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Biological Control

Characterization of Argentinian Endemic *Aspergillus flavus* Isolates and Their Potential Use as Biocontrol Agents for Mycotoxins in Maize

Boris X. Camiletti,† Juan Moral, Claudia M. Asensio, Ada Karina Torrico, Enrique I. Lucini, María de la Paz Giménez-Pecci, and Themis J. Michailides†

First author: Microbiología Agrícola, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba (FCA-UNC), CONICET, 5009 Córdoba, Argentina and Instituto de Patología Vegetal, Centro de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria (IPAVE-INTA), 5020 Córdoba, Argentina; second author: Departamento de Agronomía, University of Córdoba, Campus de Rabanales, Edif. C4, 14071 Cordoba, Spain and Kearney Agricultural Research and Extension Center, University of California, Davis (UC-Davis) 93648; third author: Química Biológica, FCA-UNC, CONICET; fourth and sixth authors: IPAVE-INTA; fifth author: Microbiología Agrícola, FCA-UNC; and seventh author: Kearney Agricultural Research and Extension Center, UC-Davis.
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ABSTRACT

Maize (*Zea mays* L.) is a highly valuable crop in Argentina, frequently contaminated with the mycotoxins produced by *Aspergillus flavus*. Biocontrol products formulated with atoxigenic (nontoxic) strains of this fungal species are well known as an effective method to reduce this contamination. In the present study, 83 *A. flavus* isolates from two maize regions of Argentina were characterized and evaluated for their ability to produce or lack of producing mycotoxins in order to select atoxigenic strains to be used as potential biocontrol agents (BCA). All of the isolates were tested for aflatoxin and cyclopiazonic acid (CPA) production in maize kernels and a liquid culture medium. Genetic diversity of the nonaflatoxigenic isolates was evaluated by analysis of vegetative compatibility groups (VCG) and confirmation of deletions in the aflatoxin biosynthesis cluster. Eight atoxigenic isolates were compared for their ability to reduce aflatoxin and CPA contamination in maize kernels in coinoculation tests. The *A. flavus* population was composed of 32% aflatoxin and CPA producers and 52% CPA producers, and 16% was determined as atoxigenic. All of the aflatoxin producer isolates also produced CPA. Aflatoxin and CPA production was significantly higher in maize kernels than in liquid medium. The 57 nonaflatoxigenic strains formed six VCG, with AM1 and AM5 being the dominant groups, with a frequency of 58 and 35%, respectively. In coinoculation experiments, all of the atoxigenic strains reduced aflatoxin from 54 to 83% and CPA from 60 to 97%. Members of group AM1 showed a greater aflatoxin reduction than members of AM5 (72 versus 66%) but no differences were detected in CPA production. Here, we described for the first time atoxigenic isolates of *A. flavus* that show promise to be used as BCA in maize crops in Argentina. This innovating biological control approach should be considered, developed further, and used by the maize industry to preserve the quality properties and food safety of maize kernels in Argentina.

Maize (*Zea mays* L.) is a high-value crop in Argentina, with a growing area divided into nine regions (I to IX) according to country's agroecological conditions (INTA 1997). This crop is planted on 5.0 million ha and reaches a production of over 36.5 million metric tons per year. Almost 65% of the corn produced in Argentina is exported to many different countries, generating a revenue of approximately US$4 billion per year (Ustarroz et al. 2010). The quality of this crop is affected by the presence of aflatoxins (Atehnkeng et al. 2008a; Perrone et al. 2014b; Probst et al. 2007; Wu and Guclu 2012). The aflatoxin group includes approximately 20 chemically related metabolites, and four major types have been identified as B_1, B_2, G_1, and G_2 (Wu et al. 2013). These aflatoxins have been classified as class I toxins by the International Agency for Research on Cancer (IARC 1993), with aflatoxin B_1 (AFB_1) being the most toxic and prevalent mycotoxin (Wu et al. 2013). In Argentina, the maximum regulatory limits for total aflatoxins in maize is 20 ng g^{-1} (Wu and Guclu 2012). Maize contaminated with aflatoxins above the local standard reached 30% in 2011 according to the maize industry (M. P. Giménez-Pecci, personal communication).

†Corresponding authors: T. J. Michailides; E-mail: tjmichailides@ucanr.edu; and B. X. Camiletti; E-mail: bcamiletti@agro.unc.edu.ar

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Aflatoxins are produced by a diversity of species within the *Aspergillus* sect. *Flavi*, with *Aspergillus flavus* being the most common species associated with AFB_1 contamination in maize worldwide (Atehnkeng et al. 2008a; Mauro et al. 2013; Ortega-Beltran et al. 2015; Perrone et al. 2014b; Probst et al. 2007). The *A. flavus* strains commonly produce type B aflatoxins (B_1 and B_2) but some uncommon isolates of this species can simultaneously produce aflatoxins of type G (G_1 and G_2) (Cotty and Cardwell 1999; Vaamonde et al. 2003). The strains of *A. flavus* also vary in the aflatoxin production, from strains producing large amounts of toxins to strains that do not produce any aflatoxins, called nonaflatoxigenic or atoxigenic (Bayman and Cotty 1993; Mauro et al. 2013). *A. flavus* strains can produce other mycotoxins such as cyclopiazonic acid (CPA) (Chang and Ehrlich 2011; Dorner et al. 1984). CPA is an indole-tetramic acid that produces a wide range of adverse effects on livestock (Cullen et al. 1988; Lomax et al. 1984) and humans, where it is been associated with "Kodua poisoning" by the consumption of millet contaminated with CPA (Rao and Husain 1985).

Based on morphological characteristics, the strains belonging to *A. flavus* are divided into two distinct sclerotial variants: S strains, which produce a large amount of sclerotia with size < 400 µm on Czapek-Dox (CZ) culture medium, and L strains, which produce a few sclerotia with size > 400 µm in this medium (Cotty 1989). Later, a third type of isolate, called nonsclerotia producers (NSP), was described in different areas of Argentina (Novas and Cabral 2002; Pildain et al. 2004).

The ratio between toxigenic and atoxigenic strains of *A. flavus* in a given area is important to determine the risk of mycotoxin contamination in susceptible crops. Equally important is to measure the mycotoxin-producing ability of the toxigenic strains, because

rare high-mycotoxin producers may have a more important causative role in a contamination episode than common low-mycotoxin producers (Cotty et al. 2008; Probst and Cotty 2012).

Several solid and liquid culture media are used to evaluate the mycotoxin-producing ability of *A. flavus* isolates (Cotty 1989; Ortega-Beltran et al. 2015; Perrone et al. 2014b; Vaamonde et al. 2003). However, the relationship between aflatoxin production in culture medium and maize kernels is a matter of concern, because Probst and Cotty (2012) observed a weak correlation between both substrates. Moreover, differences between aflatoxin production on autoclaved and viable maize has been also reported (Brown et al. 1993). Furthermore, in the case of CPA, the effect of the substrate on its production has been poorly studied.

Partial or complete deletion of the aflatoxin cluster is one of the mechanisms responsible for the loss of aflatoxin production in *A. flavus* (Chang et al. 2005; Donner et al. 2010; Mauro et al. 2013). Recently, Callicott and Cotty (2015) developed several pairs of specific primers that allow the identification of the deletions in the aflatoxin biosynthesis cluster using a simple polymerase chain reaction (PCR). At the moment, this technique has been only used to study *A. flavus* populations from United States and Mexico (Callicott and Cotty 2015; Ortega-Beltran et al. 2016).

A biological control strategy based on the application of naturally occurring atoxigenic isolates of *A. flavus* is well known as an effective method to reduce aflatoxin contamination on cotton, maize, peanut, pistachio, and other crops (Atehnkeng et al. 2014; Cotty 1990; Dorner et al. 1992; Doster et al. 2014). Two commercial biopesticides formulated with nonaflatoxigenic isolates (*A. flavus* AF36 and AflaGuard) are currently registered in the United States for using in maize and other crops (Doster et al. 2014; Mehl et al. 2012).

Two individuals that possess identical alleles at the *het* loci can form a stable heterokaryon with gene flow between them and, subsequently, belong to the same vegetative compatibility group (VCG) (Leslie 1993). Members that belong to the same VCG commonly share the ability to produce aflatoxin (Bayman and Cotty 1993; Pildain et al. 2004). *A. flavus* populations are composed of multiple VCG with relative frequencies fluctuating among crops, regions, and years (Cotty 1997; Jaime-Garcia and Cotty 2006; Mauro et al. 2013; Pildain et al. 2004). This oscillation can be managed to reduce mycotoxin contamination by increasing frequencies of nonaflatoxigenic VCG across the season and years (i.e., by displacing the toxic population of the fungus) (Cotty and Bayman 1993; Mehl et al. 2012). Thus, understanding the genetic diversity and environmental distribution of certain VCG facilitate selection of potential biocontrol agents within an *A. flavus* population (Atehnkeng et al. 2016).

The objectives of this work were to, first, characterize the *A. flavus* population present in maize ears grown in two regions of Argentina; second, measure aflatoxin and CPA production in maize kernels and liquid medium by native *A. flavus* strains; third, investigate the genetic diversity within nonaflatoxigenic isolates and characterize the aflatoxin biosynthesis cluster; and, fourth, evaluate predominant atoxigenic strains as potential biocontrol agents.

MATERIALS AND METHODS

Fungal isolates and inoculum preparation from single spore cultures. Samples of commercial maize cultivars were collected from regions I ($n = 25$) and IV ($n = 33$) of the previously described agroclimatic regions of Argentina (Fig. 1) (INTA 1997). Average maximum temperatures vary from 30 to 34°C in region I and from 26 to 30°C in region IV at maize flowering time (from December to March). Both regions have an average annual rainfall distribution of 200 to 500 mm during the same period (Cravero et al. 2017). Ten ears were randomly collected within fields immediately before harvest. Samples were transported in paper bags and stored at 4°C until analysis (de Oliveira-Rocha et al. 2012). Sample of kernels obtained by threshing of ears were surface sterilized by dipping in sodium hypochlorite (1%) solution for 5 min and rinsing three times in sterile water. Fungal populations present in maize grains were determined by direct plating of kernels (200 kernels/sample) on dichloran rose-bengal chloramphenicol agar medium (Merck, Darmstadt, Germany). In total, 83 *A. flavus* isolates were identified by observing morphological characteristics and following taxonomic schemes of the genus *Aspergillus* (Klich 2002; Pitt and Hocking 2009). To obtain monosporic cultures, serial dilutions containing spores for each isolate were prepared and seeded on water-agar medium (1.5% agar; pH 7). Petri plates were incubated in a dark incubator at 31°C for 72 h. After incubation, a single germinated conidium was selected using the ×20 magnification of a stereomicroscope (Bausch & Lomb, Bridgewater, NJ), transferred to potato dextrose agar medium (PDA) (Microtech Scientific, Orange, CA), and incubated at 31°C in darkness for 7 days. After incubation, conidia were transferred into glass vials containing 5 ml of water and the concentration of the spore suspension was adjusted to 10^6 conidia ml^{-1} using a Neubauer chamber. The vials were stored at 4°C in the dark until use. Before their use, the identity of all isolates was confirmed using morphological characteristics (Klich 2002; Pitt and Hocking 2009) and their yellowish-orange reaction on *Aspergillus* differentiation agar (Sigma-Aldrich, St. Louis), which is characteristic of the species *A. flavus* and *A. parasiticus* due to the production of aspergillic acid (Assante et al. 1981).

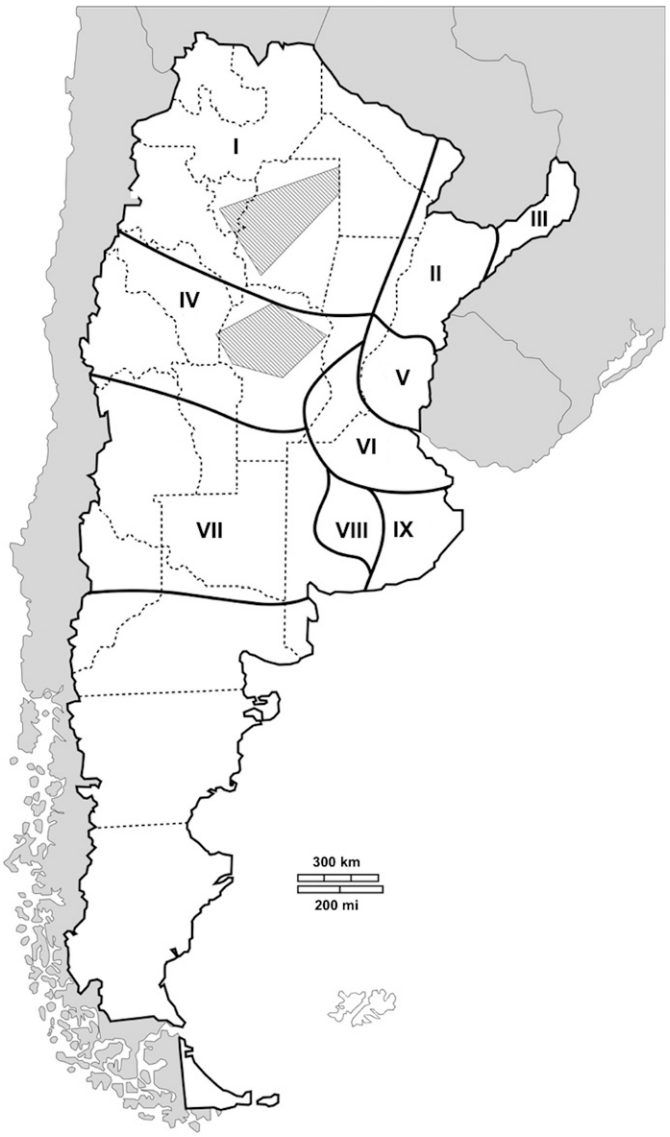

Fig. 1. Maize regions of Argentina, extracted from INTA (1997). Shaded areas within the country indicate zones in which maize ears were collected.

Sclerotial characterization. The size of sclerotia was evaluated according to Novas and Cabral (2002), with some modifications. Each strain was seeded in a centered well at 10 µl of spore suspension per Petri plate with CZ agar (Difco, Detroit) and incubated at 31°C in darkness for 14 days. Sclerotia were recovered by adding 5 ml of Tween solution (0.1% Tween 20) and filtered through Whatman filter paper number 2. Twenty sclerotia per strain were chosen randomly and their diameter was measured at ×100 magnification, using a microscope (Zeiss, Oberkochen, Germany). The *A. flavus* strains were classified as L (average diameter of sclerotia > 400 µm) or S (average diameter of sclerotia < 400 µm) according to Cotty (1989). The strains that did not produce sclerotia after the period of incubation were inoculated on corn meal agar (Difco) media and sterilized maize kernels and incubated at the same conditions as those described above to induce the production of this resistance structure. Finally, the strains that did not produce sclerotia in any of the three substrates were determined to be NSP.

Aflatoxin production in culture medium. Eighty-three Argentinean strains of *A. flavus* were evaluated for aflatoxin production. Briefly, glass vials (20 ml) were prepared with 5 ml of yeast-sucrose medium (2% yeast extract and 15% sucrose, pH 6.5) and aseptically seeded with 100 µl of a conidial suspension (10^6 conidia ml^{-1}), according to Probst and Cotty (2012). Vials were incubated in a culture chamber at 31°C in the dark for 7 days. At the end of the incubation period, 3 ml of acetone were added to stop aflatoxin production (Cotty and Cardwell 1999). Vials were gently shaken and placed in darkness for 1 h at room temperature (22 to 24°C) to allow the release of aflatoxins contained in the mycelium. Subsequently, 3 ml of methyl chloride and 5 ml of sterile deionized water were added to each vial and they were again placed to rest in darkness. Finally, 1.5 ml of the bottom layer was transferred to 2-ml glass vials and evaporated in a hood. The dry residue of each tube was dissolved in methanol/water (70:30) and filtrated through a 0.2-µm nylon filter (VWR, Radnor, PA). Extracts (4 µl) were spotted directly onto a thin-layer chromatography (TLC) plate (Silicagel 60; Merck) using a micropipette. Plates were developed in chloroform/acetone (88:12) for 40 min and air dried. The presence of aflatoxin was detected by scanning fluorescence densitometry under a 365-nm UV light and quantified using a CAMAG TLC Scanner 3 (Muttenz, Switzerland) with winCATS 1.4.2 software (Cardwell and Cotty 2002; Ortega-Beltran et. al. 2015; Probst and Cotty 2012). Standards curves were calculated with different levels of aflatoxin B_1, B_2, G_1, and G_2 purchased from Sigma-Aldrich.

Aflatoxin production in autoclaved maize grain. Maize kernels (cultivar BH8551 RR) were supplied by BH Genetics (Ganado, TX). Erlenmeyer flasks (250 ml) were prepared with 10 g of healthy maize grain and autoclaved at 121°C for 60 min. A representative set of kernels was placed on PDA medium and incubated at 31°C for 7 days to confirm sterilization of the maize kernels. Water percentage was determined with an MT-PRO grain moisture tester (John Deere, Moline, IL) and adjusted to 25% with sterile water containing 10^6 conidia $flask^{-1}$ (Probst and Cotty 2012; Probst et al. 2011). Flasks were plugged with AirOtop (Thomson Instrument Company, Clear Brook, VA) enhanced seals to allow gas exchange and put into plastics bins containing 1 cm of standing water to prevent humidity loss. Maize kernels were incubated at the same condition as described above. After the incubation period, 50 ml of methanol/water (70:30) was added to each flask and homogenized for 60 s in a mixer Waring Blender (Conair Corporation, East Windsor, NJ) according to Probst and Cotty (2012). The mixture was filtered through 415 filter paper (VWR) and aflatoxins were quantified as previously described.

Nonaflatoxigenic strain identification. Samples in which aflatoxins were not detected using TLC methodology were evaluated by high-performance liquid chromatography (HPLC) in an HP 1050 series system (Doster et al. 2014). Chromatographic separation was performed with a C18 100-by-3.9-mm Nova Pak (4-µm particle size) column. A solution of methanol/water (60:40) at 0.8 ml min^{-1} flow-rate was used as mobile phase. Aflatoxins were detected using a fluorescence detector (360 and 340 nm for excitation and emission, respectively). A photochemical reactor for an enhanced detection system (Aura Industries, New York) was used for postcolumn photolytic derivatization to increase sensitivity and selectivity of the detector. Aflatoxins were quantified using standards curves calculated as previously reported. The limits of detection for aflatoxins B_1, B_2, G_1, and G_2 were all <1 ng g^{-1}. When aflatoxins were not detected using HPLC, the strain was classified as nonaflatoxigenic (Doster et al. 2014).

CPA production analysis. All of the strains were assessed for CPA production in both a culture medium and maize kernels. The CPA extraction was performed as was described for aflatoxin extraction. The production of CPA was analyzed by HPLC using an Agilent 1100 Series apparatus (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector (280 nm), an autosampler system, a quaternary pump, and a ZORBAX Eclipse Plus C18 column (4.6 by 100 mm, 3.5-µm particle size) kept at 50°C. The mobile phase consisted of methanol and 4 mM zinc sulfate (50:50; pH 3.5) and was pumped at 0.8 ml min^{-1}. The injection volume was 50 µl. CPA was quantified using a standard curve calculated with different levels of CPA purchased from Sigma-Aldrich. Samples were considered positive when they showed a peak with retention time and spectra similar to the standard (da Motta and Valente Soares 2000). The limit of detection that allowed a recognizable spectrum was 20 ng g^{-1}.

VCG analysis. Nitrate-nonutilizing (*nit*) mutants were obtained according to Mauro et al. (2013). Petri dishes were prepared with CZ medium supplemented with potassium chlorate at 25 g $liter^{-1}$ and rose Bengal at 0.05 g $liter^{-1}$, pH 7.0. Plates were inoculated with an 8-µl spore suspension (10^6 conidia ml^{-1}) and incubated at 31°C in darkness until mycelia with a ghost-like growth pattern appeared. At least four *nit* mutants were recovered from these chlorate-resistant sectors. Phenotypes were determined by growth on CZ and two other mediums where sodium nitrate was replaced by sodium nitrite and hypoxanthine. Mutants were classified as *niaD*, *nirA*, or *cnx* according to previous bibliographies (Bayman 1991; Mauro et al. 2013; Pildain et al. 2004). Initially, a group of 12 tester pairs was obtained by self-compatibility of *niaD* with either *nirA* or *cnx* on starch medium (Cotty and Taylor 2003). All *nit* mutants were tested for compatibility to establish the VCG. If a mutant did not show compatibility with any group, a new tester pair was obtained and a new VCG formed. All of the VCG obtained were tested for complementation with VCG AF36. The diversity index was calculated as the number of VCG obtained divided by the number of isolates, and was used to express VCG diversity among the nonaflatoxigenic *A. flavus* population in maize ears (Mauro et al. 2013).

Evaluation of the reduction of mycotoxin contamination in a maize kernel assay. Eight atoxigenic members of the two dominant VCG were evaluated to test the ability to reduce AFB_1 and CPA production by toxigenic strains in coinoculations of viable maize kernels. All of the atoxigenic strains were evaluated against one mycotoxin-producing strain of each region (AS08811 for region I and AS05322 for region IV), selected by its consistent production of AFB_1 and CPA in maize kernels. Among different methods, the one selected to sterilize the maize kernel was by submerging the kernels in sodium hypochlorite (10%) solution for 2 min and, subsequently, immersing them in ethanol (70%) solution for another 2 min. Maize kernels were then air dried for 1 h on an aseptic surface in a biological safety cabinet. Erlenmeyer flasks (250 ml) containing 10 g of disinfested maize kernels were prepared (Mauro et al. 2015; Probst et al. 2011). Conidia were collected from the PDA surface of 7-day-old cultures (31°C) and suspended in sterile distilled water. Spore density was determined using a Neubauer chamber and the inoculum suspension prepared in Tween 20 (0.1%) for each treatment. The selected treatments were maize kernels simultaneously inoculated with atoxigenic and toxigenic isolates

(10^5 conidia flask^{-1} each) and maize kernels that were independently inoculated (10^5 conidia flask^{-1}) with the toxigenic or the atoxigenic strains (e.g., positive and negative controls, respectively) based on previous research (Atehnkeng et al. 2008b; Mauro et al. 2015; Probst et al. 2011). In addition, the registered nonaflatoxigenic strain AF36 was included in the analysis to compare its performance with the Argentinean atoxigenic strains. Inoculated kernels for all treatments were incubated at 31°C for 7 days in darkness, as indicated above. After the incubation period, mycotoxins were extracted and quantified as previously described.

A second independent set of treatments was prepared as previously described, with the most competitive atoxigenic isolates inoculated 48 h in advance of inoculation with the mycotoxin producers, to evaluate the effect of atoxigenic on mycotoxin production. Mycotoxins were extracted and quantified as previously described.

Cluster amplification patterns analysis. Deletions in the aflatoxin biosynthesis cluster were investigated following the methodology described by Callicott and Cotty (2015), with some modifications. Briefly, DNA was obtained from *A. flavus* isolates growing in 5% V8 juice and 2% agar, pH 6.0, as described above. Spores were collected in 1.5-ml Eppendorf tubes with 450 µl of lysis buffer (30 mM Tris, 10 mM EDTA, and 1% sodium dodecyl sulfate; pH 8.0). Samples were placed in a 60°C water bath, shaken after 30 min, and put again in the water bath. After centrifugation, 370 µl of the supernatant was transferred to a new tube. DNA was precipitated by adding 370 µl of ammonium acetate (4 M) and cleaned with 740 µl of ice-cold ethanol. Independent PCR were carried out using the pairs of primers SC01, IC01, AC01, AC02, AC03, AC04, AC05, AC06, AC07, AC08, AC09, AC10, AC11, AC12, AC13, and IC02 that amplify the markers for the aflatoxin cluster and nearby gene regions (Callicott and Cotty 2015). Reactions were developed using each primer at 10 nmol liter^{-1}, 1× AccuPower PCR PreMix (Bioneer, Alameda, CA), and 20 ng of genomic DNA, reaching a final volume of 25 µl. Samples were placed in a thermocycler programmed to 1 min at 94°C; 30 cycles each of 30 s at 94°C, 90 s at 62°C, and 90 s at 72°C; with a final elongation of 10 min at 72°C. PCR products were revealed on 1% agarose in 1× Tris-acetate-EDTA buffer.

Statistical analysis. Mycotoxin production experiments were conducted using three repetitions while coinoculation analyses were conducted twice with three repetitions. Data from repetitions of each experiment were combined after checking for homogeneity of the experimental error variances by the F test. Prior to statistical analyses, AFB_1 and CPA data were transformed by the equation $Y = \log$ (1+ µg of mycotoxin) when necessary to homogenize the variances of the treatments (Atehnkeng et al. 2008a; Doster et al. 2014; Mehl and Cotty 2010; Probst et al. 2011). Proportions of toxigenic strains between regions were compared using the χ^2 test. Student's t test was carried out to compare toxicity between isolates from different regions, as well as to compare mycotoxin production between substrates. Analysis of variance was performed to obtain the effect of the significance of the independent variables. Significant differences between the treatments were determined using Fisher's protected least significant difference test at $P < 0.05$ (Atehnkeng et al. 2008a; Doster et al. 2014). Finally, Pearson's correlation analyses were used to evaluate relationships between medium and maize kernels according to mycotoxin production, as well as between both AFB_1 and CPA. Data analysis was performed using the InfoStat software, version 2017 (InfoStat 2008).

RESULTS

Aflatoxin production and sclerotial characterization. Isolates that produced aflatoxin in either substrate were named as aflatoxigenic strains, while those that did not produce detectable quantities of aflatoxin in maize kernels or liquid media were considered nonaflatoxigenic (Table 1). In both regions, most of the strains were determined as nonaflatoxigenic, although the percentage of toxigenic strains was significantly ($P = 0.015$) higher in region IV (41%, $n = 52$) than in region I (19%, $n = 31$). Conversely, when the ability to produce aflatoxins of aflatoxigenic isolates between the two regions was compared, there was no significant difference ($P = 0.2742$) between them (Table 1). Even so, the highest values for aflatoxin production were detected among isolates from region IV. The distribution of the *A. flavus* isolates according to their ability to produce aflatoxins in maize was skewed (nonsymmetric) to the left (skew parameter $k = 2.1$), which showed the prevalence of nonaflatoxigenic isolates (relative frequency = 0.67) (Fig. 2). Furthermore, aflatoxigenic strains produced significantly ($P < 0.001$) higher amounts of AFB_1 in maize kernels than in the liquid media (Table 1). Aflatoxin production averaged 13.6 ± 1.6 and 4.0 ± 1.9 µg g^{-1} in maize kernels and the liquid medium, respectively. Pearson's correlation analysis between AFB_1 production in maize kernels and liquid media showed a moderate relationship ($r = 0.421$, $P < 0.001$). In total, seven false negatives were detected in liquid media. These isolates preidentified as nonaflatoxigenic produced AFB_1 at 0.5 to 17 µg g^{-1} in maize kernels. In total, 16 toxigenic strains (61%) produced sclerotia, with 2 isolates being classified as S strains. These S strains had AFB_1 production similar to that of L or NPS strains. All of the nonaflatoxigenic (atoxigenic) strains were defined as NSP in the sclerotial characterization.

CPA production. Thirteen *A. flavus* isolates, which did not produce aflatoxin or any detectable quantities of CPA on maize kernels or liquid media either, were determined to be atoxigenic and considered as possible biocontrol agents (Table 1). Isolates that produced CPA (84%) on either liquid media or maize kernels were classified as CPA producers. Most of our isolates produced CPA at <6 µg g^{-1} (Fig. 2) (i.e., again the frequency histogram was nonsymmetric; skew parameter $k = 2.9$). No significant differences were found in CPA production by CPA produced between the maize regions. The production of this mycotoxin had behavior similar to that of aflatoxins, presenting significantly ($P < 0.001$) higher average values on maize kernels (4.1 ± 0.6 µg g^{-1}) than in liquid media (1.1 ± 0.5 µg g^{-1}). In total, 16 strains did not produce detectable quantities of CPA in maize kernels but produced small amounts on liquid media.

All of the aflatoxigenic isolates simultaneously produced CPA with averages of 7.6 ± 1.2 µg g^{-1} in maize kernels and 1.8 ± 0.9 µg g^{-1} in the liquid medium. In general, their CPA production was significantly ($P < 0.001$) lower than aflatoxin production on maize (CPA at 7.6 µg g^{-1} versus AFB_1 at 13.6 µg g^{-1}) and in liquid media (CPA at 1.8 µg g^{1} versus AFB_1 at 4.0 µg g^{-1}), albeit the strain AS09411 produced a significantly higher amount of CPA than AFB_1. The 76% of the nonaflatoxin-producing strains produced CPA. In maize kernels, the average CPA production by the nonaflatoxigenic strains was significantly lower than the average production of this mycotoxin by the aflatoxin producers (2.0 ± 0.2 and 7.6 ± 1.2 µg g^{-1}, respectively). No significant differences were found in liquid media. In contrast to aflatoxins, seven isolates were classified as nonproducers on maize kernels. These false negatives produced from 0.03 to 0.9 µg g^{-1} in liquid media.

***Nit* mutants and VCG.** The entire population of nonaflatoxigenic isolates was included in the VCG analysis (Table 1). Eight isolates did not complement themselves or other isolates and were excluded from the analysis. In total, 265 mutant sectors were obtained from the remaining 48 isolates. The phenotypes of mutant produced were 52, 41, and 7% for *niaD*, *nirA*, and *cnx*, respectively. Based on complementation tests, six VCG were established for the evaluated isolates. VCG were named with the AM prefix for "Argentine Maize" followed by a progressive number in order of discovery. Four VCG were represented by only a single isolate. The prevalent VCG AM1 and AM5 had a frequency of 58 and 35%, respectively, and were present in both maize regions with no significant differences. None of the aflatoxigenic isolates belonged to these VCG. However, frequencies of CPA producers were 82 and 66% in AM1 and AM5, respectively. None of the Argentinean

isolates belonged to the VCG of AF36, the registered strain for use on cotton, maize, and pistachio (Doster et al. 2014). The diversity index was 0.12 for the endemic nonaflatoxigenic strains under study.

Reduction of mycotoxin contamination in maize kernels. Coinoculation experiments were conducted using the mycotoxin (both AFB_1 and CPA) producer strains AS05322 and AS08811 as representative strains of regions I and IV, respectively. The toxigenic isolates produced significantly ($P < 0.001$) higher levels of AFB_1 and CPA on viable maize than on autoclaved kernels. All of the atoxigenic strains tested in the coinoculation experiment caused a significant reduction of the quantity of both AFB_1 and CPA (Table 2). AFB_1 reduction ranged from 54 to 84% while CPA production showed reductions of 59 to 75%. On average, members of AM1 caused a significantly ($P < 0.001$) higher AFB_1 reduction than members of AM5 (73 ± 11 versus 66 ± 14%, respectively). Among the AM1 members, significantly higher reductions ($P < 0.001$) in AFB_1 contamination were obtained by coinoculation with AS03145 (78 to 83%) or AS04322 (78 to 79%). Pearson's correlation analysis between AFB_1 and CPA reductions did not show a significant relationship ($r = -0.224$, $P = 0.411$). The mycotoxin production (AFB_1 and CPA) by toxigenic isolates was similarly affected by the coinoculation with atoxigenic isolates. Coinoculations with the commercial isolate AF36 resulted in reduction of AFB_1 contamination of 55 to 70%, depending of the toxic isolate. The AF36 strain was not effective in decreasing CPA production in coinoculation with the mycotoxin producers.

TABLE 1. Vegetative compatibility groups (VCG), sclerotial characterization, and mycotoxin production by Argentinian endemic *Aspergillus flavus* isolates

		Production (μg g^{-1})[x]			
		AFB_1 (SE)		CPA (SE)	
VCG, MR, strain[y]	Type[z]	Kernels	Medium	Kernels	Medium
NI					
I					
AS11623	L	1.0 (0.3) d	1.4 (0.4) de	6.1 (1.3) abcd	0.5 (0.1) abc
AS04722	L	8.6 (2) bcd	0.3 (0.1) e	8.1 (2.3) abc	1.0 (0.2) abc
AS04822	L	17 (4) abc	0 (0)	10 (3) abcd	4.0 (0.6) a
AS05122	S	12 (3) abc	0 (0)	14 (2) ab	0.4 (0.1) bcd
AS05322	L	3.4 (0.2) cd	0 (0)	5.8 (1.3) bcde	4.2 (1.3) a
AS04922	NSP	0.5 (0.2) d	0 (0)	2.9 (0.5) bcde	0 (0.0)
IV					
AS10013	L	20 (5) abc	3.6 (0.8) abcd	8.7 (1.6) abc	0.5 (0.1) abc
AS10516	L	9.9 (2.4) abc	4.4 (0.2) abc	0 (0)	0.5 (0.1) abc
AS10919	L	2.4 (0.9) cd	0 (0)	1.6 (0.2) cde	0.3 (0.01) bcd
AS09611	L	2.8 (0.4) cd	0.8 (0) bcde	0 (0)	0.3 (0.01) bcd
AS09813	L	4.0 (0.6) cd	0.2 (0) e	4.0 (1.2) bcde	0.3 (0.01) bcd
AS02808	L	2.4 (0.5) cd	0.9 (0.1) de	0 (0)	0.1 (0.01) cd
AS07604	S	27 (5) ab	1.7 (0.4) de	14 (4) ab	0.7 (0.2) abc
AS07804	L	27 (6) ab	11 (3) ab	0.1 (0) f	0.3 (0.1) bcd
AS08004	L	35 (3) a	2.0 (0.3) bcde	5.3 (1.4) abcd	0.4 (0.01) bcd
AS08811	L	11 (3) abc	1.1 (0.1) cde	22 (1) a	0.2 (0.01) bcd
AS09011	L	5.3 (0.8) bcd	1.5 (0.2) bcde	0 (0)	0.2 (0.03) bcd
AS08105	NSP	1.8 (0.3) abcd	0 (0)	1.7 (0.1) cde	0.1 (0.01) cd
AS08508	NSP	26 (7) abc	1.2 (0.1) bcde	0 (0)	0.2 (0.03) bcd
AS08608	NSP	22 (2) ab	14 (3) a	0 (0)	0.2 (0.02) bcd
AS08711	NSP	25 (7) abc	2.9 (0.5) abcd	0 (0)	0.8 (0.2) abc
AS09311	NSP	16 (2) abc	22 (6) a	0 (0)	0.3 (0.01) bcd
AS09411	NSP	1.1 (0.4) d	0 (0)	8.3 (1.1) abc	0.3 (0.01) bcd
AS09511	NSP	14 (3) abc	0.6 (0.1) de	0 (0)	0.3 (0.01) bcd
AS11215	NSP	11 (2) abc	1.1 (0.1) de	0 (0)	0.4 (0.01) bcd
AS11415	NSP	14 (2) abc	2.8 (0.3) abcd	2.4 (0.6) cde	1.2 (0.2) ab
AS11515	NSP	8.8 (1.4) bcd	1.7 (0.2) bcde	2.2 (0.4) cde	0.5 (0.2) bcd
AM1					
I					
AS10302	NSP	0 (0)	0 (0)	1.0 (0.1) cde	0 (0)
AS10402	NSP	0 (0)	0 (0)	1.8 (0.3) cde	0 (0)
AS11821	NSP	0 (0)	0 (0)	0.8 (0.1) de	0 (0)
AS03145	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS03802	NSP	0 (0)	0 (0)	0 (0)	0 (0.)
AS04222	NSP	0 (0)	0 (0)	5.0 (0.2) abcd	0 (0.)
AS04322	NSP	0 (0)	0 (0)	0 (0)	0 (0.)
AS04522	NSP	0 (0)	0 (0)	1.0 (0.1) cde	0 (0.)
AS05022	NSP	0 (0)	0 (0)	1.5 (0.5) cde	0.7 (0.2) abc
AS05222	NSP	0 (0)	0 (0)	2.7 (0.8) cde	0.02 (0.01) d
IV					
AS12114	NSP	0 (0)	0 (0)	0 (0)	0.3 (0.02) bcd
AS12214	NSP	0 (0)	0 (0)	7.5 (1.3) abc	0 (0)
AS12306	NSP	0 (0)	0 (0)	2.6 (0.5) bcde	0 (0)
AS12410	NSP	0 (0)	0 (0)	1.7 (0.3) cde	0 (0)
AS12506	NSP	0 (0)	0 (0)	0 (0)	0.03 (0.01) d

(*Continued on next page*)

[x] AFB_1 = aflatoxin B_1, CPA = cyclopiazonic acid, and SE = standard error. For each column (kernels or medium) values with the same letter are not significantly different according to least significant difference test protected of Fisher ($P < 0.05$).

[y] MR = maize production regions I and IV in Argentina. NI = not investigated and NA = not available due to self-incompatibility.

[z] S and L isolate types produce sclerotia of <400 and >400 μm on average, respectively. Nonsclerotia producers (NSP) are isolates that did not produce sclerotia under laboratory conditions.

Results from the second set of treatments showed that mycotoxins were not detected in maize kernels inoculated with atoxigenic strains 48 h before the mycotoxin-producer inoculation.

Aflatoxin biosynthesis pathway. PCR amplification was obtained for 16 pairs of primers used to test regions of the aflatoxin cluster of the strain AS08608, which was used as a positive control, as well as for all of the atoxigenic members of the two major VCG (AM1 and AM5). In addition, all of the studied regions were detected for two other single isolates, AS11108 and AS00001, belonging to VCG AM4 and AM6, respectively. Interestingly, no PCR amplification was obtained for the isolate AS00019. Two of the five isolates with unknown VCG showed deletions, and three had all of the studied regions. Similar patterns were observed for members that belonged to the same VCG (Table 3).

DISCUSSION

In Argentina, 65% of the maize production is exported, while 35% of it is used in domestic markets to feed cattle, produce human food, and as an ingredient for different products (Ustarroz et al. 2010). Toxic species of *A. flavus* decrease the nutritional value and quality of maize kernels as a consequence of mycotoxin contamination (Varga et al. 2009). *A. flavus* strains differ in morphological characteristics, mycotoxin-producing ability, toxigenic profile, and competitive ability

TABLE 1. (*Continued from previous page*)

		Production (μg g^{-1})[x]			
		AFB_1 (SE)		CPA (SE)	
VCG, MR, strain[y]	Type[z]	Kernels	Medium	Kernels	Medium
AS12606	NSP	0 (0)	0 (0)	0.0 (0)	0.03 (0.01) d
AS12707	NSP	0 (0)	0 (0)	2.8 (0.4) bcde	0.03 (0.01) d
AS12909	NSP	0 (0)	0 (0)	2.9 (0.5) bcde	0 (0)
AS13009	NSP	0 (0)	0 (0)	0 (0)	0.1 (0.03) cd
AS03917	NSP	0 (0)	0 (0)	2.2 (0.4) cde	0 (0)
AS04050	NSP	0 (0)	0 (0)	0.0 (0)	0 (0)
AS05639	NSP	0 (0)	0 (0)	2.2 (0.2) cde	0.2 (0.01) bcd
AS05739	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS06659	NSP	0 (0)	0 (0)	1.0 (0.3) de	0 (0)
AS07517	NSP	0 (0)	0 (0)	1.0 (0.1) de	0.1 (0.01) cd
AS07617	NSP	0 (0)	0 (0)	1.5 (0.5) cde	0 (0)
AS07704	NSP	0 (0)	0 (0)	2.2 (0.4) cde	0 (0)
AS10616	NSP	0 (0)	0 (0)	4.1 (0.8) abcde	0.4 (0.03) bcd
AM2					
IV					
AS12807	NSP	0 (0)	0 (0)	1.3 (0.1) cde	0.1 (0.01) cd
AM3					
I					
AS00019	NSP	0 (0)	0 (0)	0.8 (0.1) de	0 (0)
AM4					
IV					
AS11108	NSP	0 (0)	0 (0)	0.0 (0)	1.0 (0.13) abc
AM5					
I					
AS10202	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS11622	NSP	0 (0)	0 (0)	2.0 (0.3) cde	0.02 (0.01) d
AS04001	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS04122	NSP	0 (0)	0 (0)	2.2 (0.1) cde	0 (0)
AS04422	NSP	0 (0)	0 (0)	0.6 (0.1) e	0 (0)
AS04622	NSP	0 (0)	0 (0)	0.5 (0.1) e	0 (0)
AS04755	NSP	0 (0)	0 (0)	2.9 (0.3) bcde	0 (0)
AS00018	NSP	0 (0)	0 (0)	0 (0)	0.1 (0.01) cd
IV					
AS10816	NSP	0 (0)	0 (0)	1.5 (0.3) cde	0 (0)
AS12012	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS03326	NSP	0 (0)	0 (0)	1.4 (0.1) cde	0 (0)
AS03426	NSP	0 (0)	0 (0)	1.3 (0.1) cde	0 (0)
AS06441	NSP	0 (0)	0 (0)	0.7 (0.1) e	0.1 (0.01) cd
AS07417	NSP	0 (0)	0 (0)	1.7 (0.3) bcde	0 (0)
AS07424	NSP	0 (0)	0 (0)	2.7 (0.5) bcde	0 (0)
AS09913	NSP	0 (0)	0 (0)	1.7 (0.4) cde	0.4 (0.12) bcd
AM6					
IV					
AS00001	NSP	0 (0)	0 (0)	1.2 (0.1) cde	0 (0)
NA					
I					
AS01153	NSP	0 (0)	0 (0)	0.7 (0.1) e	0.4 (0.13) bcd
AS01756	NSP	0 (0)	0 (0)	0 (0)	0.03 (0.01) d
AS02257	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS04848	NSP	0 (0)	0 (0)	1.0 (0.1) de	0.03 (0.01) d
AS05254	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS05544	NSP	0 (0)	0 (0)	0 (0)	0 (0)
IV					
AS06240	NSP	0 (0)	0 (0)	0 (0)	0 (0)
AS07024	NSP	0 (0)	0 (0)	0 (0)	0 (0)

(Cotty et al. 2008). These features have been investigated and exploited to develop biocontrol strategies to reduce mycotoxin contamination in maize crops using atoxigenic strains of the same fungal species in different countries (Atehnkeng et al. 2014; Bandyopadhyay et al. 2016; Mehl et al. 2012) but not in Argentina.

Of the 83 *A. flavus* isolates tested for mycotoxin-producing ability, 13 (16%) did not produce either aflatoxin or CPA and they were identified as atoxigenic. This low percentage of atoxigenic strains in maize ears is mainly because of the high ratio (84%) of isolates that produce CPA. At present, CPA production by *A. flavus* isolates has been considered a challenge in producing safe maize because the registered biocontrol agent AF36 was reported to produce this mycotoxin in inoculated maize kernels (Abbas et al. 2011). Thus far, there is no evidence for high levels of CPA in crops treated with the commercial product AF36 (T. J. Michailides, unpublished data). High percentages of CPA producers (73 to 94%) were isolated from other substrates such as peanut, soybean, and wheat growing in Argentina (Vaamonde et al. 2003). Conversely, nonaflatoxigenic *A. flavus* isolates are commonly found as part of the native populations present in several crops (Alaniz-Zanon et al. 2013; Mauro et al. 2013; Mehl and Cotty 2010). In our study, there was a low percentage (2.4%) of S-type strains that are mainly highly toxic (Cotty et al. 2008). Similarly, a high prevalence of L-type strains of *A. flavus* has been described in Nigeria, where the incidence of aflatoxigenic strains was lower than 40% (Atehnkeng et al. 2008a). In our study, we also described, for the first time, NSP (80%) isolates of *A. flavus* from maize in Argentina, although this type of strain was previously observed in peanut seed in the same country (Novas and Cabral 2002). All of our isolates that produced aflatoxin also simultaneously produced CPA (ratios approximately 2:1), which is in agreement with results reported by Horn et al. (1996). In fact, aflatoxigenic *A. flavus* isolates that do not produce a detectable amount of CPA were found at a low frequency (2.2%) in Argentinean populations (Vaamonde et al. 2003). Therefore, the natural occurrence of this mycotoxin in Argentine maize kernels is a matter of concern and needs to be evaluated.

Mycotoxin-production ability varies widely depending on the environmental conditions, genotype, origin, and substrate (Perrone et al. 2014a; Vaamonde et al. 2003). It is known that, within the same populations, some *A. flavus* isolates produce much higher levels of aflatoxin than others (Cotty et al. 2008). Our results indicated that the tested isolates could be classified into different groups

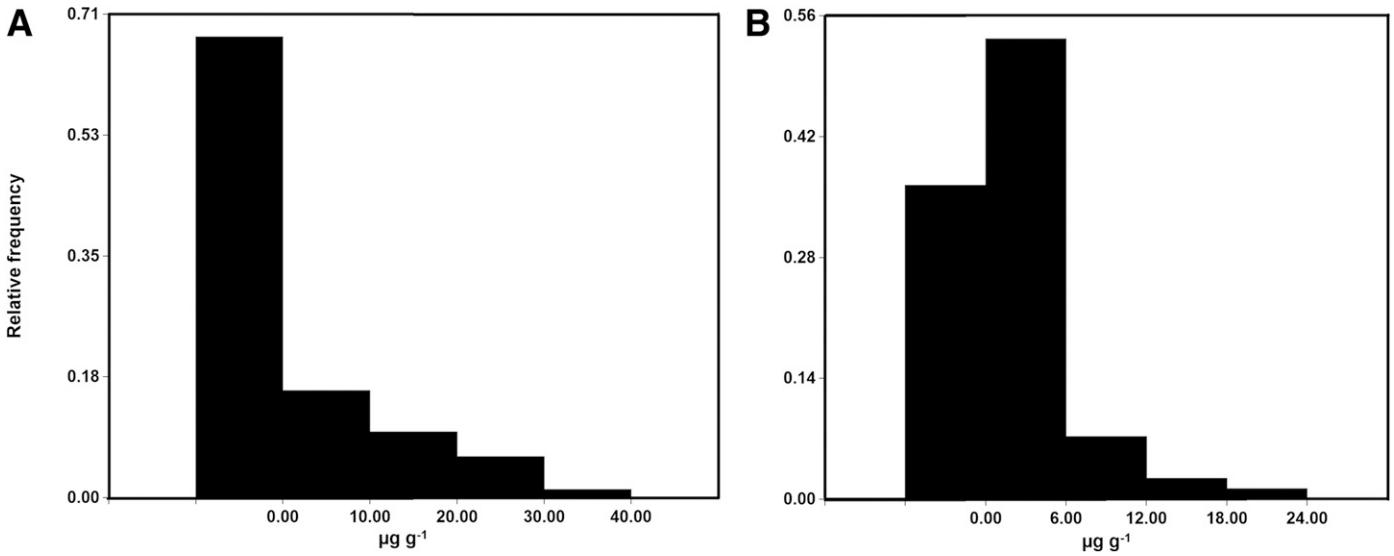

Fig. 2. Frequency histograms of the mycotoxin producing *Aspergillus flavus* isolates from maize ears grown in Argentina. **A,** Aflatoxin B_1 and **B,** cyclopiazonic acid.

TABLE 2. Ability of selected atoxigenic *Aspergillus flavus* isolates endemic to Argentina to reduce cyclopiazonic acid (CPA) and aflatoxin B_1 (AFB_1) production in maize kernels

	CPA[y]				AFB_1[y]			
	AS08811		AS05322		AS08811		AS05322	
VCG, isolate[z]	Prod (SE)	R% (SE)	Prod (SE)	R% (SE)	Prod (SE)	R% (SE)	Prod (SE)	R% (SE)
Control	32 (1.8)		57 (5)		23 (1.3)		6 (0.5)	
AM1								
AS03145	9 (2.6)	71 (6) a	13 (1.3)	74 (3) bc	4 (0.4)	84 (2) a	1 (0.3)	78 (4) a
AS04322	8 (0.6)	75 (2) a	16 (1.6)	73 (2) bc	5 (0.4)	80 (1) a	1 (0.1)	79 (1) a
AS03802	11 (1.8)	68 (5) a	23 (3.9)	60 (5) d	9 (0.9)	64 (3) cd	2 (0.3)	64 (5) bc
AS04050	9 (1.3)	69 (4) a	16 (0.7)	67 (1) bcd	10 (2.3)	61 (10) cd	3 (0.1)	60 (1) bc
AS05739	8 (1.0)	76 (3) a	19 (2.7)	68 (3) bcd	6 (1.0)	77 (4) ab	2 (0.3)	74 (4) ab
AM5								
AS10202	12 (2.6)	65 (8) a	18 (1.6)	73 (2) bcd	6 (0.6)	74 (3) abc	2 (0.2)	75 (3) ab
AS12012	9 (1.8)	71 (6) a	1 (0.4)	97 (1) a	10 (1.5)	60 (6) cd	3 (0.9)	54 (5) c
AS04001	8 (1.8)	76 (5) a	14 (4.9)	78 (7) b	8 (0.7)	68 (3) bc	2 (0.4)	65 (7) bc
AF36								
AF36	38 (1.8)	0.0 (0) a	32 (1.9)	32 (4) e	11 (1.4)	55 (6) d	2 (0.2)	70 (4) abc

[y] Prod = production (µg g^{-1}) and R% = percentage of mycotoxin reduction = [1 – (total mycotoxin in coinoculation/total mycotoxin in inoculations with only the toxigenic strains)] ×100. SE = standard error. Mean values in a column not followed by the same letter indicate significant differences according to Fisher's least significant difference test ($P < 0.05$).

[z] VCG = vegetative compatibility group. Control = AFB_1 and CPA production by toxigenic strains.

according to their mycotoxin-producing ability. In agreement with previous studies, our isolates produced higher AFB_1 and CPA quantities in maize kernels than in culture media (Chalivendra et al. 2017; Georgianna et al. 2010; Probst and Cotty 2012). The aflatoxins produced on both substrates were moderately correlated, in accordance with Probst and Cotty (2012). In addition, weak correlations were observed when the CPA production on different substrates was studied or when CPA and AFB_1 productions were correlated. AFB_1 and CPA were demonstrated to respond differently to carbon and nitrogen sources (Georgianna et al. 2010). Traditionally, liquid media containing organic nitrogen and simple sugars have been used for identifying and quantifying mycotoxin production (Davis et al. 1966; Trucksess et al. 1994; Vaamonde et al. 2003). For these reasons, liquid media could be considered to conduct preliminary screenings of *A. flavus* strains. Chalivendra et al. (2017) recently suggested that virulence of *A. flavus* in maize is related to CPA production. Here, we observed that most of the aflatoxigenic NSP isolates did not produce CPA in maize but did in a liquid medium. The presence of this type of isolate in maize kernels may suggest that CPA is not always a key pathogenicity factor within an *A. flavus* population.

The toxigenic isolates AS08811 and AS05322 produced higher levels of AFB_1 and CPA on viable maize than in autoclaved kernels. Specific constitutive proteins with antifungal activity confer resistance to *A. flavus* in some maize cultivars but were absent or at low levels in susceptible maize cultivars (Brown et al. 1999; Chen et al. 1998; Guo et al. 1998). Viable embryos were reported to synthetize certain proteins that confer moderate resistance to fungal colonization but need to be induced by germination (Chen et al. 2001; Guo et al. 1997). It was previously reported by Brown et al. (1993) that autoclaving of susceptible maize does not affect the amount of AFB_1 synthetized. Probst and Cotty (2012) compared aflatoxin production by several *A. flavus* strains on autoclaved and viable susceptible maize and reported similar levels, except for L-type strains, which produced less of this mycotoxin on autoclaved kernels, in agreement with our results.

The percentage of aflatoxigenic strains was higher in region IV than in region I, while the aflatoxin-producing ability of the isolates appears to have a similar behavior. Pildain et al. (2004) reported opposite results for peanut samples collected from these regions, where the frequency of aflatoxigenic isolates was higher in region I. In general, cooler temperatures are negatively associated with the presence of toxigenic strains and their aflatoxin-producing ability (Atehnkeng et al. 2008a; Cotty 1997; Horn 2003; Wicklow et al. 1998). However, these types of studies considered wider ranges of temperature than the current work.

The identity and frequency of VCG in an *A. flavus* population differ among agroecosystems, soil, and crop environments. Members of the same VCG share phenotypic characteristics and, in consequence, are treated as epidemiological units (Mehl et al. 2012). The low genetic diversity (diversity index = 0.12) observed in this study diverges from the high degree of diversity (diversity index from 0.45 to 0.67) present in an Italian nonaflatoxigenic *A. flavus* population isolated from maize kernels (Mauro et al. 2013). Frequencies of the VCG formed in the current study indicated the presence of two dominant genotypes (AM1 and AM5). In all, 93% of the nonaflatoxigenic *A. flavus* population in maize ears grown in Argentina belonged to these VCG. This finding is in agreement with previous results in maize kernels grown in Louisiana in the United States, where most *A. flavus* isolates (88%) consisted of two VCG (Sweany et al. 2011). The VCG to which the isolate AF36 belongs is one of the most common groups among the nonaflatoxigenic *A. flavus* VCG naturally occurring in cotton fields in Arizona and tree nut orchards in California (Cotty 2006; Doster et al. 2014; Picot et al. 2018). This VCG was not found among the *A. flavus* strains isolated from Argentinean maize ears in this study. Our results might provide additional evidence about specialization of some VCG or isolates to colonize maize ears, as indicated by Sweany et al. (2011). High variability among *A. flavus* isolates in production of hydrolytic enzymes for degradation of plant tissues may indicate differential adaptation to plant hosts (Mellon et al. 2007). The dominant nonaflatoxigenic-producing VCG contained isolates that produce CPA as well as members without CPA-producing ability. The lack of association for this mycotoxin between isolates of the same VCG suggests that members of the same VCG are not always clones. Abbas et al. (2011) determined that the *A. flavus* strain K49 isolated from maize does not produce CPA and is vegetatively compatible with the CPA producer AF36. In a later study, the authors determined that strain K49 has a single nucleotide mutation in the *dmaT* gene needed for CPA biosynthesis (Chang et al. 2012). Correll (1991) indicated that VCG phenotypes and DNA change independently and at different rates. Progeny obtained from sexual reproduction were demonstrated to differ from their parents in CPA production (Olarte et al. 2012). However, *A. flavus* was reported to be predominantly clonal in Argentina, and sexual recombination occurs at low rates (Moore et al. 2013). On the other hand, some of this variability can be explained by the high lability of CPA that may have resulted in degradation.

TABLE 3. Characterization of the aflatoxin cluster in Argentinean *Aspergillus flavus* isolates

		Aflatoxin cluster[y]															
Isolate	VCG[z]	SC	IC01	AC01	AC02	AC03	AC04	AC05	AC06	AC07	AC08	AC09	AC10	AC11	AC12	AC13	IC02
AS00019	AM3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AS02257	NA	–	X	–	X	X	–	–	X	X	X	X	–	X	–	X	–
AS06240	NA	X	X	X	X	X	X	X	X	–	X	X	X	X	X	X	X
AS12807	AM2	X	X	–	X	X	X	X	X	–	X	X	X	X	X	X	X
AS05739	AM1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS03802	AM1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS04322	AM1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS04050	AM1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS03145	AM1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS11108	AM4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS12012	AM5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS04001	AM5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS10816	AM5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS03326	AM5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS10202	AM5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS00001	AM6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS05254	NA	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS05544	NA	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS07024	NA	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AS08608	TOX	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

[y] Symbols: X or – indicate presence or absence, respectively, of polymerase chain reaction products of the tested genes.
[z] VCG = vegetative compatibility groups. NA = not available and TOX = aflatoxigenic strain.

Aflatoxin prevention technologies based on the application of atoxigenic strains as biocontrol agents in commercial agriculture have been practiced for over a decade in several states of the United States (Cotty et al. 2008). This strategy is currently under development worldwide for numerous crops and agroecosystems (Alaniz-Zanon et al. 2016; Doster et al. 2014; Mauro et al. 2015; Mehl et al. 2012). The Argentinean atoxigenic isolates were compared for their ability to disrupt aflatoxin biosynthesis in maize kernels coinfected by toxigenic strains. Our potential biological control isolates (AS04322 and AS03145) showed a similar ability to reduce aflatoxin contamination that previously was reported for nonaflatoxigenic isolates from Africa (Probst et al. 2011), Italy (Mauro et al. 2015), and North America (Mehl and Cotty 2010). Both isolates were also effective against two toxic strains from different regions and belonged to a widely distributed VCG, which may indicate that they have utility in diverse communities (Atehnkeng et al. 2016; Mauro et al. 2015). Coinoculation tests in the laboratory are commonly used as an initial tool to comparatively identify possible biocontrol agents (Atehnkeng et al. 2008b; Brown et al. 1991; Cotty 1990; Mauro et al. 2013; Mehl and Cotty 2010; Probst et al. 2011). Under field conditions, the strategy of using atoxigenic strains exploits the ability of the applications to displace aflatoxin producers from crop environments in addition to their ability to interfere directly with aflatoxin biosynthesis during coinfection (Cotty and Bayman 1993; Mehl and Cotty 2010). A tiny percentage of kernels become infected with high levels of aflatoxin in naturally contaminated maize samples (Lee et al. 1980). Therefore, a low incidence of aflatoxin-producer isolates can prevent aflatoxin contamination in maize (Cotty et al. 2008). The results obtained in the laboratory assay may not exactly reflect what happens in the field, where the competitive exclusion strategy contributes more to the aflatoxin reduction in the crop (Cotty et al. 2007). When the atoxigenic isolates were inoculated 48 h before the toxic isolates, no mycotoxins were detected in maize kernels. Our results agree with similar studies carried out in maize (Brown et al. 1991) and cottonseed (Cotty 1990). *A. flavus* contamination events occur in two distinct phases, with the first phase taking place during crop development and the second phase at any time after maturation (Cotty and Jaime-Garcia 2007). Significant infections during the first phase are associated with dry, hot conditions or wounding of the crop that favor fungal growth and crop susceptibility (Cotty and Jaime-Garcia 2007). The commercial product *A. flavus* AF36 can be applied as a single application from the V7 growth stage until emergence of the silk, according to the directions stated on its label. Application of biopesticides that ensure early colonization of maize under development by the atoxigenic strains could improve their performance as biocontrol agent, as demonstrated in this study. Studies of atoxigenic isolates as potential biocontrol agents are typically focused on evaluating only their ability to reduce aflatoxins (Cotty et al. 2008; Mauro et al. 2015; Mehl and Cotty 2010). Here, we also provide novel information on CPA reductions with similar values to those corresponding for aflatoxin reduction.

The atoxigenic isolates identified in the current study and evaluated as potential biocontrol agents did not produce sclerotia under laboratory conditions. This resistance structure serves for surviving during adverse environmental conditions (Cotty 1989; Wicklow et al. 1993). However, *A. flavus* also can survive and overwinter as mycelium in plant residues and serve as a source of new conidia (Abbas et al. 2008). Jaime-Garcia and Cotty (2004) indicated that maize cobs are the major source of *A. flavus* inoculum, with high levels of propagules after 2 years. Furthermore, high levels of inoculum have been observed in nonhost vegetative tissues such as soybean (Abbas et al. 2009). Therefore, abundant inoculum can be maintained on the soil of no-till maize fields. In Argentina, maize is commonly followed by soybean as a part of the crop rotation scheme and may favor the presence of NSP isolates in maize ears. Nevertheless, this should be considered in further studies of applying biocontrol agents in the field.

The selection of an atoxigenic *A. flavus* candidate strain for biocontrol should also consider other factors in addition to the mycotoxin-reduction ability. Knowledge of the molecular changes leading to atoxigenicity in *A. flavus* isolates is useful for tracking it after application in the agroecosystem, and for evaluating its stability in the crop field (Callicott and Cotty 2015). In *A. flavus*, deletions or loss-of-function mutations in the aflatoxin cluster define two distinct evolutionary linages (Moore et al. 2009). Four of the six nonaflatoxigenic VCG obtained in the current study presented the entire aflatoxin cluster. Furthermore, atoxigenic members of the dominant VCG for this work had remnants of all of the genes in the aflatoxin cluster as well as other isolates under study. This type of isolate seems to be predominant within the nonaflatoxigenic *A. flavus* population present in maize ears grown in regions I and IV of Argentina. This result differs from a nonaflatoxigenic population isolated from peanut crop soil in Argentina, where 73% (n = 48) of the isolates had partial or complete deletions in the aflatoxin cluster (Moore et al. 2013). Donner et al. (2010) studied several nonaflatoxigenic VCG isolated from maize grown in Africa and indicated that most of them had the entire aflatoxin cluster. In these strains, diverse polymorphisms resulted in the inability to produce aflatoxins (Donner et al. 2010). A single-nucleotide polymorphism in the polyketide synthase gene is associated with the loss of aflatoxin production in the VCG YV36 harboring the AF36 isolate and other biocontrol agents (Chang et al. 2012; Ehrlich and Cotty 2004). This nucleotide change results in a defective gene which is needed for aflatoxin biosynthesis (Ehrlich and Cotty 2004). None of the native isolates belong to this VCG, as was demonstrated in the current study. However, this nucleotide change may be found in isolates that belong to different VCG, suggesting that alleles in the *het* loci change independently of specific mutations in the aflatoxin cluster (Chang et al. 2012). On the other hand, AflaGuard is formulated with an isolate where the aflatoxin biosynthesis gene cluster is entirely deleted (Chang et al. 2005). The absence of any positive PCR for isolate AS00019 could be due to the lack of the entire aflatoxin cluster. This strain belonged to the single-isolate VCG AM3 determined in this study. Although isolate AS00019 was characterized as a CPA producer in maize kernels, it should be investigated as a potential biocontrol agent to reduce aflatoxin contamination. Other nonaflatoxigenic isolates have one or several distinct deletions within the aflatoxin cluster that explain the lack of aflatoxin production in accordance with previous data (Donner et al. 2010; Mauro et al. 2013).

In summary, based on this study, native atoxigenic isolates endemic to Argentina have the potential to be used as active ingredients in a biopesticide formulation to reduce both AFB_1 and CPA in maize. Our native isolates belong to a VCG well adapted to the target regions. It may result in an increased efficacy and a greater carryover among crops. The use of atoxigenic *A. flavus* isolates can be an effective option to reduce mycotoxin contamination in this country. This alternative should be considered by the corn industry and other industries of susceptible crops (i.e., peanut) to preserve the quality properties and food safety of maize kernels.

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