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Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *flavi* from different substrates in Argentina

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Abstract

Aspergillus section *flavi* strains isolated from peanuts, wheat and soybean grown in Argentina were screened for aflatoxins (type B and G) and cyclopiazonic acid (CPA) production. *Aspergillus flavus* was the predominant species in all substrates, although there was almost the same proportion of *A. flavus* and *Aspergillus parasiticus* in peanuts. *Aspergillus nomius* was not found. Incidence of aflatoxigenic *A. flavus* strains was higher in peanuts (69%) than in wheat (13%) or soybeans (5%) while the ratio of CPA producers *A. flavus* isolated from all substrates was very high (94% in peanuts, 93% in wheat and 73% in soybeans). Isolates of *A. flavus* able to produce simultaneously aflatoxins type B and CPA were detected in all substrates, suggesting the possibility of co-occurrence of these toxins. Almost all isolates of *A. parasiticus* resulted aflatoxins (type B and G) producers but did not produce CPA. Five of sixty-seven strains isolated from peanuts showed an unusual pattern of mycotoxin production (aflatoxins type B and G simultaneously with CPA). These strains also produced numerous small sclerotia like S strains of *A. flavus* detected in cottonseed in Arizona and in soils of Thailand and West Africa. The atypical strains are not widely distributed in Argentina and were found uniquely in peanuts.

Keywords: *Aspergillus* section *flavi*; Aflatoxins; Cyclopiazonic acid; Peanut; Wheat; Soybean

1. Introduction

Aspergillus section *flavi* includes three species (*Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*) producing aflatoxins, highly toxic and carcinogenic compounds of concern in food safety. *A. flavus* also produces other mycotoxins such as cyclopiazonic acid (CPA), an indole-tetramic acid (Luk et al., 1977). CPA occurs naturally in corn (Gallagher et al., 1978) and peanuts (Lansden and Davidson, 1983; Fernández Pinto et al., 2001) as co-contaminant with aflatoxins and may have contributed to the "Turkey X" syndrome in England in 1960 (Cole, 1986).

Continuous concern about potential effects of these mycotoxins in the human diet has led to an increasing interest in fungi within *Aspergillus* section *flavi*. *A. flavus*, *A. parasiticus* and *A. nomius* are closely

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related species but they display some physiological differences even in mycotoxin production. According to Pitt (1993), *A. flavus* isolates produce type B (B_1 and B_2) aflatoxins (AFB), or CPA, or both or neither. Generally, type G (G_1 and G_2) aflatoxins (AFG) are not produced by this species although in some reports it has been mentioned AFG production by isolates identified as *A. flavus* (Saito et al., 1986; Klich and Pitt, 1988; Vaamonde et al., 1995). "Atypical" strains of *A. flavus* have been isolated from soils in Thailand (Saito et al., 1986) and from cotton seed and soils in Arizona (Cotty, 1989). These isolates, called S strains, produced abundant small sclerotia (<400 μm in diameter) and large quantities of aflatoxins in contrast with normal, or L strains, which produce fewer but larger sclerotia (>400 μm in diameter) and less aflatoxins. Typical isolates (L strains) may produce only AFB or no aflatoxins at all (Egel et al., 1994), while all *A. flavus* S strains produce relatively large quantities of aflatoxins, some producing only AFB and others producing both B and G aflatoxins (Saito et al., 1986; Egel et al., 1994; Cotty and Cardwell, 1999).

Isolates of *A. parasiticus* consistently produce both B and G aflatoxins but they do not produce CPA (Pitt, 1993). *A. nomius* is morphologically very similar to *A. flavus* but produces both B and G aflatoxins like *A. parasiticus*. Up to now, *A. nomius* isolates are not reported to produce CPA (J.I. Pitt, personal communication).

Data from different geographical areas demonstrate a great variability in the mycotoxin-producing potential of *A. flavus* and closely related species. According to Horn and Dorner (1999), knowledge of regional differences in the toxigenicity of *A. flavus* populations as well as knowledge of the association of these populations with the dominant crop in a region may be important in determining which control measures are most effective in reducing preharvest aflatoxin contamination.

The aim of the present work was the screening of *Aspergillus* section *flavi* isolates from several crops grown in Argentina for aflatoxins and CPA production. Strains isolated from substrates with different susceptibility to aflatoxin contamination such as peanuts, soybeans and wheat were included. Based on the combination of mycotoxins produced, five *A. flavus* chemotypes were established and the rate of isolates belonging to each chemotype in the different substrates was determined.

2. Materials and methods

2.1. Fungal isolates

The strains of *A. flavus* and *A. parasiticus* used in this study were isolated from peanuts cultivated in the peanut-growing area in the province of Córdoba (30 samples), wheat from the province of La Pampa (10 samples), and soybeans purchased in the local market (10 samples).

2.2. Isolation and identification of fungi

Strain isolation was performed by direct plating method in Dichloran-18% glycerol agar (DG18) and Dichloran-chloramphenicol peptone agar (DCPA) (Pitt and Hocking, 1997). One hundred kernels for sample were placed on 10 Petri dishes with each media. Plates were incubated at 25 °C for 5 days. After isolation, strains were identified according to Pitt and Hocking (1997).

2.3. Mycotoxin production

The agar plug methods for extracellular and intracellular mycotoxins (Filtenborg et al., 1983) were used for toxin extraction of the colonies grown on Czapek-yeast extract agar (CYA). An agar plug was cut out of the colony with a cork borer. Using a needle, the plug was placed onto a TLC plate with the medium side towards the gel for detection of aflatoxins, and the mycelium side towards the gel for CPA. In this case, one or two drops of chloroform was added to the mycelium prior to the application onto the TLC plate. After application, the spot was allowed to dry and the plate was developed. For CPA the plate was previously dipped in a 2% solution of oxalic acid in methanol followed by air-drying.

Mycotoxin detection was performed using thin-layer chromatography on silicagel G60 plates (20 × 20 cm, 0.25 mm thick, Merck 5721, Germany). Chloroform/acetone (90:10) was used as developing solvent for aflatoxins and ethyl acetate/2-propanol/ammonium hydroxide (40:30:20) for cyclopiazonic

acid (Fernández Pinto et al., 2001). Aflatoxins were visualized under longwave UV light (366 nm) and CPA in daylight after treatment of the plates with Erlich's reagent (1 g of 4-dimethylaminobenzaldehyde in 75 ml ethanol and 25 ml concentrated HCl), with subsequent development of blue spots. The detection limit is 0.1 ppm for aflatoxins and 2.0 ppm for CPA. Doubtful or negative results for aflatoxins or CPA production were confirmed by culturing isolates in rice by the method of Shotwell et al. (1966).

3. Results and discussion

This study includes 130 strains isolated from different substrates (67 from peanuts, 43 from soybeans and 20 from wheat). *A. flavus* was predominant in all substrates, although there was almost the same proportion of *A. flavus* (37 strains) and *A. parasiticus* (30 strains) in peanuts. Almost all isolates of *A. parasiticus* from all substrates resulted AFB and AFG producers (Table 1). Isolates of *A. parasiticus* are typically aflatoxigenic, producing both type B and G aflatoxins but not CPA. Other authors have reported that nontoxigenic isolates of *A. parasiticus* are extremely rare (Blaney et al., 1989; Horn et al., 1996; Tran-Dinh et al., 1999). In the present study only two *A. parasiticus* isolates from peanuts were nontoxigenic. These native non-aflatoxigenic strains could be organisms of interest in order to develop biocontrol strategies for reducing aflatoxin contamination in peanuts cultivated in the region. Highly competitive atoxigenic strains might be applied to agricultural fields as biocompetitive agents. *A. nomius*, a species relatively infrequent in nature, was not isolated. Regarding *A. flavus*, considerable variability in their mycotoxin-producing potential was found in concordance with others (Hesseltine et al., 1970; Bayman and Cotty, 1993; Egel et al., 1994; Cotty and Cardwell, 1999; Horn and Dorner, 1999). Based on the combination of mycotoxins produced (AFB+/−, AFG+/− and CPA+/−) five *A. flavus* chemotypes can be considered (Table 2). The chemotype II might be considered unusual because, in general, CPA is produced by *A. flavus*, alone or in combination with type B aflatoxins, but not in conjunction with type G aflatoxins.

Table 3 shows the distribution of *A. flavus* strains isolated from the different substrates among the five chemotypes. Incidence of aflatoxigenic *A. flavus* strains was higher in peanuts (73%) than in wheat (13%) or soybeans (5%). Differences among crops in the percentage of aflatoxin producers have been found by other workers. Schroeder and Boller (1973) examined aflatoxin production by *A. flavus* isolates from peanuts, cottonseed, rice and sorghum, and they reported a higher percentage of aflatoxin-producing strains in peanuts. In general, the proportion of aflatoxigenic isolates of *A. flavus* from peanuts and peanut soils is high (Lisker et al., 1993; Horn et al., 1996). A lower rate of *A. flavus* strains capable of producing aflatoxins was found in the other grains analyzed, wheat and soybeans, which are less susceptible to aflatoxin contamination than peanuts. Another substrate with low natural aflatoxin contamination is

Table 1
Toxigenic potential of *A. parasiticus* isolates

Substrate	Number of isolates	Toxigenic[a]	Nontoxigenic
Peanuts	30	28	2
Soybeans	2	2	–
Wheat	5	5	–
Total	37	35	2

[a] Produce AFB_1, AFB_2, AFG_1 and AFG_2.

Table 2
Chemotypes of *A. flavus* based on aflatoxins and CPA production

Chemotype	Mycotoxins		
	AFB	AFG	CPA
I	+	–	+
II	+	+	+
III	+	–	–
IV	–	–	+
V	–	–	–

Table 3
Incidence of *A. flavus* chemotypes in the different substrates

Substrate	Isolates	Number of isolates of each chemotype (%)[a]				
		I	II	III	IV	V
Peanuts	37	20 (54%)	5 (13%)	2 (6%)	10 (27%)	–
Soybeans	41	2 (5%)	–	–	28 (68%)	11 (27%)
Wheat	15	2 (13%)	–	–	12 (80%)	1 (7%)

[a] Percentage related to the total number of *A. flavus* isolates in each substrate.

amaranth grain. A survey of toxigenic fungi in this product (Bresler et al., 1995) demonstrated a low potential for aflatoxin production by *A. flavus* since only 4 of 34 strains were able to produce the toxin. It could be hypothesized that the aflatoxin-producing potential of fungi in the mycota is an important factor that influences the natural aflatoxin contamination in agricultural products.

The ratio of CPA producers among *A. flavus* strains isolated from all substrates was very high (94% in peanuts, 93% in wheat and 73% in soybeans). Other surveys conducted on CPA production by *A. flavus* isolated from peanuts showed percentages of CPA-producing isolates as high as 89% (Blaney et al., 1989) and 93% (Horn et al., 1996). Resnik et al. (1996) found that 33 of 34 *A. flavus* isolates from corn produced CPA and only 5 of these strains produced aflatoxin B_1 simultaneously. In the present work, strains belonging to the chemotype I (AFB+, CPA+) were quite frequent in peanuts (63%) and were also isolated from soybeans (5%) and wheat (13%). These results suggest the possibility of co-occurrence of aflatoxin and CPA in these agricultural commodities as it was reported for peanuts grown in Argentina by Fernández Pinto et al. (2001). Although surveys on CPA production by *A. flavus* strains are more limited than those referred to aflatoxin production, it is evident that a high proportion of the isolates is capable of producing CPA.

A. flavus isolates that produced aflatoxin B but not CPA (chemotype III) were rare, amounting the 2.2% of the total of strains analyzed. This result is in concordance with those of Horn and Dorner (1999) who detected 0.6% of this type of strains, and Blaney et al. (1989), who reported that only 1 of 38 *A. flavus* isolates (2.6%) produced only AFB_1.

Five strains isolated from peanuts belonged to chemotype II (Table 2) which, as has been mentioned above, might be considered atypical. These strains are morphologically similar to *A. flavus* but produce aflatoxins B and G in conjunction with CPA. Several authors have previously mentioned that certain isolates of *A. flavus* produced type B and G aflatoxins (Hesseltine et al., 1970; Saito et al., 1986; Klich and Pitt, 1988; Saito and Tsuruta, 1993; Vaamonde et al., 1995; Cotty and Cardwell, 1999) but they do not make reference to CPA production. All isolates belonging to chemotype II in the present study correspond to S type according to the number and size of sclerotia (Table 4). The surface and underside of the colonies appeared brown due to the enormous numbers of microsclerotia. Very scarce conidiophores were formed and the colour of the few conidia was similar to those of *A. flavus* (yellow green). Strains with similar traits were isolated from soils in Thailand (Saito et al., 1986) and named as *A. flavus* var. *parvisclerotigenus* (Saito and Tsuruta, 1993), but the type isolate of this taxon was a nonproducer of G aflatoxins. Most of the G aflatoxin-producing strains of *A. flavus* have been related with the S phenotype. In a survey of North American and West African S isolates, Cotty and Cardwell (1999) found that more than 40% of West African S isolates produce both B and G aflatoxin while North American S strains produce only B aflatoxins.

The unusual pattern of mycotoxins production displayed by our chemotype II strains (AFB+, AFG+, CPA+) had previously been detected by Blaney et al. (1989) in a few isolates from peanuts in Queensland, Australia. It has also been reported by Geiser et al. (2000), who performed a phylogenetic analysis of *A.*

Table 4
Characteristics of atypical strains

Strain number	Sclerotia mean (#/cm^2)	Sclerotium diameter ± S.D. (μm)*	AFB_1	AFB_2	AFG_1	AFG_2	CPA
M32N4/BAFC490	207.8[a]	297.8 ± 70.9[a]	+	–	+	–	+
M38N3/BAFC657	242.6[a]	215.71 ± 76.7[a]	+	–	+	–	+
M35N2/BAFC589	268.67[a]	245.14 ± 54.0[a]	+	–	+	+	+
M11N2	230.3[b]	282.4 ± 53.4[b]	+	–	+	+	+
M5N11	189.69[b]	232.29 ± 89.0[b]	+	–	+	+	+

BAFC: Culture Collection of Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.

[a] Data from Novas and Cabral (2002).

[b] M.V. Novas, personal communication.

*Means based on 26 sclerotia.

flavus and *A. oryzae*, including strains from different geographic origins (California, Texas, Nigeria, Australia).

Three of our isolates belonging to chemotype II (strains BAFC 490, BAFC 657 and BAFC 589) were included in a study of vegetative compatibility (VC) of *A. flavus* isolates from peanuts in Argentina (Novas and Cabral, 2002) and they formed a single and exclusive VC group, suggesting that they have a common clonal origin.

A. flavus populations are extremely genetically diverse (Bayman and Cotty, 1993; Egel et al., 1994; Geiser et al., 2000). Phylogenetic studies based on characterization of DNA sequences from five different coding gene regions in isolates from Australian peanut fields demonstrated the existence of two subgroups called groups I and II (Geiser et al., 1998). These results were confirmed by further phylogenetic analysis of 33 S and L strains of *A. flavus* collected from various regions around the world (Geiser et al., 2000). Tran-Dinh et al. (1999) examined the genetic relationship between toxigenic and nontoxigenic isolates using RAPD analysis and found a similar separation of *A. flavus* isolates into two distinct groups with both toxigenic and nontoxigenic isolates occurring in each group. Group II includes the type S strains producing B and G aflatoxins and CPA although a few isolates within this group do not produce G aflatoxins. Results obtained by Geiser et al. (2000) demonstrated that *A. flavus*, as it is currently defined, represents a non-monophyletic assemblage including at least two major groups (I and II) and suggested that taxonomic changes are necessary. These authors concluded that group II deserves recognition as a new species.

Our atypical strains were recently studied at molecular level and all of them belong to group II (D. Carter, personal communication). These isolates were not frequent (only 5 of 130 isolates) and were found uniquely in peanuts. Reports about isolates with the same characteristics are limited. Egel et al. (1994) suggested that these clones are relatively rare and less frequently detected in niches related to agriculture to which the more frequently detected clones may be better adapted. Up to now, G aflatoxin-producing group II strains of *A. flavus* had been isolated in Australia, Southeast Asia and Africa but not in North America (Geiser et al., 2000). Results of the present work demonstrate the presence of this taxon in Argentina, confirming its prevalence in the Southern Hemisphere. Furthermore, it seems that this type of isolates is mainly associated with peanuts and peanut field soils.

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