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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

at 25 °C. After the incubation period, external and internal lesions were evaluated. The degree of pathogenicity was assessed by calculating the lesion extension and was confirmed according to Koch's postulates. In the pathogenicity test, three different levels were established according to the lesion size: isolates slightly pathogenic (lesion includes a quarter or less of the blueberry's surface), isolates moderately pathogenic (lesion includes more than a quarter and up to half of the blueberry's surface), and isolates very pathogenic (lesion includes more than half to the whole surface of the blueberry). The host specificity was determined in agreement to the capacity of the strain to produce or not the lesion. Statistical analysis on data was performed by the Kruskal–Wallis test, a non parametric significance test, using Statistix v9.0 (Analytical Software, 2008, FL, USA).

3. Results and discussion

In order to assess the influence of the storage temperature on the species distribution and toxicogenic potential of the isolates, the blueberries were incubated following two schemes: i) incubation for 7 days at 25 °C and ii) incubation for 30 days at 5 °C. The *Alternaria* strains isolated under each scheme were compared in terms of toxigenicity and pathogenicity potential.

The genus *Alternaria* was present in 100% of the samples analyzed and was the main component of the blueberry mycobiota (95%). Other species were isolated in minor proportion: *Phoma* spp. (4%) and *Penicillium* spp. (1%). A total of 134 *Alternaria* strains were isolated (76 with incubation at 5 °C and 58 with incubation at 25 °C). According to their sporulation patterns, 127 (72 isolated at 5 °C and 55 isolated at 25 °C) belonged to the *A. tenuissima* species-group, 5 (4 isolated at 5 °C and one isolated at 25 °C) to the *A. alternata* species-group and 2 to the *A. arborescens* species-group. The last species-group was not isolated at 5 °C.

Tournas and Katsoudas (2005) found *Alternaria* strains in 46% of blueberry and 8% of strawberry samples. White-grayish mycelium growing relatively slowly appeared after several days of incubation. Schilder et al. (2006) and Figueroa et al. (2010) also reported *Alternaria* growth on blueberries. The high *Alternaria* contamination of blueberries may be partially explained by the fact that faster-growing molds such as *Rhizopus* spp. and *B. cinerea* were not always present in these fruits as they are in other berries such as strawberries, where the growth of *Alternaria* is inhibited by their presence (Tournas and Katsoudas, 2005).

The high incidence of *Alternaria* in fresh vegetables may be due also to this organism's capability of growing at the low temperatures used during transport and marketing (Tournas and Katsoudas, 2005). Although the optimum growth temperatures reported for *Alternaria* ranged from 22 to 30 °C, minimum growth temperatures from 2.5 to 6.5 °C and temperatures as low as 0 °C to −5 °C from isolates from colder regions have also been reported (Samson et al., 2000).

The toxigenic profiles of *Alternaria* strains isolated from blueberries at 5 °C and 25 °C are shown in Table 1. AOH, AME and TA production was tested. No correlation was found between isolation scheme and toxigenic potential of the species. TA was the toxin produced at higher levels by the strains belonging to the *A. tenuissima* and the *A. alternata* species-group. AOH and AME were produced at low levels by all the isolates of the *A. alternata* species-group. The two isolates belonging to the *A. arborescens* species-group produced all the toxins in low quantities.

The mycotoxin analysis showed that 82 (61%) of the 134 isolates were able to produce at least one of the mycotoxins in autoclaved rice. From the toxicogenic strains, 80 (97%) produced AOH in a range from 0.14 to 119.18 mg/kg, 78 (95%) produced AME in a range from 1.23 to 901.74 mg/kg and 54 (65%) produced TA in a range from 0.13 to 2778 mg/kg. Fifty two of the isolates were able to co-produce the three mycotoxins showing a potential risk of synergistic effects. These results are in accord with several reports on *Alternaria* toxin production

Table 1
Toxigenic profile of *Alternaria* strains isolated at 5 °C and 25 °C from blueberries cultivated in Argentina.

Isolation temp.	*Alternaria* species-group	No. of isolates	Mycotoxin	No. of positive isolates	Average (mg/kg)	Range (mg/kg)
5 °C	*A. tenuissima* sp-grp.	72	TA	27 (38%)	345	0.13–2778
			AME	36 (50%)	182	1.79–806
			AOH	38 (53%)	25	0.14–119
	A. alternata sp-grp.	4	TA	4	254	0.13–988
			AME	4	26	2.16–57
			AOH	4	15	6.54–28
25 °C	*A. tenuissima* sp-grp.	55	TA	19 (35%)	345	0.13–2606
			AME	34 (62%)	156	2.21–902
			AOH	34 (62%)	20	3.30–63
	A. alternata sp-grp.	1	TA	1	334	
			AME	1	19	
			AOH	1	7.3	
	A. arborescens sp-grp.	2	TA	2	7.4	1.10–14
			AME	2	73	1.23–144
			AOH	2	22	1.63–42

isolated from different hosts. Most of the strains produced AOH and AME at mean values similar than those reported in the literature, such as 600 and 100 ppm respectively for *A. alternata* isolated from tomato (Bottalico and Logrieco, 1998), 178 and 98 ppm for *A. alternata* strains from Chinese wheat (Li et al., 2001), and 20 and 20 ppm for *A. alternata* from mandarin fruits (Logrieco et al., 2003). In the present work TA was produced in higher amounts than AOH and AME; this result was also in agreement with data from the literature. Bottalico and Logrieco (1998) reported up to 4200 ppm of TA production by *A. alternata* from tomatoes; Li et al. (2001) up to 3563 ppm for wheat strains and Logrieco et al. (2003) up to 6800 ppm for *A. alternata* isolated from mandarin fruits.

Strains of *Alternaria* isolated from blueberries were reported by Stinson et al. (1980) to produce AOH in a range from 0.25 to 72.5 mg/kg, AME in a range from 1.2 to 209 mg/kg and TA in a range from 80 to 202 mg/kg when they were inoculated on surface disinfected blueberries at 21 °C for 21 days. In contrast, Tournas and Stack (2001) reported that *Alternaria* strains did not produce AOH and AME on blueberries although they produced these toxins on strawberries, grapes and apples. In the two studies, different pretreatments were applied to the fruits. Stinson et al. (1980) reported that the fruits were mechanically broken and steam disinfected. This process allowed the colonization of the berries by the pathogen because the cuticle of the fruit was injured and could also cause substantial changes in their composition, particularly in those metabolites related to defense against fungal infection. On the contrary, Tournas and Stack (2001) used intact surface disinfected blueberries where the mechanical barrier was not damaged and the fruit defenses were not inactivated by high temperature.

In the present work, autoclaved rice was used as a substrate for mycotoxin testing of *Alternaria* isolates from blueberries. Since rice has been used in the literature for assessment of mycotoxin production for many years, it enables comparison of toxigenic potential of strains isolated from different substrates and geographical regions (Bottalico and Logrieco, 1998; Bruce et al., 1984; Li et al., 2001; Solfrizzo et al., 2005; Visconti et al., 1986; Visconti et al., 1992). In autoclaved rice there are no mechanical barriers and many of the grain defenses, that are thermolabile, have been suppressed. For this reason it provides conditions in which the isolates are capable of expressing their whole toxigenic potential, which may or may not be expressed in the substrate of study. The mycotoxin levels found in autoclaved rice are slightly higher (especially regarding TA production) but similar to those found by Stinson et al. (1980).

In surveys of the occurrence of *Alternaria* toxins in berry by-products, AME and AOH were found in cranberry nectar (0.7 and

Table 2
Pathogenic profile of *Alternaria* strains isolated from Argentinean blueberries at 5 °C and 25 °C.

Isolation temp.	*Alternaria* species-group	Isolates tested	Blueberry variety	Number of isolates		
				Very pathogenic	Moderately pathogenic	Slightly pathogenic
5 °C	*A. tenuissima* sp-grp.	17	O'Neal	6	8	3
			Misty	10	6	1
			Jewel	2	12	3
	A. alternata sp-grp.	4	O'Neal	1	3	–
			Misty	3	1	–
			Jewel	–	4	–
25 °C	*A. tenuissima* sp-grp.	17	O'Neal	4	11	2
			Misty	9	8	–
			Jewel	2	15	–
	A. alternata sp-grp.	1	O'Neal	–	1	–
			Misty	1	–	–
			Jewel	–	1	–
	A. arborescens sp-grp.	2	O'Neal	–	2	–
			Misty	1	1	–
			Jewel	–	2	–

5.6 μg/l) and in cranberry juice at very low levels (≤0.0015 μg/l) (Lau et al., 2003; Scott et al., 2006). The occurrence of *Alternaria* toxins in fruit juices is of concern because they are commonly consumed in many countries as healthy drinks.

Pathogenicity tests showed no differences between species-group, fruit variety susceptibility, and the temperature at which the strains were isolated ($p<0.05$) (Table 2). The level of aggressiveness of the different strains toward blueberries ranged from moderate to very pathogenic.

Three different levels were established according to the lesion size: isolates slightly pathogenic (lesion includes a quarter or less of the blueberry's surface), isolates moderately pathogenic (lesion includes more than a quarter and until a half of the blueberry's surface) and isolates very pathogenic (lesion includes more than half to the whole surface of the blueberry). The percentage of isolates in each pathogenicity level was 12%, 61% and 27% on O'Neal blueberries; 2%, 39% and 59% on Misty blueberries and 7%, 83% and 10% on Jewel blueberries, respectively.

No significant differences in pathogenicity could be detected for the *Alternaria* strains isolated at different temperatures. In contrast, small differences were found for the variety of blueberry on which the test was performed. Lesions due to moderate pathogenicity were found in the highest proportion (83%) in the Jewel variety whereas on Misty variety a high number of strains (59%) produced largest lesions, indicating that this variety could be more prone to *Alternaria* contamination. Comparing lesion development among isolates of the different *Alternaria* isolates, smaller lesions, indicating weak pathogenicity, were found only for *A. tenuissima* species-group.

This study was an attempt to investigate the variability of *Alternaria* strains through a polyphasic approach, taking into account morphological characteristics, toxigenic capability and pathogenicity.

The present work showed the high incidence and the toxicogenic potential of the *Alternaria* isolates from blueberries, which indicates the potential risk of *Alternaria* toxins accumulation in the fruits. More work should be done in order to evaluate the health risk posed by the presence of the *Alternaria* toxins in blueberries and in the manufactured by-products because of the lack of information existing in our country and worldwide to this respect. Taking into account that no regulations exist for *Alternaria* toxins, efforts must be made to advise blueberry producers on the employment of good practices in pre- and postharvest periods in order to prevent mold development and mycotoxin contamination.

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