A polyphasic study of non-aflatoxigenic *Aspergillus flavus* Link, isolates from maize in the Chaco semi-arid region of Argentina

Estudio polifásico de aislados no aflatoxigénicos de *Aspergillus flavus* Link, en maices de la región del Chaco semiárido argentino

Javier Barontini [1, 2, 3, 4 *], María Silvina Alaniz Zanon [6], Ada Karina Torrico [5], Marcelo Druetta [7], Ignacio Martín Luna [7], Agustina Ruiz Posse [8], Sofía Noemí Chulze [6], María de la Paz Giménez Pecci [5]

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Abstract

Maize (*Zea mays* L.) is one of the most widely planted crops globally with Argentina leading world production and exportation. Santiago del Estero province, east of Tucumán and north of Córdoba encompasses eight agro-climatic zones in the Chaco Semi-arid region, agro-ecologically characterized by a wide temperature range and frequent drought periods that expose the crop to pathogens, particularly *Aspergillus flavus*. This pathogen is responsible for ear rot and grain contamination with mycotoxins such as aflatoxin B_1 and cyclopiazonic acid. This study obtained fungal isolates from ears of maize and characterized them according to toxigenic capability and morphotype of sclerotia (S < 400 µm, associated with high levels of aflatoxins and L > 400 µm, related to variable levels of aflatoxins). In addition, those not producing aflatoxins were studied to determine phylogenetic relationships based on sequences of a segment of the *CaM* gene. Fifty-eight isolates were obtained in eight localities representing each agro-climatic zone, 30 of which were non-aflatoxigenic, 28 aflatoxigenic, and all producers of ciclopiazonic acid. Six isolates did not produce sclerotia, 51 were L and only one was S, the latter being a non-producer of aflatoxins. The number of sclerotia was positively correlated with the production of aflatoxin B_1, while size was negatively correlated. The *CaM* gene sequences corroborated that the isolates belonged to the *A. flavus* clade and the high nucleotide similarity among them (99.4% to 100%) revealed almost zero genetic diversity in this geographic region. No significant differences were observed in the proportion of isolates between growing seasons or among agroclimatic districts. This research revealed characteristics of fungus populations in this agricultural region of north Argentina.

Keywords
non-aflatoxigenic isolate • cyclopiazonic acid • sclerotia production • phylogenetic relationships

1 Universidad Nacional de Cuyo. Facultad de Ciencias Agrarias. Cátedra de Fitopatología. Almirante Brown 500. Chacras de Coria. Mendoza. Argentina. M5528AHB. * jbarontini@fca.uncu.edu.ar
2 Instituto Nacional de Tecnología Agropecuaria (INTA). Estación Experimental Mendoza. San Martín 3853. Luján de Cuyo. Mendoza. Argentina. 5507
3 Instituto de Biología Agrícola de Mendoza (IBAM). Almirante Brown 500. Chacras de Coria. Mendoza. Argentina. M5528AHB.
4 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Godoy Cruz 2290. Ciudad Autónoma de Buenos Aires. Argentina. C1425FQB.
5 INTA. Instituto de Patología Vegetal (IPAVE). Centro de Investigaciones Agropecuarias (CIAP). Av. 11 de Septiembre 4755. Córdoba. Córdoba. Argentina. X5014MGO.
6 Instituto de Investigación en Micología y Micotoxicología (IMICO). CONICET. Universidad Nacional de Río Cuarto (UNRC). Ruta Nac. 36 km 601. Río Cuarto. Córdoba. Argentina. X5804BYA.
7 Estación Experimental Agropecuaria. Este de Santiago del Estero. Instituto Nacional de Tecnología Agropecuaria (EEA - ESE - INTA). Ruta Prov. N° 6 Km 9. Quimilí. Santiago del Estero. Argentina. G3740CDA.
8 Unidad de Fitopatología y Modelización Agrícola (UFyMA). INTA. CONICET. Av. 11 de Septiembre 4755. Córdoba. Córdoba. Argentina. X5014MGO.

Resumen

El maíz (*Zea mays* L.) es uno de los cultivos más sembrados en el mundo, siendo Argentina líder mundial en producción y exportación. La Provincia de Santiago del Estero, el este de Tucumán y el norte de Córdoba abarcan ocho zonas agroclimáticas en la región Chaco semiárida, la cual se caracteriza por un amplio rango de temperatura y frecuentes sequías que exponen al cultivo a patógenos, particularmente *Aspergillus flavus,* responsable de la podredumbre de la espiga y la contaminación de granos con micotoxinas como aflatoxina B_1 y ácido ciclopiazónico. Se obtuvieron aislados del hongo desde espigas de maíz y se caracterizaron según capacidad toxigénica y morfotipos de esclerocios (S < 400 µm, asociados con elevados niveles de aflatoxinas y L > 400 µm, relacionados con niveles variables de aflatoxinas). Además, aquellos que no producían aflatoxinas se estudiaron para determinar sus relaciones filogenéticas sobre la base de un segmento del gen *CaM*. Cincuenta y ocho aislados fueron obtenidos en ocho localidades representativa de cada zona agroclimática, 30 de los cuales resultaron no aflatoxigénicos, 28 aflatoxigénicos y todos productores de ácido ciclopiazónico. Seis aislados no produjeron esclerocios, 51 fueron L y solo uno fue S, siendo este último no aflatoxigénico. El número de esclerocios correlacionó positivamente con la producción de aflatoxina B_1, mientras que el tamaño lo hizo de manera negativa. Las secuencias del gen *CaM* corroboraron que los aislados pertenecen al clado *A. flavus* y la alta similitud nucleotídica (99,4% a 100%) reveló casi nula diversidad genética en esta región. No se observaron diferencias en la proporción de aislados entre campañas agrícolas estudiadas ni distritos agroclimáticos. Esta investigación reveló características de las poblaciones de hongos en esta región agrícola del norte argentino.

Palabras clave

aislado no aflatoxigénico • ácido ciclopiazónico • producción de esclerocios • relaciones filogenéticas

Introduction

Argentina ranks sixth in maize production, with approximately 60 million tons a year. The region encompassing Santiago del Estero province, east of Tucumán and north of Córdoba, in the Chaco Semi-arid region, produces about 6 million tons (58). This geographical area is a critical climatic zone, where climatic factors are highly variable (57). Thus, maize crop development in these agroecosystems is constrained by stressful environmental factors that expose the crop to different pathogens, such as those responsible for ear rot (27, 67). The most dangerous of these pathogens is *Aspergillus flavus* Link, a globally distributed filamentous, cosmopolitan pathogen that causes opportunistic infections in animals and plants (12, 42). This pathogen is commonly present in air and soil mycobiota. Under certain water and thermal stress conditions, some isolates produce mycotoxins like aflatoxins and cyclopiazonic acid (CPA). Aflatoxins are secondary metabolites with strong hepatotoxic, teratogenic, immunosuppressive and mutagenic activity when inhaled, ingested or absorbed through animal and human skin (51); *A. flavus* is the main driver of aflatoxin contamination in maize in the world (45). While CPA produces liver necrosis, convulsions myocardium lesions in animals (62, 63), the specific exposure response to this toxin differs among species, without sufficient evidence about natural contamination of foods (81).

The maximum safety limit of aflatoxins for maize commercialization varies between countries, being 20 ng. g^{-1} in Argentina, Brazil and USA, 4 ng. g^{-1} in the European Union, and 15 ng. g^{-1} in African countries like Ghana (4, 9, 16, 34, 77).

Species of the genus *Aspergillus* produce sclerotia, resistance structures consisting of darkly pigmented and hardened hyphal mats (50) conferring different phenotypic characteristics (1). Thus, *Aspergillus* species can be classified into two morphotypes: S isolates, which produce numerous sclerotia under 400 µm in diameter, associated with high levels of aflatoxins, and L isolates, with fewer sclerotia, diameter over 400 µm, and related to variable levels of aflatoxins (75). A third type of isolate not producing sclerotia under laboratory conditions, has been identified in Argentina and Italy (13, 38).

The variability among *A. flavus* populations can be estimated by establishing phylogenetic relationships based on morphological and molecular markers (51), as in the case of the calmodulin gene (*CaM*) (36). Calmodulin (*CaM*) is a highly conserved polypeptide present in eukaryotic cells. In the genus *Aspergillus*, *CaM* is important in phosphorylation and dephosphorylation of proteins involved in the biosynthesis of aflatoxins (47).

Aflatoxin contamination usually starts in the field, during harvest of infected grains, becoming more severe during storage (35). In the field, spores of *A. flavus* are transported by biotic agents (insects, birds) and/or abiotic agents (wind, rain splash) to the maize ear. Spores enter through the silk during flowering and through the wounds of the ear cover or of developing kernels (66). Water stress, high temperatures, insect attack, certain sowing dates and high crop density favor contamination with the pathogen and the possible production of aflatoxins (48).

Some members of *A. flavus* were found unable to biosynthesize aflatoxins being, therefore, plausible biocontrol tools (23). Biological control applied in maize and peanut (6, 73), among other crops, is based on the competition for infection sites and essential nutrients between non-aflatoxigenic and aflatoxigenic isolates. Thus, non-aflatoxigenic isolates displace aflatoxigenic ones and reduce the latter's capacity to produce aflatoxins (70, 79).

Non-aflatoxin producing strains selected as biocontrol agents must meet some requirements, such as being native to the area of application, being able to displace the toxicogenic isolates in the ecosystem and thereby reduce infections (23) and be genetically incompatible with the remaining fungal population. Therefore, studies of the native fungal population are crucial (42).

This work aimed to study the diversity of *A. flavus* populations in the Chaco Semi-arid region of Argentina through the morphological, toxicological and molecular characterization of isolates obtained from maize ears.

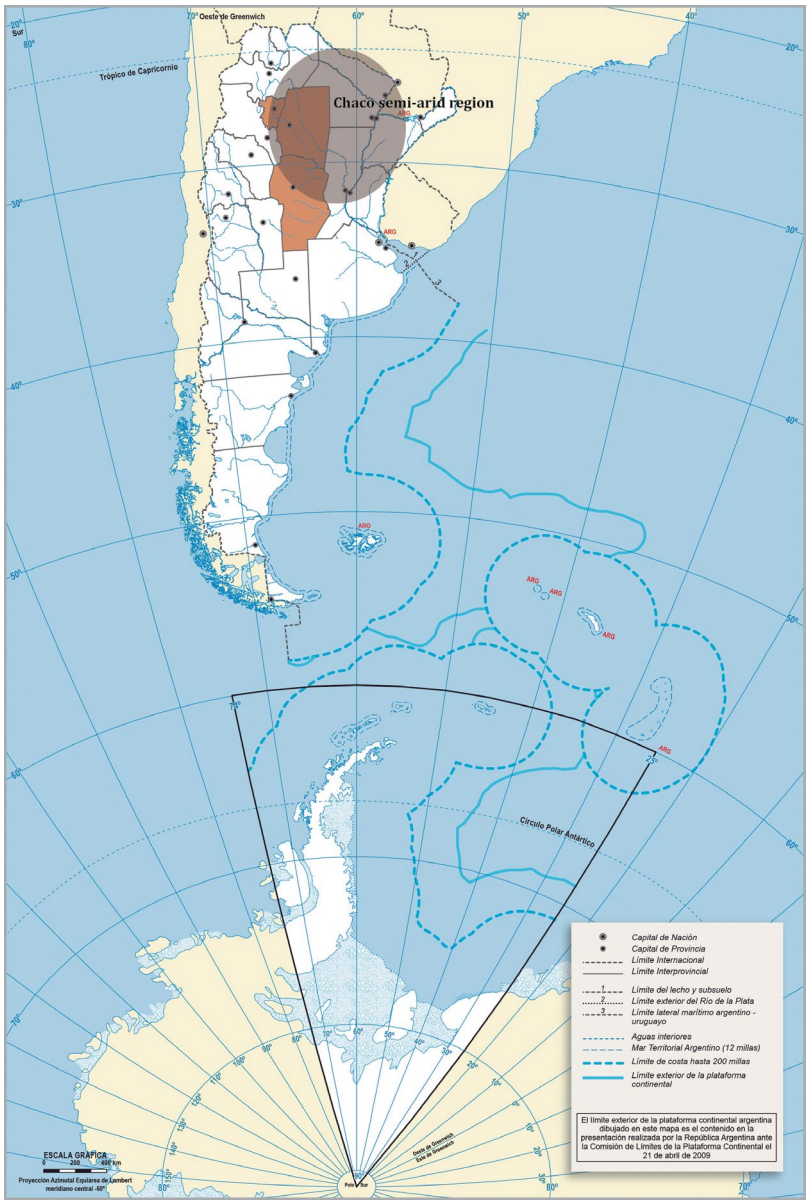
Materials and methods

Sampling

Sampling was conducted during the 2015/16 and 2016/17 growing seasons in representative localities of eight agro-climatic zones: Sachayoj (Zone 4: Río Muerto), Quimilí (Zone 5: Hickmann), Bandera (Zone 17: Bandera), Santiago del Estero (Zone 18: Monteagudo), Sumampa (Zone 37: Soto), Santa Rosa de Leales (Zone 18A: Monteagudo), Rayo Cortado (Zone 37C: Soto-north of Córdoba) and Villa de Tulumba (Zone 61B: Deán Funes), located between -30.38 and -26.55 S and -61.82 and -65.27 W (25) (figure 1, page 61). Ten ears were randomly collected along a diagonal line in the plot, at plant physiological maturity before harvest. They were threshed to form a composite sample and oven-dried at 38°C for 72 hours until final humidity was under 12 %, therefore, minimizing fungus development (26).

Isolation and morphological and molecular identification

From each sample, 100 kernels were taken, surface-desinfested with a 1% sodium hypochlorite wash followed by three washes with distilled water and plated in DG-18 medium. Kernels were incubated for 7 days at 25°C and cultures with characteristics similar to those of *A. flavus* were transferred to MEA medium. They were incubated for 7 days at 25°C (65) and then identified through taxonomic keys (48). Spores of each isolate were serially diluted, and the most diluted concentration was cultured in 2 % Water - Agar (WA) medium and incubated for 18 hours at 27°C (65). The germinated conidia were identified under Nikon SMZ-10 stereoscopic microscope (15X). Two conidia were extracted from each cultured Petri dish, along with a portion of culture medium, and transferred to Petri dishes containing MEA medium. They were incubated for 7 days at 25°C. Then, one of them was stored to obtain the single spore cultures corresponding to each isolate.



Sampling localities / Localidades evaluadas: Sachayoj. Quimilí. Santiago del Estero. Bandera. Sumampa (Santiago del Estero province). Santa Rosa de Leales (Tucumán province). Rayo Cortado. Villa de Tulumba (Córdoba province).

Figure 1. Sampling localities in the Chaco Semi-arid region of Argentina.

Figura 1. Localidades evaluadas en la región del Chaco semiárido argentino.

The identity of *A. flavus* was confirmed by PCR using the primers FLA1, 5'-GTAGG-GTTCCTAGCGAGCC-3'; FLA2 5'-GGAAAAAGATTGATTTGCGTTC-3' (41), with the ITS region as target, using the isolate *A. flavus* CCC 101-92 < NRRL 3251 as positive control, and the isolate *A. parasiticus* NRRL 2999 as negative control. Each single-spore isolate was cultured in MEA and incubated for 7 days at 25°C. Then, an aliquot of mycelium was taken, and DNA was extracted (55). For each reaction, 1 µL (final concentration of 20-100 ng of DNA.µL^{-1}, quantified in Nano Drop, Thermo Fisher Scientific, USA) of DNA solution was mixed with 24 µL of a solution composed of 5 µL 5x Green GoTaq® reaction buffer (Promega®, Madison, WI, USA), 0.5 µL of a mixture of dNTP (10 mM of dATP, dCTP, dGTP and dTTP), 1 µL of the primers (20 µM), 1 µL of polymerase enzyme GoTaq® (5U.µL^{-1}, Promega®, Madison, WI, USA) and 16.5 µL DEPC-treated water. The PCR reaction was performed following González Salgado *et al*. (2011). PCR products were revealed by agarose gel electrophoresis, visualized in UV transilluminator, with previous staining in GelRed™ Biotium solution (2.5 ng. µL^{-1}) and confirmed by the expected band size of 500 pb. Molecular size of the DNA fragments was estimated using the "qLadder 100 pb precision" marker (PB-L®, Bs As, Argentina).

Sclerotia production

Each isolate was cultured in 6-cm Petri dishes containing Czapeck dox (Cz) medium. They were inoculated in the center of the dish with 10 µL of a spore suspension and incubated for 14 days at 30°C (64). Sclerotia were recovered, their diameters measured under a microscope (Nikon eclipse Cs1 spectral) and characterized following Cotty (1989) into L or S morphotypes or isolates not producing (NP) sclerotia (60). Isolates not producing sclerotia after an incubation period were cultured in 5/2 medium for 5 to 7 days at 31°C to induce their production (38).

Toxigenic capability

Considering Aflatoxin B_1 (AFB_1) was one of the greatest frequency and toxicity of the four most important aflatoxins identified, its production was quantified (23, 82). *A. flavus* isolates were inoculated in duplicate on MEA slants for 7 days at 28°C. These cultures were used to prepare spore suspensions, following the method described by Alaniz Zanon *et al*. (2013). Spore concentration was measured in a Neubauer chamber and adjusted to 10^5 spores. mL^{-1}. Four-milliliter vials containing 1 mL broth from a medium containing 150 g sucrose, 20 g yeast extract, 10 g soytone, and 1 L distilled water, were inoculated with 100 µL of each spore suspension. Medium pH was adjusted to 5.9 with HCl. Cultures were incubated for 7 days at 30°C. Vial cultures were extracted according to Horn *et al.* (1996), by adding 1 mL of chloroform to each vial and vortexing for 30 s.

The first group of dried extracts was evaluated in their capacity to produce aflatoxins by HPLC according to Horn *et al.* (1996). Aflatoxins were analyzed by injecting 50 µL of extract from each vial into an HPLC system consisting of a Hewlett Packard model 1100 pump (Palo Alto, CA) connected to a Hewlett Packard model 1046A programmable fluorescence detector and a data module Hewlett Packard Kayak XA (HP ChemStation Rev.A.06.01). Chromatographic separations were performed on a stainless steel, C18 reversed-phase column (150 mm × 4.6 mm i.d., 5 µm particle size; Luna Phenomenex, Torrance, CA, USA) connected to a precolumn Security Guard (20 mm × 4.6 mm i.d., 5 µm particle sizes, Phenomenex). The mobile phase was water:methanol:acetonitrile (4:1:1, v/v/v) at a flow rate of 1.5 mL. min^{-1}. Pure aflatoxin solutions were used as external standards (Sigma-Aldrich, St. Louis, MO, USA).

The second group of dried extracts was resuspended in 500 µL of mobile phase of acetonitrile: $ZnSO_4$ 4 mM buffer solution (65:35, V/V) and CPA was determined in the HPLC system. Chromatographic separations were performed in Agilent ZORBAX RX-SIL column (250 mm x 4.6 mm i.d., 5 µm particle size) connected to a pre-column Security Guard (20 mm x 4.6 mm i.d., 5 µm particle sizes, Phenomenex) and 50 µl of each sample were analyzed at a flow velocity of 0.8 mL. min^{-1}.

The quantitative analysis was performed by normalization of peak areas. A calibration curve was elaborated from the areas obtained for the different concentrations of standards of aflatoxins and CPA, as applicable (Sigma Aldrich, St. Louis, MO, USA). The AFB_1 and CPA detection limit was 1 ng. g^{-1} (24, 32).

Sequencing and Phylogenetic relationships

Molecular variability of the non-aflatoxigenic isolates was explored using a 688-pb fragment of the *CaM* gene. We studied only this type of isolate given their potential for biocontrol strategies. Spores of the isolates cultivated in MEA medium were suspended for 7 days at 25°C. Conidia ($1x10^6$ conidia. mL^{-1}) were inoculated in 100 mL of lixiviated medium of potato glucose agar (10% of potato infusion from 200 g potato, 2% glucose, 4.5 pH). They were incubated in orbital shaker at 150 rpm for 48 hours at 25°C. Mycelium was collected by filtration and powdered with liquid N_2 (28). This culture medium allows one to obtain enough amount of mycelium. DNA was extracted using the cetyl-trimethylammonium bromide (CTAB) method (54), quantified by spectrophotometry and a segment of the *CaM* was amplified with the primers CL1, 5'- GA(GA)T(AT)CAAGGAGGCCTTCTC -3'; CL2A, 5'- TTTTTGCATCATGAGTTGGAC -3' (61). The obtained fragments of the expected size (688 pb) were purified using DNA columns of Wizard® SV Gel columns and PCR clean - Up system (Promega, Madison, WI, USA), following manufacturer instructions. Then, they were quantified by spectrophotometry. Both DNA chains were sent for sequencing by Sanger

method (Macrogen, Seoul, South Korea). The obtained sequences were aligned using the software Clustal X2 (54). The software MEGA version 7 (53) was used to select the best nucleotide substitution model and to obtain the phylogenetic trees using Neighbor-Joining (NJ) (74) and Maximum-Likelihood (ML) methods (39, 76), both with 10,000 replicates bootstrap. The sequences were compared with the reference NW_002477238.1 and *A. niger* MH645004.1 was used as outgroup, both from the GenBank.

Statistical analysis

Data on sclerotia morphotypes, toxigenic capacity and variability of the sequences of the *CaM* genes were analyzed using InfoStat statistical software as well as correlation analyses (29). Mean values were obtained using analysis of variance (ANOVA) and differences between means were compared using Fisher's LSD test ($P< 0.05$).

Results and discussion

Morphological and molecular identification

A total of 58 isolates were obtained in the sampling localities from the eight agro-climatic zones (table 1, page 63-64) with *A. flavus* morphological characteristics, according to Klich (2002) (figure 2, page 64). *A. flavus* identity was confirmed using the specific primers FLA1 and FLA2 through amplification of a 500-pb band (41) (figure 3, page 65). The presence of isolates in the eight zones, each one being considered a different agroecological environment given different meteorological scenarios at each growing season shows the broad distribution of the fungus in the study region, as reported for other regions of the country (8, 17) and the world (11, 30, 32).

Table 1. Isolates of *Aspergillus flavus* from maize ears collected from representative localities of eight agro-climatic zones of the Chaco Semi-arid region of Argentina. Production of aflatoxin B_1, cyclopiazonic acid and sclerotia morphotype during the 2015/16 and 2016/17 growing seasons.

Tabla 1. Aislados de *Aspergillus flavus* de espigas de maíz colectadas en localidades representativas de ocho zonas agroclimáticas del Chaco semiárido argentino. Producción de aflatoxina B_1, ácido ciclopiazónico y morfotipo de esclerocios durante las campañas agrícolas 2015/16 y 2016/17.

Isolate	Agro-climatic Zone	Locality	Sampling year	Sclerotia Morphotype	AFB_1 (ng. g^{-1})	CPA (ng. g^{-1})
ASRC1	37C	Rayo Cortado	2017	L	6.4	3.3
ASRC2	37C	Rayo Cortado	2017	NP	0.0	6.2
ASRC3	37C	Rayo Cortado	2017	NP	0.0	0.6
ASVT1	61B	Tulumba	2017	L	278.4	0.3
ASVT2	61B	Tulumba	2017	L	0.0	2.8
ASVT3	61B	Tulumba	2017	L	405.1	8.6
ASVT4	61B	Tulumba	2017	L	60.6	2.3
ASBA12	17	Bandera	2016	L	16.3	9.9
ASBA3	17	Bandera	2016	L	0.0	2.8
ASBA4	17	Bandera	2016	L	7.2	9.2
ASBA8	17	Bandera	2016	L	0.0	4.7
ASBA1	17	Bandera	2017	L	0.0	2.3
ASBA10	17	Bandera	2017	L	5.9	9.3
ASBA11	17	Bandera	2017	L	0.0	2.3
ASBA2	17	Bandera	2017	L	0.0	2.3
ASBA5	17	Bandera	2017	L	0.0	6.7
ASBA6	17	Bandera	2017	L	90.7	2.9
ASBA7	17	Bandera	2017	L	6.7	5.4
ASBA9	17	Bandera	2017	NP	0.0	6.9

Agro-climatic zones: 4: Río Muerto, 5: Hickmann, 17: Bandera, 18 and 18A: Monteagudo, 37: Soto, 37C: Soto - North of Córdoba, 61B: Dean Funes.

L: sclerotia of > 400 µm; S: sclerotia of < 400 µm; NP: non-producer of sclerotia.

AFB1: Aflatoxin B; CPA: cyclopiazonic acid.

Zonas agroclimáticas: 4: Río Muerto, 5: Hickmann, 17: Bandera, 18 y 18A: Monteagudo, 37: Soto, 37C: Soto - Norte de Córdoba, 61B: Deán Funes.

L: esclerocios de tamaño > 400 µm; S: esclerocios de tamaño < 400 µm; NP: no productor de esclerocios.

AFB_1: Aflatoxina B; CPA: ácido ciclopiazónico.

Isolate	Agro-climatic Zone	Locality	Sampling year	Sclerotia Morphotype	AFB_1 (ng. g^{-1})	CPA (ng. g^{-1})
ASQU1	5	Quimilí	2016	L	7.2	5.6
ASQU10	5	Quimilí	2016	L	0.0	54
ASQU11	5	Quimilí	2016	L	2521.5	7.5
ASQU12	5	Quimilí	2016	L	17.6	5.8
ASQU13	5	Quimilí	2016	L	5.6	1.2
ASQU14	5	Quimilí	2016	L	5.6	8.6
ASQU15	5	Quimilí	2016	L	0.0	3.2
ASQU16	5	Quimilí	2016	L	0.0	4.2
ASQU17	5	Quimilí	2016	L	0.0	8.7
ASQU18	5	Quimilí	2016	L	0.0	8.7
ASQU19	5	Quimilí	2016	L	0.0	0.6
ASQU2	5	Quimilí	2016	L	0.0	0.6
ASQU20	5	Quimilí	2016	L	0.0	7.1
ASQU21	5	Quimilí	2016	L	499.1	5.9
ASQU3	5	Quimilí	2016	L	34	0.4
ASQU4	5	Quimilí	2016	L	9.3	8.1
ASQU5	5	Quimilí	2016	L	6.3	16.4
ASQU6	5	Quimilí	2016	L	6	6.2
ASQU8	5	Quimilí	2016	L	5.7	6.9
ASQU9	5	Quimilí	2016	L	0.0	8.6
ASQU7	5	Quimilí	2017	L	0.0	1.6
ASSA1	4	Sachayoj	2016	L	9.8	8.5
ASSA3	4	Sachayoj	2016	L	13.2	9.9
ASSA4	4	Sachayoj	2016	L	0.0	7.6
ASSA6	4	Sachayoj	2016	L	9.1	9.1
ASSA2	4	Sachayoj	2017	L	70.1	7.9
ASSA5	4	Sachayoj	2017	L	0.0	3.8
ASSE5	18	Sgo del Estero	2016	L	5.8	8.9
ASSE1	18	Sgo del Estero	2017	S	0.0	1.8
ASSE2	18	Sgo del Estero	2017	L	0.0	0.6
ASSE3	18	Sgo del Estero	2017	L	48958.1	2.2
ASSE4	18	Sgo del Estero	2017	L	290.9	4.9
ASSE6	18	Sgo del Estero	2017	NP	0.0	4.9
ASSE7	18	Sgo del Estero	2017	NP	0.0	0.6
ASSE8	18	Sgo del Estero	2017	L	0.0	2.0
ASSE9	18	Sgo del Estero	2017	L	0.0	0.8
ASSU1	37	Sumampa	2017	NP	0.0	2.9
ASSU2	37	Sumampa	2017	L	12.3	5.1
ASLE1	18A	Leales	2017	L	0.0	1.3

Agro-climatic zones: 4: Río Muerto, 5: Hickmann, 17: Bandera, 18 and 18A: Monteagudo, 37: Soto, 37C: Soto - North of Córdoba, 61B: Dean Funes.

L: sclerotia of > 400 µm; S: sclerotia of < 400 µm; NP: non-producer of sclerotia.

AFB1: Aflatoxin B; CPA: cyclopiazonic acid.

Zonas agroclimáticas: 4: Río Muerto, 5: Hickmann, 17: Bandera, 18 y 18A: Monteagudo, 37: Soto, 37C: Soto - Norte de Córdoba, 61B: Deán Funes.

L: esclerocios de tamaño > 400 µm; S: esclerocios de tamaño < 400 µm; NP: no productor de esclerocios.

AFB_1: Aflatoxina B; CPA: ácido ciclopiazónico.

Figure 2. *Aspergillus flavus* Link colony on malt extract agar (MEA) culture medium.
Figura 2. Colonia de *Aspergillus flavus* Link en medio de cultivo agar extracto de malta (MEA).

M: molecular marker (100-pb DNA Ladder); **+**: *Aspergillus flavus* NRRL 3251 (positive control); -: *A. parasiticus* NRRL2999 (negative control); 1: ASSA2; 2: ASQU7; 3: ASQU21; 4: ASQU10; 5: ASQU15; 6: ASRC2; 7: ASQU12; 8: ASQU6; 9: ASBA3; 10: ASVT1.

M: marcador molecular (100-pb DNA Ladder); **+**: *Aspergillus flavus* NRRL 3251 (control positivo); -: *A. parasiticus* NRRL2999 (control negativo); 1: ASSA2; 2: ASQU7; 3: ASQU21; 4: ASQU10; 5: ASQU15; 6: ASRC2; 7: ASQU12; 8: ASQU6; 9: ASBA3; 10: ASVT1.

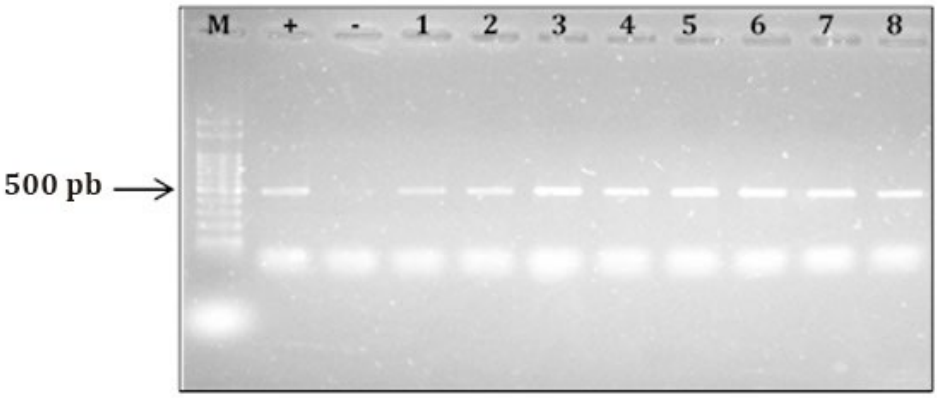


Figure 3. Electrophoresis gel showing PRC products obtained using the specific primers FLA1 and FLA2 (González Salgado *et al.*, 2011).

Figura 3. Gel de electroforesis mostrando productos de PCR obtenidos utilizando los iniciadores específicos FLA1 y FLA2 (González Salgado *et al.*, 2011).

Sclerotia production

Of the 58 *A. flavus* isolates identified, 6 (10%) produced no sclerotia, whereas 52 (90%) produced sclerotia. Of the latter, 51 (98%) were identified as L morphotype (figure 4), with > 400 µm in diameter, and only isolate, ASSE1, (2%) corresponded to S morphotype, with < 400 µm in diameter (table 1, page 63-64). Similar proportions between L, S and NP morphotypes in maize kernels were reported by Moreno (2004) in Mexico and by Pildain *et al.* (2005) in Argentina in peanut isolates. The low or null presence of S morphotype in maize was also reported by Giorni *et al*. (2007) in Italy. These authors identified one S isolate out of 70 isolates, with the remaining ones being NP. Similarly, Alaniz Zanon *et al*. (2018) reported a high proportion of L morphotype in Argentina with respect to NP, with absent S morphotype.

Sclerotia production was reported in isolates from all the agro-climatic zones and in both evaluated growing seasons. Dominance of L morphotype among isolates obtained in the Chaco Semi-arid region agrees with previous records in Argentina (7, 18) and in other countries, like Brazil, Portugal, Nigeria and Sub-Saharan Africa regions (5, 20, 33, 68, 72).

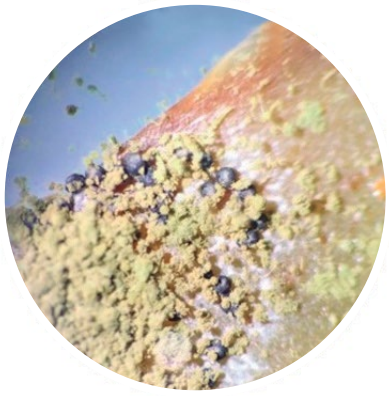


Figure 4. Sclerotia L morphotype in aflatoxigenic isolate of *Aspergillus flavus.* Observation under stereo microscope Nikon SMZ-10 (15X).

Figura 4. Esclerocios morfotipo L en aislado aflatoxigénico de *Aspergillus flavus.* Observación bajo lupa estereoscópica Nikon SMZ-10 (15X).

The high incidence of S morphotype is associated with regions of low precipitation and high temperatures, where high number of small sclerotia can be a survival trait facing rapid temperature and humidity fluctuations (19). However, and even though the study region is dry and hot, this was not observed.

Toxigenic capability

Based on the production of AFB_1 and CPA of the 58 isolates obtained, we identified 30 (52%) non-aflatoxigenic and 28 (48%) aflatoxigenic. This similarity of proportions was indicated by Martins *et al.* (2017) in peanut crop in Brazil and Camiletti *et al.* (2018) in maize ears in Argentina.

Aflatoxin-producer and non-producer isolates were obtained in all the agro-climatic zones and growing seasons, except Santa Rosa de Leales, where the only isolate found was non-producer.

Of the 28 isolates producing AFB_1, 26 (93%) had concentrations below 500 ng. g^{-1}, whereas ASQU11 and ASSE3 were higher aflatoxin producers of 2,521.5 ng. g^{-1} and 48,958.1 ng. g^{-1} respectively (table 1, page 63-64). Although the capacity to produce mycotoxins may depend on geographical origin and environmental conditions (62), our results do not show any pattern associated with zones or growing seasons. This agrees with Bayman and Cotty (1993), who did not detect differential patterns between nearby zones.

All isolates were CPA producers, indicating toxicity importance (78) and plausible use as aflatoxin biocontrol strategies. This is the case of the AF36 agent used in competitive exclusion strategies and found responsible for CPA increase in maize kernels inoculated in the field, and peanuts (2, 31). By contrast, Camiletti *et al*. (2018) detected 19% of CPA non-producer isolates in maize kernels in Argentina. On the other hand, Vaamonde *et al*. (2003) studied wheat, soybean and peanut in Argentina and found between 6% and 27% of isolates not producing this mycotoxin. In Italy, Giorni *et al*. (2007) reported 39% CPA non-producer isolates in maize kernels, whereas, in organic nut plantations in Brazil, Reis *et al*. (2014) found that 34% of the *A. flavus* isolates were non-producers of that mycotoxin. In addition, Jamali *et al*. (2012) identified 19 % of the isolates as CPA non-producers in soil samples of pistachio plantations in Czech Republic.

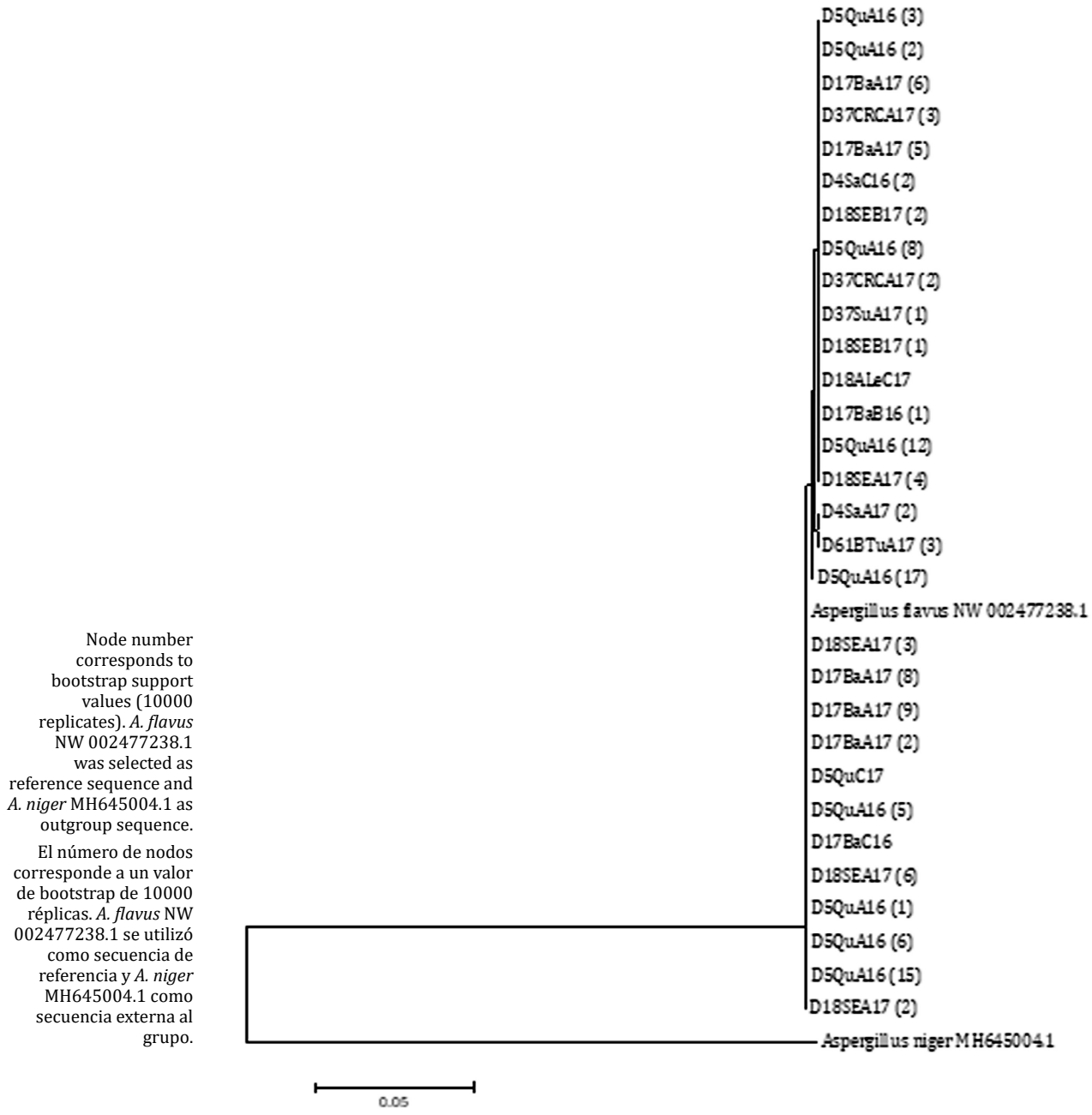
The presence of isolates producing both CPA and aflatoxins was reported in Argentina and other parts of the world (8, 18, 37, 71). The isolates with the highest AFB_1 production obtained in the Chaco Semi-arid region do not correspond to those with the highest CPA production.

Correlations between AFB_1 production and the number (r= 062) and size of sclerotia (r= -0.38) indicate that the produced amount of AFB_1 increases as sclerotia size decreases. These results are similar to those reported by Arrúa Alvarenga *et al*. (2012) and Pildain *et al*. (2005), but differ from Bouti *et al*. (2020), who did not find any correlation between sclerotia and aflatoxin production. No correlation was observed between CPA production and sclerotia morphotype.

The only S morphotype identified in this study (ASSE1), collected in 2016/17 growing season, did not produce AFB_1. While the presence of non-aflatoxin-producing isolates was also reported for USA, Ghana and Brazil (3, 22, 40, 44), in general, S morphotype isolates produce higher aflatoxin concentrations than L or NP morphotypes and are identified as major causal agents of severe contamination in maize and of most human deaths due to aflatoxicosis (13, 23). The S isolates are worldwide distributed, associated with aflatoxins B production in the USA and Africa (68), and of both B and G aflatoxins (3, 38).

Sequencing and phylogenetic analysis

All the *CaM* gene sequences studied in this work, using both NJ and ML, belong to the *A. flavus* clade (figure 5, page 67 and figure 6, page 68). The high similarity among isolates for that character showed limited genetic diversity in this geographic region. In addition, the only S morphotype is located together with L morphotype isolates, all of which are aflatoxin non-producers. This finding may be attributed to the fact that these are young populations and, therefore, may have not undergone sufficient mutations or recombination events leading to variability among isolates from the region (42, 62). However, only the northern zones of the region (4 and 5) correspond to land recently converted to agriculture, whereas in the remaining districts, both commercial hybrids and maize for self-consumption have been cultivated for several decades.



Node number corresponds to bootstrap support values (10000 replicates). *A. flavus* NW 002477238.1 was selected as reference sequence and *A. niger* MH645004.1 as outgroup sequence.

El número de nodos corresponde a un valor de bootstrap de 10000 réplicas. *A. flavus* NW 002477238.1 se utilizó como secuencia de referencia y *A. niger* MH645004.1 como secuencia externa al grupo.

Figure 5. Phylogenetic tree built using the Neighbor-Joining (NJ) statistic method based on the relationship among segments of the sequence of the *CaM* gene from *A. flavus* isolates from agro-climatic zones of the Chaco Semi-arid region in Argentina.

Figura 5. Árbol filogenético realizado con el método estadístico Neighbor-Joining (NJ) basado en las relaciones entre las secuencias de segmentos del gen *CaM* de aislados de *A. flavus* provenientes de zonas agroclimáticas del Chaco semiárido argentino.

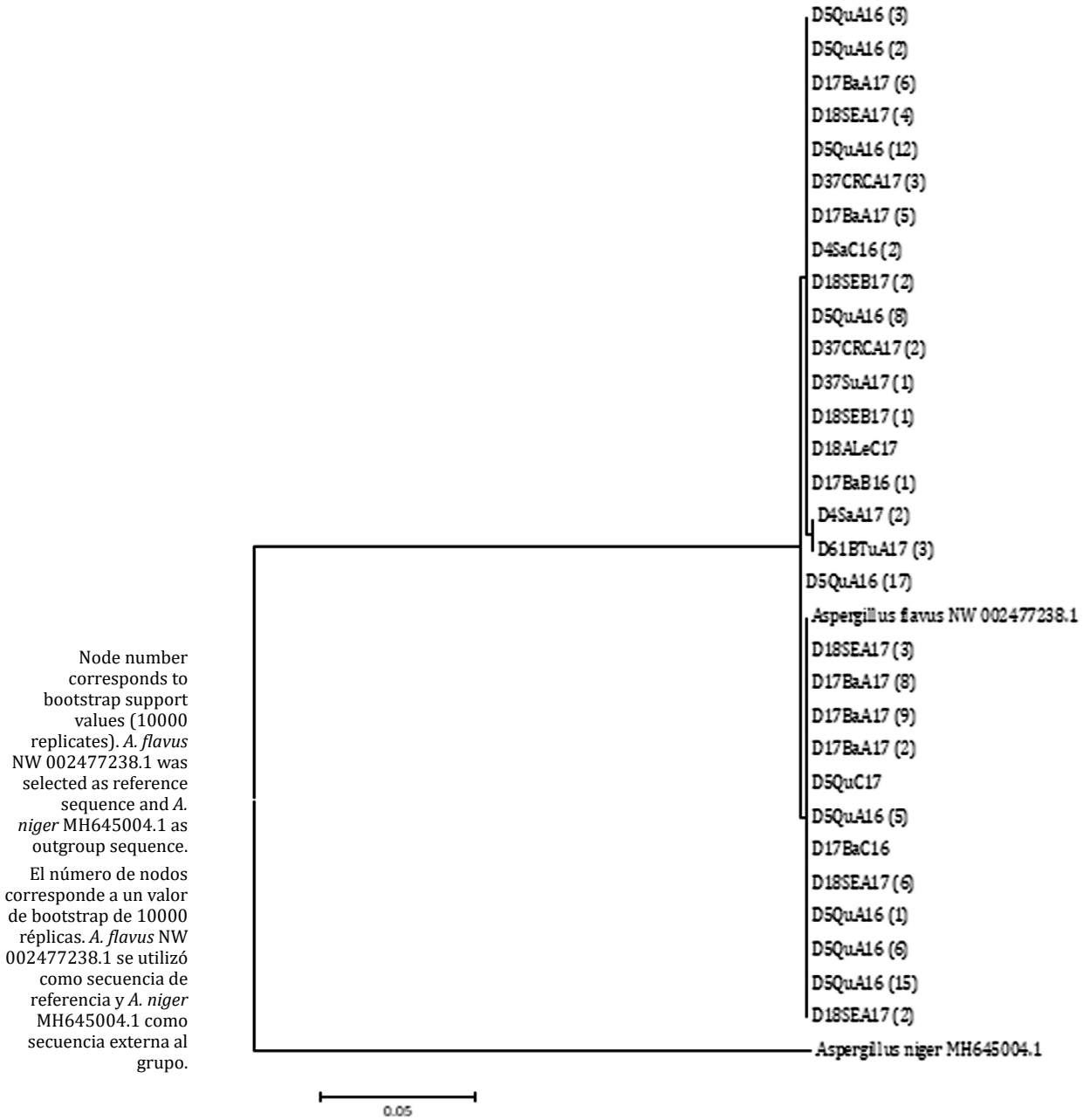


Figure 6. Phylogenetic tree built using the Maximum-Likelihood (ML) statistical method based on the relationship among segments of sequences of the *CaM* gene from *A. flavus* isolates from agro-climatic zones in the Chaco Semi-arid region in Argentina.

Figura 6. Árbol filogenético realizado con el método estadístico Maximum-Likelihood (ML) basado en las relaciones entre las secuencias de segmentos del gen *CaM* de aislados de *A. flavus* provenientes de zonas agroclimáticas del Chaco semiárido argentino.

Conclusions

A. flavus is present in all localities of the sampled agro-climatic zones and both growing seasons, evidencing the wide distribution of the pathogen in the Chaco Semi-arid region.

The population of *A. flavus* exhibits diversity in terms of sclerotia morphotypes and production of AFB_1 and CPA.

Low genetic variability among the isolates was observed, all belonging to the *A. flavus* clade.

In the future, genes from more variable regions will be used to perform phylogenetic analyses among *A. flavus* isolates.

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