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Non-aflatoxigenic *Aspergillus flavus* as potential biocontrol agents to reduce aflatoxin contamination in peanuts harvested in Northern Argentina

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ABSTRACT

Biological control is one of the most promising strategies for preventing aflatoxin contamination in peanuts at field stage. A population of 46 native *Aspergillus flavus* nonaflatoxin producers were analysed based on phenotypic, physiological and genetic characteristics. Thirty-three isolates were characterized as L strain morphotype, 3 isolates as S strain morphotype, and 10 isolates did not produce sclerotia. Only 11 of 46 non-aflatoxigenic isolates did not produce cyclopiazonic acid. The vegetative compatibility group (VCG) diversity index for the population was 0.37. For field trials we selected the non-aflatoxigenic *A. flavus* AR27, AR100G and AFCHG2 strains. The efficacy of single and mixed inocula as potential biocontrol agents in Northern Argentina was evaluated through a 2-year study (2014–2015). During the 2014 peanut growing season, most of the treatments reduced the incidence of aflatoxigenic strains in both soil and peanut kernel samples, and no aflatoxin was detected in kernels. During the 2015 growing season, there was a reduction of aflatoxigenic strains in kernel samples from the plots treated with the potential biocontrol agents. Reductions of aflatoxin contamination between 78.36% and 89.55% were observed in treated plots in comparison with the un-inoculated control plots. This study provides the first data on aflatoxin biocontrol based on competitive exclusion in the peanut growing region of Northern Argentina, and proposes bioproducts with potential use as biocontrol agents.

1. Introduction

Aspergillus flavus has the ability to infect crop species including maize, cotton and peanuts (CAST, 2003; Horn et al., 2009). Peanut is one of the most susceptible host crops to *A. flavus* invasion and subsequent aflatoxin contamination (Torres et al., 2014). *A. flavus* populations include isolates with two morphologically distinct sclerotium size variants, L strains with average sclerotium size >400 μm and S strains with sclerotium <400 μm (Cotty, 1989; Perrone et al., 2014). Both occur frequently but they differ in some characteristics including aflatoxin production (Barros et al., 2005; Cotty, 1994, 1997; Horn and Dorner, 1999, Mauro et al., 2015). Aflatoxins are carcinogens and genotoxins that directly alter the DNA structure (Williams et al., 2004). Regulatory agencies have established very low tolerance for aflatoxins in food, including peanut and peanut products. The European Union established the upper limit for aflatoxins in peanuts at 2 ng/g for aflatoxin B_1 and 4 ng/g for total aflatoxins ($B_1 + B_2 + G_1 + G_2$) (EC, 2010), while Argentina has a tolerance of 20 ng/g for total aflatoxins according to Mercosur Resolution 25/02 (Mercosur, 2002). Cyclopiazonic acid (CPA) is a toxic indole tetramic acid reported for the first time in cultures of *A. flavus* by Luk et al. (1978). Aflatoxins and CPA commonly co-occur in contaminated agricultural commodities such as maize and peanut (Abbas et al., 2008, 2011; Urano et al., 1992).

In the last decade, peanut exports from Argentina have exceeded 400,000 t/year, making it the largest peanut exporter in the world (Torres et al., 2014). Most of the peanut production is located in the Centre-South region of Córdoba Province, where climatic conditions are generally unfavourable for aflatoxin contamination at pre-harvest stage. However, recent soybean expansion and a progressive deterioration of soils have generated a displacement of many regional crops, including peanuts. Consequently, peanut cultivation has expanded to new agro-ecological areas of Northern Argentina, such as Salta Province, where climatic conditions may increase the risk of aflatoxin production (Cámara Argentina del Maní, 2015).

Biological control involves the application to soil of naturally occurring non-aflatoxigenic *A. flavus* strains that interfere with the proliferation of indigenous aflatoxigenic strains under aflatoxin production conditions (Abbas et al., 2011; Cotty and Bayman, 1993). In Argentina, a previous study showed significant reductions of aflatoxin levels in peanut kernels harvested in the peanut core area of the country treated with a biocontrol agent based on the native non-aflatoxigenic *A. flavus* AFCHG2 strain (Alaniz Zanon et al., 2013). In general, the formulations used to prevent aflatoxin production included a single *A. flavus* strain

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as antagonist. However, Probst et al. (2011) suggested that isolate mixtures could compete more effectively than individual isolates in a greater diversity of environmental niches. In this sense, the aims of the present study were to characterize native non-aflatoxigenic *A. flavus* strains isolated from the main peanut growing region of Argentina based on phenotypic, physiological and genetic characteristics; and to evaluate selected strains as biological control agents as single or mixed inocula to reduce aflatoxin accumulation in peanuts harvested in Northern Argentina.

2. Materials and methods

2.1. Characterization of the A. flavus population

2.1.1. Fungal isolates and culture conditions

Forty-six *A. flavus* evaluated as non-aflatoxigenic strains by HPLC according to Horn et al. (1996) and isolated from the major peanut-growing region of Argentina were identified using the methodology of Klich (2002). Twenty-eight of these strains were isolated from peanut kernels, 16 from soil samples and 2 from insects (Table 1). All isolates were cultured in malt extract agar (MEA) (3% malt extract, 0.5% mycological peptone, 1.5% agar, pH 7.6, 1000 ml of distilled water) at 30 °C for 7 days. During the experiment, strain agar plugs were maintained at 4 °C in vials (4 ml) containing sterile distilled water, while for long-term storage, strains were maintained as spore suspensions in glycerol 15% (w/v) at −80 °C in the culture collection at the Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina.

2.1.2. Sclerotium production

Sclerotium production by the isolates was evaluated on plates containing Czapek-Dox medium (Cz) (3% sucrose, 0.2% $NaNO_3$, 0.1% K_2PO_4H, 0.05% $MgSO_4$, 0.05% KCl, 0.001% $FeSO_4$, 1.5% agar, pH 7.3, 1000 ml of distilled water), inoculated with mycelia obtained from a 7-day old culture on MEA medium. Cultures were incubated in darkness at 30 °C for 15–21 days. Individual strains that did not produce sclerotia on Cz agar were cultured on a Petri dish with 5/2 agar (5% V8 juice, 2% agar, pH 5.2, 1000 ml of distilled water) at 30 °C for 30 days and visually examined for presence of sclerotia. The isolates were characterized as S or L morphotypes following the methodology proposed by Cotty (1989).

2.1.3. Cyclopiazonic acid analyses

The non-aflatoxigenic strains were evaluated for CPA production. All the isolates were induced to sporulate on MEA slants at 28 °C for 7 days. At the end of the incubation period, 5 ml of distilled water with Tween 80 (0.1%) was added to the slants, and the spores were harvested by vigorous agitation. The spore concentration was measured with a Neubauer chamber and adjusted to 10^5 spores/ml. This conidia concentration was used to inoculate 4-ml vials containing 1 ml of sucrose yeast extract soytone medium (Horn and Dorner, 1999). The cultures were incubated at 30 °C for 7 days in darkness. CPA production was analysed by thin layer chromatography (TLC) on silica gel 60 pre-coated glass plates (Merck 5735, Darmstadt, Germany) according to Lansden and Davidson (1983). CPA concentrations were determined by visual comparison with a CPA standard (St. Louis, MO, USA). The detection limit for CPA was 1 μg/ml.

2.1.4. Vegetative compatibility group (VCG) analysis

Nit mutants were obtained on Petri dishes with Czapek-Dox medium containing 25 g/l potassium chlorate (Bayman and Cotty, 1991) with unadjusted pH, incubated at 30 °C. At least two different *nit* mutants were obtained from each isolate and complementary pairings of *niaD*, *nirA*, and *cnx* were initially made to test the self-compatibility. VCGs were established by pairing mutants from each strain in all possible pairwise combinations (2209 combinations) according to the methodology described by Barros et al. (2006).

Table 1
Location, source, cyclopiazonic acid production and sclerotium morphotype of the 46 non-aflatoxigenic isolates evaluated.

Isolate	Location/isolation year	Source	CPA[a]	Sclerotium morphotype[b]
AR63	Charras/2001	Soil	ND	L
AR107	Charras/2001	Soil	(+)	L
AR111	Charras/2001	Soil	ND	S
AR113	Charras/2001	Soil	ND	L
AR114	Charras/2001	Soil	(+)	L
AR116	Charras/2001	Soil	(+)	L
AR119	Charras/2001	Soil	ND	S
AR122	Charras/2001	Soil	(+)	L
AR151	Charras/2001	Soil	(+)	L
ARCH084	Charras/1999	Soil	(+)	NSP
ARCH094	Charras/1999	Soil	(+)	L
ARGD070	Gral. Deheza/2001	Soil	(+)	L
ARGD090	Gral. Deheza/2001	Soil	(+)	L
ARGD113	Gral. Deheza/2001	Soil	(+)	L
ARGD116	Gral. Deheza/2001	Soil	(+)	L
ARRC140	Río Cuarto/2001	Soil	(+)	L
AR160i	Charras/2008	Insect	ND	L
AR164i	Charras/2008	Insect	ND	L
AR85G	Charras/2008	Peanut	(+)	L
AR87G	Charras/2008	Peanut	(+)	L
AR92G	Charras/2008	Peanut	ND	L
AR94G	Charras/2008	Peanut	(+)	L
AR98G	Charras/2008	Peanut	(+)	L
AR100G	Charras/2008	Peanut	(+)	L
AR108G	Charras/2008	Peanut	(+)	S
AR121G	Charras/2008	Peanut	ND	L
AR153G	Charras/2008	Peanut	(+)	L
AR13	Santa Eufemia/2009	Peanut	(+)	L
AR23	Santa Eufemia/2009	Peanut	(+)	NSP
AR24	Santa Eufemia/2009	Peanut	(+)	L
AR26	Santa Eufemia/2009	Peanut	(+)	L
AR27	Santa Eufemia/2009	Peanut	ND	L
AR30	Santa Eufemia/2009	Peanut	(+)	NSP
AR31	Santa Eufemia/2009	Peanut	(+)	NSP
AR32	Santa Eufemia/2009	Peanut	(+)	NSP
AR46	Santa Eufemia/2009	Peanut	ND	L
AR47	Santa Eufemia/2009	Peanut	(+)	NSP
AR48	Santa Eufemia/2009	Peanut	(+)	L
AR60	Santa Eufemia/2009	Peanut	ND	NSP
AR61	Santa Eufemia/2009	Peanut	(+)	NSP
AR62	Santa Eufemia/2009	Peanut	(+)	L
AR64	Santa Eufemia/2009	Peanut	(+)	NSP
AR65	Santa Eufemia/2009	Peanut	(+)	L
AR555	Santa Eufemia/2009	Peanut	(+)	L
AR578	Santa Eufemia/2009	Peanut	(+)	NSP
AFCHG34	Charras/1999	Peanut	(+)	L

[a] CPA = cyclopiazonic acid production (ND = not detected <1 μg/ml), (+) = CPA production.
[b] Sclerotium morphotype = L strain produce sclerotia >400 μm in diameter, S strain produce sclerotia <400 μm in diameter, NSP = Non-sclerotium producers.

2.2. Field assays

2.2.1. Strain selection

Three non-aflatoxigenic and non-CPA *A. flavus* producers were selected for the field trials, including the competitive strain AFCHG2 evaluated in a previous study (Alaniz Zanon et al., 2013), and strains AR27 and AF100G (Table 1). These strains belong to a VCG that comprises only non-aflatoxigenic strains. These 3 strains have also been shown to be competitive when in situ assays in peanut kernels were carried out, reducing aflatoxin contamination levels produced by a toxigenic *A. flavus* strain (Alaniz Zanon et al., 2014).

2.2.2. Inoculum preparation

The *A. flavus* strain inocula were produced by solid-state fermentation on autoclaved long grain rice according to Alaniz Zanon et al.

(2013). Briefly, the substrate was conditioned in plastic bags; the moisture content in the rice was 35–40%. The bags were inoculated with 10^7 conidia and hand-shaken daily. After 4 days at 30 °C, the substrate was dried in a forced air draft oven. Under these conditions, conidia were not observed. Each isolate was prepared individually for treatments consisting of a mixture of two strains. After drying, the two strains were combined to achieve an equal concentration. Before field trials, the viable count of *A. flavus* was determined by homogenising prepared inocula (10 g) in peptone water (90 ml) and dilutions were plated in triplicate on Dichloran 18% Glycerol (DG18) agar (Pitt and Hocking, 1997, 2009). The viable count of the different inocula based on non-aflatoxigenic *A. flavus* strains was 10^8 cfu/g.

2.2.3. Field trial design

The field trials were performed in commercial fields with previous history of peanut cultivation during the 2014 and 2015 growing seasons in Tartagal, Salta Province, Northern Argentina. Fields had not previously been used for biocontrol assays. The experiments were established as split plot designs. In both 2014 and 2015 field trials each plot consisted of 180 m × 17.5 m divided into three 60 m × 17.5 m sub-plots. The buffer area among plots consisted of ten rows without any inoculation. The peanut cultivar (Runner type) was planted in rows at 70 cm distance.

The planting dates were January 14, 2014 and January 10, 2015. The inocula were applied with machines used to dispense fertilizer at a rate of 25 kg inoculum/ha in each trial 50 days after planting. The control and treatment sub-plots were monitored with temperature sensor devices to register soil temperature at 5 cm depth during the last stages of peanut growth. During the 2014 peanut growing season, the field assay consisted of the following controls and treatments: (1) un-inoculated control; (2) AFCHG2 inoculation; (3) AR27 inoculation; and (4) AFCHG2 + AR27 inoculation. During 2015 field trial controls and treatments were: (1) un-inoculated control; (2) AFCHG2 inoculation; (3) AR27 inoculation; (4) AR100G inoculation; (5) AFCHG2 + AR27 inoculation; (6) AR27 + AR100G inoculation; and (7) AR100G + AFCHG2 inoculation. The digging dates were June 2, 2014 and May 15, 2015. At harvest stage (June 17, 2014 and May 26, 2015) after drying, kernels from the control and treatment plots from both field trials shelled by hand. Climatic data during both field trials were obtained from the Instituto de Clima y Agua (INTA, 2016).

During 2014 and 2015 field trials the efficacy of the various biocontrol agents was monitored using the shift of the toxigenic/non-toxigenic ratio in the treated plots in relation to control plots.

2.2.4. Fungal population in soil

2.2.4.1. Soil sampling. In order to determine the *A. flavus* populations, ten soil samples were taken in two diagonal transects extending from opposing corners in each sub-plot at two different times: 1) immediately after planting, and 2) after pod maturation prior to digging. Each soil sample (approximately 100 g) was a pool from 5 sub-samples taken with a trowel from the top 5 cm of soil. For each sample, the five subsamples were combined in a paper bag and air-dried for 1–2 days at 25–30 °C. Samples were thoroughly mixed and passed through a testing sieve (2 mm mesh size).

2.2.4.2. Fungal isolation and identification. From each soil sample 10 g were diluted with 90 ml of peptone water 0.1% (w/v). This mixture was homogenised by orbital shaker (20 min, 150 rpm) and decimal dilutions were prepared in peptone water 0.1% (w/v). A 0.1 ml aliquot of each dilution per sample was spread on Dichloran Rose Bengal Chloramphenicol (DRBC) agar modified with NaCl 3% (Alaniz Zanon et al., 2013) and DG18 agar (Pitt and Hocking, 1997, 2009). The plates were incubated in darkness for 5–7 days at 30 °C. Data were expressed as colony forming units per gram of soil (cfu/g). Fungal colonies that resembled *Aspergillus* section *Flavi* were sub-cultured on MEA medium for further identification according to Klich (2002).

2.2.4.3. Toxigenic profile of A. flavus isolates. Aspergillus flavus isolates were inoculated on MEA medium. After the incubation period, spores were harvested, counted, and the concentration was adjusted to 10^5 spores/ml and used to inoculate 1 ml of the sucrose yeast extract soytone medium according to Horn and Dorner (1999). Preliminary analysis of the extracts to screen for aflatoxin production was carried out using TLC following the methodology described by Geisen (1996). Aflatoxins were quantified by HPLC according to Horn et al. (1996).

2.2.5. Fungal population from peanuts

From each sub-plot, approximately 3 kg of peanut kernels were analysed. This sample was mixed thoroughly and 100 kernels (2 replicates) were selected to determine fungal infection. The remaining sample was ground to obtain a sub-sample of 25 g (3 replicates) for aflatoxin analysis. Peanut kernels from each sub-plot were surface disinfected for 1 min in 1% sodium hypochlorite solution, rinsed with sterile distilled water, and transferred to Petri dishes containing DRBC modified with 3% NaCl and DG18. Plates were incubated at 25 °C for 7 days. The incidence of toxigenic isolates of *A. flavus* in peanut kernels was determined by testing all the isolates for toxigenicity as described above (item 2.2.4.3).

2.2.6. Aflatoxin analysis in peanut kernels

The aflatoxin analysis was performed using the method of Trucksess et al. (1994). Peanut kernel samples were ground and homogenised, and 25 g (3 replicates) were mixed with acetonitrile: H_2O (84:16, v/v). The mixture was shaken for 30 min, and filtered using N°4 Whatman filter paper. Cleaning columns MycoSep®224 AflaZon (Romer Laboratories, USA) were used for extract cleaning step and 2 ml of the purified extract were collected and evaporated to dryness. Aflatoxins were quantified by injecting 50 μl of the extract from each sample 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 pre-column Security Guard (20 mm × 4.6 mm i.d., 5 μm particle size, Phenomenex). The mobile phase was water:methanol:acetonitrile (4:1:1, v/v/v) at a flow rate of 1.5 ml/min and the limit of detection was 1 ng/g of B_1 and G_1 and 0.8 ng/g of B_2 and G_2. Reference aflatoxin standards were used (Sigma-Aldrich, St. Louis,MO, USA).

2.2.7. Statistical analysis

Data of fungal populations were log-transformed prior to analysis of variance (ANOVA). Mean separation and comparisons were made by Fisher's least significant difference (LSD) test at a probability level of $p < 0.05$. To compare the different treatments, aflatoxin concentrations were subjected to nonparametric Kruskal–Wallis test followed by Dunn's nonparametric multiple comparison test. The statistical analyses were performed using SigmaStat for Windows version 2.03 (SPSS Inc., San Jose, CA).

3. Results and discussion

3.1. Characterization of the A. flavus population

Most of the 46 non-aflatoxigenic strains ($n = 33$) were characterized as L strain morphotype. Only 3 isolates were identified as S strain morphotype and 10 isolates did not produce sclerotia (Table 1). These results agree with Moore et al. (2013), who determined that approximately 60% (48/80) of the *A. flavus* L isolates sampled in Argentinean peanut soil were non-aflatoxigenic. Similar results were obtained in Italy and Nigeria, where around half of the *A. flavus* L strain isolates did not produce aflatoxin (Atehnkeng et al., 2008; Mauro et al., 2013).

Only 11 out of 46 non-aflatoxigenic isolates did not produce CPA (Table 1). The evaluation of CPA production by non-aflatoxigenic *A. flavus* intended to be used as biocontrol agent is a matter of concern, since Abbas et al. (2011) observed that, in the evaluation of two strains registered for biocontrol of aflatoxin in USA, AF36 (Afla −/CPA +) and NRRL 21882 (AflaGuard®) (Afla −/CPA −), CPA was reduced in crops treated with AflaGuard®, but the levels of this mycotoxin increased when maize and peanut were treated with AF36.

All isolates included in the VCG analysis formed two or three complementary *nit* mutants. Nearly all colonies of *A. flavus* sectored at least once after 15 days. Among the 46 isolates, 1342 mutants were obtained and the types of mutants produced were 76.2% *niaD* ($n = 1023$), 20.3% *nirA* ($n = 273$) and 3.4% *cnx* ($n = 46$). Only 18 of the 46 isolates evaluated produced *cnx* mutants. The proportion of each kind of mutant was similar to that obtained in previous studies on *A. flavus* populations (Barros et al., 2006; Novas and Cabral, 2002). The mechanism by which the proportion of *nit* mutants is unequal is not yet well understood; it was postulated that there might exist some association to the physical size of the genes involved, or that some loci could be more susceptible to mutations (Barros et al., 2006; Klittich and Leslie, 1988). All isolates that formed complementary mutants were self-compatible. Based on complementation tests, 17 VCGs were obtained from the 46 isolates evaluated. Nine VCGs contained two or more isolates and 8 VCGs included only a single isolate. In the 9 multimember VCGs, two groups had 7 isolates, two VCGs included 4 isolates, one VCG had 3 isolates and four VCGs contained 2 isolates. The VCG diversity index for the non-aflatoxigenic *A. flavus* population, expressed as the number of groups divided by the total number of isolates, was 0.37. The genetic diversity of the *A. flavus* population under study was lower than that observed (0.56) in an *A. flavus* population that included toxigenic and non toxigenic strains isolated from the major peanut growing region of Argentina (Barros et al., 2006).

3.2. Field trials

3.2.1. 2014 growing season

Density of total filamentous fungi before the application of the bioproducts in the field was homogeneous across all the soil samples, with an average count around 10^4 cfu/g of soil. The inoculum level of native *Aspergillus* section *Flavi* in soil at planting time were also homogeneous and similar among plots, around 10^2 cfu/g of soil. The *Aspergillus* section *Flavi* mean count was 212 cfu/g. In comparison with peanut field trials carried out in Córdoba Province during previous growing seasons, the density of *Aspergillus* section *Flavi* was lower in the field located in Salta Province selected for the present study (Barros et al., 2005, 2006; Alaniz Zanon et al., 2013). In soil samples statistical differences ($p < 0.05$) in the percentages of native toxigenic strains were observed among the control and the treatment plots: 67% (un-inoculated control), 50% (AFCHG2), 29% (AFCHG2 + AR27) and 23% (AR27).

At harvest time, the incidence of peanut kernels infected with *Aspergillus* section *Flavi* ranged from 9 to 46% and there were significant differences ($p < 0.05$) between treated and control plots. The peanut kernel infection percentage with *A. flavus* was higher in treated plots (mean 45%) than in the control ones (9%). The double inoculum AFCHG2 + AR27 showed a higher kernel infection level (46%) compared to the single inoculum treatments. AFCHG2 single inoculum showed a percentage infection with *Aspergillus* section *Flavi* significantly different to the mixture inoculum. However, there were no significant differences ($p < 0.05$) among peanut kernels from plots treated with AFCHG2 + AR27 inoculum and AR27 single inoculum. In general, the treatments assayed showed a decrease in the percentage of aflatoxigenic strains detected. A reduction in the percentage of aflatoxigenic isolates from treated plots was observed in comparison with the control plots, mainly when the mixture of two strains inoculum was applied (44% vs.17%, respectively). Reduction of toxigenic strains was observed both in soil samples and in peanut kernels in the treatment based on the mixed inoculum (AFCHG2 + AR27).

The climatic conditions may influence the aflatoxin production, as reported by Kebede et al. (2012) and Diao et al. (2015), and during the 2014 peanut growing season, the climatic conditions in the agro-ecological area under study were not favourable for aflatoxin production through the different stages of the peanut development. The mean temperatures were around 21.4 °C and the cumulative rainfall since the planting stage until harvest was 587 mm (INTA, 2016) (Fig. 1). During the last period of peanut growth, the mean soil temperature registered was 19.4 °C. No aflatoxins were detected in peanut kernels at harvest stage (detection limit of 1 μg/kg). However, a promising result of this trial was the competitive ability of the evaluated strains to reduce toxigenic strains in both soil and peanut kernels at the inoculum level applied (25 kg/ha). The biocontrol strains were able to interact among themselves in the ecosystem, simultaneously influencing the peanut kernel invasion. Some inocula were more competitive than others and showed higher kernel infection rates and greater reduction in aflatoxigenic strains.

3.2.2. 2015 growing season

During the 2015 peanut growing season densities of total fungi and of toxigenic *Aspergillus* section *Flavi* soil populations were determined 50 days after planting and at harvest stage. The count of total filamentous fungi in soil samples collected 50 days after planting was similar among the plots (10^4 cfu/g), with exception of the plot to be treated with the mixture AFCHG2 + AR27 inoculum. In this plot, 2.12×10^5 cfu/g of soil was observed (Table 2). These data are similar to the count obtained in the soil samples evaluated during the previous growing season (2014) in the same agro-ecological area. The initial *Aspergillus* section *Flavi* counts were significantly different among plots for the different treatments, except those plots to be inoculated with AFCHG2 + AR100G double inoculum. Plots destined for single inocula AFCHG2 and AR27 showed the lowest *Aspergillus* section *Flavi* counts (10^2 cfu/g), while the others showed values around 10^3 cfu/g (Table 2). Soil samples collected at pod maturation stage showed fungal densities around 10^4 cfu/g. In general in the soil samples, no changes were observed in the total fungal counts with respect to the initial fungal densities. Also no significant differences ($p < 0.05$) in *Aspergillus* section *Flavi* densities were observed when comparing data from soil sampled 50 days after planting and at harvest stage.

During the 2015 peanut growing season the cumulative rainfall exceeded 625 mm and the mean temperature was 24.2 °C (Fig. 1), with 35–37 °C being the maximum temperatures registered in a few days of the first 3 weeks after planting (INTA, 2016). A high percentage of peanut infection was observed in kernels from the different treatments. In the control plot a significantly lower infection level ($p < 0.05$) was observed in comparison with the treatments AR27 and AR100G single inocula and the mixture of AFCHG2 + AR100G inoculum (Table 2). The infection percentages of peanut kernels from treatments consisting of single inocula (AFCHG2, AR27 and AR100G) were lower than those treated with an inoculum comprising a mixture of two strains (AFCHG2 + AR27, AFCHG2 + AR100G and AR27 + AR100G).

The mean soil temperature registered at different field points during the last month prior to harvest was 19 °C (Fig. 1). However, at the digging stage precipitation was abundant, so the relative humidity was elevated, this situation creating a risk of aflatoxin production (Dorner et al., 1989). All plots assayed showed different aflatoxin contamination levels. It is probable that aflatoxin contamination detected in peanut kernels was produced at the post-harvest stage, when pods had been dug from the soil, inverted in the field and exposed to favourable climatic conditions for aflatoxin production. With exception of the plots in which *A. flavus* AR27 was evaluated as a single inoculum, all the treatments showed a significant reduction of aflatoxin contamination, around 86% (range 78.36–89.55%), in comparison to the un-inoculated control plots (Fig. 2, Table 2). The most effective inocula were those based on the combination of AFCHG2 + AR27 and AR27 + AR100G strains. These data indicate that *A. flavus* AR27 was not effective to compete with native aflatoxigenic strains and to control aflatoxin

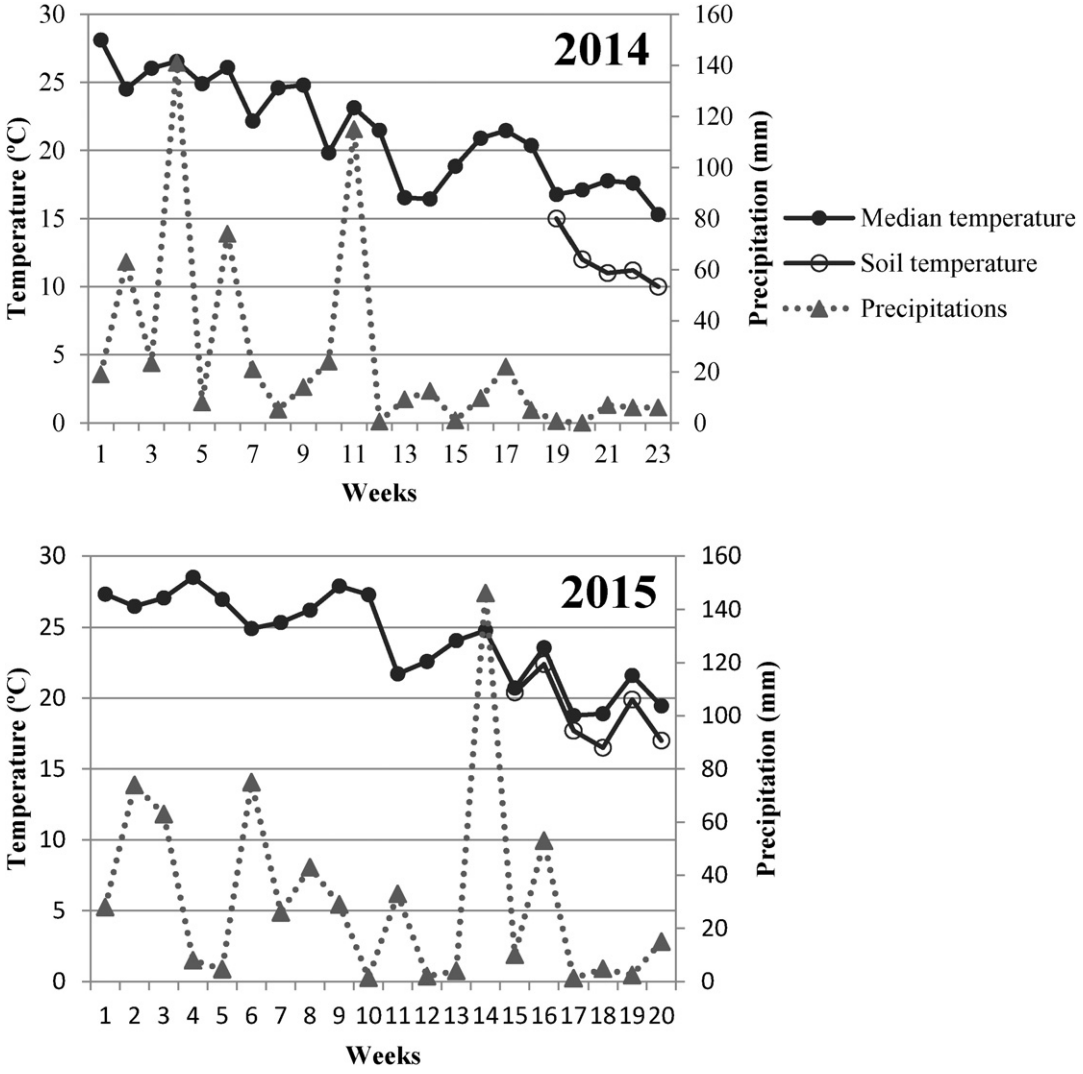

Fig. 1. Climatic conditions and soil temperatures during the 2014 and 2015 field trials.

accumulation when applied as a single inoculum. However, *A. flavus* AR27 strain showed better results when applied as a two strain inocula in combination with other non-aflatoxigenic isolates (AFCHG2 + AR27 and AR27 + AR100G).

Regarding the inoculum application rate, the dosage employed was similar to the rates used on peanuts in the USA (Dorner et al., 1998; Dorner and Cole, 2002; Dorner and Horn, 2007), and lower than those evaluated in Australia (Pitt and Hocking, 2006) and in the Centre-

Table 2
Total mycoflora and *Aspergillus* section *Flavi* from soil samples collected at planting and harvest time, infection of peanut kernels and aflatoxin contamination at harvest stage during the 2015 field trial.

	Soil				Peanut kernels		
	Planting stage		Harvest stage		Harvest stage		
Treatment	Total mycoflora[a]	*Aspergillus* section *Flavi*[a]	Total mycoflora[a]	*Aspergillus* section *Flavi*[a]	Peanut infection[b]	Aflatoxigenic strains[c]	Aflatoxins[d] (μg/kg)
(1) Un-inoculated Control	5.38×10^4 a[e]	4.13×10^3 f	1.64×10^5 h	1.25×10^2 a	70.50 d	86.21 h	40.2 e
(2) AFCHG2	2.85×10^4 a	3.75×10^2 a	2.29×10^4 b	1.13×10^3 d	53.00 b	24.24 f	5.8 c
(3) AFCHG2 + AR27	2.27×10^5 b	5.75×10^3 g	6.49×10^4 f	2.13×10^3 f	50.00 a	7.69 c	4.4 a
(4) AR27	3.30×10^4 a	7.50×10^2 b	1.13×10^5 g	5.13×10^2 b	75.60 e	6.38 b	41.4 f
(5) AR27 + AR100G	2.94×10^4 ab	1.50×10^3 d	2.78×10^4 c	1.25×10^2 a	65.00 c	15.38 d	4.2 a
(6) AR100G	2.80×10^4 a	1.13×10^3 c	2.94×10^4 d	9.00×10^2 c	87.00 f	6.25 a	4.7 b
(7) AR100G + AFCHG2	2.34×10^4 a	1.75×10^3 e	4.20×10^4 e	1.25×10^3 de	99.50 h	17.65 e	8.7 d

All the data represent the average values of three replicates.
[a] The counts are expressed as colonies forming units per gram of soil (cfu/g).
[b] Peanut infection is expressed as the percentage of peanut kernels infected with *Aspergillus* section *Flavi*.
[c] These data are expressed as the percentage of the aflatoxigenic strains.
[d] Aflatoxin levels are expressed as parts per billion (ppb) or μg/kg (ND = not detected;<1 μg/kg).
[e] Within a column, values not sharing a common letter are significantly different ($p < 0.05$).

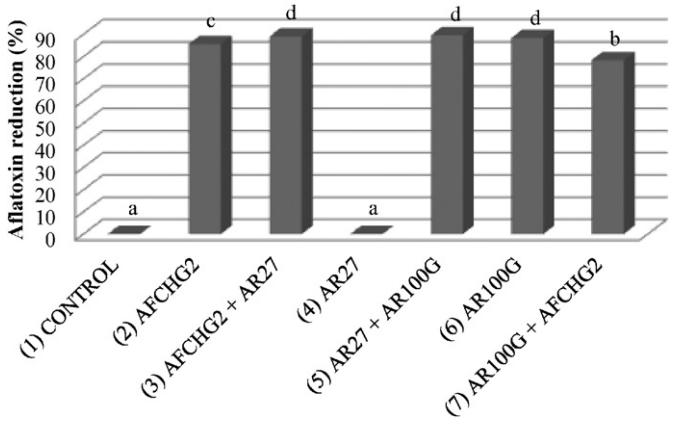

Fig. 2. Aflatoxin reduction in peanut kernels harvested during 2015 field trial. Different letters indicate significant differences between treatments ($p < 0.05$). LSD Fisher test.

South of Córdoba Province, Argentina (Alaniz Zanon et al., 2013). This aspect is relevant both from the economic and environmental point of view since low inputs of biocontrol agent were needed to reduce the aflatoxin accumulation without altering the native mycoflora.

This study provides the first data on aflatoxin biocontrol based on competitive exclusion in peanut crop in the peanut growing region of Northern Argentina. An inoculum comprising a mixture of two non-toxigenic *A. flavus* strains proved to be effective in the reduction of aflatoxin accumulation in peanut kernels.

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References

Abbas, H.K., Zablotowicz, R.M., Bruns, H.A., 2008. Modeling of colonization of maize by toxigenic and non-toxigenic *Aspergillus flavus* strains: implication for biological control. World Mycotoxin J. 1, 333–340.

Abbas, H.K., Zablotowicz, R.M., Horn, B.W., Phillips, N.A., Johnson, N.A., Jin, X., Abel, C.A., 2011. Comparison of major biocontrol strains of non-aflatoxigenic *Aspergillus flavus* for the reduction of aflatoxins and cyclopiazonic acid in maize. Food Addit. Contam. 28, 198–208.

Alaniz Zanon, M.S., Barros, G.G., Cotty, P.J., Chulze, S.N., 2014. SSR analysis and competition assay in peanut and maize using non-aflatoxigenic *Aspergillus flavus* strains in Argentina. International Mycotoxin Conference 2014, p. 184 (Beijing, China. Poster 16).

Alaniz Zanon, M.S., Chiotta, M.L., Giaj-Merlera, G., Barros, G.G., Chulze, S.N., 2013. Evaluation of potential biocontrol agent for aflatoxin in Argentinean peanuts. Int. J. Food Microbiol. 162, 220–225.

Atehnkeng, J., Ojiambo, P.S., Ikotun, T., Sikora, R.A., Cotty, P.J., Bandyopadhyay, R., 2008. Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 25, 1264–1271.

Barros, G., Torres, A., Chulze, S., 2005. *Aspergillus flavus* population isolated from soil of Argentina's peanut growing region. Sclerotia production and toxigenic profile. J. Sci. Food Agric. 85, 2349–2353.

Barros, G., Torres, A., Rodriguez, M., Chulze, S., 2006. Genetic diversity within *Aspergillus flavus* strains isolated from the peanut-cropped soils in Argentina. Soil Biol. Biochem. 38, 145–152.

Bayman, P., Cotty, P.J., 1991. Improved media for selecting nitrate nonutilizing mutants in *Aspergillus flavus*. Mycologia 83, 311–316.

Cámara Argentina del Maní, 2015. Caracterización cluster manisero Argentino. Available at: http://www.camaradelmani.com.ar.

CAST, Council for Agricultural Science and Technology, 2003. Mycotoxins: Risks in Plant, Animal and Human Systems. In: Richard, J.L., Payne, G.A. (Eds.), Task Force Report 139. Ames, IA, USA.

Cotty, P.J., 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79, 808–814.

Cotty, P.J., 1994. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *Aspergillus flavus* infecting cotton bolls and on aflatoxin content of cottonseed. Phytopathology 84, 1270–1277.

Cotty, P.J., 1997. Aflatoxin-producing potential of communities of *Aspergillus* section *Flavi* from cotton producing areas in the United States. Mycol. Res. 101, 698–704.

Cotty, P.J., Bayman, P., 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. Postharvest Pathol. Mycotoxins 83 (12), 1283–1287.

Diao, E., Dong, H., Hou, H., Zhang, Z., Ji, N., Ma, W., 2015. Factors influencing aflatoxin contamination in before and after harvest peanuts: a review. J. Food Res. 4, 148–154.

Dorner, J.W., Cole, R.J., 2002. Effect of application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* on subsequent aflatoxin contamination of peanuts in storage. J. Stored Prod. Res. 38, 329–339.

Dorner, J.W., Horn, B.W., 2007. Separate and combined applications of nontoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* for biocontrol of aflatoxin in peanuts. Mycopathologia 163, 215–223.

Dorner, J.W., Cole, R.J., Blankenship, P.D., 1998. Effect of inoculum rate of biological control agents on preharvest aflatoxin contamination of peanuts. Biol. Control 12, 171–176.

Dorner, J.W., Cole, R.J., Sanders, T.H., Blankenship, P.D., 1989. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. Mycopathologia 105, 117–128.

EC, European Commission, 2010. Commission Regulation (EU) No 165/2010. Amending Regulation (EC) no 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins.

Geisen, R., 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Syst. Appl. Microbiol. 19, 388–392.

Horn, B.W., Dorner, J.W., 1999. Regional differences in production of aflatoxin B_1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Appl. Environ. Microbiol. 65, 1444–1449.

Horn, B.W., Moore, G.G., Carbone, I., 2009. Sexual reproduction in *Aspergillus flavus*. Mycologia 101, 423–429.

Horn, B.W., Greene, R.L., Sobolev, V.S., Dorner, J.W., Powell, J.H., 1996. Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii*. Mycologia 88, 574–587.

INTA, Instituto Nacional de Tecnología Agropecuaria, 2016. Instituto de Clima Y Agua. Estación Experimental Castelar, Argentina.

Kebede, H., Abbas, H.K., Fisher, D.K., Bellaloui, N., 2012. Relationship between aflatoxin contamination and physiological responses of corn plants under drought and heat stress. Toxins 4 (11), 1385–1403.

Klich, M.A., 2002. Identification of Common *Aspergillus* Species. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Klittich, C.J.R., Leslie, J.L., 1988. Nitrate reduction mutants of *Fusarium* moniliforme. Genetics 118, 417–423.

Lansden, J.A., Davidson, J.I., 1983. Occurrence of cyclopiazonic acid in peanuts. Appl. Environ. Microbiol. 45, 766–769.

Luk, K.C., Kobbe, B., Townsend, J.M., 1978. Production of cyclopiazonic acid by *Aspergillus flavus* link. Appl. Environ. Microbiol. 33, 211–212.

Novas, M.V., Cabral, D., 2002. Association of mycotoxin and sclerotia production with compatibility groups in *Aspergillus flavus* from peanut in Argentina. Plant Dis. 86 (3), 215–219.

Mauro, A., Battilani, P., Callicott, K.A., Giorni, P., Pietri, A., Cotty, P.J., 2013. Structure of an *Aspergillus flavus* population from maize kernels in northern Italy. Int. J. Food Microbiol. 162, 1–7.

Mauro, A., Battilani, P., Cotty, P.J., 2015. Atoxigenic *Aspergillus flavus* endemic to Italy for biocontrol of aflatoxins in maize. BioControl 60, 125–134.

Mercosur/Gmc/Res. N° 25/02, 2002. Reglamento Técnico Mercosur Sobre Límites Máximos de Aflatoxinas Admisibles en Leche, Maní y Maíz. (Derogación de la Res. GMC N° 56/94). Buenos Aires, Argentina.

Moore, G., Elliott, J., Singh, R., Horn, B., Dorner, J., Stone, E., Chulze, S., Barros, G., Naik, M., Wright, G., Hell, K., Carbone, I., 2013. Sexuality generates diversity in the aflatoxin gene cluster: evidence on a global scale. PLoS Pathog. 9, 1–12.

Perrone, G., Gallo, A., Logrieco, A.F., 2014. Biodiversity of *Aspergillus* section *Flavi* in Europe in relation to the management of aflatoxin risk. Front. Microbiol. 5, 8–12.

Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage. Blackie Academic & Professional, London, United Kindom.

Pitt, J.I., Hocking, A.D., 2006. Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. Mycopathologia 162, 233–243.

Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage. Springer. Third edition. Dordrecht, The Netherlands, p. 523.

Probst, C., Bandyopadhyay, R., Price, L.E., Cotty, P.J., 2011. Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. Plant Dis. 95, 212–218.

Torres, A.M., Barros, G.G., Palacios, S.A., Chulze, S.N., Battilani, P., 2014. Review on pre- and post-harvest management of peanuts to minimize aflatoxin contamination. Food Res. Int. 62, 11–19.

Trucksess, M.W., Stack, M.E., Nesheim, S., Albert, R.H., Romer, T.R., 1994. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1 and G_2 in corn, almonds, Brazil nuts, peanuts, and pistachio nuts: collaborative study. J. AOAC Int. 77, 1512–1521.

Urano, T., Trucksess, M.W., Beaver, R.W., Wilson, D.M., Dorner, J.W., Dowell, F.E., 1992. Co-ocurrence of cyclopiazonic acid and aflatoxins in corn and peanuts. J. AOAC Int. 75, 838–841.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M., Aggarwal, D., 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am. J. Clin. Nutr. 80, 1106–1122.