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RESEARCH ARTICLE

In vitro evaluation of sorghum genotypes for their resistance to aflatoxin B_1 contamination

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Abstract

Sorghum is Ethiopia's main staple meal and the most significant nutritional security crop. Aflatoxin contamination in sorghum grains has been documented all across the world, including Ethiopia. Therefore, the use of resistant genotype is considered to be the most feasible means to mitigate the problem. Thus, 20 sorghum genotypes were evaluated *in vitro* for their reaction to aflatoxin contamination at Haramaya University School of Plant Sciences, crop protection laboratory. Mycotoxin analysis was done using enzyme-linked immunosorbent assay (ELISA). The findings showed that the sorghum genotypes tested for aflatoxin B_1 responses had considerably varied responses. Long Muyera was identified as the most sensitive genotype to aflatoxin B_1 contamination (34.1g kg^{-1}); with contamination levels significantly beyond the maximum tolerated limit (10g kg^{-1}). From the highland genotypes with a contamination level of 6.9g kg^{-1}, genotype W-5 (Weger-5) was relatively resistant to aflatoxin B_1.Teshale genotype was the least resistant of the lowland sorghum genotypes examined. Except for genotype Long Muyera, highland sorghum genotypes were less sensitive to aflatoxin B_1 contamination than lowland sorghum genotypes. As a result, it is worthwhile to choose and use genotypes that remain resistant in certain areas.

Keywords

Aflatoxin; ELISA; Genotypes; Resistance; Susceptible

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Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the world's fifth most significant cereal crop, following maize, rice, wheat, and barley (1).It is an important food security crop in Sub-Saharan Africa; provide food for more than 300 million people. It is grown in semi-arid, drought-prone areas where other crops cannot survive consistently. Sorghum is a major staple food crop in Ethiopia, placing in second only to maize in terms of overall production. It ranks third in terms of productivity per hectare, trailing only wheat and maize, and third in terms of area cultivated, trailing only teff and maize. It is grown in practically every region, covering almost 1.9million acres in total (2). Ethiopia is the origin and diversification centre for sorghum and the crop has been grown for many thousand years(3).Furthermore, Ethiopia, along with northern Sudan, is the greatest producer of sorghum in eastern and southern Africa.

Sorghum is grown in all regions of Ethiopia at altitudes ranging from 400m to 2500m, and it is one of the most important staple crops farmed in the country's poorest and most food insecure regions (4).It is Ethiopia's most significant nutritional security crop. In comparison to other major cereal crops, sorghum is also drought tolerant. However, the production has become affected by numerous diseases and has been contaminated by a variety of field

and storage mould-producing fungi. One of the most important groups of fungi impacting sorghum in the field and during postharvest periods, including storage, is Aspergillus(5-9).

Aflatoxin contamination and Aspergillus infection have been detected in sorghum seeds or grains all over the world(10), particularly in Ethiopia(7,11). Aflatoxins are naturally occurring toxic secondary metabolites of storage fungi (*Aspergillus flavus* and *A. parasiticus*) that form in the majority of agricultural commodities stored in hostile habitats, temperatures, and water activities. These two species are widespread and common throughout the world's tropical and subtropical climates. Aflatoxins have been identified as a pollutant in agricultural food products, specifically grains and animal feeds(12). Aflatoxin exposure poses the greatest danger in tropical and subtropical areas, where *Aspergillus* spp. thrive, and poses the least risk in places with both cooler and drier off seasons (13).Consumption of aflatoxin-contaminated grain can result in aflatoxicosis(14), which are among the most common mould-related disorders worldwide.

The introduction of resistant crop cultivars is thought to be the most practicable way to offset damage caused by grain moulds and subsequent mycotoxin contamination(15). A field experiment was carried out in Ethiopia with different agro ecologies to evaluate sorghum genotypes for resistance to aflatoxin and fumonisin-producing fungi as well as to determine variation among sorghum genotypes as substrates for fungal invasion(9), and they reported variation of sorghum genotypes in their reaction. Menkir *et al.* (15) conducted an experiment to determine the relative contribution of fungal species to grain mould damage and the level of diversity in sorghum for grain mould resistance. Their findings suggest that it is possible to discover sources of resistance to certain fungus in various sorghum germplasm banks. However, data on the sensitivity of the currently extensively farmed sorghum genotypes to aflatoxin contamination in Ethiopia is insufficient. As a result, it is prudent to assess the sensitivities of released and adapted sorghum varieties to aflatoxin contamination. Thus, the current experiment was carried out with the particular goal of evaluating sorghum genotypes for their reactivity to aflatoxin *in vitro*.

Materials and Methods

Description of experimental treatments and experimental design

The experiment was conducted at Haramaya University, School of Plant Sciences; Crop Protection Laboratory. Twenty sorghum grain genotypes widely cultivated in both lowland and highland of the country obtained from Ethiopian Agricultural Research Centres (Melkassa and Sirinka) and Haramaya University, Ethiopia (Table 1) were evaluated *in vitro* for their resistance to the contamination of Afaltoxin B_1.The experiment was laid out in a complete block design with three replications.

Inoculum preparation and inoculation

Aspergillus flavus was isolated from heavily infested sorghum grain, tested positive for aflatoxin B_1 contamination, and grown on AFPA (*Aspergillus flavus* and *parasiticus* agar) media. The twenty examined sorghum grain genotypes were individually soaked in water overnight (1kilogram), washed, transported to one litre containers, and autoclaved. Each autoclaved sorghum grain sample received 250g of pure fungal cultures cultivated on AFPA medium with spore densities of $10^6 ml^{-1}$ (5ml).The inoculated sorghum grain was incubated at 27°C for 7days with three replications till the fungus colonised the grain (Figure 2).

Table 1. Description of the tested sorghum varieties

No	Variety Name	Pedigree	Maintainer	Year of release	Altitude
1	Abesher	P-9403	MARC	2000	Lowland
2	Alemaya	Al-70	MARC	1970	Highland
3	Birhan	PSL5061	SARC	2002	Lowland
4	Chelenko	ETS 1176	MARC	2005	Highland
5	ETS3255	ETS3255	MARC	1978	Highland
6	Fendisha Red		HU	2015	Highland
7	Fendisha White		HU	2015	Highland
8	Gambella	Gambella 1107	MARC	1976	Lowland
9	Gubeye	P-9401	MARC	2000	Lowland
10	Hormat	ICSV 1112 BF	SARC	2005	Lowland
11	Long Muyera		HU	2009	Highland
12	Meko	M-36121	MARC	1997	Lowland
13	Melekam	WSV-387	MARC	2009	Lowland
14	Misker	PGRC/E#69441x P-9401	SARC	2007	Lowland
15	Muyera 1	EST-1005	HU	2010	Highland
16	Muyera 2		HU	2010	Highland
17	Raya	M36-121	SARC	2007	Lowland
18	Teshale	3443-2-0P	MARC	2002	Lowland
19	W-3 (Weger 3)	Farmers variety	-	-	Highland
20	W-5 (Weger 5)	Farmers variety	-	-	Highland

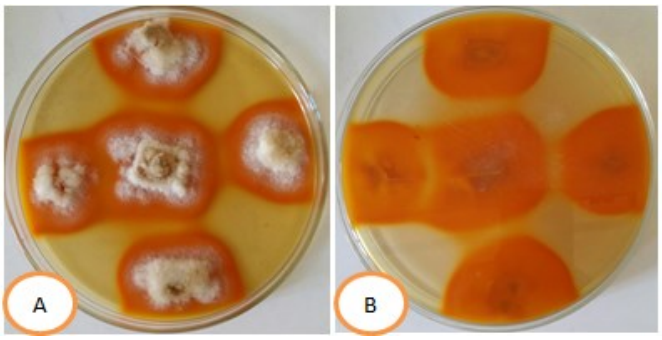

Figure 1. *Aspergillus flavus* isolated from highly infested and aflatoxin B_1 tested positive sorghum grain on AFPA media. A, front side and B is back side of the culture

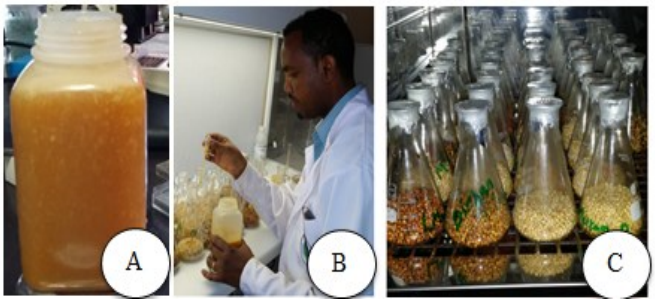

Figure 2. Inoculums preparation, inoculation and incubation. A) *Aspergillus flavus* inoculum at concentrations of 10^6 ml^{-1}, B) Inoculation and C) Incubation of inoculated sorghum genotypes

Aflatoxin B_1 extraction and analysis

After completely mixing the infected and fully infested sorghum grain genotypes separately, a representative subsample of 100g sorghum grain was obtained and ground to fine particle size for aflatoxin B_1 analysis using a Thomas-Wiley laboratory mill (Model 4, Thomas Scientific TM; USA). For aflatoxin B_1 (AFB_1) extraction, 5g of thoroughly mixed ground grain samples were separately diluted with 25 ml of 70% methanol, CH_3OH (1:5 w/v). The diluted samples were shaken for 2-3minutes before being filtered through Whatman No.1 filter paper (Whatman Inc., Clifton, NJ, USA) and used for analysis without any further dilution. Aflatoxin B_1 concentration in the sample was determined using enzyme-linked immunoassay (ELISA). This technique has been used for analyses of aflatoxin B_1 (16).There were no clean-up procedures carried out during the process (17).

Aflatoxin B_1 assay procedure

The ELISA was performed according to the manufacturer's instructions (Helica Biosystems Inc, Santa Ana, CA). Wondimeneh *et al.* (11) provide a brief explanation of the assay technique. Before placing the relevant microwell cartridges and antibody-coated microtiter wells into the microwell holder for each standard and sample to be analysed, all reagents were warmed to room temperature. For aflatoxin B_1, 200µl conjugate was pipetted into the dilution well, and 100µl aflatoxin B_1 standard solutions and sample extracts were pipetted and mixed three times with a priming pipettor into the conjugate-containing dilution wells. The 100µl mixed solution from each dilution well was transferred to the appropriate antibody-coated microliter well and incubated at room temperature for 15 minutes. After incubation, the liquid was emptied from the microwell, and the residual liquid was collected by tapping the microwells holder upside down on a clear filter towel. The wells were filled with distilled water, then drained, and the residual liquid was evacuated as before. For aflatoxin B_1, this washing technique was repeated five times. To remove residuals, the microwell holder was tapped upside down on a clear filter towel after each washing stage. Finally, a 100µl substrate reagent was added to each well and incubated at room temperature for 5minutes before covering with aluminium foil to avoid direct light.

The optical density (OD) was evaluated using a microtiter plate reader and a 45nm filter after 100µl of the stop solution was added to each well. The amount of aflatoxin B_1 in the sample extracts was evaluated using an absorbance data calibration curve for standards with concentrations of 0, 1, 2.5, 5.0, 10.0, and 20.0g kg^{-1} for AFB_1. The optical density (OD) of the sample was multiplied by the OD of the zero standards to obtain the mean absorbance of standards or samples/mean absorbance of negative controls (%B/Bo). The standard concentrations were plotted on a log scale along the x-axis, with the corresponding %/Bo values plotted on the y-axis. Correlation values (R_2) ranged between 0.960 and 0.982. The limit of detection was approximated to obtain a%B/Bo by taking the mean of 20 repetitions of blanks and subtracting 2 times the standard deviation of the blanks. Detection limits ranging from 0.01-0.03µg kg^{-1} were obtained by interpolating from the standard curve.

Data analysis

SAS software was used to do analysis of variance (ANOVA). The least significant difference (LSD) at the 0.05 probability level was used to separate treatment means.

Result and Discussion

Reaction of tested sorghum genotype for Aflatoxin B_1 contamination

The analysis of variance revealed a highly significant ($p<0.05$) difference between the sorghum genotypes tested *in vitro* for their resistance for aflatoxin B_1contamination (Table 2).The level of aflatoxin B_1 contamination ranged from 6.9µg kg^{-1} to 34.1µg kg^{-1}.The highest (34.1µg kg^{-1}) aflatoxin B_1 contamination was recorded from sorghum genotype Long Muyera whereas the smallest (6.9µg kg^{-1}) aflatoxin B_1 contamination level was recorded from sorghum genotype W-5 (Weger 5). Fendisha Red was the next highly (29.0µg kg^{-1}) contaminated genotype and followed by sorghum genotype ETS3255 (27.2µg kg^{-1}). The variation of the tested genotypes in their aflatoxin B_1 contamination were also observed when they are tested under field condition by Wondimeneh *et al.*(9).They reported as Long Muyera had higher level of aflatoxin B_1 contamination in two consecutive years of experiment. However, the current study's findings are consistent with those of Prom *et al.*(18),who found no substantial contamination severity for most sorghum lines inoculated with toxigenic fungus species. This *in vitro* and the previous field experiment result reported by Wondimeneh *et al.* (9)

Table 2. Reaction of sorghum grain genotypes for the contamination of Aflatoxin B_1 under *in vitro* experiment.

Sorghum genotypes	Aflatoxin B_1 concentration (µg kg^{-1})
Abesher	22.9bc +3.6
Alemaya	24.6bc +3.4
Birhan	17.4 cdef +9.7
Chelenko	24.5bc +2.6
ETS3255	27.2ab +3.6
Fendisha Red	29.0ab +2.3
Fendisha White	14.2 efg +3.8
Gambella	25.5b +2.9
Gubeye	23.3bc +3.2
Hormata	24.3bc +5.0
Long Muyera	34.1a +3.0
Meko	15.6 efg +6.5
Melekam	11.0 efg +1.4
Misker	26.3b +3.0
Muyera 1	12.2 efg +4.8
Muyera 2	21.9 bcd +4.9
Raya	13.2 efg +6.4
Teshale	10.2 fg +3.8
W-3 (Weger 3)	18.0 cde +4.1
W-5 (Weger 5)	6.9 g +2.3
LSD	7.2768
CV	21.921

indicated that sorghum genotype Long Muyera is most susceptible genotype for aflatoxin B_1 contamination. The aflatoxin B_1 concentration recorded from Long Muyera in both *in vivo* and *in vitro* experiments was above the maximum (10g kg-1) standard in East African Community goods designed for direct human consumption(19). In this *in vitro* study, among the tested sorghum genotypes, except sorghum genotype W-5 (Weger 5), all other exhibited aflatoxin B_1 contamination level above the maximum limit in foods intended for direct human consumption in East African Community.

The sorghum genotypes used in this experiment were cultivated in two different agro ecologies of Ethiopia (Fig. 3); ten of them were cultivated in lowland and others were cultivated in mid to highland agro ecologies. In average, the lowland sorghum genotypes were relatively contaminated with aflatoxin B_1 lower (18.97µg kg^{-1}) than genotypes cultivated in mid to highland area (21.26µg kg^{-1}). The highest and lowest aflatoxin B_1 contamination levels were recorded from sorghum genotype Long Muyera and W-5 (Weger 5), respectively both from highland sorghum genotypes. From the lowland sorghum genotypes Teshal and Misker showed lowest and highest records of aflatoxin B_1 contamination, respectively. This genotype response result against aflatoxin B_1 contamination with respect to the location of the cultivation agro ecologies of the test genotypes is in

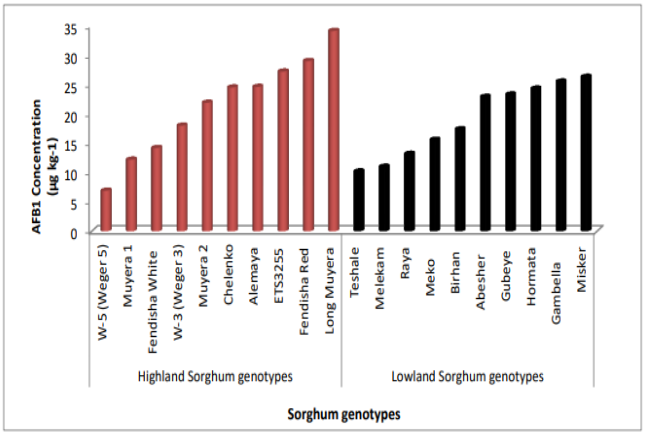

Figure 3. Reaction of highland and lowland sorghum genotypes for Aflatoxin B_1 contamination under *in vitro* condition

agreement with the result reported by (9) as they tested the genotypes *in vivo* condition. Long Muyera, which was highly contaminated with aflatoxin B_1, is also local and widely cultivated sorghum genotype cultivated in high and mid-altitude of eastern Ethiopia farmers and they store the produce for a minimum of six months in underground pit storage. If harvested sorghum grain is stored for an extended period of time, fungal infection and aflatoxin contamination will persist, particularly if the storage environment is humid and warm (20). Hell *et al*. (21) showed increased aflatoxin contamination of maize grain with storage duration, and Gemede (22) in sorghum.

Conclusion

In this *in vitro* investigation,20 sorghum genotypes were examined for their response to aflatoxin contamination and sorghum genotype W-5 (Weger-5) was found promising for resistance reaction to aflatoxin B_1 contamination from sorghum grain genotypes cultivated in highland areas due to its relatively low level of contamination (6.9g kg^{-1}). Teshale and Melkam sorghum genotypes may be suitable for lowland settings since them are resistant to aflatoxin B_1 contamination. Long Muyera was the most sensitive genotype examined, with aflatoxin B_1 contamination above the maximum acceptable limit of 10g kg^{-1}. Long Muyera is the local and commonly cultivated genotype in eastern Ethiopia's high- and mid-altitude farmers, who keep the crop for at least six months in underground pit storage. According to study, storing sensitive sorghum grain for an extended period of time causes fungal infection and aflatoxin contamination, especially if the storage environment is moist, humid, and warm. As a result, selecting sorghum genotypes resistant to aflatoxin B_1 contamination is worthwhile.

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Compliance with ethical standards

Conflict of interest: The author has no conflicts of interest to declare.

Ethical issues: None.

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