

Distribution of *Aspergillus* Section *Flavi* among Field Soils from the Four Agroecological Zones of the Republic of Bénin, West Africa

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ABSTRACT

Cardwell, K. F., and Cotty, P. J. 2002. Distribution of *Aspergillus* section *Flavi* among field soils from the four agroecological zones of the Republic of Bénin, West Africa. *Plant Dis.* 86:434-439.

Certain members of *Aspergillus* section *Flavi* produce carcinogenic and immunotoxic metabolites called aflatoxins. These fungi perennate in soils and infect maize grain in the field and in storage. The distribution of *Aspergillus* section *Flavi* across the four different agroecologies of Bénin Republic was determined. The four agroecological zones range from humid equatorial tropics in the south to the dry savanna near the Sahara desert in the north. Soil samples collected in 1994 to 1996 from 44 different maize fields in Bénin were assayed over 3 years (88 samples total) for fungi in *Aspergillus* section *Flavi*. All soils tested contained *A. flavus*. Isolates (1,454 total) were collected by dilution plate from the soils and existed in populations ranging from <10 to >200 CFU/g of soil. CFU counts did not differ from year to year or change significantly with cropping systems within a zone, but differed significantly among zones. Incidence of *A. flavus* strain isolations varied from south to north, with greater number of CFU of L strain isolates in southern latitudes and higher numbers of CFU of S strain isolates found in the north. The L strain isolates occurred in 81 of 88 samples, whereas S strain isolates were in only 41 of 88 soil samples. Of 96 L strain isolates tested, 44% produced aflatoxins. Only B toxins were produced, and toxigenic isolates averaged over 100 µg of aflatoxin B₁ per 70 ml of fermentation medium (~1.4 ppm). All S strain isolates produced both B and G aflatoxins, averaging over 557 µg of aflatoxin B₁ per 70 ml (8 ppm) and 197 µg of aflatoxin G₁ per 70 ml of fermentation medium (2.8 ppm). *A. parasiticus* and *A. tamarii* were present in less than 10% of the fields and were not associated with any particular agroecological zone.

Maize has been adopted as a primary staple human food in Africa. In parts of West Africa, it is consumed up to three times a day and is used as weaning food for babies (1,21). Maize is vulnerable to degradation by mycotoxic fungi, but in the developing world and in sub-Saharan Africa in particular, official monitoring of mycotoxin contamination levels is rare. In West Africa, 98% of people test serologically positive for aflatoxin exposure (32), and in Bénin Republic and Nigeria high concentrations of aflatoxins have been found in pre- and postharvest maize (15,27,30,31). Aflatoxin is one of the most potent natural toxins that people may consume without knowledge of potential health impacts (2,4).

All aflatoxin producing fungi are contained within *Aspergillus* section *Flavi*. Fungal isolates within section *Flavi* vary widely in aflatoxin producing ability (10,13,17). Interest in this variability has increased because of recent suggestions that atoxigenic strains of *A. flavus* and *A. parasiticus* might be applied to agricultural fields in order to reduce the risk of aflatoxin contamination (5,9,12). The species most commonly implicated in contamination events, *A. flavus*, can be divided into two strains on the basis of morphological, physiological, and genetic criteria (3,7). Typical or L strain isolates vary widely in aflatoxin producing ability, and a significant percent of L strain isolates are atoxigenic (produce no aflatoxins). S strain isolates have a tendency to produce greater quantities of smaller sclerotia than L strain isolates. S strain isolates also produce more aflatoxin than L strain isolates, and atoxigenic S strain isolates are rare (7,10,17). The S strain has been further divided into S_B isolates that produce only B aflatoxins and S_{BG} isolates that produce both B and G aflatoxins (11,25). Initial comparison of S strain isolates from West Africa and North America revealed that

only S_B isolates occurred in North America, whereas in West Africa only S_{BG} occurred (11,16). Description of the variability within *Aspergillus* section *Flavi* across the distinct agroecological zones of West Africa would facilitate efforts to define the etiology of aflatoxin contamination in that region. The current study looks at the distribution and toxigenicity of species and strains within *Aspergillus* section *Flavi* across four agroecological zones in Bénin Republic.

MATERIALS AND METHODS

Survey methods. Surveys were conducted from 1994 to 1996 to evaluate distribution of *Aspergillus* section *Flavi* in cultivated soils immediately prior to harvest in Bénin Republic. Samples were collected from each of the four agroecological zones of Bénin (Fig. 1). The coastal savanna (CS) (south of 7.5° latitude) has two growing seasons (April to July and September to November), average rainfall between 1,300 and 1,500 mm, and mean temperatures from 25 and 35°C. The southern Guinea savanna (SGS) (between 7.5 and 8.5° latitude) has the same bimodal seasonal pattern as the CS, rainfall ranging from 1,000 to 1,300 mm, and temperature maxima from 26 to 38°C. The northern Guinea savanna (NGS) (8.5 to 10.5° latitude) is characterized by one growing season (April to September), average rainfall of 1,000 to 1,100 mm, and temperature maxima varying from 28 to 40°C. The northernmost Sudan savanna (SS) also has one growing season (May to September), rainfall between 900 and 1,000 mm, and temperature maxima of 28 to 45°C (Fig. 1). Thus, rains decrease and temperatures increase with increasing latitude with CS southernmost, followed by SGS, then NGS, and SS northernmost. Cropping systems were recorded in each field (Table 1).

Maize fields were selected arbitrarily in each agroecological zone by the criteria that the maize had cobs (dough stage or older) and that they were no closer than 5 km from the previous field. Soils were collected from 40 fields in 1994, 22 fields in 1995, and 26 fields in 1996. Across the years, 22 fields were sampled more than once to assess the fungal population over time relative to the cropping system. A

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Accepted for publication 21 September 2001.

Publication no. D-2002-0215-01R

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total of 22 fields were sampled from the CS, 29 from the SGS, 22 from the NGS, and 15 from the SS.

In each field, five subsamples were randomly taken within each of four quadrants with a probe to a depth of 23 to 30 cm, and the 20 subsamples were pooled, oven-dried for 5 days at 40°C, and stored in sealed plastic bags at room temperature (22 to 26°C) until used. Soil pH was measured in H₂O (20% soil, wt/vol).

Strain isolation and CFU quantitation. CFU were estimated by dilution plate technique on Modified Rose Bengal Agar (MRBA), a selective medium for *Aspergillus* section Flavi (8). *Aspergillus* section Flavi colonies were identified by characteristic growth pattern, retention of rose Bengal in mycelia, and production of characteristic conidiophores after 3 days on MRBA (8). All *Aspergillus* section Flavi colonies were transferred to 5/2 agar (5% V8 juice and 2% agar, pH 5.2). After 5 days incubation (unilluminated, 31°C) on 5/2 agar, isolates were classified into species and strain by observing colony characteristics and, when necessary, with microscopic verification of conidial morphology as previously described (8). Several 3-mm plugs of sporulating culture were transferred to 4-dram vials containing 5 ml of sterile distilled H₂O. These conidial suspensions were stored for further study at 8°C.

Aflatoxin production from soil isolates. In order to determine relative frequency of toxic to nontoxic strain distribution across the country, a subset of 2 to 3 L strain isolates was selected randomly from each soil sample in 1994. Aflatoxin production of 96 of these L strain isolates was measured in the liquid medium of Adye and Matales (A&M), as previously described (8,20). To assess variance in toxin production among strains, aflatoxin production by 10 additional L strain isolates was compared with 10 S strain isolates in three replicates of fermentation per isolate in modified A&M medium. A pair of one L strain and one S strain isolate was randomly selected from the isolate conidial suspensions from each of 10 fields. In order to detect the S_{BG} phenotype, fermentations were performed in A&M medium containing 22.5 mM urea as the sole nitrogen source (11). Fermentations were replicated three times. Erlenmeyer flasks (250 ml) containing 70 ml of medium were inoculated with 100 µl of conidial suspension (5,000 to 7,000 conidia). Flasks were incubated at 30°C in the dark on an orbital shaker (150 rpm) for 5 days, after which 70 ml of acetone was added to each flask to lyse fungal cells and extract the aflatoxins from the mycelium.

Culture filtrates containing 50% acetone (vol/vol) were filtered through Whatman No. 4 filter paper. One hundred milliliters of filtrate was added with an equal volume of water to a 250-ml separatory funnel, and

the solution was extracted twice with 25 ml of methylene chloride. Extracts were filtered through 50 g of anhydrous sodium sulfate to remove residual water, and the sodium sulfate was rinsed with an additional 25 ml of methylene chloride after filtration. The methylene chloride fractions were combined and dried in a fume hood. The residue was redissolved in 4 ml of methylene chloride and either diluted or concentrated to allow accurate densitometry. Extracts and aflatoxin standards were separated on thin-layer chromatography

plates (silica gel 60, 250 mm) by development with diethyl ether-methanol-water (96:3:1) (29). Aflatoxins were quantified using a scanning densitometer (model cs-930, Shimadzu Scientific Instruments, Inc., Tokyo) (24).

Data analysis. All analyses were done with SAS version 2000, SAS Institute, Cary, NC. Pearson correlation coefficients were generated to assess relationships of ecological and biological variables. A general linear models (GLM) procedure was used to analyze variance of the main and

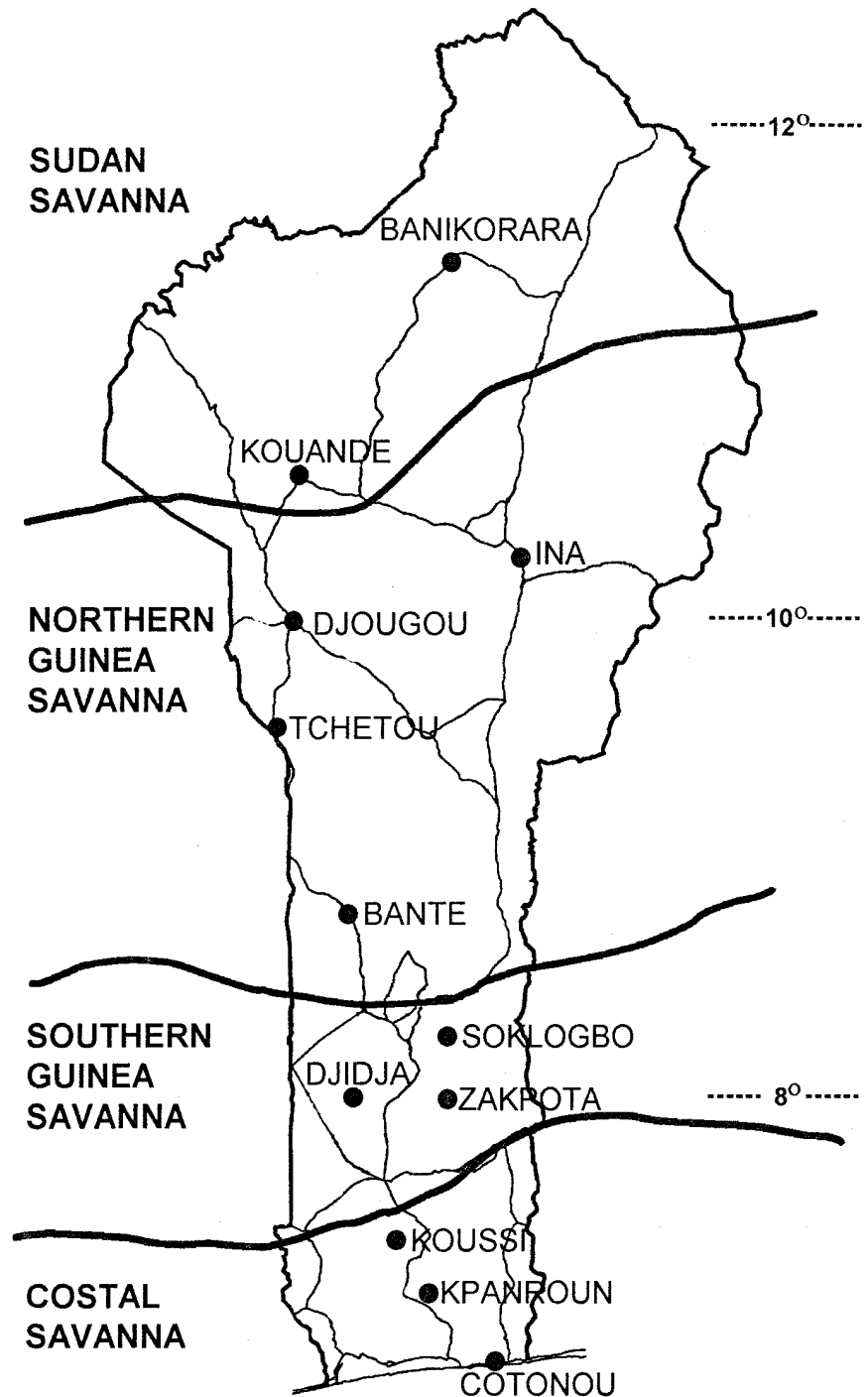


Fig. 1. Map of Bénin Republic with agroecological zones and villages where soil samples were collected.

interactive effects of location and crop system over time on CFU of *A. flavus*, and least significant differences (LSD) means analyses were performed on log₁₀ (count +1) and arcsine square root (%) transformed data. Zones and crop systems were treated as class variables.

RESULTS

Aspergillus section Flavi was detected in soil from each of the 88 field samples. *A. flavus* was predominant (90% of section Flavi), followed by *A. tamarii* (7%) and *A. parasiticus* (2%). One percent of the iso-

lates could not be assigned to a previously described species. A total of 1,454 section Flavi colonies were transferred from MRBA to 5/2 agar. Of the *A. flavus*, 25% were S strain isolates. Although *A. flavus* was detected in all 88 fields, L strain isolates were detected in all but 7, and S strain isolates were found in only 41 fields. Of 96 L strain isolates tested, 42 were positive for aflatoxin B₁ production, with the B₁-positive isolates producing just over 100 µg per 70 ml of fermentation medium (1.4 ppm) (data not shown). No L strain isolate produced aflatoxin G₁. Five *A. parasiticus*

isolates were tested for aflatoxin production in liquid medium, and all produced over 1,000 µg of aflatoxin B₁ (14.3 ppm) and over 150 µg of aflatoxin G₁ per 70 ml of fermentation medium (2.1 ppm) (data not shown). All 10 S strain isolates tested produced both B and G aflatoxins averaging over 557 µg of aflatoxin B₁ (8.0 ppm) and 197 µg of aflatoxin G₁ per 70 ml of fermentation medium (2.9 ppm) (Table 1). Of the 10 L strain isolates tested in triplicate, no G aflatoxins were produced and only 50% produced aflatoxin B₁. The average B₁ aflatoxin produced by L strain iso-

Table 1. Aflatoxin^x production (µg per 70 ml of fermentation medium) by selected isolates of *Aspergillus flavus* S and L strains in liquid fermentation

Isolate ^y	Strain	B ₁	G ₁	ISOLATE	Strain	B ₁	G ₁
BN005K	S	149.05	103.90	BN005F	L	24.06	0.00
BN027S	S	434.20	87.02	BN027A	L	399.00	0.00
BN031L	S	25.67	12.90	BN031R	L	0.00	0.00
BN033L	S	83.41	39.06	BN033A	L	0.00	0.00
BN035E	S	132.08	75.53	BN035I	L	0.00	0.00
BN039A	S	1,443.36	496.63	BN039B	L	164.72	0.00
BN047C	S	981.38	758.66	BN047A	L	0.00	0.00
BN053G	S	34.86	24.61	BN053N	L	90.00	0.00
BN059A	S	1,821.75	222.70	BN059K	L	0.00	0.00
BN066G	S	458.72	149.26	BN066A	L	36.97	0.00
Mean ^z	S	556.45 a	197.03 a		L	62.48 b	0.00 b

^x Means of three replications.

^y Paired isolate accessions were selected for demonstration purposes only. Isolate identification, BN = Bénin, 000-060 refers to field number, letters A to T refer to isolate within field.

^z LSD mean comparison at $\alpha = 0.5$ compares aflatoxin B₁ S versus L strains and aflatoxin G₁ S versus L strains. Mean quantities of both aflatoxin B₁ and G₁ produced by S strain isolates differ significantly from those produced by L strain isolates.

Table 2. Cropping system, soil pH, and CFU per g of soil of *Aspergillus* section Flavi of fields near selected villages across four ecozones in Bénin during 1994 to 1996

Village	Crop system	Year	Zone ^a	pH	% <i>A. flavus</i>	CFU/g ^b
Kouande	Maize + cowpea	1994	SS	6.5	100	11
	Maize + sorghum	1995			100	91
Banikora	Maize sole	1994	SS	7.2	100	15
	Maize + cotton	1995			100	6
Glazoue	Maize + cowpea	1996	NGS	7.2	40	32
	Maize + groundnut	1995			87	33
	Cotton	1996			100	140
Banté	Maize sole	1995	NGS	6.8	100	16
	Maize + cotton	1996			95	48
Guessou Sud	Maize	1995	NGS	5.7	71	15
	Maize + sorghum	1996			100	77
Ina	Maize	1995	NGS	6.4	100	184
	Maize	1996			100	56
Tchetou	Maize	1995	NGS	6.4	100	1,262
	Soybean	1996			100	61
Djidja(1)	Maize sole	1994	SGS	7.5	86	111
	Maize + groundnut	1995			91	50
	Maize + cowpea	1996			100	15
Soklogbo	Maize + cotton	1995	SGS	6.5	93	19
	Maize + cassava	1996			95	123
Zakpota	Maize + groundnut	1994	SGS	7.5	95	320
	Maize	1995			91	190
Houngomè	Maize + groundnut	1994	SGS	6.6	100	230
	Maize	1995			100	75
	Maize + cowpea	1996			100	39
Koussi	Maize + groundnut	1995	CS	6.9	100	7,868
	Maize + cassava	1996			92	61
Kpanroun	Maize + cassava	1994	CS	7.2	100	82
	Maize + cassava	1995			100	128
	Maize + cassava	1996			100	101
Avegame	Maize + cassava	1994	CS	7.8	95	4,271
	Maize sole	1995			100	113
	Maize sole	1996			95	203

^y From north to south: SS = Sudan, NGS = northern Guinea, SGS = southern Guinea, and CS = coastal savannas.

^z Log₁₀(CFU) used in analysis, untransformed data presented. LSD means analysis revealed no differences between crop systems.

lates, 62.5 µg per 70 ml (0.9 ppm), was significantly lower than the production by the S strain isolates (Table 1).

Significant differences among years were not seen in the quantity of propagules (CFU) per gram of soil or incidence of particular section Flavi species (Tables 2 and 3). Mean CFU, percent *A. flavus* and percent *A. parasiticus* were not significantly correlated with either zone or crop system (Table 2). However, incidence of S strain isolates differed significantly among zones, and incidence of *A. tamarii* differed significantly among both zones and crop systems (Table 3).

Maize was found intercropped (grown simultaneously in the same field) with several other crops. Maize with cassava was predominant in the south (Coastal Savanna), maize with groundnut or cotton was more frequent in the Guinea savannas, and maize with cotton, sorghum, or cow-

pea was common in the Sudan savanna (Table 2). Fields having intercropped maize with cotton, groundnut, and/or sorghum had significantly higher CFU counts of *A. tamarii* (n = 5, data not shown), resulting in a significant *F* value in a GLM analysis of variance for crop system effect on the quantity of *A. tamarii* in soils (Table 3) that could not be explained by effect of agroecological zone (Table 4). There was no apparent effect of crop system on *A. flavus* or *A. parasiticus* counts when differences due to zone were accounted for (crop system analyzed as a nested variable within zone).

Soil pH decreased significantly from south to north in Bénin, ranging from an average of 6.98 in the CS to 6.55 in the SS (Table 4). Mean CFU of *A. flavus* per gram of soil was extremely variable, ranging from 6 to 28,000. CFU counts were significantly higher in the SGS than the SS

(Table 4). *A. flavus*, *A. parasiticus*, and *A. tamarii* were found distributed across all agroecological zones, but none were significantly associated with any particular zone (Table 4). The S strain isolates of *A. flavus* were significantly more frequent in northern latitudes, while L strain isolates were significantly more frequent in southern latitudes (Tables 4 and 5). Incidence of the S strain was negatively correlated, and incidence of the L strain was positively correlated, with soil pH. However, this was not a strong effect and was confounded with latitude (Table 5).

DISCUSSION

Aspergillus section Flavi is widely distributed across Bénin. Studies from Israel and North America show similar frequencies of *A. flavus*, and in those regions *A. parasiticus* occurs in frequencies similar to those found in the current study

Table 3. Effect of crop system (cropps), agroecological zone, and year on mean CFU per g of soil^y, and percent *Aspergillus* section Flavi^z group isolates in soils across Bénin

	df	Mean CFU		% <i>A. flavus</i>		% S strain		% <i>A. parasiticus</i>		% <i>A. tamarii</i>	
		F value	P > F	F value	P > F	F value	P > F	F value	P > F	F value	P > F
Year	2	0.04	0.84	3.43	0.07	3.75	0.06	0.01	0.92	2.28	0.14
Zone	3	0.47	0.70	1.52	0.93	8.94	0.0001	0.09	0.97	2.78	0.05
Year*zone	4	0.75	0.53	1.45	0.24	2.52	0.07	0.23	0.87	4.66	0.006
Cropps (zone)	18	0.44	0.97	1.52	0.12	0.80	0.70	0.24	0.99	4.39	0.0001
Year*cropps (zone)	3	0.35	0.79	0.27	0.85	2.37	0.08	0.13	0.94	0.07	0.98

^y Mean CFU was log transformed for general linear model (GLM) analysis.

^z Percent data were arcsine square root transformed for GLM analysis.

Table 4. Analysis of differences among agroecological zones for soil pH, CFU of *Aspergillus* section Flavi in soils, percent *Aspergillus* spp., and S and L strain isolates of *A. flavus* (LSD, $\alpha = 0.05$)¹

Zone ^a	N ^v	Rainfall ^w	Temp ^x	pH	CFU ^y	%Af ^z	%At ^z	%Ap ^z	%L ^z	%S ^z
SS	15	900	45°C	6.55 b	65.1 b	91	7	2	31 b	60 c
NGS	21	1,100	40°C	6.82 ab	106.2 ab	92	7	1	47 b	40 b
SGS	29	1,300	38°C	7.22 a	222.9 a	91	6	3	89 a	2 a
CS	22	1,500	35°C	6.98 a	90.2 ab	91	6	3	88 a	3 a
MSE				0.36	0.51	0.07	0.05	0.03	0.13	0.11

¹ All % data were arcsine transformed for analysis; means within a column followed by a different letter are significantly different.

^a CS = coastal, SGS = southern Guinea, NGS = northern Guinea, and SS = Sudan savannas.

^v N = number of fields.

^w Rainfall maxima in cm/year.

^x Temperature maxima in °C/year.

^y CFU = back transformed from log₁₀(mean CFU/zone).

^z %Af = mean % *Aspergillus flavus*; %At = mean % *A. tamarii*; %Ap = mean % *A. parasiticus*; %L = mean % *A. flavus* L strains; and %S = mean % *A. flavus* S strains isolated from the soil community.

Table 5. Correlations^x of percent S and L strain isolates of *Aspergillus flavus*, percent *A. parasiticus* (APAR) and *A. tamarii* (ATAM) in the field^y, total CFU^z isolates per g of soil, relative to soil pH, latitude (LAT), and longitude (LON), N = 79

	Soil pH	LAT	LON	%S	%L	%ATAM	%APAR	CFU
Soil pH	1.00							
LAT	-0.29**	1.00						
LON	-0.05	0.22	1.00					
%S	0.16	0.62***	-0.03	1.00				
%L	-0.22*	-0.57***	-0.05	-0.88***	1.00			
%ATAM	0.15	0.05	-0.03	-0.13	-0.26	1.00		
%APAR	-0.02	-0.09	0.00	-0.09	-0.14	0.01	1.00	
CFU	0.09	-0.19	-0.07	-0.14	0.09	-0.09	0.09	1.00

^x Correlation coefficients significant with $P < 0.001 = ***$, $0.01 \geq P > 0.001 = **$, $0.05 \geq P > 0.01 = *$.

^y All % data were arcsine square root transformed prior to analysis.

^z CFU data were log₁₀ (count+1) transformed.

(10,18,19,26). The aflatoxin producing potential of *Aspergillus* communities is higher when S strain is present, as L strain isolates produce on average only 33% as much toxin as S strain isolates (10). All North American S strain isolates express the S_B phenotype and produce no G aflatoxins (10,17). In West Africa, S strain isolates similarly produce greater quantities of aflatoxins than L strain isolates. By contrast, in the current study and as previously reported (11), the West African S strain isolates all express the S_{BG} phenotype producing both B and G aflatoxins. Certain regions in the United States have high incidences of *A. parasiticus*, and in those regions, contamination of crops with G aflatoxins may be reliably attributed to *A. parasiticus* (10). However, from results of the current study, it is more likely that crop contamination with G aflatoxins in north Bénin is caused by the S strain of *A. flavus*.

The high aflatoxin producing potential of African S strain isolates, along with their dominance within fungal communities in parts of Bénin, suggest this strain may play an important role in contamination of foods in West Africa. In vitro and in vivo inoculation studies have shown that maize can be infected by West African S strain isolates, and both B and G aflatoxins are expressed in maize (6). Nevertheless, it is not known whether the *A. flavus* S strain readily infects maize in the field under normal management conditions. Out of 227 aflatoxin-positive maize samples from Bénin analyzed at IITA in 1993 through 1995, only 7 were positive for G aflatoxins (14), 6 being from the far northern Sudan savanna and 1 being from the northern Guinea savanna. The current survey suggests that these G toxin-containing samples were more likely to have been contaminated, not by *A. parasiticus*, but by S_{BG} isolates of *A. flavus*. The relative virulence of these strains to maize needs to be examined.

The *A. flavus* communities resident in different regions of Bénin differed in strain composition. The L strain isolates, although present throughout Bénin, were more prevalent in the south, while S strain isolates were found more frequently in the north. Thus, this study revealed that in addition to climatic and crop system variables influencing crop contamination, there is a gradient in the inherent toxigenicity of the endemic *Aspergillus* communities also influencing the aflatoxin content of crops in Bénin. Orum et al. (23) disassociated crop sequence from variations in the incidence of S strain isolates in Arizona and postulated that temperature variation has a greater impact on *A. flavus* community composition. In that study, the incidence of S strain isolates peaked during the warmest periods and rapidly declined during cool periods. This agrees with results from the current study, where the S strain isolates

were most prevalent in the agroecological zones bordering the Sahara desert where the highest average temperatures occur. High incidences of S strain isolates are also frequently found in relatively low rainfall, high temperature regions of North America (8,17,22). Production of many small sclerotia may be a survival trait for an organism adapted to rapid and extreme fluctuations in moisture and temperature. Such fluctuations are routine on the edges of deserts.

Geographical divergence among communities of section Flavi has been described in North America (10,17,22), with resident section Flavi communities differing among regions in incidences of both *A. tamarii* and the S strain of *A. flavus*. In North America, S strain isolates were most common in alkali soils and rare in acidic soils. The relationship between soil pH and S strain isolates incidence in Bénin was opposite this and may be an additional reflection of physiological differences between North American and Beninese S strain isolates. Nevertheless, the inverse correlation between frequencies of the L strain isolates and pH was statistically weak and there was no significant correlation of pH with frequency of S strain, while the effect of zone/latitude on both strains was highly significant. Another interpretation is that soil pH may have nothing to do with *A. flavus* population dynamics and that climate is a more important determinant.

The severity of aflatoxin contamination of maize may more closely relate to the aflatoxin producing potential of the resident fungi infecting the crop than to the quantity of infections. This may partly explain the lack of correlation between aflatoxin content and kernel infection in Bénin (15,27,28). Lack of correlation between frequencies of L strain isolates and aflatoxin production in mixed strain infections in cotton has been reported (9).

The climatic gradient across agroecological zones from very wet, 9-month bimodal rainfall distribution in the south to semiarid 3-month rain distribution in the north provides a range of biophysical conditions in which aflatoxin contamination of grain may occur. Nevertheless, the risk of encountering aflatoxins in grain is not directly related to climatic variables (15). Management factors also contribute to risk of contamination, and crop management practices are zone specific (14,15,28,31). Variability in the average aflatoxin producing potential of communities of fungi in *Aspergillus* section Flavi is documented (10) and clearly a factor contributing to variability in aflatoxin contamination of maize across Bénin (15). Different management practices may be required to reduce contamination caused by the various *A. flavus* morphotypes. Understanding factors influencing patterns of *A. flavus* strain composition may help in

the assessment of the risk of aflatoxin contamination on a pan-regional scale.

ACKNOWLEDGMENTS

This work was supported in part by the BMZ (German Ministry of Co-operation), Germany, and by Specific Cooperative Agreement no. 58-6435-7-F054 with the Foreign Agricultural Service, U.S. Department of Agriculture. We thank M. Sétamou, D. Downey, N. Hurban, E. Tedihou, and M. Koubé for technical assistance. This is IITA MS no. 99/021/JA.

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