Hindawi
International Journal of Agronomy
Volume 2018, Article ID 5468602, 7 pages
https://doi.org/10.1155/2018/5468602

Research Article

Identification of Sources of Resistance for Peanut *Aspergillus flavus* Colonization and Aflatoxin Contamination

Richard Moise Alansou Dieme,[1] **Issa Faye**,[2] **Yedomon Ange Bovys Zoclanclounon**,[3] **Daniel Fonceka**,[3] **Ousmane Ndoye**,[4] **and Papa Madiallacke Diedhiou**[1]

[1]*Université Gaston Berger, BP 211 Saint-Louis, Senegal*
[2]*Centre National de Recherches Agronomiques de Bambey, Institut Sénégalais de Recherches Agricoles, BP 211 Bambey, Senegal*
[3]*Centre d'Etudes Régional pour l'Amélioration de l'Adaptation à la Sécheresse, Route de Khombole, BP 3320 Thiès, Senegal*
[4]*Conseil Ouest et centre Africain pour la Recherche et le Développement Agricoles, Avenue Bourguiba, BP 48 Dakar RP, Senegal*

Correspondence should be addressed to Richard Moise Alansou Dieme; alansou7dieme@gmail.com

Received 25 May 2018; Revised 7 August 2018; Accepted 13 September 2018; Published 1 October 2018

Academic Editor: Glaciela Kaschuk

Peanut aflatoxin contamination caused by *Aspergillus flavus* is a serious constraint for food safety and human health in Senegal. The present study aimed to identify sources of resistance for *A. flavus* colonization and aflatoxin contamination. Thus, seeds from 67 peanut genotypes were tested under laboratory conditions. Aqueous conidial suspension of an aflatoxinogenic strain of *A. flavus* was used for inoculation in Petri dishes containing ten seeds of each genotype, and data on incidence and severity were recorded. Total aflatoxin concentration in seeds was determined on 15th day after inoculation using mReader® method. Results showed a significant ($p < 0.001$) variation of aflatoxin, incidence and severity among the tested peanut genotypes. Incidence ranged from 0 to 70% with a mean of 20.36 ± 0.8%. Out of the 67 genotypes, eight showed incidence less than 10%. Severity ranged from 0 to 44% with a mean value of 8.82 ± 0.45%. The genotype 12CS_104 showed aflatoxin concentration level in conformity with the European standard (4 ppb). Out of three clusters revealed by hierarchical classification based on disease incidence and severity, the cluster 1 contained 33 genotypes characterised by low incidence and severity values. These genotypes can be tested under field conditions to confirm their resistance to *A flavus*.

1. Introduction

Peanut (*Arachis hypogaea* L.) is an important staple crop in Senegal. The national peanut production was estimated at 1,050,042 tons during the rainy season of 2016 [1]. This crop is mainly produced in Fatick, Kaolack, Kaffrine, Louga, and Thies regions, with more than 60% of the national peanut production [1]. Peanut seeds are widely used for food consumption and play a significant economic role for small-scale farmers and food industries in Senegal [2]. However, pre- and postharvest aflatoxin contamination in peanut is a serious threat for food safety and human health in Senegal [3]. It is one of the major constraints limiting sustainable and good quality seed production in the world [4]. Aflatoxin contamination is due to *Aspergillus flavus* (Link ex Fries, Teleomorph: *Petromyces flavus*) [5]. Damages caused by this facultative plant pathogen in maize, peanut, and sesame were reported in Senegal [6]. Considerable economic losses caused by this bacterium are mainly due to crop quality value and international trade restrictions on food stuffs charged in aflatoxin [7].

Aflatoxin is the name of a group of toxin known as G1, G2, B1, B2, M1, and M2 that produced the plant pathogen [8]. These toxins occur naturally and have been found in a wide range of commodities, including peanuts used for animal and human consumption [9]. Aflatoxins are toxic, mutagenic, and carcinogenic compounds [10]. Depending on their levels, toxins can severely affect the liver and induce immune-suppressing effects [9].

To handle this issue, a wide range of preharvest aflatoxin contamination management methods were developed. Application of atoxinogenic isolates of *A. flavus* [11] and

host genetic resistance were tested [12]. In Senegal, previous studies reported that varieties 55-437 and 73-3 were resistant to *A. flavus* [13]. Identification of new sources of resistance merits to be investigated for efficient peanut breeding program. First step of host genetic resistance is the seed colonization test. Therefore, the present study was undertaken to identify promising peanut genotypes under laboratory conditions.

2. Materials and Methods

2.1. Plant Materials. The plant material consisted of 67 genotypes including 58 chromosomal substitutions lines [14] and nine national released varieties. The chromosomal substitution lines belong to a cross between Fleur 11 and a synthetic amphidiploid parent (Table 1).

2.2. Isolation of Aspergillus flavus, Sporangial Suspension Preparation, and Inoculation. Aflatoxinogenic strain provided from peanut seeds were purified by successive cultures on 5/2 agar medium. The aflatoxin concentration level was checked using the Reveal® Q^+ Aflatoxin test kit (accesso peanut enterprise corporation, USA). The spore suspension of *A. flavus* was obtained by soaking colonized seeds in 50 ml of sterile distilled water. Then, one drop of Tween 20 was added to the solution and thoroughly mixed for 10 minutes. Inoculation was carried out by introducing 100 μl of the supernatant of the spore suspension into each Petri dish.

2.3. Seed Colonization Test. The seed colonization test was conducted following a modified Mehan and McDonald procedure. For each genotype, 50 seeds were sterilized and rinsed properly in sterile distilled water. Then, the seeds were hydrated to about 20% moisture content. The 50 seeds of each genotype were placed in 5 Petri dishes containing 10 seeds, and each Petri dish was considered as a replication. The seeds were inoculated with a conidial suspension (60 μL containing approximately $1 \times 10^8\,\mathrm{mL}^{-1}$ conidia of the aflatoxigenic strain of *A. flavus*). This preparation was kept at laboratory conditions ($25 \pm 0.12^{\circ}$C and $82 \pm 0.42\%$ relative humidity) for fifteen days.

2.4. Data Collection. The seeds' colonization was observed during two weeks, and aflatoxin concentration was measured using the Reveal® Q^+ Aflatoxin test kit (accesso peanut enterprise corporation, USA). The incidence was calculated using the following formula:

$$\text{incidence}\,(\%) = \frac{\text{number of seeds showing pathogen colonizaton}}{\text{total number of seeds}} \times 100. \quad (1)$$

Table 1: Peanut material used in this study.

No	Genotypes	Description	Country of origin
1	12CS_001	CSL*	Senegal
2	12CS_004	CSL	Senegal
3	12CS_006	CSL	Senegal
4	12CS_007	CSL	Senegal
5	12CS_008	CSL	Senegal
6	12CS_009	CSL	Senegal
7	12CS_010	CSL	Senegal
8	12CS_011	CSL	Senegal
9	12CS_012	CSL	Senegal
10	12CS_016	CSL	Senegal
11	12CS_018	CSL	Senegal
12	12CS_020	CSL	Senegal
13	12CS_021	CSL	Senegal
14	12CS_022	CSL	Senegal
15	12CS_023	CSL	Senegal
16	12CS_024	CSL	Senegal
17	12CS_027	CSL	Senegal
18	12CS_028	CSL	Senegal
19	12CS_031	CSL	Senegal
20	12CS_032	CSL	Senegal
21	12CS_033	CSL	Senegal
22	12CS_034	CSL	Senegal
23	12CS_036	CSL	Senegal
24	12CS_037	CSL	Senegal
25	12CS_039	CSL	Senegal
26	12CS_041	CSL	Senegal
27	12CS_042	CSL	Senegal
28	12CS_047	CSL	Senegal
29	12CS_048	CSL	Senegal
30	12CS_050	CSL	Senegal
31	12CS_051	CSL	Senegal
32	12CS_052	CSL	Senegal
33	12CS_053	CSL	Senegal
34	12CS_054	CSL	Senegal
35	12CS_055	CSL	Senegal
36	12CS_059	CSL	Senegal
37	12CS_060	CSL	Senegal
38	12CS_061	CSL	Senegal
39	12CS_062	CSL	Senegal
40	12CS_063	CSL	Senegal
41	12CS_066	CSL	Senegal
42	12CS_070	CSL	Senegal
43	12CS_072	CSL	Senegal
44	12CS_075	CSL	Senegal
45	12CS_076	CSL	Senegal
46	12CS_078	CSL	Senegal
47	12CS_079	CSL	Senegal
48	12CS_084	CSL	Senegal
49	12CS_085	CSL	Senegal
50	12CS_090	CSL	Senegal
51	12CS_091	CSL	Senegal
52	12CS_095	CSL	Senegal
54	12CS_096	CSL	Senegal
55	12CS_100	CSL	Senegal
56	12CS_111	CSL	Senegal
57	12CS_112	CSL	Senegal
58	12CS_118	CSL	Senegal
59	12CS_119	CSL	Senegal
60	55-33	Variety	Senegal
61	55-437	Resistant control	Senegal

Table 1: Continued.

No	Genotypes	Description	Country of origin
62	73-30	CSL	Senegal
63	73-33	Resistant control	Senegal
64	78-936	Variety	Senegal
65	Fleur11	Susceptible control	Senegal
66	GC-8-35	Variety	Senegal
67	L27	Variety	Senegal

*Chromosomal substitution lines.

The severity scale of aflatoxin on seeds was estimated using a modified Tonapi et al. [15] scale. It was defined as follows: 0, noninfected seeds; 1, seeds whose surface covered by the fungus is less than 20%; 2, 20%–40% seed surface covered by the fungus; 3, 40%–60% seed surface covered by the fungus; 4, 60%–80% seed surface covered by the fungus; and 5, 80%–100% seed surface covered by the fungus. The severity calculation based on Tonapi et al. [15] formula was as follows:

$$\text{severity}\,(\%) = \frac{\sum_{i=1}^{n}(N_i \times i)}{\text{total of seeds} \times (n-1)}, \quad (2)$$

where $p < 0.001$ i is severity scale from 0 to 5 and N_i is the number of seed corresponding to scale i of severity.

2.5. Data Analysis. Data analysis was performed with the open-source statistical software R version 3.4.5 [16]. Descriptive statistics of recorded data were generated with pastecs package [17]. In order to find out variability of incidence and severity according to tested genotypes, data were subjected to Poisson regression analysis using glm (generalized linear model) function of package stats implemented in the R. Spearman's rank correlation test was performed to highlight relationship between incidence, severity, and aflatoxin concentration levels using correlation test function of package stats. Identification of different groups of genotypes based on incidence and severity was performed based on a principal component analysis and a hierarchical clustering with the functions PCA and HCPC of package FactoMineR [18], respectively. The Euclidean distance and Ward classification method were used to classify tested genotypes. The function fviz_pca_biplot [19] was used to plot the principal components analysis biplot in different clusters based on hierarchical classification.

3. Results

3.1. Reaction of Peanut Genotypes to Aspergillus flavus. Analysis of variance revealed highly significant ($p < 0.001$) variation of aflatoxin incidence and severity among the tested peanut genotypes (Table 2).

The severity ranged between 0 and 44%, respectively, with a mean of $8.82 \pm 0.45\%$. The recorded incidence ranged from 0 to 70% with an average value of $20.36 \pm 0.80\%$ (Table 3).

One genotype (12CS_104) showed aflatoxin concentration level less than 4 ppb. A total of 34 genotypes presented aflatoxin concentration level up to 2000 ppb (Figure 1).

Table 2: Deviance values from the Poisson regression model on incidence and severity.

Source of variation	Degree of freedom	Incidence	Severity
Replication	4	33.9 (ns)	2.04 (ns)
Genotype	66	7242.2***	190.08***

***Significant chi-squared test at 0.001 level of probability; ns = not significant.

Out of the 67 genotypes, eight showed incidence less than 10% while 33 showed incidences between 10 and 20% and 16 with incidences ranged from 20 to 30% (Figure 2).

3.2. Correlation between Incidence, Severity, and Aflatoxin Concentration Level. Spearman's rank correlation test revealed a strong relationship ($r = 0.93$, $p < 0.001$) between incidence and severity of peanut genotypes. Positive and significant correlations were detected between aflatoxin concentration levels and disease incidence ($r = 0.28$, $p < 0.01$) and aflatoxin concentration levels and disease severity ($r = 0.35$, $p < 0.05$) (Table 4).

3.3. Classification of the Tested Genotypes according to Sensibility and Aflatoxin Concentration Level. The factorial axes 1 and 2 explained 60.5 and 39.5% of overall variability, respectively (Figure 3). Hierarchical classification performed on principal component analysis revealed three clusters of genotypes based on disease incidence and aflatoxin concentration levels (Figure 3). The clusters 1, 2, and 3 grouped 33, 20, and 14 genotypes, respectively. The incidence and aflatoxin concentration are significantly ($p < 0.001$) associated to cluster 1 (Table 4).

Mean values of these two variables in this cluster are less than the overall mean. Therefore, cluster 1 is characterized by desirable genotypes which combine low incidence values and aflatoxin concentration levels. Cluster 2 is significantly ($p < 0.001$) related to the aflatoxin concentration level (Table 5).

The mean value of aflatoxin concentration in cluster 2 (4075.5 ppb) is 190% which is higher than the overall mean (2143.8 ppb). Thus, this second cluster is characterized by genotypes with high level of aflatoxin. Incidence is linked to cluster 3 (Table 5). Mean value of this variable (35%) in cluster 3 is superior to overall mean (20.35%). Thus, the cluster 3 encompasses the most susceptible genotypes to *A. flavus*.

Based on the closest distance between each genotype and the respective cluster centres, 12CS_039, 12CS_010, and 12CS_050 were the first representative genotypes (paragon) of cluster 1, 2, and 3, respectively (Table 4). Based on the farthest distance from a genotype projected point in a cluster to the centres of the two others, clustering revealed that cluster 1, 2, and 3 were characterised by the genotypes 12CS_104, 78-936, and 12CS_021, respectively (Figure 3, Table 5). Based on results, out of 67 genotypes, 33 promising genotypes (cluster 1) were noted (Figure 3).

Table 3: Means of incidence and severity of the tested lines.

Lines	Incidence		Severity	
	Mean	Std deviation	Mean	Std deviation
12CS_001	44	13.42	1.02	0.44
12CS_004	30	12.25	0.74	0.23
12CS_006	30	15.81	0.46	0.29
12CS_007	18	8.37	0.34	0.21
12CS_008	12	16.43	0.22	0.27
12CS_009	24	8.94	0.58	0.33
12CS_010	18	8.37	0.4	0.27
12CS_011	18	14.83	0.42	0.48
12CS_012	32	13.04	0.82	0.43
12CS_016	34	8.94	0.82	0.54
12CS_018	38	13.04	0.66	0.33
12CS_020	22	10.95	0.5	0.34
12CS_021	52	10.95	1.64	0.49
12CS_022	16	15.17	0.36	0.43
12CS_023	18	13.04	0.28	0.24
12CS_024	28	10.95	0.5	0.21
12CS_027	18	8.37	0.26	0.19
12CS_028	18	8.37	0.3	0.20
12CS_031	16	5.48	0.4	0.28
12CS_032	14	11.40	0.16	0.11
12CS_033	10	12.25	0.2	0.23
12CS_034	14	15.17	0.28	0.34
12CS_036	14	5.48	0.26	0.15
12CS_037	16	5.48	0.32	0.19
12CS_039	14	8.94	0.22	0.23
12CS_041	24	15.17	0.64	0.38
12CS_042	24	18.17	0.42	0.42
12CS_047	18	13.04	0.4	0.22
12CS_048	6	8.94	0.08	0.13
12CS_050	34	11.40	0.76	0.29
12CS_051	34	20.74	0.72	0.36
12CS_052	30	15.81	0.76	0.67
12CS_053	36	15.17	0.98	0.59
12CS_054	26	11.40	0.68	0.44
12CS_055	18	8.37	0.48	0.54
12CS_059	24	16.73	0.48	0.48
12CS_060	12	13.04	0.4	0.39
12CS_061	14	11.40	0.24	0.19
12CS_062	18	8.37	0.26	0.15
12CS_063	18	14.83	0.42	0.53
12CS_066	32	22.80	0.8	0.76
12CS_070	22	19.24	0.56	0.53
12CS_072	14	13.42	0.26	0.28
12CS_075	28	13.04	0.66	0.36
12CS_076	12	4.47	0.18	0.08
12CS_078	10	12.25	0.16	0.18
12CS_079	18	16.43	0.34	0.34
12CS_084	14	5.48	0.3	0.21
12CS_085	22	8.37	0.5	0.32
12CS_090	14	13.42	0.34	0.50
12CS_091	26	11.40	0.64	0.48
12CS_095	12	8.37	0.26	0.27
12CS_096	16	8.94	0.48	0.58
12CS_100	40	12.25	0.96	0.34
12CS_104	6	8.94	0.14	0.26
12CS_111	6	8.94	0.1	0.14
12CS_112	18	16.43	0.26	0.32
12CS_118	20	10.00	0.3	0.20
12CS_119	20	7.07	0.42	0.28
55-33	4	5.48	0.04	0.05
55-437	12	10.95	0.18	0.19
73-30	6	5.48	0.06	0.05
73-33	16	11.40	0.28	0.19
78-936	12	4.47	0.24	0.31
Fleur_11	28	16.43	0.5	0.17
GC-8-35	22	10.95	0.6	0.24
L27	10	0.00	0.16	0.13
Mean		20.36		8.82
Standard error		0.80		0.45

Table 4: Spearman's rank matrix correlation performed on incidence, severity, and aflatoxin concentration levels data.

	Incidence	Severity	Aflatoxin concentration levels
Incidence	1.00		
Severity	0.93***	1.00	
Aflatoxin concentration levels	0.28*	0.35**	1.00

*Significant Spearman's rank correlation test at 0.05 level of probability.
**Significant Spearman's rank correlation test at 0.01 level of probability.
***Significant Spearman's rank correlation test at 0.001 level of probability.

4. Discussion

In the present study, a wide phenotypic variation was observed among the tested genotypes for incidence, severity, and aflatoxin concentrations. This variation can be explained by the variability of seed coat structure of the tested genotypes. In fact, the seed coat can constitute a barrier to *A. flavus* seed invasion depending on its thickness and/or permeability [20], and Zhou and Liang [21] studies showed that genotypes seed coat with smaller hilum, more compact arrangement and thicker testa showed more resistance to *A. flavus*. In addition, implication of wax and cutin layers of seed coat was demonstrated to be related to genotypes resistance [22]. Another explanation of this wide variation in incidence, severity, and aflatoxin rate can be biochemical compounds' differential variability in the tested seeds. Lindsey and Turner [23] demonstrated that the presence of polyphenol compounds, specifically, tannins in seed can have inhibitor effect against *A. flavus*. Amaya et al. [24] and Liang et al. [25] showed the difference among seed coat biochemical compounds to determine sensibility to *A. flavus*. Liang [22] demonstrated that the presence of trypsin in seeds can also be related to resistance to *A. flavus*. Turner et al. [26] isolated and identified the 5,7-dimethoxyisoflavone as an inhibitor for *A. flavus* invasion in peanut seed.

12CS_104 was the most resistant genotype to aflatoxin contamination with an aflatoxin level lower than the European Union standards (4 ppb). However, except 12CS_104, all the genotypes have their aflatoxin concentration level higher than the Chinese (20 ppb) standards.

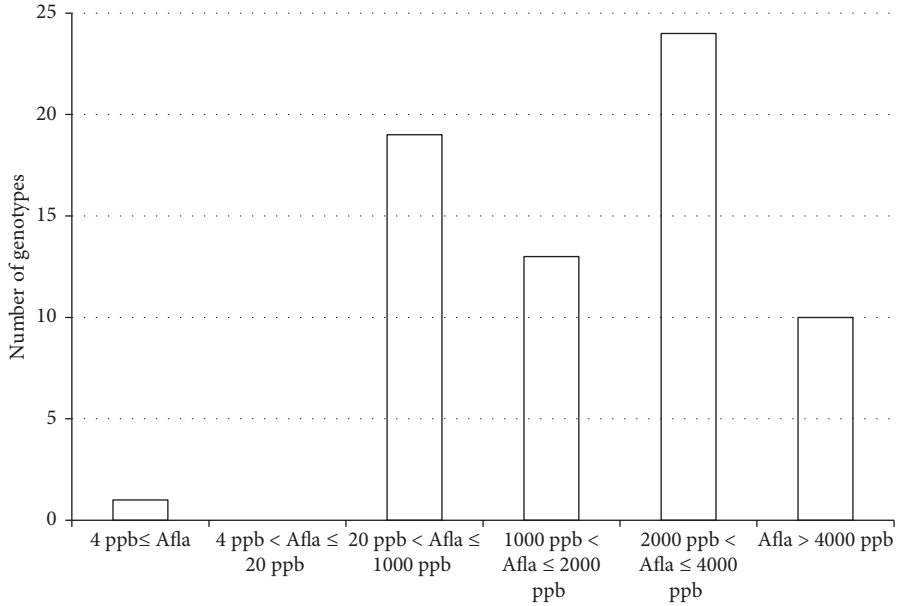

Figure 1: Number of peanut genotypes according to aflatoxin concentration level interval values.

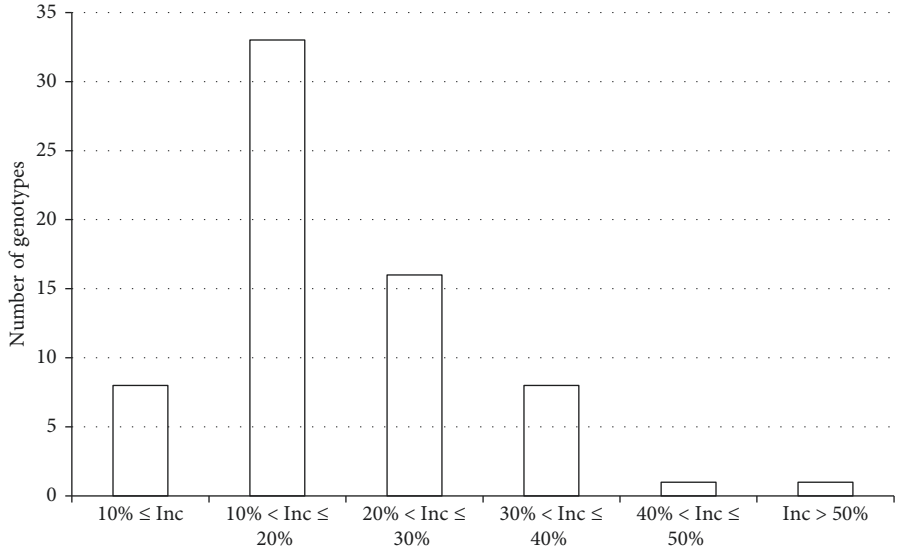

Figure 2: Number of peanut genotypes showing aflatoxin presence according to incidence intervals.

Indeed, the highest aflatoxin concentration level was observed with genotype 78-936. The contrasting genotypes observed in this study can be used as positive and negative checks, respectively, for accurate field experiment. Furthermore, these contrasted genotypes can be used to develop mapping population for genetic study such as inheritance of aflatoxin and identification of quantitative trait loci (QTL). The varieties 55-437 and 73-30 showed incidence less than 15% as reported by the previous study realized 30 years ago by Zambettakis et al. [13], but their aflatoxin concentration levels were largely up to the European Union standards.

The correlation test showed a positive relationship between *A. flavus* colonization and aflatoxin contamination. This confirmed that the presence of *A. flavus* induced aflatoxin production in seeds. Hierarchical classification highlighted three clusters according to incidence, severity, and aflatoxin concentration levels. The relatively low values of incidence observed on the 33 genotypes belonged to cluster 1 should be confirmed under field conditions. These genotypes can be evaluated in different locations on infested fields.

5. Conclusion

This study uncovered that the lines 12CS_104 exhibited low values of incidence and severity. Furthermore, its aflatoxin

Figure 3: Principal component analysis biplot showing peanut genotypes clustering based on their severity and incidence.

Table 5: Description of each cluster based on incidence, aflatoxin concentration levels, and paragon and singular genotype per cluster.

	Cluster 1			Cluster 2			Cluster 3		
	Mean	Overall mean	p value[†]	Mean	Overall mean	p value	Mean	Overall mean	p value
Description of clusters by variables									
Incidence	15.09	20.35	$<10^{-3}$	18.8	20.35	>0.05	35.00	20.35	$<10^{-3}$
Aflatoxin	915.65	2143.89	$<10^{-3}$	4075.50	2143.89	$<10^{-3}$	2279.57	2143.89	>0.05
Description of clusters by distances									
Top 3 representative genotypes[††]	12CS_039 (0.11)	12CS_090 (0.16)	73-33 (0.29)	12CS_010 (0.12)	12CS_079 (0.22)	12CS_027 (0.24)	12CS_050 (0.15)	12CS_066 (0.41)	12CS_016 (0.42)
Top 3 characteristic genotypes[†††]	12CS_104 (2.82)	55-33 (2.75)	12CS_111 (2.74)	78-936 (3.68)	12CS_028 (2.39)	12CS_031 (2.28)	12CS_021 (3.45)	12CS_001 (3.00)	12CS_100 (2.59)

[†]Correlation signification of variables with a cluster. [††] Based on the closest distance between each genotype and the respective cluster centres. [†††] Based on the farthest distance from a genotype projected point in a cluster to the centres of the two others.

concentration level was smaller than standards. This genotype represents a relevant tool for the breeding program for resistance to *A. flavus* as a potentially resistant gene donor.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors sincerely thank the West Africa Agricultural Productivity Program (WAAPP) for financial support and facilities help.

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