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Combining ability and heritability for host resistance to *Aspergillus flavus* and Aflatoxin accumulation in tropical mid-altitude maize

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Abstract

Aflatoxins are toxic, and highly carcinogenic secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*. They pose a serious health hazard to humans and animals that consume contaminated grain. Recently, the National Maize Breeding Program at National Crop Resources Research Institute (NaCRRI) incorporated breeding for resistance *to A. flavus* infection and aflatoxin accumulation in its initiative to improve maize grain quality in Uganda. The breeding strategy had three major components: (i) identifying locally adapted, elite germplasm with resistance to *A. flavus* and reduced aflatoxin accumulation, (ii) improving locally adapted, elite germplasm for host resistance, and (iii) formation of new populations for pedigree breeding. To identify sources of resistance, we first compared effectiveness of media plating and media free techniques for assessment of kernel infection rate (KIR) on various germplasm. We generated 144 three-way test crosses and screened them together with their parental inbred lines and 4 single cross testers for host resistance to *A. flavus and* reduced aflatoxin accumulation. Using line by tester analysis, we identified 5 resistant inbred lines and 7 hybrids. We used the resistant inbred lines to make crosses with new sources of resistance from International Center for Maize and Wheat Improvement (CIMMYT) and International Institute for Tropical Agriculture (IITA) in diallel design. To strengthen this work, we conducted a survey to assess farmers' knowledge on *A. flavus* and aflatoxins and their management practices. Results obtained provided us with a foundation for development and deployment of new germplasm with resistance to *A. flavus* and aflatoxin accumulation for improved grain quality for both domestic consumption and export.

Key words: Aflatoxins, *Aspergillus flavus*, combining ability, heritability, Kernel Infection Rate (KIR)

Introduction

Maize production in Uganda and other parts of the world faces a number of constraints, including low yields due to reduced soil fertility, drought, pests and diseases. Furthermore, post-harvest losses due to fungal infestations result in substantial losses. Fungi cause ear rots, discoloration of grain, and accumulation of mycotoxins (Chandrashekar, 2000). The action of various fungi on maize grain leads to accumulation of various forms of mycotoxins, the most common being aflatoxins. These are toxic, and highly carcinogenic secondary metabolites of *A. flavus* and *A. parasiticus* (Agag, 2004). They pose a serious health hazard to humans and animals that consume contaminated maize with these (Brown *et*

al., 1999). Four major groups of aflatoxins, B1, B2, G1 and G2, are classified as Group 1 human carcinogens. Aflatoxin M1, a metabolite of aflatoxin B1, found in the milk of animals eating contaminated grain, is classified as a Group 2B human carcinogen (Agag, 2004).

Initially, aflatoxin contamination was considered a post-harvest problem due to improper handling and storage. However, research has indicated that infection by *A. flavus* and subsequent contamination with aflatoxins also occurs prior to harvest (Moreno and Kang, 1999; Agag, 2004; Wrather and Sweets, 2006). Aflatoxin contamination is more serious in tropical and subtropical regions of the world, where temperature and relative humidity favor the growth of *A. flavus*, and make conventional control strategies costly and ineffective. Uganda's temperatures and humidity provide an optimal environment for growth of *A. flavus* and resulting production of aflatoxins (Royes and Yanong, 2002; Kaaya and Warren, 2005). Developing host by breeding strategies is possible, and it is the most desirable and cost-effective strategy (Widstrom, 1996; Brown *et al.*, 1999; Moreno and Kang, 1999). Screening for resistance to *A. flavus*, on the other hand, is more difficult than for other diseases because: (i) there is no standard resistant material for use as a control in comparison, (ii) inoculation methods often do not result in an adequate level of infection or aflatoxin to differentiate between genotypes, (iii) the undependability of natural infection hinders repeatability across different locations and years and, (iv) rapid and inexpensive assays of fungal infection and aflatoxin levels are not available (Brown *et al.*, 1999).

A brief review of aflatoxin research in Uganda

Research on aflatoxins in food crops in Uganda started in the 1960's and continued into the early seventies. These studies revealed that consumers were exposed to aflatoxins, evidenced by the presence of aflatoxins in food samples and their linkage to liver cancer among consumers (Alpert *et al.*, 1971; Kaaya and Warren, 2005). However, this research was interrupted from 1971 to 1990, and even then was focused mainly on the incidence of post-harvest contamination of produce (Kaaya and Warren, 2005). A few studies on pre-harvest contamination conducted later focused on the general incidence and severity of maize ear fungal rots and factors responsible for their occurrence (Bigirwa *et al.*, 2007). Selection for low rates of ear rot infections has been a routine activity in the National Maize Breeding program, and the current research is the first deliberate attempt to address the problem of aflatoxin in maize by breeding for host resistance. On-going work is focused on finding sources of resistance among locally adapted germplasm and introductions from other breeding programs, to form novel resistant source of germplasm and varieties for deployment to farmers.

Identification of locally adapted, elite germplasm with resistance to resistance to Aspergillus flavus and aflatoxins

Evaluation of local elite germplasm for resistance included three activities: (i) screening inbred lines, testers, testcrosses and commercial varieties to identify sources of resistance, (ii) estimating general combining ability (GCA), specific

combining ability (SCA) and heritability for *A. flavus* resistance, and (iii) determining the relationship between *A. flavus* infection and agronomic traits. Screening for resistance involved artificial field inoculation followed by laboratory assessment of infection with *A. flavus* by determining rate of kernel infection and quantifying the amount of aflatoxin accumulation in the grain. Kernel infection rate (KIR) was evaluated in three ways as: percentage of kernel infection (PKI), incidence of severely infected kernels (ISIK) and percentage of severely infected kernels (PSIK). Grain aflatoxin levels were determined after the assessment of KIR. Additionally, evaluation for agronomic performance was conducted in three experimental sites.

Materials and methods

Germplasm

In 2009, at NaCRRI, 40 inbred lines and four single-cross yield testers of heterotic groups A (CML312/CML442) and B (CML202/CML395; CML395/CML444; CML384/CML444) were screened for *A. flavus* kernel infection rate and aflatoxin accumulation. The inbred lines were from diverse sources and included 15 weevil-resistant lines, 5 CKLs (CIMMYT, Kenya elite lines), 10 NMLs (Namulonge elite lines), and 10 CMLs (CIMMYT released lines), of which 4 were QPM (quality protein maize) lines.

Inoculation technique

Aspergillus flavus was grown on Potato-Dextrose Agar (PDA) for 14 days at 28^0C with 12 hours of light. Colonies, together with the media, were blended with distilled water and filtered. Conidial concentration was estimated using a hemacytometer, and adjusted to a concentration of 1×10^6 spores per ml with distilled water. Two drops of Tween-20 per 100 ml were added and each ear was inoculated with 5 ml of spore suspension at 28 days after mid-silk, using a non-wounding technique. In this technique, the husks were slightly parted by hand to expose developing kernels and, immediately following inoculation, were pulled back over the kernels. Then the ear was covered with a polythene bag to create an environment conducive for the fungus (Zuber *et al.*, 1978; Windham and Williams, 1998; Li, 2004).

Assessment of Kernel Infection rate (KIR)

We initially used two methods: Media plating technique and a Media-free-isolated-kernel-incubation (MIKI) technique (Li, 2004) to determine their effectiveness and later adopt that which proved more amenable. We found that the MIKI technique gave more consistent results due to limited cross infection of kernels, and that the fungus growing solely on the kernel made assessment of the degree of kernel infection easier. In the MIKI method, ears from inoculated plants were harvested, hand-shelled and bulked by plot, then dried in paper packs. Kernels were then surface sterilized with running distilled water, 70% ethanol and 1.25% sodium hypochloride for 1 minute each, rinsed and incubated at 95-100% relative humidity at 31°C for 7 days. Each kernel was placed in a small plastic cap to avoid cross contamination. Kernel infection levels were later recorded by counting the number of infected kernels and noting the number of those with vigorous fungal growth covering over 50% of its surface. The 3 KIR measurements of percent-of-kernels-infected (PKI), incidence of

severely-infected kernels (ISIK), and percent-severely-infected kernels (PSIK) were computed as follows:

PKI =

Number of infected kernels x 100% ..... (1)
Total number of kernels incubated

ISIK =
Number of severely infected kernels x 100% ..(ii)
Total number of incubated kernels

PSIK =
Number of severely infected kernels x 100% (iii)
Total number of infected kernels

Aflatoxin quantification

After determining KIR, total aflatoxin levels from grain samples were determined using the VICAM AflaTest (VICAM, Watertown, MA, USA) (VICAM 2001, VICAM, 1999, Eduardo *et al*., 2005), immune-affinity fluorometric method. This method produced an aflatoxin recovery of $\geq$ 85% and a detection limit of 1 ppb. A logarithmic transformation of the aflatoxin measurements was used to equalize variance and normalize the data.

Statistical analysis

Three major analyses were conducted using GENSTAT 12th edition (VSN International Ltd. www.vsni.co.uk): analysis of variance (ANOVA), analysis of combining ability, and correlation analysis. Since field experiments were in an alpha lattice design, ANOVA was conducted using the Linear Mixed Model selection of the Restricted Maximum Likelihood (REML) procedure. Means were compared by Fisher's Protected Least Significant Difference (LSD) test at $P \leq 0.05$. Combining ability for line by tester mating design was analyzed using genotype means as input data. The skeleton line x tester analysis of variance used is outlined in Table 1.

The relative importance of GCA and SCA was evaluated using a ratio of variance components, calculated as $2\delta^2GCA/(2\delta^2GCA+\delta^2SCA)$, as recommended by Baker (1978), and hereafter referred to as "Baker's ratio". Since parents were considered to be a fixed effect, heritability estimates were not appropriate, but the analogous broad-sense and narrow-sense coefficients of

Table 1. Line x tester analysis of variance

Source	df	MS	EMS	F-test denominator
Location (E)	(E-1)		-	M7
Line (males)	(m-1)	M1	$\delta^2_e + f\delta^2_{Em} + (4x3)\delta^2_m$	M2
Loc x Line	(L-1)(m-1)	M2	$\delta^2_e + f\delta^2_{Em}$	M7
Tester (female)	(f-1)	M3	$\delta^2_e + m\,\delta^2_{Ef} + (mx3)\,\delta^2_f$	M4
Loc x Tester	(E-1)(f-1)	M4	$\delta^2_e + 36\,\delta^2_{Ef}$	M7
Line x Tester	(m-1)(f-1)	M5	$\delta^2_e + \delta^2_{Efm} + 3\,\delta^2_{fm}$	M6
Loc x Line x Tester	(m-1)(f-1)(E-1)	M6	$\delta^2_e + \delta^2_{Efm}$	M7
Pooled Error	E(mf-1)	M7	δ^2_e	

genetic determination were estimated (Hall, 2002; Li *et al.,* 2004; Adefris and Becker, 2005):

$$H^2 = \frac{2\delta^2_{GCA} + \delta^2_{SCA}}{2\delta^2_{GCA} + \delta^2_{SCA} + \delta^2_e} \quad \text{..... (iv)}$$

$$h^2 = \frac{2\delta^2_{GCA}}{2\delta^2_{GCA} + \delta^2_{SCA} + \delta^2_e} \quad \text{..... (v)}$$

Correlations between various traits were obtained using genotype means, and the significance of correlation coefficients was determined using the degrees of freedom for each pair wise comparison.

Results

Results obtained indicated a highly significant variation ($P \leq 0.001$) in the percent-kernel-infection (PKI) among inbred lines and testers, while testcrosses showed marginally significant variability ($P \leq 0.05$). The incidence of severely-infected-kernels (ISIK) and percent-severely infected-kernels (PSIK) were not significant among hybrids, but significant differences were observed among inbred lines and testers ($P \leq 0.001$). The major measure of kernel infection rate - PKI, ranged from 4% to 89% for local inbred lines, with aflatoxin concentration ranging from 0 to 63 ppb (Table 2). The mean PKI for test crosses was 38.3%, with a very wide range of values between 7.0% - 84.2% and, the standard error of genotype means was 13.8%. There were few hybrids in the low (n = 18, 14%) and high categories of infection (n = 15, 12%), with the highest proportion in the moderately-low category (n = 58, 46%) (Table 3). Eighteen hybrids were selected that had a low PKI, low general ear rot infection rate, high yield, and Aflatoxin levels less than 20 ppb (Table 4).

Combining ability and heritability estimates for resistance

Line x tester analysis of combining ability indicated that inbred lines (male parents) had significant GCA ($P \leq 0.01$) for PKI while testers showed significant GCA for both PSIK ($P \leq 0.01$) and ISIK ($P \leq 0.05$). Specific combining ability was significant ($P \leq 0.01$) for the Incidence of severely-infected kernels (ISIK) (Table 5).

General combining ability effects were variable for response to field inoculation. Two inbred lines had significant GCA effects for low percent-kernel-infection (NML141 = -17.3* and WL118-15 = -14.1*), while four inbred lines (CKL-14, NML17, NML3, WL118-9 and WL429-37 had significant GCA effects for high PKI. A number of inbred lines showed significant GCA effects for incidence of severely-infected kernels (ISIK). Inbred lines showed varying GCA effects for yield in different locations. Across site general combining ability effects revealed that crosses from CMLI81 (0.88***), CML182 (0.38*), WL118-15 (0.49*), WL429-35 (0.89**), WL429-43 (0.57**) and NML3 had higher yields (Table 6). GCA values for tester A indicated that it contributed to increased severity of kernel infection while testers B1 and B2 contributed to increased yield (Table 7).

A variance component ratio (Baker's ratio > 0.5) revealed that GCA was more predominant than SCA for PKI while SCA was more predominant than GCA for both PSIK and ISIK (Baker's ratios < 0.5). Heritability estimates from variance components were low for PKI (h^2 = 19%,

Table 2. Estimated kernel infection rate of selected maize inbred lines and their aflatoxin concentration

Inbred line	[a]PKI (%)	[b]PSIK (%)	[c]ISIK (%)	[d]Aflatoxin (PPB)	[d]Aflatoxin (PPB) (log10)
CML384	4.0	0.0	0.0	19.0	1.28
WL429-30	4.7	0.0	0.0	30.0	1.48
CML182	5.5	0.0	0.0	22.0	1.34
NML3	6.5	0.0	0.0	0.0	0.00
WL429-36	7.3	12.5	1.1	26.0	1.41
WL429-37	8.4	20.0	2.9	0.0	0.00
WL118-15	8.7	0.0	0.0	30.0	1.48
NML141	8.8	0.0	0.0	23.0	1.36
CML181	10.6	0.0	0.0	1.1	0.04
WL118-1-1	14.7	75.0	10.3	25.0	1.40
CML395	17.6	16.7	4.4	28.0	1.45
WL429-14	17.9	35.7	6.2	63.0	1.80
CML202	18.5	31.1	6.5	0.0	0.00
WL429-16	19.3	6.7	2.2	20.0	1.30
WL118-26	22.8	44.4	12.0	16.0	1.20
NML166	23.5	35.0	7.4	24.0	1.38
NML56	25.2	79.8	20.1	18.0	1.26
WL429-43	28.9	41.9	13.9	20.0	1.30
CKL-11	34.8	42.8	14.6	18.0	1.26
CKL-31	48.2	32.2	16.1	12.0	1.08
CML444	51.4	37.8	20.7	20.0	1.30
NML89	52.2	26.3	18.5	64.0	1.81
CKL-7	52.9	29.6	15.3	21.0	1.32
NML88	57.6	17.0	9.8	20.0	1.30
CML312	60.3	39.0	23.5	41.0	1.61
CKL-26	65.1	72.9	49.0	20.0	1.30
NML85	72.1	45.5	33.8	17.0	1.23
WL429-38	77.2	38.5	29.4	29.0	1.46
CKL-18	89.1	59.8	53.3	29.0	1.46
Mean	30.6	28.6	12.1	22.6	1.19
SEM[e]	10.6	13.9	7.4	9.7	0.33
CV (%)[f]	45.9	24.8	65.0	42.8	28.0

[a]PKI, Percent-kernel-infection calculated, [b]PSIK, Percent-severely infected kernels, [c]ISIK, Incidence of severely infected kernels, [d] Aflatoxin concentration was log-transformed to normalize the variance (Steel *et al.*,1997)

Table 3. Frequency of percent-kernel-infection means for hybrids

[a]PKI class	Resistance level	Frequency	Percentage of hybrids	[a]PKI class mean
0-19.9	Low	18	14.3	15.5
20-39.9	Moderately low	58	46.0	30.8
40-59.9	Moderately high	35	27.8	49.6
60-79.9	High	13	10.3	66.0
80-99.9	Very high	2	1.6	83.3
Total		126	100	

[a]PKI, Percent-kernel-infection calculated as number of infected kernels x 100/ total number of incubated kernels

Table 4. Percent-Kernel-Infection estimated using media-free isolated-kernel method, yield and ear rot damage of three-way test cross hybrids evaluated in 2009B

Three-way test cross	[a]Percent kernel infection	Yield (t/ha)	[b]Percent ear rot damage
TA/WL429-43	7.0	6.0	2.65
TB2/WL118-15	9.8	5.2	5.79
TB/WL429-30	14.9	5.3	6.21
TA/WL429-35	17.1	6.9	6.70
TB2/WL429-43	17.8	5.4	8.27
TB2/NML141	19.1	5.5	8.73
TA/NML56	19.2	5.4	6.92
TB/NML166	19.4	5.1	3.55
TB/WL118-15	19.7	5.4	8.28
TB/NML85	20.4	5.1	0.00
TB1/WL429-36	20.8	5.3	2.45
TB2/NML97	20.9	5.2	6.45
TB1/CKL-10	22.0	5.2	6.58
TA/CML181	26.1	6.2	2.09
TB1/NML141	27.0	5.1	9.93
TA/WL429-36	28.7	5.2	7.07
TB1/WL118-15	29.2	5.8	4.14
TB1/WL429-16	29.9	5.6	7.30
Mean of all crosses	38.3	5.1	9.2
SEM	13.79	0.90	8.64

[a] Hybrids Percent-kernel-infection means of less than 30%
[b]Hybrids with number of less than 10% of the ears in a plot showing visible fungal growths

Table 5. Combining ability for percent kernel infection, percent severely infected kernels and incidence of severely infected kernels

Source	d.f.	Mean squares		
		[a] PKI	[b] PSIK	[c] ISIK
Crosses	120	282.8*	489.9	0.03*
GCA_{female}	3	207.7	1995.0**	0.05*
GCA_{male}	35	379.9**	437.7	0.02*
SCA	82	244.2	457.2	0.03**
Error	66	189.7	345.3	0.01
F-value (GCA female)		1.1	5.8**	4.0*
F-value (GCA male)		2.0**	1.3	1.8*
F-value (SCA)		1.3	1.3	2.0**

H^2 = 37%) and PSIK (h^2 = 15%, H^2 = 36%) but broad sense heritability was high for ISIK (h^2 = 13%, H^2 = 57%) (Table 8).

Agronomic performance

The hybrids used showed significant variability for all agronomic traits across locations except anthesis-silking interval. Few traits showed significant genotype by environment interactions (G x E), but the interaction was highly significant ($P \leq 0.001$) for maize streak virus (MSV), Turcicum leaf blight (TLB), ear rots and husk cover, ear borer damage, and marginally significant ($P \leq 0.05$) for ear borer damage and ear aspect (Table 9).

Relationship between A. flavus infection and other traits among local elite germplasm

Correlation analysis showed a significantly positive relationship of PKI to aflatoxin content of grain for inbred lines (r = 0.47, $P \leq 0.05$; r^2 = 0.22). Correlations with ear traits showed that a more dent type grain texture was correlated with more kernel infection (ISIK, r = 0.51, $P \leq 0.001$; and PSIK, r = 0.23, $P \leq 0.05$), while PKI did not show significant relationship to grain texture. More complete husk cover was not correlated with *A. flavus* infection indicators, but was associated with a lower percent of general ear rots (ER%, r = 0.49, $P \leq 0.01$) (Table 10).

For the testcross hybrids, percent of ears with rot from A. *flavus* was higher in later-flowering genotypes, but kernel infection measurements (PKI, PSIK, and ISIK) were not related to the flowering time. There was a highly significant negative correlation between yield and *Aspergillus ear* rot (r = -0.35***), but not between yield and kernel infection rate assessments. Damage by ear borers had strongly positive correlations with *Aspergillus* ear rot (r = 0.27**), TPS (0.28**) and KIR score (r = 0.23**). Tight husk cover was significantly associated with higher PKI (r = -0.19*). Grain with softer texture had both a higher incidence of *Aspergillus* ear rot (r = 0.18*) and higher severity (r = 0.23*). Not surprisingly, inferior ear aspect correlated with more *Aspergillus* ear rot (r = 0.34***), since visible ear rot is a considered in assigning ear aspect scores (Table 11).

Table 6. General combining ability effects of inbred lines (male parents) for kernel infection rate and yield

Inbred line	Kernel infection rate			Yield			
	[a]PKI	[b]PSIK	[c]ISIK	NaCRRI	NaSARRI	Bugi ZARDI	Across-site
CKL-10	2.41	-9.7	-0.06	-0.56	0.67	-0.71	-0.20
CKL-11	-6.29	-16.2	-0.13*	0.12	-0.09	-0.18	-0.05
CKL-14	20.51**	5.0	0.65***	0.15	0.05	-0.45	-0.08
CKL-18	-25.38	2.2	-0.17	-0.29	-1.71*	-1.93*	-1.31***
CKL-26	-12.98	13.4	0.09	-0.29	-0.17	-0.68	-0.38
CKL-7	4.51	-3.9	0.10	-0.71	-0.84*	-0.05	-0.53*
CKL-8	4.61	18.2	0.54***	0.91*	-0.39	-0.06	0.15
CML144	5.61	-13.6	-0.14*	-0.47	1.06***	-0.01	0.19
CML159	-1.29	-6.2	0.11	0.31	-0.47	-0.13	-0.10
CML181	7.31	17.2	0.81***	1.17***	0.62[#]	0.86	0.88***
CML182	6.31	-12.1	0.11	0.50	0.39	0.26	0.38*
NML141	-17.28*	-15.4	-0.22***	0.31	-0.40	-0.10	-0.06
NML156	0.81	25.4	0.18*	0.09	-0.45	-1.95**	-0.77**
NML166	-5.69	7.3	0.29***	0.65*	0.06	0.03	0.25
NML17	14.11*	0.0	0.36***	-0.38	-0.45	-0.18	-0.34
NML3	18.71*	6.2	0.51***	-0.87*	0.99*	1.09*	0.40
NML56	-5.49	-2.2	-0.15*	-0.43	-1.03**	-0.77	-0.74***
NML85	-14.09	14.2	-0.04	-1.01*	0.17	0.63	-0.07
NML88	0.71	13.8	0.55***	0.40	0.37	-1.17*	-0.13
NML89	-6.39	1.4	0.02	-0.23	0.05	1.03*	0.28
NML97	-2.49	19.5*	0.41***	-0.37	-0.64	0.44	-0.19
WL118-1-1	11.51	3.7	0.41***	-0.23	-0.54	0.19	-0.19
WL118-13	-10.99	0.8	-0.05	-1.18**	-0.53	-0.42	-0.71**
WL118-15	-14.09*	-7.4	-0.12*	0.72*	0.13	0.62	0.49*
WL118-26	-10.59	4.6	0.08	0.36	-0.27	0.09	0.06
WL118-7	9.31	-8.7	0.03	0.04	-0.26	-0.09	-0.11
WL118-9	24.11**	1.4	0.24***	-0.73*	-0.75	0.05	-0.48*
WL429-14	-5.99	-23.8*	-0.19**	0.03	-0.25	0.05	-0.06
WL429-16	4.21	8.3	0.15*	0.40	-0.01	-0.20	0.06
WL429-30	-1.89	6.5	-0.04	-0.09	0.29	-0.10	0.03
WL429-33	-3.69	10	0.69***	-0.21	0.19	-0.32	-0.11
WL429-35	-13.49	1.9	-0.22*	0.62	1.02	1.01	0.89**
WL429-36	-10.29	-9.9	-0.18**	-0.06	-0.36	0.45	0.01
WL429-37	16.51*	-16.1	0.06	-0.06	0.43	0.62	0.33
WL429-38	-4.29	0.5	0.02	-0.10	0.34	0.37	0.20
WL429-43	-2.69	-10.6	-0.02	0.61	1.02**	0.08	0.57**

Table 7. General combining ability effects of testers (female parents) for kernel infection rate and yield

Tester	Kernel infection rate			Yield			
	PKI	PSIK	ISIK	NaCRRI	NaSARRI	Bugi ZARDI	Across-site
A (CML312 × CML442)	-1.97	10.90**	0.052*	-0.08	0.07	-0.21	-0.07
B (CML202 x CML 395)	0.81	-4.84	-0.004	-0.23	-0.41**	-0.14	-0.26***
B1(CML395 x CML 444)	-1.97	-6.27	-0.046	0.20	0.33*	0.09	0.21**
B2(CML 384 x CML 444)	2.86	-1.55	-0.014	0.15	0.08	0.29	0.18*

***significant at P≤0.001, *significant at P≤0.05

Table 8. Variance components, Baker's ratios and heritability estimates for kernel infection rate measurements

Trait	Variance components			Baker's ratio[a]	Coefficient of genetic determination	
	δ^2_{Line}	δ^2_{tester}	δ^2_{SCA}		Narrow sense[b]	Broad sense[c]
PKI	56.60	0.59	54.46	0.51	19.0	37.1
PSIK	27.50	54.44	111.90	0.42	15.2	36.0
ISIK	0.003	0.001	0.013	0.23	13.3	56.7

[a]Ratio of variance component calculates as $[\delta^2_{GCA(male)} + \delta^2_{GCA\ (female)}] / [\delta^2_{GCA(male)} + \delta^2_{GCA\ (female)+} \delta^2_{(SCA)}]$ according to Baker (1978)

[b]Narrow sense coefficient of genetic determination calculated as $[\delta^2_{GCA\ (male)} + \delta^2_{GCA(female)}] / [\delta^2_{GCA(male)} + \delta^2_{GCA(female)+} \delta^2_{(SCA)} + \delta^2_e]$

[c]Broad sense coefficient of genetic determination calculated as $[\delta^2_{GCA\ (male)} + \delta^2_{GCA(female)} + \delta^2_{(SCA)}] / [\delta^2_{GCA(male)} + \delta^2_{GCA\ (female)} + \delta^2_{(SCA)} + \delta^2_e]$

Discussion

Genotypic variations for kernel infection, yield and other agronomic traits

The existence of significant variation among testers, inbred lines, and their hybrids for *A. flavus* infection and agronomic performance offers an opportunity for genetic improvement in the local breeding program. Most studies reported only the incidence of kernel infection indicated by percent-kernel-infection (PKI), but in this work, the incidence of severely-infected kernels (ISIK) and the percent-severely-infected kernels were included as additional measures of kernel infection rate. During screening for host resistance, evaluation of agronomic performance cannot be

Table 9. Mean squares for across 3 sites of secondary traits of three-way cross hybrids in 2009B

Trait	Mean square		
	Genotype	G x E	Pooled error
Anthesis days	16.48***	7.46	7.23
Silking days	24.69***	10.46	10.24
ASI[a]	2.22	1.95	1.77
Plant aspect[b]	0.44***	0.32	0.27
Ear aspect[c]	0.77***	0.32*	0.26
Plant height	929.60***	349.9	360.6
Ear height	512.20***	230.2	200.8
MSV[d]	207.19***	106.9***	74.8
TLB[e]	0.30***	0.20***	0.11
Percent ear borer damage [f]	26.39*	24.43*	19.12
Percent ear rot damage [g]	246.78***	192.7***	106.7
Percent ear husk cover [h]	384.15***	183.6	156.0
Husk cover score [i]	1.46***	0.33***	0.23
Grain texture [j]	2.22***	0.39	0.33
Number of ears per plant	0.05*	0.04	0.04
Yield[k]	2.44***	1.25	1.05

[a] ASI, anthesis-silking-interval measured as days to silking less days to anthesis , [b] Plant aspect (1 = Good, 5 = Poor)
[c] Ear aspect (1 = Good, 5 = Poor), [d] MSVDD, Maize Streak Virus Disease measured as the percentage number of plants with symptoms in a plot, [e] TLB, *Turcicum* leaf blight (1 = no symptoms, 5 = Severe symptoms), [f] Percent number of ears with ear borer damage per plot, [g] Percent number of ears with ear rot damage per plot, [h] Percent ear husk cover given as the percentage number of ears with open husks in a given experimental plot, [i] Extent of ear husk cover taken in a quantitative score for the whole (1 = good, 5 = poor), [j] Grain texture (1 = totally flint, 5 = totally dent), [k] Yield calculated at 12.5% moisture content
***, **, * value significant at P≤0.001, P≤ 0.01 and P≤0.05 respectively

ignored if commercially acceptable hybrids are the goal. However, in this study and earlier studies such as Brown *et al.* (1999) and Shirley *et al.* (2001) few genotypes we found to have both resistance to *A. flavus* and desirable agronomic traits.

Heritability

Since resistance to *A. flavus* is known to be quantitatively inherited and strongly influenced by environmental conditions, the coefficient of genetic determination (analogous to heritability when a fixed set of parents is used) was used to estimate the proportion of the variability attributed to genetic effects (Holland *et al.*, 2003; Melanie, 2006). This coefficient was found to be low for kernel infection. This corroborates past findings that attributed the low heritability to large environmental effects (Melanie, 2006). Resistance is also polygenic and involves the integration of several physiological processes. Thus, the low heritability for kernel infection implies little transmissibility of characters between

Table 10. Correlations between ear traits, *Aspergillus flavus* infection and aflatoxin concentration for 40 inbred lines

	PKI[a]	PSIK	ISIK	Aflatoxin	*Asp*. ER (%)	Ear rot (%)	GT(1-5)	HC (1-5)
PSIK[b]	0.44*	-						
ISIK[c]	0.87***	0.71***	-					
Aflatoxin (PPB)	0.47*	0.17	0.16	-				
Asp ER (%)[d]	0.68***	0.16	0.45*	0.19	-			
Ear rot% [e]	0.53**	0.11	0.39*	0.13	0.57**	-		
Grain texture (1-5)[f]	0.31	0.32	0.51**	0.04	-0.07	-0.10	-	
Husk Cover (1-5)[g]	-0.16	-0.06	-0.12	-0.24	0.16	0.49**	-0.13	-

*significant at P≤0.05, **significant at P≤0.01, ***significant at P≤ 0.001. [a] PKI, Percent-Kernel-Infection calculated, [b] PSIK, Percent-severely infected kernels, [c] ISIK, Incidence of severely infected kernels, [d] Percentage number of ears with *A. flavus* rot, [e] Percentage number of ears with general ear rots [f] HC (1-5). Extent of ear husk cover, [g] Grain texture in a quantitative score of 1-5 (1 = flint, 5 = dent)

generations, and this prompts the need for efficient methods of selection to achieve better genetic gains (Li, 2004).

Combining ability among inbred lines for host resistance

The predominance of GCA over SCA suggests that additive genetic effects are more important than non-additive effects in the inheritance of resistance in germplasm evaluated. This is shown by a ratio of variance component (Baker's ratio) greater than 0.5. A Baker's ratio equal to 1 would mean total influence of additive genetic effects (Baker, 1978; Cravero *et al*., 2004). In this study, the significance of male GCA in percent kernel infection and in most of the secondary traits reveals a substantial contribution by inbred lines to variability among hybrids for resistance to fungal infection and agronomic performance. This implies that the chance of obtaining a superior hybrid is increased by crossing high performing inbred lines. The predominance of GCA also suggests the opportunity to attain genetic improvement by accumulating favorable alleles from inter-regional variability through selection (Li, 2004, Adefris and Becker, 2005*).*

Relationship of Aspergillus flavus infection to other traits

The main aim of the correlation analysis was to identify candidate traits for indirect selection for resistance to *A. flavus* (Melanie, 2006). The presence of visible fungal growth (*Aspergillus* ear rot) in the field confirms field infection and may be useful in selecting resistant genotypes. However, visual selection of plants or genotypes with low *Aspergillus* ear rot is inadequate for genetic and physiological studies of resistance, since fungal infection usually occurs without visible symptoms

Table 11. Correlation of ear rots and *Aspergillus flavus* infection with agronomic traits of hybrids

Trait	Natural ear rots [a]			Measures of *A. flavus* infection			
	NaCRRI	NaSARRI	Bugi ZARDI	*A. flavus* ear rot[b]	PKI[c]	PSIK[d]	ISIK[d]
Days to anthesis	0.04	-0.03	0.28**	0.36***	0.06	0.04	0.00
Days to silking	0.05	0.04	0.29**	0.43***	0.05	0.09	0.05
ASI	0.16	0.24**	0.11	0.10	0.03	-0.01	0.05
Yield	-0.17*	-0.16	-0.46***	-0.35***	-0.10	-0.08	-0.06
Percent ear borer	0.45***	0.88***	0.23**	0.27**	0.16	0.16	0.28**
Percent bad husk cover	-0.05	0.20*	0.51***	-0.13	-0.15	0.01	-0.01
Husk cover score (1=good)	-0.14	0.24**	0.15	-0.14	-0.19*	0.09	0.00
Grain Texture (1=flint)	0.02	0.05	0.12	0.18*	0.01	0.23*	0.16
Ear Aspect (1=good)	0.16	0.23*	0.63***	0.34***	0.13	0.10	0.13

*** Significant at P≤ 0.001, **significant at P≤0.01, *significant at P≤ 0.05, [a] Percentage number of ears with natural fungal rots, [b] Percentage number of ears with *A. flavus* ear rot, [c] PKI, percent-kernel-infection, [d] PSIK, Percent-severely infected kernels, [e] ISIK, Incidence of severely infected kernels

(Koenning and Payne, 1999). Moldy growth occurs in the field when plants are very susceptible or when there are favorable conditions and grain injury, such as by ear borers. Measurement of kernel infection rate is therefore more reliable, as stated by Kang *et al*. (2002). This was confirmed in this work, where there was significant variation between genotypes for kernel infection rate, yet there were no significant differences for *Aspergillus* ear rot among the same genotypes. Since *A. flavus* had a strongly positive correlation with general ear rot attack, genotypes with high ear rot infection were more likely to be susceptible to infection by *A. flavus*. In this work, unlike in others, the percent-kernel-infection showed no relationship to grain texture, which is probably a reflection of low means for infection and little variation for grain texture, since most of the genotypes were of flint texture. However, the incidence of severely-infected-kernels, percent-severely-infected-kernels and *Aspergillus* ear rot did show a significant increase in softer endosperms. This is because it is easier for the fungus to grow and spread quickly in softer kernels (Chandrashekar *et al*., 2000). It is also possible that a softer kernel will be more prone to both mechanical damage and heat injury during drying. A bruised kernel offers entry points for the fungus, and hence increased likelihood of infection (Munkvold, 2003). This also applies to infection following attack by ear borers that create entry points for the fungus, as seen in many studies (Betr'an *et al*., 2002; Betr'an and Isakeit, 2004). This study confirms these findings, as there was a significant relationship between *A. flavus* ear rots, grain texture and ear borer damage.

Infection was found to be higher in late-maturing crosses as shown by the strongly positive correlation of flowering dates with *Aspergillus* ear rots. This was also applicable to other ear rots in this study. Strongly negative correlation between yield and fungal infection has also been reported in a number of studies. This has been attributed to the fact that stressed or weak plants are low yielding and very susceptible to kernel infection (Moreno and Kang, 1999; Tubajika *et al.*, 1999; Betr'an and Isakeit, 2004).

Kernel infection and accumulation of aflatoxin

Percent-Kernel-Infection (PKI) showed a significantly positive correlation with aflatoxin concentration in the grain. This implies increased kernel infection led to increased accumulation of aflatoxin. Selecting inbred lines with lower PKI increases the chance of obtaining cultivars with high resistance to aflatoxin accumulation (Zhang *et al*., 1997). Unlike percent-kernel-infection, percent-severely-infected-kernels (PSIK) and the Incidence of severely-infected–kernels (ISIK) did not show any relationship to aflatoxin accumulation. This implies that accumulation of aflatoxin may be more affected by the number of grains infected than it is by the severity of infection on individual kernels. It also suggests that the use of percent-kernel-infection (PKI) is more important in selecting for low aflatoxin content in maize genotypes. The possible reason for this is that fungal colonization has been reported to occur in the kernel embryo before it appears in the endosperm tissues, and kernel infection and accumulation of aflatoxin occurs in the field (Zhang *et al*., 1997). The relationship between percent-kernel-infection and accumulation of aflatoxins was significant, but not strong ($r^2 = 0.22$). This could possibly be due to the large

effects of micro-environmental conditions affecting both kernel infection and aflatoxin accumulation in different ways. Also, the distribution of aflatoxin in a grain lot is not even. Fungi tend to develop in isolated pockets, which results in a very uneven distribution of infection and aflatoxin accumulation within a grain lot (Zheng *et al*., 2006). Moreover, aflatoxin from infected kernels can accumulate on adjacent uninfected kernels (Li, 2004). These, together with errors during sampling, may affect the relationship between kernel infection rate and aflatoxin accumulation.

It was further observed in this study that some genotypes had a low kernel infection with high aflatoxin concentration while others had a low aflatoxin concentration and high kernel infection. This may be attributed to the effect of resistance proteins in the kernel. A protein with a molecular mass of 28kDa may be higher in genotypes with less kernel infection because it reduces fungal growth, while the 14 kDa trypsin inhibitor and the 100kDa protein may be present in high concentrations in genotypes with lower aflatoxin content (Huang *et al.*, 1997; Chen *et al.*, 2005; Baker *et al.*, 2009). It is therefore relevant to select genotypes first for low kernel infection, and secondly for low accumulation of aflatoxin. It also important to study the protein profiles of the locally adapted lines

Introgression of resistance into locally adapted germplasm

Results from evaluation of elite inbred lines indicated that general combining ability for resistance was more important than specific combining ability. Our results showed that resistant inbred lines used as the male parent in three-way testcrosses contributed more to resistance that the single-cross testers. This implied that the local breeding program could focus on screening a wide range of inbred lines and widen the genetic base for resistance by including inbred lines derived from regional and international populations in order to achieve greater genetic improvement in locally adapted germplasm. For this reason, in the current season, we accessed 14 additional known resistant inbred lines from CIMMYT and 5 lines from IITA. We have used the inbred lines for introgression into local adapted lines and also for crosses to develop new resistant populations for improved grain quality.

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