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Aspergillus section Flavi populations from field maize in Argentina

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Aims: Populations of *Aspergillus* section Flavi were studied from a commercial field of maize in Río Cuarto, Córdoba, Argentina.

Methods and Results: The *Aspergillus* species were isolated from soil, debris and insects during three periods: pre-planting, growing maize and post-harvest. The colony count from non-rhizospheric soil in the pre-planting period was higher than in growing maize and the post-harvest period. Debris samples analysed during all periods showed similar infection percentages for *Aspergillus* section Flavi. The samples of insects collected during the maize-growing period showed a lower percentage of *Aspergillus* isolates than the samples from soil and debris. Aflatoxigenic strains were present in lower levels in each component of the agroecosystem studied. All the strains that produced sclerotia were L strains.

Conclusions: In this field agroecosystem, the only strains with a high probability for transfer to the storage agroecosystem were L strains with low toxigenic potential.

Significance and Impact of the Study: Maize pre-harvest contamination with aflatoxigenic inoculum was not significant.

INTRODUCTION

Maize (*Zea mays* L) is one of the main crops in Argentina. During the harvest season of 1999/2000, the production of maize reached 20 million tons, of which 48% was for the external market and the remaining for internal consumption (Bolsa de Cereales 1999). Contamination by aflatoxin occurs both during maize development and after harvesting. Aflatoxins are potent carcinogenic and teratogenic metabolites produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (IARC 1993). These toxins have been detected in various agricultural commodities, including peanuts, cottonseed and corn (Van Egmond 1995). Studies of naturally-occurring aflatoxins in maize have demonstrated that the levels of contamination are variable. In some years, these levels can be higher than those established by the international regulatory guidelines (Chulze *et al.* 1989; Resnik *et al.* 1996). Populations of *Aspergillus* section Flavi have been isolated from different agroecosystems such as cotton, peanut and maize (Cotty 1997; Horn and Dorner 1998; Wicklow *et al.* 1998), and its presence pre-harvest has been documented. *Aspergillus* has been detected in soil, debris and insects (Angle 1982; Diener and Davis 1987; McMilliam 1987; Wicklow 1988; Lussenhop and Wicklow 1990; Wicklow 1991; Horn *et al.* 1995; Olanya *et al.* 1997). The integrated pest management of aflatoxin producers in maize cultivation requires identification of the potential sources of *A. flavus* inoculum within maize fields, and then establishment of which of these populations are associated with aflatoxin-contaminated grain at harvest (Wicklow *et al.* 1998). It is also important to know the characteristics of toxigenic strains. *Aspergillus* section Flavi can be grouped by morphological and genetic characteristics into the species *A. flavus*, *A. parasiticus* and *A. nomious* (Kurtzman *et al.* 1987; Klich and Pitt 1988). The aflatoxin producers can be classified as L or S strains according to sclerotial morphology. S strains produce numerous small sclerotia (< 400 μm) and fewer conidia than L strains; L strains produce fewer, larger sclerotia (Cotty 1989). S strains produce relatively high levels of aflatoxins, while L strains produce only B_1 and B_2, or are atoxigenic (Egel *et al.* 1994).

In order to develop effective prevention strategies, it was important to know where and when the inoculum of section

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Flavi was present in commercial maize fields in one of the main productive areas in Argentina. In this study, populations of *Aspergillus* section Flavi resident in a commercial maize field were studied. Potential aflatoxin producers were examined on the basis of sclerotial and toxigenic characteristics.

MATERIALS AND METHODS

Sampling site

Commercial fields of maize in Rio Cuarto, Córdoba, Argentina were selected. Samples were collected during the periods September (pre-planting), December (growing maize) and May (post-harvest).

Collection procedures

Non-rhizospheric soil samples were collected from a commercial field of maize during pre-planting, maize-growing and post-harvest periods. Fifteen samples at each period were collected. Each of the samples consisted of a mixture of 10 soil samples (5–10 g each) taken from the top 3 cm of soil at different places within the field. The samples were taken in diagonal section at 100 m intervals. Sub-samples of each sample were combined in a paper bag and air-dried for 1–2 days at 25–30°C. Samples weighing 100 g were thoroughly mixed, passed through a testing sieve (2 mm mesh size) and the soil separated from the debris. Soil samples were stored at 5°C.

Mycological studies

Aspergillus section Flavi isolation from soil. Enumeration of fungal propagules was carried out on solid medium, using the surface spread method, by blending 10 g soil of each sample with 90 ml 0·1% peptone water solution. Serial dilutions of 10^{-1} to 10^{-3} from each sample and 0·1 ml aliquots were inoculated in triplicate on *Aspergillus flavus* and *parasiticus* agar (AFPA) medium. The Petri dishes were incubated at 30°C for 48 h (Pitt and Hocking 1997).

Fungal identification. Macroscopic examination of fungal colonies that looked like *Aspergillus* section Flavi were subcultured on malt extract agar medium (MEA) for further identification. *Aspergillus* species were identified according to taxonomic schemes proposed by Pitt and Hocking (1997) and Klich and Pitt (1994).

Aspergillus section Flavi isolation from debris. Fifteen debris samples at each period, collected from a maize field with soybean rotational treatment, were processed according to McGee *et al*. (1996) with some modifications. Five pieces were taken arbitrarily from each sample and cut into small sections (1 cm). These sections (50) were surface-disinfected for 1 min in 1% chlorine solution, rinsed three times in sterile distilled water and transferred to Petri dishes containing AFPA medium; they were then incubated at 30°C for 48 h. Identification was carried out as described above.

Aspergillus section Flavi isolation from insects. Strains were isolated from the maize earworm which attacks the aerial part of the maize plant in this zone. *Helicoverpa zea* was collected during the period of grain maturation using wind-orientated funnel traps. Insects (100) were killed by freezing at −20°C and were then washed twice with sterile distilled water containing Tween 80 (0·01%) (McGee *et al*. 1996). The insects were cultivated individually on AFPA medium and incubated at 30°C for 48 h. The isolates were identified according to Pitt and Hocking (1997).

Sclerotia characterization. Each *Aspergillus* section Flavi strain isolated was transferred to Petri dishes with agar medium 5/2, according to Cotty (1989). The Petri dishes were incubated at 30°C for 7–10 days. The *A. flavus* isolates producing typical sclerotia (average diameter $< 400\ \mu m$) were assigned to the S strain; all other *A. flavus* isolates were assigned to the L or typical strain.

Aflatoxin production. Aflatoxin analyses were performed following the methodology proposed by Geisen (1996) with some modifications. The *A. flavus* strains were incubated in Petri dishes with malt extract agar at 30°C for 5 days. One colony of each strain was transferred to an Eppendorf tube and 500 μl chloroform were added. The mixture was agitated at 4000 rev min^{-1} for 20 min. The mycelium was extracted at room temperature and the chloroform extract dried with nitrogen. The residue was re-dissolved in 10 μl chloroform for TLC screening. Positive samples were quantitatively determined by HPLC, following the methodology of detection proposed by Trucksess *et al*. (1994). An aliquot (200 μl) was derivatized with 700 μl trifluoroacetic acid/acetic acid/water (20:10:70). The derivatized aflatoxin (50 μl solution) was analysed using a reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of a Hewlett Packard 1100 pump (Palo Alto, CA, USA) connected to a Hewlett Packard 1046[a] programmable fluorescence detector, interfaced to a Hewlett Packard Chem Station. Chromatographic separations were performed on a stainless steel Supelcosil LC-ABZ C_{18} reversed-phase column (150 × 4·6 mm i.d., 5 μm particle size; Supelco). Water/methanol/acetonitrile (4:1:1) was used as the mobile phase at a flow rate of 1 ml. Fluorescence of aflatoxin derivatives was recorded at

excitation and emission wavelengths of 360 and 440 nm, respectively. Standard curves were constructed with different levels of AFB_1 and AFG_1. These toxins were quantified by correlating peak heights of sample extracts with those of standard curves. The limit of detection of the analytical method was 5 ng g^{-1}.

Statistical analysis

Statistical analyses were performed on log-transformed data. To compare the independent variables (sampling periods) with dependent variables, data for *A. flavus* section Flavi populations were analysed by ANOVA, followed by Tukey's test.

RESULTS

Aspergillus section Flavi isolated from non-rhizospheric soil, debris and insects

Mycological analysis from non-rhizospheric soil samples showed that the colony count during the pre-planting period was higher than in the growing and post-harvest periods. The values were 1×10^4–2×10^5 in the first period while in the other periods, the count varied over the range 1×10^2 to 1×10^3. The debris analysed during the maize-growing period showed a similar percentage of infection of *Aspergillus* section Flavi in all three periods. The samples of insects collected during the maize-growing period showed a lower isolation percentage than the samples of soil and debris (Table 1). Figures 1 and 2 show the incidence of *A. flavus* and *A. parasiticus*. The source of inoculum for both strains in the first and third period was soil. The source of inoculum in growing maize changed; *A. flavus* and *A. parasiticus* were present predominantly on debris. Insects appeared not to be an important source of infection.

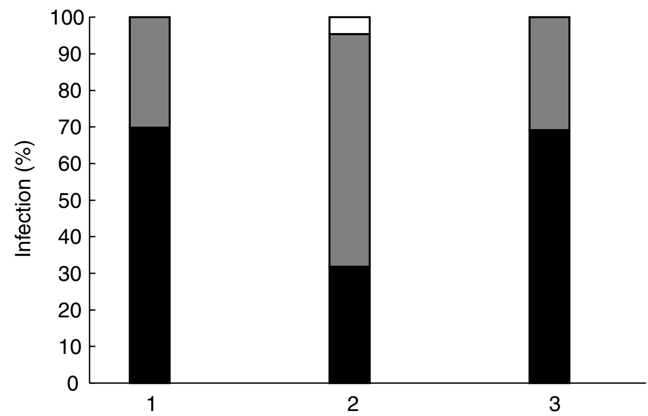

Fig. 1 *Aspergillus flavus* populations at three periods: (1) pre-planting; (2) maize growing; (3) post-harvest. (□) Debris; (■) soil; (▒) insects

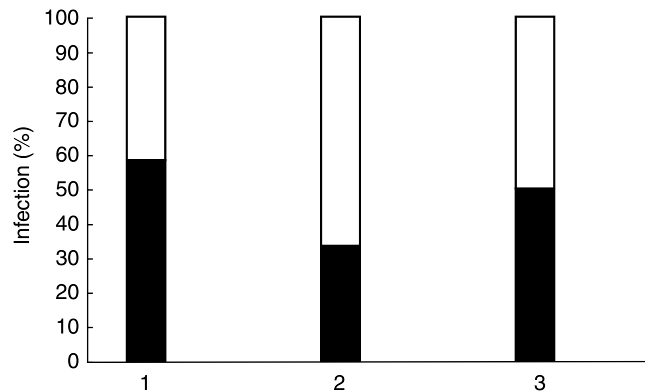

Fig. 2 *Aspergillus parasiticus* populations at three sampling periods: (1) pre-planting; (2) maize growing; (3) post-harvest. (□) Debris; (■) soil

Aflatoxin production and sclerotia characterization

Aspergillus flavus and *A. parasiticus* strains showed a variable capacity to produce aflatoxin (Table 2). The proportion of *A. flavus* strains toxigenic in soil samples was 18, 15 and 12%, respectively, during the three periods. In debris samples, the proportion of toxigenic strains was 10, 30 and 37%. The highest percentage of non-toxigenic *A. flavus* (82%) was isolated from the pre-planting samples. However, the most toxigenic strains belonged to this period, with levels of AFB_1 between 0·5 and 3·65 μg g^{-1} mycelium. The

Table 1 *Aspergillus* section Flavi population in soil, debris and insects during three sampling periods

Sampling period	Count medium AFPA	Soil samples $\log_{10}$ cfu g^{-1}*	Debris samples % infection*	Insect samples % infection†
Pre-planting		4 ± 0·5‡	30 ± 18‡	
Maize growing		2 ± 0·1§	32 ± 0·8‡	3 ± 0·1
Post-harvest		2 ± 0·5§	37 ± 0·8‡	

*Values are means ± S.D. of 50 samples.
†Values are means ± S.D. of 100 samples.
Significant differences were found between ‡ and § ($P < 0·05$) Tukey's test.

Table 2 Aflatoxin production by *Aspergillus flavus* and *A. parasiticus* at three sampling periods from soil, debris and insects

Samples	Sampling period	AFB_1 and AFG_1 Range (μg g^{-1} mycelium)	
Soil	1	*A. flavus*	
		41 strains	NT
		10 strains	0·05–3·65*
		A. parasiticus	
		2 strains	NT
		5 strains	0·22–6·39*
			0·16–2·5†
	2	*A. flavus*	
		6 strains	NT
		1 strain	2·45*
		A. parasiticus	
		1 strain	1·4*
			0·85†
	3	*A. flavus*	
		16 strains	NT
		2 strains	0·35–0·82*
		A. parasiticus	
		1 strain	0·33*
			0·29†
Debris	1	*A. flavus*	
		20 strains	NT
		2 strains	0·21–0·35*
		A. parasiticus	
		2 strains	NT
		3 strains	0·89–25·08*
			0·95–0·98†
	2	*A. flavus*	
		11 strains	NT
		3 strains	0·45–0·65*
		A. parasiticus	
		1 strain	NT
		1 strain	4·13*
			3·72†
	3	*A. flavus*	
		3 strains	NT
		5 strains	0·58–2·06*
		A. parasiticus	
		1 strain	0·68*
			0·62†
Insects	2	*A. flavus*	
		1 strain	8·33*

*μg^{-1} AFB_1.
†$\mu g^{-1}$$AFG_1$.
NT = Non-toxigenic.

major percentage of toxigenic *A. parasiticus* strains (71%) was isolated in the pre-planting period from soil. Analysis of the debris samples showed that the *A. flavus* strains producing the most aflatoxin were found in the post-harvest period, with levels of AFB_1 between 0·58 and 2·06 μg g^{-1}, while strains of *A. parasiticus* were found before planting with levels of AFB_1 between 0·89 and 25·08 μg g^{-1} and levels of AFG_1, between 0·95 and 0·98 μg g^{-1}. Comparatively, the most toxigenic *A. flavus* strains were isolated from soil and insects, while the most toxigenic *A. parasiticus* strains were isolated from debris. If the aflatoxin production capacity of both species is compared, it appears that only 11% of the isolates of *A. flavus* are toxigenic, while 97% of *A. parasiticus* strains are aflatoxin producers. In the first period of maize growth, only 26% of strains isolated from soil and debris samples produced sclerotia. In the second and third periods, the percentage of strains isolated with sclerotial production capacity was 18 and 34%, respectively. All the strains that produced sclerotia during the different periods of maize growing were L strains. These data reveal the low potential for aflatoxin production in all the L strains isolated.

DISCUSSION

The data indicate that potentially toxigenic *Aspergillus* section Flavi strains were extensively distributed in low levels in all components of the agroecosystem studied. Similar values were found by Shearer *et al.* (1992) in Iowa crop fields and by Wicklow *et al.* (1998) in an Illinois field. McGee *et al.* (1996), in a study carried out in Iowa during non-epidemic years, found lower levels of *Aspergillus* section Flavi contamination. According to Zummo and Scott (1990), *A. flavus* has a greater ability to survive on debris, and this was confirmed for the second sampling period in our study.

The proportion of toxigenic strains agrees with data from the USA in non-epidemic years (Shearer 1992).This author found that the percentage of toxigenic isolates was high in 1988 (55%) following an aflatoxin outbreak, but declined substantially in 1990 in a non-epidemic year. Since only 1·5% of strains were aflatoxin and sclerotial producers in laboratory culture, the results of our study agree with Bennett *et al.* (1979) and Shearer *et al.* (1992), who did not observe a correlation between aflatoxin production and sclerotia.

The potential to produce aflatoxin contrasted with the high levels of aflatoxins produced by S strains (Cotty 1989; Egel *et al.* 1994). S strains occur in the USA (Cotty 1989; Doster and Michailides 1994), Thailand (Saito *et al.* 1986) and Africa (Hesseltine *et al.* 1970). The behaviour of toxigenic strains in the field studies agreed with the results found at harvest in 1999. Only one sample of maize was contaminated with aflatoxin B_1 at a level of 5 p.p.b. Indeed, all samples of corn meal prepared from maize from this productive area were negative for aflatoxins (Etcheverry *et al.* 1999).

The results presented here indicate that in this field agroecosystem, the only strains with a high probability of transference to storage were L strains with low toxigenic potential. This knowledge is important for the management of prevention strategies in storage.

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REFERENCES

Angle, J.S., Dunn, K.A. and Wagner, G.H. (1982) Effect of cultural practices on the soil population of *Aspergillus flavus* and *Aspergillus parasiticus*. *Soil Science Society of America Journal* **46**, 301–304.

Bennett, J.W., Horowitz, P.C. and Lee, L.S. (1979) Production of sclerotia by aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. *Mycologia* **71**, 415–422.

Bolsa de Cereales (1999) Número Estadístico 1999/2000. Buenos Aires. Argentina Cereal Office. Government Printing Office.

Chulze, S., Bertinetti, C., Dalcero, A. *et al.* (1989) Incidence of aflatoxin, zearalenone and deoxynivalenol on corn in Argentina. *Mycotoxin Research* **5**, 9–12.

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

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

Diener, U.L. and Davis, N.D. (1987) Biology of *A. flavus* and *A. parasiticus*. In *Aflatoxin in Maize. A Proceeding of the Workshop* ed. Zuber, M.S., Lillehoj, E.B. and Renfro, B.L. pp. 33–40. Mexico City: CIMMYT.

Doster, M.A. and Michailides, T.J. (1994) Development of *Aspergillus* molds in litter from pistachio trees. *Plant Disease* **78**, 393–397.

Egel, D.S., Cotty, P.J. and Elias, K.S. (1994) Relationships among isolates of *Aspergillus* sect. *Flavi* that vary in aflatoxin production. *Phytopathology* **84**, 906–912.

Etcheverry, M., Nesci, A., Barros, G., Torres, A. and Chulze, S. (1999) Occurrence of *Aspergillus* section Flavi and aflatoxin B_1 in corn genotypes and corn meal in Argentina. *Mycopathologia* **147**, 37–41.

Geisen, R. (1996) Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Systematic and Applied Microbiology* **19**, 388–392.

Hesseltine, C.W., Shotwell, O., Smith, M., Ellis, J.J., Vandegraft, E. and Shannon, G. (1970) Production of various aflatoxins by strains of the *Aspergillus flavus* series. In *Proceedings of the First U.S Japan Conference on Toxic Microorganisms* ed. Herzberg, M. pp. 202–210. Washington: Government Printing Office.

Horn, B.W. and Dorner, J.W. (1998) Soil population of *Aspergillus* species from section Flavi along a transect through peanuts-growing region of the United States. *Mycologia* **90**, 767–776.

Horn, B.W., Greene, R.L. and Dorner, J.W. (1995) Effect of corn and peanut cultivation on soil populations of *Aspergillus flavus* and *A. parasiticus* in Southwestern Georgia. *Applied and Environmental Microbiology* **62**, 2472–2475.

IARC (1993) International Agency for Research on Cancer. *Monograph on the Evaluation of Carcinogenic Risk to Human* **56**, 257–263.

Klich, M.A. and Pitt, J.I. (1988) Differentiation of *Aspergillus flavus* from *Aspergillus parasiticus* and other closely related species. *Transactions of the British Mycological Society* **91**, 99–108.

Klich, M.A. and Pitt, J.I. (1994) *A laboratory Guide to Common Aspergillus Species and their Teleomorphs.* ed. Kilch, M.A. and Pitt, J.I. Australia: Commonwealth Scientific and Industrial Research.

Kurtzman, C.P., Horn, B.W. and Hesseltine, C.W. (1987) *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. *Antonie Van Leeuwenhoek* **53**, 147–158.

Lussenhop, J. and Wicklow, D.T. (1990) Nitidulid beetles as a source of *Aspergillus flavus* infective inoculum. *Transactions of the Japanese Mycology Society* **31**, 63–74.

McGee, D.C., Olanya, O.M., Hoyos, F.M. and Tiffany, L.H. (1996) Populations of *Aspergillus flavus* in the Iowa cornfield ecosystem in years not favorable for aflatoxin contamination of corn grain. *Plant Disease* **80**, 742–746.

McMilliam, W.W. (1987) Relation of insects to aflatoxin contamination in maize grown in the southeastern USA. In *Aflatoxin in Maize. A Proceeding of the Workshop* ed. Zuber, M.S., Lillehoj, E.B. and Renfro, B.L. pp. 194–200. Mexico City: CIMMYT.

Olanya, O.M., Hoyos, G.M., Tiffany, L.H. and McGee, D.C. (1997) Waste corn as a point of inoculum for *Aspergillus flavus* in the corn agroecosystem. *Plant Disease* **81**, 576–581.

Pitt, J.I. and Hocking, A.D. (1997) *Fungi and Food Spoilage* Vol. II, ed. Pitt, J.I. and Hocking, A.D. London: Blackie Academic and Professional.

Resnik, S., Neira, S., Pacin, A., Martinez, E., Apro, N. and Latreite, S. (1996) A survey of the natural occurrence of aflatoxins and zearalenone in Argentina field maize: 1983–94. *Food Additives and Contaminants* **13**, 115–120.

Saito, M., Tsuruta, O., Siriacha, P., Kawasugi, S., Manabe, M. and Buangsuwon, D. (1986) Distribution and aflatoxin productivity of the atypical strains of *Aspergillus flavus* isolates from soil in Thailand. *Proceedings of the Japan Association of Mycotoxicology* **24**, 41–46.

Shearer, J.F., Sweets, L.E., Baker, N.K. and Tiffany, L.H. (1992) A study of *Aspergillus flavus/parasiticus* in Iowa crop fields: 1988–90. *Plant Disease* **76**, 19–22.

Trucksess, M.W., Stack, M.E., Nesheim, S., Albert, R.H. and Romer, T.R. (1994) Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1, G_2 in corn, almonds, Brazil nuts, peanuts and pistachio nuts: collaborative study. *Journal of AOAC International* **6**, 1512–1521.

Van Egmond, H.P. (1995) Mycotoxins: regulations, quality assurance and reference materials. *Food Additives and Contaminants* **12**, 321–330.

Wicklow, D.T. (1988) Patterns of fungal associations within maize kernels in North Carolina. *Plant Disease* **72**, 113–115.

Wicklow, D.T. (1991) Epidemiology of *Aspergillus flavus* in corn. In *Aflatoxin in Corn: New Perspectives* ed. Shotwell, O.L. and Hurburg, C.R. Jr pp. 315–328. Research Bulletin 599. Ames, Iowa: Iowa Agriculture and Home Economics Experiment Station.

Wicklow, D.T., McAlpin, C.E. and Platis, C.E. (1998) Characterization of the *Aspergillus flavus* population within an Illinois maize field. *Mycological Research* **102**, 263–268.

Zummo, N. and Scott, G.E. (1990) Relative aggressiveness of *Aspergillus flavus* and *A. parasiticus* in maize in Mississipi. *Plant Disease* **74**, 978–981.