Screening methods, genetic variability and correlation studies for *Aspergillus flavus* resistance in sub-tropical maize (*Zea mays* L.)

Pooja Kumari[1], Robin Gogoi[2]*, Basavaraj Y. B[2], Srinivasa N[2], Ashwini Kumar[2], Meena Shekhar[3], Mallikarjuna MG [4]*.

1 Division of Plant Quarantine, ICAR-National Bureau of Plant Genetic Resources, New Delhi 110012, India

2 Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India

3 Unit office, ICAR-Indian Institute of Maize Research, New Delhi 110012, India

4 Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India

*Corresponding author: E-mail: r.gogoi@rediffmail.com

Keywords: *Aflatoxin B1, Aspergillus flavus, ELISA, Maize, Kernel Screening Assay, Side Needle Spore Suspension Inoculation.*

Abstract

Maize is an important cereal crop and a significant contributor to global food and nutritional security. The consumption and nutritional qualities of maize are severely affected by aflatoxin B1. Aflatoxin B1 is a potent carcinogenic, mutagenic and teratogenic secondary metabolite produced by *Aspergillus flavus*. Nonchemical and sustainable management of aflatoxin B1 contamination necessitates resistant maize inbred lines for aflatoxigenic *A. flavus* and subsequent production of aflatoxin B1. In the present investigation, we have screened thirty diverse Indian maize inbred against aflatoxigenic isolates AF1 and AF3 using kernel screening assay (KSA) method and indirect competitive ELISA (Enzyme-Linked Immunosorbent Assay). The results showed the lower *A. flavus* colonization (13.33-23.33%) in five maize inbred lines viz., M6, M7, M9, M17 and M22. Similarly, the inbred lines M3, M8, M14, M15 and M17 showed lower aflatoxin B1 concentration (0.09-7.03 µg/g) under both AF1 and AF3 infections. We showed the association and reliability between the rapid lab based KSA screening Side Needle Spore Suspension (SNSS) inoculation methods with five tolerant inbred lines and susceptible lines M2, M4 and M5 and check line M24. The five inbred lines viz., M6, M7, M9, M17 and M22 showed the least infection in field condition (1-2%) compared to M2, M4, M5 and check M24 (92%). Our investigation showed that KSA could be employed for rapid screening of maize genotypes for aflatoxin tolerance and the SNSS inoculation method is more effective in the field. Further, five inbred lines M6, M7, M9, M17 and M22, identified as the resistant source to aflatoxin, could be employed as candidates for basic and applied maize research in the Indian maize breeding programme.

Abbreviations

AF1– aflatoxigenic isolate 1
AF3– aflatoxigenic isolate 3
AFB1– aflatoxin B1
ANOVA– analysis of variance
CFU– colony forming unit
ELISA– enzyme-linked immunosorbent assay
IARC– International Agency for Research on Cancer
ICAR– Indian Council of Agricultural Research
IIMR– Indian Institute of Maize Research
KSA– kernel screening assay
MSS– mean sum of square
PBS– phosphate-buffered saline
PCoA– principal co-ordinate analysis
RBD– randomized block design
SNSS– side needle spore suspension
TSS– total sum of square
WIM– whorl inoculation method

Introduction

Aspergillus flavus is ubiquitously present in many ecological niches and produces a range of toxins called aflatoxins. Aflatoxin is a concern for global food safety (Dorner 2002). Many agricultural commodities infected with *A. flavus* and aflatoxin contamination are extremely harmful to human and animal health. The aflatoxins are carcinogenic, mutagenic and potent immunosuppressive agents and ubiquitous contaminants of food in the developing world (Kamkar *et al.* 2014). Among aflatoxins, AFB1, AFB2, AFG1 and AFG2 are major aflatoxins and aflatoxins M1 and M2 are respective metabolic products (Samuel *et al.* 2013; Squire 1981). The International Agency for Research on Cancer (IARC)

Table 1 - The list of diverse maize inbred lines used for screening against aflatoxigenic isolates of *A. flavus*, AF1 and AF3 through kernel screening assay colonization and Aflatoxin B1.

S. No.	Inbred lines	Genotypes
1.	M1	CM 123-1-1-1-⊗-1-1-1
2.	M2	(6- Mixture purple)-2-⊗ -1-1
3.	M3	PFSR (White)- ⊗-1
4.	M4	CML249-1-2-1-1-1-1-⊗-2-1-2
5.	M5	PFSR (Y)-C0-3⊗-1-2-1-⊗-1-1-1
6.	M6	Yellow grains-1- ⊗-2-1
7.	M7	CM 115-4-2-3-2-2-1-2-2-1- ⊗- 1-1-2
8.	M8	V338-1⊗-1-1-1-1-⊗-1-1-1
9.	M9	CM 105-2-1-1-⊗-1-1
10.	M10	TL02A-1184A-32-4-1-1-1-2-2-1- ⊗-3-1-1-2
11.	M11	V338-1⊗-1-1-1-1- ⊗-1-1
12.	M12	CM 142-1-2-1- ⊗ -1-1-2-1
13.	M13	PFSR (Y)-C0
14.	M14	CML 297-1-2- ⊗-1-1-1 A
15.	M15	CML 342-1-1-2-⊗-1-1-2
16.	M16	Indimyt-100
17.	M17	Deep Orange S-1(1-Y Cob S3)-2-⊗-2-1-2 B
18.	M18	(6-Mixture purple) -3- ⊗-1-3
19.	M19	CM501
20.	M20	Indimyt-100 ⊗-1-1-1-1-1-1
21.	M21	CM 111-1-1-1-⊗-1-1-2
22.	M22	Indimyt-345 (dent)
23.	M23	PFSR (Y) -C1-A-B1 White heart
24.	M24	Yellow-1- ⊗-1-1
25.	M25	Cream Yellow (P) -1- ⊗- 2-1-1-1
26.	M26	JCY3-7-1-2-1-'b-6-1-2-1-1-1-1-2- ⊗-1-1
27.	M27	CM 202-2-1-1- ⊗ - 1-1-1
28.	M28	AF-04-B-5796-A-7-1-2-2-1-2-1-1- ⊗-1-1-1 (B)
29.	M29	CML163-1-1-⊗-2-1-1
30.	M30	(6-Mixture purple)-1- ⊗-1-2

classified AFB1 as a primary group of carcinogenic compounds (Reddy *et al.* 2009; Tavakoli *et al.* 2013). Farm animal mortality and reduced productivity are associated with the consumption of aflatoxin B1 (AFB1) contaminated feed. Consumption of AFB1 contaminated foodstuff leads to liver cancer development in humans (Hsieh 1989; Payne and Widstrom 1992). Additionally, grains contaminated with AFB1 can even lead to death in acute cases. The detrimental effect of AFB1 on human and animals have been reported for decades. However, effective management of aflatoxin contamination remains challenging.

Maize is a crop of global importance owing to its contribution to food and nutritional security (Agrawal *et al.* 2018). Globally, various abiotic and biotic stresses limiting maize production and quality are reported in literature (Babu *et al.* 2020; Jeevan *et al.* 2020; Gioi *et al.* 2017; Karjagi *et al.* 2017; Mallikarjuna *et al.* 2020). Ear rot in maize caused by *A. flavus* one of the significant contributors to food insecurity due to aflatoxin contamination. Notably, *A.flavus* infection in maize is a major problem in developing nations where no proper monitoring for pre-harvest, post-harvest and storage techniques of agricultural commodities are adopted. Maize kernels serve as a potential substrate for *A. flavus* infection and the production of AFB1. The unpredicted fluctuation in the weather parameters in the era of climate change can escalate *A. flavus* infection and aflatoxin production under field conditions.

The causal agent *A. flavus* prefers maize silk for its colonization and later invades maturing maize kernels under field condition (Payne *et al.* 1988). Subsequently, infection develops before harvesting itself, and it continues till storage. Therefore, it is always advisable to follow pre-harvest measures to control aflatoxin contamination. However, it is quite challenging to follow and practice the pre-harvest controlling methods in maize. Therefore, the use of host-plant resistance is the best management strategy to overcome this acute problem. Various biochemical and morphological factors govern

Table 2 - The descriptive statistics of *A. flavus* colonization and aflatoxin B1 concentration in the kernels of diverse maize inbred lines.

	Kernel Screening Assay				Aflatoxin B1(µ/g)	
	Isolate AF1		Isolate AF3		Isolate AF1	Isolate AF3
	Colonization (%)	Scale	Colonization (%)	Scale		
Min.	16.66	1.0	13.33	1.0	0.09	0.34
Max.	83.33	5.0	83.33	5.0	7.02	7.03
Mean	53.66	3.0	50.66	3.0	2.11	2.52
SE (±)	1.96	-	2.28	-	0.22	0.20
N	30	30	30	30	30	30

resistance to maize kernels against *A. flavus* infection and subsequent AFB1 synthesis. Resistant maize inbred lines inhibit *A.flavus* colonization, and subsequent aflatoxin accumulation in maize kernels (Brown *et al.* 2003). Presently, the search for available resistance sources in maize against *A. flavus* is getting attention for sustainable and eco-friendly management of aflatoxin contamination. In India, few reports are available on the screening of maize genotypes for *A. flavus* colonization and AFB1 production. However, much of these studies are conducted i) by collecting randomly genotypic samples from different geographic regions (Bhat *et al.* 1997; Vijayasamundeeswari *et al.* 2009; Shekhar *et al.* 2011) or ii) by simple lab-based assays (Hajare *et al.* 2006; Shekhar *et al.* 2011). The selection of aflatoxin resistant maize inbred lines for the derivation of genetic stocks, lines and new hybrids necessitates the planned screening of maize inbred lines. Therefore, the present investigations were framed to perform 1) an in-vitro screening of maize inbred lines against *A. flavus*, 2) to validate the efficiency of the in-vitro method with in-vivo screening in the field and 3) to identify promising maize inbred lines against *A. flavus* colonization and subsequent AFB1 accumulation

Material and methods

Maize inbred lines

The seeds of thirty-five maize inbred lines were collected from the ICAR-Indian Institute of Maize Research (IIMR), Ludhiana, India. The inbred lines were sown in *Kharif*-2018 at Research Farm, ICAR-Indian Agricultural Research Institute, New Delhi (216 m above mean sea level; 28°36' 50" N, 77°12' 32" E). The lines showing uniformity were chosen for subsequent experimentation (Table 1). The kernels of the selected 30 inbred lines were properly cleaned, labelled and stored at 4°C

Fungal isolates

Two potential isolates of aflatoxigenic *Aspergillus flavus* named AF1 and AF3 were used in the experiment (Kumari *et al.* 2020). AF1 isolate was collected from Hyderabad belonged to the peninsular zone of India (Zone-IV). Whereas, AF3 isolate collected from Assam

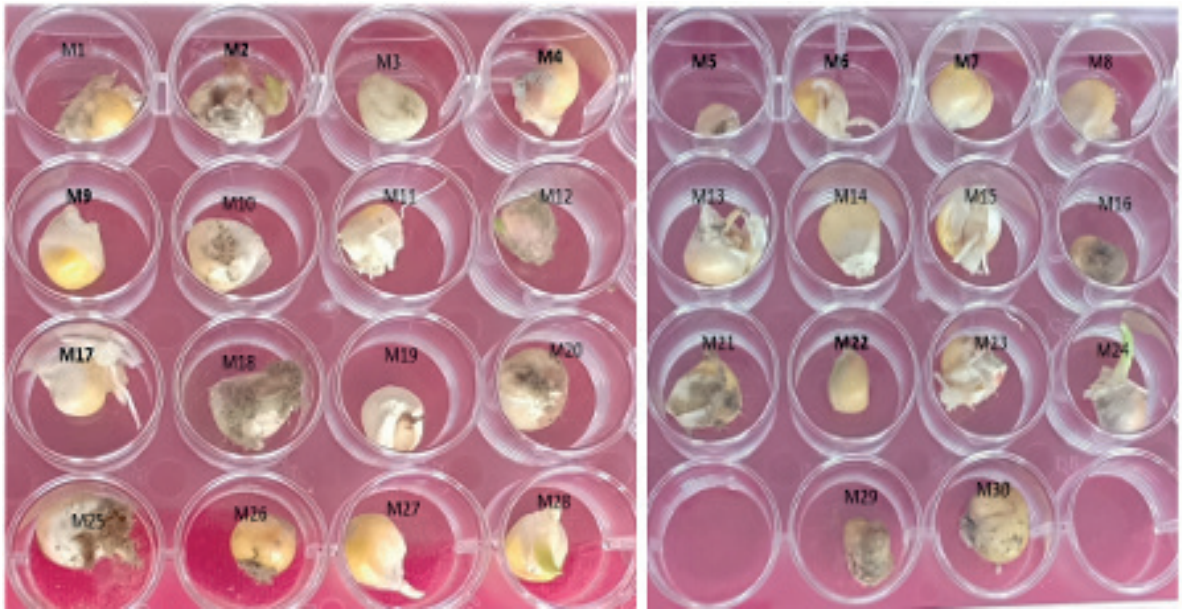

Fig. 1 - Colonization of aflatoxigenic isolate of *Aspergillus flavus*, AF1 on the kernels of inbred maize lines namely M1 to M30.

Table 3 - Analysis of variance for *A. flavus* colonization in Kernel Screening Assay (KSA) and Side Needle Spore Suspension Inoculation (SNSS) inoculation method under field condition.

Source	Kernel Screening Assay					Side Needle Spore Suspension Inoculation		
	AF1		AF3			AF1		
		TSS	MSS	TSS	MSS	DF	TSS	MSS
Block	2	46.66	23.33^{ns}	106.66	53.33^{ns}	2	10.12	5.06^{ns}
Gen	29	27823.33	959.42***	37693.30	1299.77***	8	29539.85	3692.48***
Error	58	2820.00	48.62^{ns}	3760.00	64.83^{ns}	16	55.70	3.48^{ns}
Total	89	30690.00		41560.00		26	29605.68	

Note: TSS: total sum of square; MSS: mean sum of square; ns: non-significant; *** $p < 0.001$.

represented Northern Hill Zone (Zone-I). Both AF1 and AF3 are potent aflatoxin B1 (AFB1) producers with 43.87 and 33.17 µg/g of aflatoxin production, respectively (Kumari *et al.* 2020).

Kernel screening assay

The kernel screening assay (KSA) of all thirty inbred lines was performed as reported by Tubajika and Damann (2001). For each replication of an inbred line, ten healthy dried kernels were taken, surface disinfected with 0.5% sodium hypochlorite and rinsed with sterile distilled water. Afterwards, these kernels were dipped into spore suspension of *A. flavus* with spores counts of 105 spores/ml for 20 minutes. Spore treated kernels were individually placed in culture plate wells (24-well Nunc multidish, Nunclon, Denmark). Plate incubation was done at 26 ± 2°C for 15 days, at a relative humidity of 95 ± 2%. At the final stage of incubation, kernels were enumerated based on fungal colonization, and the maize inbred lines were rated from 1-5 (1=1-20%, 2=21-40%, 3=41-60%, 4=61-80% and 5=81-100%) of the kernels infected by fungus bearing conidia (Hajare *et al.* 2006). The experiment was repeated three times.

Detection of AFB1 by indirect competitive ELISA

The kernels treated with the AF1 and AF3 isolates were subjected to AFB1 estimation. Maize kernels (100 mg) were ground in 1 ml coating buffer, centrifuged at 8000 rpm for 3 min, and the supernatant was separated. The supernatant was used to detect AFB1 level by indirect competitive enzyme-linked immunosorbent assay (ELISA) (Reddy *et al.* 2001). Briefly, 150 µl of supernatant was used to coat wells of microtiter plates. AFB1 standard (3 µl) dissolved in 150 µl of coating buffer used as a positive control, whereas negative control was prepared separately with healthy maize kernels. After coating, ELISA plates were incubated at 37°C for 1 hr. The plates were washed three times with phosphate-buffered saline (PBS) containing tween-20 (PBST) at 5 min interval. After the washing step, blocking was done using PBST-BSA (300 µl to each well) followed by ELISA plate incubation at 37°C for 1 hr. The plates were washed three times with phosphate-buffered saline (PBS) containing tween-20 (PBST) at 5 min interval. AFB1 primary antibody was dissolved in PBST-PO (1X PBST, 1% PVP and 0.1% ova albumin) in 1:1000 dilution. Subsequently, 100 µl of primary antibody was coated to each well and plates were incubated at 37°C for 1 hr. Plates washing with PBST was repeated three times at 5 min interval. This step was followed by the addition of alkaline phosphatase labelled goat antirabbit IgG conjugate diluted to 1:30,000 in PBST-PO. Plates were incubated at 37°C for 1 hr, followed by three times washing with PBST. The solution of p-nitrophenyl phosphate prepared in 10% diethanolamine was used as substrate. Later 100 µl of substrate buffer was added to each well, and plates were incubated at room temperature and then read in an ELISA reader at 405 nm. The concentration of AFB1 in the samples was calculated as per Shekhar *et al.* (2011).

Validation under field condition

Total five maize inbred lines viz., M6, M7, M9, M17, and M22 showed minimal *A. flavus* growth and aflatoxin B1along with other susceptible lines (M2, M4 and M5) and check (M24) were tested during Kharif 2019. The experiment was laid out in randomized block design (RBD) with three replications. The spacing was 45 × 10 cm (row × plant) in each row was maintained. The standard package of practices was followed as per Jeevan *et al.* (2020).

Mass multiplication and preparation of inoculum

The aflatoxigenic isolate of *A. flavus* inoculum was prepared for whorls inoculation and side needle inoculation methods. For whorls inoculation, the highly aflatoxigenic isolate of *A.flavus*, AF1, was used for mass multiplication. The inoculum of *A. flavus* was developed on wheat seeds as per Bock and Cotty (1999). Clean wheat seeds were properly washed and soaked overnight. Then 1/4th of the volume of 250 ml conical flasks were filled with wheat seeds, and they were autoclaved two times on the same day at 15 lbs for 30 min. A week-old fresh culture of *A.flavus* isolate AF1 was used to inoculate the autoclaved wheat seeds by transferring a bit of actively growing mycelium. The flasks were incubated at 29°C for six days with

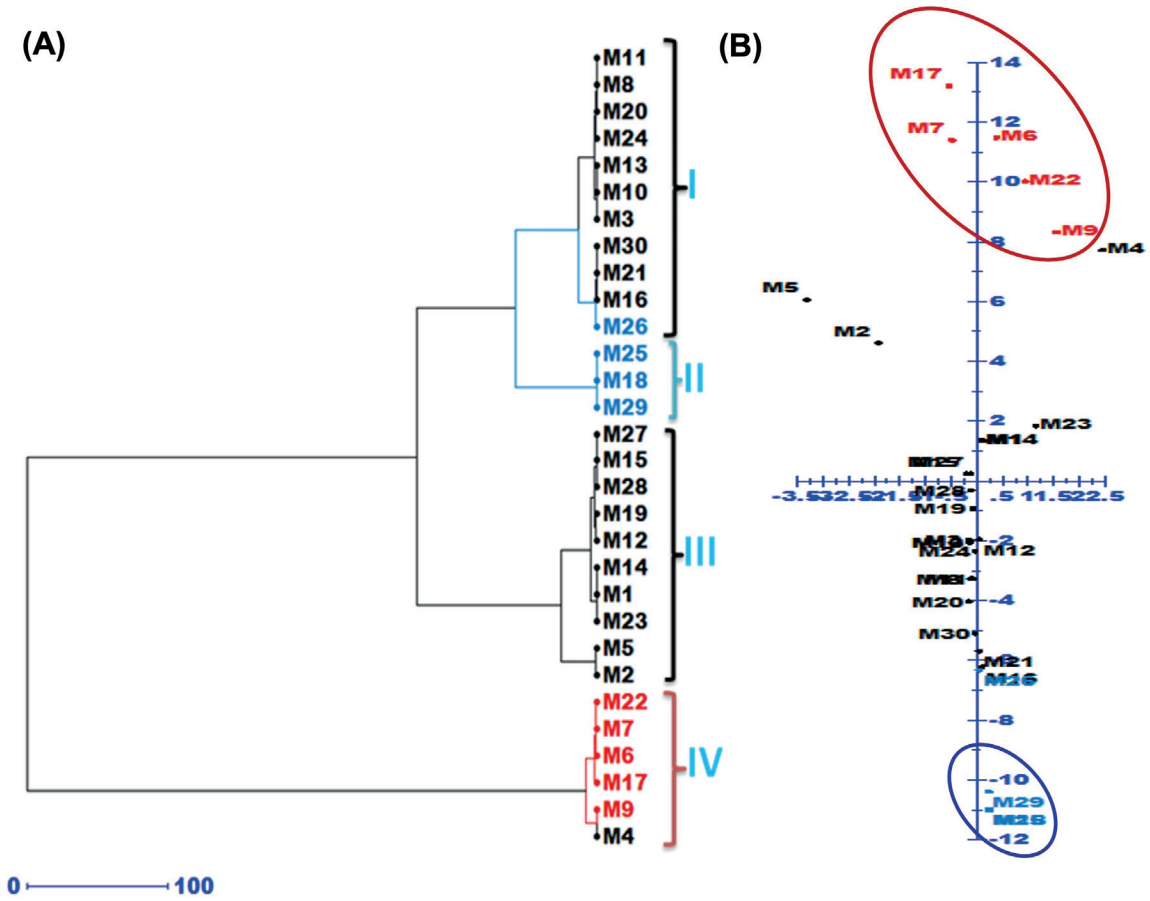

Fig. 2 - Genetic relation of thirty maize inbred lines response to *A. flavus* infection. (A) The dendrogram showing hierarchical relationship genetic relationship of maize inbred lines. The highly resistant and susceptible lines are colored with red, blue color, respectively. (B) The principal co-ordinate analysis showing grouping of maize inbred lines based on disease scoring parameters. The resistant lines are marked with red text and red eclipse, susceptible lines were marked with blue text and blue circle.

intermittent shaking of the seeds for every two-day interval to ensure uniform growth of *A.flavus*. After six days, the seeds covered with fungi were taken out, and the infected wheat kernels were placed in a sterile blotter paper sheet and dried in the incubator at 37°C for two days. The dried seeds were stored at room temperature in the airtight plastic container.

For side needle inoculation, pure culture of *A.flavus* isolates (AF1) was inoculated into 100 ml sterile 2% sucrose solution and incubated at 28°C for 10 days. When conidia were formed, the suspension was filtered using a sterile cheesecloth. The concentration of spore suspension was adjusted to $9x10^7$ CFU/ml with a haemocytometer and stored at 4°C for subsequent inoculation

Field inoculation methods

Two methods viz., whorls inoculation and side needle spore suspension inoculation methods used for artificial inoculation studies of *A.flavus* on maize under field condition. Each method was executed with three replications along with the control. In the whorl inoculation method (WIM), each plant was inoculated with infected wheat kernels inoculum (1 g) into the whorl of maize plants at 40 days after sowing. In the side needle spore suspension (SNSS) inoculation method, the primary ear of each plant was injected with 3.4 ml of the spore suspension by using a repeater syringe at 7-10 days after silking (Zummo and Scott 1989). The scoring of the *A. flavus* infection was done as percent infected cobs to inoculated cobs with the scale of Shekhar *et al.* (2012) (Table S1).

Statistical analysis

The descriptive statistics, correlation and t-tests were performed using SAS v 9.2. The analysis of variance of randomised complete block design was performed as per the following model: $Y_{ij}=\mu+\alpha_i+\beta j+\varepsilon_{ij}$; where μ is the mean fungal colonization, α_i is the i^{th} genotype effect, βj is the j^{th} block effect, and ε_{ij} is the random error. The diversity and principal co-ordinate analyses

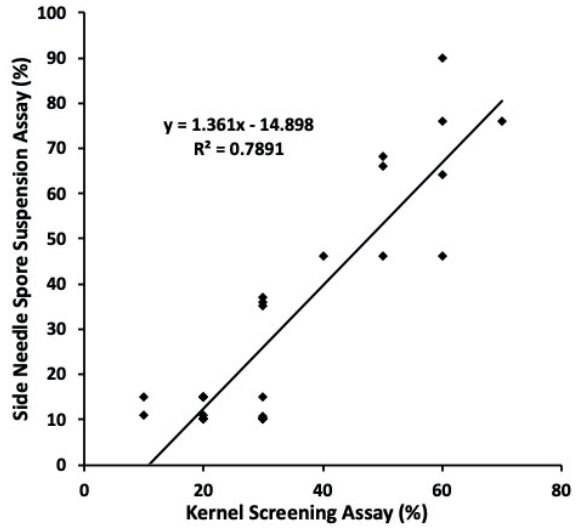

Fig. 3 - The correlation graph depicting the association between lab-based kernel screening assay (KSA) and side needle spore suspension assay (SNSS) in field condition.

(PCoA) were performed with DARwin v. 6.0 (Perrier *et al.* 2003).

Results and Discussion

Aflatoxin contamination is ubiquitous in the developing world. Several research findings highlighted the prominent contamination of agricultural commodities, including maize and food products with *Aspergillus flavus* and aflatoxin B1 (Berthier and Valla 1998; Reddy *et al.* 2004; Bennett and Klich 2003). In India, aflatoxin contamination in maize has become an important issue due to increased maize consumption pattern over the years. The major proportion of maize in the south and south-east Asia is diverted to feed and food (Agrawal *et al.* 2018; Mallikarjuna *et al.* 2014). The intake of aflatoxin-contaminated maize by farm animals, birds and humans allows entry of aflatoxin to food and environmental chain, which subsequently adversely affect the consumers health (Begum and Samajpati 2000). Exploitation of host genetic resistance is one of the reliable approaches to manage aflatoxin contamination in maize and necessitates reliable phenotyping methods and resistance sources. Therefore, the present investigation was framed to assess the screening methods and to identify the aflatoxin resistance sources in subtropical maize germplasm. The results are discussed under various section as follows

Descriptive statistics and diversity analysis for A. flavus colonization and aflatoxin B1

Genetic variability is the cornerstone for the improvement of the target trait. The genetic variability of 30 maize inbreds for *A.flavus* resistance was analysed with KSA using two highly toxigenic AF1 and AF3 isolates. The descriptive statistics of *A.flavus* colonization and aflatoxin B1 showed significant variability across the maize inbred lines (Table 2). The *A.flavus* colonization on maize seeds was ranged from 16.66-83.33% and 13.33-83.33% for toxigenic isolates of AF1 and AF3, respectively. Similarly, the AF1 and AF3 showed Aflatoxin B1 content ranging from 0.013-0.981 and 0.048-0.982, respectively. Among the maize inbred lines under investigation, M6, M7, M9, M17 and M22 inbred lines showed the lowest *A.flavus* colonization percentage against both the aflatoxigenic isolates AF1 and AF3 (13.33-30.00%) with a resistance reaction of < 2. On the other hand, M18, M25, M26 and M29 showed higher colonization percentage (70.00-83.33%) with the highly susceptible reaction on disease scale (5.0) (Fig. 1). The identified tolerant (M6, M7, M9, M17 and M22) and susceptible (M18, M25, M26 and M29) maize inbred lines could be targeted for line development, basic and strategic research, and hybrid breeding. Further, Hajare *et al.* (2006) also showed a significant variation for *Aspergillus* colonization in Indian maize germplasm through KSA approach.

Further, the diversity analysis was employed to visualize the divergence of maize inbred lines in response to *A.flavus* infection. The coefficient of genetic dissimilarity for *A.flavus* infection traits varied from 0.21 to 35.41 with a mean of 11.98. The maximum dissimilarity was observed between the inbred lines M17 and M29, suggesting a contrast response to *A.flavus* isolates AF1 and AF3 infection. The contrasting lines combinations could be effectively used in developing a mapping population to map genomic regions for *A.flavus* resistance. Further, the dendrogram showed the grouping of maize into four clusters. Interestingly, cluster II (M18, M25 and M29) and cluster IV (M6, M7, M9, M17 and M22) encompass susceptible and resistant inbred lines, respectively (Fig. 2A). In PCoA, the first two co-ordinates explained a total of 86.23 % variation. The results of PCoA are consistent with the clustering pattern of the dendrogram, where highly resistant and highly susceptible inbred lines were grouped separately (Fig. 2B).

Analysis of variance for A. flavus colonization KSA and SNSS inoculation method

Analysis of variance for KSA and SNSS inoculation methods was revealed a preponderance of genotype effects on the total variation for disease incidence (Table 3). The genotype contribution was significant ($p < 0.001$) for KSA with AF1 and AF3 isolates, whereas SNSS inoculation method under field condition with AF1 isolate. The significant and higher contribution of genotypes for *A.flavus* infection and colonization

suggesting the genetic basis of resistance/susceptible reaction with major genomic regions and could be exploited for further genetic improvement. The preponderance of genotypic variation (>90%) indicating the minimal influence of the environment and presence of major loci/QTLs for aflatoxin tolerance in maize. Supporting to our results, researchers identified major QTLs explaining more than 10% of the variation for *A.flavus* resistance even in early segregating generations (F2:3) in maize (Brooks *et al.* 2005; Womack *et al.* 2020; Warburton *et al.* 2011).

Association and correlation analysis between A. flavus isolates colonization and aflatoxin B1

The AF1 and AF3 isolates showed a positive and significant correlation coefficient for colonization efficiency ($r = 0.87$; $p < 0.0001$). The high degree of correlation between the isolates suggests that the inbred lines under investigation show similar responses to both the aflatoxigenic isolates, i.e., AF1 and AF3. The t-test was computed between the mean fungal colonization pattern of isolates, showed that the AF1 and AF3 colonization patterns were on par with each other (t: 0.60; $p = 0.55$). Similarly, aflatoxin B1 production from both isolates were also found non-significant (t: – 0.13; $p = 0.18$). The genotypes showing the similar responses to both aflatoxigenic isolates AF1 and AF3, may harbours common genetic Host-pathogen interaction machinery for colonization and aflatoxin production or the isolates AF1 and AF3 may could share the common virulent genes. Interestingly, among the Indian isolates, AF1 and AF3 shared six the afl genes viz., aflO, aflP, aflQ, aflM, aflD and aflS, which could be a basis for similar colonization efficiency in both the isolates (Kumari *et al.* 2020).

Similarly, correlation coefficients were computed for the *A.flavus* colonization percentage and respective aflatoxin B1 level. For AF1 isolate, a positive and significant correlation was observed between colonization and aflatoxin B1 level ($r = 0.24$, $p < 0.02$). However, correlation was not significant between colonization level and aflatoxin B1 for AF3 isolate ($r = 0.03$, $p = 0.80$). Ehrlich *et al.* (2011) showed the inverse correlation between the ability to produce aflatoxin and Aspergillus colonization on maize seeds. On the contrary, Diame *et al.* (2018) reported a positive and significant correlation between aflatoxin concentration and Aspergillus incidences on peanuts. The differential association between both colonization pattern and aflatoxin production could be associated with the host-pathogen interaction mechanisms which mainly relay on genetic background of maize germplasm and aggressiveness with more toxins synthesis potential of the Aspergillus isolates.

Efficiency and association of screening methods for A. flavus in maize

The t-test computed for the mean disease incidences by whorl inoculation method and side needle suspension methods showed the independent behaviour of Aspergillus infection from both the methods ($t = 3.60$; $p < 0.001$). The SNSS inoculation method was found efficient to screen maize inbred lines to aflatoxin tolerance under an artificial epiphytic condition in the field. All the cobs inoculated with the SNSS inoculation method showed Aspergillus infection ranging from 1-95%. However, the whorl inoculation method was found inefficient to cause Aspergillus infection in the inoculated plants. In the whorl inoculation method, only susceptible check M24 showed the disease to the extent of 34% only. Therefore, the SNSS inoculation method is more efficient for screening the maize germplasm against *A.flavus* under field condition.

The rapid screening techniques are pre-requisite to identify aflatoxin resistance in maize. Thus, there is a need to develop and validate the most rapid, durable, efficient and eco-friendly approaches to screen a large number of maize genotypes. The reliability of any rapid and high throughput screening protocols depends on association with field-level screening methods. The association between lab-based rapid KSA with field-based SNSS inoculation method was tested by computing the correlation coefficient between both approaches. A very high positive and significant correlation was observed between the results of KSA and SNSS inoculation methods ($r = 0.89$; $p < 0.001$) (Fig. 3). The SNSS inoculation method was found efficient to develop typical maize cob rot symptoms under field conditions, unlike the whorl inoculation method. Additionally, Williams *et al.* (2011 and 2013) showed the efficiency of the SNSS inoculation method in creating *A.flavus* disease incidence under artificial epiphytotic condition. The high degree of significant positive correlation between the KSA and SNSS inoculation methods indicate the feasibility of these methods for efficient and reliable screening of maize genotypes under lab and field conditions, respectively.

Conclusions

The rapid screening techniques and resistance germplasms are pre-requisite for breeding disease resistant cultivars. The stepwise investigation to screen the subtropical Indian maize germplasm against aflatoxigenic isolates AF1 and AF3 revealed significant variability for aflatoxin resistance. The five inbred lines (M6, M7, M9, M17 and M22) showed resistance to maize cob rot and aflatoxin B1 in both *in-vitro* and *in-vivo* screening could be utilized by maize breeders in afla-

toxin resistance breeding programme. The present study projected light on kernel screening assay, ELISA, followed by field validation. We have shown the association and feasibility of KSA and SNSS inoculation methods in screening the maize germplasm against *A.flavus*, the most dominant aflatoxin producing fungus.

Acknowledgements

The primary author is thankful to Director ICAR-NBPGR to grant PhD study leave, and work was conducted in the Division of Plant Pathology, ICAR-IARI, New Delhi. The authors are very thankful to the Head and Professor, Division of Plant Pathology, ICAR-IARI, New Delhi, for facilitation.

Authors contribution

RG, MS, SN and MGM conceived and conceptualized the experiment. RG and SN supervised the experiment. PK performed the experiments and written the draft. MS shared the germplasm for the investigation and improved the draft. MGM performed statistical analysis. SN, AK and YB involved in performing the experiments. PK, SN, RG and MGM finalized the manuscript.

Declaration

The authors declare no conflict of interest.

References

Agrawal PK, Mallikarjuna MG, Gupta HS, 2018. Genetics and applied genomics of quality protein maize for food and nutritional security. In: Biotechnologies of Crop Improvement, Volume 3 (pp. 151-178). Springer, Cham..

Babu BS, Lohithaswa HC, Mallikarjuna N, Pandravada A, Balasundara DC, 2020. Genetic characterization of maize doubled haploid lines for Fusarium stalk rot caused by *Fusarium verticillioides*. J Genet 99:1-5.

Berthier J, Valla G, 1998. Moisissures-mycotoxines et aliments: du risque à la prévention, (pp.05-20). Université Claude Bernard, Lyon.

Begum F, Samajpati N, 2000. Mycotoxin production on rice, pulses and oilseeds. Naturwissenschaften 87:275-277.

Bennett JW, Klich M, 2003. Mycotoxins. Clin Microbiol Rev 16:497-516.

Bhat RV, Vasanthi S, Rao BS, Rao RN, Rao VS, Nagaraja KV, Bai RG, Prasad CK, Vanchinathan S, Roy R, Saha S, 1997. Aflatoxin B1 contamination in maize samples collected from different geographical regions of India-a multicentre study. Food Addt Cont 14:151-156.

Bock CH, Cotty PJ, 1999. Wheat seed colonized with atoxigenic *Aspergillus flavus*: characterization and production of a biopesticide for aflatoxin control. Biocontrol Sci and Tech 9:529-543.

Brooks TD, Williams WP, Windham GL, Willcox MC, Abbas HK, 2005. Quantitative trait loci contributing resistance to aflatoxin accumulation in the maize inbred Mp313E. Crop Sci 45:171-174.

Brown RL, Zhi-Yuan C, Menkir A, Cleveland TE, 2003. Using biotechnology to enhance host resistance to aflatoxin contamination of corn. Afr J Biotechnol 2:557-562.

Dieme RMA, Faye I, Zoclanclounon YAB, Fonceka D, Ndoye O, Diedhiou PM, 2018. Identification of sources of resistance for Peanut *Aspergillus flavus* colonization and Aflatoxin contamination. Int J Agronomy 2018:5468602. https://doi.org/10.1155/2018/5468602

Dorner JW, 2002. Simultaneous quantitation of *Aspergillus flavus/A. parasiticus* and aflatoxins in peanuts. J AOAC Int 85:911-916.

Ehrlich KC, Qijian W, Deepak B, 2011. Inverse correlation of ability to produce aflatoxin and Aspergillus colonization of maize seed. Food Nutr Sci 2:486-489

Gioi HA, Mallikarjuna MG, Shikha M, Pooja B, Jha SK, Dash PK, Basappa AM, Gadag RN, Rao AR, Nepolean T, 2017. Variable level of dominance of candidate genes controlling drought functional traits in maize hybrids. Front Plant Sci 8:940. https://doi.org/10.3389/fpls.2017.00940

Hajare SS, Hajare SN, Sharma A, 2006. Screening of Indian corn varieties for aflatoxin resistance. BARC News Lett 273:218-230.

Hsieh DPH, 1989. Potential human health hazards of mycotoxin. In: Natori S, Hashimoto K, Ueno Y (eds): Mycotoxins and Phycotoxins, (pp 69-80) Elsevier Amsterdam.

Jeevan B, Gogoi R, Sharma D, Manjunatha C, Rajashekara H, Ram D, Mishra KK, Mallikarjuna, MG, 2020. Genetic analysis of maydis leaf blight resistance in subtropical maize (*Zea mays* L.) germplasm. J Genet 99:89. https://doi.org/10.1007/s12041-020-01245-3

Kamkar A, Yazdankhah S, Mohammadi Nafchi A, Mozaffari Nejad AS, 2014. Aflatoxin M1 in raw cow and buffalo milk in Shush city of Iran. Food Addit Contam Part B. 7:21-24.

Karjagi CG, Sekhar JC, Lakshmi SP, Suby SB, Kaur J, Mallikarjuna MG, Kumar P, 2017. Breeding for resistance to insect pests in maize. In Breeding insect resistant crops for sustainable agriculture,

(pp 201-229). Springer, Singapore.
Kumari P, Gogoi R, Srinivasa N, Shekhar M, 2020. Characterization of aflatoxigenic *Aspergillus flavus* associated with aflatoxin B1 (AFB1) production in maize kernel in India. Indian Phytopathol 74(1):103-112.
Mallikarjuna MG, Bhat JS, Hossain F, Veeraya P, Tyagi A, Karjagi CG, Lohithaswa HC, 2020. Genetic enhancement of heat tolerance in maize through conventional and modern strategies. In Heat stress in food grain crops: plant breeding and omics research (pp 28-66). Bentham. https://doi.org/10.2174/9789811473982120010004
Mallikarjuna MG, Nepolean T, Hossain F, Manjaiah KM, Singh AM, Gupta HS, 2014. Genetic variability and correlation of kernel micronutrients among exotic quality protein maize inbreds and their utility in breeding programme. Indian J Genet Plant Breed 74:166-173.
Payne GA, Hagler Jr, WM, Adkins CR, 1988. Aflatoxin accumulation in inoculated ears of field-grown maize. Plant Dis 72:422-424.
Payne GA, Widstrom NW, 1992. Aflatoxin in maize. Crit Rev Plant Sci 10:423-440.
Perrier XAFP, Flori A, Bonnot F, 2003. Data analysis methods. In: Hamon P, Seguin M, Perrier X, Glaszmann JC, Ed., Genetic diversity of cultivated tropical plants. (p.76). Enfield, Science Publishers. Montpellier.
Reddy SV, Kiran Mayi D, Uma Reddy M, Thirumala-Devi K, Reddy DVR, 2001. Aflatoxins B1 in different grades of chillies (*Capsicum annum* L.) in India as determined by indirect competitive-ELISA. Food Addt Contam 18:553-558.
Reddy CS, Reddy KRN, Kumar RN, Laha GS, Muralidharan K, 2004. Exploration of aflatoxin contamination and its management in rice. J Mycol Plant Pathol 34:816-820.
Reddy KRN, Reddy CS, Muralidharan K, 2009. Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. Food Control 20:173-178.
Samuel SM, Aiko V, Panda P, Mehta A, 2013. Aflatoxin B-1 occurrence, biosynthesis and its degradation. J Pure Appl Microbiol 7:965-971.
Shekhar M, Khan AA, Kumar S, Velazhahan R, 2011. Genotypic variability in maize for aflatoxin contamination. Arch Phytopathol Plant Prot 44:520-527.
Shekhar M, Kumar S, 2012. Inoculation methods and disease rating scales for maize diseases. Directorate of maize research. second edition (revised), (pp 31). ICAR-DMR, New Delhi (India)
Squire RA, 1981. Ranking animal carcinogens: a proposed regulatory approach. Sci 214:877-880.
Tavakoli H, Kamkar A, Riazipour M, Mozaffari Nejad AS, Rafati H, 2013. Assessment of aflatoxin M1 levels by enzyme-linked immunosorbent assay in yoghurt consumed in Tehran, Iran. Asian J Chem 25:2836-8.
Tubajika KM, Damann KE, 2001. Sources of resistance to aflatoxin production in maize. J Agric Food Chem 49:2652-2656.
Vijayasamundeeswari A, Mohankumar M, Karthikeyan M, Vijayanandraj S, Paranidharan V, Velazhahan R, 2009. Prevalence of aflatoxin B1 contamination in pre-and post-harvest maize kernels, food products, poultry and livestock feeds in Tamil Nadu, India. J. Plant Protection Res 49:221-224.
Warburton ML, Brooks TD, Windham GL, Williams WP, 2011. Identification of novel QTL contributing resistance to aflatoxin accumulation in maize. Mol. Breed 27: 491-499.
Williams WP, Ozkan S, Ankala A, Windham GL, 2011. Ear rot, aflatoxin accumulation, and fungal biomass in maize after inoculation with *Aspergillus flavus*. Field Crops Res 120:196-200.
Williams WP, Alpe MN, Windham GL, Ozkan S, Mylroie JE, 2013. Comparison of two inoculation methods for evaluating maize for resistance to *Aspergillus flavus* infection and aflatoxin accumulation. Int J Agron 2013:972316. https://doi.org/10.1155/2013/972316
Womack ED, Williams WP, Windham GL, Xu W, 2020. Mapping quantitative trait loci associated with resistance to aflatoxin accumulation in maize inbred Mp719. Front Microbiol 11: 45.
Zummo N, Scott GE, 1989. Evaluation of field inoculation techniques for screening maize genotypes against kernel infection by *Aspergillus flavus* in Mississippi. Plant Dis 73:313-316.

Table S1 - The disease rating scale for ear rot of maize caused by *Aspergillus flavus* under field condition (Shekhar and Kumar, 2012).

Scale	Percent infection (%)	Disease reaction
1	No infection	HR
2	1 to 3 % infection	R
3	>3 to 25%	MR
4	>26 to 50	MS
5	>50 to 75	S
6	>75	HS

Shekhar M, Kumar S, 2012. Inoculation methods and disease rating scales for maize diseases. Directorate of maize research. second edition (revised), (pp 31). ICAR-DMR, New Delhi (India).