

Association of Mycotoxin and Sclerotia Production with Compatibility Groups in *Aspergillus flavus* from Peanut in Argentina

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ABSTRACT

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

Vegetative compatibility (VC) of *Aspergillus flavus* isolates from peanut seed was studied to evaluate preliminary diversity and its association with mycotoxin production and sclerotia production and number. *A. parasiticus* isolates also were included as a comparative group. Isolates were divided into five categories based on mycotoxin production combination. Five of the *A. flavus* isolates were considered atypical because they simultaneously produced aflatoxins B, G, and cyclopiazonic acid (CPA). Vegetative compatibility groups (VCGs) were determined through complementation tests between nitrate-nonutilizing mutants. Sclerotia diameters and the number of sclerotia produced per square centimeter were determined for each isolate. Out of 32 isolates of *A. flavus*, 25 combined in 13 VCGs, whereas the remaining could not be assigned to any particular group. Each VCG included isolates of the same mycotoxin category, with only one exception. Also, all isolates within the same VCG were characterized by their ability to produce or not produce sclerotia. Isolates between VCGs showed significant differences in number of sclerotia per square centimeter, but differences in sclerotia size were not evident. Atypical isolates simultaneously producing aflatoxins B, G, and CPA formed a single and exclusive VCG.

Aflatoxins are carcinogenic secondary metabolites that occur in foods and feeds worldwide (3). *Aspergillus flavus* Link:Fr., *A. parasiticus* Speare, and *A. nomius* Kurtzman, Horn, & Hesselstine produce a number of aflatoxins in agricultural commodities such as peanut seed (15,22,36). Furthermore, *A. flavus* frequently produces cyclopiazonic acid (CPA; 17,26). Conventional methods for the prevention of aflatoxin contamination are not reliable on a commercial scale for any of the crops affected (11). There is interest in developing a biological control agent that can increase crop safety by decreasing aflatoxin content. Such a method may depend on the displacement of toxigenic isolates using atoxigenic isolates of the same species. However, before the employment of a mycotoxin biological control, it is necessary to study the genetic diversity of the population of interest. Toxicological studies on CPA have shown that it appears to act primarily as an entero-nephrotoxin in chickens and pigs and as a hepatotoxin in

rats (17). *A. flavus* produces B aflatoxins (B₁ and B₂), CPA, both, or none of these toxins (22,33,36). *A. parasiticus* typically produces, in addition to B aflatoxins, aflatoxins G₁ and G₂, but not CPA. *A. nomius* exhibits the same mycotoxin pattern as *A. parasiticus*, but morphologically resembles *A. flavus*. However, "atypical" isolates of *A. flavus* simultaneously producing B and G aflatoxins have been reported (4,16,26,36).

A. flavus isolates that produce sclerotia can be divided into two types of strains, L and S, on the basis of physiological, morphological, and genetic criteria (3,10). S strain isolates produce numerous small sclerotia (<400 µm in diameter) and fewer conidia than L strain isolates. Typical isolates (L) of *A. flavus* may produce only B aflatoxins or no aflatoxins at all and all S strain isolates (named atypical isolates by Saito et al.; 34) reported to date produce large quantities of aflatoxins, some producing only B aflatoxins and others producing both B and G aflatoxins. Relationships among *A. flavus* isolates were inferred from restriction site variability in a portion of the Taka amylase A gene (18). In this study, two isolates that simultaneously produced B and G aflatoxins (21) were determined as intermediate between *A. flavus* and *A. parasiticus* by a phenetic analysis and as ancestral to both species by a cladistic analysis (18).

A. flavus has no sexual stage; however, some of its variability can be examined by sorting isolates into vegetative compatibil-

ity groups (VCGs). VCGs have been associated with many morphological and physiological features, such as sclerotia size, mycotoxin production, and spatial distribution, in this genus (1,23,32). Heterokaryon formation can only occur between strains with identical alleles at each of several loci governing vegetative compatibility (19,31). *Aspergillus* strains produce no clear signs of anastomosis or antagonism when they meet as wild strains; therefore, complementary nitrate-nonutilizing (*nit*) mutants are commonly used to identify compatible isolates (1,32). Once identified, complementary mutants are paired. Complementation is indicated by a zone of dense, wild-type growth and heavy sporulation at the intersection of the two colonies due to hyphal anastomosis and nutritional complementation in the heterokaryon. Heterokaryons can form only between *nit* mutants of vegetative compatible isolates. Thus, isolates that are vegetative compatible belong to the same VCG.

In Argentina, 99% of peanut production is localized in the center of Córdoba province. Isolates of *A. flavus* and *A. parasiticus* were previously obtained from peanut seed in this region to examine their mycotoxin production (36). Five isolates identified as *A. flavus* by their morphological characteristics simultaneously produced aflatoxins B and G and also CPA as reported by Blaney et al. (4). Each of the isolates also produced sclerotia.

The objectives of this preliminary study were to (i) determine for the first time in Argentina and in South America VCG diversity in *A. flavus* isolates from peanut seed that may enable a future development of a biocontrol strategy and (ii) evaluate any relationship between VCGs and mycotoxin production and sclerotia size and number, mainly between typical and atypical isolates.

MATERIALS AND METHODS

Isolates. Isolates of *A. flavus* ($n = 32$) and *A. parasiticus* ($n = 11$) had been obtained from peanut seed from 19 peanut fields in Córdoba province, Argentina, in a previous study (36). Categories into which *Aspergillus* isolates were placed (Table 1), based on toxin production, have been modified from Vaamonde et al. (37). Once the present study was finished, all "atypical" isolates and one isolate per VCG were placed in the Culture Collection, Facultad de Ciencias Exactas y Naturales (BAFC

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This research was supported by PRHIDED-CONICET (Publication no. 145) and University of Buenos Aires.

Accepted for publication 27 September 2001.

Publication no. D-2002-0103-01R
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cultures) of the University of Buenos Aires (Table 2).

VCGs. The *nit* mutants of *A. flavus* and *A. parasiticus* were produced and their phenotypes were determined by the methods developed by Cove (12) and modified by Bayman and Cotty (2). Plugs of mycelia of *Aspergillus* isolates were transferred from potato dextrose agar (PDA) to Czapek-Dox (Cz) plates containing potassium chlorate at 25 g/liter with nonadjusted pH. Cultures were incubated at 30°C and the margins of the colonies with restricted growth were examined regularly for fast-growing sectors, consisting of sparse my-

celium. *Aspergillus nit* mutants grow as wild-type isolates in Cz containing ammonium; therefore, hyphal tips from fast-growing sectors were transferred to plates of Cz in which ammonium, added as ammonium tartrate, was used instead of NaNO₃. The *nit* mutant phenotypes were determined by growing them on several media amended with different nitrogen sources (12). Three classes of mutants were recovered: *niaD* (nitrate nonutilizing), *nirA* (nitrite and nitrate nonutilizing), and *cnx* (hypoxanthine and nitrate nonutilizing). At least two different *nit* mutants were obtained from each strain. The source of nitrogen in the selection medium influenced the ratio of mutant classes (2). In *A. flavus* isolates, *nirA* mutants appeared frequently on glutamate (2); therefore, when it was not possible to obtain at least two different mutants from an isolate, we replaced the chlorate with this medium. To test for complementation, pairs of complementary mutants were inoculated 15 mm apart in the center of Cz plates and incubated for 7 to 14 days at 30°C. Compatibility was identified by a line of wild-type growth at the zone of interaction. Initially, complementary pairings of *niaD*, *nirA*, and *cnx* mutants within

each isolate were made to assess self-compatibility. Isolates that did not demonstrate self-compatibility were excluded from the analyses. For each isolate, an *niaD*, an *nirA*, and, when available, a *cnx* were selected as testers for pairing with new *nit* mutants. To establish VCGs, testers were paired in all possible combinations. As each group of new *nit* mutants was generated, they were paired against all testers for VCGs recognized at that time. All positive reactions were repeated for verification.

Sclerotial production. For sclerotia production, plates (60 by 15 mm) containing Cz were inoculated with mycelia. Cultures were incubated in darkness at 30°C for 14 days (23). Sclerotia were obtained by scraping the surface of the plate (three replicate plates per isolate) over a no. 2 Whatman filter paper during irrigation with water containing Tween 20 (100 µl/liter), followed by rinsing with running tap water. Sclerotia were further cleaned in a beaker with repeated rinses and decanting, and later were air dried.

When an isolate, formerly identified as a sclerotia producer, did not produce any sclerotia under the culture conditions of this study, we cultured it in the same media used for the identification, malt extract

Table 1. Characterization of *Aspergillus flavus* and *A. parasiticus* isolates on the basis of mycotoxin production combination

Species, category	Aflatoxin B	Aflatoxin G	CPA ^z
<i>A. flavus</i>			
I	+	-	+
II	+	+	+
III	+	-	-
IV	-	-	+
<i>A. parasiticus</i>			
V	+	+	-

^z Cyclopiazonic acid production.

Table 2. *Aspergillus flavus* and *A. parasiticus* vegetative compatibility group (VCG) and isolate characteristics^a

Species, culture ^v	VCG	Category	Sclerotia mean ^w	Sclerotium diameter (µm) ± SD ^x	Strain isolate ^y
<i>A. flavus</i>					
M35N1A	A	IV	None	None	...
M34N2	A	IV	None	None	...
M15N3	A	I	None	None	...
M11N1/544 ^z	A	IV	None	None	...
M11N1X	A	IV	None	None	...
M12N1	B	I	15.78 a	435 ± 103 e	L
M6N1/117 ^z	B	I	20.4 a	345.23 ± 80.99 d	L
M32N1	B	I	17 a	640.88 ± 189.94 g	L
M38N1	B	I	6.6 a	453.44 ± 114.07 e	L
M29N1	B	I	7.1 a	423 ± 97.8 e	L
M32N4/490 ^z	C	II	207.8 c	297.8 ± 70.9 c	S
M38N3/657 ^z	C	II	242.6 d	215.71 ± 76.7 a	S
M35N2/589 ^z	C	II	230.3 cd	282.4 ± 53.4 bc	S
M36N1/294 ^z	D	I	2 a	636.2 ± 153.9 g	L
M3N1X	D	I	11 a	520.8 ± 242.14 f	L
M22N3/172 ^z	E	IV	134.8 b	258 ± 65.9 b	S
M36N2	E	IV	155.3 b	299 ± 70.03 c	S
M2N1	F	I	14.8	552.6 ± 113.2	L
M10A	G	I	10.4	580.2 ± 128.67	L
M10N3	H	I	11.3	584.4 ± 128.4	L
M11N3	I	I	None	None	...
M14N1	J	I	None	None	...
M14N2	K	I	None	None	...
M29N3	L	III	None	None	...
M40N1	M	IV	201.1	265.11 ± 73.2	S
<i>A. parasiticus</i>					
M15N1	N	V
M15N2/611 ^z	N	V
M3N1/617 ^z	O	V
M12N3	O	V
M25N1	P	V
M25N3	Q	V

^a None = no sclerotia were produced. Only VCGs with two or more isolates were statistically analyzed. Means in a column not sharing a common letter are significantly different ($P < 0.05$) according to a least significant difference test.

^v *Aspergillus* sp. and culture designation.

^w Mean per square centimeter, based on three square centimeters, chosen randomly, from each of three replicate plates per isolate on Czapek-Dox.

^x Means based on 26 sclerotia, chosen randomly, from each of three replicates plates per isolate.

^y Strain S isolates produce sclerotia that average <400 µm in diameter; strain L isolates produce sclerotia >400 µm in diameter.

^z BAFC cult: Culture Collection number, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.

agar (MEA). We also incubated this isolate on peanut seed previously autoclaved with distilled water. If all these procedures failed, we considered that this isolate had lost its ability to produce sclerotia.

Sclerotial size and number. To estimate diameters and number of sclerotia, three replicate observations were made per isolate using a gridline. A grid of lines was marked on the bottom of a 6-by-15-mm dish to form squares of 1 cm² each. Samples were observed under a stereomicroscope. To assess diameters, sclerotia were spread out on the petri dish and measurements of 26 sclerotia per replicate, which intersected a line that had been previously chosen randomly, were recorded. It was assumed that sclerotium shape was approximately a prolate spheroid. Numbers of sclerotia were enumerated by counting three squares of 1 cm² each, chosen randomly, from each replicate.

Data analysis. Only VCGs with two or more isolates were statistically analyzed. Sclerotia data (number and diameter) were analyzed using an unbalanced hierarchically two level nested analysis of variance design, followed by least significant difference mean separation tests ($P = 0.05$). The software used was the Statistical Package BIOM (F. James Rohlf) 1982.

RESULTS

***nit* Mutants.** Most isolates formed two or three complementary mutants. When we replaced KClO₃ with glutamate to increase the frequency of *nirA* mutants, we did not obtain the expected results. Out of 39 isolates, from which we recovered at least two types of *nit* mutants, 31 were self-compatible.

We obtained many *niaD* mutants, one or two *nirA* of each isolate, and we also recovered one *cnx* in seven of these isolates. Isolates were paired according to complementation tests to study self-compatibility. As previously mentioned, compatibility was identified by a line of wild-type growth at the zone of interaction. A positive reaction between two different mutants types of isolate M15N2 is shown in Figure 1A. Most of the isolate pairings failed to generate the heterokaryon; therefore, we chose only the *nit* mutants that showed a positive reaction as testers of the isolates.

In some cases, we noticed that pairings previously defined as positive turned out negatively and that it was coincident with a phenotype change in one of the testers, expressed as an increase in sporulation and aerial mycelia.

VCGs. Complementation tests revealed 13 VCGs among the 32 *A. flavus* isolates named A to M. The largest VCGs (A and B) included five isolates each, one included three (C), four VCGs had two isolates (D and E), and the last eight were represented by a single isolate (F-M; Table 2). *A. parasiticus* isolates grouped into four VCGs, two composed by two isolates each

(N and O) and two represented by single isolates (P and Q; Table 2). All isolates that would be expected to complement with each other, based on interactions with a third isolate, did complement; however, we did test all possible combinations. None of the VCGs included isolates of the two species used in this study, *A. flavus* and *A. parasiticus*.

Eight isolates were self-incompatible; therefore, they were excluded from the analyses. The negative reaction between isolates M35N1B (self-incompatible) and M11N1 is shown in Figure 1B.

Some pairs of isolates produced positive and negative reactions in different combinations of *nit* mutants.

All of the isolates grouped in the same VCG produced the same combination of mycotoxins and sclerotia production, with the exception of M15N3, which differed in the combination of mycotoxins (Table 2).

As previously mentioned, atypical isolates M11N2 and M5N9 were excluded from the analyses. The other three isolates were vegetatively compatible with each other and vegetatively incompatible with the rest of the isolates, so we placed them in the same VCG (C).

We determined VCG diversity of *A. flavus*, expressed as the number of groups divided by the total number of isolates (22), obtaining an index of 0.52.

Most isolates placed in the same VCG had been collected from different fields and those fields were distant from each other.

Sclerotial production. In a previous study (36), 22 isolates of *A. flavus* were identified as sclerotial producers. All isolates produced sclerotia under our culture conditions, except for isolate M4N1. When we cultured this isolate on MEA and on peanut seed, no sclerotia were produced.

All isolates produced some sclerotia with diameters >400 μm; therefore, they could be classified as L strain isolates by the Cotty criterion (10). However, the average was <400 μm in some isolates (Table 2).

Analyses of variance showed significant differences among VCGs ($P < 0.0001$) in number of sclerotia per square centimeter and in sclerotia size. However, isolates within a VCG were significantly different in sclerotia size ($P > 0.0001$) but not in number of sclerotia per square centimeter ($P = 0.334$) (Table 2).

DISCUSSION

In the present study, *A. flavus* VCG diversity from peanut seed and its correlation with mycotoxin and sclerotia production was determined for the first time in Argentina and in South America.

As expected, none of the VCGs included isolates of the two species studied. As has been stated (22), although *A. flavus* and *A. parasiticus* are closely related based on their DNA complementation (28,29), the lack of any vegetative compatibility between these species suggests that they are genetically isolated from each other.

We obtained approximately 90% of *niaD*, 8% of *nirA*, and 2% of *cnx* mutants. This proportion of different mutant types matches results obtained in other studies. The relationship found by Horn and Greene (22) for the three mutants types was *niaD* > *nirA* > *cnx*. Bayman and Cotty (2) detected 80 to 90% of *niaD*, 10% of *nirA*, and 10% of *cnx* mutants. Most mutants of the same isolate failed to complement with each other; also, some pairs of isolates produced a positive and negative reaction in different combinations of *nit* mutants. These results are similar to those of Brooker et al. (6), who observed that each isolate also sectoried mutants that were unable to complement with any of the other mutants derived from the same isolate. In addition, some mutants could complement with only some of the other compatible isolates. Results obtained in *nirA* mutants of three isolates (*data not shown*) indicate that these mutants lack the ability to form heterokaryons. It had been suggested that the use of mutants could introduce the possibility of a negative pairing reaction as a result of other noncomple-

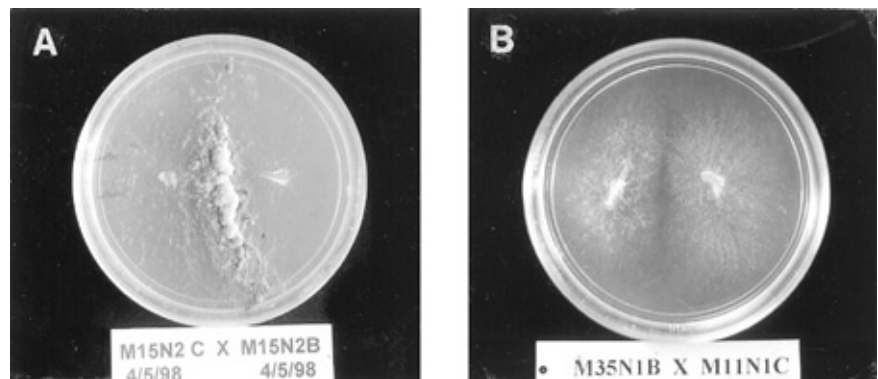


Fig. 1. Complementation among nitrate-nonutilizing (*nit*) mutants of *Aspergillus flavus*. As a result of complementation, dense hyphal growth occurred between two different *nit* mutants of **A**, M15 N1 isolate (self-compatible isolate), and **B**, no complementation is observed between M35N1B and M11N1 isolates.

mentary mutations (20). Therefore, we paired the isolates in all possible combinations with each other to avoid a false negative.

Some isolates were self-incompatible and, therefore, could not be assigned to any VCG. Heterokaryon self-incompatibility (HSI) isolates already have been noticed in *A. flavus* (22,32) and also in other species, such as *Fusarium oxysporum* (8,25), *F. moniliforme* (9), *Colletotrichum* spp. (6), *Rhizoctonia solani* (24), *Verticillium albo-atrum* (7), and *V. lecanii* (27). The explanation of this phenomenon is still uncertain (9,32).

The high *A. flavus* diversity index that we obtained (0.52), agrees with the results of other studies (1,22). Horn et al. (23) proposed that the high VCG diversity of *A. flavus* in corn (32), cottonseed (1), and peanut (22) is similar to nonpathogenic soil isolates of *F. oxysporum* (20). In contrast, many populations of *F. oxysporum* pathogenic to plants have low VCG diversities due to the selective advantage of some VCGs in infecting a specific host (5,30). Therefore, they suggested that none of the *A. flavus* VCGs from agricultural produce is selectively adapted for invading crops under field condition.

We found that VCGs were strongly associated with mycotoxin and sclerotia production. Vegetatively compatible isolates shared the same mycotoxin combination and number of sclerotia per square centimeter of production. The only exception is isolate M15N3, which differed in mycotoxin production from the other isolates of the VCG A. It seems that there is a stronger association between belonging to a certain VCG and sclerotia production rather than mycotoxin production. VCGs already had been associated with many morphological and physiological features. However, association between mycotoxin combination and sclerotia production among isolates belonging to the same VCG is found for the first time. Papa (32) found no apparent relationship between VCGs and aflatoxin or sclerotium production. Bayman and Cotty (3) detected that, with the exception of one VCG, groups contained either S or L isolates. They also found significant differences in aflatoxin B₁ production among VCGs, but they did not attempt to study aflatoxin G or CPA. Horn et al. (23) found positive correlation between total aflatoxin (B₁ and B₂) and CPA in *A. flavus*. The number of sclerotia per plate was negatively correlated with kojic acid production and also detected that VCGs contained either S or L isolates.

Cotty (10) considered that differentiating strains by the occurrence of sclerotia is ambiguous and proposed that sclerotia size is important as a physiological trait. He stated that "isolates producing numerous small sclerotia were designated strain S ('small') isolates...isolates with any sclerotia greater than 400 µm in diameter were

designated strain L ('large') isolates". According to his criterion, all isolates used in our study belong to L strain. However, we observed substantial differences in sclerotia number and size of isolates between VCGs, particularly isolates included in VCGs C and E and the isolate M40N1, which also produced very small sclerotia and in larger quantities. We believe that these five isolates could correspond to the S strain, although all of them produced some sclerotia over 400 µm in size (Table 2). The statistical analysis showed significant differences between isolates within the same VCG in sclerotia size but not in number of sclerotia per square centimeter. This suggests a better correlation between VCGs and the number of sclerotia than between VCGs and sclerotia size. Perhaps it would be better to consider the average size instead of the size of single sclerotia. Finally, we propose that it is not enough to differentiate S and L strain types by merely using sclerotia size, but that it is necessary to define a combination of traits that could take into account most of the diversity found.

Genetic diversity of a fungal population may be of major importance in developing a biocontrol strategy. Tran-Dinh et al. (35) suggested that, if genetic exchange can occur, the introduction of a more aggressive nontoxic isolate as a biocontrol agent could result in a recombinant with both increased aggressiveness and toxicity. In the present study, we found that aflatoxigenic and nonaflatoxigenic isolates grouped in different VCGs. Heterokaryon formation can only occur between vegetatively compatible isolates; therefore, these results enable the implementation of a biological control using a nonaflatoxigenic isolate belonging to a VCG different from the aflatoxigenic ones.

In populations of *Aspergillus* spp., each VCG may be a single clone; therefore, VCGs would be series of clones that were derived from a common ancestor (14). This idea of clonality has been supported by comparison of VCGs and restriction fragment length polymorphism of the mitochondrial genome (13) and analyses of random amplified polymorphic DNA (3). Information about the close relationships between isolates belonging to the same VCG is continuously increasing. In the present study, VCGs corresponded clearly with mycotoxin combination and sclerotia production by the isolates. Atypical isolates, simultaneously producing aflatoxins B, G, and CPA, grouped in a single VCG, indicating that the particular mycotoxin combination produced by these isolates could have arisen once in the region studied. It is necessary to continue the study of these atypical isolates, implementing new techniques to complement the information we have compiled. It would be of interest to study compatibility between our atypical isolates and those cited by Blaney et al. (4)

from Australia. If these sets of isolates prove to be compatible with each other, we could propose a common origin, probably in the Southern Hemisphere.

The search for and study of new *A. flavus* isolates in Cordoba and other provinces is currently being conducted.

ACKNOWLEDGMENTS

We thank G. Vaamonde and others for making available the *Aspergillus flavus* and *A. parasiticus* isolates; J. E. Wright and G. Vaamonde for the critical review of the manuscript; and J. Calcagno for statistical advice.

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