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ORIGINAL ARTICLE

Reaction of Maize Varieties to *Aspergillus flavus* and Aflatoxin Production

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KEYWORDS

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ABSTRACT: Aflatoxins, are very toxic mixture and having the potential to cause cancer, produce by some the fungi, mostly *Aspergillus flavus* and *Aspergillus parasiticus*, during their growth, harvest and storage on crops such as wheat, corn, cottonseed, peanut, pistachio, etc. For appraising the reaction of maize varieties to *A. flavus* growth and produced aflatoxin, five varieties of maize, namely KSC400, KSC403, KSC600, KSC703, KSC704, were selected. An isolate *A. flavus* that produce afalatoxin was used for inoculation of maize kernels (as *in vitro* conditions). In a statistic completely random design with three replications, 10 gr of kernels of selected cultivars were inoculated with 1×10^6 spores/ml of fungal spore suspension. After eight days, the percentage of fungal growth and the colonization of maize kernels were calculated. The aflatoxin B1 produced in contaminated all maize cultivars were measured with high performance liquid chromatography (HPLC). Statistical analyses showed a significant difference (α=0.05) between the fungal growth percentages and the amount of aflatoxin B1 in the kernels of tested maize cultivars. Also, the results showed that among mentioned varieties, KSC600 was the least susceptible variety to the growth of *Aspergillus*. The amount of produced aflatoxin B1 was variable among the varieties. KSC600 had the least whereas KSC403 had the highest rate of aflatoxin B1 content.

INTRODUCTION

Aflatoxins are harmful substances generated by some kinds of fungi that are exist naturally anywhere; they can pollute food products such as wheat, maize, and nuts. They also cause significant economic damage, an estimated 25% or more of the world's food products loss annually [1]. The contamination of aflatoxins in agricultural crops is a drastic threat to both humans and domestic animal health which is why the United States Food and Drug Administration (FDA) and similar organizations in many other countries

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have strict regulations on aflatoxins contamination in food and feeds [2].

The FDA has set a permissible threshold for total aflatoxins in human food as 20 ppb and as 0.5 ng/ml of aflatoxin type M1 in milk. The European Union has even imposed stricter rules on imported agricultural goods. Threshold levels for aflatoxins have also been determined for different animal feed categories. Unfortunately, backward or developing countries in many regions of the world, such as some African ones, cannot afford to pay for aflatoxin monitoring and reductions in food and feed products. This has increased the risk of aflatoxin exposure and consequently the prevalence of acute aflatoxin poisoning (aflatoxicosis) [3] and an increase in complications in children who are stunted and malnourished (Kwashiorkor) [4, 5].

Aflatoxins, an important group of toxic secondary fungal metabolites, are produced by some Aspergillus species such as *A. flavus*, *A. tamarii*, *A. parasiticus*, *A. nomius*, and *A. bombycis* [6], among them, *A. flavus* and *A. parasiticus* are the most common fungi associated with aflatoxin production in susceptible crops. Due to their considerable presence in natural products as well as their toxic and carcinogenic characteristics, aflatoxins are known as the main mycotoxins. So far, several types of aflatoxins have been identified, the most popular ones are B1, B2, G1 and G2 [7]. Among them, aflatoxin B1 is the most toxic ones [8].

In addition to harmful effects of aflatoxin existing in crops on human and animal health, there are also considerable economic costs in order to reduce aflatoxin content in products. Estimates show that direct losses in annual crop yields in the United States could reach tens of millions of dollars, and in years with severe contamination of corn, losses could reach hundreds of millions of dollars [9]. Of course, considering the decline in revenue from other factors such as export market losses, sampling and testing, and adverse effects on human and animal health, total costs attributable to aflatoxin is much higher [10].

Pre-harvest aflatoxin contaminations in crops are very complex problems influenced by numerous living and non-living agents. So, a multidisciplinary approach may be needed to control aflatoxin contamination when field conditions are conducive to fungal infection. A scope for in-depth study on aflatoxin contamination control is pre-harvest resistance in the host plant [11, 12].

Because *A. flavus* infects crops before harvest, the host-resistance strategy may be easier for the grower to integrate into different crop management systems to prevent aflatoxin pre-harvest contamination. So far, several maize lines have been bred with increased resistance properties to *A. flavus* growth and aflatoxin production, this enables the identifying natural resistance resources [13-16]. However, research has showed that resistance to aflatoxin production is multigenic. Also, it must be mentioned the attempts to transfer the resistance from modified lines into commercial ones with desirable agronomic properties has been fulfilled slowly due to unavailability of biomarkers to facilitate the transition of resistance genes [17].

Unlike corn, cotton has a limited variety of germplasms and to date no species with natural resistance to *A. flavus* have been identified. For this reason, it is very important to develop seed-based resistance in cotton. A number of potential corn resistance-associated proteins (RAPs) and their encoding genes have been identified and some of these genes have been introduced into cotton for resistance incidence evaluation [18-20]. However, more investigations are needed to explain the biochemical mechanisms that reveal resistance phenotypes in corn grains or other sources until they can be used to strengthen resistance through marker-based breeding methods in corn or genetic engineering in cotton [15, 21-24].

Today, one of the most important problems of the global health community is the aflatoxin contamination of agronomic products. Different countries have set special rules for the production, consumption and importation of food and pharmaceutical product to deal with the serious dangers posed by mycotoxins [25]. In USA, food or drugs materials containing more than 20 ng/g of aflatoxins are legally prohibited for sales, import and export [7, 26].

Since 1960s decade, the time of aflatoxin discovery, *A. flavus* has been usually noted as the most prevalent fungus affecting food crops. This is enough to show its economic importance. Around the world, this fungus is prevalently seen as an air and soil mycoflora agent as well as on dead

or alive animal and plant organisms. The fungus is especially interested in establishing and growing on nut, grains, and oily products, maize, wheat, rice, peanuts, pistachios and almonds are the main products infected with this fungus [27].

The strategic importance of maize crop is clear, and of course the basic need to protect and optimize its products to maintain a safe margin in global trade. Contamination of maize by *Aspergillus* species and their mycotoxins is one of the most serious challenges for the production, consumption and export of maize all over the world. Mycotoxin contamination of corn is greatly influenced by weather and stress conditions [28].

In a research, pistachio varieties showed different sensitivity to artificial inoculation of *A. flavus*, the fungal establishment on pistachio kernel and produced aflatoxin concentration. The highest fungal settlement in the kernel was related to the Ahmadaghaie and Ohadi cultivars, while the lowest colonization was related to Akbari and Kaleh-Ghouchi cultivars. The Abbasali, Shahpasand Fakhri and Kalkhandan, cultivars showed the highest and lowest aflatoxin concentration in kernels, respectively [8].

Obviously, all various aspects of infection by *A. flavus* and contamination by aflatoxin must be surveyed comprehensively and integrated manner. One of the best ways to handle this is evaluating of the sensitivity or resistance of different cultivars of a given crop or garden plant, and selecting of the most resistant cultivars against to the growth of fungus and natural production of aflatoxin. Using these cultivars in breeding programs, the infection of crops to the fungus and their probable contamination to aflatoxin is reduced. The present study is dedicated to the evaluation of the sensitivity of maize cultivars against *A. flavus* growth and its aflatoxin production.

MATERIALS AND METHODS

Aspergillus flavus isolate and maize cultivars

One strain of aflatoxigenic *A. flavus* (isolated from infected maize kernels) was used to study the growth rate of the pathogen and aflatoxin B_1 production on maize seeds (as *in vitro* conditions). This isolate could only produce aflatoxins B1 and B2 and was not able to producing aflatoxin G1 and G2. For evaluating the sensitivity of maize cultivars to *A. flavus* establishment and colonization, and the production of aflatoxin, five cultivars of maize, namely KSC400, KSC403, KSC600, KSC703 and KSC704, were selected. These cultivars were received from Seed and Plant Research Improvement Institute (Karaj, Iran).

Growth rates of Aspergillus flavus on different maize cultivars

To calculate the growth and colonization rate of the fungus on the kernels of different maize cultivars, 10 gram of kernels of each cultivar (in a completely random design with three replications) were surface-sterilized by 0.5% (w/v) sodium hypochlorite (NaOCl) solution and then soaked in sterilized distilled H_2O for 10 minutes to remove chlorine effect and to absorb the moisture required for germination. Then, the seeds of each cultivar were separately placed in a petri dish and 1ml of the fungal suspension (with a density of 1×10^6 spore/ml) was added to inoculate the kernels. The petri dishes were placed inside plastic containers filled with sufficient distilled water to provide the required moisture. They were then incubated at 26°C. Eight days after inoculation, the mean percent of growth and colonization of *A. flavus* on the kernels of different maize cultivars were calculated based on colonized kernel surface. The average colonization percentages of different cultivars were compared and analyzed by SPSS software and Duncan's Multiple Range Test.

Extracting and measuring the aflatoxin B1 produced in contaminated maize kernels

After calculation of fungal colonization of contaminated maize kernels (eight days post-inoculation), the kernels were dried by an oven to stop further growth of the fungus and toxin production. Afterwards, the aflatoxin content of each maize sample was measured by HPLC.

Quantification of aflatoxins produced in contaminated kernels

Assessment of aflatoxin production in maize cultivars were performed by using Waters e2695 (USA) HPLC, consisting of a chromolith C18, 100 mm × 4.6 mm, column (Phenomenex, USA) equipped by a fluorescence detector (Waters 2475, USA).The moving phase was water/methanol/ acetonitrile (60:20:20) with a flow rate of 2.5 ml/min. For aflatoxins (AFs) detection, the 365 nm and 435 nm were used as the excitation and emission wavelengths, respectively. The limit of detection (LOD) for AFs was 0.3 mg/ml. For this purpose, maize samples were slurried up with water in a ratio of 1/3 for 15 minutes, then slurried samples were extracted (30 g) with 90 ml of pure methanol on a Waring blender (Waring, USA) for 3 minutes and filtered through Whatman paper No. 4. Filtrates (8 ml) were mixed with phosphate buffer (42 ml). Immunoaffinity columns were used for purification of samples. First, 20 ml of phosphate buffer was passed (transmitted) through the column to ready it, then 25 ml of the extract mixed with the phosphate buffer was passed (transmitted) through the column; and the column was again washed with 20 ml of phosphate buffer. After drying the column, 1500 µl of methanol (with the purity special for liquid chromatography) was passed through the column. By one minute, 750 µl of methanol was again passed through the column. After collecting the total methanol phase, 1750 µl of water was added to it, and finally 200 µl of the preparation was syringed into the HPLC machinery. The quantification of aflatoxins B1, B2, G1 and G2 were fulfilled by comparison of the peak areas with the calibration curves prepared by aflatoxin pure standard solutions (Sigma-Aldrich, Milan, Italy). The linearity of the analytical response was checked by analyzing the calibration standards and using seven concentrations over the range 0.4–2.7 ng/ml aflatoxins B1. In the case of mobile phase HPLC, the methanol/water (40/60) used for the derivation of potassium bromide, nitric acid and Kobra cell. The chromolite column (10cm) with an internal diameter of 4.6mm (Partisil 5 ODS3, USA) was used. The column temperature was set to 35ºC with a moving phase of 2.5 mL/min. Fluorescent detector was adjusted at wavelengths ex=365 nm and em=435 nm.

RESULTS

Growth rate of Aspergillus flavus on different maize cultivars

Eight days after inoculation, the rate of *A. flavus* growth and colonization on kernels of different Maize cultivars were measured (Table 1). The results showed the growth and colonization rates are significantly different at a 5% level. Among all tested cultivars, KSC600 and KSC403 had the lowest and the highest rates of *A. flavus* growth, respectively. Therefore, KSC600 and KSC403 were the most and the least resistant to *A. flavus* colonization, respectively.

Table 1. Comparison of *Aspergillus flavus* growth on the seeds of tested maize cultivars

Maize cultivar	Average Growth (%)	Statistical Grouping* (α=0.05)
KSC 600	37.99	A
KSC 400	65.67	B
KSC 704	66.45	B
KSC 703	69.30	B
KSC 403	71.38	B

* Different letters following the averages show significant difference at 5% level (Duncan's Multiple Range Test).

Production of aflatoxin B1 in kernels of different maize cultivars

Quantities of aflatoxin B1 produced in maize kernels were measured by HPLC and presented in Table 2. The results showed the rates of aflatoxin B_1 production in different maize cultivars were significantly different at a 5% level eight days after inoculation. Among all tested cultivars, KSC403 and KSC600 had the highest and the lowest rates of aflatoxin B1 production, respectively.

Table 2. Comparison of average production of aflatoxin B1 in the seeds of tested maize cultivars.

Maize cultivar	Average aflatoxin B1 production (ng/g)	Statistical grouping* (α=0.05)
KSC 600	1054.66	A
KSC 703	1201.66	A
KSC 400	1409	A
KSC 704	2889.66	B
KSC 403	8366.33	C

*Different letters following the averages show significant difference at 5% level (Duncan's Multiple Range Test).

DISCUSSION

Aflatoxins are produced and released as secondary metabolites by some species of *Aspergillus*, including *A. flavus*, *A. parasiticus* and *A. nomius* under special conditions. Among these species, *A. flavus* is the most common species and has a particular economic importance [29]. *A. flavus* grows and contaminates many oilseeds and nuts, including peanuts, cottonseed, corn, and pistachios as desirable substrates. The use of resistant cultivars is always considered as the most basic way to manage this problem. Evaluating the resistance and susceptibility of different cultivars of a particular product and selecting cultivars resistant to *A. flavus* and aflatoxin are the challenges of selecting the appropriate cultivar. In breeding programs, these cultivars can use for reducing the contamination rate of food crops to aflatoxin.

The production of aflatoxins is controlled by some identified factors, including the genetic characteristics of toxigenic fungi and their physicochemical environment [30]. In other words, factors influencing the aflatoxigenic process include fungal properties, chemical composition of affected food materials, temperature, moisture and time. The most important factors are fungal properties and the chemical composition of food materials. Extensive research has been conducted in many countries on the role of various chemical and physical factors in the growth of fungi and the production of aflatoxins in food, and successful results have been obtained in this field.

In Lata *et al.* study on peanut, among 21 different genotypes , the four ones, namely IC-48, J-11, ICGV 89104 and ICGS-76, had the lowest rates of aflatoxin content (<25 ppb) and the highest rate of phenol (>1300 µg/g). Aflatoxin production had a negative correlation with phenol contents of peanut kernels (r^2=-0.42) and leaves (r^2=-0.37, $p<0.05$) [31].

In the other hand identification of cultivars resistant to growth and development of aflatoxigenic strains of *A. flavus* in different agricultural products has been done in the many countries and during numerous researches. Genetic resistance has been identified in aflatoxin-sensitive crops, including maize [32, 33], cotton [34], and peanut [35].

The best way to mitigate aflatoxin contamination is to use host resistance. Genetic diversity has been reported among peanut cultivars [36]. The results of the study of Ghewande *et al.* on the resistance of peanut cultivars to the growth of *A. flavus* and aflatoxin production showed that the level of resistance in various cultivars are different. This study showed that the amount of AFB1 in different cultivars was also very variable [36].

Gradziel and Wang also conducted a study on the susceptibility of California almond cultivars to *A. flavus* and found that the susceptibility of different maize cultivars to *A. flavus* is different [37]. Aim of the present research was determination of resistant maize cultivars to the *A. flavus* and aflatoxins production. For sustainable maize production, continuous identification of resistant maize cultivars and use of complementary sources of resistant genotypes is essential.

One of the problems in evaluating the resistance of maize cultivars to aflatoxin is the lack of a rapid screening tool in the laboratory. The kernel screening assay (KSA), has been developed for the study of aflatoxin resistance in cereals, including maize, GT-MAS:gk [38, 39]. Seeds are the primary target of aflatoxin-producing fungi. So seed-based resistance as the main target indicates host resistance. Accordingly, the KSA has the ability to separate sensitive seeds from resistant ones [38, 39].

The results obtained from the KSA study showed the two level of resistance, at the pericarp level and sub pericarp level. Injury to the pericarp causes a slight loss of resistance in the corn population. Significant expression of resistance has been observed even in injured kernels. This indicates that the sub pericarp is a resistance source. Further research emphasized the role of pericarp waxes in kernel resistance [40, 41] and showed qualitative and quantitative differences between pericarp waxes of GT-MAS:gk and sensitive genotypes [41, 42].

The KSA emphasized resistance sources in 31 inbreed assayed during a field experiment in Illinois [16, 39]. The KSA is potentially capable of detecting aflatoxin-resistant maize germplasm among inbred lines selected in Africa for ear-rot resistance, for inclusion as parents [43, 44].

The goal of this program is to combine ear-rot resistant lines in Central and West Africa with lines that resistant to other pathogens such as *A. flavus* and *Fusarium verticillioides* with resistance in inbred lines from the U.S. with the aim of developing resistant lines with desirable agronomic traits useful in U.S. plant breeding programs and in national programs of Central and West Africa [17]. The KSA method has comparative advantages over traditional screening methods [39]: (1) With this method, it is possible to perform experiments repeatedly and out of season; (2) it requires a small amount of kernels.; (3) detection of various mechanisms of resistance in the kernels is possible; (4) it is possible to disagree or approve field assessment (identify escapes); and (5) correlation of *in vitro* and *in vivo* has been described.

Therefore the KSA method can be an appropriate complement to standard breeding practices for germplasm initial assessment. However, further experiments in the fields are required for the resistance final confirmation.

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