Interactions Between *Fusarium verticillioides*, *Aspergillus flavus*, and Insect Infestation in Four Maize Genotypes in Lowland Africa

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ABSTRACT

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An experiment was designed to compare cycles of selection of four maize genotypes for ear- and grain-quality characteristics, interactions with *Aspergillus flavus* and *Fusarium verticillioides* infection, and insect ear infestation in two seasons. Mean infection levels by *A. flavus* and *F. verticillioides* were significantly higher in inoculated rows than in the controls. The *F. verticillioides*-inoculated rows had significantly more coleopteran beetles and lepidopteran borers per ear than the controls and *A. flavus*-inoculated rows. Genotypes and cycles of selection within genotype were not different with respect to number of insects or percent fungal incidence in the ear, but they were different for husk extension, field weight, 100-grain weight, and grain density. Inoculation with either fungus resulted in significantly higher percentage of floaters (i.e., loss of grain density) and lower grain weight than the controls. Aflatoxin (B1 and B2) in *A. flavus*-inoculated rows averaged 327 ppb in the first season and 589 ppb in the second (dryer) season. Fumonisin levels in *F. verticillioides*-inoculated rows did not differ between seasons, with an average of 6.2 ppm across seasons. In the noninoculated control rows, fumonisin was significantly higher in the first (5.3 ppm) than in the second (3.1 ppm) season. For all genotypes, husk extension and yield parameters decreased in the fungal-inoculated treatments. General ear-rot scoring was significantly correlated with incidence of *F. verticillioides* in kernels and grain-weight loss but not with *A. flavus* in the grain.

Additional keywords: *Eldana saccharina*, maize postharvest pests, *Mussidia nigrivenella*, *Sesamia calamistis*, stem borers, West Africa.

Maize is particularly vulnerable to degradation by mycotoxigenic fungi (23,31). Two mycotoxigenic fungi that are prevalent in maize in Africa are *Aspergillus flavus* Link:Fr., which produces aflatoxins (11,12,42,48), and *Fusarium verticillioides* Sacc. (Nirenberg) (synonym *F moniliforme* Sheld.), which produces the toxin fumonisin (16,26). Many fungi enter the ear through the silk channel (20), often carried in by *Lepidoptera* and *Coleoptera* spp. (3,9,36). *A. flavus* spores land on the silk, germinate, and enter the cob just prior to pollination and subsist on senescent silks within the husks indefinitely (8,27). *Fusarium* spp. spores land on the silk, germinate, and enter the ear after pollination (17,31,36). Both may invade kernels directly through weak spots in the pericarp, such as silk scars and stress cracks, through the pedicel, or through damage due to insect feeding sometime before harvest (15,35, 39,50).

Lepidopterous borers are considered among the most important insect pests of maize in Africa. Three stem borer species, *Sesamia calamistis* Hampson (Noctuidae), *Busseola fusca* Fuller (Noctuidae), and *Eldana saccharina* Walker (Pyralidae) are known to cause significant yield loss in West Africa (5). These borer species feed on stems of plants as well as maize kernels. The maize cob borer, *Mussidia nigrivenella* Ragonot (Pyralidae), causes serious damage to maize ears, especially in the Guinea savannas of West Africa (4,42). Larvae of *M. nigrivenella* start their damage from the tip of the ear and bore into kernels, producing abundant frass and silk. The maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae) is an important pest of stored maize grain in West Africa (22). Infestations of the maize weevil often begin in the field before maize is harvested. Other coleopteran species, such as *Cathartus quadricollis* Guerin-Meneville (Sylvanidae) and *Carpophilus* sp. (Nitidulidae), also are commonly detected on maize just before harvest (42). Numerous insect species have been implicated in facilitating the dispersal of *A. flavus* and subsequent aflatoxin contamination (3,28,41,42,50) and of *F. verticillioides* and fumonisin contamination in maize (10).

At least four genetically controlled characteristics modulate resistance of maize to the fungi *F. verticillioides* and *A. flavus* and the toxins they produce: (i) resistance to the infection process through physical barriers, (ii) resistance to fungal growth and toxin production after infection has occurred, (iii) resistance to insect damage, and (iv) tolerance of environmental stress (13,44,50). At the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, maize breeders work to provide a range of maize maturity groups and grain types to meet the demands of different end users throughout West Africa. While developing these alternatives, efforts have been made to improve the ear and to eliminate generic "ear rot" for all breeding materials, without specific attention to the etiology of the rot. There has been no specific screening against susceptibility to *A. flavus* or *F. verticillioides*; nevertheless, it was expected that constant selection for better agronomic characteristics (better husk cover, reduced ear rot, stress tolerance, and stem borer resistance) would improve the maize populations and concomitantly reduce *A. flavus* infection and aflatoxin contamination and susceptibility to Fusarium ear rot.

The current study was conducted to assess the effect of cycles of selection of four maize genotypes on fungus infection, insect infestation, and mycotoxin contamination. Currently used maize screening criteria for general ear rot and ear damage by insects were assessed for efficacy in reducing *A. flavus*, *F. verticillioides*, and insects during successive breeding cycles.

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MATERIALS AND METHODS

Seasons. In much of West Africa below latitude 10° north, the annual rainfall pattern is bimodal, allowing for at least two crops during the year. Our experiment was conducted twice at IITA in Ibadan, Nigeria, in 1996. The first trial was planted on 17 May (season A) and the second on 27 August (season B). The experiment in season A was exposed to the most rainfall (774 mm), which could be expected to enhance growth of both fungi (15,30). The season B trial had a shortage of rainfall (474 mm) and required irrigation during the last 45 days.

Maize genotypes. Early and advanced cycles of selection of each of four experimental maize genotypes were evaluated (Table 1). The genotypes were divided into late and early maturity categories. The late genotypes were planted 7 days ahead of the early genotypes, so silking occurred at approximately the same time in all accessions. Genotype Tropical Zea Early (TZE) composite 4, with broad adaptation in the lowland tropics, had undergone four cycles of selection for high yield and good ear characteristics. Genotype Tropical Zea Borer Resistant (TZBR) Eldana 1 was formed in 1988 and selected for resistance to the borer *E. saccharina*, using ear and stem damage and husk cover as selection criteria (18). Genotype Pool 16 was originally a population developed at the Centro International de Mejoramiento de Maiz y Trigo (CIMMYT), Mexico, D.F., that exhibited drought tolerance in West Africa, and was improved within the subregion for resistance to maize streak virus and adaptation to the semiarid lowland tropics. Genotype Gbogbe, a local, floury-endosperm cultivar, known for good storability and husk cover, was backcrossed for two generations with the IITA population Tropical Zea Streak Resistant White 1 (TZSR-W-1) to improve yield (19). Selection for the husk cover and milling quality of Gbogbe was made during backcrossing and subsequent selection. For the remainder of the paper, this population will be referred to simply as Gbogbe.

Field design. The trial was a split-plot design conducted during two seasons with three replicates per season. The main plot was a factorial arrangement of two factors: maize genotype and cycle of selection. The subplot was four inoculation treatments. Each genotype-cycle treatment was planted in plots of six rows, 5 m long. Rows one and six were border rows; row two was inoculated with *A. flavus*; row three was the *A. flavus* control row; row four was the *F. verticillioides* control row; and row five was inoculated

TABLE 1. Maize genotypes

Population[a]	Cycles of selection	Maturity, grain color, milling quality [b]	Selection emphases
TZE composite 4	C0 and C2	E, W, SD	Yield, drought tolerance, husk cover, ear rot resistance
TZBR Eldana 1	C0 and C5	L, M, F/D	*Eldana* resistance
Pool 16 SR	Gusau 1981 and Acr 1990	E, M, D	Streak resistance, drought tolerance, yield, husk cover
Gbogbe × TZSR-W-1	C0 and C3	L, Y, F/Fl	Yield, husk cover, weevil resistance, milling quality

[a] TZE = genotype Tropical Zea Early, TZBR = genotype Tropical Zea Borer Resistant, and TZSR-W-1 = Tropical Zea Streak Resistant White 1.
[b] E = early, L = late, W = white, M = mixed, Y = yellow, F = flint, Fl = floury, SD = semident, and D = dent.

TABLE 2. Least significant means (LSM) of ear- and grain-quality variables by cycles of selection and fungal inoculation treatments[a] in seasons A and B in Ibadan, Nigeria, in 1996, and pairwise differences across seasons

Cycles and treatments	Af[b] (%)	Fv[b] (%)	Other fungi[b] (%)	Insect damage[c]	No. of borers[d]	No. of beetles[d]	Extension[e]	Floaters (%)	100-grain wt (g)	Consumable-grain wt[f]	Field wt[g] (kg/ha)	Ear rot rating[h]
Season A												
Cycle E	31.6	30.6	2.0	1.9	4.7	14.9	2.7	45.9	28.3	24.8	3,769	2.9
Cycle A	33.4	32.7	3.0	2.0	6.1	17.1	2.3	41.6	28.5	24.2	3,841	3.0
Trt Af	77.4	8.9	0.9	1.8	3.7	9.9	2.5	46.7	27.9	22.7	3,774	3.1
Trt C1	27.0	14.2	6.2	1.7	5.5	13.2	2.5	40.1	30.0	26.9	4,019	2.6
Trt C2	18.8	32.1	2.5	1.5	4.8	13.5	2.4	37.2	27.9	27.4	3,933	2.5
Trt Fv	6.8	71.4	0.4	2.8	7.8	27.4	2.6	51.0	28.8	20.9	3,477	3.7
Season B												
Cycle E	14.8	22.8	0.1	1.3	0.0	13.7	2.6	41.5	25.4	23.4	3,173	2.8
Cycle A	18.2	24.4	0.1	1.3	0.4	14.0	2.3	31.1	29.7	24.8	3,894	3.4
Trt Af	35.2	8.7	0.0	1.5	0.0	7.4	2.6	40.4	28.5	21.2	3,191	3.8
Trt C1	23.7	9.5	0.2	1.0	0.2	5.9	2.3	36.0	30.0	26.0	3,886	2.3
Trt C2	4.2	1.7	0.1	1.3	0.1	8.7	2.4	30.9	27.4	26.3	3,415	2.4
Trt Fv	2.9	74.4	0.1	1.5	0.4	33.4	2.5	38.0	27.4	22.8	3,243	4.0
LSM[i]												
Cycles E to A	7.0	1.9	0.5	0.0	0.7	1.6	–0.2**	–5.2**	1.3*	0.3	400**	0.0
Af to C1	–28.1***	3.0	–2.7***	–0.3*	1.0	–1.1	–0.2**	–2.7	–0.8	4.4***	404**	–1.0***
Af to C2	–42.5***	8.8***	0.8	–0.2	0.7	0.4	–0.1*	–5.0**	–0.1	4.8***	–411**	1.0***
Af to Fv	–49.0***	65.3***	–0.2	0.5***	2.3**	17.4***	0.0	–0.9	–0.1	–0.1	–141	0.3*
C1 to C2	–14.4***	5.8**	–1.9***	0.0	0.2	1.5	0.0	–2.3	0.7	0.4	6	0.0
C1 to Fv	20.9***	–62.3***	2.9***	–0.8***	–1.3	–18.5***	–0.2**	–1.7	–0.7	4.6***	546***	–1.2**
C2 to Fv	6.5**	–56.5***	1.1*	–0.7***	–1.6*	–7.0***	–0.1*	–4.1*	0.1	5.0***	553***	–1.2**

[a] Means of 48 observations per cycle and 24 observations per fungal treatment. Cycle E = early cycle of selection; cycle A = advanced cycle of selection. Treatment (Trt) Af = inoculation with *Aspergillus flavus*; Fv = inoculation with *Fusarium verticillioides*; C1 = control adjacent to Af treatment; and C2 = control adjacent to Fv treatment.
[b] Percent *A. flavus*, *F. verticillioides*, and other fungi in 100 kernels.
[c] Visible ear damage at harvest (1 to 5 scale, where 1 is low and 5 is high).
[d] Mean counts of insects per five ears at harvest.
[e] Extension of husk beyond end of cob (1 to 5 scale, where 1 is good and 5 is poor).
[f] Consumable grain weight = 100-grain weight – discolored and insect-damaged grain.
[g] Field weight = mean row cob weight.
[h] Rating of kernels (1 to 5 scale, where 1 = sound and 5 = damaged).
[i] Pairwise differences across seasons. Comparisons ($P > t$) are means of 96 cycle and 48 treatment observations. Levels of significance: *** = 0.001, ** = 0.01, and * = 0.05.

with *F. verticillioides*. Fertilizer (N-P-K, 60-30-30) was applied at a low rate to create plant stress and simulate conditions in African farm fields. Apron plus (Novartis Crop Protection, Basel, Switzerland) was applied to all seeds to prevent downy mildew infection.

Inoculation methods. The *A. flavus* isolate was obtained from a naturally infected ear found at the IITA station, Ibadan, Nigeria, and maintained on acidified potato dextrose agar (PDA) for the first inoculation (internal reference isolate number IITA MP00196.NAF). The fungus was reisolated from inoculated ears for growth on acidified PDA and used for inoculation during the second season (internal reference isolate number IITA MP00296.NAF). In both seasons, at 5 and 10 days past mid-silk, 2 ml of an *A. flavus* conidial suspension (1×10^6/ml) was atomized on the silk of all primary cobs in one row of each main plot (27,47).

The *F. verticillioides* isolate was obtained from a naturally infected ear found in a farmer's field near Ibadan, Nigeria (internal reference isolate number IITA MP00196.NFM), and species identification was made by observing *Fusarium* spp. macro- and microconidia and single-filament microconidial chains when cultured on KCl (34). The fungus was reisolated from inoculated ears, cultured, and used for inoculation during in the second season (internal reference isolate number IITA MP00296.NFM). Inoculum was grown on acidified PDA, and microconidia were washed into a suspension with distilled water. At 10 and 15 days past mid-silk, 2 ml of *F. verticillioides* inoculum (1×10^6/ml) was atomized on the silks of all primary cobs in one row of each main plot (9).

The silks of different genotypes were inoculated with suspensions of *A. flavus* or *F. verticillioides*, and the controls were inoculated with water. All spray suspensions and water controls were amended with 1% (vol/vol) Triton X-100 to increase adhesion. All sprayings were carried out between 5:30 and 6:30 p.m. The sprayed cobs were covered for 24 h with a pollination bag to prevent rain or dew from dislodging the spores and to prevent cross-contamination. The control rows were sprayed first and covered to avoid contamination at each inoculation.

Ear and grain characteristics. A husk-cover rating was done per row on a scale of 1 to 5 just prior to harvest, as described by Kossou et al. (22), where 1 = husk well extended beyond the ear tip and 5 = ear tip exposed. From each row, five ears were set aside for laboratory analyses, and the rest of the ears were harvested and dehusked. An ear rot rating was done on the bulk of the harvested cobs on a scale of 1 to 5, based on a visual assessment of grain color and development, where 1 = sound, unblemished kernels and 5 = kernels damaged, covered with fungus, or discolored. An insect-damage rating on a scale of 1 to 5 also was made on the row, where 1 = 0 to 5%, 2 = 6 to 25%, 3 = 26 to 50%, 4 = 51 to 75%, and 5 = 76 to 100% insect-damaged kernels (5). Moisture content was assessed with a Dickey John moisture tester.

Grain density, which is an indirect measure of the ratio of hard to soft endosperm texture, was determined by a flotation test (53). A sodium nitrate solution with specific gravity of 1.25 at ambient temperature was used to measure the percentage of floating kernels from 50 kernels in 500 ml of solution. The specific gravity of the solution was checked with a hydrometer before, during, and after measurement. A Stenvert grain hardness test was conducted (37).

TABLE 3. Effects of season, maize genotype, cycle of selection, and inoculation treatment on fungal and insect variables

Variable	*Aspergillus flavus*[a] (%)	*Fusarium verticillioides*[a] (%)	Other fungi[a] (%)	Insect damage[b]	No. of borers	No. of beetles
ANOVA[c]						
Season	***	ns	**	*	**	ns
Genotype	ns	ns	ns	ns	ns	ns
Season × genotype	ns	ns	ns	ns	ns	*
Cycle	ns	ns	ns	ns	ns	ns
Treatment	***	***	***	***	*	***
Treatment × season	***	***	***	***	ns	ns
Treatment × genotype	*	ns	ns	ns	ns	ns
ANOVA by fungal treatment[d]						
Af treatment						
Season	**	ns	ns	ns	**	ns
Genotype	ns	ns	ns	ns	*	ns
Season × genotype	ns	ns	ns	*	*	ns
Cycle	ns	ns	ns	ns	**	ns
Cycle × season	ns	ns	ns	ns	**	ns
C1						
Season	ns	ns	*	*	**	*
Genotype	**	*	ns	ns	ns	ns
Season × genotype	ns	ns	ns	ns	ns	ns
Cycle	ns	ns	ns	ns	ns	ns
Cycle × season	ns	ns	ns	ns	ns	**
C2						
Season	***	**	ns	ns	ns	ns
Genotype	**	ns	ns	ns	ns	ns
Season × genotype	***	ns	ns	ns	ns	ns
Cycle	ns	ns	ns	ns	ns	ns
Cycle × season	ns	ns	ns	ns	ns	ns
Fv treatment						
Season	**	ns	ns	*	*	ns
Genotype	ns	ns	ns	ns	ns	ns
Season × genotype	ns	ns	ns	ns	ns	ns
Cycle	ns	ns	ns	ns	ns	ns
Cycle × season	ns	ns	ns	ns	ns	ns

[a] Percent kernel damage by fungi is arcsine square-root transformed.
[b] Visible ear damage on a 1 to 5 scale, where 1 is low and 5 is high.
[c] Analysis of variance ($P > F$); levels of significance: *** = 0.001, ** = 0.01, * = 0.05, and ns = not significant. No interactive effect with cycle was significant, and no higher order interaction terms were significant.
[d] Af = inoculation with *A. flavus*; Fv = inoculation with *F. verticillioides*; C1 = control adjacent to Af treatment; and C2 = control adjacent to Fv treatment. No other interactions were significant.

Insect counts. At harvest, five ears with husk were selected at random and removed from each row, placed in a cloth bag, and taken to the laboratory for insect counts. Each ear was dehusked separately, and lepidopterous borers were immediately identified to species and counted by species. Beetles were collected with an aspirator, pooled, and stored in a freezer for later counting. Numbers of beetles collected from five ears were analyzed as a pooled sample, and species were identified.

Fungal quantification. The five ears assessed for insect infestation were shelled by hand. Five kernels per ear were selected at random for a total of 25 kernels per sample. The kernels were surface-disinfected for 1 min in 3.5% NaOCl, rinsed in sterile distilled water, and plated on sterile filter paper. Plated kernels were incubated at 26°C and fungal species were assessed directly on the plates after 7 days. Only *A. flavus* and *F. verticillioides* were identified to species and recorded, while all other fungi were grouped and recorded as "other."

Mycotoxin analysis. The remainder of the kernels from the five ears were bulked and ground in a mill (Romer Labs Inc., Union, MO) and 50-g subsamples each were assayed for aflatoxin and fumonisin. Parts per billion of aflatoxin B1 and B2 were assessed by extraction and thin-layer chromatography for quantification (46). Sensitivity ranged from 3 to 500 ppb for this analysis. For fumonisin analysis, a fumonisin Veratox kit (Neogen Corp., Lansing, MI) was used, and optical density was scanned with a microplate reader (MR250; Dynatech Laboratories, Chantilly, VA) with a 650-nm absorbency filter. This assay was sensitive to 0.5 ppm. Due to logistics, aflatoxin analyses were performed only on the *A. flavus*-inoculated row and its control, and fumonisin analysis was performed only on the *F. verticillioides* row and its control.

Grain-loss assessment. The weight of a 100-grain sample (subsampled from five ears) was taken in the laboratory. Grains damaged by insects and those discolored by fungi were sorted out of the 100 grains, leaving the consumable grain, which was weighed. The percentage of loss due to biotic factors was calculated as the difference between the total 100-grain weight and the consumable-grain weight (7).

Data analysis. Data was analyzed with a Statistical Analysis Systems program (version 6.12, SAS Institute, Cary, NC). A mixed-model analysis of variance (ANOVA) was used to test the random effect replicate (season) and the fixed effects of season, genotype, cycle of selection, and inoculation treatments on ear- and grain-quality parameters (25). A multivariate ANOVA procedure was run but did not provide further information. A correlation analysis of the relationships among the ear rot pathogens, insects, and ear and grain parameters by row was performed using season A data only. Percent fungal kernel infection data were arcsine square-root transformed prior to analysis. Untransformed means are shown in figures and tables.

RESULTS

Seasonal factors. Averages of ear- and grain-quality variables for each season and mean differences among treatments are shown

TABLE 4. Effects of season, maize genotype, cycle of selection, and inoculation treatment on husk cover, grain hardness, ear appearance, and yield parameters[a]

Parameter	Husk cover[b] ($P > F$)	Floaters (%) ($P > F$)	100-grain wt (g) ($P > F$)	Consumable-grain wt[c] ($P > F$)	Field wt ($P > t$)	Ear rot[d] ($P > t$)
ANOVA						
Season	ns	*	ns	*	ns	*
Genotype	***	***	*	ns	*	ns
Season × genotype	ns	***	ns	ns	ns	ns
Cycle	**	**	*	ns	**	ns
Cycle × season	ns	**	ns	ns	ns	ns
Cycle × genotype	ns	**	ns	ns	ns	ns
Treatment	***	*	ns	***	***	***
Season × treatment	ns	ns	ns	ns	*	***
Genotype × treatment	ns	ns	ns	ns	ns	*
ANOVA by fungal treatement[e]						
Af treatment						
Season	ns	ns	ns	*	*	**
Genotype	**	**	ns	ns	*	ns
Season × genotype	ns	ns	ns	ns	ns	ns
Cycle	*	ns	ns	ns	ns	ns
C1						
Season	ns	ns	ns	ns	ns	ns
Genotype	**	ns	**	*	ns	*
Season × genotype	ns	ns	ns	ns	ns	ns
Cycle	ns	ns	*	ns	ns	ns
Cycle × genotype	ns	ns	*	ns	ns	ns
C2						
Season	ns	ns	*	ns	ns	ns
Genotype	***	*	ns	ns	ns	ns
Season × genotype	ns	*	ns	ns	ns	ns
Cycle	**	*	ns	ns	ns	ns
Cycle × season	ns	*	ns	ns	ns	ns
Fv treatment						
Season	ns	ns	*	ns	ns	ns
Genotype	**	*	ns	ns	ns	ns
Season × genotype	ns	**	ns	ns	ns	ns
Cycle	*	**	ns	ns	*	ns
Cycle × season	ns	**	ns	ns	ns	ns

[a] In analysis of variance (ANOVA; $P > F$) no higher order interactions were significant. Pairwise comparisons ($P > t$) were means of six observations. Interactions with cycle not shown were not significant. Levels of significance: *** = 0.001, ** = 0.01, * = 0.05, and ns = not significant.

[b] Husk cover rating (1 to 5 scale, where 1 = husk well extended beyond the ear tip and 5 = ear tip exposed).

[c] Weight per 100 kernels.

[d] Ear rot rating (1 to 5 scale, where 1 = sound and 5 = damaged).

[e] Af = inoculation with *Aspergillus flavus*; Fv = inoculation with *Fusarium verticillioides*; C1 = control adjacent to Af treatment; and C2 = control adjacent to Fv treatment.

in Table 2. ANOVA for the variables are shown in Tables 3 and 4. In the general ANOVA, there was a significant seasonal effect on percentage of *A. flavus* and other fungi in kernels, ear damage rating, number of ear borers, percent floaters, grain hardness, and ear rot ratings (Tables 3 and 4). The percentage of kernels infected with *A. flavus* dropped from a mean infection of 77.4% in the *A. flavus*-inoculated plots in season A to 35.2% in season B, although this was not significant (Fig. 1). In the *Fusarium* spp.-inoculated rows, *F. verticillioides* infection was 71.4% in season A and 74.4% in season B (Table 2). Percentages of other fungi were significantly higher in the control rows in the first compared with the second season. There was no seasonal effect detected in the ear damage ratings (scale of 1 to 5) or beetle numbers (Table 3). Ear borer numbers declined significantly from the highest mean (in the *F. verticillioides* inoculation) of 7.8 borers per 5 ears in the A season to less than 1 borer per 5 ears in season B. In contrast, beetle numbers increased in the *F. verticillioides*-inoculated rows from 27.4 to 33.4 beetles per 5 ears. Husk-cover ratings were not affected by season, while grain density (percent floaters) did change significantly (Table 4).

Aflatoxin (parts per billion of B1 + B2) in the *A. flavus*-inoculated rows increased from 327 ppb in the first season to 589 ppb in the second (Fig. 1), although the difference was not significant. In the *F. verticillioides*-inoculated rows, fumonisin levels were not significantly different between seasons, with 6.3 and 6.1 ppm in the first and second seasons, respectively. However, in the noninoculated control rows, fumonisin differences between seasons were significant, with 5.3 ppm fumonisin in the first season and 3.1 ppm in the second (Fig. 1).

Genotype effects. Maize genotype did not have a significant effect on toxin production or fungal or insect-related pest variables (Table 3), but grain and ear characteristics were significantly different among genotypes (Table 4).

Fungal treatments. The inoculation treatments were highly effective as indicated by significant treatment effects (Tables 2 and 3). The mean infection levels by *A. flavus* and *F. verticillioides* were inversely related in the respective treatments (Table 2). The noninoculated controls showed some signs of contagion by the inoculated neighbor. Control 1 (C1), which was adjacent to the *A. flavus*-inoculated plot, had a higher percentage of kernels infected with *A. flavus* than either control 2 (C2) or the *F. verticillioides* treatment. C2 had significantly more *F. verticillioides* than C1 or the *A. flavus* treatment (Table 2).

There was a significant interaction between genotype and inoculation treatment for percentage of *A. flavus*. Significant differences in *A. flavus* incidence among maize genotypes could only be seen in the ANOVA by fungal inoculation treatment (Table 3). There

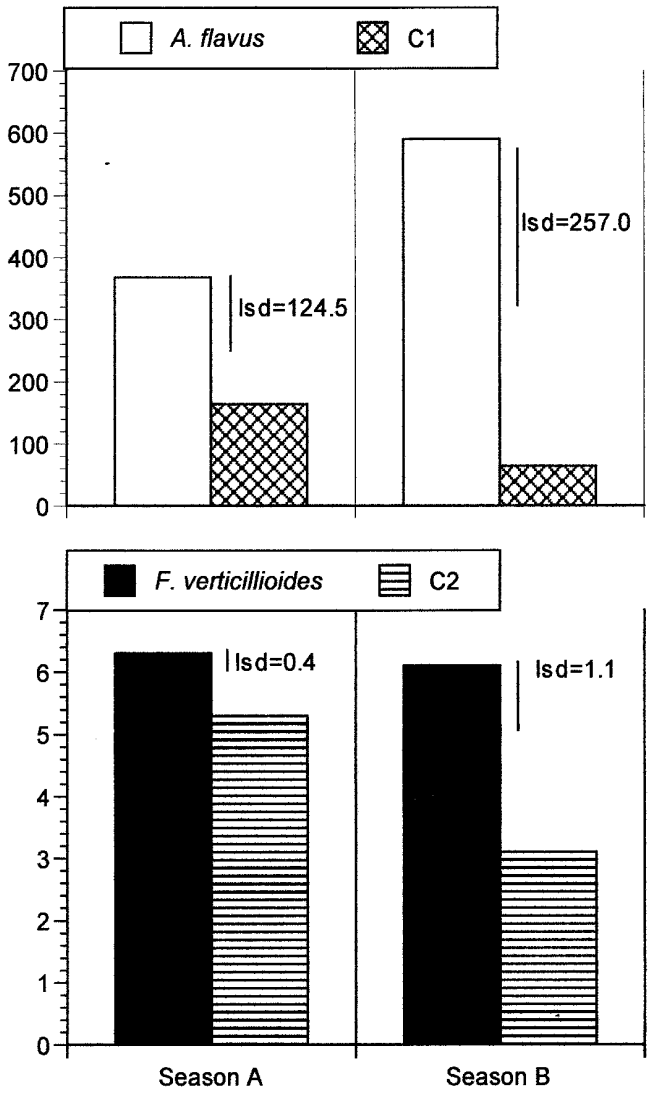

Fig. 1. Effect of season and inoculation with *Aspergillus flavus* on parts per billion of aflatoxin (B1 and B2) production relative to the noninoculated control (C1) and inoculation with *Fusarium verticillioides* on parts per million of fumonisin production relative to the noninoculated control (C2). Season least significant difference (LSD) for aflatoxin = 143 and for fumonisin = 0.7.

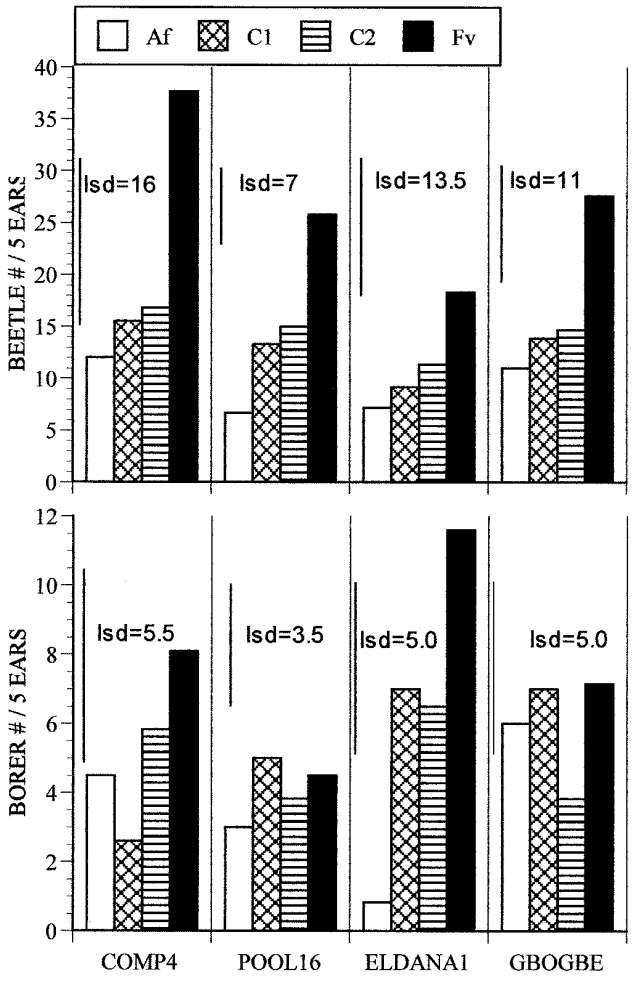

Fig. 2. Effect of inoculations with *Aspergillus flavus* (Af) and *Fusarium verticillioides* (Fv), and controls with no fungal inoculations (C1 and C2, respectively) on the number of beetles and ear borers per five ears in four maize genotypes. Insect numbers are means of six observations in season A. Least significant difference (LSD) across genotypes for beetles = 6.4 and for borers = 2.9.

were consistent differences in both seasons for *A. flavus* infection among genotypes in the control treatments (Table 3). The least significant mean (LSM) genotype infection with *A. flavus* in the C1 treatment across seasons was Eldana 1, 32% > Gbogbe, 23% > composite 4, 22% > Pool 16, 21% ($P > t$ for Eldana 1 versus composite 4 = 0.006, and for Eldana 1 versus Pool 16 = 0.002). This genotype ranking did not change between seasons. Although differences among genotypes for percentage of *F verticillioides* were generally not significant, Eldana 1 also had significantly more of this fungus than the other genotypes in the C1 treatment.

Insect variables. The four species of ear borers encountered, listed in descending order of frequency, were *M. nigrivenella*, *E. saccharina*, *Sesamia calamistis*, and *Cryptophlebia leucotreta* Meyrick (Tortricidae). Beetles listed in descending order of frequency were *Sitophilus zeamais*, *Cathartus quadricollis*, a *Carpophilus* sp., *Tribolium castaneum* (Tenebrionidae), a *Palorus* sp. (Tenebrionidae), and a *Cryptolestes* sp. (Laemophloeidae).

In the general ANOVA, season and fungal inoculation had a significant effect on the rating of ear damage by insects, counts of borers, and number of beetles per five ears (Table 3). The pairwise differences in the LSM of the insect variables showed that the *F. verticillioides* inoculation resulted in significantly higher insect damage ratings and beetle numbers than either the controls or the *A. flavus* treatment (Table 2).

In the ANOVA by *A. flavus* treatment, maize genotypes were significantly different in the number of borers per five ears in the *A. flavus* treatment (Table 3). This effect occurred in season A, when borer pressure was highest and was not seen in the other inoculation treatments. In the rows where *A. flavus* was introduced into the ears, the borer-resistant genotype Eldana 1 had significantly lower borer numbers than the other genotypes (Fig. 2).

To better understand the relationship between borers and the fungal inoculations, the borer counts were separated by species (Fig. 3). Eldana 1 had *Sesamia calamistis* only in the *F. verticillioides*-inoculated ears and *E. saccharina* only in the *F. verticillioides*-inoculated and neighboring control (C2) rows. Gbogbe had significantly more *S. calamistis* than the other genotypes, particularly when inoculated with *F. verticillioides*. Composite 4 had significantly more *E. saccharina* in the *F. verticillioides* treatment than in the other inoculation treatments. The genotype Eldana 1 had significantly more *M. nigrivenella* in the *F. verticillioides*-inoculated row and significantly less in the *A. flavus*-inoculated row. The other genotypes had no apparent fungus by Mussidia interaction.

Ear and grain characteristics. There were significant genotype and cycle-of-selection reactions for all of the ear and grain characteristics except consumable-grain weight and the ear-rot rating (Table 4). In both seasons, the rows inoculated with either fungus had a higher rating for husk extension (poorer husk cover and decreased extension) than the controls (Table 2, Fig. 4). Percent kernel flotation as an inverse measure of grain density was inherently different among genotypes. In advanced cycles of selection, percent flotation was reduced although, with fungal inoculation, the percentage increased. Weight (100 grain) was significantly different among genotypes and increased significantly with cycle of

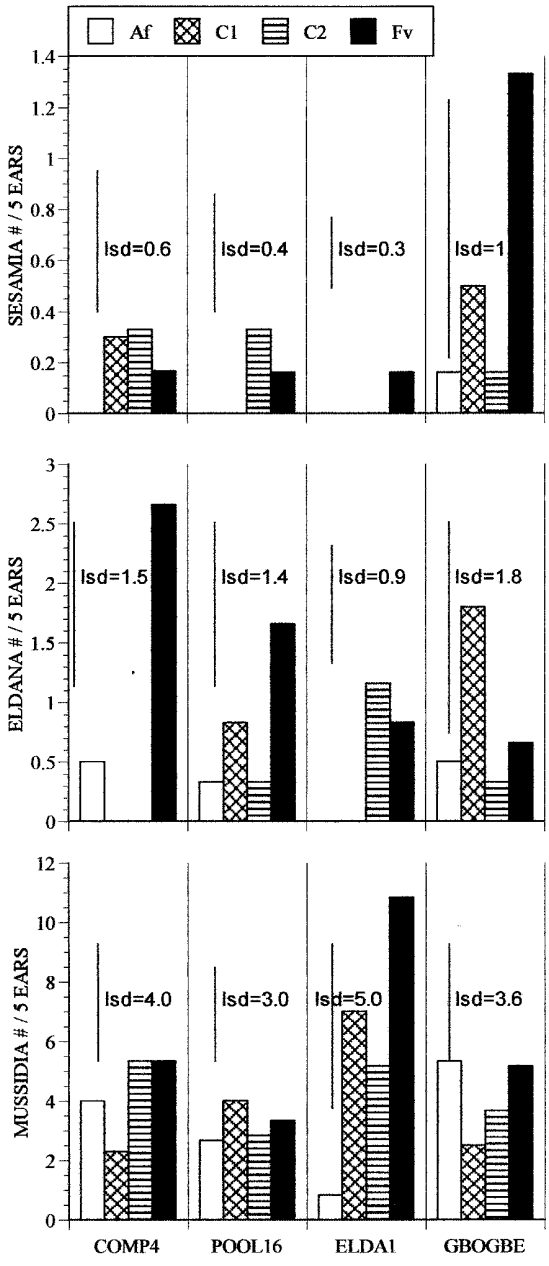

Fig. 3. Effect of inoculations with *Aspergillus flavus* (Af) and *Fusarium verticillioides* (Fv), and controls with no fungal inoculations (C1 and C2, respectively) on the number of ear borers per five ears of four maize genotypes in season A: *Sesamia calamistis*, least significant difference (LSD) across genotypes = 0.5; *Eldana saccharina*, LSD = 1.1; and *Mussidia nigrivenella*, LSD = 2.8. Borer numbers are means of six observations.

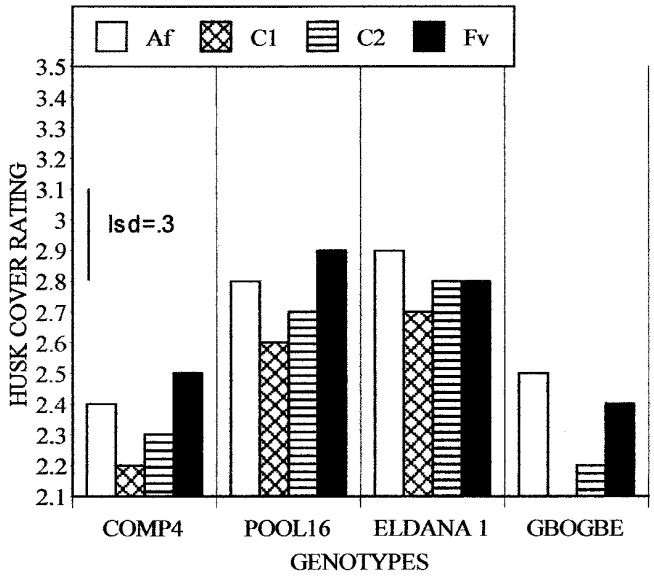

Fig. 4. Effect of inoculations with *Aspergillus flavus* (Af) and *Fusarium verticillioides* (Fv), and controls with no fungal inoculations (C1 and C2, respectively) on husk cover extension rating (1 = good and 5 = poor) in four maize genotypes. Least significant difference (LSD) across genotypes = 0.13; bars show means of 12 ratings.

selection, although consumable-grain weight of 100 grains did not change with cycles of selection. Consumable-grain weight was significantly affected by inoculation treatments. This was linked to ear rot ratings, which also were not affected by genotype or improved with cycle of selection (Tables 2 and 4). The advanced cycle of all genotypes had significantly better field weight (kilograms per hectare), but fungal inoculation decreased yield significantly.

Mycotoxins. Within both seasons, aflatoxin contamination in the *A. flavus* inoculated ears was significantly higher ($P \geq F = 0.0001$) than in the controls (C1) but was not significantly different between cycles of selection or among genotypes in either the first or second season (Fig. 1). The trend, however was that Pool 16 consistently had the lowest mean aflatoxin levels in the inoculated rows, with 354 ppb, compared with 533 ppb in composite 4. Likewise, within both seasons, the *F. verticillioides*-inoculated row had significantly higher levels of fumonisin than the control (C2). Again, the trend was that Pool 16 had the lowest mean fumonisin level across seasons, with 5.5 ppm, compared with the highest contamination level, 6.6 ppm, in Gbogbe.

Correlations. The negative correlation between percent incidence of *A. flavus* and *F. verticillioides* in kernels was highly significant (Table 5) but mostly an effect of experimental design. Significant negative correlations occurred between *A. flavus* and all insect variables: number of kernels damaged per 100 kernels, ear damage, and numbers of borers and beetles. The presence of *F. verticillioides* in the ear was negatively correlated with all other fungi but significantly positively correlated with all insect and damage variables. Both the ear damage rating (1 to 5 scale used by entomologists) and the ear rot rating (1 to 5 scale used by breeders) were positively related to *F. verticillioides*, grain-weight loss, and insect counts. Counts of discolored grain at harvest were significantly negatively correlated with other fungi, but this was probably an artifact of experimental design, because other fungi were present primarily in the control rows in much lower incidence than *F. verticillioides*. Count of insect-damaged grain at harvest was correlated with discolored grain and autocorrelated with grain-weight loss (damage was a criterion for sorting out consumable grain). Beetle and borer numbers were correlated with each other and with damage counts, while only beetle numbers were correlated to grain discoloration. Grain-weight loss was correlated with the two damage-rating scales, beetle counts, and *F. verticillioides* incidence. Borers were positively related with damaged-kernel counts, beetles and ear damage rating. Beetles were positively correlated with *F. verticillioides* and all other damage measurements. The husk-cover rating correlated negatively with field weight but was positively correlated with percent floaters, indicating that husk cover and grain hardness increased with yield. There were significant negative correlations between field weight and grain-weight loss, ear rot rating, and percent floaters. Grain density (the inverse of percent floaters) was negatively correlated with insect ear-damage rating and increased with field weight. Grain hardness and percent floaters were inversely related, and grain hardness was positively correlated with percent discoloration of grain and grain-weight loss.

DISCUSSION

Fungal interactions. Silk inoculation was consistently successful, because high levels of infection were obtained in both seasons. The control rows were used to determine genotypic response under more moderate inoculum pressure, as well as to assess the effects of fungal infection in the inoculated rows. The ear rot ratings (1 to 5 scale used by breeders) and ear damage ratings (used by entomologists) were good predictors of levels of *F. verticillioides*, the general category of other fungi, and insects at harvest; however, they were not good indicators for *A. flavus*.

Fungus-insect interactions. In this study, mean numbers of both beetles and borers tended to be lower in *A. flavus*-treated rows. The relationship between insects and *A. flavus* is not necessarily straightforward. Insects have vectored *A. flavus* (3,50), and a strong relationship between insect feeding in the ear and aflatoxin contamination has been demonstrated (3,24,41,42). Conversely, the larvae of *Chilo partellus* (Pyralidae) were killed and mummified by *A. flavus* (2). A reduction in the survival rate of *Heliothis zea* and *Spodoptera frugiperda* (Noctuidae) was observed when reared on a diet treated with spores of *A. flavus* and *A. parasiticus* (52). On the other hand, *M. nigrivenella* was not sensitive to aflatoxin or *A. flavus* in the diet (52). *F. verticillioides*, likewise, may be introduced into the stem and ear via insects (21,29,31), although the cause and effect relationship is not clear. In this research, numbers of borers and beetles in the ear at harvest were significantly higher in the *F. verticillioides*-inoculated rows (Fig. 2).

F. verticillioides has been variously described as an entomopathogen and as an insect-growth promoter (1,14,38,45). It has been identified as an entomopathogen of *Heliothis virescens* (1), on forest pests (38) and on the rice brown planthopper (45) and has been described as a growth promoter of storage coleopteran infestation of cereals (14). It is unclear in this experiment if the higher numbers of insects were due to an attraction to the inoculated cobs or simply higher survival rates. Subsequent experiments have shown significantly higher survivorship rates of *E. saccharina* in *F. verticillioides*-infected maize stems than in non-infected stems (K. F. Cardwell and F. Schulthess, *unpublished data*), but all of the interactions have not been explored.

TABLE 5. Correlations among fungus, insect, and ear and grain characteristics in season A (n = 96 rows)[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1.00	…	…	…	…	…	…	…	…	…	…	…	…	…
2	–0.66***	1.00	…	…	…	…	…	…	…	…	…	…	…	…
3	–0.19	–0.30**	1.00	…	…	…	…	…	…	…	…	…	…	…
4	0.15	0.41***	–0.34***	1.00	…	…	…	…	…	…	…	…	…	…
5	–0.25*	0.56***	–0.13	0.43***	1.00	…	…	…	…	…	…	…	…	…
6	0.04	0.45***	–0.34***	0.81***	0.54***	1.00	…	…	…	…	…	…	…	…
7	–0.26*	0.54***	–0.13	0.35***	0.67***	0.47***	1.00	…	…	…	…	…	…	…
8	–0.21*	0.31**	–0.12	0.08	0.38***	0.24	0.53***	1.00	…	…	…	…	…	…
9	–0.33**	0.49***	–0.10	0.28**	0.50***	0.28**	0.45***	0.22*	1.00	…	…	…	…	…
10	0.04	0.16	–0.10	0.10	0.07	0.10	0.14	0.17	0.01	1.00	…	…	…	…
11	0.04	–0.17	0.05	–0.10	–0.15	–0.24	–0.19	–0.15	–0.20	–0.26*	1.00	…	…	…
12	–0.06	0.45***	0.37***	0.36***	0.36***	0.46***	0.36***	0.19	0.26*	0.18	–0.31**	1.00	…	…
13	–0.02	0.19	–0.16	0.13	0.09	0.19	0.22*	0.18	–0.06	0.39***	–0.44***	0.32**	1.00	…
14	0.01	0.15	–0.12	0.33***	–0.15	0.27**	0.07	–0.02	0.07	0.05	–0.03	0.15	–0.28**	1.00

[a] Partial correlation coefficients are significant at * = 0.05, ** = 0.01, and *** = 0.001. Characteristics: 1 = number/100 kernels with *Aspergillus flavus*; 2 = number/100 kernels with *Fusarium verticillioides*; 3 = number/100 kernels with other fungi; 4 = number/100 kernels discolored at harvest; 5 = number/100 kernels damaged by insects at harvest; 6 = grain weight loss [1 – (total 100-grain wt – damaged grain wt/total 100-grain wt) × 100]; 7 = ear insect damage (1 to 5 scale, where 1 is low and 5 is high); 8 = number of borers/five ears; 9 = number of beetles/five ears; 10 = husk cover rating (1 to 5 scale, where 1 = good and 5 = poor); 11 = field weight (kg/ha); 12 = ear rot (1 to 5 scale, where 1 = sound and 5 = damaged); 13 = percent floaters; and 14 = grain hardness.

Fungus-insect-cultivar interactions. The presence of *F. verticillioides* had different effects on the various borers, depending on the host genotype (Fig. 3). The stem borer-resistant population Eldana 1 showed low numbers of both *E. saccharina* and *Sesamia calamistis* in our trials, and neither ear borer was present in ears inoculated with *A. flavus* or its adjacent control. This maize population was formed from lines known to exhibit resistance to multiple stem- and ear-boring insects (6,43) and has been selected in the presence of both *E. saccharina* and *S. calamistis* over the years. Nevertheless, in the presence of *F. verticillioides*, the numbers of these borers were higher and the population became significantly more vulnerable to *M. nigrivenella*. In Eldana 1, *A. flavus* reduced *M. nigrivenella* numbers significantly. This is in contrast with other findings that *M. nigrivenella* was not sensitive to *A. flavus* in vitro and that it increased with increasing *A. flavus* and aflatoxin in the field (42). In this experiment, the high dose of fungal inoculum may have overcome the tolerance.

Gbogbe × TZSR-W-1 is a population developed from a Benin local cultivar that has been shown to have good storability, with low beetle infestation (22), at least in part because of good husk cover (29). In the presence of *F. verticillioides*, it had a significantly reduced husk cover and more beetles than in the other treatment rows. This strong response could be responsible for some of the controversy over the inherent storability of this cultivar. In one study, Gbogbe was significantly more resistant to *Sitophilus zeamais* (22) while in another (29) no differences were seen between this cultivar and others.

The number of beetles was always highest in the *F. verticillioides*-infected rows, regardless of genotype. The interactions we describe were not considered in past stem-borer or beetle-resistance breeding programs. If the level of susceptibility to these two fungi are different, or the fungi are a hidden environmental effect of location, it may be difficult to make progress in breeding for insect resistance or determine the cause of the observed variability.

Interaction of fungi and insects with ear and grain characteristics. Maize kernels differ in the amount of void space within the endosperm in relation to the starch/protein ratio, so floury endosperm has less protein content than flinty endosperm (33). With cycles of selection, we saw significant increases in 100-grain weight and in grain density. There was a loss of density in the presence of fungi, which was not unexpected, because fungal metabolism within maize kernels decreases dry-matter content and, thus, kernel density (40).

An exposed cob is more vulnerable than one enclosed in the husk, and good husk cover is considered key to protecting the ear from fungi and insects (32,49,51). Yet, in this trial, husk rating correlated only with percent floaters and not with other damage scores. The husk extension was negatively correlated with field weight, showing that healthy ears were more likely to have long husks. Husk extension in the inoculated rows was decreased compared with the controls; thus, when selecting for good husk cover, it is necessary to be aware of possible genotype-fungus interactions. How the fungal inoculations resulted in reduced husk cover is unclear, but the effect was significant in the two genotypes with the best husk cover, composite 4 and Gbogbe. The phenomenon could be an artifact of the experimental design if a high dose of fungus in the ear at an early stage could compete for resources needed for development.

The efficacy of field evaluation methods for selection of better ears. The 1 to 5 ear-damage rating scale was a good indicator of both insect damage and *F. verticillioides* infection. The 1 to 5 ear rot score was as effective as the insect damage score, and it also was a predictor of field-weight loss; however, the ear rot and insect damage scores did not reflect *A. flavus* in the ear. Cycles of selection had improved yield, husk-cover rating, and grain density. With improved husk cover and harder grain, it would be expected to have improved resistance to ear and grain biota. Nevertheless, in these trials, we found no improvements in these characteristics with the cycle of selection. It is possible that, after a certain level of improvement in husk cover, the benefits in terms of protection of the ear do not continue to improve. Grain density when the grain is still filling may not be as important as the postharvest dried-kernel density for discouraging invasion. Therefore, two important points are derived from this experiment: (i) passive selection for generally improved ear appearance, husk cover, and grain hardness and density is not a guarantee that the ear will be of good quality and storability after many cycles of selection; and (ii) when working on maize resistance to stem and ear borer, postharvest beetle, and milling characteristics, the presence of fungi, particularly *F. verticillioides*, in the plant will affect the variability of the system.

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