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Toxigenic *Alternaria* species from Argentinean blueberries

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ABSTRACT

Blueberries are traditionally consumed in North America, some European countries and Japan. In Argentina, the blueberry crop is profitable because production starts in November, when the northern hemisphere lacks fresh fruit. Fungal contaminants can grow and produce mycotoxins in fresh fruit. The aims of this work were to identify the main genera of the mycobiota of blueberries grown in Argentina and to determine the toxicogenic potential, pathogenicity and host specificity of the species isolated. The genus *Alternaria* was the main component of the blueberry mycobiota (95%); minor proportions of *Phoma* spp. (4%) and *Penicillium* spp. (1%) were also isolated. According to their sporulation patterns, 127 *Alternaria* isolates belonged to the *Alternaria tenuissima* species-group, 5 to the *Alternaria alternata* species-group and 2 to the *Alternaria arborescens* species-group. The last mentioned species-group was not isolated at 5 °C. Of the 134 isolates, 61% were toxicogenic in autoclaved rice; 97% of these produced alternariol (AOH) in a range from 0.14 to 119.18 mg/kg, 95% produced alternariol methylether (AME) in a range from 1.23 to 901.74 mg/kg and 65% produced tenuazonic acid (TA) in a range from 0.13 to 2778 mg/kg. Fifty two isolates co-produced the three mycotoxins. According to the size of the lesion that they caused on blueberries, the isolates were classified as slightly pathogenic, moderately pathogenic and very pathogenic. No significant differences in pathogenicity were found on different blueberry varieties.
In this work, high incidence and toxicogenic potential of the *Alternaria* isolates from blueberries were demonstrated. Thus, more studies should be done to evaluate the health risk posed by the presence of the *Alternaria* toxins in blueberries and in the manufactured by-products.

1. Introduction

Fruits, and in particular berries, have been the focus of recent interest among researchers and health professionals for their role in prevention of chronic diseases (Venketeshwer Rao and Snyder, 2010). In recent years, several berries such as strawberry, blueberry, cranberry, and black raspberry have been studied for their beneficial effects on human health. These benefits include prevention of certain types of cancer, cardiovascular diseases, type II diabetes, obesity, neurodegenerative diseases associated with aging, and infections (Dembinska-Kiec et al., 2008; Pappas and Schaich, 2009; Takikawa et al., 2010). Blueberries are traditionally consumed in North America, some European countries and Japan, as fresh fruits, frozen or manufactured into juices, cakes, jams, sauces, yoghurts, ice creams, etc. The USA is the main consumer, producer, exporter and importer of blueberries in the world (Dansa, 2008). In Argentina, the blueberry crop, with a cultivated area of 3700 ha, is profitable because production starts in November, when the northern hemisphere lacks fresh fruit. The production has doubled annually during the last decade, reaching 11,500 t in 2009 (Pérez et al., 2008; Santillán, 2009). Ninety percent of the production is exported, mostly to USA, the European Union (EU), the UK, and in minor proportion to Asia and Canada.

Fruits are particularly susceptible to fungal spoilage and a wide variety of molds is capable of growing and spoiling the various types of berries. It is important to identify fungal contaminants in fresh fruits because some molds can grow and produce mycotoxins on these commodities (Stinson et al., 1980; Tournas and Stack, 2001; Tournas and Katsoudas, 2005). Controlling fruit rot is especially important for fresh berries that are held in refrigerated storage between harvest and peak demand. Losses in storage are correlated with the incidence of fruit rot at harvest. *Alternaria* fruit rot can be a major post harvest problem on fresh blueberries (Wright et al., 2004). With warm and humid weather, fruit rots in blueberries are of concern, particularly anthracnose fruit rot (*Colletotrichum acutatum*) and *Alternaria* fruit rot. Post-harvest rot can occur on berries that look fine at harvest, but carry fungal spores that can infect and develop in the fruits during storage and processing (Schilder et al., 2006; Schilder, 2011). In Argentina blueberries are marketed principally fresh, and fruits are very prone to the development of fungal diseases during the postharvest period. There are few studies on postharvest diseases of this crop

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in Argentina. The reported fungi associated with blueberry diseases were *Alternaria tenuissima*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Curvularia* sp., *Fusarium solani*, *Fusarium* sp., *Humicola grisea* sp., *Pestalotiopsis guepini*, *Phoma* sp., *Phomopsis vaccinii*, *Phytophthora* sp, *Pucciniastrum vaccinii*, *Pythium* sp., *Rhizoctonia* sp., *Sclerotinia sclerotiorum*, *Sclerotium bataticola* and *Stemphyllium* sp. (Wright et al., 2004; 2005).

The growth of *Alternaria* species in blueberries is especially problematic since it could result in accumulation of mycotoxins (Stinson et al., 1980). The major *Alternaria* mycotoxins belong to three structural classes: the tetramic acid derivative, tenuazonic acid (TA); the dibenzopyrone derivatives, alternariol (AOH), alternariol methylether (AME) and altenuene; and the perylene derivatives, the altertoxins (Andersen et al., 2002). The toxicity of TA has been reported in plants, in chick embryos and several other animal species, including guinea pigs, mice, rabbits, dogs, and rhesus monkeys. AOH and AME are mutagenic and cytotoxic to bacterial and mammalian cells, and are suspected to be carcinogenic. Both AOH and AME cause weakly acute toxicity but show synergistic effects (Visconti and Sibilia, 1994; da Motta and Valente Soares, 2000; Ostry, 2008; Logrieco et al., 2009).

Taking into account that postharvest fruit spoilage results in significant economic losses and that, if the spoiling fungi are toxigenic, they could pose a health risk for the consumer, the aims of this work were:

1. To identify the principal genera of the mycobiota of blueberries grown in Argentina.
2. To determine the toxicogenic potential, pathogenicity and host specificity of the species isolated.
3. To determine the influence of the storage temperature on the species distribution, toxicogenic potential and pathogenicity of the isolates.

2. Materials and methods

2.1. Blueberry samples

Thirty blueberry samples of the O'Neal variety were assessed for fungal contamination. Blueberries of Misty and Jewel varieties were also used to test the pathogenic potential of the strains. All the samples were cultivated in Buenos Aires province, Argentina. The samples were collected from farms of 10 districts of the region during the 2009 harvest.

2.2. Isolation and identification of fungi

Two hundred (200) symptomless berries of each sample were plated on Dichloran Rose Bengal Chloramphenicol Agar (DRBC). One hundred (100) berries were incubated at 25 °C for 7 days and one hundred (100) at refrigeration temperature (5 °C) for 30 days. Molds capable of causing postharvest spoilage were isolated from visibly affected berries and taxonomic identification of the different species was made according to Samson et al. (2004), Pitt and Hocking (2009) and Simmons (2007).

2.3. Alternaria species-group determination

From single-conidial cultures, the *Alternaria* isolates were cultured on Potato-Carrot-Agar (PCA) for 7 days at 25 °C under an alternating light/dark cycle consisting of 8 h of daylight and 16 h of darkness. After the incubation period, the isolates were examined and identified according to sporulation patterns and conidial morphology (Simmons, 2007).

2.4. Mycotoxin production and analysis

Fungal cultures were grown on 12.5 g of autoclaved polished rice (La Capilla, Arrosur, Buenos Aires, Argentina) with 40% moisture in 250 ml flasks. Each flask was inoculated with agar plugs of one-week old *Alternaria* cultures. The flasks were incubated in the dark at 25 °C for 21 days (Li et al., 2001). The method for the detection of *Alternaria* toxins in rice was described by Li et al. (2001). The culture material was homogenized with 30 ml of methanol and filtered through filter paper (Whatman no. 1, Whatman Ltd., UK). Briefly, the filtrate was clarified with 60 ml of 20% ammonium sulfate and divided into two parts. One part (40 ml) was extracted three times with 10 ml of chloroform. The organic phases were combined, evaporated to dryness, and dissolved in 4 ml of methanol for AOH and AME analysis by high performance liquid chromatography (HPLC). Another part (20 ml) was adjusted to pH 2 with 6 N HCl and extracted twice for TA with 15 ml of chloroform. TA was then partitioned into 10 ml of 5% sodium bicarbonate that was successively acidified to pH 2, and extracted twice with 10 ml of chloroform. The chloroform extracts were combined, washed with 7.5 ml of water by liquid–liquid extraction, and evaporated to dryness. The residue was redissolved in 4 ml of methanol and analyzed for TA by HPLC. The HPLC system consisted of Shimadzu LC-142 CA liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20 µl loop and a Shimadzu SPD M10Avp UV photodiode array detector. The analytical column was Jupiter 4.6×250mm 5 µ C18 (Phenomenex, USA). Standards of TA (as a copper salt), AME and AOH were purchased from SIGMA Chemical Company (St. Louis, MO, USA). From all solid standards, individual stock solutions of 0.5 mg/ml were prepared in methanol and stored at −18 °C. The copper salt was reconverted to tenuazonic acid as described by Scott and Kanhere (1980). Working standard solutions of 5 µg/ml of each toxin were then prepared. The mobile phase was methanol/water (80:20) containing 300 mg ZnSO4.H2O/l, for AOH and AME, and methanol/water (85:15) containing 300 mg of ZnSO4.H2O/l for TA. A flow rate of 0.4 ml/min was used. The wavelength for recording chromatograms was 258 nm for AOH and AME, and 280 nm for TA. A calibration curve was constructed for quantification purposes using the toxins standards and correlating peak-area versus concentration. A linear response was observed in the range of 0.25–25 ng for AOH and AME and in the range of 1–50 ng for TA. The spectra were acquired in the range of 200–300 nm. Reference spectra were acquired during the elution of associated standards and used for peak identification by comparison after spectra normalization. The detection limits of the DAD detector were determined as three times the baseline standard deviation (signal-to-noise ratio of 3) and were 11 µg/kg for TA, 2 µg/kg for AME, and 5 µg/kg for AOH, respectively. Each analysis was performed by duplicate.

2.5. Pathogenicity

A set of 41 representative strains obtained from blueberries, 21 isolated at 5 °C and 20 isolated at 25 °C (Table 2), was evaluated for pathogenicity and host specificity on fruits of the O'Neal, Misty and Jewel varieties, by means of the toothpick-inoculation technique (Serdani et al., 2002). In particular, 34 strains of the *A. tenuissima* species-group, 5 strains of the *Alternaria alternata* species-group and 2 strains of the *Alternaria arborescens* species-group were tested. Halved toothpicks were autoclaved five times in distilled water, and once in potato dextrose broth and then placed on 90 mm Petri dishes containing PDA. The toothpicks were inoculated with the *Alternaria* strains and were incubated at 25 °C for two weeks to allow complete colonization. Each blueberry was inoculated with toothpicks, one of which was free of fungal growth as control. For each strain, two repetitions were done on each variety. Inoculated blueberries were placed on trays, covered with plastic bags, and incubated for 7 days