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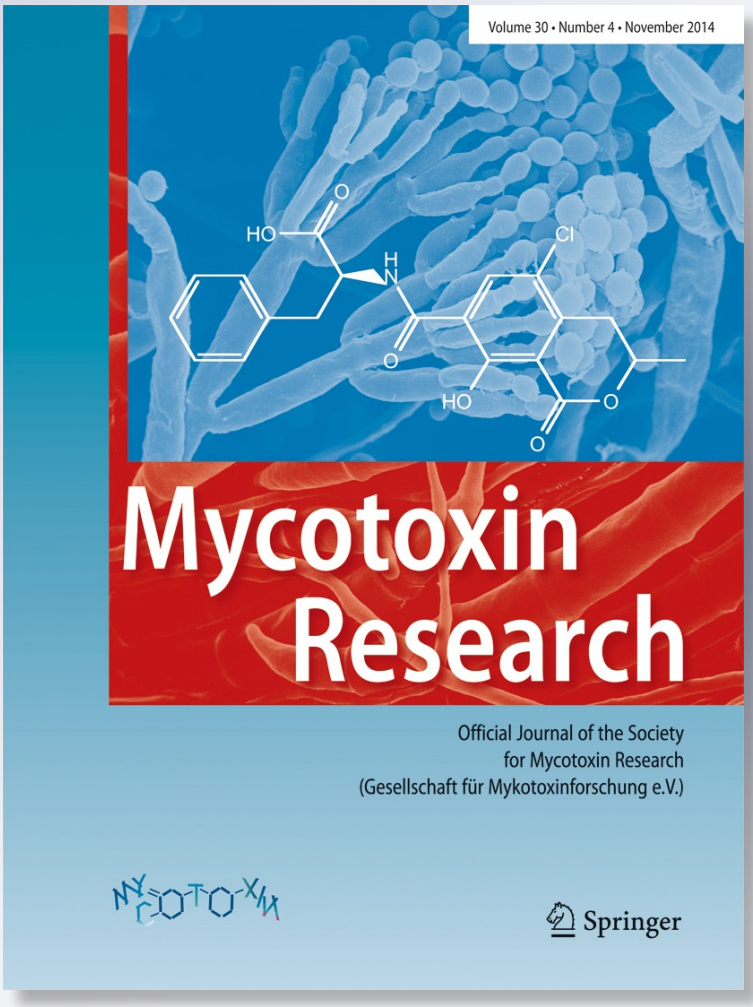

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ORIGINAL PAPER

Mycotoxigenic potential of fungi isolated from freshly harvested Argentinean blueberries

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Abstract *Alternaria alternata*, *A. tenuissima*, *Fusarium graminearum*, *F. semitectum*, *F. verticillioides*, *Aspergillus flavus*, and *Aspergillus* section *Nigri* strains obtained from blueberries during the 2009 and 2010 harvest season from Entre Ríos, Argentina were analyzed to determine their mycotoxigenic potential. Taxonomy status at the specific level was determined both on morphological and molecular grounds. Alternariol (AOH), alternariol monomethyl ether (AME), aflatoxins (AFs), zearalenone (ZEA), fumonisins (FBs), and ochratoxin A (OTA) were analyzed by HPLC and the trichotecenes deoxynivalenol (DON), nivalenol (NIV), HT-2 toxin (HT-2), T-2 toxin (T-2), fusarenone X (FUS-X), 3-acetyl-deoxynivalenol (3-AcDON), and 15-acetyl-deoxynivalenol (15-AcDON) by GC. Twenty-five out of forty two strains were able to produce some of the mycotoxins analyzed. Fifteen strains of *Aspergillus* section *Nigri* were capable of producing Fumonisin B_1 (FB_1); two of them also produced Fumonisin B_2 (FB_2) and one Fumonisin B_3 (FB_3). One of the *F. graminearum* isolated produced ZEA, HT-2, and T-2 and the other one was capable of producing ZEA and DON. Two *A. alternata* isolates produced AOH and AME. Four *A. tenuissima* were capable of producing AOH and three of them produced AME as well. One *Aspergillu flavus* strain produced aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), and aflatoxin G_1 (AFG_1). To our knowledge, this is the first report showing mycotoxigenic capacity of fungal species isolated from blueberries that include other fungi than *Alternaria* spp.

Keywords Blueberries · Mycotoxins · *Alternaria spp* · *Aspergillus spp* · *Fusarium spp*

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Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is a small fruit that belongs to the Ericaceae family. Blueberries are widely consumed as fresh and dried fruits, jams, sauces, and juices in diets. In recent years, considerable attention has been paid on the health benefits of this fruit, which include antioxidant, anticancer, anti-neurodegenerative, and anti-inflammatory activities (Takikawa et al. 2010).

Blueberries are consumed mostly in North America, some European countries and Japan. USA is the main consumer, producer, and trader of blueberries in the world (Dansa 2008). In Argentina, its production has increased considerably during the last decade, reaching 11,500 tn in 2009 (Santillán 2009).

The production areas are located in Entre Ríos, Tucumán, and Buenos Aires Provinces. Entre Ríos accounts for more than 50 % of the blueberry local production, with over 50 % of the fields located in the Concordia County (Bruzone 2007, Munitz et al. 2013). Approximately 90 % of Argentinean production is exported as fresh fruit, mainly to the USA and Canada, during the northern hemisphere winter season.

Blueberries, like most fruits, are prone to fungal spoilage. Contamination by different molds can occur during pre-harvesting, harvesting, and blueberry processing. The most significant fungal pathogens associated with blueberry are *Alternaria tenuissima*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, *Phomopsis vaccinii*, and *Monilinia vaccinii-corymbosi* (Gabler et al. 2004, Rivera et al. 2009, Tarnowski et al. 2008, Tournas and Katsoudas 2005, Wharton and Schilder 2005, Wright et al. 2004, 2008a, 2008c). In Argentina, additional fungal diseases have been reported in blueberries, produced by *Rhizoctonia solani*, *Fusarium solani*, *Pestalotiopsis guepini*, *Pucciniastrum vaccinii*, *Dothichiza caroliniana*, *Nigrospora sacchari*, *Nigrospora sphaerica*, *Rhizopus stolonifer*, *Botryosphaeria* spp., *Fusicoccum* spp., *Dothiorella* spp., *Phomopsis* spp., *Cylindrocladium* spp., *Curvularia* spp., *Phoma* spp., *Phytophthora* spp., *Aspergillus* spp., and *Penicillium* spp. (Pérez et al. 2007, Rivera et al. 2009, Wright et al. 1998, 2008b). Nevertheless, there are few reports on postharvest diseases of blueberries in our country (Munitz et al. 2013, Rivera et al. 2009, Wright et al. 2008c).

It is important to identify fungal contaminants in fresh fruits because some molds can grow and produce mycotoxins on this commodity (Tournas and Katsoudas 2005). Mycotoxins are secondary metabolites produced by fungi that are found in food and fodder. Greco et al. (2012) reported mycotoxins in Argentinean blueberries which were produced by *Alternaria* spp. Little is known about the accumulation of other mycotoxins in Argentinean blueberries beyond the cited study. None of the named mycotoxins have regulations in blueberries in Argentina. Toxicological information for them has been described by JECFA (2014).

There is a wide variety of molds that grow on blueberries, and among them some are potentially mycotoxin producers, such as *Alternaria alternata*, *A. tenuissima*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium graminearum*, *Fusarium semitectum*, and *Fusarium verticillioides* (Cappellini et al. 1972, Cappellini and Ceponis, 1977, Cline and Milholland 1995, Smith et al. 1996, Cline 1997, Luan et al. 2007, Rivera et al. 2009, Wright et al. 2004, 2008a, 2008c, Wright 2009, Munitz et al. 2013). Considering the fungal pathogens previously detected on this crop species, it is not unlikely that other mycotoxins of great significance are being produced by other groups, including *Aspergillus* and *Fusarium* species.

It is the aim of the present paper to assess the mycotoxigenic potential of a wider range of fungal species isolated from blueberry samples, based on previous work by Munitz et al. (2013).

Materials and methods

Specific assignment of mycotoxicogenic isolates

Potentially mycotoxicogenic strains were morphologically characterized for taxon-specific diagnostic characters (Ellis 1971, Klich 2002, Nelson et al. 1983, Pitt and Hocking 2009, Samson et al. 2004, and Simmons 2007), identified, and grouped according to its putative species. Representative samples from a specific group were further subjected to PCR analysis in order to validate the morphological identification. For each representative isolate, a fragment of the rDNA ITS1–5.8S–ITS2 region was amplified, using the universal primers ITS5/ITS4 (White et al. 1990). Each PCR reaction contained 10 ng of template DNA, 0.4 μM of each primer, 200 μM dNTPs, 1.5 mM MgCl2, 1.0 U Taq DNA polymerase, and 1× IB Taq polymerase buffer (Taq DNA polymerase Invitrogen™). Temperature cycling was conducted with the following program: denaturation at 95 °C for 4 min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, plus a final elongation period at 72 °C for 5 min. PCR products were purified using ExoSAP-IT® (USB, Cleveland, OH, USA) and forward and reverse- sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Products were run on an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were quality-edited and mounted into contigs using the program Genetool v. 1.0. The correspondence between morphology-based and sequence-based identification was verified on the basis of sequence identity and *E* value (probability of the alignment occurring by chance) of local isolates with sequence information from specimens for the respective putative species. Sequence alignment analysis was carried out against the curated, non-redundant RefSeq database (NCBI) using the program BLASTn (Altschul et al. 1997).

Strains, culture conditions, and mycotoxin induction

Two isolates of *F. semitectum*, one of *F. verticillioides*, and two of *F. graminearum* were transferred to carnation leaf agar slants and incubated under 12 h fluorescent light and 12 h darkness for 7 days at 25 °C to stimulate conidial formation (Megalla et al. 1987). Eight isolates of *A. flavus* and nineteen of *A.* section *Nigri* were grown on potato dextrose agar slants and incubated at 28 °C for 7 days. Four isolates of *A. alternata* and six of *A. tenuissima* were grown on potato dextrose agar slants and incubated at 25 °C for 7 days. The suspensions of all fungal strains were prepared by adding (5.0 ml) sterile

distilled water to each slant and gently scraping the agar surface with a wire loop to give a turbid suspension (1×10^5 conidia/ml).

In order to induce the production of deoxinivalenol (DON), nivalenol (NIV), fusarenone X (FUS-X), 3-acetyl-deoxynivalenol (3-AcDON), 15-acetyl-deoxynivalenol (15-AcDON), T-2 toxin (T-2), HT-2 toxin (HT-2), and zearalenone (ZEA), rice media was inoculated with 50 μl conidial suspension of *F. semitectum* and *F. graminearum*, respectively, and were incubated at 25 °C for 3 weeks (Ferreira Geraldo et al. 2006, He et al. 2007, Sampietro et al. 2013). For testing aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2), rice media was inoculated with 50 μl of the conidial suspension of *A. flavus* and incubated for 1 week, at 25 °C (Resnik et al. 1996, Moltó et al. 1997). Rice medium was prepared with distilled water added with up to 40 % of polished rice in 250 ml Erlenmeyers, and autoclaved at 121 °C for 30 min. Autoclaved flasks were shaken by hand. For ochratoxin A (OTA) production, 5 ml of the conidial suspension of *A.* section *Nigri* were inoculated in 25 ml of YES media following the methodology described by Magnoli et al. (2003); flasks were incubated stationary at 25 °C for 28 days in the dark. To test for the production of Fumonisin B_1 (FB_1), Fumonisin B_2 (FB_2), and Fumonisin B_3 (FB_3), 500 μl of the conidial suspension of *A.* section *Nigri* and *F. verticillioides* were inoculated in the rice media and incubated at 25 °C for 3 weeks (Hinojo et al. 2006). To test for the production of alternariol (AOH) and alternariol monomethyl ether (AME), 500 μl of the conidial suspension of *Alternaria* spp. were inoculated in the rice media and incubated at 25 °C for 2 weeks (Torres et al., 1998).

At the end of the incubation period, the toxins concentrations were determined by high performance liquid chromatography (HPLC) or gas chromatography (GC).

Mycotoxins analysis

Every procedure for mycotoxin analyses, except for AME and AOH, follows standardized regulations and has been ISO 17025 certified. Acetonitrile (ACN), toluene, and methanol (MeOH) were purchased from Sintorgan (Argentina); hexane and sodium bicarbonate ($NaHCO_3$) from J.T Baker (Mexico); trifluoroacetic anhydride (TFA) from Tedia Company (USA); acetic acid (HAc), formic acid, sodium hydroxide (NaOH), and ethyl acetate from Merck Química Argentina (Argentina); *4*-(N,N-Dimethylamine) pyridine (DMAP) and heptafluorobutyric acid anhydride (HFBA) from Sigma-Aldrich (Switzerland) and orthophosphoric acid from Merck (Germany); deepoxy-deoxynivalenol (E-DON) from Biopure, 1.0 μg/ml (Tulin, Austria); and 2-amino-5-chlorobenzophenone (ACBP) from Sigma (Germany). The phosphate buffer saline (PBS) was prepared with a mixture of 0.26 g of monoacid sodium phosphate, 1.14 g of diacid sodium phosphate, 7.02 g of sodium chloride, 0.201 g of potassium chloride (Merck Química, Argentina), and 0.5 g of sodium azide (J.T Baker, Mexico) and then diluted to a 1-l with bi distilled water and adjusted the pH of the solution to 7.4. Fumonisins, trichothecenes, and ZEA standards were purchased from Biopure (Tulin, Austria); aflatoxins, AOH, AME, and OTA standards were obtained from Sigma Chemical Company (USA).

Analysis of aflatoxins (AFs) and ZEA involved 25 g of rice extracted with 105 ml of ACN blending for 3 min at high speed with a blender (Osterizer, USA). Approximately 5 ml of the supernatant were passed on through an extraction column (PuriTox TC-M 160, Trilogy). Subsequently, the filtrate was divided in three fractions, two of 1 ml (0.2 g) and one of 2 ml (0.4 g), and evaporated under vacuum in a water bath at 60 °C until dryness. HPLC reversed-phase analysis was performed to quantify aflatoxins (AFB_1, AFB_2, AFG_1 y AFG_2). The evaporated extract (1 ml) was reconstituted in 200 μl hexane and was derivatized with a solution of 50 μl TFA, shacked for 30 s, and after 5 min neutralized with 950 μl of ACN/water (1:9), where the water phase was separated. The HPLC equipment used (Agilent 1100 series) included a degasser (G1322A), an autosampler (G1313A), a fluorescence detector (G1321A), quaternary pump (G1311 A), and a temperature controller (G1316A). A Microsorb-MV C18 reverse phase column (150 mm×4.6 mm×15 μm) was used. The mobile phase was H_2O/ACN/MeOH (70:15:15 *v*/*v*/*v*), the flow rate was 1 ml/min, and the injection volume of 100 μl. Fluorescence excitation and emission wavelengths were set at 360 and 440 nm, respectively. Detection and quantification limits (LOD and LOQ, respectively) for all AFs were 0.2 and 0.3 μg/kg.

For the analysis of ZEA, the evaporated extract of 0.2 g was resuspended in 1 ml of MeOH:H_2O (7:3, *v*/*v*). The HPLC equipment used a Thermo-hypersil BDS C18 (250 mm× 4.6 mm×5 μm) column. The mobile phase was H_2O/MeOH/ACN (50:23:27, *v*/*v*/*v*), the flow rate was 1 ml/min and the injection volume of 25 μl. Fluorescence excitation and emission wavelengths were set at 236 and 460 nm. Detection and quantification limits (LOD and LOQ) were 4.7 and 9.7 μg/kg, respectively.

Samples were analyzed to determine the presence of FB_1, FB_2, and FB_3. Twenty-five grams of the sample were extracted with methanol/water to maintain a relation of 75/25 *v*/*v* by blending for 2 min at high speed with a blender (Osterizer, USA). The extract was filtered through Whatman N° 4 paper, adjusted to pH=6 with NaOH 1 M, and centrifuged during 10 min (500 G). For the clean-up step, quaternary amine solid phase extraction column was used (Strata SAX, Phenomenex), previously conditioned by the successive passage of 5 ml of MeOH and 5 ml of MeOH/H_2O (3:1, *v*/*v*). Then, 20 ml of the filtered were applied to the column, washed with 5 ml MeOH/H_2O (3:1, *v*/*v*) and 5 ml of MeOH. The

fumonisins (FBs) were eluted with HAc/MeOH (1:99) at speed of 1 ml/min, and the elutes were collected and evaporated to dryness at 60 °C under a stream of nitrogen. The residue was resuspended in 2 ml of HAc/MeOH (1:99, *v*/*v*), separated in two aliquots of 800 μl, evaporated to dryness at 60 °C under a stream of nitrogen, and resuspended in 1 ml of ACN/H_2O (1:1, *v*/*v*). The HPLC equipment used a reverse phase C18 (250 mm×4.6 mm×5 μm) column (Thermo Hypersyl C18) at 23 °C. The mobile phase was sodium phosphate 1 N 1,000 ml/3,348 ml MeOH/24 ml orthophosphoric acid at pH=3.3; the flow rate was 1 ml/min. Sample was online derivatized with 17 μl OPA+20 μl sample+17 μl OPA and injected. Fluorescence excitation and emission wavelengths were set at 335 and 440 nm. Detection and quantification limits (LOD and LOQ) for FB_1 were 10 and 18 μg/kg, for FB_2 6 and 30 μg/kg, and for FB_3 12 and 30 μg/kg, respectively. The HPLC equipment used (Agilent 1100 series) included a degasser (G1322A), an auto sampler (G1313A), a fluorescence detector (G1321A), quaternary pump (G1311 A), and a temperature controller (G1316A).

For the OTA analysis, the whole volume of the sample was blended during 2 min and after centrifugation, the solid supernatant was separated. Twenty grams of the solid phase were added with 48 ml of ACN and 32 ml of the supernatant, blended for 2 min and filtrated. An aliquot of 4 ml was taken and 44 ml of buffer PBS at pH 7.4 were added and centrifuged during 10 min (1,600 G). For the clean-up step, an immunoaffinity column (OCHRAPREP®, R-Biopharm Rhône LTD) at a flow rate of 2–3 ml/min was used. The column was washed with 20 ml of buffer PBS pH 7.4 at a flow rate of 5 ml/min. OTA was eluted with 1.5 ml of HAc/MeOH (2:98, *v*/*v*) by gravity, backflushing three times before being evaporated until dryness at 50 °C. The residue was resuspended in 250 μl of mobile phase, ACN/H_2O/HAc (421.5:570:8.5, *v*/*v*/*v*). The HPLC equipment used a reverse phase Hypersil BDS C18 (125 mm×4 mm×5 μm) column, the mobile phase was set at a flow rate of 1 ml/min, and the injection volume of 100 μl. Fluorescence excitation and emission wavelengths were set at 333 and 460 nm at a temperature of 40 °C. LOD and LOQ for OTA were 0.01 and 0.07 μg/kg, respectively. The HPLC equipment used (Agilent 1100 series) included a degasser (G1322A), an auto sampler (G1313A), a fluorescence detector (G1321A), quaternary pump (G1311 A), and a temperature controller (G1316A).

For trichothecenes determination, 25 g of samples were extracted with 105 ml of ACN, blending for 3 min at high speed with a blender (Osterizer, USA). Approximately 8 ml of the supernatant were passed through an extraction column (PuriTox TC-M 220, Trilogy). Subsequent, the filtrate was divided in two fractions of 1 ml (0.2 g), and evaporated under vacuum in a water bath at 60 °C until dryness. After that, 200 μl of ethyl acetate/methanol (19:1) were added to the residue and shaked for 15 s in vortex and 150 μl of this solution were incorporated to a derivatization tube (internal standards ACBP and E-DON) and evaporated to dryness under a stream of nitrogen at temperature lower than 60 °C. Then, 100 μl of toluene/acetonitrile (80:20, *v*/*v*) with 2 mg/ml of the catalyst DMAP were added to the residue and shacked for 15 s with vortex followed by the addition of 50 μl of the HFBA, shacked again for 15 s and put in a bath sand at 60–65 °C during 30 min. After this time, 1.2 ml of $NaHCO_3$ 5 % and 400 μl of toluene were added, shacked for 30 s with vortex and cooled at room temperature before centrifuged at 2,000 rpm for 2 min. An aliquot of 300 μl from the toluene phase was separated and put into an insert to be processed by GC. The GC (Agilent Technology 7890A) used an HP-5 (30 m×0.32 mm×0.25 μm) column and had autosampler (Agilent 7693), nitrogen as carrier and auxiliary gas and microelectron capture detector (μECD), working at 300 °C. LOD and LOQ were 4 and 10 μg/kg for DON; 2.4 and 6 μg/kg for FUS-X; 6.4 and 15.6 μg/kg for NIV; 6 and 9 μg/kg for T-2; 2 and 5 μg/kg for HT-2; and 3.2 and 8 μg/kg for 3-AcDON and 15-AcDON.

For the AME and AOH extraction, the rice culture media was extracted with 50 ml of ethyl acetate/formic acid (99:1, *v*/*v*) by shaking at 200 rpm for 50 min in darkness. Then, the solid particles were separated from the extract centrifuging at 3,200 rpm during 10 min. The solids where extracted two more times with 50 ml of ethyl acetate/formic acid (99:1, *v*/*v*), following the same steps of the first extraction. The extract was then mixed and evaporated. Before dry under vacuum in a water bath at 40 °C until dryness, the residue was filtered through nylon filter 0.45 μm.

The residue was suspended in 500 μl of mobile phase, methanol/phosphoric acid 1 % (65:35, *v*/*v*). For HPLC separation, the analytical column used was a Thermo Hypersyl C18 reversed phase column (250 mm×4.6 mm×5 μm) with a guard column Hypersyl C18 (10×4 mm×5 μm). Constant flow was 1 ml/min. Ultraviolet detection was made at 256 nm. Detection was performed at 256.4 nm; column temperature was 40 °C and sample temperature was 10 °C. LOD and LOQ for AOH were 6 and 10 μg/kg, and LOD and LOQ for AME were 2 and 4 μg/kg, respectively. The HPLC equipment used (Agilent 1100 series) included a degasser (G1322A), an auto sampler (G1313A), a UV detector (G1314 D), quaternary pump (G1311 A), and a temperature controller (G1316A).

Results and discussion

Based on morphological and colony appearance, six potentially mycotoxicogenic groups of species were determined: *A. flavus*, *A.* section *Nigri*, *A. tenuissima*, *A. alternata*, *F. graminearum*, and *F. verticillioides*. Specific assignment for *A.* section *Nigri*, *F. graminearum*, *F. verticillioides*, and *A. flavus* further validated using molecular information as

Table 1 rDNA region sequences from micotoxicogenic specific groups obtained from blueberry varieties in Entre Ríos Province (Argentina)

Morphological identification	Consensus sequence data for the ITS1-5.8S-ITS2 region of morphologically ambiguous groups in the present study
Aspergillus flavus	TGATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGATCGGA CGCGGTGCCGCCGCTGCCTTTGGGGCCCGTCCCCCCCGGAGAGGGGACGACGACCCAACACACAAGCCGT GCTTGATGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCA AAGACTCGATGATTCACGGAATTCTGCAATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCC GGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCGATACAATCAACTCAGACTTCACTAGATCAGA CAGAGTTCGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTGAGAGCCCCCGGCGGCCATGAATGGCGGGC CCGCCGAAGCAACTAAGGTACAGTAAACACGGGTGGGAGGTTGGGCTCGCTAGGAACCCTACACTCGGTA ATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTA
Aspergillus section *Nigri*	TTATTGATATGCTTAAGTTCAGCGGGTATCCNTNCNTGATCCGAGGTCAATCTGAGAAGATTGGGGGTCGAGGC AAGCCCCGGCCGGGCCCATAGAGCGGGTGACAGAGCCCCATACGCTCGAGGACCGGACGGTGCCGCCGTT TCTCTCGAGGCCCGCCCCCGGGGGGGCGCGGCCCAACAACCAGCGGGGCTGGAGGGGAGAAATGACGC TCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAAT TCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTG AAAGTTTTAACTGATTGGTATCAATCAACTCAGACTGCACGCTTTCAGACAGTGTTCCATTGGGGTCTCCGG CGGGCGCGGTCCCGGGGGCAGGCCCCGGGCCGCCCGAAGGCGGGCCCGCCGAAGCAACAGGGTACGGT AAGCACGGGTGGGAGGTTGGGCCCCGAAGGACCCANCACTCGGTAATGATCCTTC
Fusarium graminearum	AGGTCACATTCNGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAACGATGTGTAAATTACTAC GCTATGGAAGCTCGACGTGACCGCCAATGTATTTGGGGAGTGCAGCAGGACTGCAGCTCCCAACACCAAG CTGGGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGT TCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTTGCTGCGTTCTTCATCGATGC CAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTGTTTTTTATACTCAGAAGTTCCACTAAAAACA GAGTTTAGGGTTCCTGCGGCGGGCCGTCCCTTTTTACGGGGCGCGGGCTGATCCGCCGAGGCAACATAAG GTATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTG TTA
Fusarium verticillioides	TCCGAGGTCACNTTCNGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTA CTACGCTATGGAAGCTCGACGTGACCGCCAATCAATTTGGGGAACGCGATTTGACTCGCGAGTCCCAACA CCAAGCTGGGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATG TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTTGCTGCGTTCTTCATC GATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTATGGTTTTACTCAGAAGTTACATATAG AAACAGAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAAC AATTGGTATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGA CCTTGT

indicated above. The ITS consensus sequences for *F. verticillioides*, *F. graminearum*, *A. flavus*, and *A.* section *Nigri* isolates from the present study are shown in Table 1. No conflict between morphological and sequence information was detected; in all cases, *E* value was 0.0, and similarity scores with sequences from the corresponding putative species at the RefSeq database was always above 98 %. According to morphological analysis, all the black aspergilla

Table 2 Mycotoxigenic strains over total analyzed strains

Mycotoxin	OTA	FB_1	FB_2	FB_3	AFB_1	AFB_2	AFG_1	AFG_2	AOH	AME	ZEA	DON	NIV	HT-2	T2	FUS-X	3-AcDON	15-AcDON
Aspergillus niger	0/19	15/19	2/19	1/19	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Aspergillus flavus	n.a.	n.a.	n.a.	n.a.	1/8	1/8	1/8	0/8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Alternaria alternata	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2/4	2/4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Alternaria tenuissima	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	4/6	3/6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Fusarium graminearum	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2/2	1/2	0/2	1/2	1/2	0/2	0/2	0/2
Fusarium semitectum	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0/2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Fusarium verticillioides	n.a.	1/1	0/1	0/1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

n.a. not analyzed

Table 3 FBs concentration (μg/kg) produced by different *Aspergillus* section *Nigri* strains recovered from blueberries

CIM No.	FB_1	FB_2	FB_3
31346	42,255	nd	nd
31363	6,375	nd	nd
31355	3,188	nd	nd
31364	310	nd	nd
31360	545	nd	nd
31365	9,880	nd	nd
31366	259	nd	nd
31367	1,807	nd	nd
31347	182	nd	nd
31356	595	nd	nd
31345	1,262	nd	nd
31361	969	87	23
31362	826	nd	nd
31369	105	nd	nd
31371	1,061	41	nd

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nd not detected

isolated were bisseriate and should therefore be included within *A.* section *Nigri* (Soares et al. 2013). Again, this information was compatible with sequence data from the ITS region. Nevertheless, neither morphology nor sequence information allowed for taxonomic status assessment of those isolates at the specific level, which therefore remained identified as "*A.* section *Nigri*".

Table 2 shows that 25 strains out of forty two isolates analyzed were capable of producing some of the mycotoxins studied. Only one strain of *A. flavus* out of eight was able to produce aflatoxins AFB_1, AFB_2, and AFG_1. Its specific status was verified using ITS data. Vaamonde et al. (2003) indicated that this particular chemotype should be type II, and for that reason, the capability of producing cyclopiazonic acid should be studied. Other authors would classify this strain into chemotype VI or VII depending on the cyclopiazonic acid production capacity (Razzaghi-Abyaneh et al. 2006, Giorni et al. 2007, Sánchez-Hervás et al. 2008, Varga et al. 2011).

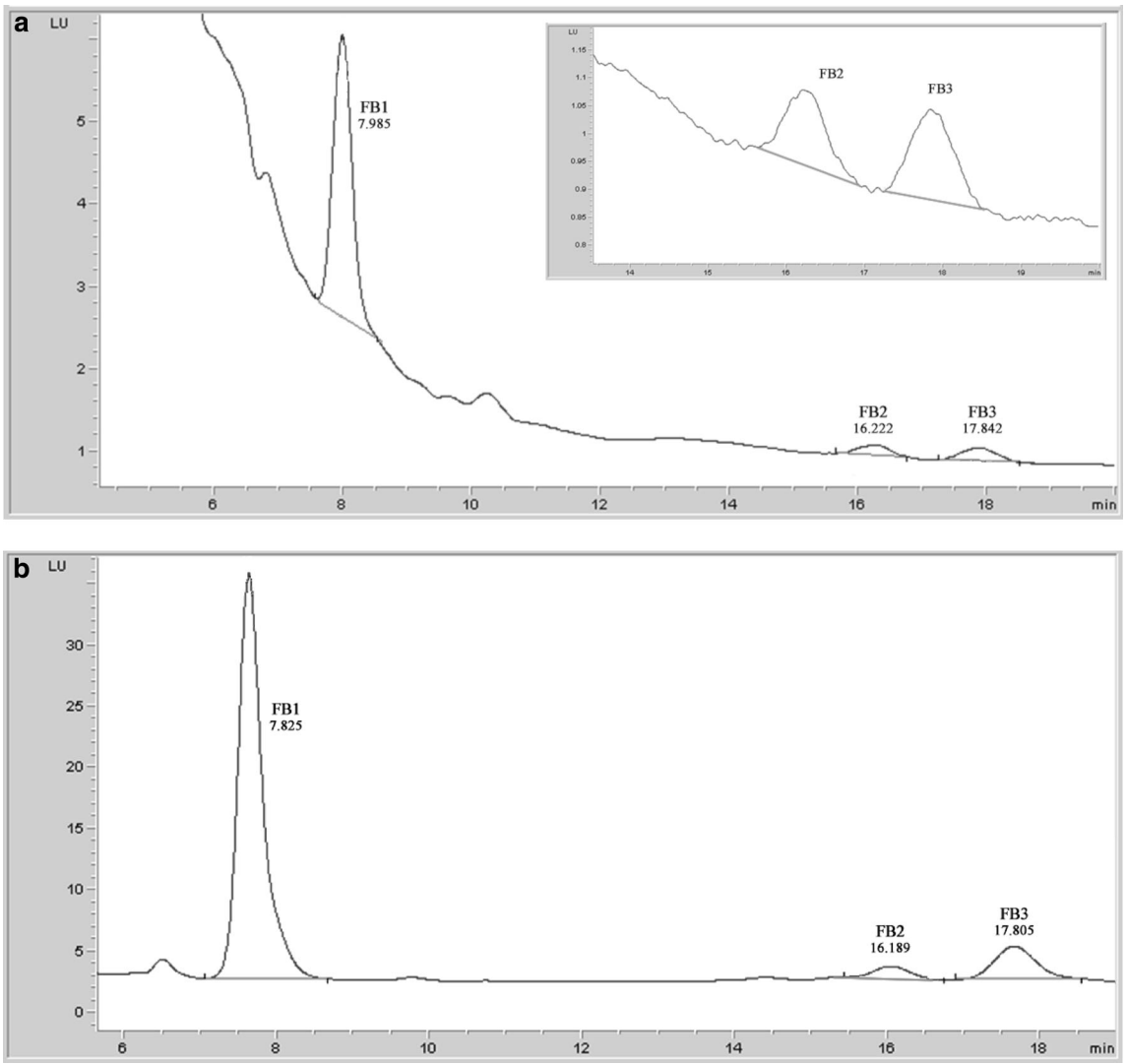

Fig. 1 **a** Fumonisins B1, B2, and B3 standards **b** Sample 31361 contaminated by FB1, FB2, and FB3

Table 4 AOH and AME (μg/kg) produced by species of *Alternaria* recovered from blueberries

CIM No.	Species	AOH	AME
32959	*A. alternata*	14,112	15,159
32963	*A. alternata*	4,649	7,372
32958	*A. tenuissima*	9,695	nd
32960	*A. tenuissima*	9,789	13,549
32961	*A. tenuissima*	1,099	624
32966	*A. tenuissima*	2,508	4,109

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nd not detected

Vaamonde et al. (2003) showed high frequency of toxigenic strains of *A. flavus*, isolated from peanut (93 %), soybean, and wheat (100 %). The *A. flavus* strain characterized in the present work produced 3,932 μg/kg of AFB_1, 165 μg/kg of AFB_2, and 119 μg/kg of AFG_1, and was therefore a higher AFs producer than those reported by Vaamonde et al. (2003) and Astoreca et al. (2011).

None of the 19 strains of *A.* section *Nigri* was able to produce OTA under the studied conditions. Riba et al. (2008) have found that 28 % of Algerian isolates identified as *A.* section *Nigri* obtained from wheat were low OTA producers. Based on the information of Varga et al. (2010) regarding the ability of *A.* section *Nigri* to produce fumonisin, 19 strains of this fungi isolated from blueberries were studied (Table 3). Several authors are discussing the production of FB_1 and FB_3 by *A.* section *Nigri* (Nielssen and Logrieco 2011; Varga et al. 2012). On the other hand, it is also necessary to confirm that the fumonisin quantified in this work as FB_1 is that toxin, FB_6 or a combination of both toxins (Mansson et al. 2010). Figure 1 shows a chromatogram of sample 31361 (*Aspergillus* section *Nigri*) depicting FB_1, FB_2, and FB_3 production, along with the respective standards chromatogram.

Two strains of *A. alternata* out of four analyzed had the ability of producing AOH and AME (Table 4). Three strains of *A. tenuissima* were able to produce AME and four out of six strains produced AOH. Greco et al. (2012) found that 61 % of the 134 isolates of *Alternaria* species isolated from Argentinean blueberries were mycotoxin producers; among them, 97 % were able to produce AOH, 95 % AME, and 65 % tenuazonic acid.

Table 5 Mycotoxin concentration (μg/kg) produced by different species of *Fusarium* recovered from blueberries

CIM No.	Species	ZEA	DON	HT-2	T-2	FB_1	FB_2	FB_3
30424	*F. graminearum*	1,617	nd	142	173			
30425	*F. graminearum*	138	5,868	nd	nd			
31339	*F. verticillioides*					1,544	nd	nd

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nd not detected

Within the *F. graminearum* isolated, two of them were able to produce ZEA. Table 5 shows that one of those strains was able to produce both H-T2 and T2, and the other produced DON as well. None of them were NIV, FUS-X, 3-AcDON, or 15-AcDON producers. None of the two *F. semitectum* analyzed had the ability of producing ZEA. During the 2010 harvest season, only one *F. verticillioides* strain was isolated from Misty cultivar, and it showed only a low FB_1 fumonisin production ability.

Conclusion

More than half of the strains analyzed in the present study have shown mycotoxicogenic potential. These results suggest a considerable intoxication risk through consumption of this small fruit and point at the necessity of studying the natural occurrence of mycotoxins in blueberry and its byproducts. In order to further refine and optimize taxonomic identifications, with regards to the respective specific toxicogenic profiles, further molecular analysis for specific assignment is currently underway, incorporating additional nuclear and mitochondrial loci (Calmodulin, β-tubulin, Transcription Elongation Factor 1-alpha, Cytochrome B oxidase).

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Conflicts of interest None.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Astoreca AL, Dalcero AM, Fernández Pinto V, Vaamonde G (2011) A survey on distribution and toxigenicity of *Aspergillus* section flavi in poultry feeds. Int J Food Microbiol 146:38–43

Bruzone I. 2007. Frutasfinas: berries. http://www.minagri.gob.ar/SAGPyA/economias_regionales/_frutas/_cadenas/Frutas_finas_berrie_11_07.htm. Accessed January 2013

Cappellini RA, Ceponis MJ (1977) Vulnerability of stem-end scars of blueberry fruits to postharvest decays. Phytopathol 67:118–119

Cappellini RA, Stretch AW, Maiello JM (1972) Fungi associated with blueberries held at various storage times and temperatures. Phytopathol 62:68–69

Cline WO (1997) Fruit rot diseases of blueberry. http://www.ces.ncsu.edu/depts/pp/notes/Fruit/blueberryinfo/berryrots.htm Accessed December 2010

Cline WO, Milholland RD (1995) Diseases of blueberry fruit at harvest in North Carolina. J Small Fruit Viticult 3:219–225

Dansa AM (2008) Dirección de Mercados Agroalimentarios, Bs. As., Argentina. http://www.fruticulturasur.com.ar/upload/articulos/archivos/Arandanos.pdf. Accessed January 2013

Ellis M (1971) Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew

Ferreira Geraldo MR, Tessmann DJ, Kemmelmeier C (2006) Production of mycotoxins by Fusarium graminearum isolated from small cereals (wheat, triticale and barley) affected with scab disease in Southern Brazil. Braz J Microbiol 37:58–63

Gabler J, Kačergius A, Jovaišienė Z (2004) Detection of *Phomopsis vaccinii* on blueberry and cranberry in Europe by direct tissue blot immunoassay and plate-trapped antigen ELISA. J Phytopathol 152: 630–632

Giorni P, Magan N, Pietri A, Bertuzzi T, Battilani P (2007) Studies on *Aspergillus* section flavi isolated from maize in northern Italy. Int J Food Microbiol 113:330–338

Greco M, Patriarca A, Terminiello L, Fernández Pinto V, Pose G (2012) Toxigenic *Alternaria* species from Argentinean blueberries. Int J Food Microbiol 154:187–191

He J, Yang R, Zhou T, Tsao R, Young JC, Zhu H, Li X, Boland GJ (2007) Purification of deoxynivalenol from Fusarium graminearum rice culture and mouldy corn by high-speed counter-current chromatography. J Chromatogr A 1151:187–192

Hinojo MJ, Medina A, Valle-Algarra FM, Gimeno-Adelantado JV, Jiménez M, Mateo R (2006) Fumonisin production in rice cultures of *Fusarium verticillioides* under different incubation conditions using an optimized analytical method. Food Microbiol 23:119–127

JECFA (2014) Joint FAO/WHO Expert Committee on Food Additives http://apps.who.int/food-additives-contaminants-jecfa-database/search.aspx. Accessed February 2014

Klich MA (2002) Identification of common *Aspergillus* species. Centraalbureau voor Schimmel cultures, Delft

Luan YS, Feng L, Xia XY, An LJ (2007) First report of *Alternaria tenuissima* causing disease on blueberry in China. Plant Dis 91:464

Magnoli C, Violante M, Combina M, Palacio G, Dalcero A (2003) Mycoflora and ochratoxin-producing strains of *Aspergillus* section Nigri in wine grapes in Argentina. Lett Appl Microbiol 37:179–184

Mansson M, Klejnstrup M, Phipps R, Nielsen K, Frisvad J, GotfredsenC LT (2010) Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. J Agric Food Chem 58: 949–953

Megalla SE, Bennett GA, Ellis JJ, Shotwell OL (1987) Production of deoxynivelenol and zearalenone by isolates of *Fusarium graminearum*. J Food Protect 50:826–828

Moltó GA, González HHL, Resnik SL, Pereyra González A (1997) Production of trichothecenes and zearalenone by isolates of *Fusarium* spp. from Argentinian maize. Food Add Contam 14: 263–268

Munitz MS, Garrido CE, Gonzalez HHL, Resnik SL, Salas PM, Montti MIT (2013) Mycoflora and potential mycotoxin production of freshly harvested blueberry in Concordia, Entre Ríos Province, Argentina. Int J Fruit Sci 13:312–325

Nelson PE, Toussoun TA, Marasas WFO (1983) *Fusarium* species: an illustrated manual for identification. The Pennsylvania State University, University Park

Nielssen KF, Logrieco AF (2011) Letter to the editor on "Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin". Int J Food Microbiol 143:143–149

Pérez BA, Murillo F, Divo de Sesar M, Wright ER (2007) Occurrence of *Fusarium solani* on blueberry in Argentina. Plant Dis 91:1053

Pitt JI, Hocking AD (2009) Fungi and food spoilage. Blackie Academic & Professional, London

Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Allameh A, Kazeroon-Shiri A, Ranjbar-Bahadori S, Mirzahoseini H, Rezaee M (2006) A survey on distribution of *Aspergillus* section Flavi in corn field soils in Iran: populations patterns based on aflatoxins, cyclopiazonic acid and sclerotia production. Mycophatol 161:183–192

Resnik SL, González HHL, Pacin A, Viora M, Caballero GM, Gros E (1996) Cyclopiazonic acid and aflatoxins production by *Aspergillus flavus* isolated from Argentinian corn. Mycotoxin Res 12:61–66

Riba A, Mokrane S, Mathieu F, Lebrihi A, Sabaou N (2008) Mycoflora and ochratoxin A producing strains of *Aspergillus* in Algerian wheat. Int J Food Microbiol 192:85–92

Rivera MC, Wright ER, Pérez BA, González Rabelino P, Pérez JA (2009) Enfermedades del arándano. In: Wright ER (ed) Guía de Enfermedades, Insectos y Malezas del Arándano. Orientación Gráfica Ed, Buenos Aires, pp 1–68

Sampietro DA, Apud GR, Belizán MM, Vattuone MA, Catalán CA (2013) Toxigenic potential of *Fusarium graminearum* isolated from maize of northwest Argentina. Braz J Microbiol 44:417–422

Samson RA, Hoekstra ES, Frisvad JC (2004) Introduction to food and airborne fungi. Centraalbureau voor Schimmelcultures, Delft

Sánchez-Hervás M, Gil JV, Bisbal F, Ramón D, Martínez-Culebras PV (2008) Mycobiota and mycotoxin producing fungi from cocoabeans. Int J Food Microbiol 125:336–340

Santillán P (2009) Balance de la temporada de arándanos del Hemisferio Sur 2008–2009. http://www.fruticulturasur.com.ar/fichaNota.php?articuloId=762.Accessed February 2013

Simmons EG (2007) *Alternaria*. An Identification Manual. CBS Biodiversity Series 6. Utrecht

Smith BJ, Magee JB, Gupton CL (1996) Susceptibility of rabbiteye blueberry cultivars to postharvest diseases. Plant Dis 80:215–218

Soares C, Calado T, Venâncio A (2013) Mycotoxin production by *Aspergillus niger* aggregate strains isolated from harvested maize in three Portuguese regions. Rev Iberoam Micol 30:9–13

Takikawa M, Inoue S, Horio F, Tsuda T (2010) Dietary anthocyanin-rich blueberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP activated protein kinase in diabetic mice. J Nutrition 140:527–533

Tarnowski TLB, Savelle AT, Scherm H (2008) Activity of fungicide against *Monilini vaccinii-corymbosi* in blueberry flowers treated at different phenological stages. Plant Dis 92:961–965

Torres A, González HHL, Etcheverry M, Resnik SL, Chulze S (1998) Production of alternariol and alternariol mono-methyl ether by isolates of *Alternaria* spp. from Argentinian maize. Food Add Contam 15:56–60

Tournas VH, Katsoudas E (2005) Mould and yeast flora in fresh berries, grapes and citrus fruits. Int J Food Microbiol 105:11–17

Vaamonde G, Patriarca A, Fernández Pinto V, Comerio R, Degrossi C (2003) Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section flavi from different substrates in Argentina. Int J Food Microbiol 88:79–84

Varga J, Kocsubé S, Suri K, Szigeti G, Szekeres A, Varga M, Tóth B, Bartók T (2010) Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin. Int J Food Microbiol 143:143–149

Varga J, Frisvad JC, Samson RA (2011) Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud Mycol 69:57–80. doi:10.3114/sim.2011.69.05

Varga J, Kocsubé S, Tóth B, Bartók T (2012) Letter to the editor: response to letter to the editor on "Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin" published in Int J Food Microbiol 143:143–149". Int J Food Microbiol 152:46–48

Wharton PS, Schilder AC (2005) Effect of temperature on apothecial longevity and ascospore discharge by apothecia of *Monilinia vaccinii-corymbosi*. Plant Dis 89:397–403

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelgard DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, New York, pp 315–322

Wright ER (2009) Enfermedades en cultivos para frutos pequeños en la República Argentina. Horticom News. "http://www.horticom.com/pd/print.php?sid=73288". Accessed December 2010

Wright ER, Rivera MC, Flynn MJ (1998) First report of *Pestalotiopsis guepini* and *Glomerella cingulata* on blueberry in Buenos Aires (Argentina). EPPO Bul 28:219–220

Wright ER, Rivera MC, Esperón J, Cheheid A, Rodríguez Codazzi A (2004) *Alternaria* leaf spot, twig blight, and fruit rot of highbush blueberry in Argentina. Plant Dis 88:1383

Wright ER, Fernández RL, Benva M, Pérez JA, Rivera MC, Roncoroni S, Nicolini F, Vásquez PE, Ciurca P, Pérez BA (2008a) Deterioro poscosecha de arándano en Entre Ríos y Buenos Aires, Argentina. In: Divo de Sesar M, Rocca M, Vilella F (eds) Avances en cultivos frutales no tradicionales. Arándanos–Cerezas–Frutillas–Granadas. Buenos Aires Univ, Buenos Aires, pp 63–68

Wright ER, Folgado M, Rivera MC, Crelier A, Vásquez P, López SE (2008b) *Nigrospora sphaerica* causing leaf spot and twig and shoot blight on blueberry: a new host of the pathogen. Plant Dis 92:171

Wright ER, Pérez BA, Roncorini S, Nicolini F, Pasut F, Moreno A, Vásquez PE, Lara M, Fernández RL, Rabellino M, Benva M, Borsarelli M, Cornellati D, Pérez JA, Ciurca P, Divo de Sesar M, Asciutto K, Trejo E, Colavolpe B, Rivera MC (2008c) Distribución e incremento de patógenos dearándano en Buenos Aires y Entre Ríos, Argentina, en 2007/2008. In: Divo de Sesar M, Rocca M, Vilella F (eds) Avances en cultivos frutales no tradicionales. Arándanos–Cerezas–Frutillas–Granadas. Buenos Aires Univ, Buenos Aires, pp 39–45