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Fungal screening and aflatoxin production by *Aspergillus* section *Flavi* isolated from pre-harvest maize ears grown in two Argentine regions

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ABSTRACT

Aflatoxin contamination in maize kernels grown in central Argentina has increased since 2008. Pre-harvest maize ear samples were collected during two growing seasons to determine prevalent fungal genera as well as aflatoxin production capacity by *Aspergillus* sect. *Flavi* in two maize regions. A direct planting procedure was performed for fungal screening. *Aspergillus* section *Flavi* was identified via a morphological analysis and some colonies were isolated. Strains were subjected to molecular analysis and aflatoxin production was studied. *Fusarium* was the fungus genus with the highest severity, followed by *Penicillium* and *Aspergillus* in both regions. *Aspergillus* sect. *flavi* were detected in 27 (73%) samples. PCR amplifications were observed in 94% of the isolates. *Aspergillus* sect. *flavi* severity varied significantly between years. A great proportion of toxigenic strains were observed in a native *Aspergillus* sect. *flavi* population (95%) producing B- and G-type aflatoxins. Maize ears grown in regions I and IV in Argentina are prone to degradation by fungi. Toxigenic strains are predominant in native *Aspergillus* sect. *Flavi* populations. Aflatoxin contamination of maize kernels is not restricted to post-harvest; rather, it is a serious issue that begins with colonization by *Aspergillus* sect. *Flavi* in ears.

1. Introduction

Maize (*Zea mays* L.) is widely grown in Argentina in nine regions (I to IX) under different agro-ecological conditions (INTA, 1997). In regions I and IV, the crop production has greatly increased due to expansion of the agricultural frontier and the adoption of technology (Viglizzo et al., 2012).

Environmental conditions influence the fungal colonization of maize ears during flowering (Diener, 1989; Perrone et al., 2014b). This period usually spans December to March in Argentina, and varies among planting regions (Cirilo, 2004). Fungi may affect maize directly by mechanical damage causing yield losses, or indirectly, by secreting and spreading mycotoxins (Iheanacho et al., 2014). *Fusarium* Link. is the most important pathogenic fungal genus causing spoilage of maize, with *F. verticillioides* (Sacc.) and *F. graminearum* Schwabe Nirenberg being the most common species frequently isolated from maize kernels (Etcheverry et al., 1999; Presello and Botta, 2004). *Penicillium* Link species are also present in maize ears and produce a wide range of secondary metabolites, including ochratoxin, citrinin and scalonic acid D (Pitt and Hocking, 2009). Aflatoxins are secondary metabolites produced in nature by several *Aspergillus* Fr.:Fr. species. *A. flavus* Link and *A. parasiticus* Speare are the most common species associated with aflatoxin contamination in crops (Perrone et al., 2014a). Recently, other species of *Aspergillus* section *Flavi* were reported as responsible for aflatoxin contamination in maize (Perrone et al., 2014b; Soares et al., 2012). Previous studies in Argentina indicated the presence of *Aspergillus* sect. *Flavi* in stored maize grains as well as in soil, debris and insects from maize fields (Nesci and Etcheverry, 2002; Nesci et al., 2006).

Maize regions I and IV are characterized by extreme temperatures and hot dry weather during the maize growing period (SMN,

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2015). The *Aspergillus* sect. *Flavi* population increases in crops grown under these climate conditions (Bhatnagar-Mathur et al., 2015). High levels of aflatoxins in maize grown in regions I and IV have been reported by the Argentinian maize industry. More than 30% of the crop exceeds the limit of 20 parts per billion of total aflatoxins established by international regulations (personal communication, June 9, 2011).

Some *Aspergillus* species are similar and identification to species level using schemes based on morphological characteristics requires expertise. Effective management of disease caused by *Aspergillus* species requires proper identification of the fungal pathogen (Gherbawy and Voigt, 2010). Molecular identification of fungi was reported to be a reliable method to confirm the identity of strains previously identified using the conventional method based on morphological characteristics (Iheanacho et al., 2014). Devi et al. (2013) developed a marker for accurately differentiating *A. flavus* isolates from other *Aspergillus* species by polymerase chain reaction (PCR). This primer pair has not been tested in strains obtained from Argentine crops.

Populations of *Aspergillus* sect. *Flavi* exhibit extremely diverse toxigenic characteristics. *A. flavus* produces B-type aflatoxins (B_1 and B_2) and often cyclopiazonic acid (CPA) depending on the geographic origin, substrate, genotype and environmental conditions. *A. parasiticus* produces G-type aflatoxins (G_1 and G_2) in addition to B-type aflatoxins (Perrone et al., 2014b; Vaamonde et al., 2003). Recently, new aflatoxigenic species that share morphological characters with *A. flavus* were reported to produce both B-type and G-type aflatoxin but the exact taxonomic designations remain unclear (Donner et al., 2009; Perrone et al., 2014b). Vaamonde et al. (2003) proposed classifying *Aspergillus* sect. *Flavi* isolated from several Argentine crops into groups according to the production of B- and G-type aflatoxins. All aflatoxins are teratogenic and carcinogenic, with Aflatoxin B1 being the metabolite with the highest toxigenic effect on humans and animals (Peraica et al., 1999).

An appropriate disease management strategy to reduce the presence of mycotoxins in maize crops requires knowledge about the incidence and toxicity of *A. flavus* strains present in ears. Kernels infected during the growing season remain as inoculum. Under improper storage conditions, fungi grow and aflatoxin contamination increases during the post-harvest period (Bhatnagar-Mathur et al., 2015; Williams et al., 2011). In order to develop disease management strategies for maize regions I and IV in Argentina, the aims of this work were to: study the severity of the main toxigenic genera (*Fusarium*, *Penicillium* and *Aspergillus*) present in corn ears; record the severity of *Aspergillus* sect. *Flavi* identified using morphological criteria; molecularly characterize native *Aspergillus* sect. *Flavi* strains by PCR; estimate the percentage of toxigenic *Aspergillus* sect. *Flavi* strains, and quantify their aflatoxin production.

2. Materials and methods

2.1. Sampling

Samples of different commercial maize cultivars were collected from the maize regions I and IV in Argentina during the 2012/13 and 2013/14 growing seasons (Fig. 1) (INTA, 1997). Region I lies approximately between latitudes 20°00′ and 30°00′ S, with average maximum temperatures varying from 30 to 34 °C during the maize flowering period. Region IV is located approximately between latitudes 30°00′ and 34°00′S, with average maximum temperatures ranging from 26 to 30 °C during the same period. Both regions are located between longitudes 62°00′and 67°00′W, where rainfall is between 200 and 500 mm during the flowering period (SMN, 2015). Weather conditions varied remarkably between the growing seasons studied (Table 1) (INTA, 2016). Ten ears were randomly selected from fields at the end of the growing season, immediately before harvest. Samples were transported in paper bags and dried in a forced air oven (38 °C for 72 h) to reduce moisture content to below 12%. Samples were stored at 4 °C until analysis (Oliveira Rocha et al., 2012; Smart et al., 1990).

2.2. Fungal screening

A direct planting procedure for internal infestation was carried out to determine the main fungal genera present in maize ears (Pitt and Hocking, 2009). Sample grains (200 grains/sample), obtained by threshing of ears, were surface-disinfected by dipping in 1% sodium hypochlorite solution for 5 min and rinsed three times in sterile water. Each sample was analyzed in two culture media, Dichloran Rose-Bengal Chloramphenicol Agar (DRBC) and Dichloran Glycerol Agar (DG18) (Merck, Darmstadt, Germany), using 10 Petri dishes per medium. Ten grains per dish were planted directly on the medium surface and incubated at 25 ± 2 °C in darkness for 7 days (Giorni et al., 2007). After the growing period, samples were examined and the number of grains with fungus colonies exhibiting morphological characteristics consistent with those of *Aspergillus*, *Penicillium*, *Fusarium* and other fungus species was counted (Perrone et al., 2014b). Results are expressed as severity (percentage of infected grains) and incidence (percentage of infected samples).

2.3. Morphological identification of Aspergillus sect. Flavi

Spores from colonies that looked like *Aspergillus* sect. *Flavi* were transferred and sub-cultured on malt extract agar medium (MEA) at 25 °C in darkness during 7 days for further identification (Nesci and Etcheverry, 2002). *Aspergillus* sect. *Flavi* species were determined by observing colony characteristics and conidial morphology, following taxonomic schemes proposed by Pitt and Hocking (2009). The presence of species of *Aspergillus* sect. *Flavi* was expressed as severity and incidence. Colonies were isolated for further molecular characterization and determination of aflatoxin production.

2.4. Molecular characterization of Aspergillus sect. Flavi isolates

2.4.1. Fungal DNA extraction

Culture medium (20% potato leachate; 2% sucrose; pH 4.5) was prepared and transferred (100 mL) to 250-mL flasks. Spore suspensions were prepared from previously grown *Aspergillus* sect. *Flavi* isolates (at 25 °C in darkness for 7 days) and added to the medium (1×10^5 conidia/mL). Flasks were placed on a shaker at 250 rpm and incubated at 25 °C for 48 h. The mycelia were harvested by filtration through Whatman N° 1 filter, rinsed three times with sterile water and stored lyophilized at −40 °C (Devi et al., 2013). DNA was extracted following the procedure described by Doyle and Doyle (1990). *A. niger* isolated from maize kernels was used as a negative control. *A. flavus* 114116 provided by the Instituto Nacional de Enfermedades Infecciosas (Buenos Aires, Argentina) was used as a positive control. *A. parasiticus* NRRL 2999 provided by Universidad Nacional de Rosario (Santa Fe, Argentina) was also tested as an additional control in order to determine if the primer studied is suitable for distinguishing this species from *A. flavus*.

2.4.2. Primers and PCR conditions

The primers used in the reaction had the following nucleotide sequence: Asp f1, 5′-CCCGTGAAGTTGCCCAGGT-3'; Asp r2, 5′-GTCGTTTGGTGAGTGGGAA-3′ (Devi et al., 2013). Reagents were

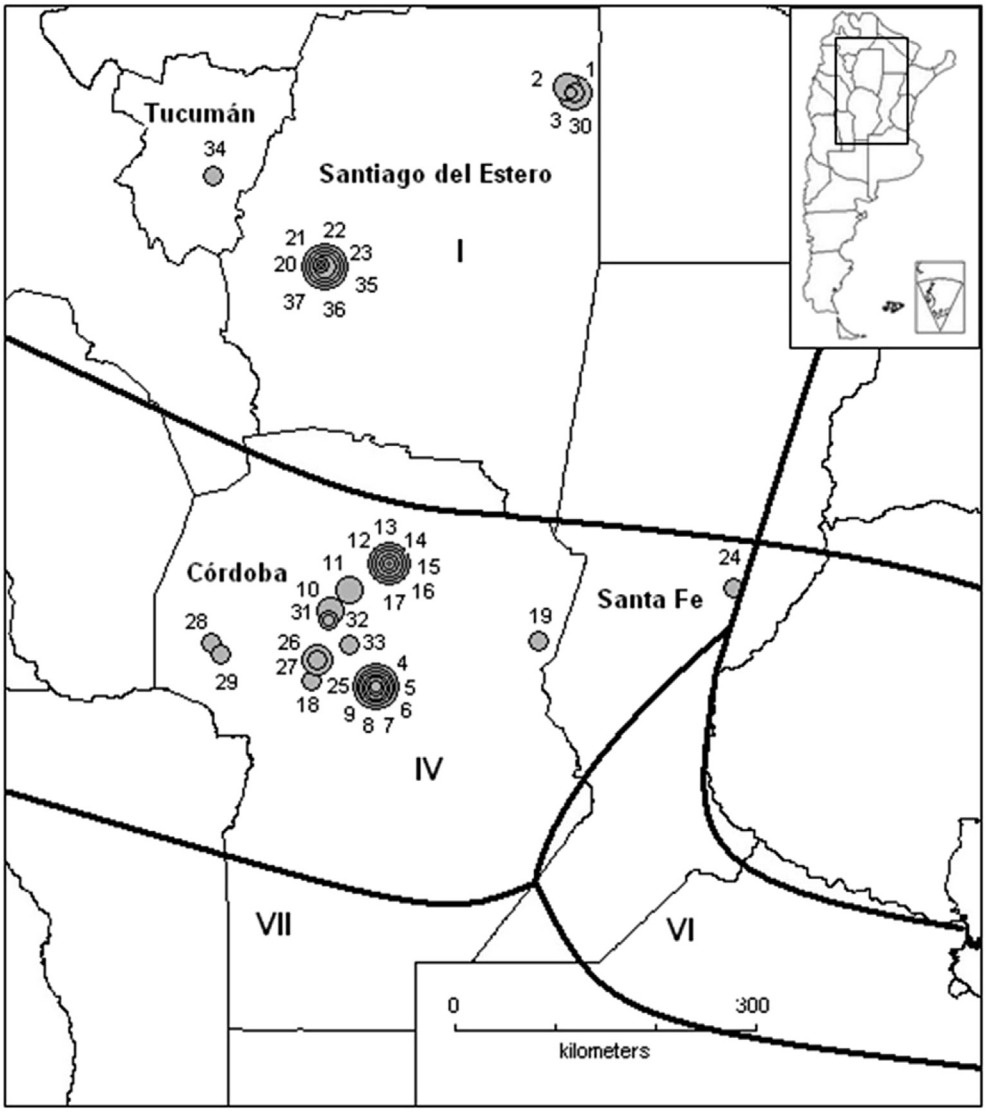

Fig. 1. Maize growing regions of Argentina, extracted from INTA (1997), and location of samples collected during the 2012/13 and 2013/14 growing seasons. I and IV indicate the regions studied.

Table 1
Weather conditions in the maize region during the flowering period in the two growing seasons.

Growing seasons	Temperature (°C) [a]				Precipitation (mm) [b]	
	Maximum		Minimum			
	I	IV	I	IV	I	IV
2012/2013	37.5	31.3	21.8	16.3	134.8	279.9
2013/2014	35.8	33.3	21.6	17.8	719.6	455.5

[a] Mean values obtained from data registered during the period from December to March.
[b] Total precipitation registered during the period from December to March.

purchased from Promega (Madison, WI, USA). For each individual reaction, 1 μL (20–100 ng DNA/μL final concentration) of DNA sample was mixed with 24 μL of PCR solution containing 5 μL of 5 × Green GoTaq® Reaction Buffer, 0.5 μL of dNTP mixture (10 nM of dATP, dCTP, dGTP and dTTP), 1 μL of the primers (2.5 μM), 1 μL of GoTaq® DNA Polymerase, and 16.375 μL of Nuclease Free Water. PCR cycling was carried out in a thermocycler, according to Devi et al. (2013), with some modifications: one cycle at 94 °C for 4 min, 40 cycles at 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1.5 min, with a final extension at 72 °C for 11 min. PCR products were electrophoresed and observed under UV illumination. Molecular sizes were estimated with 100bp DNA ladder marker.

2.5. Aflatoxin production by Aspergillus sect. Flavi

Aflatoxin was extracted using the methodology proposed by Trucksess et al. (1994). Eppendorf tubes containing 1 mL of YES medium (20% sucrose, 2% yeast extract, pH 5.9) were inoculated with conidial suspension to obtain 10^5 spores/mL and incubated at 30 °C in darkness during 7 days. The extraction was carried out by adding 1 mL of chloroform and shaking vigorously for 1 min. The chloroform phase was extracted and evaporated. Samples were resuspended in methanol/water (70:30) and filtered through a 0.22-μm nylon filter before analysis. Aflatoxin identification and quantification was performed using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS), according to Arroyo-Manzanares et al. (2015) with

some modifications. The analyses were conducted in a Thermo scientific ™ system consisting of a degasser, quaternary pump, column oven and an LTQ XL™ ion trap mass spectrometer. Chromatographic separations were performed with a C18 100 × 2.1 mm Hypersil™ ODS (5 μm particle size) column. A solution of ammonium formate in acetonitrile (10 mM) was used as a mobile phase. Samples (10 μL) were analyzed at a flow rate of 0.2 mL/min at 45 °C. Aflatoxin standards were purchased from Sigma-Aldrich (San Luis, MO, USA) and standard curves were calculated with different levels of aflatoxin B_1, B_2, G_1 and G_2. Limits of detection and limits of quantification were defined as the concentration at which the signal-noise ratio was close to 3 and 10, respectively (Rubert et al., 2012).

2.6. Statistical analysis

Data were analyzed using InfoStat software version 2014 (Di Rienzo et al., 2014). Mean values were obtained via ANOVA ($P < 0.05$) and significant differences between means were determined using Fisher's LSD test. Aflatoxin concentration data were transformed using the equation $y = \log10$ (1+μg of aflatoxin per g of mycelium) to homogenize variances (Atehnkeng et al., 2008).

3. Results and discussion

3.1. Fungal screening

The occurrence of *Aspergillus*, *Penicillium* and *Fusarium* genera in kernels was assessed in 37 samples (Table 2). The genus *Fusarium* exhibited the highest incidence and severity in both regions. This is partially explained because most Fusarium species commonly present in maize ears have an optimum growth temperature near 25 °C (Pitt and Hocking, 2009). The predominance of *Fusarium* was also indicated by Aguaysol et al. (2013), who reported a similar severity (50%) in maize kernels of different genotypes grown in region I during the 2011/12 growing season. *Fusarium* predominance was also reported in stored kernels in Australia and Saudi Arabia, but with a lower severity (close to 30%) (Burgess et al., 1981; Mahmoud et al., 2013). *Fusarium* severity did not show significant differences ($P < 0.05$) between either regions or growing seasons. *Penicillium* showed a remarkable incidence. In region I, *Penicillium* severity was significantly higher ($P < 0.05$) in the 2013/14 growing season than in the 2011/12 growing season. However, no significant differences were observed between growing seasons in region IV. Our results are consistent with findings reported by Mahmoud et al. (2013), who mentioned *Penicillium* as one of the prevalent fungal genera in maize and sorghum kernels, with severities ranging from 13 to 15%. By contrast, high *Penicillium* severity values (mean = 67%) were recorded in stored maize kernels in Argentina (Etcheverry et al., 1999). *Aspergillus* contamination was observed in this study, without significant differences ($P < 0.05$) between maize regions. *Aspergillus* severity was significantly higher ($P < 0.05$) during the 2012–2013 growing season than during the previous season. Etcheverry et al. (1999) reported high *Aspergillus* severity values (close to 60%) in stored maize kernels in Argentina. Data from Nigerian markets indicated *Aspergillus* as the predominant genus, with similar severity in unprocessed maize kernels (Atehnkeng et al., 2008; Donner et al., 2009).

We also recorded the presence of the genera *Nigrospora*, *Eurotium*, *Cladosporium*, *Ulocladium*, *Verticillium*, *Trichoderma*, *Rhizopus*, *Absidia*, *Bispora*, *Alternaria* and *Basipetospora*, with severities ranging between 1 and 2%. *Trichoderma*, *Cladosporium* and *Alternaria* species were indicated as part of the soil fungal population in a pre-harvest maize ecosystem in region IV (Nesci et al., 2006). The presence of *Trichoderma* species in the kernel econiche suggests their potential use for biocontrol of *A. flavus* (Atehnkeng et al., 2008). *Eurotium* spp. were found to be common in stored maize kernels, while *Ulocladium* and *Absidia* were present at low severity (1%) in stored maize kernels (Pitt and Hocking, 2009). These authors isolated *Nigrospora* and *Rhizopus* from stored maize kernels in Thailand and the Philippines, with an average severity of 2%. The present work is the first report of the isolation of *Bispora, Basipetospora* and *Verticillium* species from maize ears grown in Argentina.

3.2. Incidence and severity of Aspergillus sect. Flavi

Aspergillus sect. *Flavi* was detected in 27 of the 37 samples collected in both regions (Table 3). Significant differences were observed in *Aspergillus* sect. *Flavi* incidence and severity between growing seasons. All samples (24) collected from different geographical regions during the 2012–2013 growing season were infected with *Aspergillus* sect. *Flavi*. Severity ranged from 0.5 to 48.0% (median = 0.5%) in samples from region I, and from 0.5 to 3.5% (median 1.5) in samples from region IV, with mean values of 8.3 and 1.4%, respectively. Of the 12 samples collected in the

Table 2
Incidence and severity of three fungal genera in samples taken from maize ears from two maize production regions of Argentina during the 2012–2013 and 2013–2014 growing seasons.

Growing season	Region I				Region IV			
	Incidence[b]	Severity[c]			Incidence[b]	Severity[c]		
		Mean	Median	Range		Mean	Median	Range
Fusarium[a]								
2012–2013	100.0	58.4a	61.1	29.0–98.0	100.0	60.7a	75.0	4.0–95.5
2013–2014	100.0	72.1a	59.0	50.0–100.0	100.0	72.5a	70.5	32.0–100.0
Penicillium[a]								
2012–2013	75.0	6.1b *	1.0	0.0–35.5	100.0	33.0a *	25.5	1.0–69.5
2013–2014	100.0	24.7a	22.0	11.0–47.0	87.5	20.2a	19.5	0.0–51.0
Aspergillus[a]								
2012–2013	100.0	10.73a	4.0	0.5–48.0	100.0	13.5a	10.6	0.5–61.0
2013–2014	20.0	1.0b	0.0	0.0–5.1	25.0	0.5b	0.0	0.0–2.0

The same letter within a column for the dependent variable indicates no significant differences between growing seasons for each fungus genus ($P < 0.05$). (*) indicates that differences between regions were significant ($P < 0.05$).
[a] Fungal genera studied: *Fusarium*, *Penicillium* and *Aspergillus*.
[b] Incidence: percentage of infected samples (n = 25 in region I; n = 12 in region IV).
[c] Severity: percentage of infected kernels (n = 200).

Table 3
Severity of *Aspergillus* sect. *Flavi* and strains isolated from maize ears collected from two maize production regions of Argentina during the 2012–2013 and 2013–2014 growing seasons.

Sample	Region[a]	Growing season	Maize genotype	Severity (%)[b]	Strain isolated
1	I	2012–2013	DS 120 PW	0.5	40
2			DS 120 PW	2.1	103, 104, 102, 38
3			DK 747 VT3P	48.0	–
20			P 1780HR/Y	0.5	–
21			P 3115H	0.5	118
22			DK 747 VT3P	6.5	41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53
23			P 30F35H/HR	0,5	116
30		2013–2014	DK 747 VT3P	0.0	–
35			P 1780HR/Y	0.0	–
36			P 3115H	5.0	–
37			P 30F35H/HR	0.0	–
34			NK 138 TD MAX	0.0	–
4	IV	2012–2013	P 2053Y/YR	2.0	78, 80, 77, 76
5			L4674xL4671	0.5	81
6			P 1780HR/Y	1.0	123, 125, 126
7			P 1780HR/Y	1.0	128, 127
8			AX 852 MGRR	2.0	82, 85, 86
9			AX 852 MGRR	1.5	129, 130
10			DK 747 VT3P	1.0	124
11			SPS 2756 TD Max	3.5	87, 94, 93, 90, 96, 95
12			ACA 468 MGRR2	1.5	119, 120
13			LT 632 MGRR2	2.0	97, 98, 99, 100
14			Unknown	1.0	121, 122
15			DM 2749MGRR2	2.0	112, 114, 115
16			DS 120 PW	1.5	105, 106, 108,
17			DK 747 VT3 Pro	2.0	39, 74, 75, 76
18			AG 9008 TD Max	0.5	111
19			MC 210	0.5	109
24			Unknown	1.0	70, 71
25		2013–2014	P 31Y05H/HR	0.0	–
26			DS 510 PW	1.0	34, 33
27			DS 510 PW	0.0	–
28			LT 611 MGRR2	0.0	–
29			P 31Y05H/HR	2.0	–
31			DK 747VT3 Pro	0.0	–
32			DK 747 MGRR2	0.0	–
33			SW 5148, SW 5160, SW 5147	0.0	–

[a] I and IV: maize production regions in Argentina.
[b] Severity: percentage of infected kernels (n = 200).

2013–2014 growing season, only three were contaminated with *Aspergillus* sect. *Flavi*. Wicklow et al. (1998) reported an average severity of *Aspergillus* sect. *Flavi* of 2% over four years in pre-harvest kernels grown in Illinois (USA). The variation in *Aspergillus* sect. *Flavi* severity is explained by the difference in precipitation between growing seasons. High *Aspergillus* infections are expected in maize ears when droughts occur during the flowering period (Presello and Botta, 2004). Although maize grown in high-temperature regions has increased susceptibility to *Aspergillus* sect. *Flavi* infection during the flowering period (Atehnkeng et al., 2008), no significant differences ($P < 0.05$) in incidence or severity were found between regions I and IV. This lack of differences may be due to the rainfall distribution, which was similar in both regions. The presence of *Aspergillus* sect. *Flavi* in maize ears occurs mainly due to colonization through the stylar canal. Wounding by insects provides additional infection courts and increases vulnerability of kernels to fungal growth (Diener, 1989). Maize debris, soil and insects in maize agro-ecosystems from region IV were reported as a source of inoculum because they were found to contain a high frequency of *Aspergillus* sect. *Flavi* spores (Nesci and Etcheverry, 2002; Nesci et al., 2006). This fungus can grow under environment conditions of low water activity, infecting kernels at post-harvest (Presello and Botta, 2004). As a result, high severity of *Aspergillus* sect. *Flavi* was reported in stored maize kernels from Argentina (Sepúlveda and Piontelli, 2005). Previous studies indicated that *Aspergillus* sect. *Flavi* present in stored maize in Argentina was composed of *A. flavus* (78%) and *A. parasiticus* (22%) (Etcheverry et al., 1999). Other authors (Atehnkeng et al., 2008; Donner et al., 2009; Perrone et al., 2014b) indicate that *A. flavus* is the main species (over 90% of isolates) of *Aspergillus* sect. *Flavi* colonizing maize worldwide.

3.3. Molecular characterization of Aspergillus sect. Flavi isolates

Primers reported by Devi et al. (2013) were used to characterize local *Aspergillus* sect. *Flavi* strains isolated from maize ears. A total of 66 isolates identified using morphological characteristics (Table 3) were analyzed by PCR. The PCR produced an amplicon of 490 bp (Fig. 2) in 62 *A. flavus* strains as well as in the *A. parasiticus* strain used primarily as an additional control (Table 4). The negative control, *A. niger*, and four strains did not show the expected band. Although the sequence analyzed encodes the HATPase_c domain protein of *A. flavus* (Wang et al., 2012), PCR using these primers was not able to distinguish *A. flavus* from *A. parasiticus* in local strains. These species are morphologically similar (White, 1999). Several isolates previously misidentified as *A. flavus* were reassigned to *A. parasiticus* by Hesseltine et al. (1970). Nevertheless, this PCR assay was useful to differentiate these species of *Aspergillus* sect. *flavi* from other *Aspergillus* species isolated from maize. Molecular identification has become the most usual tool to confirm fungal identity (Iheanacho et al., 2014).

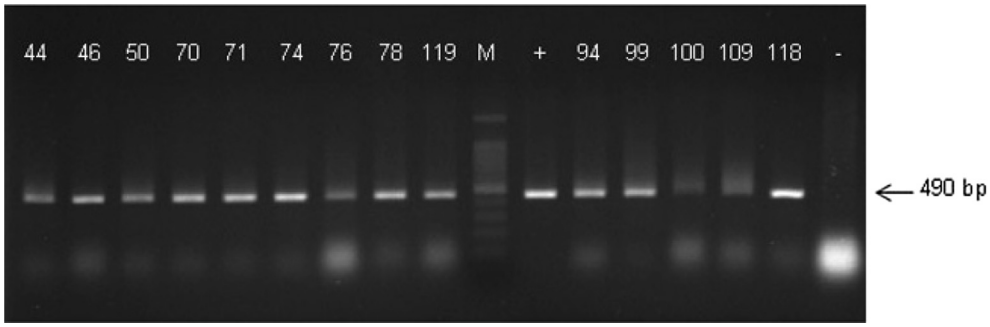

Fig. 2. Gel electrophoresis image showing PCR products. M: molecular weight marker (100 bp DNA Ladder); +: *Aspergillus flavus* 114116 (positive control); -: *Aspergillus niger* (negative control); 44, 46, 50 and 118: *Aspergillus* sect. *Flavi strains* isolated from region I;70, 71, 74, 76, 78, 94, 99, 100, 109 and 119: *Aspergillus* sect. *Flavi strains* isolated from region IV.

3.4. Aflatoxin production by Aspergillus sect. Flavi

Aflatoxin production varied among ***Aspergillus* sect. *Flavi*** strains, as indicated by the results of the quantification of aflatoxin B_1, B_2, G_1 and G_2 (Table 4).

Aspergillus sect. *Flavi* strains were classified into chemotypes based on the combination of the aflatoxins produced, according to Vaamonde et al. (2003) (Table 5).

Only three strains (4.5%) isolated from regions I and IV in both growing seasons did not produce any detectable aflatoxins, and were therefore determined as atoxigenic. Nowadays, PCR methods are commonly used for identifying and monitoring indels on the aflatoxin biosynthetic pathway in order to confirm atoxigenicity in *Aspergillus* sect. *Flavi* strains (Callicott and Cotty, 2015). Interest in studying the presence of atoxigenic strains in *Aspergillus* sect. *Flavi* populations has increased recently because of their potential use as biological control agents. These strains may displace toxigenic strains present in crop environments, reducing the risk of aflatoxin contamination (Perrone et al., 2014a). The atoxigenic *A. flavus* strain AF36 is registered in USA to be used in cotton, maize and pistachio (Mehl and Cotty, 2010). Similar studies were carried out in maize fields in Africa, achieving up to 95% of aflatoxin reduction (Atehnkeng et al., 2014).

A remarkable proportion (79.0%) of *Aspergillus* sect. *Flavi* strains were classified as chemotype I (B- and G-aflatoxin producer). "Typical" *A. flavus* strains were characterized as producers of only B-type aflatoxins due to deletions in the G-type aflatoxin biosynthesis cluster. On the other hand, *A. parisiticus* strains produce both types of aflatoxins (Perrone et al., 2014a; Probst et al., 2012). However, "atypical" strains that share morphological characters with *A. flavus* and produce both aflatoxins B and G have been reported in the southern hemisphere in Nigeria, Thailand, Argentina, and Australia (Pildain et al., 2004; Probst et al., 2012; Vaamonde et al., 2003; Varga et al., 2009). Probst et al. (2012) confirmed the sequence alignment of these strains with the full gene required for G-type aflatoxin production. These strains were also characterized by producing abundant S-type sclerotia (<400 μm) (Perrone et al., 2014a; Probst et al., 2012). Thus, Saito and Tsuruta (1993) suggested these strains as a new variety, named *A. flavus* var. *parvisclerotigenus*; more recently, Pildain et al. (2008) assigned a novel phylogenetically distinct group called *A. minisclerotigenes* for these species, and Donner et al. (2009) named this population s S_{BG}. However, the exact taxonomic designation of this population still remains unclear (Perrone et al., 2014a). Total aflatoxins had a mean of 59.78 μg g^{-1} (range = 0–747 μg g^{-1}); this result supports findings reported by Probst et al. (2012), who indicated that *Aspergillus* sect. *Flavi* strains producing B- and G-type aflatoxin generate high aflatoxin levels (over 10 μg g^{-1}).

On the other hand, data indicated the presence of a great number of toxigenic strains in *Aspergillus* sect. *Flavi* populations from regions I and IV (95.0 and 95.5%, respectively) (Table 6).

There are no previous data about the toxigenicity of these populations in maize kernels grown in these regions of Argentina. A similar study was carried out in peanut samples from region IV by Vaamonde et al. (2003), who reported that more than 70% of *Aspergillus* sect. *Flavi* strains produce aflatoxins. By contrast, Donner et al. (2009) indicated that 61% of *Aspergillus* sect. *Flavi* strains present in Nigerian maize were atoxigenic. Cotty (1997) suggested that, in low-latitude zones with high annual average temperature, toxigenic strains are more frequently isolated than non-toxigenic strains. Thus, Wei et al. (2014) reported that the severity of atoxigenic strains decreases in zones at low latitudes. In this work, no significant differences ($P < 0.05$) in aflatoxin production capacity were observed between maize regions, even though aflatoxin production depends on temperature and water activity (Pildain et al., 2004). Variability in aflatoxin production between growing seasons was not analyzed because only one *A. flavus* strain was isolated from samples collected during the 2013–2014 growing season. Production of aflatoxin B_1 and G_1 was significantly higher than that of aflatoxin B_2 and G_2 in both maize regions. These results support the assignment of subscript numbers 1 and 2 to indicate major and minor compounds, respectively (Pitt and Hocking, 2009). Further studies of morphological, molecular and aflatoxicogenic characteristics should be carried out to characterize Argentine *Aspergillus* sect. *Flavi* strains from maize ears.

4. Conclusions

The ears of maize grown in regions I and IV in Argentina are prone to degradation by fungi, including *Fusarium*, *Penicillium* and *Aspergillus* as the fungal genera with the highest incidence and severity values. *Aspergillus* Sect. *Flavi* presents similar severity and incidence in both regions, showing variability between growing seasons, and toxigenic strains are present in a great proportion of its native populations, producing B- and G-type aflatoxins. This seems to be the reason why high levels of aflatoxins are detected in freshly harvested maize. The molecular assay carried out in this study is proposed as a rapid molecular method to identify *Aspergillus* sect. *Flavi* strains responsible for aflatoxin contamination in maize. However, further studies are needed on these strains for a proper taxonomic designation at species level. *Aspergillus* sect. *Flavi* colonization in ears is confirmed as the onset of aflatoxin contamination of maize kernels; management strategies should therefore be oriented to avoid fungal colonization during the maize

Table 4
Characterization based on PCR analysis and aflatoxin production of *Aspergillus* sect. *Flavi* strains isolated from ears of maize grown in two maize production regions of Argentina during the 2012–2013 and 2013–2014 growing seasons.

Strain	PCR[a]	Aflatoxins (μg g^{-1})[b]			
		B_1	B_2	G_1	G_2
40	+	–	–	76.939	1.443
103	+	1.193	0.044	0.390	0.007
104	+	22.542	3.925	4.535	0.61
102	+	2.953	0.796	10.134	6.290
38	+	39.479	7.762	14.107	3.319
78	+	0.046	0.069	0.010	0.086
80	+	0.158	0.120	0.584	0.086
77	+	4.907	16.066	29.198	4.142
76	+	2.761	1.653	6.577	1.260
81	+	29.947	5.286	7.782	1.772
125	+	72.563	17.607	567.463	10.878
126	+	–	–	2.176	0.923
123	+	13.294	2.500	3.270	1.412
128	+	27.674	–	–	5.506
127	+	1.552	2.869	9.239	1.155
86	–	0.027	0.086	0.005	0.114
85	+	0.051	0.164	0.321	0.131
82	+	0.289	0.526	–	0.199
129	+	10.882	2.061	–	0.333
130	+	–	–	–	–
124	+	–	–	22.606	0.331
87	+	3.693	5.903	26.366	13.509
94	+	6.767	3.728	19.038	11.005
93	–	0.747	0.100	0.370	0.054
90	+	0.175	0.070	0.275	0.043
96	–	0.044	0.098	0.038	0.120
95	+	0.048	0.064	0.020	0.074
119	+	1.418	11.063	7.619	1.237
120	+	0.882	1.106	–	0.092
97	+	0.037	0.069	0.019	0.094
98	+	2.934	0.086	2.175	0.013
99	+	0.500	1.199	2.462	0.664
100	+	0.067	0.138	0.508	0.114
121	+	–	–	10.869	3.752
122	+	472.755	89.542	78.512	14.285
112	+	0.027	0.137	0.006	0.180
114	+	0.018	0.136	0.236	0.164
115	+	0.072	0.085	0.131	0.097
105	+	0.383	0.123	0.364	0.096
106	+	8.716	2.264	–	–
10	+	19.968	27.029	145.973	69.795
39	+	138.306	29.029	39.569	5.543
74	+	2.650	2.494	5.901	4.506
75	–	8.307	1.744	–	–
76	+	11.937	1.698	3.529	0.633
111	+	1.852	10.248	9.848	1.181
109	+	1.895	1.780	11.698	3.153
118	+	3.454	0.661	18.596	0.272
41	+	4.314	3.147	17.993	7.534
42	+	4.935	0.909	2.075	0.524
43	+	–	–	15.496	2.120
44	+	11.033	2.040	–	0.416
45	+	93.273	105.100	455.782	93.709
46	+	–	–	–	–
47	+	1.993	5.674	8.195	3.905
48	+	0.039	0.070	0.011	0.090
49	+	64.850	13.835	–	–
50	+	19.740	97.784	129.256	18.040
51	+	0.066	0.073	0.037	0.093
52	+	0.028	0.089	–	–
53	+	86.160	0.082	2.593	–
116	+	7.573	1.470	–	–
71	+	4.983	1.063	–	–
70	+	38.409	9.321	9.170	1.260
34	+	5.940	6.075	31.200	15.905
33	+	–	–	–	–

[a] [+]: PCR amplification present; [−]: PCR amplification absent.
[b] Limits of quantification: 0.3 μg kg^{-1} for B_1 and G_2, 0.4 μg kg^{-1} for B2 and G_1. Limits of detection: 0.1 μg kg^{-1} for all aflatoxins.

Table 5
Chemotypes of *Aspergillus* sect. *Flavi* strains based on aflatoxin production in kernels of maize grown in Argentina.

Chemotype	Mycotoxins[a]		Percentage of strains (n = 66)
	B-aflatoxins	G-aflatoxins	
I	+	+	79.0
II	+	–	9.0
III	–	+	7.5
IV	–	–	4.5

[a] B-aflatoxins: B_1 and B_2; G-aflatoxins: G_1 and G_2.

Table 6
Distribution of toxigenic and atoxigenic *Aspergillus* sect. *Flavi* strains and their aflatoxin production in maize ears collected from two maize production regions.

Region[a]	Strain[b]		Aflatoxin mean values (μg g^{-1})[c]			
	AT	TS	B_1	B_2	G_1	G_2
I	1	19	0.92a (1.97)	0.60a (2.03)	1.04a (2.66)	0.53a (1.98)
IV	2	43	0.65a (2.68)	0.50a (1.96)	0.76a (2.75)	0.40a (1.85)

Values shown in parenthesis represent upper limits of aflatoxin ranges. Same letter within a column indicates no significant differences between maize production regions ($P < 0.05$).
[a] I and IV: maize production regions in Argentina.
[b] TS: number of toxigenic strains, AT: number of atoxigenic strains.
[c] Aflatoxin mean values are based on transformed data of toxigenic strains using the equation $y = \log_{10}(1+\mu g$ of aflatoxin per g of mycelium).

growing season in order to reduce aflatoxin levels in maize kernels. In other words, aflatoxin contamination of maize kernels grown in Argentina is not just a post-harvest problem.

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