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Fusarium branch blight on highbush blueberry in Argentina

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In Argentina, highbush blueberry (*Vaccinium corymbosum*) is grown in Tucumán, Entre Ríos and Buenos Aires provinces. In the 2010 to 2011 cropping season, a new disease with 10% incidence was observed on the foliage of "Emerald" plants in Concordia, Entre Rios. Symptoms included acropetal blight and premature branch death. Leaves remained attached and showed tan to brown discoloration. A fungal species was isolated from diseased plant parts and identified as belonging to *Fusarium* section Gibbosum. Based on its growth and pigmentation on potato-dextrose-agar, and characters on carnation leaf-piece agar and Spezieller Nährstoffarmer agar, the strain was identified as *Fusarium acuminatum.* Additionally, the internal transcribed spacer (ITS) regions of nuclear ribosomal genes were amplified by PCR, sequenced and the DNA sequence was compared with those in GenBank. The NCBI-Blastn search showed 100% identity of the DNA sequence with GenBank Accession No U85533 sequence for *F. acuminatum*. Pathogenicity was confirmed on wounded branches of potted blueberry plants. To our knowledge, this is the first report of *F. acuminatum* causing branch blight on highbush blueberry in Argentina and worldwide.

Key words: Fruit crops, berries, *Vaccinium corymbosum* L., fungi, DNA sequence.

INTRODUCTION

Blueberries are traditionally consumed in North America and European countries which cannot satisfy their demand from October to November (Bañados, 2009). Consequently, fruit production in the Southern Hemisphere constitutes a profitable export activity. In Argentina, highbush blueberry (*Vaccinium corymbosum* L., Ericaceae) crops are mainly located in the provinces of Tucumán, Entre Ríos and Buenos Aires. The district of Concordia, Entre Ríos, reaches 37% of the national planting area (Argentinean Blueberry Committee, 2012). The exportation of fresh and frozen blueberries in 2012 reached 19,993 t with peaks in October and November (Ministerio de Agricultura, Ganadería y Pesca, 2014). Since 1995, researchers of the University of Buenos

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Aires conduct crop health surveys in that area leading to the characterization of several plant pathogens. The list of fungi already identified includes *Pestalotiopsis guepinii* (Desm.)

Steyaert (Wright et al., 1998), *Glomerella cingulata* (Stoneman) Spauld. and H. Schrenk (Wright et al., 1998; Pérez et al., 2012), *Alternaria tenuissima* (Kunze) Wiltshire (Wright et al., 2004), *Botrytis cinerea* Pers. (Vasquez et al., 2007), *Nigrospora sphaerica* (Sacc.) E.W. Mason (syn=*Khuskia oryzae* H.J. Huds.) (Wright et al., 2008), *Bipolaris cynodontis* (Marignoni) Shoemaker (syn=*Cochliobolus cynodontis* R.R. Nelson) (Sisterna et al., 2009), *Sclerotinia sclerotiorum* (Lib.) de Bary (Pérez et al., 2011b), *Fusarium solani* (Mart.) Sacc. (Pérez et al., 2007) and *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach and Nirenberg (Pérez et al., 2011a). *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips was the only pathogen reported to cause acropetal blight (Wright et al., 2012). Recently, unusual aerial symptoms appeared not associated to the above mentioned microorganisms. The objective of this study was to identify the etiology of this disease.

MATERIALS AND METHODS

Collection of samples

Plant disease surveys were conducted in Concordia, located at 31°23′32″S 58°1′1″W, 21 m.a.s.l., with annual rainfall of 1300 mm. Disease incidence was estimated as the proportion of diseased plants. Samples of blighted “Emerald” blueberry branches were collected and taken to the laboratory for further analysis. Small symptomatic branch fragments were surface-disinfected by immersion in ethanol:water (7:3 vol.) for 1 min, 2% (vol.) of Cl as NaOCl for 1 min, rinsed in sterile distilled water, blotted dry, plated on culture medium, and incubated at 22˚C for seven days under fluorescent light with a 12 h photoperiod.

Growth of fungal isolate

The mycelium was sub-cultured by successive transfers of hyphal tips from colony margins onto 2% water agar (WA). The isolate was first identified by biometric and cultural methods, and then subjected to polymerase chain reaction (PCR) assay. The isolate was grown on potato-dextrose-agar (PDA), carnation leaf-piece agar (CLA), and Spezieller Nährstoffarmer agar (SNA) to assess cultural characters by eye and microscopic examinations. On PDA, colony morphology, pigmentation of aerial mycelium and culture media, and mycelium growth at 25 and 35°C were observed. On CLA and SNA, color of sporodochia, shape and size of macroconidia, presence and type of conidiogenous cells (mono or polyphialides), presence/absence of microconidia, chlamydospores and perithecia were recorded (Leslie and Summerell, 2006).

Fungal DNA isolation, amplification and sequencing

Disks of mycelia were excised from a 5-day-old-PDA plate and ground in liquid nitrogen. DNA was extracted using DNeasy Plant Mini kit, quantified and checked by agarose gel electrophoresis. To amplify the internal transcribed spacer (ITS) regions of nuclear ribosomal genes, PCR reaction was carried out in a 20 µl reaction mix containing 25 ηg fungal DNA, 100 µm each of dNTP´s (dATP, dCTP, dGTP, dTTP), 25 µmoles of ITS1 forward (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 reverse (5´-TCCTCCGCTTATTGATATGC-3´) oligonucleotide primers (White et al., 1990) and 1 unit of *Taq* DNA polymerase, in 1X PCR reaction buffer. PCR amplifications were carried out in a thermocycler programmed for initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 30 s, and a 3 min final extension at 72°C. PCR products were checked by electrophoresis on 1.5% agarose gel in TBE buffer. Amplification products of the ITS region were purified with QIAquik PCR purification kit, and sequenced with amplification primers by the dideoxy chain-termination method at INTA Genomic Service Unit (http://www.inta.gob.ar). The sequence was edited manually, and compared with GenBank (http://www.ncbi.nlm.nih.gov/genbank/) database. Similar nucleotide sequences were aligned using Crustal Omega program (Thompson et al., 1994).

Pathogenicity tests

Koch’s postulates were performed on potted two-year-old “Blue Crisp” blueberry grown in INTA-Concordia Experimental Station, as there were no “Emerald” plants available. Market blueberry fruits and plants of different plant species such as carrot *(Daucus carota* var. *sativus* Hoffm.*)*, garlic *(Allium sativum* L.*)* and onion *(Allium cepa* L.) were included in the inoculations. The purified *Fusarium* isolate was increased on culture media at 22°C during seven days. Inoculation methods included: i) conidia suspension prepared by adding sterilized distilled water to PDA slants and shaking for 1 min in a rotary shaker, filtered through sterilized surgical gauze, and adjusted to $3x10^6$ conidia/mL. The suspension was sprayed onto 10 plants with needle-injured branches and foliage. Controls were equally treated but sprayed with sterilized distilled water. The plants were individually enclosed in polyethylene bags for three days and maintained at 22°C in a climatic chamber; ii) plugs of WA with fungal development were placed on needle-wounded branches of 10 plants and attached with pieces of wet cotton. On control plants, WA pieces without the fungus were placed on the wounded branches. The inoculated and control plants were incubated under greenhouse conditions; iii) WA plugs of 4 mm diameter with fungal development were placed on market blueberry fruits which were previously disinfected by immersion in 70% ethanol and 2% bleach, rinsed with distilled water, blotted dry by a laminar flow cabinet, and placed in plastic containers (10 blueberry fruits/container, 4 replicates) in humid chambers or on WA plates (3 fruits/plate, 4 replicates) kept under laboratory conditions. WA plugs without the fungus were used on control fruits, iv) WA plugs of 4 mm diameter with fungal development were placed on surface disinfected carrot taproots and bulbs of garlic and onion, and kept separately in plastic containers under humid conditions in the laboratory. WA plugs without the fungus were placed on control taproots and bulbs. In all cases, inoculated and control plants, fruits, roots and bulbs were monitored regularly for the presence of disease symptoms. The fungus was reisolated from symptomatic inoculated organs after surface disinfection as described.

RESULTS

In October 2010, a new disease was recorded on “Emerald” blueberry plants, with an incidence of 10%. Acropetal blight developed from the base toward the apex of the branches and led to the death of leaves,

Figure 1. Acropetal stem blight on an “Emerald” blueberry plant.

branches and plants. Leaves became tan to brown and remained attached to the branches, which also became tan to brown (Figure 1). A *Fusarium* isolate was obtained from diseased branches. Macroconidia were large, ventrally and dorsally curved with a pronounced foot-shaped basal cell. The isolate was identified as belonging to section “Gibbosum”. On PDA plates, fungal growth was profuse; the aerial mycelium was initially white, developing a rose to burgundy red pigmentation in the center of the colony, and brown to burgundy red pigments in the agar. After two days at 25 and 35°C, colony growth was 15 and 10 mm, respectively. The isolate grew faster at 25°C covering the 9 cm culture plates after 7 days while it required nine days to cover the plate at 35°C (Figure 2). According to this data, it was considered a fast-growing strain at 25°C. On CLA culture medium, macroconidia formed in orange sporodochia were slender, broadly falcate, thick walled, with 3 to 5 septa (mostly 3), a curved elongation in the apical cell and a distinct foot shape, and averaged 48.4 µm (45 to 52 µm) x 3.7 µm (3 to 4 µm). Microconidia were rare, 0 to 1 septate, reniform, born in monophialides. Aseptate conidia were rare, 7.4 µm (6 to 9 µm) x 2 µm while more frequent 1-septate microconidia measured 14.6 µm (13 to 16 µm) x 2 µm. After 5 days on CLA and SNA, round chlamydospores formed abundantly, mainly in chains (2 to 9) averaging 13.2 µm (10 to 15 µm). No perithecia were observed. Based on morphological and cultural characters, the isolate was identified as *Fusarium acuminatum* Ellis and Everh. (teleomorph: *Gibberella acuminata* Wollenw.) (Nelson et al., 1983; Crous et al., 2004; Leslie and Summerell, 2006). The isolate was deposited in the IMYZA Microbial Collection as INTA-IMC582. The 507 bp DNA sequence was deposited in GenBank with the accession number KF250347. The Blastn analysis of the ITS region of nuclear ribosomal DNA showed 100% identity with the DNA sequence for *F. acuminatum* (teleomorph *Gibberella acuminata*), accession No U85533 (O'Donnell, 1997). This sequence was cited as reference for *F. acuminatum* by Leslie and Summerell (2006). Figure 3 shows the results of agarose gel electrophoresis of the strain.

In the pathogenicity tests, symptoms on plants sprayed with conidia suspensions (i) appeared 15 days after the treatment. Stem blight developed acropetally from the wounds. The response varied greatly among and within plants. At day 21, 20% of them had died, and the others showed branch blight that ranged between 1 cm long and branch death (Figure 4a). In 30 days, 70% of the plants had died. Six days after inoculation with WA plugs (ii), the plants showed 0.6 x 0.4 cm dark brown necrotic lesions on the branches. Symptoms were not observed on control plants. After 15 days, necrotic lesions longer than 1 cm developed acropetally on branches (Figure 4b). Plant death was observed at 21 days. Control plants did not show disease symptoms. The fungus developed on the surface of inoculated blueberry fruits (iii) after 14 days (Figure 4c). After 9 days, inoculated carrots (iv) showed dark red discoloration with rotten areas of 2.5 cm diameter covered with profuse mycelia (Figure 4d). No symptoms were recorded on the controls, and inoculated garlic and onion bulbs. *F. acuminatum* was reisolated from inoculated organs that developed disease symptoms.

DISCUSSION

The presence of a new species of *Fusarium* is reported on blueberry in this paper. There was a coincidence of the morphological traits and growth at 25 and 30°C of our strain with the descriptions for *F. acuminatum* (Crous et al., 2004; Leslie and Summerell, 2006; Nelson et al., 1983). Besides, the GenBank DNA sequence comparison of ITS region of ribosomal genes indicated complete identity with the sequence U85533 for *F. acuminatum* (O'Donnell, 1997), which was cited as a reference for this species by Leslie and Summerell (2006). Williams et al. (2002) developed a species-specific primer set to identify *F. acuminatum* such as FAC-F (5´-GGGATATCGGGCCTCA-3´) and FAC-R (5´-GGGATATCGGCAAGATCG-3´). However, Kikot et al. (2011) reported that this primer set was non-specific and showed cross-reaction with *F. graminearum*. Harrow et al. (2010) differenciated *F. acuminatum*, *Fusarium*

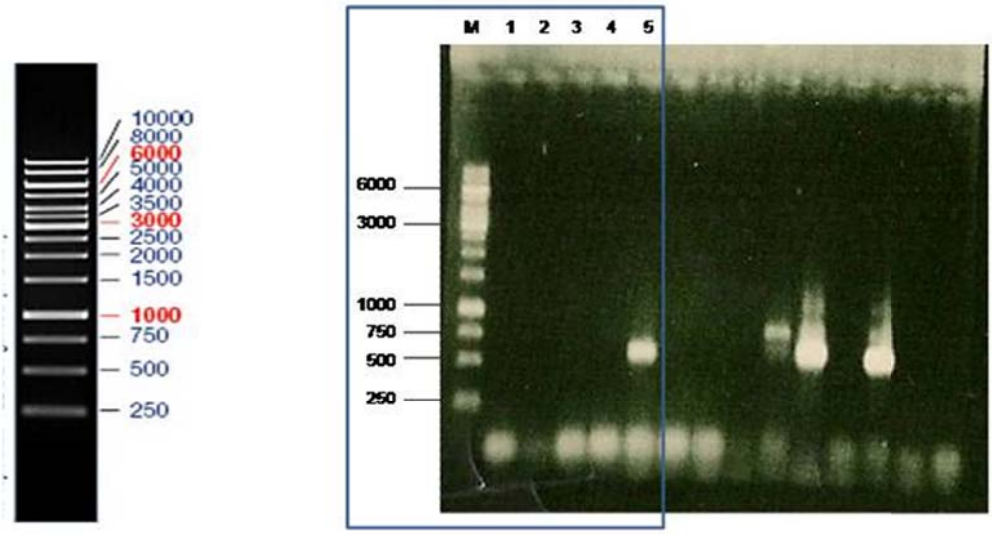

Figure 3. Agarose gel electrophoresis of F. acuminatum ribosomal ITS region amplified by PCR with primers ITS1 and ITS4 (White et al., 1990). M: molecular weight marker GeneRuler 1 kb DNA Ladder (thermo scientific).

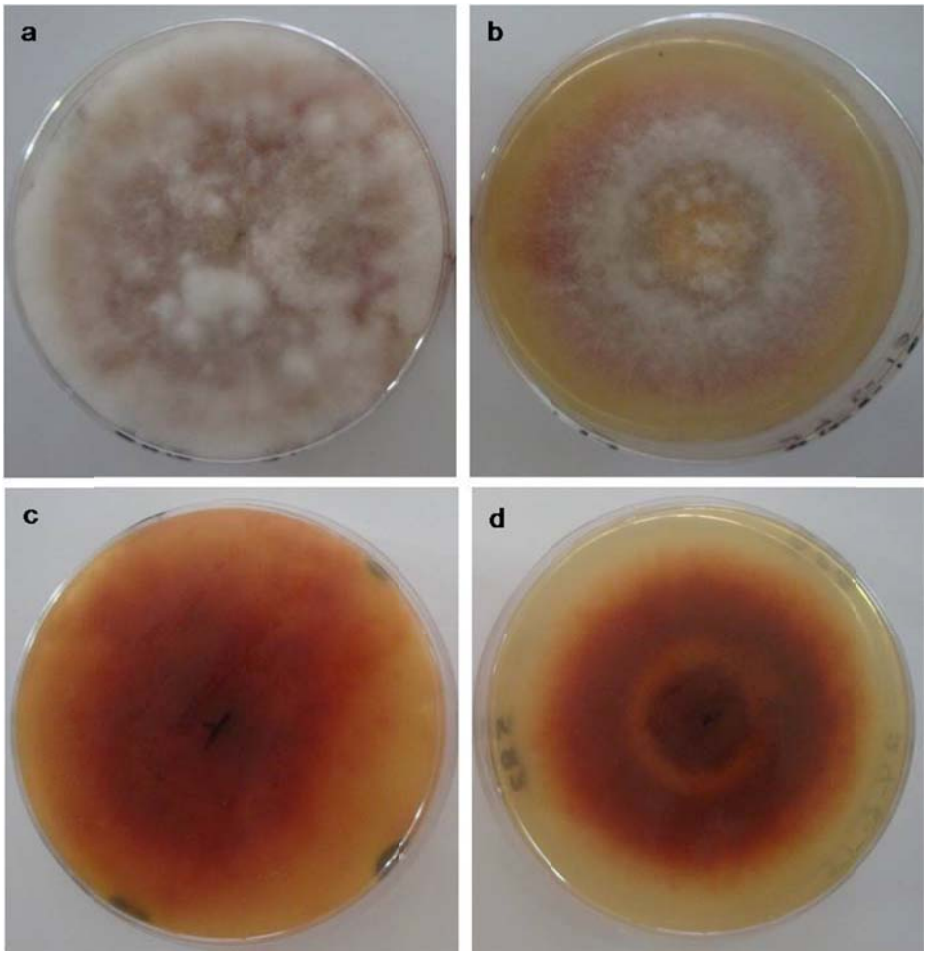

Figure 2. Growth of *Fusarium acuminatum* after 7 days on PDA at different temperatures. **(a)** Colony at 25˚C. **(b)** Colony at 35°C. **(c)** Reverse at 25˚C. **d)** Reverse at 35˚C.

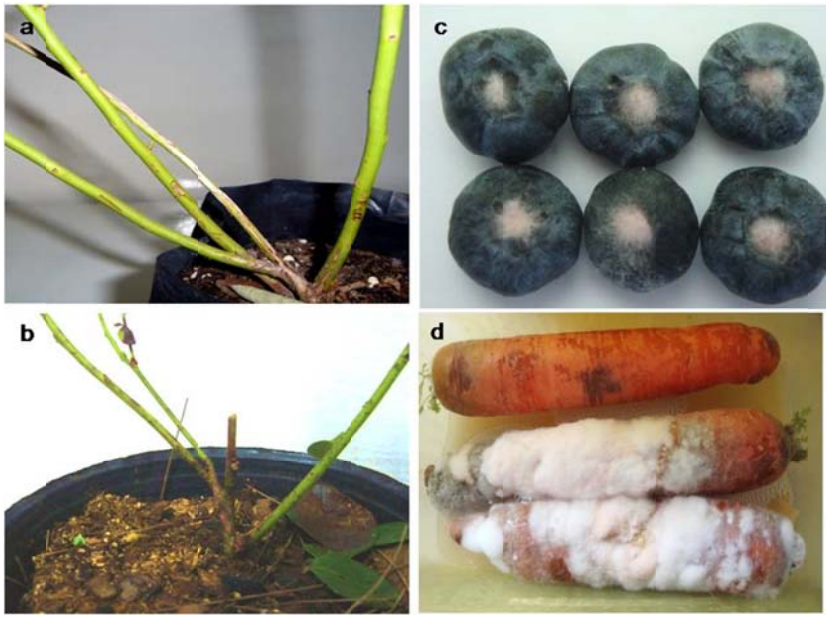

Figure 4. Results of target inoculations with *F. acuminatum.* **(a, b)** Necrotic spots and blight on blueberry branches. **(c)** *F. acuminatum* growth on blueberry fruits. **(d)** Rot and pathogen development on carrots.

avenaceum and *Fusarium tricinctum* isolates using βTUB and EF1α. The identification of *Fusarium* species is important to predict the potential mycotoxigenic risk of a strain (Sampietro et al., 2010, Wing et al., 1993; Altomare et al., 1997; Visconti et al., 1989). As some *F. acuminatum* isolates may be toxin-producers (Logrieco et al., 1992; Marín et al., 2012), it deems necessary to continue research on this subject. We observed that the isolate of *F. acuminatum* was able to colonize blueberry fruits and carrot taproots. However, fruit infection was not observed either in field conditions or after harvest.

The fungus *F. acuminatum* has been cited in temperate regions usually as a soil saprophyte or associated with root and crown diseases on numerous plants (Leslie and Summerell, 2006). A total of 235 species has been included in its host list by Farr and Rossman (2014). Additionally, 83 and 11 records were added for the synonym *Fusarium scirpi* var. *acuminatum* and the teleomorph stage *Gibberella acuminata*, respectively. This pathogen has been isolated from roots, stems, leaves, flowers, seeds and fruits (Esquivel, 1991; Elmer, 1996; Farr and Rossman, 2014). Although it has been mainly associated to root and collar rot (Rai and Singh, 1981; Mathew et al., 2010; Lazreg et al., 2013; Borrego-Benjumea et al., 2014), there are several reports on aerial organs of plants such as banana (Esquivel, 1991), durum wheat (Fakhfakh et al., 2011), onion (Parkunan and Jin, 2013), pumpkin (Elmer, 1996), and pigeonpea (Sharma et al., 2014). In spite of the wide host range of *F. acuminatum*, highbush blueberry (*V. corymbosum*) has not been previously cited as a host. Consequently, this is the first report of *F. acuminatum* as pathogen of *V. corymbosum* in Concordia, Argentina, and to our knowledge, the first citation worldwide. *Amaranthus caudatus* var. *mantegazzianus* (Pass.) Hanelt, *Aspidosderma quebracho-blanco* Schltdl., *Cucurbita ficifolia* Bouché, *C. maxima* Duchesne ex. Lam. subsp. *maxima*, *Glicine max* (L.) Merrill, *Hordeum distichon* L., *Olea europea* L., *Pinus elliottii* Engelm., *Pinus ponderosa* Dougl ex Lawson and P. Lawson, and *Pinus taeda* L. are the species recorded as hosts of *F. acuminatum* in Argentina (Nome Huespe et al., 2014). As blueberry is grown in this country near vegetable, cereal and oil crop fields which are susceptible to *F. acuminatum* as cited in foreign literature, this new host may be important as inoculum source. In addition, even though no natural infections have been reported in Argentina, the isolate was able to infect carrots, causing rot.

F. acuminatum has been reported as one of the *Fusarium* species with ice-nucleating activity (INA) which can be important for the frost damage to the host before infection (Pouleur et al., 1992). This INA characteristic may have intrinsic host origin, or be caused by extrinsic ice (frost, snow, frozen soil or parts of plants or INA microorganisms (Pouleur et al., 1992; Richard et al., 1996; Humphreys et al., 2001; Lundheim, 2002). The INA of intrinsic origin has been studied *in vitro* in blueberry cultivars (Kishimoto et al., 2014). Also, INA has been associated with bacteria, lichens, free-living fungi, among others (Pouleur et al., 1992; Richard et al., 1996; Lundheim, 2002). The species *Fusarium acuminatum*, *F. avenaceum*, *F. moniliforme*, F. *oxysporum*, and *F. tricinctum* have been reported as ice nucleator fungi (Pouleur et al., 1992; Richard et al., 1996; Humphreys et

al., 2001; Seifi et al., 2014). Also, it has been suggested that beside morphological traits, INA characteristic may add useful data to identify *Fusarium* species (Richard et al., 1996). The INA ability of some strains of *F. acuminatum* may be of significance as frost damage of plants may facilitate pathogen infections and disease development under cold weather conditions. The studied disease was detected during the spring. Winter surveys and additional research will be needed in case disease symptoms are observed after cold weather, associated or not to INA activity of the pathogen.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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