complex. Many species have been described, and a number of morphologically distinct groups, especially of small-spore-catenulate *Alternaria,* have been isolated as complexes from different host plants (Hong et al., 2006; Pryor and Michailides, 2002; Skiles, 1953; Simmons, 2007). An important limitation to traditional classification of the genera is the absence of sexual stages. Characterizing and determining the relative importance of each *Alternaria* species infesting onion, using traditional microscopic or morphological methods, is challenging. Traditional characterization, based on cultural characteristics, conidial morphology and ontogeny, is usually time consuming and difficult in the absence of reliable reference materials and expertise. Morphological criteria are affected by culture media, culture age and environmental cultural conditions (Rotem, 1994; Simmons, 1992). Pathogenicity tests to separate pathogenic from saprophytic species may also yield inconclusive results under different growing conditions and with different cultivars. Correct identification of species of *Alternaria* affecting onion in tropical conditions in the Caribbean Basin, particularly in Puerto Rico, may require the use of diverse strategies, such as morphological criteria and the use of molecular techniques. Analysis of random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), PCR amplification and sequencing of the nuclear internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) gene, and amplified fragment length polymorphism (AFLP) fingerprinting have complemented traditional taxonomy (Chou and Wu, 2002; Pérez-Martínez et al., 2004; Pryor and Michialides, 2002; van der Waals et al., 2004).

Few studies have been published on the characterization of species of *Alternaria* causing diseases of onions under tropical conditions. Our objectives were to determine the incidence of *Alternaria* at the different stages of the onion life cycle under field conditions in Puerto Rico; to characterize these species on the basis of morphology, pathogenicity and DNA sequence data; and to determine phylogenetic relationships among *Alternaria* isolates associated with onion foliage under tropical conditions. The results will help in understanding the relative impor-

tance of the population diversity and dynamics of *Alternaria* as an onion pathogen under tropical conditions.

MATERIALS AND METHODS

Incidence of Alternaria spp. in onion fields

Alternaria spp. incidence in onion foliage was determined during two consecutive growing seasons: November 2003 to January 2004, and February to April 2004 at the municipalities of Santa Isabel and Juana Díaz in southern Puerto Rico. Onions lots were planted following technological package for onion production (UPR-Agricultural Experiment Station, 1999). Onions rows and lots were separated by cultivar. Lot sizes were approximately between 2.9 and 3.7 ha. Lots were located at road PR-1, Bo. Jauca, Sector Destino, at Santa Isabel, Puerto Rico, [(N17° 58' 39. 8"/W66° 22' 47. 1" at 22 m above sea level (Map Datun, Puerto Rico) (GPS 12 XL, 12 channel, Garmin 1996-2000)][7], and at the UPR-Agricultural Experiment Station, Road 510 km 3.0 Sabana Llana, Juana Díaz, Puerto Rico [(N18° 01' 39. 1"/ W66° 31' 33. 2" at 34 m above sea level (Map Datun, Puerto Rico) (GPS 12 XL, 12 channel, Garmin 1996-2000)].

Samples were collected every 14 days from four different plots planted with cultivars Mercedes (Seminis®) or Excalibur (Sunseeds®). A systematic zig-zag sampling technique was used during the survey. Onion leaves were randomly collected from every eighth row for a total of 50 samples per lot. Incidence was determined by counting elliptical brownish to purple foliar lesions from the field sampled. Samples were placed inside plastic bags in an ice box and transported from the field to the Plant Pathology Laboratory on the University of Puerto Rico, Mayagüez Campus. Temperature, precipitation and relative humidity data were collected at a meteorological station located at UPR-Agricultural Experiment Station, Juana Díaz. Precipitation and temperature data were taken with Land Surface (N18° 01'58.2"/W 66° 31'53.2") and Weskler (N18 01.584 W 066 31. 531') instruments, respectively, of the National Climatic Data Center, United States Weather Bureau.

A correlation analysis ($\alpha = 0.05$) based on *Alternaria* spp. incidence (%) was performed. Correlation coefficient was calculated by using Pearson's method and InfoStat/Student 2.0 from the National University of Córdoba.

[7]Company or trade names in this publication are used only to provide specific information. Mention of a company or 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.

Fungal isolates

For fungal isolation, five sections (0.5 cm) from each leaf sample were superficially disinfested with 70% ethyl alcohol, followed by 0.05% sodium hypochlorite, then rinsed twice with de-ionized double distilled sterile water. Leaf sections were transferred to acidified (25% lactic acid) potato dextrose agar (APDA) (Difco) amended with streptomycin (Sigma®). Petri plates (60 × 15 mm) were incubated for seven days at ± 27° C under a 10 to 12 h cool-white fluorescent dark/light cycle for one week. Thirty-five *Alternaria* isolates were selected on the basis of colony appearance for further morphological characterization (Table 1). Morphological characterization was based upon criteria established by Simmons (1992) using single-spore colonies of *Alternaria*, transferred to potato-carrot agar (PCA) and V8 juice agar (V8). Sporulation pattern and conidium morphology were examined after five to seven days at 27° C for a 12-12 h dark-light regime. Colony characteristics were described from six different culture media: corn meal agar (CMA), Czapeck agar, dichloran rose bengal yeast extract sucrose agar (DRYES), oatmeal agar (OA), PCA and V-8 agar (V8A). Fungal cultures were stored on PCA plates at 4° C and in lyophilized at -20° C.

Morphotype isolates of various *Alternaria* 'species groups' were obtained from E.G. Simmons [*Alternaria infectoria* Simmons (EGS27-193), *A. alternata* (EGS34-016), *A. arborescens* Simmons (EGS39-128), *A. tenuissima* (Nees & T. Nees: Fr.) Wiltshire (EGS34-015), and *A. porri* (EGS48-147)]. *Pleospora eturmiuna* Simmons (teleomorph of *Stemphylium eturmiunum*) were used for reference comparisons.

Pathogenicity tests

Pathogenicity tests were conducted in vitro in the laboratory and under field conditions at the Juana Díaz Agricultural Experiment Station. Onion cultivars Mercedes (Seminis®), Excalibur (Sunseeds®), and Candy (Petoseed®) were used as test plants. For the in vitro tests, asymptomatic leaves collected from 60- to 70-day-old plants were treated as before described for fungal isolation, and placed in sterile humid chambers on petri plates (100 × 15 mm) 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, and all treatments were repeated three times. Nineteen *Alternaria* spp. isolates grown on PCA for seven to ten days at 12 h daylight regime were used during the pathogenicity tests (Table 1). Conidial suspension was prepared by flooding the cultures with sterile di-ionized distilled water and rubbing the fungal colony with a sterile crystal rod. Conidia concentration was adjusted to 10^5 CFU's/ml by using a hemacytometer. Inoculations were made at the middle region of

TABLE 1.—Alternaria *spp. isolated from onion foliage of cvs. Mercedes and Excalibur from two locations in southern Puerto Rico and their GenBank accession numbers used for phylogenetic studies.*

Isolate no.	Species group[1]	Onion cultivar	Locality	GenBank Accession no.
Alt1*[2]	*Alternaria destruens*	Mercedes	Santa Isabel	DQ323681
Alt2*	*A. destruens*	Excalibur	Santa Isabel	DQ323680
Alt3*	*A. tenuissima*	Excalibur	Santa Isabel	—
Alt4*	*A. tenuissima*	Mercedes	Juana Díaz	DQ323692
Alt5*	*A. tenuissima*	Excalibur	Santa Isabel	DQ323695
Alt6*	*A. tenuissima*	Excalibur	Santa Isabel	DQ323684
Alt7*	*A. tenuissima*	Excalibur	Santa Isabel	—
Alt8*	*A. palandui*	Mercedes	Santa Isabel	DQ323686
Alt9*	*A. palandui*	Mercedes	Santa Isabel	DQ323702
Alt10*	*A. palandui*	Mercedes	Juana Díaz	DQ323682
Alt 11	*A. palandui*	Excalibur	Santa Isabel	—
Alt 12*	*A. palandui*	Excalibur	Santa Isabel	—
Alt 13	*A. palandui*	Mercedes	Santa Isabel	—
Alt14*	*A. palandui*	Excalibur	Juana Díaz	DQ323687
Alt15	*Alternaria* sp. **	Mercedes	Santa Isabel	—
Alt16	*Alternaria* sp. **	Mercedes	Santa Isabel	DQ323688
Alt17*	*Alternaria* sp. **	Mercedes	Santa Isabel	DQ323689
Alt18*	*Alternaria* sp. **	Mercedes	Santa Isabel	DQ323690
Alt19	*Alternaria* sp. **	Mercedes	Santa Isabel	DQ323707
Alt20	*Alternaria* sp. **	Mercedes	Santa Isabel	DQ323701
Alt21	*Alternaria* sp. **	Mercedes	Santa Isabel	DQ323709
Alt22	*Alternaria* sp. **	Excalibur	Santa Isabel	DQ323694
Alt23*	*Alternaria* sp. **	Mercedes	Santa Isabel	—
Alt24	*Alternaria* sp. **	Excalibur	Santa Isabel	DQ323708
Alt25	*Alternaria* sp. **	Mercedes	Santa Isabel	—
Alt26	*Alternaria* sp. **	Mercedes	Santa Isabel	DQ323685
Alt27*	*Alternaria* sp. **	Excalibur	Santa Isabel	DQ323691
Alt28	*Alternaria* sp. **	Excalibur	Santa Isabel	DQ323696
Alt29	*Alternaria* sp. **	Excalibur	Santa Isabel	DQ323693
Alt30	*Alternaria* sp. **	Mercedes	Juana Díaz	DQ323703
Alt31*	*Alternaria* sp. **	Excalibur	Santa Isabel	—
Alt32	*Alternaria* sp. **	Mercedes	Santa Isabel	—
Alt33	*Alternaria* sp. **	Excalibur	Santa Isabel	DQ323704
Alt34*	*A. allii*	Excalibur	Juana Díaz	DQ323705
Alt35*	*A. allii*	Mercedes	Juana Díaz	DQ323683

[1]Species groups corroborated by Dr. Emory G. Simmons, Crawfordsville, IN. (pers. comm., 2004) on the basis of morphological characterization on Potato Carrot Agar.

[2]* = Isolates selected for pathogenicity tests; ** = a taxonomically undescribed group of small-spore *Alternaria* spp. that has an *A. arborescens* intermediate sporulation pattern.

the leaves with or without wounding. Leaf tissue was wounded with a sterile dissecting needle and inoculum was applied with a sterile cotton swab soaked in the conidial suspension. Control plants were inoculated with sterile distilled water and handled as previously described. As for those *Alternaria* isolates that did not produce conidia on PCA, mycelial plugs (4 mm) were taken from the growing margin of the fungal colony (7 to 10 d old) and used for inoculation. Sterile

PCA plugs were used as control. Pathogenicity tests were conducted twice. After inoculations, leaves were examined for disease symptoms and data of virulence were taken by measuring lesions in cm. Pathogens were re-isolated to complete Koch postulates.

A field experiment was arranged in a randomized complete block design at the Juana Díaz Agricultural Experiment Station. Insecticides such as avermectin and cipermetrin were applied after the second and sixth weeks of planting, respectively. Variables and inoculation methods were as described before.

Genomic DNA Extraction and Amplification of ITS region

Total genomic DNA was extracted from selected *Alternaria* spp. single-spore isolates (n = 25) including four morphotypes provided by E.G. Simmons and *Pleospora eturmiuna* (Tables 2 and 3). *Alternaria* isolates were grown in 50 ml of sterile potato dextrose broth (PDB, Difco). Cultures were placed on a rotary shaker at 120 rpm and incubated in the dark at 27° C for seven days. Mycelia mats from cultures were harvested by vacuum filtration with a Buchner funnel and sterile filter paper (Fisher Scientific, P8) and transferred to sterile plastic tubes to be stored at -20° C. Genomic DNA was extracted from mycelium by using the FastDNA®Kit (Q-Biogene, Irvine, CA) according to the manufacturer's instructions in Fast PrepTM Bio 101 equipment (Thermo Electron Corporation Milford, MA). DNA samples were diluted in TE buffer (10 mM Tris-HCL; 1 mM EDTA) to a concentration of 25 ng/µl and stored at -20° C.

For sequence analysis, the nuclear rDNA gene ITS region, including ITS1, ITS2 and the 5.8S ribosomal gene, was amplified by using polymerase chain reaction (PCR) with a thermocycler (Perkin Elmer, Model 2400, Wellesley, MA) and primers ITS1 and ITS4 (White et al., 1990). Polymerase chain reaction was performed in a total volume of 25 µl containing 2.5 µl 10X of TermoPol buffer (New England, BioLabs.), 200 µM of each dNTP's (Roche®, USA), 1 µM of each single

TABLE 2.—*GenBank accession numbers of morphotypes of* Alternaria *spp. and* Pleospora eturmiuna *used for reference and comparison in phylogenetic studies.*

Isolate no.	Species group	GenBank Accession no.
Riv-St1	*Pleospora eturmiuna*[1]	DQ323706
EGS27-193[2]	*A. infectoria*	DQ323697
EGS34-015[2]	*A. tenuissima*	DQ323698
EGS34-016[2]	*A. alternata*	DQ323699
EGS48-147[2]	*A. porri*	DQ323700

[1]*Pleospora eturmiuna* (teleomorph of *Stemphylium eturmiuna*).

[2]Species groups obtained from Dr. Emory G. Simmons, Crawfordsville, IN. (pers. comm., 2004).

TABLE 3.—Alternaria, Embellisima *and* Ulocladium *sequences obtained from the GenBank and used for phylogenetic studies.*

Species	GenBank Accession no.
Alternaria alternata	DQ156341
A. arborescens	AY154706
A. destruens	AY278836
A. longipes	AY154684
A. macrospora	AY154689
A. porri	AF229470
A. sesamicola	AF314588
A. solani	AY154716
A. tenuissima	AY751455
A. zinniae	AY154696
Embellisima sp	AF212307
Ulocladium sp.	AY943384
Stemphylium botryosum	Y17068
S. vesicarium	AF229484

primer, 1 U/µl of *Taq* DNA polymerase (New England, BioLabs, MA) and 50 ng/µl of template DNA. Polymerase chain reaction conditions were as follows: 94° C for 4 min, followed by 35 cycles of 94° C for 2 min, 55° C for 30 s and 72° C for 1 min, with a final extension at 72° C for 4 min. Two microliters of the PCR product was separated on 1.2% agarose gel (Fisher Scientific, New Jersey) prepared with 1X sodium bromide (SB) (Brody and Kern, 2004) and 4 µl of ethidium bromide (1 µg/1 µl, Sigma®, St. Louis, MO). Polymerase chain reaction products were visualized with a UV illuminator (Quantity One® 4.5 2003, Bio-Rad Laboratory, Inc., Japan).

Polymerase chain reaction products were purified by using MinELute PCR Purification Kit (Qiagen®, Maryland) according to the manufacturer's instructions. The ITS region was sequenced by using forward primer ITS1 and reverse primer ITS4. The DNA was sequenced at the Molecular Resources Facilities of the New Jersey Medical School (http://www. umdnj.edu/mrfweb/).

Analysis of sequence data

Alternaria spp. sequences of the ITS region of the rDNA were edited and assembled by using Chromas Lite version 2.0 (www.technelysium.com.au) and aligned by ClustalX program version 1.83. Distance analysis was performed by using the Phylo_win program (*Genome Populations Interactions*, University of Montpellier, France) with the Neighbor-Joining method. Kimura's two-parameter model of evolution was used and the number of bootstraps was 1,000 replicates. Similarity values between sequences were calculated by pairwise comparisons. Sequences from other species such as *A. arborescens*, *A. longipes*, *A. macrospora*, *A. sesamicola*, *A. solani*, *A. zinniae* and related genera

(i.e., *Embellisia* sp., *Stemphylium botryosum*, *S. vesicarium*, *Ulocladium* sp.) were obtained from the GenBank database (Table 3).

RESULTS

Onion leaf blight was commonly observed in the southern region of Puerto Rico. Long elliptical purple and brown lesions with distinctive chlorotic halos were observed in the field (Figures 1A and 1B). Emerging *Alternaria* spp. conidiophores were frequently produced on infected tissue and easily observed under the dissecting microscope. Similar lesions were observed associated with herbicide and insect damage, especially leaf miners (*Liriomyza* sp.) (Figures 1C and 1D). Eventually, lesions caused the collapse or folding of the leaves affecting plant development and production (Figures 1E and 1F).

Alternaria's incidence

Alternaria spp. lesions were first observed between 18 to 31 days after planting (DAP) (Table 4). Incidence increased as plants of both cultivars developed. Higher incidence, 42% for Excalibur, 52% for Mercedes, occurred 60 to 102 days after planting (Table 4). During our studies, temperature ranged from 23.8 to 26.3 °C; precipitation was low, ranging from 0 to 0.29 cm, and the relative humidity (RH) ranged from 68 to 88% (Table 5). Disease incidence varied in response to RH. A positive correlation was observed between RH and *Alternaria*'s incidence only in cultivar Mercedes ($r = 0.7$) at $\alpha = 0.05$. Minimal fluctuations in temperature and precipitation occurred but correlation coefficients with incidence were not statistically significant (Table 5). Overall, no major differences were observed between onion cultivars in purple blotch incidence and colonies of *Alternaria* spp. recovered. Disease incidence in Juana Díaz was lower than in Santa Isabel (data not shown). At Juana Díaz, higher incidence was observed in 46- to 87-day-old plants ranging from 25 to 33% for the two cultivars evaluated.

Alternaria species

Two hundred and eighty *Alternaria* isolates associated with leaf blight of onion (cvs. Mercedes and Excalibur) were collected. Thirty-five isolates were selected as representative groups for further characterization (Table 1). Based on morphology, four species groups were identified: *Alternaria destruens*, *A. tenuissima*, *A. palandui* (Ayyangar), and *A. porri* (Figure 2). Sixty-two percent of the selected isolates belong to a taxonomically undescribed group of small-spore *Alternaria* spp. having an *A. arborescens* intermediate sporulation pattern. Thus, a complex of five different *Alternaria* species is associated with onion leaf blight on the island.

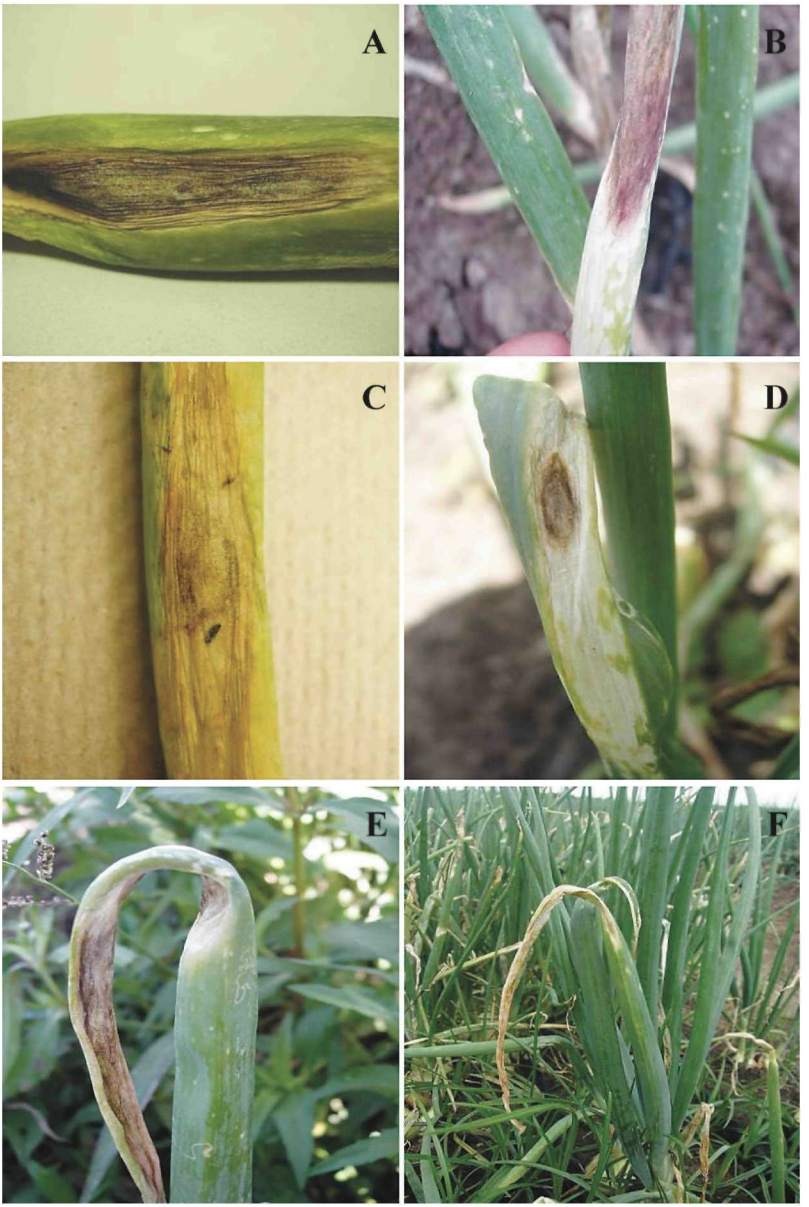

FIGURE 1. Common foliar lesions observed in onion in the southern region of Puerto Rico. A) Brown and B) purple lesions; *Alternaria* sp. growing on lesions from damage caused by C) leaf miners and D) herbicide; E) Collapse of the leaf caused by *Alternaria* spp.; and F) leaf blight in the field.

TABLE 4.—Alternaria *incidence (%) during onion plant life cycle at commercial plots located at Santa Isabel, Puerto Rico.*

	Onion Cultivars			
	Mercedes		Excalibur	
DAP[1]	C[2]	%	C	%
4-17	0	0	0	0
18-31	14	28	4	8
32-45	7	14	14	28
46-59	2	4	0	0
60-73	23	46	24	48
74-87	14	28	24	48
88-102	26	52	21	42
103-116	8	16	3	6

[1]DAP = Days after planting, 2004.
[2]C = Number of *Alternaria* colonies isolated from 50 samples.

Alternaria spp. colonies developed in six culture media were examined and their mycelial growth described (Table 6). Species were morphologically characterized on the basis of conidium size, number and type of septa, and catenulation (Table 7) (Figure 2). Isolates Alt34 and Alt35, belonging to the *A. porri* group, did not sporulate in culture media and were characterized on the basis of colony development, pathogenicity and DNA analysis. The narrow ellipsoid long-beaked conidia, typical of *A. porri,* were rarely observed (Figure 2E). These isolates produced an ochreous pigment on culture media similar to that which was described for *A. allii* by Nolla in 1927 (Figures 2F and 3B). These two isolates, Alt34 and Alt35, will be referred to as *A. allii* from now on.

Isolates of *A. destruens* from Puerto Rico produced beakless amber conidia in simple chains of four to eight conidia characteristic of the

TABLE 5.—*Temperature (°C), relative humidity and precipitation during sampling period. Data taken at UPR-Agricultural Experiment Station, Juana Díaz, Puerto Rico. Average data from the second season.*

DAP[1]	Temperature (°C)	Relative humidity (%)	Precipitation (cm)
4-17	26.29	77.78	0
18-31	25.71	76.73	0
32-45	24.46	77.43	0.14
46-59	24.45	68.39	0
60-73	25.09	88.38	0
74-87	25.44	74.08	0
88-100	23.84	84.27	0.29
103-116	26.13	81.13	0.29
Average	25.18	78.52	0.09

[1]Days after planting during January to April, 2004.

TABLE 6.—*Colony descriptions of* Alternaria *spp. isolated from onion and grown in different culture media.*

Alternaria spp.	Culture media[1]					
	V-8	Czapeck	OA	DRYES	PCA	CMA
A. palandui	Reddish brown colony, cream aerial mycelium	Dark olive green colony, grey aerial mycelium	Light green to grey colony, white to cream aerial mycelium	Sandy cream colony	Dark olive green colony, scarce mycelium	Dark brown colony, scarce mycelium
A. destruens	Dark brown colony, no aerial mycelium	Reddish brown colony, no aerial mycelium	Dark olive green colony, no aerial mycelium	Cream to light yellow colony, pinkish superficial mycelium	Amber colony, abundant amber color conidia produced in media	Dark brown colony, abundant light brown conidia was produced in media
A. tenuissima	Reddish brown colony, cream and brown patches of aerial mycelium	Dark olive green to brownish colony, pink to cream center	Dark olive green colony	Amber colony with cream color areas	Brown colony, abundant conidia	Aerial mycelium cream to brown in color
A. tenuissima Alt7 & Alt8	Light brown to greenish colony	Light greenish grey colony, dark center	Light green colony, scarce white aerial mycelium	Amber to yellow colony, white margins	Dark green colony with a darker center, abundant conidia	White to cream colony, scarce mycelium
Alternaria sp.	Brown colony, light grey aerial mycelium	Light grey with darker center colony	Dark grey colony	Cream with white margin colony	Light brown colony, abundant conidia	Cream with dark center colony, scarce mycelium
A. allii	Dark green to reddish colony, scarce pink aerial mycelium, ocherous pigment on culture media	Dark olive green with pink aerial mycelium	Light green colony with ash grey mycelium	Dark green to yellow colony, concentric rings, light green margins	Scarce mycelium light green	Scarce mycelium dark brown

[1]CMA= corn meal agar; DRYES = dichloran rose bengal yeast extract sucrose agar; OA= oatmeal agar; PCA= potato carrot agar.

TABLE 7.—*Morphological characterization of* Alternaria *species group isolated from onion foliage.*

				Conidial Size[3] (µm)			
		Num. Septa[2]		L		W	
Alternaria species group	Catenulation[1]	T	L-(O)	I	A	I	A
Alternaria destruens	4-8	3-6	0-(2)	(22-55)	36	(10-15)	11
A. tenuissima	6-13	4-8	0-(2)	(32-76)	56	(10-18)	13
A. palandui	5-12	3-8	0-(2)	(25-75)	55	(8-12)	10
Alternaria sp.[4]	4-10	4-8	0-(3)	(35-68)	47	(10-16)	12
A. tenuissima[5]	6-10	4-8	0-(3)	(22-68)	55	(10-16)	12
A. arborescens[6]	4-6	2-4	0-(1)	(17-27)	21	(8-11)	10
A. allii[7]	N/A	N/A		N/A		N/A	

[1]Number of conidia produced in chains (catenulation) on potato carrot agar.
[2]Number of transverse (T), longitudinal (L) and oblique (O) septa.
[3]Measurements in micrometers (µm): L = length, W = width, I = interval and A = Average.
[4]*Alternaria* sp. a taxonomically undescribed group (Simmons, pers. comm.).
[5]Pathogenic isolate.
[6]Isolate 39-128 (E. G. Simmons included as reference).
[7]Does not apply (N/A). These isolates did not sporulate in culture media.

species (Figure 2A). Conidial size ranged from 22 to 55 µm long and 10 to 15 µm wide with three to six transverse and two oblique septa (Table 7). Isolates of *A. palandui* from Puerto Rico produce narrow apical beak conidia, dark brown when mature with wall and septa contrastingly darker (Figure 2B). Conidial size ranged from 25 to 75 µm long and 8 to 12 µm wide with three to eight transverse and two oblique septa. Simple chains of five to 12 conidia were observed in PCA (Table 7).

Alternaria tenuissima produced dark brown verrucose conidia ranging in size from 22 to 68 µm long and 10 to 16 µm wide with four to eight transverse septa and three oblique septa in PCA. *Alternaria tenuissima* also showed conidial wall and septa that were contrastingly darker. Conidial chains of six to 10 spores were observed in PCA (Table 7).

A large number of isolates (62%) from onion fields were included in a taxonomically undescribed group of small-spore *Alternaria* spp. having an *A. arborescens* intermediate sporulation pattern. Light brown ornamented (verrucose) conidia were short, ovoid to ellipsoid. Conidial size ranged from 35 to 68 µm long and 10 to 16 µm wide with four to eight transverse and three oblique septa. Conidial chains of four to 10 spores were observed as tufts in PCA (Table 7). Abundant sporulating hyphae were produced; these were long, and erect conidia production occurs aerially on the branching tips of the hyphae.

Other fungal species identified associated with onion foliar lesions were: *Aspergillus niger*, *Curvularia* sp., *Fusarium* sp., *Glomerella*

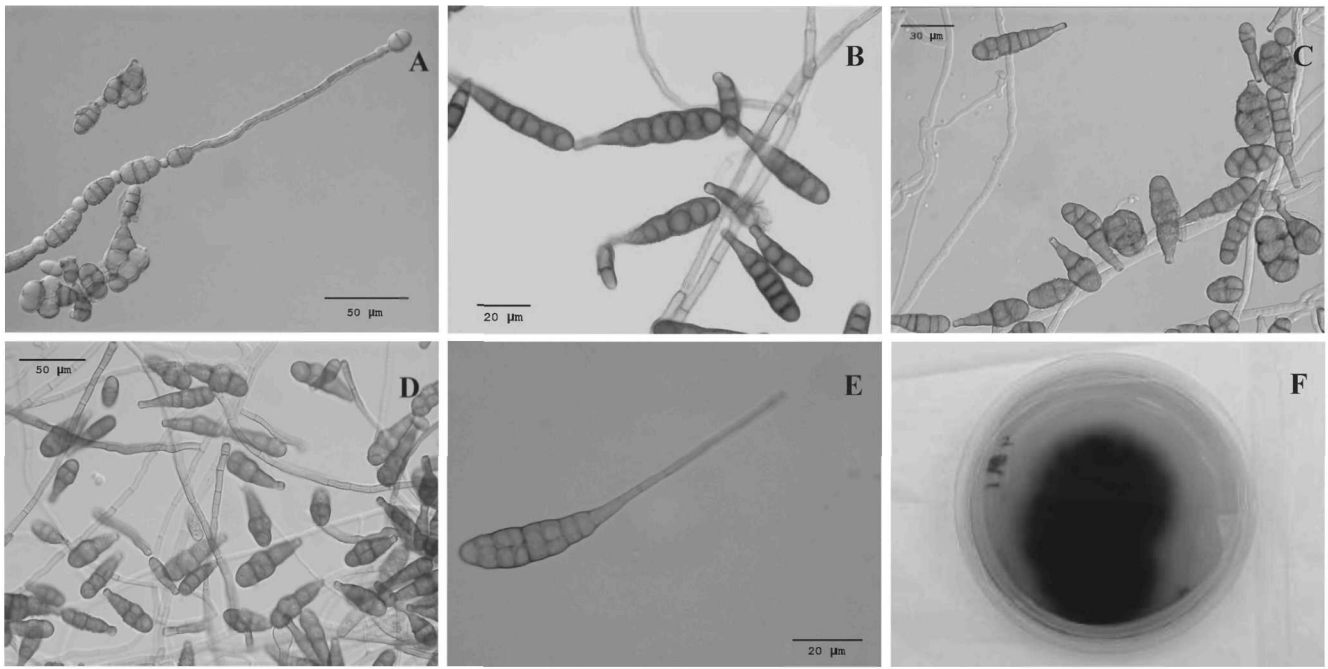

FIGURE 2. Conidia of A) *A. destruens*; B) *A. palandui*; C) *Alternaria* sp., an undescribed species with *A. arborescens* intermediate sporulation pattern; D) *A. tenuissima*; E) *A. allii*; and F) *A. allii* in culture media, observed ochreous pigment in PDA.

cingulata (teleomorph of *Colletotrichum gloeosporioides*), *Helminthosporium* sp., *Nigrospora* sp., *Penicillium* sp., *Pleospora eturmiuna* Simm. (teleomorph of *Stemphylium eturmiunum*) and *Verticillium* sp. (Fernández and Rivera, 2006).

Pathogenicity tests

Out of 19 *Alternaria* isolates only five were pathogenic to onion foliage of cultivars Mercedes, Excalibur and Candy under laboratory (in vitro) and field conditions (Figure 3A; Table 8). These were two isolates of *A. allii,* two isolates belonging to *A. tenuissima* and one isolate of *Alternaria* sp. Isolates Alt34 and Alt35, belonging to *A. allii*, were the most virulent, causing larger lesions in wounded and unwounded leaves in both laboratory (in vitro) and field conditions in all cultivars evaluated. Lesion size ranged from 1.3 to 5.0 cm long and 0.9 to 1.2 cm wide. Typical elliptical foliar lesions were purple or dark brown with a chlorotic halo (Figure 3A). In the field, lesions were often observed extending from the tip of the leaf causing a brownish blight.

Isolates Alt6 and Alt7 belonging to the *A. tenuissima* species group developed foliar lesions only in wounded tissues on all cultivars evaluated. Lesions were smaller at 3.0 to 3.6 cm long and 0.6 to 0.8 cm wide. *Alternaria tenuissima* species group isolates were less virulent than *A. allii*, producing small light brown to reddish lesions which cause collapse of the leaves.

Alternaria sp. with an *A. arborescens* intermediate sporulation pattern, evaluated in this study, caused irregular lesions in wounded tissues of the three cultivars evaluated in the field. Elliptical brown to cream color lesions, ranging from 4.2 to 6.2 cm long and 0.7 to 1.0 cm wide were observed. Tissue collapsed at the center of the lesions.

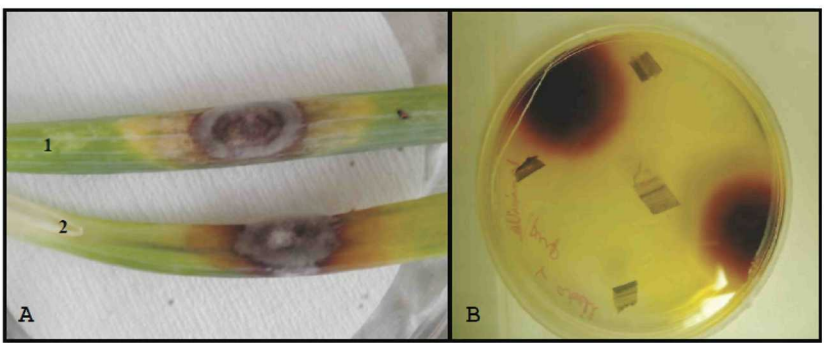

FIGURE 3. A) Purple foliar lesions developed during in vitro pathogenicity tests by isolate Alt34 (1) with wounding and (2) without wounding; B) Reisolation on culture media after pathogenicity tests to fulfill Koch postulates. Note ochreous pigment on culture media.

TABLE 8.—*In vitro and field pathogenicity tests conducted on three different onion cultivars, Mercedes, Excalibur, and Candy, with different* Alternaria *spp. isolates.*

		Mercedes				Excalibur				Candy			
		Field		*In vitro*		Field		*In vitro*		Field		*In vitro*	
Isolates	Species Group	W[1]	UW	W	UW	W	UW	W	UW	W	UW	W	UW
Alt6	*A. tenuissima*	+[2]	-	-	-	+	-	-	-	+	-	-	-
Alt7	*A. tenuissima*	+	-	-	-	+	-	-	-	+	-	-	-
Alt27	*Alternaria* sp.[3]	+	-	-	-	+	-	-	-	+	-	-	-
Alt34	*A. allii*	+	+	+	+	+	+	+	+	+	+	+	+
Alt35	*A. allii*	+	+	+	+	+	+	+	+	+	+	+	+

[1]Field pathogenicity tests using wounded (W) and unwounded= UW onion foliage tissue.
[2]Positive pathogenicity tests (+) = leaf blight was observed on onion tissues; Negative pathogenicity tests (-) = no symptoms were observed.
[3]*Alternaria* sp., a taxonomically undescribed group (Simmons, pers. comm.).

DNA analysis

Sequence size of the nuclear internal transcribed spacer (ITS) of ribosomal DNA (rDNA) gene of different *Alternaria* spp. ranged from 530 to 600 bp. Sequence analysis of the region showed 98 to 100% homology among *Alternaria* isolates occurring in onions when compared to that of other sequences in the GenBank (Tables 2 and 3). Phylogenetic relationships based on sequences of the ITS region from *Alternaria* isolates and other *Pleosporaceae* (*Ulocladium* sp., *Embellisima* sp., and *Stemphylium* sp.) from the GenBank distinguished three distinctive clades (Figure 4). The first clade of large filiform-beaked spores included *A.*

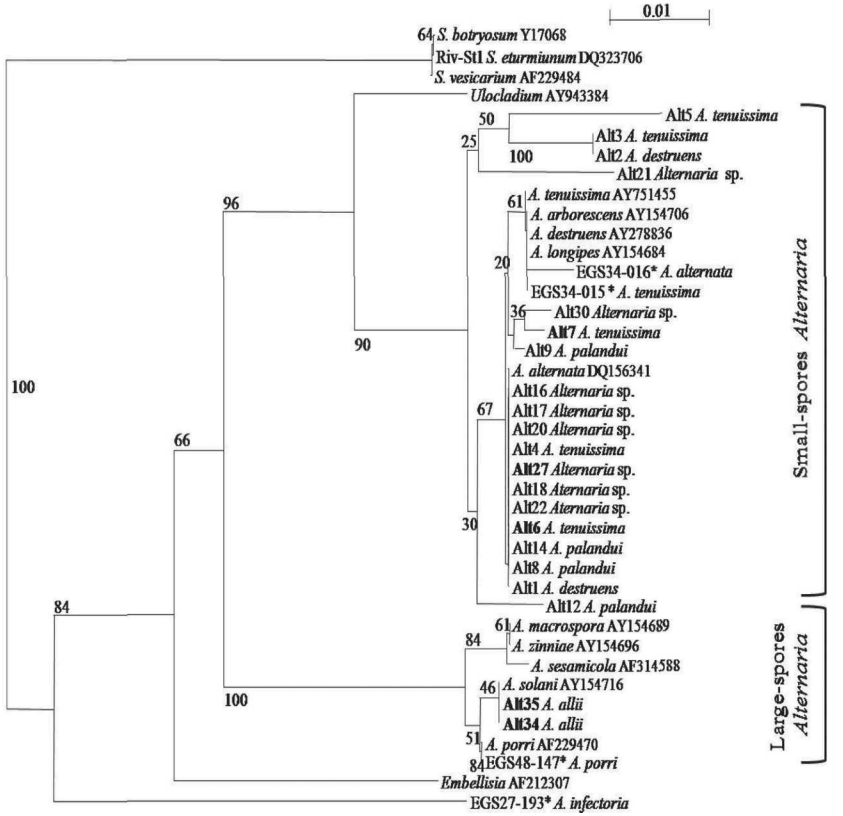

FIGURE 4. Phylogenetic tree obtained by Neighbor-Joining analysis of the nuclear ITS region of rDNA gene of *Alternaria* spp. and other Pleosporaceae. Kimura distance (parameter-2) was used with bootstraps of 1000 repetitions. * = Morphotypes isolates from Emory G. Simmons collection; pathogenic isolates in bold; *Alternaria* sp. = a taxonomically undescribed group of small-spore *Alternaria* that has an *A. arborescens*' intermediate sporulation pattern.

allii from this study, as well as isolates from the GenBank including *A. porri*, *A. solani*, *A. macrospora*, *A. zinniae* and *A. sesamicola*. These formed a monophyleic group, discrete from other members of the genus. The second clade included a diverse group of small-spore *Alternaria*, including *A. tenuissima*, *A. alternata*, *A. palandui*, *A. destruens*, and *Alternaria* spp. with an *A. arborescens* intermediate sporulation pattern; a third clade included *Stemphylium* spp. (Figure 4). Interestingly, Alt34 and Alt35, belonging to *A. allii*, were grouped along with *A. solani* and not with *A. porri* morphotype sequences obtained from the GenBank. Also the isolate of *A. infectoria* species-group segregated apart from the *A. arborescens*, *A. tenuissima*, and *A. alternata*. *Alternaria* sequences obtained during this study were deposited in the GenBank (Table 1).

DISCUSSION

Alternaria spp. incidence in onion increases as foliage matures, just before bulb development (Miller, 1983; Stavely and Slana, 1971). Similar findings were reported by Nolla in 1927 in onion fields located in the northern region of the island (Nolla, 1927). The highest RH occurred at maturity of the plant, a time which is the sensitive stage for *Alternaria* infection. Other authors reported that fungal infection has a higher incidence during periods of high relative humidity (>90%) for more than 11 h or after morning dew at temperatures ranging from 20 to 25 °C (Miller, 1975; Everts and Lacy, 1990).

Pathogenicity tests conducted in vitro and under field conditions using 19 *Alternaria* isolates showed that only five isolates belonging to three different *Alternaria* species groups were pathogenic to onions. These were *A. allii*, *A. tenuissima*, and *Alternaria* sp. (*A. arborescens* intermediate sporulation pattern). *Alternaria tenuissima* was reported pathogenic to onions in previous studies in Puerto Rico (Vélez-Rodríguez and Rivera-Vargas, 2007). *Alternaria allii*, developing lesions in unwounded tissues, was the most virulent species evaluated. Also, *A. allii* was consistently pathogenic in all trial conditions tested. Preliminary reports of these findings have been published elsewhere (Fernández et al., 2005; Fernández and Rivera, 2008).

Pathogenicity tests conducted with 14 isolates, belonging to *A. destruens*, *A. palandui*, *A. tenuissima*, and *Alternaria* sp. (*A. arborescens* intermediate sporulation pattern) species group, were negative. Small spore *Alternaria* spp., such as *Alternaria destruens* and *A. palandui*, have not been previously reported as associated with onions in the Caribbean or in the Western Hemisphere. *Alternaria destruens* was reported on a parasitic plant, *Cuscuta gronovii*, in Rochester,

Massachusetts (Simmons, 1998). *Cuscuta* sp. was commonly observed growing near onion plants in fields in Puerto Rico. Thus, this data could explain the presence of *A. destruens* in onion fields. *Alternaria palandui* was first reported affecting onion foliage of cultivar Bellary in India (Ayyangar, 1928). This group of non-pathogenic *Alternaria* isolated from typical lesions in the field, were acting as saprophytes or secondary invaders after *A. porri* (or *A. allii*) infected the tissue. Interestingly, *A. palandui* was reported pathogenic to onions in India on cultivar 'Bellary', but onion cultivars commonly grown in Puerto Rico and tested during these studies apparently were resistant to this species (Ayyangar, 1928).

In Puerto Rico, brown and purple foliar lesions were common in the field. Even though *A. porri* is commonly known as purple blotch, other species have been reported associated with similar symptoms in onions. Among them are *A. allii*, *A. alternata*, *A. iranica*, *A. palandui*, *A. tenuissima*, *A. tenuis* and *A. vanuatuensis* (Ayyangar, 1928; Nolla, 1927; Skiles, 1953; Simmons, 2007; Vélez-Rodríguez and Rivera-Vargas, 2007). *Alternaria* was also found associated with foliar damage caused by leaf miners (*Liriomyza* sp.) and herbicide treatments. Isolates belonging to *A. allii* were the most virulent, penetrating tissue with and without wounding in all cultivars evaluated. Other species examined such as *A. tenuissima* and *Alternaria* sp. were none or only weakly pathogenic. *Alternaria tenuissima* and *A. tenuis* have been found associated only with wounded onion tissues (Skiles, 1953). In Puerto Rico, further studies on the relationship between insects and *Alternaria* spp. incidence in the field are needed. In particular, studies relating onion thrips damage to *Alternaria* spp. severity in the field are important. Recent reports of *Frankliniella* thrips species affecting onions may exacerbate *Alternaria* spp. severity in the field by allowing new sites for fungal penetration, especially species such as *A. tenuissima* and *Alternaria* sp., which require open wounds to cause infection (Feliciano et al., 2008). In fact, *Thrips tabaci* damage to onion foliage and its relationship to *Alternaria* spp. severity is well documented (McKenzie et al., 1993). Effective insect control could diminish *Alternaria* infection in the field.

Analysis of the rDNA ITS region of *Alternaria* spp. showed it to be highly conserved and did not distinguish it among small-spore *Alternaria*, thus confirming previous findings with *Alternaria* population associated with late blight of pistachios and citrus (Roberts et al., 2000; Pryor and Michailides, 2002). This region was inappropriate for taxonomic resolution of these species; however, it has value in discriminating among the small-spore vs. large-spore *Alternaria*. Isolate of *A. infectoria* species-group segregated apart from the *A. arborescens*, *A.*

tenuissima, and *A. alternata*. Our results support previous findings with *A. infectoria* from pistachios (Pryor and Michailides, 2002). *Alternaria allii* were grouped with *A. solani* and not with *A. porri* morphotypes obtained from the GenBank. This piece of evidence in addition to the fact that these isolates produced an ochreous pigment on culture media, similar to what was described for *A. allii* by Nolla in 1927, make us believe that Alt34 and Alt35 are *A. allii* isolates. Future research should be focused on resolving *Alternaria* systematic by using other molecular techniques or biochemistry.

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