Research Note

LEAF BLIGHT OF ONION CAUSED BY PLEOSPORA ETURMIUNA *SIMM. (TELEOMORPH OF* STEMPHYLIUM ETURMIUNUM*) IN PUERTO RICO*[1,2]

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Various *Stemphylium* species are known as foliar pathogens of economically important crops. In Texas and Wisconsin in the United States, in India and Egypt, *S. vesicarium* (Wallr.) Simm. has been reported causing leaf blight of onions (Miller et al., 1978; Shishkoff and Lorbeer, 1989; Hassan et al., 2007). In Puerto Rico, *S. botryosum* Simm. was reported causing foliar lesions of onions (Toro, 1923). Recently, *S. herbarum* and *Stemphylium* sp. were reported causing ellipsoidal sunken lesions which eventually developed into brownish to purple lesions that extended through the tip of the leaf (Vélez-Rodríguez and Rivera-Vargas, 2007). *Stemphylium herbarum* Simm. and *Stemphylium* sp. isolates produced blight in the field very similar to that caused by *Alternaria* spp. *Stemphylium herbarum* is a foliar pathogen of alfalfa and had not been reported in onion until recently (Chaisrisook et al., 1995; Vélez-Rodríguez and Rivera-Vargas, 2007). The perfect stage of *Stemphylium* spp. is described under the Loculoascomycetes genus *Pleospora*. In 2001, *Pleospora eturmiuna* Simm., teleomorph of *S. eturmiunum*, was morphologically characterized from morphotype isolates from New Zealand affecting tomato fruits (Simmons, 2001). This species has not been previously reported in onions.

From November to January 2003 and January to April 2004, leaf blight was observed in onion foliage of cvs. Mercedes and Excalibur grown at the University of Puerto Rico-Agricultural Experiment Station in Juana Díaz and on commercial farms in Santa Isabel, Puerto Rico. Sixty days after sowing, symptoms were observed on young and mature leaves. Symptomatic leaves were collected and slides were prepared by mounting thin sections of host tissue in lactophenol with cotton blue; then tissues were examined under the microscope. Length and width measurements of conidia and of conidiophores were taken. Fungal isolates were grown on potato dextrose agar (PDA), V-8 agar and potato carrot agar (PCA) at ± 27° C under a 10 to 12 h cool-white fluorescent dark/light cycle for further characterization and identification. Semi-permanent slides were prepared to observe under a light microscope morphological features of fungal structures (i.e., mycelium, conidia, ascomata, asci and ascospores). In addition, differential interference contrast optics (Nomarski optics) was used to examine the specimens. Representative fungal isolates were identified at species level by expert taxonomist Dr. Emmory G. Simmons

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(Crawfordsville, IN, pers. comm.). A preliminary report has been published (Fernández and Rivera-Vargas, 2006).

Pathogenicity tests were conducted on foliage of 60-day-old plants of onion cultivars Mercedes (Seminis®)[5], Candy (Petoseed®) and Excalibur (Sunseeds®) under field conditions. Prior to inoculation, plant tissues were superficially disinfested with 0.5% sodium hypochlorite, 70% ethanol and sterile de-ionized-double-distilled water. Conidial suspension (10^5 conidia per milliliter) and mycelial disks (4 mm) from the edge of single spore colonies, grown on acidified PCA for a week, were used as inoculum. Conidial suspension was done by washing the surface of the colony with 50 ml sterile double-de-ionized-distilled water with three drops of Tween 20. For wounded and unwounded foliage inoculation, conidial suspension was sprayed or mycelial disks were placed at the center on the upper side of the onion leaf. For wounded treatments, incisions were made at the center of mature leaves by using a sterile dissecting needle prior to inoculation. Sterile de-ionized-double-distilled water and PCA disks were used as control. Pathogenicity tests were repeated twice.

For DNA extraction, *Stemphylium* mycelia was incubated for seven to 10 days at 27° C in 50 ml of potato dextrose broth (PDB, Difco) in a rotary shaker at 120 rpm. Mycelia was harvested by vacuum filtration through a Buchner funnel with sterile filter paper (Fisher Scientific, P8) and washed with two volumes of sterile de-ionized-double-distilled water. Total genomic DNA was extracted by using the Fast DNA® Kit (Q-BIO gene, USA) according to the manufacturer's instructions in Fast Prep™ Bio 101 equipment (Thermo Electron Coorporation Milford, MA). The concentration of DNA was estimated by absorbance at 260 nm.

The rDNA-ITS region of four *Stemphylium* isolates were amplified by Polymerase Chain Reaction (PCR) using primers ITS1 and ITS4 (White et al., 1990; Konstantinova et al., 2002). The PCR was performed in a total volume of 25 µl containing 2.5 µl 10× of TermoPol buffer (New England BioLabs.), 50 mM of each dNTP's (Roche®, USA), 20 pM of single primers, 1U/µl of Taq DNA polymerase (New England BioLabs, MA) and 50 ng/µl of template DNA. DNA was amplified by using a thermal cycler (GeneAmp, Perkin Elmer, 2400). The PCR consisted of initial denaturation of 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 µl of the PCR products were separated on 1.4% agarose gel (Fisher Scientific, NJ) prepared with 1X sodium bromide (Brody and Kern, 2004) and 4 µl of ethidium bromide (1 µg/1 µl, Sigma®, St. Louis, MO). The gel was visualized in a UV illuminator (Quantity One® 4.5 2003, BioRad Laboratory Inc., Japan).

The PCR 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 primers ITS1 and reverse primer ITS4, and compared with other sequences available on GeneBank database (http://www.ncbi.nlm.nih.gov) using the program ClustalX version 1.83. The similarity value between sequences was calculated by pair-wise comparisons.

On the basis of the morphological characters, the fungus causing leaf blight of onion was confirmed as *Pleospora eturmiuna* Simm., teleomorph of *Stemphylium eturmiunum*. The pathogen produced its sexual and asexual stages in PCA and V-8. Superficial or immersed ascomata (200 to 300 µm) developed abundantly in both culture media (Figure 1A). Simmons described the ascomata on PCA as having a small ostiolar beak and flexible hyphae that arise from its surface cells (Simmons, 2001). Dark round to ovoid asco-

[5]Trade names are mentioned to provide specific information and do not constitute a warranty of equipment or material by the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

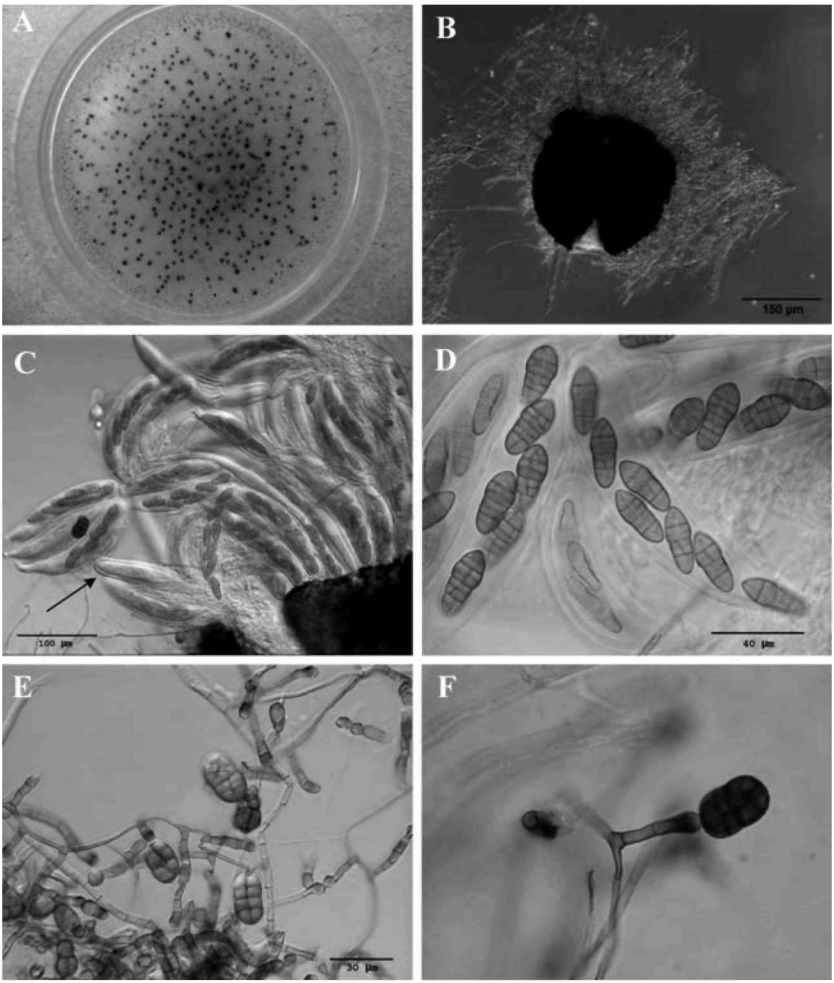

FIGURE 1. *Pleospora eturmiuna* (A) ascomata developed on PCA, (B) ascomata, (C) bitunicate asci (→), (D) brown melanized multicellular ascospores. Its anamorph *Stemphylium eturmiunum*: (E, F) conidia and conidiophores. (B, C, D, E Nomarski optics).

mata with long ovoid bitunicate asci (125 to 150 × 27 µm) containing eight ellipsoid melanized multicellular ascospores (25 to 30 × 10 to 12 µm) were observed under the microscope (Figures 1B to D). *Stemphylium eturmiunum*, anamorph of *P. eturmiuna*, has short geniculated conidiophores with one apical conidia (Figures 1E, F). Abundant brown ovoid to ellipsoid mature conidia (20 to 27 × 10 to15 µm) are produced in culture media. Conidia have one to five transverse and one to two longitudinal dark septa, and punctuated wall ornamentation (Figure 1F). Some transverse septa developed constrictions in

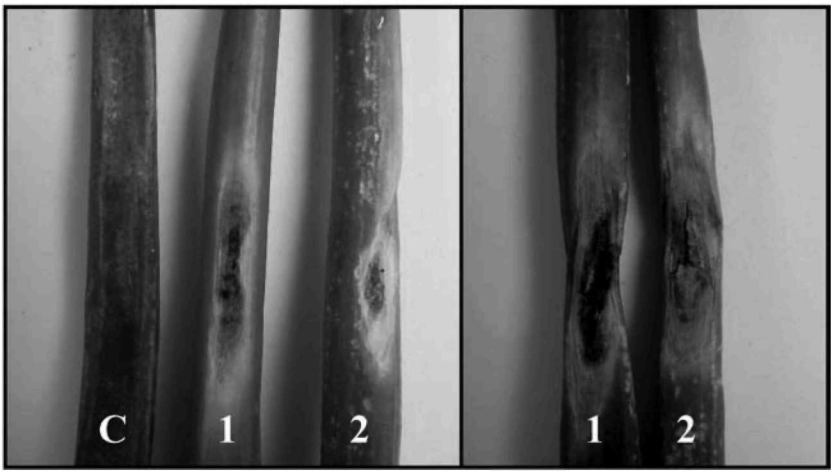

FIGURE 2. Leaf blight caused by *Pleospora eturmiuna* in onions. Control plant (C), inoculation with (1) and without wounding (2).

the spores. When the first transeptum is formed, the juvenile conidium is oblong and can measure 18 × 7 µm (Simmons, 2001).

Pleospora eturmiuna caused leaf blight of onions in plants inoculated with or without incisions, and lesions were similar to those caused by *Alternaria* spp. Ellipsoidal to ovoid leaf lesions, with distinctive chlorotic halos and brown to purple centers, developed in all onion cultivars evaluated under field conditions, resembling those previously observed in the field (Figure 2). Lesion size ranged from 4.5 to 6.5 cm long to 0.7 to 1.0 cm wide. *Pleospora eturmiuna*, teleomorph of *S. eturmiunum,* was re-isolated from diseased tissues.

Amplification of rDNA ITS region of four *P. eturmiuna* isolates produced bands that ranged from 530 to 600 bp. When we compared a 558-bp sequence of the rDNA ITS region of one *P. eturmiuna* isolate (Accession number DQ323706) with other sequences in the GeneBank, our isolate have 99% homology to *P. eturmiuna* (EGS29-099; Accession number AY329230) isolated from tomato fruit in New Zealand. In addition, our isolate has more than 90% of similarity with other species such as *Stemphylium alfalfae, S. astragali, S. botryosum, S. callistephi, S. subglobuliferum, P. gracilariae, P. herbarum, S. lancipes S. majusculum, S. nabarii, S. solani, P. tarda*, and *S. vesicarium*. Three species, *S. botryosum, P. herbarum* and *S. vesicarium*, have been reported pathogenic to onion crops (Suheri and Price, 2000; Vélez-Rodríguez and Rivera-Vargas, 2007).

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