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Martin S. Munitz $^{a\ b}$, Carolina E. Garrido $^{b\ c}$, Hector H. L. Gonzalez $^{d\ e}$, Silvia L. Resnik $^{f\ g}$, Paula M. Salas $^{b\ e}$ & Maria I. T. Montti a

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^a Facultad de Ciencias de la Alimentación, Universidad Nacional de Entre Ríos, Concordia, Entre Ríos, Argentina

^b Agencia Nacional de Promoción Científica y Tecnológica, CABA, Argentina

^c Fundación de Investigaciones Científicas Teresa Benedicta de la Cruz, Luján, Argentina

^d Consejo Nacional de Investigaciones Científicas y Técnicas, CABA, Argentina

^e Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Buenos Aires, CABA, Argentina

^f Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, CABA, Argentina

⁹ Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, La Plata, Argentina

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Mycoflora and Potential Mycotoxin Production of Freshly Harvested Blueberry in Concordia, Entre Ríos Province, Argentina

MARTIN S. MUNITZ^{1,2}, CAROLINA E. GARRIDO^{2,3}, HECTOR H. L. GONZALEZ^{4,5}, SILVIA L. RESNIK^{6,7}, PAULA M. SALAS^{2,5}, and MARIA I. T. MONTTI¹

¹Facultad de Ciencias de la Alimentación, Universidad Nacional de Entre Ríos, Concordia, Entre Ríos, Argentina

²Agencia Nacional de Promoción Científica y Tecnológica, CABA, Argentina

³Fundación de Investigaciones Científicas Teresa Benedicta de la Cruz, Luján, Argentina

⁴Consejo Nacional de Investigaciones Científicas y Técnicas, CABA, Argentina

⁵Departamento de Ingeniería Química, Facultad de Ingeniería,

Universidad de Buenos Aires, CABA, Argentina

⁶Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales,

Universidad de Buenos Aires, CABA, Argentina

⁷Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, La Plata, Argentina

Blueberries cv. Misty (2009) and cvs. Emerald, Jewel, and Misty (2010) were analyzed for mycoflora occurrence in the main production area of Argentina. Isolation frequencies and relative densities of the fungal species were statistically compared. Alternaria tenuissima was predominant and the potential presence of Alternaria toxins may pose a risk for blueberries consumption. This is the first report of Aspergillus flavus, Alternaria alternata, Alternaria vaccinii, Arthrinium phaeospermum, Cladosporium cladosporioides, Curvularia lunata, Epicoccum nigrum, Eurotium chevalieri, Fusarium graminearum, Fusarium semitectum, Fusarium verticillioides, Geotrichum candidum, Mucor racemosus, Penicillium citrinum, Trichoderma harzianum and Trichocladium spp. isolated from blueberries in Argentina.

KEYWORDS blueberry cultivars, fungi, Alternaria, mycotoxins

Address correspondence to Martin S. Munitz, Facultad de Ciencias de la Alimentación, Universidad Nacional de Entre Rios, Monseñor Tavella 1450 (CP3200), Concordia, Entre Rios, Argentina. E-mail: munitzm@fcal.uner.edu.ar

INTRODUCTION

Highbush blueberry (*Vaccinium corymbosum* L.) is a small fruit that belongs to the Ericaceae family. Its production in Argentina has been considerably increased during the last decade. The common production areas are located in Entre Ríos, Tucumán, and Buenos Aires provinces. Entre Ríos is the main production area in Argentina (Fig. 1) with more than 50% of the blueberry fields located in Concordia Department, and represents more than 50% of the blueberry local production (Bruzone, 2007). Approximately 90% of the Argentinean production is exported as fresh fruit, principally to the United States and Canada in their winter season.

This fruit has high levels of nutrients that are beneficial for health and combined with an ideal water activity and low pH value, make blueberries particularly susceptible to fungal spoilage. The fruit diseases can begin in the field by the action of plant pathogens and other fungi. However, the rot is usually produced during postharvest and storage periods. This problem implies important economic losses. On the other hand, some of these moulds could be able to generate toxins and result in a health risk for consumers. Stinson et al. (1980) found *Alternaria* metabolites (alternariol, alternariol methyl ether, altertoxins, and tenuazonic acid) in blueberries.

The most significant fruit rots of blueberry include Alternaria rot by *Alternaria tenuissima* (Cappellini et al., 1972; Cline, 1997; Cline and Milholland, 1995; Luan et al., 2007; Rivera et al., 2009; Smith et al., 1996; Wright et al., 2004, 2008a, 2008c); Botrytis blight rot or grey mould by *Botrytis cinerea* (Cappellini et al., 1972, 1983; Cappellini and Ceponis, 1977;

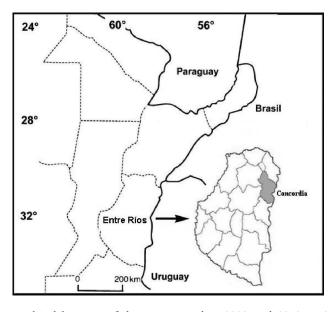


FIGURE 1 Geographical location of the zone tested in 2009 and 2010 in Concordia, Entre Ríos Province, Argentina.

Hildebrand et al., 2001; Lambert, 1990; Rivera et al., 2009; Smith, 1998; Smith et al., 1996; Tournas and Katsoudas, 2005; Vásquez et al., 2007; Wright et al., 2008a, 2008c); Anthracnose fruit rot or ripe rot caused by Colletotrichum gloeosporioides (teleomorph Glomerella cingulata) and Colletotrichum acutatum (Barrau et al., 2001; Cappellini et al., 1972; Cline, 1997; Daykin and Milholland, 1984; Guerber and Correll, 1997; Hartung et al., 1981; Lambert, 1990; Polashock et al., 2005; Rivera et al., 2009; Smith et al., 1996; Verma et al., 2006, 2007; Wharton and Schilder, 2008; Wright et al., 1998, 2008c; Yoshida et al., 2007); Phomopsis soft rot by Phomopsis vaccinii (Cline, 1997; Cline and Milholland, 1995; Gabler et al., 2004; Smith et al., 1996); and Mummy berry caused by Monilinia vaccinii-corymbosi (or Sclerotinia vaccinii-corymbosi) (Cline, 1997; Cline and Milholland, 1995; Copes et al., 2001; Cox and Scherm, 2001a, 2001b, 2001c; Lehman and Oudemans, 1997, 2000; Lehman et al., 2007; Ngugi and Scherm, 2004; Ngugi et al., 2002a, 2002b; Penman and Annis, 2005; Scherm and Copes, 1999; Scherm et al., 2001, 2004; Tarnowski et al., 2008; Wharton and Schilder, 2005). Smith et al. (1996) compared the susceptibility of several rabbiteye blueberry varieties to fungal diseases and Cappellini et al. (1972) compared three highbush blueberry cultivars. Both were carried out in the United States and differences were found between the cultivars.

In Argentina, the most common agents responsible of blueberry diseases have been identified as *Alternaria tenuissima* (Rivera et al., 2009; Wright et al., 2004, 2008a, 2008c); *Botrytis cinerea* (Rivera et al., 2009; Vásquez et al., 2007; Wright et al., 2008a, 2008c); *Dothichiza caroliniana* (Baino et al., 2007); *Pucciniastrum vaccinii* (Dal Bello and Perelló, 1998; Rivera et al., 2009; Wright et al., 2008a); *Pestalotiopsis guepini* (Rivera et al., 2009; Wright et al., 1998, 2008a); *Colletotrichum gloeosporioides* (Rivera et al., 2009; Wright et al., 1998, 2008c); *Nigrospora sphaerica* (Wright et al., 2008b); *Bipolaris cynodontis* (Rivera et al., 2009; Sisterna et al., 2009); and *Fusarium solani* (Pérez et al., 2007). Rivera et al. (2009) described other diseases produced by *Rhizoctonia solani, Botryosphaeria* spp., *Fusicoccum* spp., *Dothiorella* spp., *Phomopsis* spp., Nigrospora Sacchari, *Cylindrocladium* spp., *Curvularia* spp., *Phoma* spp., *Phytophthora* spp., *Rhizopus stolonifer*, *Aspergillus* spp., *Penicillium* spp., and *Trichoderma* spp.

The aims of this study were to identify the mycoflora present on three different commercial blueberry cultivars freshly harvested in the major Argentinean production area and to study the influence of cultivar type on fungal contamination focusing on those species able to produce mycotoxins.

MATERIALS AND METHODS

Blueberry Samples

A preliminary study to optimize the isolation and identification methodologies was carried out during December 2009 in the main production area of

blueberries in Argentina, Concordia Department, Entre Ríos province (Lat. 31° 23′ 32″ S, Long. 58° 1′ 1″ W). This production area (Fig. 1) represents more than 50% of the blueberry local production (Bruzone, 2007). Forty-two samples of the cv. Misty were collected (approximately 300 g per sample).

During 2010, ten samples each of three commercial cultivars of blueberry (Emerald, Jewel, and Misty) were collected from ten different fields each (approximately 300 g per sample), in the same zone of Concordia.

The lots (approximately 850 m²) have from 20 to 25 lines of plants with 15 to 20 bushes each. Samples were collected in zig-zag every four lines and three bushes leaving external plants that could be exposed to stress conditions.

Isolation and Identification of Fungi

For the mycoflora isolation, 100 berries per sample were cut in two pieces, eight halves per plate, and placed on disposable petri dishes (Massobact, Buenos Aires) with rose bengal-chloramphenicol agar (Biokar Diagnostic BK151, Beauvais, France) in a sterile laminar flow cabinet. This selective medium was chosen for avoiding bacterial growth (Pitt and Hocking, 2009). The plates were incubated in the dark at 28°C for 7 days and the resulting fungal colonies subcultured onto potato dextrose agar (PDA; Biokar Diagnostic BK095) and then identified. Where several fungi were isolated from a single berry, all were recorded.

Isolates of fungi were identified according to the following authorities: Ellis (1971); Klich (2002); Nelson et al. (1983); Pitt and Hocking (2009); Samson et al. (2004); and Simmons (2007).

The isolation frequency (Fr) and the relative density (RD) of genera and species were calculated according to González et al. (2008):

$$Fr(\%) = (ns/N) \times 100 \quad RD(\%) = (ni/Ni) \times 100,$$

where ns = number of samples where a fungal species occurred; N = total number of samples; ni = number of isolates of a fungal species; and Ni = total number of fungal isolates.

Statistical Analysis

Asymptotic tests for equality of proportions were used to compare relative densities (RD) of of fungal species between varieties (Devore, 1987). The Fischer exact test (Conover, 1980) was applied to analyze possible differences in the isolation frequencies (Fr) of fungal species between varieties. Significance was declared at P < 0.05 (significant differences) and P < 0.01

(highly significant differences). The analysis was performed using the Statistix 7.0 package (2000).

RESULTS AND DISCUSSION

Fungi Associated with Blueberry

Mycoflora associated with blueberries identified in Concordia during the 2009 harvest season is shown in Table 1. Based on *RD* as well as *Fr*, it can be seen that *Alternaria tenuissima*, *Colletotrichum gloeosporioides*, *Rhodotorula* spp., *Aspergillus niger*, *Epiccocum nigrum*, and *Phomopsis vaccinii* were the predominant fungi.

There is a wide variety of moulds that can grow on blueberries and are potentially mycotoxin producers. Probably these facts are due to the presence of available nutrients, an optimal water activity, and a low pH that inhibit the bacterial growth (Stinson et al., 1980).

The potentially mycotoxigenic fungi recovered from the three different blueberry cultivars during the 2010 harvested season in the same location are shown in Figure 2 and the other mycotoxigenic fungi recovered from the three cultivars in 2010 are listed in Table 2. Based on RD as well as Fr, it can be seen that $Cladosporium\ cladosporioides$ was the most prevalent fungus in Jewel cultivar, followed by $Alternaria\ tenuissima$, $Alternaria\ alternata$, $Epiccocum\ nigrum$, and $Aspergillus\ niger$. Other species recovered at low incidence levels (2 > RD% > 0.6%) were $Arthrinium\ phaeospermum$,

TABLE 1 Isolation Frequency (*Fr*) and Relative Specific Density (*RD*) of the Mycoflora Recovered from Blueberries cv. Misty Harvested in Concordia, Entre Ríos Province, Argentina in 2009

Number of isolates	1,	180	
Species	Fr (%)	RD (%)	
Alternaria alternata	31.0	3.4	
Alternaria tenuissima	100.0	18.2	
Aspergillus niger	78.6	8.9	
Aureobasidium pullulans	47.6	4.7	
Cladosporium cladosporioides	42.9	5.1	
Colletotrichum gloeosporioides	100.0	18.6	
Curvularia lunata	33.3	3.4	
Epiccocum nigrum	69.0	8.1	
Fusarium graminearum	31.0	3.8	
Fusarium semitectum	26.2	1.7	
Nigrospora sphaerica	57.1	2.1	
Penicillium citrinum	21.4	0.8	
Phomopsis vaccinii	64.3	4.7	
Rhodotorula spp.	83.3	12.3	
Trichoderma harzianum	35.7	4.2	

Fr: Frequency of isolation; RD: Relative density.

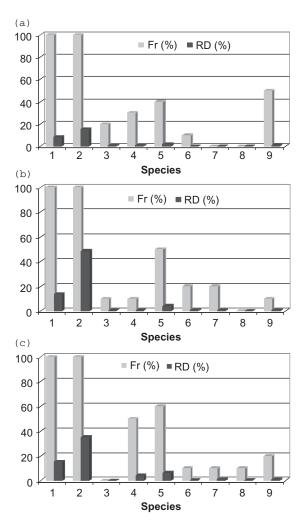


FIGURE 2 Potentially mycotoxigenic fungi recovered from blueberry cultivars: (a) Jewel; (b) Emerald; and (c) Misty, collected in Concordia, Entre Ríos province, Argentina, during 2010 harvest season. Key: 1. Alternaria alternata; 2. Alternaria tenuissima; 3. Alternaria vaccinii; 4. Aspergillus flavus; 5. Aspergillus niger; 6. Fusarium graminearum; 7. Fusarium semitectum; 8. Fusarium verticillioides; 9. Penicillium citrinum.

Trichoderma harzianum, Rhizopus stolonifer, Botrytis cinerea, Alternaria vaccinii, and Aspergillus flavus.

Alternaria tenuissima was the most prevalent species isolated from Emerald cultivar, followed by Alternaria alternata, Epiccocum nigrum, Aspergillus niger, Trichoderma harzianum, Cladosporium cladosporioides, and Phomopsis vaccinii. Other species recovered at low incidence levels were Colletotrichum gloeosporioides, Curvularia lunata, Fusarium graminearum, and Sclerotinia vaccinii.

TABLE 2 Isolation Frequency (Fr) and Relative Specific Density (RD) of Other Than Mycotoxigenic Fungi Recovered from Blueberries Harvested in Concordia, Entre Ríos Province, Argentina in 2010

Number of isolates Species	Cultivar						
	Emerald 2,705		Jewel 4,100		Misty 2,865		
	Fr (%)	RD (%)	Fr (%)	RD (%)	Fr (%)	RD (%)	
Arthrinium phaeospermum	40.0	1.5	nd	nd	nd	nd	
Aureobasidium pullulans	30.0	0.6	10.0	0.2	30.0	0.9	
Botrytis cinerea	20.0	1.4	nd	nd	20.0	0.5	
Cladosporium cladosporioides	100.0	19.2	40.0	3.3	40.0	2.1	
Colletotrichum gloeosporioides	30.0	0.4	50.0	1.7	30.0	1.4	
Curvularia lunata	40.0	0.6	40.0	0.9	10.0	0.2	
Epicoccum nigrum	100.0	4.4	80.0	8.1	100.0	13.4	
Eurotium chevalieri	10.0	0.1	10.0	0.2	nd	nd	
Geotrichum candidum	30.0	0.6	20.0	0.6	nd	nd	
Mucor racemosus	nd	nd	10.0	0.4	nd	nd	
Nigrospora sphaerica	10.0	0.4	10.0	0.4	nd	nd	
Phoma spp.	10.0	0.1	nd	nd	nd	nd	
Phomopsis vaccinii	30.0	1.0	60.0	2.2	nd	nd	
Rhizoctonia solani	10.0	0.4	nd	nd	nd	nd	
Rhizopus stolonifer	30.0	1.4	30.0	0.6	30.0	0.7	
Rhodotorula spp.	100.0	38.2	90.0	8.9	60.0	8.9	
Sclerotinia vaccinii	10.0	0.4	10.0	0.7	nd	nd	
Trichocladium spp.	nd	nd	10.0	0.2	nd	nd	
Trichoderma harzianum.	10.0	1.5	30.0	3.7	80.0	9.2	

Fr: Frequency of isolation (%); RD: Relative density (%); nd: Not detected.

Similar behavior was found in the cv. Misty, where the prevalent species was *Alternaria tenuissima*, followed by *Alternaria alternata*, *Epiccocum nigrum*, *Trichoderma harzianum*, *Aspergillus niger*, *Aspergillus flavus*, and *Cladosporium cladosporioides*. Other species recovered at low incidence levels were *Colletotrichum gloeosporioides*, *Fusarium semitectum*, *Aureobasidium pullulans*, *Penicillium citrinum*, and *Rhizopus stolonifer*. In all blueberry cultivars the only identified yeast was *Rhodothorula* spp.

Comparing the results of the present study with those observed by other authors, it can be seen that the identified genera in Argentinean blueberries were similar to those found in the bibliography, like *Alternaria* (Cappellini and Ceponis, 1977; Cappellini et al., 1983; Lambert, 1990; Tournas and Katsoudas, 2005), *Aspergillus* (Cappellini and Ceponis, 1977; Lambert, 1990; Rivera et al., 2009; Wright et al., 2008c), *Botrytis* (Smart, 1937); *Cladosporium* (Smart, 1937; Tournas and Katsoudas, 2005), *Colletotrichum* (Cline and Milholland, 1995), *Curvularia* (Rivera et al., 2009), *Epicoccum* (Cappellini et al., 1972), *Fusarium* (Cappellini et al., 1972; Rivera et al., 2009; Tournas and Katsoudas, 2005; Wright et al., 2008a), *Mucor* (Lambert, 1990; Smith et al., 1996), *Nigrospora* (Cappellini et al., 1972; Wright et al., 2008c), *Penicillium* (Cappellini et al., 1972; Lambert, 1990; Rivera et al., 2009; Smart,

1937; Tournas and Katsoudas, 2005; Wright et al., 2008c), *Phoma* (Cappellini and Ceponis, 1977; Rivera et al., 2009); *Phomopsis* (Nicolini et al., 2008; Rivera et al., 2009; Wright et al., 2008a), *Rhizopus* (Cappellini and Ceponis, 1977; Smart, 1937; Smith et al., 1996), *Rhizotocnia* (Wright et al., 2008a, 2008c) and *Trichoderma* (Cappellini et al., 1972; Lambert, 1990; Nicolini et al., 2008; Rivera et al., 2009; Tournas and Katsoudas, 2005; Wright et al., 2008c).

In reference to fungal species, similarities were found in relation to Alternaria tenuissima (Cappellini et al., 1972; Cline, 1997; Cline and Milholland, 1995; Luan et al., 2007; Rivera et al., 2009; Smith et al., 1996; Wright et al., 2004, 2008a, 2008c); Aspergillus niger (Cappellini et al., 1972), Aureobasidium pullulans (Tournas and Katsoudas, 2005), Botrytis cinerea (Cappellini and Ceponis, 1977; Cappellini et al., 1972, 1983; Hildebrand et al., 2001; Lambert, 1990; Rivera et al., 2009; Smith, 1998; Smith et al., 1996; Tournas and Katsoudas, 2005; Vásquez et al., 2007; Wright et al., 2008a, 2008c), Colletotrichum gloeosporioides (Cappellini et al., 1972; Daykin and Milholland, 1984; Hartung et al., 1981; Lambert, 1990; Rivera et al., 2009; Smith et al., 1996; Wright et al., 1998, 2008c), Monilinia vaccinii-corymbosi (Cline, 1997; Cline and Milholland, 1995; Copes et al., 2001; Cox and Scherm, 2001a, 2001b, 2001c; Lehman and Oudemans, 1997, 2000; Lehman et al., 2007; Ngugi and Scherm, 2004; Ngugi et al., 2002a, 2002b; Penman and Annis, 2005; Scherm and Copes, 1999; Scherm et al., 2001, 2004; Tarnowski et al., 2008; Wharton and Schilder, 2005), Nigrospora sphaerica (Wright et al., 2008b); Phomopsis vaccinii (Cline, 1997; Cline and Milholland, 1995; Gabler et al., 2004; Smith et al., 1996), Rhizopus stolonifer (Cappellini et al., 1972; Rivera et al., 2009), and Rhizoctonia solani (Rivera et al., 2009).

Based on an extensive analysis of the literature, in Argentina there are no previous references about *Alternaria alternata*, *Alternaria vaccinii*, *Arthrinium phaeospermum*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *Curvularia lunata*, *Epicoccum nigrum*, *Eurotium chevalieri*, *Fusarium graminearum*, *Fusarium semitectum*, *Fusarium verticillioides*, *Geotrichum candidum*, *Mucor racemosus*, *Penicillium citrinum*, *Trichoderma harzianum*, and *Trichocladium* spp., isolated from blueberries.

Some fungi that were quoted for the first time in Argentinean blueberries were not detected in 2009 and 2010 in Concordia. Baino et al. (2007) reported *Dothichiza caroliniana* causing a foliar disease affecting Jewel, Emerald, and O'Neal cultivars. In 1998, Dal Bello and Parelló described for the first time leaf rust caused by *Pucciniastrum vaccinii* in Bluegold cultivar. The same year, Wright et al. reported *Pestalotiopsis guepini* in several cultivars causing stem blight and fruit rot. Hongn et al. (2007) isolated *Corynespora casiicola* of blueberry leafs. Sisterna et al. (2009) identified blueberry plants with dieback, and bud and branch blight caused by *Bipolaris cynodontis*. Rivera et al. (2009) described *Nigrospora sacchari* responsible of stem blight. Pérez et al. (2007) evaluated the occurrence of *Fusarium solani*, responsible

for root rot in Misty and Sharp Blue cultivars; this study was made in the same region (Concordia Department) with Misty cultivar. In the present work, this *Fusarium* species was not detected because it produces a root disease and probably did not occur as berries contamination.

Performing the asymptotic tests, significant differences (P < 0.05) were found when comparing the RDs between 2009 and 2010 harvests of Misty cultivar for *Aspergillus niger* (Table 3). Highly significant differences (P < 0.01) were found for all the other fungi except for *Fusarium semitectum* and *Penicillium citrinum*.

Aspergillus flavus, Botrytis cinerea, Fusarium verticillioides, and Rhizopus stolonifer were not isolated in 2009, and Nigrospora sphaerica and Phomopsis vaccinii were not isolated in 2010. The comparison between the species Fr using the Fisher exact test is shown in Table 3. Highly significant differences (P < 0.01) were found for Alternaria alternata and Colletotrichum gloeosporioides. The differences in the contamination levels found in this work between both harvest seasons may be explained by the weather conditions registered in Concordia, principally rainfalls, but this should be studied thoroughly over several harvested seasons.

TABLE 3 P Values for the Comparison between Mycoflora Contamination of Highbush Blueberry cv. Misty Harvested during 2009 and 2010 in Concordia Department, Entre Ríos Province, Argentina

Species	P value			
	RD comparison	Fr comparison		
Alternaria alternata	0.0001	0.0001		
Alternaria tenuissima	0.0001	1.0000		
Aspergillus flavus	0.0001	0.001		
Aspergillus niger	0.0295	0.4186		
Aureobasidium pullulans	0.0001	0.4815		
Botrytis cinerea	0.0002	0.0339		
Cladosporium cladosporioides	0.0001	1.0000		
Colletotrichum gloeosporioides	0.0001	0.0001		
Curvularia lunata	0.0001	0.1316		
Epiccocum nigrum	0.0001	0.0970		
Fusarium graminearum	0.0001	0.2536		
Fusarium semitectum	0.0994	0.4199		
Fusarium verticillioides	0.0166	0.1923		
Nigrospora sphaerica	0.0001	0.0010		
Penicillium citrinum	0.8759	1.0000		
Phomopsis vaccinii	0.0001	0.0002		
Rhizopus stolonifer	0.0001	0.0054		
Rhodotorula spp.	0.0019	0.1897		
Trichoderma harzianum	0.0001	0.0154		

Fr: Frequency of isolation (%); RD: Relative density (%).

P < 0.05: differences are significant.

P < 0.01: differences are highly significant.

The comparison between Fr of the three cultivars studied in 2010 shows that significant differences (P < 0.05) exist for *Cladosporium cladosporioides* comparing Jewel and Emerald, and Jewel with Misty. Highly significant differences (P < 0.01) were observed for *Trichoderma harzianum* between Jewel and Misty cultivars.

Comparing the most important possible mycotoxigenic fungi isolated, significant differences (P < 0.05) were observed in RDs between Emerald and Misty cultivars for Fusarium graminearum, and highly significant differences (P < 0.01) between Jewel and Misty cultivars for Alternaria alternata, Alternaria tenuissima, Aspergillus flavus, and Aspergillus niger; between Jewel and Emerald cultivars for Alternaria alternata and Alternaria tenuissima; and between Emerald and Misty cultivars for Alternaria tenuissima and Aspergillus flavus. The Emerald and Misty cultivars presented the lowest contamination levels in comparison to Jewel cultivar (Table 2) and that probably may represent a difference in their resistance to fungal invasion.

CONCLUSIONS

This study provides information on the natural mycoflora present on three commercial blueberry cultivars freshly harvested in the main production area in Argentina during 2010, and the comparison of one of them with the fungal contamination observed during 2009 harvest season.

The high incidence of *Alternaria tenuissima* and *A. alternata* isolates in the mycoflora of the 2009 and 2010 harvests should be a matter of concern and the natural occurrence of *Alternaria* toxins in the Argentinean blueberries needs to be studied. Other fungal species associated with blueberry in Argentina that should be taken into account because of their toxigenic potential and prevalence include *Alternaria vaccinii*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium graminearum*, *Fusarium semitectum*, and *Penicillium citrinum*. Members of the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma* that were isolated, are capable of producing mycotoxins, for example, *Aspergillus flavus* (aflatoxins and ciclopiazonic acid); *Aspergillus niger* (ochratoxins and fumonisins); *Fusarium verticillioides* (fumonisins); *Fusarium graminearum* and *Fusarium semitectum* (trichothecenes); and *Penicillium citrinum* (citrinin). The presence of these fungal toxins in this commodity should be determined and monitored.

Based on a thorough analysis of the available literature, this is the first time that the following fungi are reported in Argentinean blueberries: Aspergillus flavus, Alternaria alternata, Alternaria vaccinii, Arthrinium phaeospermum, Cladosporium cladosporioides, Curvularia lunata, Epicoccum nigrum, Eurotium chevalieri, Fusarium graminearum, Fusarium semitectum, Fusarium verticillioides, Geotrichum candidum,

Mucor racemosus, Penicillium citrinum, Trichoderma harzianum, and Trichocladium spp.

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