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Diagnosis of mycotoxigenic fungi instored grain corn

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Abstract

Fungi in stored grains can cause harmful effects on the health of consumers due to the mycotoxins that they produce. aflatoxins are the most toxic and carcinogenic fungal metabolites that are frequently more found in nature. During 2014/2015 an investigation was conducted to analyze the interaction of storage conditions with the incidence of mycotoxigenic fungi and aflatoxin concentration in stored grain corn. 27 samples of stored corn grain from the main producers states of Mexico were taken, and the conditions in which they were stored were registered. Fungi and aflatoxins incidence were quantified in laboratory, and the interaction effect of seed conditions with those parameters. High incidence of *Fusarium* and *Aspergillu*s species were found, with 32 and 8% respectively, but with low levels of Aflatoxins. Also, we found influence between the levels of aflatoxins with the storage time and *Aspergillus* incidence. A high influence of grain moisture and volumetric weight with *Aspergillus* incidence was observed. Also we found an influence with the level of aflatoxins, storage time and *Aspergillus* incidence.

***Aspergillus*, aflatoxin, storage condition.**

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Introduction

Currently corn, along with wheat and rice are globally 75% of the produced grains. They provide 56% of carbohydrates and 50% protein food in the world, and in addition to providing nutrients for animals elements is a basic raw material for the processing industry (Suleiman et al., 2013;. Simopoulos, 2002 ; Morris and López-Pereira, 2000; FAO, 1993; Stoskopf, 1985; Malavolta et al., 1974).. Stored grain corn, depending on storage conditions and health of the culture from which they come, can be attacked by differentiated fungi pathogens into two groups. Field and store (Suleiman et al., 2013; Miller, 2008; Jouany, 2007; Carrillo, 2003; Arbeláez Torres, 1978; Christensen and Kaufmann, 1969). In Mexico, the most important fungal genera that infect grain storage are *Aspergillus*, *Fusarium* and *Penicillium* (Crhistensen and Lopez, 1962; Arrúa Alvarenga et al., 2012;. Garcia and Martinez, 2010; Hernández et al., 2007; Gallardo et al., 2006;. Bucio et al., 2005), they also are reported worldwide (Stumpf et al., 2013;. Maširević et al., 2012;. Marín et al., 2012;. Ghiasian et al., 2004;. Orsia et al., 2000). These fungi can cause harmful effects on the health of consumers by generating mycotoxins (Binder, 2007; Cabanes, 2000; Pitt, 2000; Martinez and Benavides Moreno Ocampo, 1988). 300 fungal toxins (.; Binder, 2007; Carrillo, 2003 Arroyo-Manzanares et al., 2014) are known. Aflatoxin is problem for many products, but when it comes to grains, is mainly a problem in maize (Miller, 1995).

The aflaxotinas B1, B2, G1 and G2 are the most toxic and carcinogenic fungal metabolites that are naturally more frequently, and the most important producers of *Aspergillus* species are the Flavi section, including *A. flavus, A. parasiticus* and other species (Richard, 2007; Bennett and Klich, 2003; Brasel Hussein, 2001; Widstrom, 1996; Miller, 1995).

Mexican Official Standard (NOM-188-SSA1-2002) sets a limit of 20 ppm aflatoxins in cereal grains for human consumption (Ministry of Health, 2002).

While aflatoxin limits tolerated in globally food vary from 0 to 35 ppm, more frequently from 4 to 20 ppm (FAO, 2004). The concentrations of mycotoxins are a function of fungi in stored grains developed and competition between them, which is determined the humidity, storage time, water activity, temperature, pH, substrate composition and presence of pests (Sanchis et al., 2007;. Shapira and Paster 2004). In stored grains with high humidity (>14%) and temperature (>20 ° C), and / or drying can be potentially contaminated inadequate (Ominski et al., 1994). Contamination of grains with mold and fungi is recognized as one of the most important problems in tropical countries around the world (Kaaya and Kyamuhangire, 2006), where the only viable solution to mycotoxins in the fork is to prevent growth fungal (Tefera, 2012; Carrillo, 2003).

This calls for the study of the interactions between these factors, allowing a better understanding of the influence of storage conditions on the content of mycotoxins in stored corn kernels, and therefore set design management strategies that reduce mycotoxin levels, prevention of fungal growth on stored grains.

The objective of this research was to analyze the interaction of storage conditions and the incidence of fungal mycotoxigenic and Introduction

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Materials and methods

The works were carried out in the Laboratory of pathogenic fungi of expertise in plant protection Universidad Autónoma Chapingo, located at km 38.5 of the highway Mexico - Texcoco, Chapingo, State of Mexico, and the laboratory of organic material of CINVESTAV Queretaro, located in the Real Fractionation Juriquilla, Santiago de Queretaro, Queretaro State.

The implementation period of the study was between the months of July 2014 to September 2015. corn samples were taken of grain stores of the major producing states (Table 1). They were performed in accordance with the provisions of the Official Mexican Standard (NOM-188-SSA1-2002) that sets the limits of aflatoxins in cereal grains.

State	Samples
Chiapas	6
Guanajuato	3
México	5
Morelos	2
Oaxaca	2
Puebla	4
Sinaloa	3
Tabasco	1
Veracruz	1
Total	**27**

During and immediately after sampling, the principal conditions in the grains were determined (humidity, type of storage, damaged grains, volumetric weight, storage time and genetic material) were determined.

12 seeds of each sample were used, which were initially disinfected on its surface, for 2 minutes, sodium hypochlorite at 2% rinsed with sterile distilled wáter and dried with sterile paper.

Later they were placed in petri dishes (3 seeds per Petri dish) containing medium potato-dextrose agar culture and incubated about 7 days at 27 (± 2) ° C (Hernandez et al., 2007; Carrillo, 2003; Quiñones Martinez, 2011). Isolations and purifications were performed by culturing hyphae tip of each strain of the fungus for subsequent identification (Morales et al., 2007; Ruiz Castañeda, 2001; French and Teddy, 1980).

The morphological identification of fungi was performed by microscopic observation of assemblies from developed isolates in Petri dishes on PDA culture medium and AA with carnation, with the technique of tape (transparent adhesive), (Arenas, 2003).

The observed and descriptions structures were compared with descriptive identification keys (Sampson et al., 2014;. Leslie and Summerell, 2006; Carrillo, 2003; Moreno-Martinez and Benavides Ocampo, 1988; Nelson et al., 1981; Booth, 1971) .

The biological and morphological characteristics considered during identification were: growth rate, appearance of pigmentation and aerial mycelium colony level in culture, presence or absence, arrangement, size, shape and color of micro and mesoconidias; size, shape, color and number of cells macroconidia, presence or absence of chlamydospores (Fusarium strains), size and shape of the bladder and head and presence or absence of metulae in *Aspergillus* strains.

For molecular identification as isolation frequency was considered and the eight most common strains were selected.

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For the extraction of fungal DNA, the protocol described by the company Macrogen DNeasy Plant Mini Kit Kit, Quiagen Brand, consisting of the following is followed:

Each fungus purified and incubated for 7 days in culture medium PDA, transferred 50 to 100 mg of mycelium, a microcentrifuge tube (Eppendorf), 400 uL of Buffer AP1 and 4 mL of RNase was added, stirred at vortex.

Samples were incubated in a water bath for 10 min at 65 ° C, stirring by inversion 2 or 3 times; They were added 130 mL of Buffer AP2 5 min and kept on ice. The mixture was placed in the QIAshredder mini spin column and centrifuged for 2 min at 14,000 rpm.

The liquid passed through the column to a new microcentrifuge tube, to which were added 1.5 volumes of buffer AP3 / E transferred. This solution was placed in a DNeasy Mini spin column, centrifuged for 1 min at 8,000 rpm, it was transferred to a new tube, and added 500 uL of buffer AW; He centrifuged again for 1 min at 8,000 rpm, and 500 uL of buffer AW was added, and then centrifuged for 2 min at 14,000 rpm, for the purpose of washing the DNA; finally, the DNeasy Mini spin column to a new tube was transferred, they were put 100 mL of buffer AE DNeasy for elution, incubated for 5 min at room temperature and centrifuged for 1 min at 8,000 rpm (Qiagen, 2012).

Once the DNA of the 8 strains obtained, the product was sent to Laboratories Macrogen in Korea, for the amplification of two universal regions corresponding to the ITS region and the region of the genes coding for the factor elongation.

Aspergillus strains for amplification of the ITS region was made and the universal primers ITS-4 (5'-TCCTCCGCTTATTGATATGC-3 ') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') rRNA genes were used, the subunit 18S, 5.8S and 28S, which amplify an internal intergenic spacer (ITS) and generate a product of varying size between 550 and 585 base pairs (bp) approximately (Samson et al., 2014).

Meanwhile, for strains with morphological characteristics corresponding to Fusarium, genes encoding elongation factor EF1 and EF2 GGAAGTACCAGTGATCATGTT ATGGGTAAGGAGGACAAGAC which generate a product of 700 bp (O'Donnell et al., 1998).

Quantification of aflatoxin was performed using affinity chromatography based on monoclonal antibodies, which detect aflatoxins B1, B2, G1 and G2 in grains (Aflatest / VICAM), validated by AOAC (1995). For the same the following procedure was followed: 50 grams of sample were ground and 5 g of free iodine salt, then they were mixed in a solution of methanol / distilled water 80:20 in a blender for 1 minute were added.

It later went through filter paper into a beaker; 10 mL of the obtained solution was diluted in 40 mL of distilled water. Subsequently, 2 mL of the extract filtered and the affinity column is spent per Aflatest at a rate of 1-2 drops.seg^{-1}, just as the affinity column Aflatest was washed 2 times by passing distilled water at a rate of 1- 2 drops.seg^{-1} 5mL.

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Aflatoxins were eluted from the affinity column by passing through the same 1 mL of HPLC grade methanol at a rate of 1-2 drops.seg-^{1}, collecting the resulting in a 10 mL tube; He was added 1 mL Aflatest developer, and placed for 30 seconds in a fluorometer VICAM Series 4 E, previously calibrated.

The data were sorted into a spreadsheet (Excel), and then multiple linear regression tests were performed using SAS version 9.3 software, including the frequency of isolates aflatoxinasy levels recorded storage conditions during sampling.

It was considered as a criterion variable for the repressor enter and remain in the model must have less than 0.05 type I error and where it was necessary, the intercept was not included in the model.

Results and discussion

General characteristics of the samples

Samples of corn kernels studied came from different types of establishments, either producer or commercial premises local storage, being the most part, from producing.

Storage forms varied according to the type of establishment where they came from. Those samples obtained from producers generally were stored in drums of 100 or 200 liters, or in bags, and less frequently in trojas or cozcomates. In the storage premises commonly performed in sacks, while in storage facilities was took place on grain silos with more advanced technology.

The reduced availability of technology in the grain storage by small farmers causing a greater amount of loss mainly in tropical and subtropical regions (IICA, 2012; Munkvold, 2003a; Jouany, 2007).

Mostly grains were analyzed native and hybrid varieties Asgrow7573, and lower frequencies and other hybrids like Cronos DK2060.

The stored grain moisture ranged from a range of 14.4 and 22.2%, with an average of 13.24% (Table 2). In 70% of grain moisture content was 13% or less, which according Munkvold (2003b) is recommended for storage of corn.

The storage time was also very variable (from 4-42 months), with 60% of samples stored for less than a year, with longer storage facilities producers destine their production to consumption.

Characteristics	Average	Minimum	Maximum
Humidity	13.24	10.40	22.20
Storage time (months)	11.81	4.00	42.00
Damage to the seed (%)	19.81	1.00	84.00
Volumetric weight (kg.hL-1)	72.24	46.90	80.70

Table 2 Characteristics of the samples studied grain corn. Year 2014/2015

The damage observed in the seeds were mechanical damage during harvest or insect damage, and were around 20%. Both storage time, as the level of damage having grains are determining factors in the incidence of mycotoxigenic fungi, and therefore of mycotoxins in the kernels (Christensen and Kaufmann, 1969; Binder, 2007) by.

For the volumetric weight average was 72.24 kg.hL-1, with a range that varied from 46.9 to 80.7 kg / hl, values very close to those reported by Peña Betancourt et al., (2013), who analyzed 15 samples from from different states of Mexico, Creole and hybrid grain corn varieties intended for consumption, they found that the volumetric weight fluctuated between 49 and 80 kg.hL-1.

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Frequency of isolation of fungi

The genera most frequently (incidence) were *Fusarium* and *Aspergillus*, with 32 and 8% incidence respectively.

These two genres together with *Penicillium* (1.1%) are the most common fungi in stored grain worldwide (Stumpf et al., 2013;. Maširević et al., 2012;. Marín et al., 2012;. Arrúa Alvarenga et al., 2012. ; Garcia and Martinez, 2010; Hernández et al., 2007; Gallardo et al., 2006; Bucio et al., 2005;. Ghiasian et al., 2004;. Orsia et al., 2000;. Crhistensen and Lopez, 1962).

Other genera of fungi were isolated *Cladosporium* (2.4%), *Rhizopus* (1.1%) and *Trichoderma* (1.4%) (Figure 1).

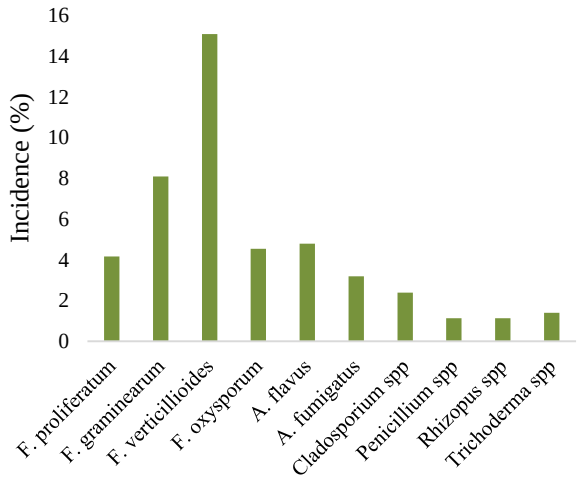

Figure 1 Frequency of isolation of fungi (%) in stored corn kernels from different states of Mexico

4 species of *Fusarium* (*F. verticillioides* 15.1%, 4.5% *F. oxysporum*, *F. proliferatum* 4.2% and 8.1% *F. graminearum*), and two species of *Aspergillus* (*A. flavus* and *A. fumigatus* 4.8% were identified 3.2%).

When multi-linear regression with a probability of type I error of 0.05 between the moisture content in the grains, the storage time, damage and volumetric seed weight, the incidence of *Aspergillus*, could generate a model, corresponding to the best fit to the following equation:

Incidence of *Aspergillus* = 2.11 (% moisture content in grain) - 0.27 (volumetric weight).

Some factors such as damage to grains either mechanically or by insects, facilitating fungal infection (Setamouet al., 1997), were part of the model when the probability of type I 0.1 error is allowed, but to reduce it 0.05 was no longer in the best-fit model, thus indicating that, although it has influenced the level of incidence of *Aspergillus*, the moisture content in the grains and the volumetric weight, had greater influence.

The grain moisture was the most influential factor in the incidence of *Aspergillus* spp. inthe evaluated samples, which coincides with Ominski et al., (1994), and together with temperature, strongly influenced the fungal invasion in stored grain.

The influence of volumetric weight is that associated with increased grain hardness (Correa et al., 2002), thus generating a greater difficulty to be invaded by microorganisms. Which partially explains the low incidence of these in stored grain.

However, none models separately acting factors, and the degree of fungal invasion depends on an interaction between them (Christensen and Kaufmann, 1969; Binder, 2007).

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Aflatoxin levels

Aflatoxin levels detected in the samples had a variation of between 0 and 68 ppm, with an average of 9.74 ppm (Figure 2). 93% of the samples had an aflatoxin concentration below the level allowed by the Official Mexican Norm (NOM-188-SSA1-2002), which sets the limits of aflatoxins in cereal grains for human and animal consumption 20 ppm.

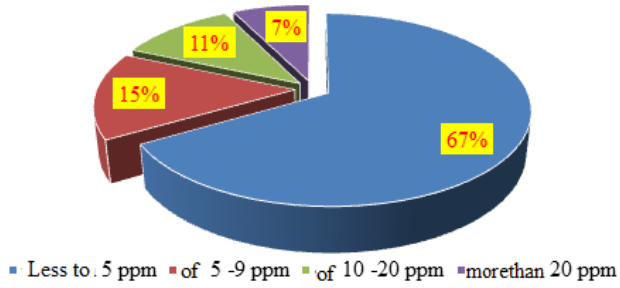

Figure 2 Levels of mycotoxins in samples stored corn kernels from different states of Mexico over the years 2014/2015

The mycotoxin levels above 20 ppm were found to a lesser extent, with a total of 7%. The best fit for this variable was the following model: aflatoxin levels (ppm) = 0.49 (storage time) + 0.68 (% incidence of *Aspergillus*).

According to background, Hell et al., (2000), in a study conducted in the countries of the east coast of Africa, found as the most influential factors in the accumulation of aflatoxins storage time, damage to the seed and use of local products or plants (excerpts) as protectants during storage.

But the degree of fungal invasion is well documented as one of the main factors causing postharvest mycotoxin content (Chulze, 2010; Bennett and Klich 2003; Swanson, 1987).

Conclusions

Fungi with the highest incidence in samples of corn grains were *F. verticillioides*, *F. oxysporum*, *F. proliferatum*, *F. graminearum*, *A. flavus* and *A. fumigatus*. As the moisture content in the grains and the volumetric weight of these factors most influence on the incidence of *Aspergillus* in the samples studied.

In 81% of the samples they were detected aflatoxin, although in lower concentrations to 20 ppm in 93% of them. Only 7% of the samples was out of the norm with concentrations greater than 20 ppmC and maximum of 49 ppm. The most influential factors in the levels of aflatoxins in grains were: storage time and the incidence of *Aspergillus* spp.

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