Association between *Aspergillus flavus* Colonization and Aflatoxins Production in Immature Grains of Maize Genotypes

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Abstract: *Aspergillus flavus* maize colonization leads to crop contamination by toxic secondary metabolites and carcinogens called aflatoxins (AF); it has negative effects in public health and has caused economic losses in agricultural activities. Eleven genotypes of immature maize grain frequently used in Mexico were inoculated *in vitro* with two indigenous toxigenic strains of *A. flavus*. The size of inoculum, temperature, humidity and presence of other phytopathogens were assessed. Genotypes Popcorn, C-526, Garst 8366, As910 and 30G40 showed resistance to rating of fungal colonization (FC) and AF accumulation, while 3002W, 30R39, Creole, C-922, HV313 and P3028W genotypes were less resistant. AFB1 had the highest concentrations (26.1 mg/kg ± 14.7 mg/kg), while AFB2, AFG1 and AFG2 showed only residual concentrations 1.6, 2.0 and 4.0 μg/kg, respectively. Concerning FC and AF, there were significant differences (P < 0.01) between strains and genotype. Both strains showed significant association (P < 0.01) between FC and the concentrations of AFB1 and AFB2 (R²: 99.5% and 93.2%; 87.2% and 73.2%, respectively). Results suggest that the level of resistance to fungus infection and AF accumulation is related to maize genotype. It emphasizes the relevance of developing *A. flavus* resistant maize genotypes as an alternative to control contamination in foodstuff intended for human and animal consumption.

Key words: AF, Aspergillus flavus, immature maize grain, resistance, Mexico.

1. Introduction

Aflatoxins (AF) are secondary toxic metabolites produced by several fungi, mainly the *Aspergillus* spp. which grows on grains and seeds, changing their texture, flavour, color and quality. Presence of AF in cereals is related mainly to *A. flavus* infection during plant development [1, 2]. Improper handling of humidity and temperature in agricultural products are factors that favor infection with *A. flavus* [3-5].

Globally, maize (*Zea mays* L.) provides 15% of the proteins and 20% of the calories in diets. Furthermore, in developing countries such as Latin America, Africa and Asia, maize is a staple food and occasionally is the only protein source in their diets [6]. Around 78% of maize samples are contaminated with AF [7]. Economic losses attributed to AF contamination are large [8, 9], mainly in developing countries that lack the appropriate regulations for the control of mycotoxin contaminated foods [10]. In Mexico, the presence of maize contaminated with *A. flavus* strains has also been documented [4, 11-15]. This is relevant due to the high national consumption of maize (20 million t/year) as well as *per capita* (329 g daily). In addition, the use of maize in animal feed is increasing,
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leading to an increase in its production in recent years [4, 13, 16].

AF is extremely toxic compounds that have carcinogenic, mutagenic, teratogenic and immunosuppressive capacities [17]. Therefore AF contamination in agricultural products is a serious public health problem, and affects productivity in domestic animals and agriculture in general [18]. For these reasons, many countries have established maximum permitted levels of AF concentration in food destined for human and animal consumption. For instance, the U.S. Food and Drug Administration established a limit of 20 ppb of AF in cereals and 0.05 ppb AFM1 in milk [14].

Because mycotoxins are unavoidable in worldwide, they have become one of the leading perils in both the feed and food industry. Strategies have been developed in order to control the presence of AF in maize, either by eliminating or reducing them to acceptable levels. For AF reduction, it is recommended: (1) to improve agricultural practices and storage conditions [19], (2) insect control [20-22], and (3) the use of natural or synthetic products to prevent toxicogenic fungi growth [14]. However these strategies have been proved to be insufficient, as approximately 25% of the agricultural production destined for consumption is contaminated with mycotoxins [23]. Therefore, concern for the use of mycotoxin-contaminated matrices dictates increased understanding about the plant and fungus interactions and presence of host-plant resistance to mycotoxin-producing fungi and mycotoxins occurrence [8].

An alternative for food contamination control is the use of maize and other cereal genotypes with genetic characteristics that provide them resistance to prevent development of phytopathogenic and toxic fungi. This seems to be a safe and economically adequate option to reduce the AF maize accumulation [24]. Maize infection with *A. flavus* and AF accumulation depends on the innate susceptibility of grain and the environmental factors which contribute to it, as well as the ability of the fungus to penetrate the grain [25]. Studies on breeding to improve resistance of maize strains have reported the importance of several factors involved in the infection process of grains with *A. flavus*: (1) presence of antifungal proteins [26], (2) regulatory factors in signal transduction [27], and (3) physical barriers [28].

Restricted development of *A. flavus* has been reported in some maize genotypes [9, 29]. Many breeding programs to evaluate the resistance to AF contamination in several maize genotypes use commercial strains of *A. flavus* which are characterized by a high production of AF [19, 30-33]. It is known that in field infections, *A. flavus* strains show variable ability to contaminate agricultural products [9]; in addition, there is little information on the capacity of commercial maize phenotypes to resist damage caused by field strains. Indigenous strains of *A. flavus* which are called Cuahutitlán and Tamaulipas, have demonstrated the ability to infect local cornfields and caused aflatoxin contamination of cereal crops [3, 4, 13] as well as the ability to damage the physiological functioning and histological structure in animals [34]. In Mexico and the State of Aguascalientes, the use of hybrid maize has increased in recent decades. However, forage maize hybrids used have been developed to improve grain yield [35], neglecting the quality of the forage [36] as well as its resistance to diseases.

The aim of this study was to evaluate the resistance of 11 maize genotypes to AF accumulation, AFs produced by two Mexican strains of *Aspergillus flavus* under controlled conditions of temperature, humidity and infective dose.

**2. Materials and Methods**

2.1 Grain Preparation

Immature maize grains of 11 genotypes (*Creole, 30R39, P3028W, HV313, Popcorn, C-526, 3002W, C-922, Garst 8366, As910, 30G40;* Fig. 1) conventionally grown in the State of Aguascalientes were used, and their main characteristics are shown in
Table 1. These genotypes were donated by the Forage Production Unit of the Aguascalientes Autonomous University in Mexico.

The maize was harvested 100 days after seeding. They were placed in paper bags for dehydration in an oven (55 °C/13 days), and the initial humidity content was calculated for each genotype. Grains were collected from dehydrated cobs and kept in hermetic containers. Before fungal inoculation, grains were allocated in glass containers with lids (400 g/container) and sterilized (121 °C, 15 min). To verify the absence of other contaminant flora, 500 seeds of each genotype were sown in MSA media (malt 2%, salt 6% and agar 2%) for eight days at 25 °C [37].

2.2 Grain Inoculation with A. flavus Spores

Cuautitlán and Tamaulipas strains of *A. flavus* considered as toxigenic [1] were used. Strains were cultured in Petri dishes with potato-dextrose agar and incubated at 27 °C for 10 days. To obtain spores, Petri dishes were washed with Tween 20 at 0.1%. Spore concentration was calculated using a hemocytometer to obtain a stock solution (5 × 10^6 spores/mL) to inoculate. Paraffin oil (1%) was added as fixer to the spore suspension [38]. Recommended security procedures for handling *A. flavus* cultures were followed [39]. Laboratory equipment was submerged for 5 min in a sodium hypochlorite solution (1:10, v/v), and working areas were sanitized with 6% sodium hypochlorite [40]. Maize grains were inoculated using a sterile non-invasive technique with 5 mL of inoculum (2.5 × 10^7 spores/g grain), and the humidity was adjusted to 15% by adding sterile distilled water. Flasks were agitated daily to prevent adhesion.

Three treatments were designed for each of the 11 maize genotypes (*n* = 20 flasks): (1) control group; (2) Cuautitlán strain; (3) Tamaulipas strain. The control group was not inoculated, but it was handled as groups 2 and 3 (humidity, spore fixer, temperature and period of assay). Each flask represents one experimental unit. Flasks were incubated at 27 °C ± 2 °C. The growth of *Aspergillus flavus* was recorded after 14 days of incubation. Fungal colonization (FC) level was expressed as the percentage of invasion on the surface of the grains, assigning levels 0, 1, 2 or 3 (0%, 1%-33%, 34%-67%, 68%-100%, respectively), according to the modified method of Guo et al. [41].

2.3 AF Quantification

Inoculated maize genotypes and controls were processed in a mill, inoculated and sieved (850 μm mesh). Flour was kept in hermetically sealed bags and maintained frozen at -20 °C until analyzed. To quantify AF concentrations (AFB1, AFB2, AFG1 and AFG2), samples were analyzed according to the Association of Official Analytical Chemists (AOAC) official methods [42]. Extraction tubes were used during the solid phase (Supelclean LC-CN, Supelco Inc., Bellefonte, PA).
### Table 1  Genotypes of immature maize grains used for inoculation with spores of *Aspergillus flavus*.

| Genotypes | Source    | Features                                      | Initial moisture (% average) |
|-----------|-----------|-----------------------------------------------|-----------------------------|
| Creole    | Local     | Natural cross, white jagged grains, low resistance to pests | 10.2                        |
| 30R39     | Pioneer   | High grain yield                              | 13.5                        |
| P3028W    | Pioneer   | Modified single cross, white jagged grains, low resistance to pests, tolerant to lodging and foliar diseases | 14.4                        |
| HV313     | Caloro    | Varietal cross, white semi-crystalline grains, high grain yield | 13.2                        |
| Popcorn   | Local     | Natural cross, smooth small yellow hard grains, low resistance to diseases | 18.0                        |
| C-526     | Hartz seed| White semi-crystalline grains, high grain yield, tolerant to *H. turcicum*, rust, *Fusarium* (stem), head smut | 19.0                        |
| 3002W     | Pioneer   | High forage yield, tolerant to diseases        | 11.0                        |
| C-922     | Hartz seed| Semi-crystalline grains, high grain yield, tolerant to diseases | 18.7                        |
| Garst 8366| Garst     | Modified single cross, white semi-crystalline grains, high grain yield | 12.8                        |
| As910     | Aspros    | Triple cross, white semi-jagged grains, high grain yield, tolerant to lodging and foliar diseases | 13.4                        |
| 30G40     | Pioneer   | Modified single cross, white semi-crystalline grains, high grain yield, tolerant to lodging and foliar diseases | 16.4                        |

Extracted eluate was derived and analyzed by a HPLC system with fluorescence detector (Varian ProStar binary pump; FP 2020 detector, Varian Associates Inc., Victoria, Australia; SupelcosilHPLC LC-18 Column, Supelco Inc.). AF concentrations were calculated by a standard curve from purified AF (B₁, B₂, G₁, G₂; Sigma) obtained by using the same methodology.

In order to perform AF extraction, 50 g of each corn samples were mixed with methanol:water (8:2, v/v), then they were eluted in solid-phase cartridges (SPE) using acetic acid 0.5%. SPE were washed with tetrahydrofuran 20% (THF), then hexane and finally THF 25%. Eluate was obtained with methylene chloride:THF 20% (99:1), it was evaporated to full dryness under nitrogen stream. To achieve an adequate identification and quantitation of AFB₁, samples were derivatized to AFB₁ hemiacetal (AFB₂a), a fluorescent compound, using trifluoroacetic acid. The AFB₂a was injected to HPLC system under following conditions: C18 column (SupelcosilHPLC LC-18 Column, 150 mm × 4.6 mm, Supelco Inc.); temperature (25 °C ± 2 °C); mobile phase acetonitrile:methanol:water (1:1:2, v/v/v); flow rate 1.0 mL/min; λ<sub>ex</sub>: 360 nm, λ<sub>em</sub>: 460 nm (Varian ProStar binary pump; FP 2020 detector, Varian Associates Inc., Victoria, Australia); injection volume 20 μL. Quantitation of AF was performed using a standard curve of purified AF (B₁, B₂, G₁, G₂; Sigma Aldrich; Fig. 2b) according to the AOAC [42]. The AFB₁ production by both strains of de *A. flavus* was determined in every established times in potato dextrose agar (PDA) media culture. Minimum detection limit was 0.3 ng/g for each AF. The quantitation
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Fig. 2  Analysis and production of AFB1. (a): chromatogram of derivatized AFB1 (AFB2a); (b): linear regression analysis of the standard curve; (c): the second-order polynomial equation describes the AFB1 production by Cuautitlan and Tamaulipas strains of *Aspergillus flavus*, points represent actual data of AFB1.

2.4 Statistical Analysis

Colonization and AF production rates were analyzed by one way analysis of variance (ANOVA). To determine the association between fungal colonization rate and AF production, linear regression analyses were performed. To correlate these two variables, a Pearson correlation analysis was carried out. AF production curves were adjusted for second-order polynomial regression (Fig. 2c). \( P < 0.05 \) was considered as significant. SAS V8 software was used (SAS Institute, Cary, NC, USA).

3. Results and Discussion

This study evaluated the resistance of 11 maize grain genotypes to FC, as well as AF accumulation from two toxicogenic *A. flavus* strains during 14 days. There were significant differences in AF accumulation, which was related with FC on the different maize genotypes. *Popcorn, Garst 8366, As910, C-526* and *30G40* showed the highest resistance to infection by the Cuautitlan strain (Fig. 3a). For the Tamaulipas strain (Fig. 3b), maize genotypes *As910, Garst 8366* and *30G40* showed resistance to fungal infection. Resistant genotypes evidenced significantly lower FC \((P < 0.01, \text{ level 1 = slow and scarce})\), compared to their respective controls. The *Popcorn* and *C-526* genotypes were resistant to the Cuautitlan strain but not to the Tamaulipas strain. This difference indicates that the Tamaulipas strain is more aggressive than the Cuautitlan strain. The control group did not show apparent FC (0 level) with any of the *A. flavus* strains.

Those maize genotypes that were susceptible to fungal infection showed a rapid and abundant FC (level 3), compared to their corresponding controls.

AFB1 showed the highest concentration (26.1 mg/kg ± 14.7 mg/kg), while types of B2, G1 and G2 showed only residual concentrations (1.6, 2.0 and 4.0 \( \mu \)g/kg, respectively) in all studied genotypes and both strains.

Maize genotypes which showed resistance to infection by the Cuautitlan fungal strain, also showed lower accumulation of AFB1 (*C-526, Popcorn, 30G40, As910* and *Garst 8366*, Fig. 3c). In addition, genotypes resistant to infection by the Tamaulipas strain were also resistant to AFB1 accumulation (*As910, Garst 8366*...
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Fig. 3  Resistance of 11 genotypes of immature maize grains to fungal colonization. (a) and (b): fungal colonization, expressed as the invasion of grain surface at level 0, 1, 2 or 3; (c)-(f): aflatoxins production (B1 and B2) from two *Aspergillus flavus* strains. Literals indicate significant differences, studentized Tukey test ($P < 0.05$; $n = 20$).
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and 30G40; Fig. 3d). Those genotypes showed the lowest concentrations ($P < 0.01$) of AFB$_1$ produced by both *A. flavus* strains.

Genotypes resistant to AFB$_2$ produced by the Cuautitlan strain were C-526, Popcorn, 30G40, Garst 8366 and As910 (Fig. 3e). For the Tamaulipas strain, resistant genotypes to AFB$_2$ accumulation were 30G40, As910 and Garst 8366 (Fig. 3f). AFB$_2$ accumulation in resistant genotypes was significantly lower ($P < 0.01$) compared to control groups of each genotype for both strains.

FC and AF accumulation B$_1$ and B$_2$ were significantly related ($P < 0.01$), probably due to the interaction between maize genotype and fungal strains. When AF production was compared in each strain, a positive correlation was observed (Fig. 4) between colonization by *A. flavus* strains and the production of AFB$_1$ and AFB$_2$ ($P < 0.01$, with Pearson coefficients of 94% to 99%). Regression analysis showed a significant influence ($P < 0.01$) of FC on AFB$_1$ and AFB$_2$ production (Figs. 4a and 4b, respectively); the determination coefficient for the Cuautitlan strain reached values of $R^2 = 99.5\%$ and 93.2\%, respectively. Whereas for the Tamaulipas strain values were $R^2 = 87.2\%$ and 73.2\%, respectively. Concerning grain colonization, the Tamaulipas strain was more aggressive than the Cuautitlan strain ($P < 0.01$), however the latter strain had the highest production levels of AFB$_1$ and AFB$_2$ from the 11 maize genotypes.

This study evaluated the resistance of 11 maize genotypes to AF accumulation, and the AF was produced by two Mexican strains of *Aspergillus flavus* under controlled conditions of temperature, humidity and infective dose. The results showed that maize genotype was associated with the level of colonization of each strain, which had significant differences in their ability to infect grains. Moreover, FC determined the accumulation of AFB$_1$ and AFB$_2$. These findings are reported for the first time using indigenous toxicogenic strains and maize genotypes widely used in Mexico. This information is highly relevant to agriculture and the food industry, since it might reduce the risk of human exposure through the production and selection of maize genotypes resistant to colonization of *A. flavus*.

This would be a complementary alternative to other strategies that have been described to diminish the impact of food contamination, such as the use of competitive non-toxicogenic strains [43], biological control agents (bacteria, yeasts) [44], insect control [45, 46], chemical and physical grain treatments [47] and the addition of sequestrants in animal diets [48, 49].

This study evaluated the resistance of 11 maize grain genotypes to FC, as well as AF accumulation from two toxicogenic *A. flavus* strains during 14 days. There were significant differences in AF accumulation, which was related with FC on the different maize
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Ankala et al. [29] and Kelley et al. [50] demonstrated that the non-commercial maize line (Mp313E, Mp04:86) is resistant to *A. flavus* infection, and suggested an association with the defense mechanisms of the plant [9]. Chen et al. [51] suggested that the main factors for resistance are the synthesis of antifungal proteins and the presence of physical barriers, such as pericarp thickness. In this study, in agreement with the Chen report, the *Popcorn* genotype characterized by its thick pericarp, showed to be resistant to infection. Other studies in endogamic maize hybrids have shown that if the pericarp is intact, the possibility of invasion by *A. flavus* and other pathogen agents is reduced [30]. In addition, Barros-Rios et al. [28] evaluated the structure and composition of the cell wall in maize grains and concluded that its thickness is a barrier which prevents grain damage caused by phytopathogens.

Maize genotypes *As910*, *Garst 8366* and *30G40* do not present a hard pericarp, which suggests that their defense mechanisms against fungi might be related to the synthesis of antifungal compounds. It is known that control of phenotypic traits, such as maize resistance to fungal colonization and AF accumulation, involves gene expression [52]. Gene expression related to maize resistance to infection has been associated to environmental factors, such as scarcity of water [53-55]. Ehrlich et al. [56] have shown that gene hypC, involved in AF synthesis is activated under conditions that inhibit fungal growth. Since this study was performed under controlled conditions of fungal growth, it is suggested that intrinsic genetic factors associated to FC resistance and AF accumulation were decisive for the results. The data show that fungi ability to produce AF (B₁ and B₂) was determined by the *A. flavus* capacity to colonize maize grains. The correlation between FC and AF accumulation was analyzed during a 14-day period, and a positive association between these two variables was found. Therefore, maize genotypes resistant to colonization (*As910*, *Garst 8366* and *30G40*) also showed resistance to AF accumulation (B₁ and B₂); meanwhile genotypes less resistant to colonization (*30R39*, *P3028W*, *HV313* and *Creole*) also showed the highest levels of AF accumulation. These results are in agreement with reports which stated that maize lines with high colonization levels also presented a significant accumulation of AF [27, 57]. Furthermore, it has been shown that mycotoxins, as secondary metabolites, are produced once the initial vegetative growth phase of the fungus has been completed after the conidia contacting the grain and are able to germinate [29, 58-60]. So if there is a delay in colonization, it also causes a delay in the buildup of AF in grains.

Significant differences were observed among the 11 maize genotypes concerning colonization capacity and AF production caused by the indigenous strains of *A. flavus*, and suggests that the invasiveness and pathogenicity of those strains are genetically determined. These results are in agreement with those who reported that difference in morphology and physiology of *A. flavus* strains is related to their ability to invade, use its resources and contaminat the grain [9, 61, 62]. These differences would explain the differential incidence of AF levels restricted to agricultural harvests produced in specific seasons and areas as well as associated to the presence of toxicogenic strains that interact with genotypic susceptibility and the environmental conditions prevailing in each agricultural cycle [30, 63, 64].

4. Conclusions

In this study, it showed that maize genotype is associated with the colonization level of maize grain by *Aspergillus flavus*. Significant differences are also observed in the capacity of the fungal strains to infect maize grains, as well as in genotype-strain interaction. In turn, colonization levels determined the concentration of accumulated AF. Only some maize genotypes (*Garst 8366*, *Popcorn*, *As910* and *C-526*) showed resistance to fungal growth and consequent AF accumulation. These data suggest that physical barriers...
and the presence of antifungal compounds in some maize genotypes confer resistance to fungal invasion. Therefore, production and selection of maize genotypes resistant to toxicogenic strains of *Aspergillus flavus* would reduce the risk of human exposure to contaminated food.

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**References**

[1] G.A. Payne, Aflatoxin in maize, Crit. Rev. Plant. Sci. 10 (1992) 423-440.
[2] Food and Agriculture Organization of the United Nations (FAO), Foodborne Diseases and Their Socioeconomic Impact, Technical Report on Agricultural and Food Engineering, Roma, France, 2009. (in Spanish)
[3] L.A. Rodríguez-del-Bosque, Impact of agronomic factors on aflatoxin contamination in preharvest field corn in Northeastern Mexico, Plant Dis. 80 (1996) 988-993.
[4] C.M. Bucio-Villalobos, D. Guzmán-de-Peña, J.J. Peña-Cabrerales, Aflatoxin synthesis in corn fields in Guanajuato, Mexico, Rev. Iberoam. Micol. 18 (2001) 83-87.
[5] G.L. Windham, W.P. Williams, Evaluation of corn inbreds and advanced breeding lines for resistance to aflatoxin contamination in the field, Plant Dis. 82 (2002) 232-234.
[6] S. Bhatnagar, F.J. Betrán, L.W. Rooney, Combining abilities of quality protein maize inbreds, Crop Sci. 44 (2004) 1997-2005.
[7] S.K. Jand, P. Kaur, N.S. Sharma, Mycoses and mycotoxicosis in poultry: A review, Indian J. Anim. Sci. 75 (2005) 465-476.
[8] P. Vardon, C. McLaughlin, C. Nardinelli, Potential economic costs of mycotoxins in the United States, in: Mycotoxins: Risks in Plant, Animal and Human Systems, CAST, Iowa, United States, 2003, pp. 136-142.
[9] DD. Deng, S. Jiang, Y. Wang, Y. Bian, J. Chen, B. Jia, Genetic analysis of the resistance to *Aspergillus flavus* infection in maize (*Zea mays* L.), Agric. Sci. China 8 (2009) 761-765.
[10] J.W. Cary, K. Rajasekaran, R.L. Brown, M. Luo, Z.Y. Chen, D. Bhatnagar, Developing resistance to aflatoxin in maize and cottonseed, Toxins 3 (2011) 678-696.
[11] E. Torres, K. Acuña, L. Naccha, J.P. Castellon, Quantification of aflatoxins in corn distributed in the city of Monterrey, Mexico, Food Add. Contam. 12 (1995) 383-386.
[12] J.A. Méndez-Albores, G. Arámbula-Villa, R.E. Preciado-Ortiz, E. Moreno-Martínez, Aflatoxins in pozol, a nixtamalized, maize-based food, Int. J. Food Microbiol. 94 (2004) 211-215.
[13] S. García, N. Heredia, Mycotoxins in Mexico: Epidemiology, management, and control strategies, Mycopathologia 162 (2006) 255-264.
[14] Information Statement of the Institute of Food Science & Technology (IFST), Mycotoxins [Online], http://www.ifst.org.
[15] G.N. Montes, C.A. Reyes, R.N. Montes, M.A. Cantu, Incidence of potentially toxigenic fungi in maize (*Zea mays* L.) grain used as food and animal feed, CyTA J. Food 7 (2009) 119-125.
[16] Food and Fisheries Information Service (SIAP) [Online], http://www.siap.gob.mx. (in Spanish)
[17] International Agency for Research on Cancer (IARC), Monograph on the Evaluation of Carcinogenic Risk to Human, IARC Press, Lyon, France, 2002, Vol. 82, pp. 223-249.
[18] W.L. Bryden, Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security, Anim. Feed Sci. Tech. 173 (2012) 134-158.
[19] W.B. Henry, maize aflatoxin accumulation segregates with early maturing selections from an S2 breeding cross population, Toxins 5 (2013) 162-172.
[20] H.K. Abbas, M.A. Weaver, B.W. Horn, I. Carbone, J.T. Monacell, W.T. Shier, Selection of *Aspergillus flavus* isolates for biological control of aflatoxins in corn, Toxin Rev. 30 (2011) 59-70.
[21] X. Ni, J.P. Wilson, G.D. Buntin, B. Guo, M.D. Krakowsky, R.D. Lee, et al., Spatial patterns of aflatoxin levels in relation to ear-feeding insect damage in pre-harvest corn, Toxins 3 (2011) 920-931.
[22] X. Ni, J.P. Wilson, M.D. Toews, G.D. Buntin, R.D. Lee, X. Li, et al., Evaluation of spatial and temporal patterns of insect damage and aflatoxin level in the pre-harvest corn fields to improve management tactics, Insect Sci., Sep. 3, 2012. DOI: 10.1111/j.1744-7917.2012.01531.x.
[23] Food and Agriculture Organization of the United Nations (FAO), Safety evaluation of certain mycotoxins in food, in: 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives, FAO Food and Nutrition Paper No. 74, 1992.
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Rome, France, 2001.

[24] W.B. Henry, G.L. Windham, M.H. Blanco, Evaluation of maize germplasm for resistance to aflatoxin accumulation, Agronomy 2 (2012) 28-39.

[25] B.W. Horn, Ecology and population biology of aflatoxicogenic fungi in soil, J. Toxicol. Toxin Rev. 22 (2003) 351-379.

[26] Z.Y. Chen, R.L. Brown, K.E. Damann, T.E. Cleveland, Identification of maize kernel endosperm proteins associated with resistance to aflatoxin contamination by Aspergillus flavus, Phytopathology 97 (2007) 1094-1103.

[27] R.L. Brown, Z.Y. Chen, M. Warburton, M. Luo, A. Menkir, A. Fakhoury, et al., Discovery and characterization of proteins associated with aflatoxin-resistance: Evaluating their potential as breeding markers, Toxins 2 (2010) 919-933.

[28] J. Barros-Rios, R.A. Malvar, H.J.G. Jung, R. Santiago, Cell wall composition as a maize defense mechanism against corn borers, Phytochemistry 72 (2011) 365-371.

[29] A. Ankala, B.K. Bolla, R. Shivaji, W.P. Williams, J.R. Wilkinson, Comparative analysis of the performance of Aspergillus flavus on resistant and susceptible maize genotypes during infection, Fungal Ecol. 4 (2011) 32-41.

[30] R.D. Walker, D.G. White, Inheritance of resistance to Aspergillus ear rot and aflatoxin production of corn from CI2, Plant Dis. 85 (2001) 322-327.

[31] F.J. Betrán, T. Isakeit, G. Odvody, Aflatoxin accumulation of white and yellow maize inbreds in diallel crosses, Crop Sci. 42 (2002) 1894-1901.

[32] H.D. Gardner, W.P. Williams, G.L. Windham, Diallel analysis of aflatoxin accumulation in maize, Field Crop Res. 102 (2007) 60-63.

[33] W.P. Williams, S. Ozkan, A. Ankala, G.L. Windham, Ear rot, aflatoxin accumulation and fungal biomass in maize after inoculation with Aspergillus flavus, Field Crop Res. 120 (2011) 196-200.

[34] A. Martínez-de-Anda, A.G. Valdivia, F. Jaramillo-Juárez, J.L. Reyes, R. Ortiz, T. Quezada, et al., Effects of aflatoxin chronic intoxication in renal function of laying hens, Poultry Sci. 89 (2010) 1622-1628.

[35] R. Gaytán-Bautista, M.I. Martínez-Gómez, N. Mayek-Pérez, Grain and forage yield in maize hybrids and their advanced F₂ generation, Agr. Tec. Mex. 35 (2009) 295-304.

[36] H.G. Núñez, G.E.F. Contreras, C.R. Faz, Important agronomic and chemicals characteristics in high energy hybrid forage corns, Tec. Pecu. Mex. 41 (2003) 37-48.

[37] J.M. Wells, J.A. Payne, Toxigenic Aspergillus and Penicillium isolates from weevil-damaged chestnuts, Appl. Microbiol. 30 (1975) 536-540.

[38] G.L. Windham, W.P. Williams, P.M. Buckley, H.K. Abbas, Inoculation techniques used to quantify aflatoxin resistance in corn, J. Toxicol. Toxin Rev. 22 (2003) 313-325.

[39] D. Shoemaker, M. Torchia, Laboratory safety, appendix B, in: Official Methods of Analysis of the Association of Official Analytical Chemist, Virginia, United States, 1995, Vol. 49, pp. 1-7.

[40] Aflatoxin Handbook, United States Department of Agriculture (USDA), Washington, United States, 2002, Vol. 2, pp. 1-9.

[41] B.Z. Guo, J.S. Russin, R.L. Brown, T.E. Cleveland, N.W. Widstrom, Resistance to aflatoxin contamination in corn as influenced by relative humidity and kernel germination, J. Food Prot. 59 (1995) 276-281.

[42] P.M. Scott, Natural toxins, AOAC. 49 (1995) 1-30.

[43] D. Bhatnagar, J.W. Cary, K. Ehrlich, J. Yu, T.E. Cleveland, Understanding the genetics of regulation of aflatoxin production and Aspergillus flavus development, Mycopathologia 162 (2006) 155-166.

[44] M.G. Etcheverry, A. Scandolara, A. Nesci, M.S. Vilas-Boas-Ribeiro, P. Pereira, P. Battilani, Biological interactions to select biocontrol agents against toxigenic strains of Aspergillus flavus and Fusarium verticillioides from maize, Mycopathologia 167 (2009) 287-295.

[45] T.E. Cleveland, P.F. Dowd, A.E. Desjardins, D. Bhatnagar, P.J. Cotty, United States Department of Agriculture—Agricultural Research Service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops, Pest Manag. Sci. 59 (2003) 629-642.

[46] H.K. Abbas, J.R. Wilkinson, R.M. Zablotsowicz, C. Accinelli, C.A. Abel, H.A. Bruns, et al., Ecology of Aspergillus flavus, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn, Toxins Rev. 28 (2009) 142-153.

[47] A. Yiannikouris, J.P. Jouany, Mycotoxins in feeds and their fate in animals: A review, Anim. Res. 51 (2002) 81-99.

[48] D.E. Diaz, W.M. Hagler Jr., B.A. Hopkins, L.W. Whitlow, Aflatoxin binders I: In vitro binding assay for aflatoxin B₁ by several potential sequestering agents, Mycopathologia 156 (2002) 223-226.

[49] D.E. Diaz, W.M. Hagler Jr., J.T. Blackwelder, J.A. Eue, B.A. Hopkins, K.L. Anderson, et al., Aflatoxin binders II: Reduction of aflatoxin M₁ in milk by sequestering agents of cows consuming aflatoxin in feed, Mycopathologia 157 (2004) 233-241.

[50] R.Y. Kelley, W.P. Williams, J.E. Mylroie, D.L. Boykin, J.W. Harper, G.L. Windham, et al., Identification of maize genes associated with host plant resistance or susceptibility to Aspergillus flavus infection and aflatoxin accumulation, PLOS ONE, 2012. DOI: 10.1371/journal.pone.0036892.

[51] Z.Y. Chen, R.L. Brown, T.E. Cleveland, Evidence for an association in corn between stress tolerance and resistance...
Association between *Aspergillus flavus* Colonization and Aflatoxins Production in Immature Grains of Maize Genotypes

[52] A. Menkir, R.L. Brown, R. Bandyopadhyay, Z. Chen, T.E. Cleveland, A USA-Africa collaborative strategy for identifying, characterizing and developing maize germplasm with resistance to aflatoxin contamination, *Mycopathologia* 162 (2006) 225-232.

[53] C. Paul, G. Naidoo, A. Forbes, V. Mikkilineni, D. White, T. Rocheford, Quantitative trait loci for low aflatoxin production in two related maize populations, *Theor. Appl. Genet.* 107 (2003) 263-270.

[54] Z. Wang, J. Liu, D. Lee, B. Scully, B. Guo, Postharvest *Aspergillus flavus* colonization in responding to preharvest field condition of drought stress and oligo-macroarray profiling of developing corn kernel gene expression under drought stress, *Phytopathology* 98 (2008) S166.

[55] M.L. Warburton, T.D. Brook, G.L. Windham, W.P. Williams, Identification of novel QTL contributing resistance to aflatoxin accumulation in maize, *Mol. Breeding* 27 (2011) 491-499.

[56] K.C. Ehrlich, P. Li, L. Scharfenstein, P.K. Chang, HypC, the anthrone oxidase involved in aflatoxin biosynthesis, *Appl. Environ. Microb.* 76 (2010) 3374-3377.

[57] Z.Y. Chen, R.L. Brown, K.E. Damann, T.E. Cleveland, PR10 expression in maize and its effect on host resistance against *Aspergillus flavus* infection and aflatoxin production, *Mol. Plant. Pathol.* 11 (2010) 69-81.

[58] J. Sekiguchi, G.M. Gaucher, Conidiogenesis and secondary metabolism in *Penicillium urticae*, *Appl. Environ. Microb.* 33 (1977) 147-158.

[59] A.M. Calvo, R.A. Wilson, J.W. Bok, N.P. Keller, Relationship between secondary metabolism and fungal development, *Microbiol. Mol. Biol. R.* 66 (2002) 447-459.

[60] K.C. Ehrlich, Q. Wei, R.L. Brown, D. Bhatnagar, Inverse correlation of ability to produce aflatoxin and *Aspergillus* colonization of corn seed, *Food Nutr. Sci.* 2 (2011) 486-489.

[61] M. Razzaghi-Abyaneh, M. Shams-Ghahtarokhi, A. Allameh, A. Kazeroon-Shir, S. Ranjbar-Bahadori, H. Mirzahosseini, et al., A survey on distribution of *Aspergillus* section Flavi in corn field soils in Iran: Population patterns based on aflatoxins, cyclopiazonic acid and sclerotia production, *Mycopathologia* 161 (2006) 183-192.

[62] J. Gao, Z. Liu, J. Yu, Identification of *Aspergillus* section Flavi in maize in Northeastern China, *Mycopathologia* 164 (2007) 91-95.

[63] H.L. Mehl, P.J. Cotty, Variation in competitive ability among isolates of *Aspergillus flavus* from different vegetative compatibility groups during maize infection, *Phytopathology* 100 (2010) 150-159.

[64] C. Probst, F. Schulthess, P.J. Cotty, Impact of *Aspergillus* section Flavi community structure on the development of lethal levels of aflatoxins in Kenyan maize (*Zea mays*), *J. Appl. Microbiol.* 108 (2010) 600-610.