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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. AFB_1 had the highest concentrations (26.1 mg/kg ± 14.7 mg/kg), while AFB_2, AFG_1 and AFG_2 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 AFB_1 and AFB_2 (R^2: 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 AFM_1 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 (2.5×10^5 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].

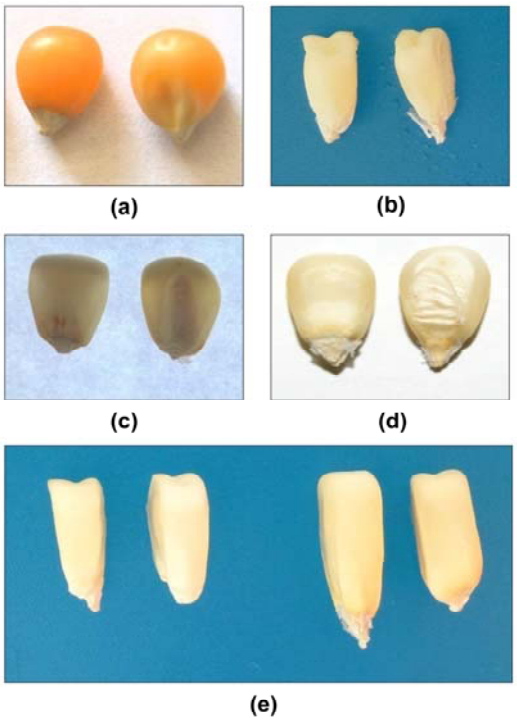

Fig. 1 Morphological characteristics of maize genotypes used. (a): yellow and non-jagged grains (*Popcorn*); (b): white, opaque and jagged gains (*Creole*, *P3028W*); (c): (translucid view) and (d): white, opaque and non-jagged grains (*HV313*, *C526*); (e): white, semi-crystalline and semi-jagged grains (*As910*, *30R39*, *3002W*, *Gartz 8366*, *30G40* and *C-922*).

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 (AFB_1, AFB_2, AFG_1 and AFG_2), 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).

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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 (B_1, B_2, G_1, G_2; 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 AFB_1, samples were derivatized to AFB_1 hemiacetal (AFB_{2a}), a fluorescent compound, using trifluoroacetic acid. The AFB_{2a} 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; λex: 360 nm, λem: 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 (B_1, B_2, G_1, G_2; Sigma Aldrich; Fig. 2b) according to the AOAC [42]. The AFB_1 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

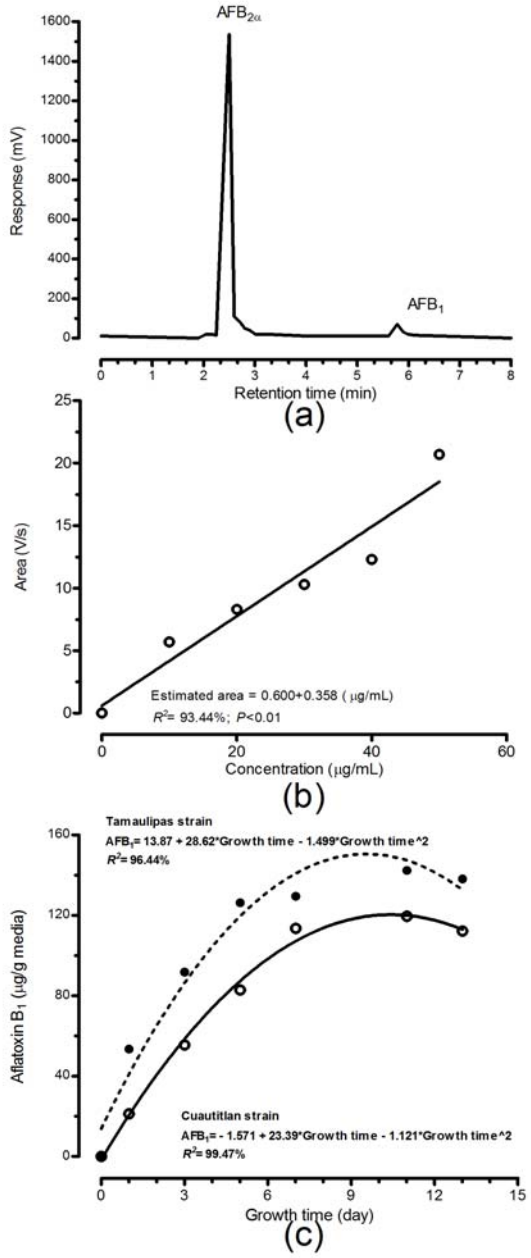

Fig. 2 Analysis and production of AFB_1. (a): chromatogram of derivatized AFB_1 (AFB_{2a}); (b): linear regression analysis of the standard curve; (c): the second-order polynomial equation describes the AFB_1 production by Cuautitlan and Tamaulipas strains of *Aspergillus flavus*, points represent actual data of AFB_1.

data were obtained via Galaxie (Ver. 1.9.302.530) software.

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, lineal regression analyzes 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 ($P < 0.01$, 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.

AFB_1 showed the highest concentration (26.1 mg/kg ± 14.7 mg/kg), while types of B_2, G_1 and G_2 showed only residual concentrations (1.6, 2.0 and 4.0 μg/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 AFB_1 (*C-526*, *Popcorn*, *30G40*, *As910* and *Garst 8366*; Fig. 3c). In addition, genotypes resistant to infection by the Tamaulipas strain were also resistant to AFB_1 accumulation (*As910*, *Garst 8366*

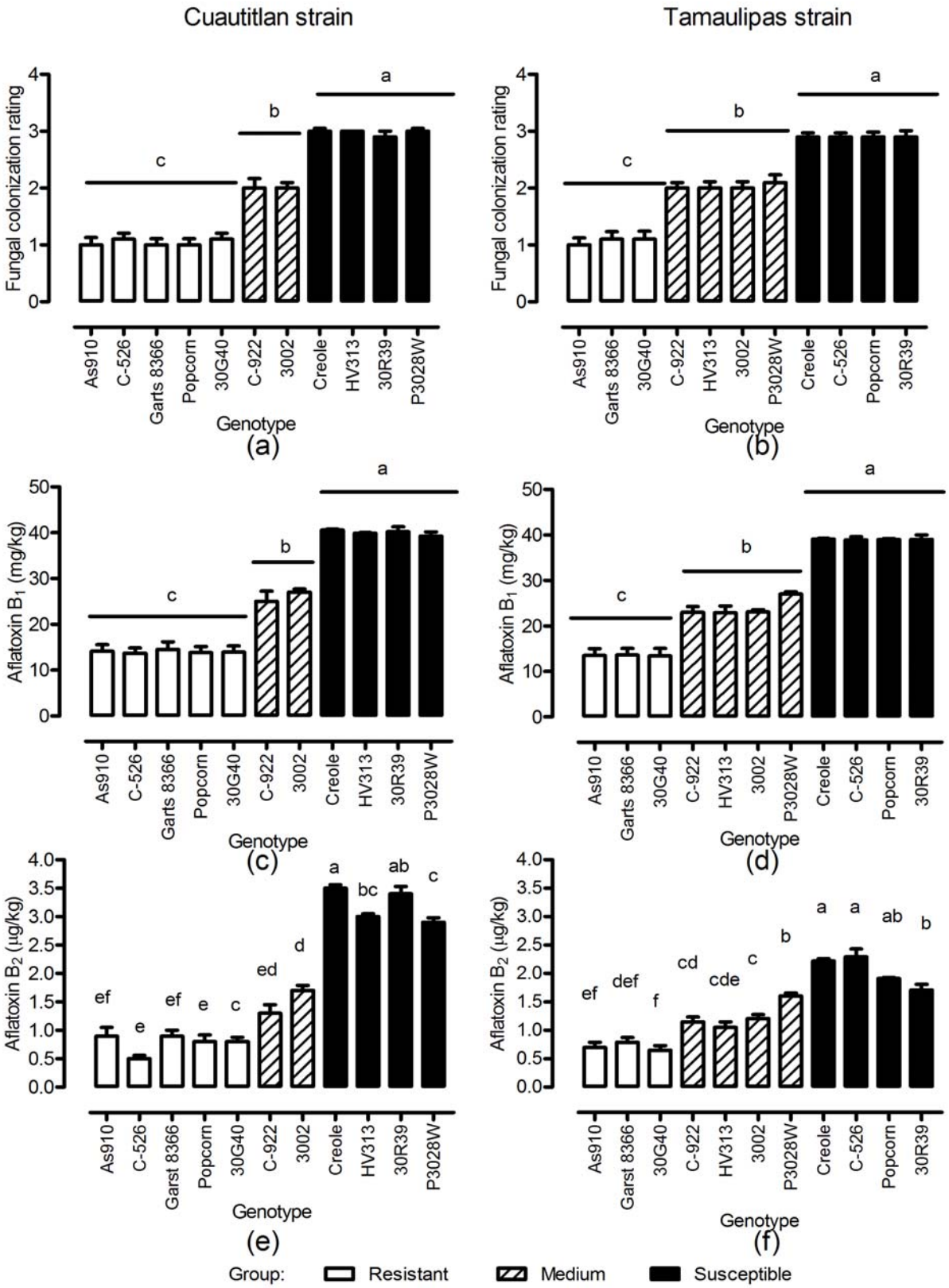

Fig. 3 Resistance of 11 genotypes of immature maize grains to fungal colonization. (a) and (b): fungal colonization, expressed as the invasion of grain surface at level 0, 1, 2 or 3; (c)-(f): aflatoxins production (B_1 and B_2) from two *Aspergillus flavus* strains. Literals indicate significant differences, studentized Tukey test ($P < 0.05$; $n = 20$).

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

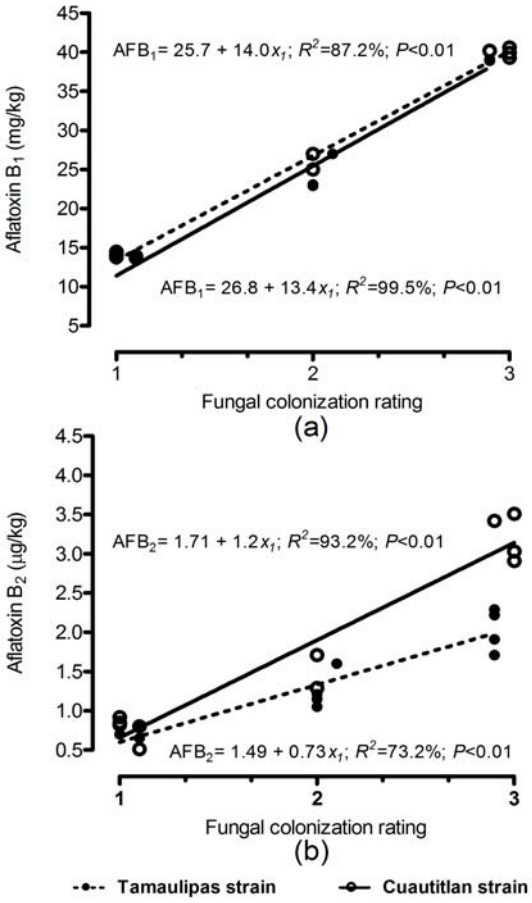

Fig. 4 Regression analysis between AFB_1 (a) and AFB_2 (b) production and fungal colonization rating (x_1), R^2 = coefficient of determination.

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

genotypes. 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 (B_1 and B_2) 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*, *Garts 8366* and *30G40*) also showed resistance to AF accumulation (B_1 and B_2); 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.

Acknowledgments

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