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RESEARCH ARTICLE / *ARTIGO*

Effects of variety, cropping system and soil inoculation with *Aspergillus flavus* on aflatoxin levels during storage of maize

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ABSTRACT

The effects of soil inoculation with *A. flavus*, variety and cropping system on the level of aflatoxin in stored maize were investigated under crop storage conditions in Benin. The experiment was organized in a factorial scheme (two varieties x two cropping system x with or without *A. flavus* soil inoculation) with eight treatments carried out in a completely randomized block design with three replications. *A. flavus* incidence in the plots soil was assessed both before and after soil inoculation. The harvested maize was stored for four months and cobs were sampled in a monthly interval and analyzed for the determination of *Fusarium* spp. colonization, *Penicillium* spp. and *A. flavus* cfu levels, insect pests population size and aflatoxin content. Multi-factorial analysis of variance and linear regression analyses with dummy variables were used to compare treatments. The concentrations of aflatoxin B_1 and B_2 in the kernels tended to increase with time during storage. Variety and fungal inoculation were the main factors influencing the levels of aflatoxins in stored maize. The improved variety showed a higher number of *A. flavus* cfu and aflatoxin B_1 and B_2 levels as compared to the local variety. Intercropping with *Vigna unguiculata* decreased the aflatoxin concentration in the improved variety but not in the local variety. The local variety had higher levels of *Penicillium* spp. and lower levels of *Fusarium* spp. than the improved variety. The treatments had no effect on the populations of the most common storage insect pests, but their levels were positively correlated with aflatoxin content. Both the initial inoculum level and the variety effect on the water content of the kernels after harvest played a significant role in *A. flavus* infection.
Key words: *Aspergillus flavus*, *Zea mays*, soil inoculation, thin layer chromatography, storage fungi, storage insects.

INTRODUCTION

In tropical Africa, maize is a staple diet of local human populations, and one of the most vulnerable crops to aflatoxin contamination. Maize is usually stored for a long period, either for self sufficiency during the dry season, or for marketing purposes. Aflatoxin contamination of maize is a serious public health issue that has been studied extensively, particularly in Western Africa (Gong et al., 2002).

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* and affect many crops, including peanut, maize (corn), cottonseed, rice, spices and other crops (Bennett & Klich, 2003). *Aspergillus flavus* is more prevalent than *A. parasiticus* in African soils and on maize kernels. Among the factors contributing to *in planta* aflatoxin production are genotype (Mehan et al., 1986), environmental factors (Sander et al., 1993), inadequate pre-harvest and storage practices (Jacques, 1988; Hell et al., 2003), the presence of storage insect pests (Dowd et al., 2005), and the occurrence of other fungi (Hill et al., 1985). The combination of some or all of these factors may ultimately results in increased risk of aflatoxin production in field crops, stored food and feed (Cardwell & Henry, 2004). In tropical agricultural systems, the threat of aflatoxin contamination is high, since most of the factors that favor both the fungal and toxin development are more prevalent.

The determinant factor for *A. flavus* infection in a maize field is the presence of primary inoculum. The presence of *A. flavus* propagules in their infective form during maize ripening, harvesting, and during storage is an essential factor for maize contamination by aflatoxin. Horn (2003) showed that the higher the inoculum of *A. flavus* in a field, the higher the risk of maize becoming contaminated (Jaime-Garcia & Cotty, 2004).

The impact of other biological factors affecting maize during different processes from the field to the storage structure has been studied extensively (Setamou et al., 1997; Hell et al., 2000). Insect colonization of grain is one of the principal factors that have a positive impact on aflatoxin development. They cause wounds on grains and cobs during their feeding leading to the removal of the natural barriers that protect maize grains and, subsequently, to an increase the accessible area for fungal colonization. The activities of insects also increase moisture levels

during storage due to their metabolism, thereby changing the environmental conditions (Beti et al., 1995). Insects can also transport fungal propagules during their movement (Beti et al., 1995). Thus, high insect population levels in maize either during production or storage significantly increases the risk of aflatoxin contamination.

The impact of other fungi on the development of *A. flavus* and aflatoxin contamination depends primarily on environmental conditions and on mold species involved. It has been reported that the presence of *Fusarium* spp. is detrimental to *A. flavus* development (Wicklow & Shotwell, 1983). However, the direct impact of *Fusarium* spp. or *Penicillium* spp. on aflatoxin production in maize has not been studied, especially not for cropping conditions in Western Africa. Most studies have focused on the correlation between aflatoxin and fumonisin contamination (Abbas et al., 2005).

Previous studies have suggested diverse levels of resistance to aflatoxin among maize genotypes (Brown et al., 1999). However, it has been difficult to breed for a resistant variety that has a good and a viable level of resistance to mycotoxins (Campbell & White, 1995). The most important causes for this are (i) the lack of identified resistance genes, (ii) the complex nature of inheritance of resistance, (iii) the varying levels of infection by *A. flavus* in maize and (iv) the high variability of maize contamination by aflatoxin within and between seasons that limits resistance transfer (Menkir et al., 2006; Munkvold, 2003). Researchers at the International Institute of Tropical Agriculture (IITA) in collaboration with the Southern Regional Research Center (SRRC) in the USA have recognized various sources of maize germplasm for breeding aflatoxin resistant genotypes (Menkir et al., 2006). Genetically, maize resistance to aflatoxin is more quantitative than qualitative (Gardner et al., 1987) and is highly influenced by environmental factors. Hence, both genetic resistance and environment need to be considered when assessing susceptibility of a given maize genotype under cropping conditions.

Maize intercropping with another crop was identified by Hell et al. (2003) as one of the possible favorable factors for aflatoxin accumulation in maize in West Africa. The most prevalent rotational systems in rural Africa are maize/cowpea, maize/cassava, and maize/peanut. In the same study, the intercropping of maize and cassava, maize and tomato as well as maize and pepper were associated with reduced amounts of aflatoxin in maize, but only for northern localities. In a previous study, Cardwell et al. (2000) found that maize intercropping with cotton and sorghum also increased the risks of aflatoxin contamination in maize. This led to the hypothesis that intercropping maize with specific crops increases the risk of aflatoxin contamination in maize kernels. Similarly, maize rotation with susceptible crops or maize monoculture on the same plot can lead to a high level of aflatoxin contamination.

In order to develop effective and efficient control methods, a good understanding of the effects of both abiotic and biotic factors that influence *Aspergillus* development and aflatoxin production is critical. The objectives of this study were two-fold: 1) to investigate the impact of maize variety, cropping system and primary inoculum levels on aflatoxin production by *A. flavus*; and 2) to determine the relationships between aflatoxin contamination and population of storage insects and other mold fungi. Understanding these interactions could be useful in predicting aflatoxin contamination in pre- or post-harvest maize, when timely applications of control measures targeting mycotoxin reduction are critical, especially in agricultural regions with limited production resources.

MATERIALS AND METHODS

Plant materials, cropping and experimental design

Two maize (*Zea mays* L.) varieties were used in the experiment: "Gbogbe", a local maize variety with a maturity cycle of 90 days, and "TZSR-W" (tropical *Zea mays* streak resistant white), an improved variety. The latter variety has a longer maturity cycle of 120 days, and was expected to accumulate less aflatoxins because of the hard tegument of its kernels that might prevent infection by *A. flavus*. Besides growing the two maize varieties in pure stands, their intercropping with Kpodjiguégué - a local variety of cowpea (*Vigna unguiculata* (L) Walp) that has a cropping cycle of 90 days was also tested. The cowpea was sown between each pair of maize rows. A completely randomized block design of the eight treatments (two varieties in two cropping systems, with and without *A. flavus* inoculation) and three replications was used in the experimental set up (Table 1). Each plot had a size of 8 m x 8 m. Maize plants within a row were separated by 25 cm and the rows were separated by 75 cm. Two weeks after planting, a NPK fertilizer (15-15-15) was applied at a rate of 160 kg per ha. At the beginning of the grain formation, urea was applied at a rate of 50 kg per ha. Weeding was carried out before each fertilizer application.

Inoculum preparation and plots soil inoculation

For inoculation, a fungal culture was used which was originally obtained from maize and collected in the coastal zone (South) of the Republic of Benin. This was purified by single spore isolation and had its capacity to produce aflatoxin demonstrated by spore suspension fermentation followed by, aflatoxins extraction from the fermented suspension followed by extraction of aflatoxins and its quantification. Half of the experiment was inoculated with this selected *A. flavus* strain. t. The fungus was grown in plates containing 5/2 medium (5% V8 juice and 2% agar) (Cotty, 1989). The plates were kept in an incubator at 31°C in the dark for 7 days. The conidia were collected and suspended in sterile distilled water. Approximately 100 µl of tween 80 were added per liter of water and the concentration of the suspension was determined using a hemocytometer. The original suspension was diluted to obtain a concentration of

TABLE 1 - Factors combinations and treatments reference of the field experiment

Number	Variety	Cropping system	Inoculation	Treatment
1	Gbogbe	Intercropped with cowpea	Inoculated	LVCP1
2	Gbogbe	Intercropped with cowpea	Non - inoculated	LVCP0
3	Gbogbe	Pure stand	Inoculated	LV1
4	Gbogbe	Pure stand	Non - inoculated	LV0
5	TZSR	Intercropped with cowpea	Inoculated	IVCP1
6	TZSR	Intercropped with cowpea	Non - inoculated	IVCP0
7	TZSR	Pure stand	Inoculated	IV1
8	TZSR	Pure stand	Non - inoculated	IV0

2.7 x 10^7 conidia per liter used for inoculating autoclaved rice paddy at the rate of 100 mL spore suspension per 1000 g of autoclaved paddy. The colonized paddy was properly mixed by shaking, stored for 5 days at 31°C in the dark in an incubator (Percival, Model I-35LL) and later spread in the experimental field. The inoculum (paddy-spore mix) was spread over the soil surface when approximately 50% of the maize plants started flowering between 35 and 44 days after sowing. For each plot, one kg of the inoculum was spread uniformly by hand over the whole area of each plot.

Soil sampling and *A. flavus* population evaluation

Soil samples were taken from the top soil layer (0 - 5 cm depth) in all 24 plots approximately 24 hours before field inoculation and two weeks after inoculation to verify the effectiveness of the inoculation treatment. Eight sub-samples were taken along diagonals, five along the medians and one in the center of each plot. The 14 sub-samples were pooled and mixed to represent one sample per plot. The soil water content was determined from differences in weight of soil samples before and after drying in the oven at 130°C for two hours.

The number of colony-forming units (cfu) was obtained by mixing 10 g of soil in 50 mL of sterile distilled water in a flask. The flask was then vigorously shaken for 30 min and 100 µL of the resulting suspension were used to inoculate a plate containing a Modified Rose Bengal Agar (Cotty, 1994). The plates were left in an incubator at 31°C in the dark for three days and all isolates belonging to the genus *Aspergillus* were subcultured onto a 5/2 medium. After seven days of incubation, isolates were identified and number of *A. flavus* colonies was recorded. When too many colonies appeared on the Petri dishes, the suspension was further diluted, replated, incubated, and recounted.

Maize evaluations at post-harvest

The maize cobs were harvested at maturity, which occurred at 110 and 125 days after planting for Gbogbe and TZSR-W, respectively. After sun drying for two days, the cob samples were collected for each treatment and the remainder of the harvest was stored in jute bags and kept in a storage room on wood pallets. A sample of 15 cobs was taken from each treatment and analyzed in the laboratory after one, two, three and four months in storage. The percentage of the de-husked cobs covered with molds was assessed visually followed by grain shelling, counting and identification of all insect species observed. Ground maize was used to determine moisture content with the method described above.

To assess the inoculation of maize kernels by *A. flavus*, 10 g of ground maize of each cob sample was mixed in 50 mL of sterile distilled water in a flask. The number of cfu was then obtained following the same steps as for soil cfu determination. The number of cfu of *Penicillium* spp. was estimated on potato dextrose agar amended with rose Bengal. *Fusarium* colonization of cobs was determined by visually estimating the percentage of the cob area covered by signs of this fungus.

Aflatoxin analysis

The aflatoxin contamination from ground maize was determined as described by Thomas et al. (1975). For each sample, 50 g of ground maize were weighed placed in a 500 mL Erlenmeyer flask containing 250 mL of methanol and water (60/40 vol/vol) and the flasks were shaken for 30 min. The suspension was filtered and separated with a mixture of saturated sodium chloride and hexane solution. A second separation procedure was performed using chloroform that binds with the toxins, and the mixture was then drained into 250 mL Erlenmeyer flasks containing 5 g of cupric carbonate. The flask was shaken for 30 seconds and filtered through a Whatman filter No. 42 containing 50 g of anhydrous sulfate. The chloroform extract was collected into a beaker and allowed to evaporate. The extract was dissolved with 1 mL of chloroform, transferred into a smaller container and stored in the refrigerator for aflatoxin quantification.

Aflatoxin was quantified by thin layer chromatography. Each sample was diluted with 1 mL of chloroform and spotted at 2 cm from the base on pre-coated silica gel TLC plates with a mixture of aflatoxins B_1, B_2, G_1 and G_2 standard. The spotted plates were developed in a mixture of diethyl ether/methanol/water (95/4/1 vol/vol/vol) for about 25 min. The plates were dried and scanned with a densitometer (CAMAG TLC Scanner 3) run with win-CATS 1.4.2 software (Camag AG). The variations in the intensity of the fluorescence were automatically used in

comparison with the standard to calculate the concentration of aflatoxins. Samples with too intense fluorescence as compared with the standard were diluted, spotted and chromatographed again.

Data analysis

A multi factorial analysis of variance was performed using the general linear model procedure in SAS (SAS Institute, 2003) with "variety", "inoculation" and "cropping system" as independent variables. The means of the treatments were separated by Fisher's least significant difference test (P = 0.05). The Pearson correlation was calculated between pairs of all variables analyzed.

The concentration c of both aflatoxins on maize kernels were transformed to $c' = \ln(c + 1)$ to create linear relationships and to achieve homogeneous variances. To test the progress of aflatoxin on maize stemming from two treatments, for instance from the local and the improved variety, the following model was fitted to the combined data of the two treatments:

$$c'(t) = \ln(c(t)+1) = (a + d \cdot \Delta a) + (b + d \cdot \Delta b) \cdot t \quad (1)$$

The dummy variable d is equal to 0 for the first treatment, e.g. for the local variety, and equal to 1 for the second treatment, for example for the improved variety. Therefore, the parameter a is the intercept of the regression line of the first treatment, Δa is the difference in intercept between the second and the first treatment. Thus ($a + \Delta a$) would be the intercept of the second treatment. Similarly, b is the slope of the first treatment, while Δb the difference in slope between both treatments. Then it was tested if Δa and Δb were significantly different from 0. If one of the two differences was not significantly different from 0, it was set to 0 and the regression analysis was repeated with one of the three-parametric models:

$$c'(t) = \ln(c(t)+1) = (a + d \cdot \Delta a) + b \cdot t \quad \text{if } \Delta b = 0 \quad (2a)$$

$$c'(t) = \ln(c(t)+1) = a + (b + d \cdot \Delta b) \cdot t \quad \text{if } \Delta a = 0 \quad (2b)$$

If both differences were not significantly different from 0, the difference with the highest P-value was set to 0 firstly. When the remaining difference in the 3-parametric model (eq. 2a or 2b) was again not significantly different from 0, it was concluded that the lines of the two treatments did not differ neither in the intercept nor in the slope.

Similar to eq. 1 in which the effect of two treatments can be compared, another function was established to simultaneously describe the data of the four treatments carried out with each cultivar:

$$c'(t) = \ln(c(t)+1) = (a + d_i \cdot \Delta a_i + d_c \cdot \Delta a_c) + (b + d_i \cdot \Delta b_i + d_c \cdot \Delta b_c) \cdot t \quad (3)$$

This equation includes two dummy variables, d_i and d_c, to identify the treatment (index i for inoculation, index c for the cropping system), and four difference terms, Δa_i, Δa_c, Δb_i, and Δb_c, to estimate the effect of treatments on the intercept a and the slope b in relation to the non-innoculated ($d_i = 0$) pure stand ($d_c = 0$). As described above, first the full model (eq. 3) was simultaneously fitted to the data of the four treatments of a cultivar, and then the model was reduced by setting non-significant differences stepwise to 0. The regression analyses were carried out using SigmaPlot10.

RESULTS

Soil sampling and *A. flavus* population evaluation

All plots had relatively dense natural populations of *A. flavus* in the soil, which ranged from 653.1 to 2062.3 cfu/g prior to the toxigenic *A. flavus* strain inoculation. Inoculation increased the level of cfu in IV1 (improved variety in pure stand and with inoculation), IVCP1 (improved variety intercropped and with inoculation), LV1 (local variety in pure stand and with inoculation) and LVCP1 (local variety intercropped and with inoculation) by 1611.1, 879.7, 1791.0 and 543.9 cfu/g, respectively. Similar increasing trends were found in the non-inoculated plots IV0 (improved variety in pure stand and without inoculation) and IVCP0 (improved variety intercropped and without inoculation), in which increases of *A. flavus* of 680.7 and 165.9 cfu/g were observed from day 0 to day 15. However, for LV0 (local variety in pure stand and without inoculation) and LVCP0 (local variety intercropped and without inoculation), the number of *A. flavus* propagules decreased by 1300.0 and 240.2 cfu/g, respectively. The analysis of treatment effects showed that inoculated plots had a significantly higher level of cfu/g of *A. flavus* than non-inoculated plots (Figure 1).

Maize evaluations at post-harvest

Water content of stored maize cobs

The water content of the two maize varieties was significantly different after one month of storage (Figure 2); the improved variety had significantly higher water content

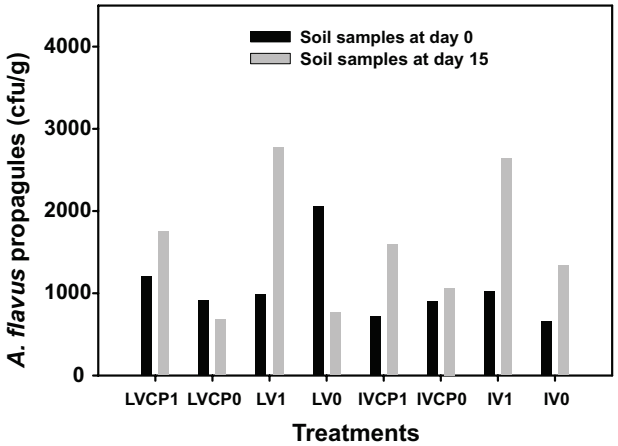

FIGURE 1 - Population density of *Aspergillus flavus* (cfu/g) in the soil of eight treatment plots (Table 1) before and after inoculation (on day 0, none of the plots were inoculated; on day 15, LVCP1, LV1, IVCP1 and IV1 were inoculated).

($P < 0.001$) as compared with the local variety. In general, the water content during the first month of storage was higher than in later months irrespective of the treatment. This may be due to the difference between the duration of the cycle for the varieties since the local variety, with a shorter cycle, was dryer at harvest.

***Aspergillus flavus* propagules in stored maize cobs**

The number of cfu/g of *A. flavus* propagules of the different treatments was variable along the sampling period. During the first month of storage, the amount of propagules did not differ between the varieties, but in the subsequent months the improved variety had a consistently higher number of cfu of *A. flavus* propagules than the local variety. The situation was different in the inoculated treatments with higher cfu of *A. flavus* compared to non-inoculated treatments, observed from the third month on. Intercropping of maize with cowpea did not lead to a measurable impact on the level of *A. flavus* propagules in maize during the four months of storage (Figure 3). Furthermore, there were no significant interactions between the three treatments (i.e. inoculation, varieties and cropping system) during the four months of storage.

***Penicillium* spp. propagules of stored cobs**

During the first month there were only differences in *Penicillium* spp. between maize varieties, with the local variety being more contaminated than the improved variety (Figure 4). Althought it appeared that a high humidity content in the local variety, as compared with the improved variety was behind the different contamination levels, this conjecture was not supported by experimental evidence because water content was not determined for the samples. Neither inoculation, nor intercropping had a significant impact on the levels of contamination with *Penicillium* spp. Only after four months of storage the cobs from the non-inoculated plot had significantly more *Penicillium* spp. propagules than the cobs from the *A. flavus* inoculated plot. The number of cfu of *Penicillium* spp. from the inoculated maize plot decreased with storage time, while the level was fairly constant for the non-inoculated plots. For maize intercropped with cowpea, the level of contamination by *Penicillium* spp. increased from the first to the third month in storage and then declined during the fourth month in storage.

***Fusarium* sp. colonization of stored cobs**

Aspergillus flavus inoculationation did not significantly affect the colonization of *Fusarium* spp. of the stored cobs. The only measurable impact on *Fusarium* spp. colonization was variety, with the improved variety having a significantly higher extent of colonization of *Fusarium* spp. during the storage period (Figure 5). The difference in *Fusarium* spp. colonization on varieties is mainly imputable to the difference in water content between varieties. In general, the colonization of *Fusarium* spp. increased significantly during storage of the maize cobs.

Insect Populations

There were no significant differences among the treatments with respect to the insect populations during the four months of storage, although the population of *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) increased significantly during storage. The population of *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) was significantly higher for the stored cobs of the local variety than for the improved variety at the end of storage period. The population of *Cathartus quadricollis* Guérin-Méneville

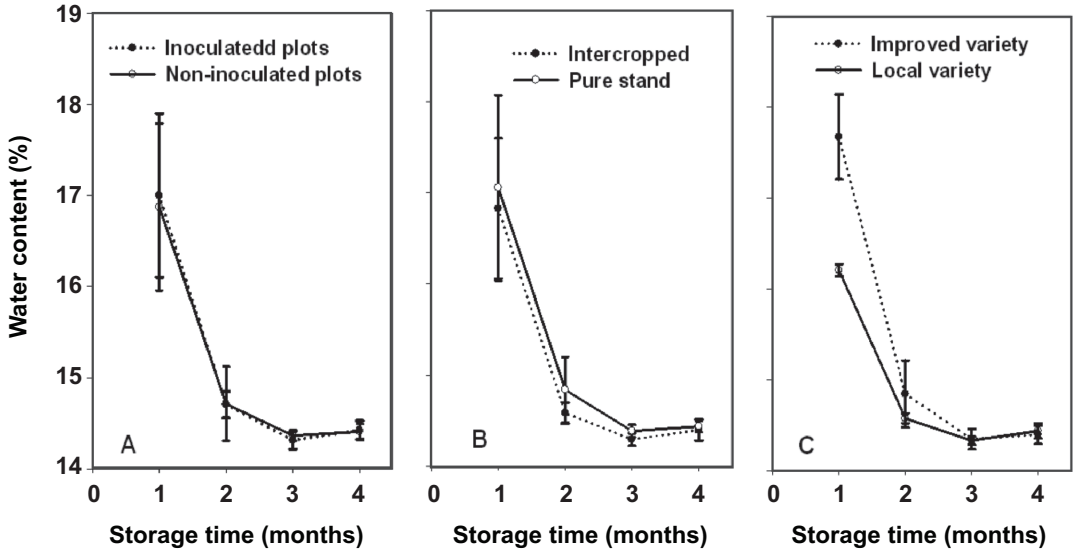

FIGURE 2 - A. Temporal progress of moisture content (%) in maize kernels during four months of storage as affected by inoculation; **B.** cropping system and **C.** variety.

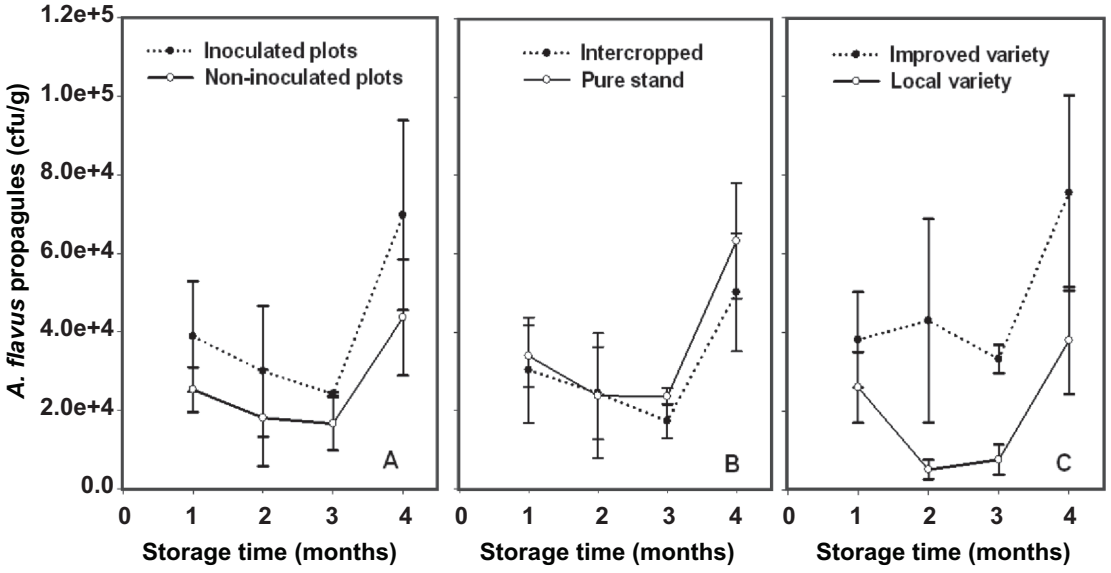

FIGURE 3 - A. Dynamics of *Aspergillus flavus* propagules (cfu/g) of the maize kernels during four months of storage depending on inoculation; **B.** cropping system and **C.** variety.

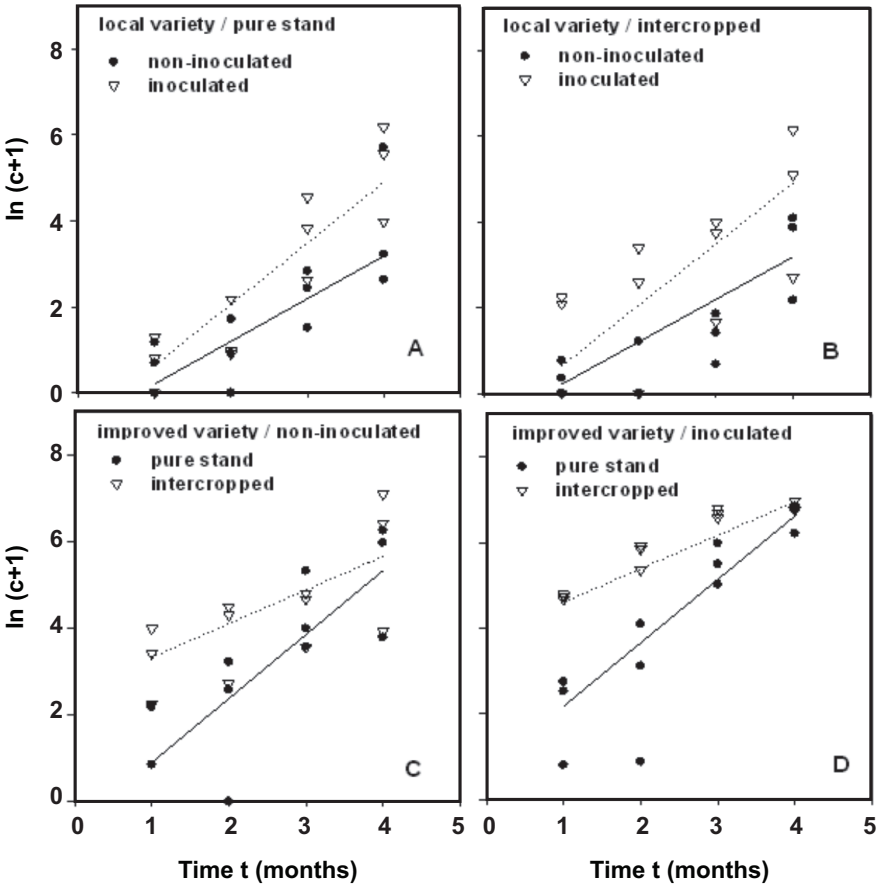

FIGURE 4 - Progress of total aflatoxin in maize kernels during four months of storage for two varieties and four treatments (see Table 1 for treatment definitions). The original concentrations *c* of the three samples in each treatment per observation date were log-transformed ($\ln(c + 1)$). The lines are the regression lines resulting from the simultaneous fitting of eq. 3 to the data of the four treatments for each cultivar.

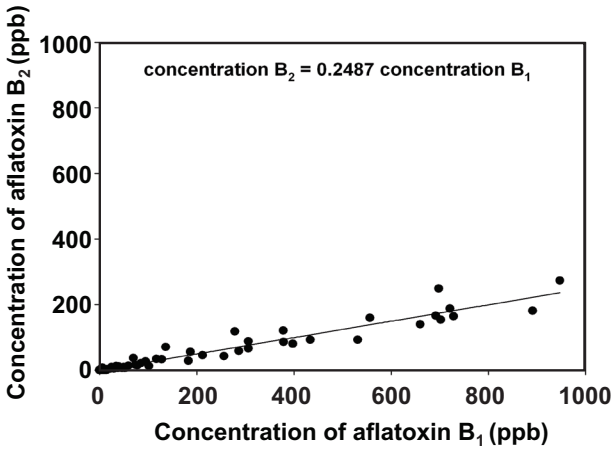

FIGURE 5 - Correlation between the aflatoxin B_1 and B_2 concentrations inthe 96 maize samples stored for four months.

(Coleoptera: Cucujidae) increased significantly between the third and the fourth month of storage irrespective of treatment. However, there were no significant differences among the inoculationation, cropping system and varietal treatments (Figure 6).

Aflatoxin analysis

Aflatoxin B_1 and B_2 in stored cobs

Only aflatoxin B_1 and B_2 were detected in the maize cobs, with aflatoxin B_1 dominating in all treatments. The concentration of total aflatoxin (B_1+B_2) increased exponentially in all treatments. The variability of the concentrations determined for the 3 samples per treatment and per months was high, even after the ln-transformation was applied (Figure 7). When eq. 3 was used to analyze the progress of total aflatoxin in the four treatments of the local variety, neither the inoculation nor the cropping system had a significant effect on the intercept, which represents the initial concentration of aflatoxin. Also the slope, i.e. the rate of increase of aflatoxin with time, was not influenced by the cropping system, but inoculation significantly increased the slope by 0.43 (Figure 7A-B). The final equation for the dynamics of the aflatoxins concentration on the local variety was (R^2 = 0.65):

$$c'(t) = -0.78 + (0.99 + d_i \cdot 0.43) \cdot t \quad (4)$$

In the joint analysis of the four treatments (with eq. 3) involving the improved cultivar, only the effect of inoculation on the slope (Δb_i) was not significantly different from 0. Therefore, the final reduced model for the progress of aflatoxin on the improved variety had 5 parameters (R^2 = 0.76):

$$c'(t) = (-0.58 + d_i \cdot 1.28 + d_c \cdot 3.13) + (1.48 - d_c \cdot 0.70) \cdot t \quad (5)$$

The intercept and the slope of the reference treatment, i.e. of the non-inoculated pure stand, were -0.58 and 1.48, respectively (Figure 7C-D). Inoculation increased the intercept by 1.28, intercropping by 3.13. Inoculation did not affect the slope, while intercropping reduced the slope by 0.70.

The two cultivars performed differently, because for the local variety the only significant effect was that of the inoculation leading to an increased slope of aflatoxins concentration progress line, while for the improved variety, inoculation as well as the intercropping increased the initial level of aflatoxins, however, intercropping reduced the slope of aflatoxin concentration progress line.

When the same treatment combinations of both cultivars were compared using eq. 1 for all four cases, the

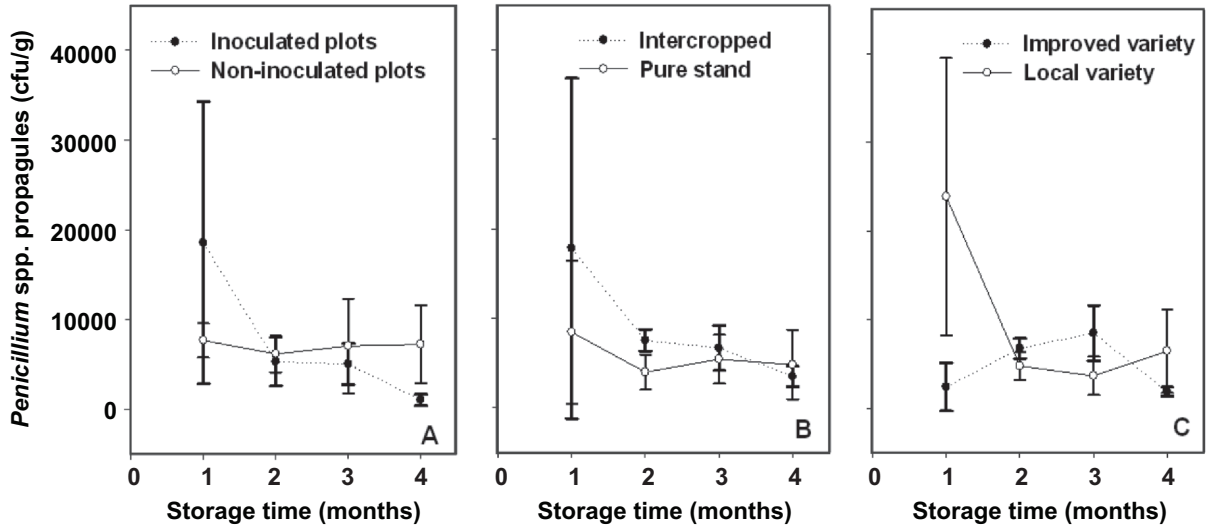

FIGURE 6 - A. Dynamics of *Penicillium* spp. propagules (cfu/g) of maize kernels during four months of storage depending on inoculation; **B.** cropping system and **C.** variety.

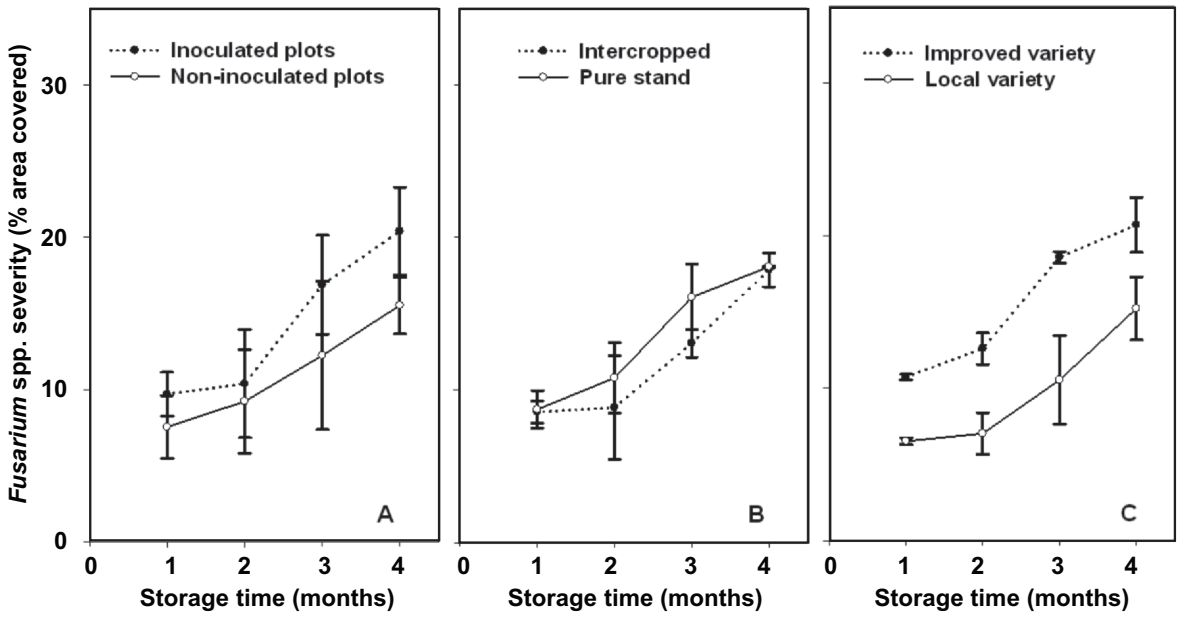

FIGURE 7 - A. Progress curves of *Fusarium* spp. colonization of maize cobs (% area covered) during four months of storage depending on inoculation; **B.** cropping system and **C.** variety.

differences between slopes were not statistically different. The local variety had always a lower intercept than the improved variety. The difference in intercept between the two varieties was significant for the intercropped plots in the full model with four parameters, for the pure stand only when a joint slope was assumed in a three-parametric model (eq. 2a).

The aflatoxin B_2 concentrations were clearly lower than the aflatoxin B_1 concentrations. In many samples, no aflatoxin B_2 was detected in the first and second sampling date. The aflatoxin B_2 concentrations in the different treatments (data not shown) also increased exponentially, but with a lower level. The non-transformed aflatoxin B_1 and B_2 concentrations of the 96 maize samples measured during the four months of storage were highly correlated with a correlation coefficient of 0.97 (Figure 8). Thus, the aflatoxin B_2 concentration of a sample can be predicted from the B_1 concentration. The regression line, calculated over all samples, had an intercept that was not significantly different from 0 so that the line could be forced through the origin with a slope of 0.2487 (Figure 8). On average, the concentration of aflatoxin B_2 in a sample was, therefore, only one fourth of the concentration of B_1.

Correlations among characteristics of stored maize cobs

A significant and positive correlation was observed between *A. flavus* cfu and aflatoxin B_1 ($r = 0.54$, $P < 0.0001$), and between *A. flavus* cfu and aflatoxin B_2 ($r = 0.52$, $P < 0.0001$) of the stored maize cobs (Table 2). There was a negative correlation ($r = -0.34$, $P = 0.0008$) between *A. flavus* cfu and *Penicillium* spp. cfu (Table 2), between *Penicillium* spp. cfu and aflatoxin B_1 ($r = -0.21$, $P = 0.05$), and between *Penicillium* spp. cfu and aflatoxin B_2 ($r = -0.16$, $P = 0.11$), although, the latter was not significant. On the stored maize cobs, the colonization of *Fusarium* spp. was positively correlated with *A. flavus* cfu ($r = 0.49$, $P < 0.0001$), with aflatoxin B_1 ($r = 0.53$, $P < 0.0001$), and with aflatoxin B_2 ($r = 0.56$, $P < 0.0001$), respectively. The positive correlation between aflatoxin and *Fusarium* spp. was due to the double positive correlation between *A. flavus* and aflatoxin and *A. flavus* and *Fusarium* spp. The level of aflatoxin was also positively correlated with the population size of the three species (Table 2).

DISCUSSION

In this study, a relatively high level of cfu of *A. flavus* was observed in the soil of non-inoculated plots in comparison with the inoculated plots. This is likely due to the history of maize cultivation in the experimental area for a few years. In West Africa, agriculture fields commonly present high levels of *A. flavus* inoculum. A survey conducted by Cardwell & Cotty (2002) in Benin showed that all 88 surveyed fields were inoculated with *A. flavus* propagules, with some samples exceeding 5000 cfu/g, while the average inoculation was around 486 cfu/g. Jaime-Garcia & Cotty (2004) identified previously grown aflatoxin susceptible crops as a major primary inoculum source that initiates new *A. flavus* infection cycles during subsequent maize cropping.

Our results also indicated that application of rice

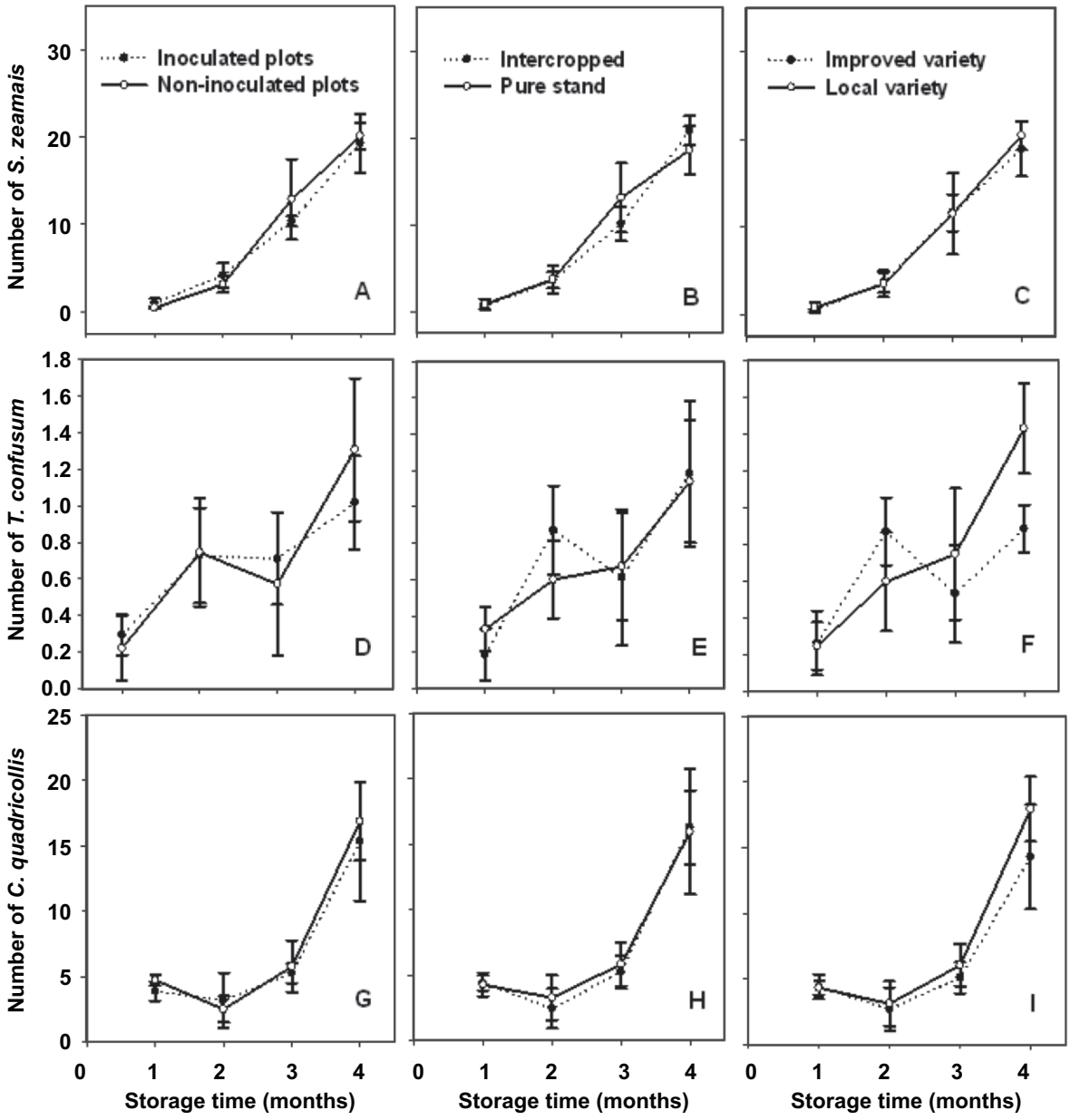

FIGURE 8 - Population dynamics of *Sitophilus zeamais* (**A.B.C.**), *Tribolium confusum* (**D.E.F.**) and *Cathartus quadricollis* (**G.H.I.**) on maize cobs during four months of storage depending on inoculation (**A.D.G.**); cropping system (**B.E.H**.) and crop variety (**C.F.I.**).

paddy carrying toxigenic *A. flavus* on fields was an effective method of inoculation, although rarely used in previous studies in which mostly used wheat-alginate pellets as carrier/substrate for *A. flavus* inoculum (Daigle & Cotty, 1995; Bock & Cotty, 1999). The fungal inoculation allowed the establishment of a toxigenic population in the inoculated plots, so that *A. flavus* cfu determination and aflatoxin level assessment was possible. However, according to Cotty & Cardwell (1999), the levels of aflatoxin contamination were usually higher if a high proportion of S-strains isolates (small sclerotia strains characterized by a higher capability for aflatoxin production in opposite to L-strains isolate that are characterized by a lower capability for aflatoxin production) are present in a given fungal population, but there can be a high variation in such levels.

The growth curve of *A. flavus* cfu of the stored maize cobs indicated that infection took place at two major stages. There was an initial decrease in cfu level from the first to the third month of storage, followed by an increase in cfu levels after the third month of storage. The high levels of aflatoxin contamination that developed during the first month indicated that *A. flavus* infection of maize already

TABLE 2 - Correlation coefficients between characteristics of stored maize cobs: *A. flavus* and *Penicillium* spp. propagules (cfu), moisture content (%), aflatoxins concentrations (ppb), *Fusarium* spp. severity (% cob area covered) and the number of insects (*Sitophilus zeamais, Tribolium confusum* and *Cathartus quadricollis*) during storage

Main observed variables	*A. flavus* propagules	Moisture content	Aflatoxin B_1 concentration	Aflatoxin B_2 concentration
Moisture content	0.13	--	-0.32 **	-0.33 **
Aflatoxin B_1 concentration	0.54 ***	-0.32 **	--	0.97 ***
Aflatoxin B_2 concentration	0.53 ***	-0.33 **	0.97 ***	--
Penicillium spp. Propagules	-0.34 ***	-0.15	-0.21 *	-0.16
Fusarium spp. Severity	0.49 ***	-0.22 *	0.53 ***	0.56 ***
S. zeamais number	0.20 *	-0.52 ***	0.50 ***	0.49 ***
T. confusum number	0.02	-0.44 ***	0.28 **	0.26 **
C. quadricollis number	0.25 **	-0.27 **	0.36 **	0.36 **

***= P<0.0001, ** = P<0.001 and * = P<0.05

occurred in the field prior to or during harvest, which is in agreement with previous reports (Setamou *et al*, 1997; Bankole & Mabekoje, 2003). The high number of cfu observed at the beginning of the first month could have also been influenced by the high water content observed in the maize kernels at the beginning of the storage (Cardwell et al., 2000) and the aggressiveness of the *A. flavus* isolates (Zummo & Scott, 1990). At harvest, the water content in maize reached its highest level which might have favored *Aspergillus* colonization. Cardwell et al. (2000) indicated that excessive water content in maize could minimize the resistance of maize kernel to aflatoxin contamination. In this study, high levels of moisture content were found in the improved maize variety. The improved maize variety plots with maize grains having higher water content that were inoculated with *A. flavus* had a higher level of *A. flavus* infection than the non-inoculated and local maize variety plots but also with dryer grains.

As their water content decreased, the maize kernels became less susceptible to fungal colonization, therefore successful *A. flavus* infection decreased from the first to the third month. The increasing level of *A. flavus* after the third month could be due to improvement in conditions for *A. flavus* development and insect activity, probably as a result of favorable temperatures and the multiplication of *Cathartus quadricollis* and *Tribolium confusum* which were recorded during this period. Furthermore, biological activity of insects might have created a microenvironment that favored *A. flavus* and other fungal growth (Picco et al., 1999). Insects can also spread *A. flavus* propagules throughout the maize bulk leading to new infection loci.

One of the hypotheses why cowpea intercropping led to lower toxin levels is that the direct spread of *A. flavus* propagules from soil to the maize cobs may be reduced by physical barriers of the non-host crop. For the local variety, neither inoculation, nor cropping system affected the values of aflatoxin concentrations at the harvest. However, inoculation increased aflatoxin contamination during storage on maize grown in pure stand as well as intercropped. This shows the importance of the amount of the initial inoculum in the soil for the final aflatoxin accumulation in maize during storage. In the case of the local variety, cowpea did not interfere with the infection of the maize plant. The results are different for the improved variety, in which intercropping and inoculation both led to a higher toxin level of maize at final harvest due to the high level of *A. flavus* infection. However, during storage, the kernel that originated from the intercropping system had a lower increase in aflatoxin. There could be two possible explanations. Firstly, because of the physical barrier posed by cowpea covering the soil under maize plants, the inoculum that led to the infection of i the maize cobs in intercropping plots may have originated from distant sources (airborne inoculum) instead of originating locally from the soil. Many studies have shown the presence of *A. flavus* propagule in the air around maize fields (Ilag, 1975; Abdalla, 1988). Secondly t the mechanism of infection of the cobs itself was of a different nature. There are several ways by which natural *A. flavus* infection in maize cobs can take place. Infection can occur through the silk and in this case *A. flavus* propagules are mainly superficial to maize kernel (Marsh & Payne, 1984a, 1984b), through the cob with access to the kernel through the spikelet (Smart et al., 1990). It can also be introduced and spread by cob borers and other insects (Drepper & Renfro, 1990; Beti et al. 1995). The timing of the infection and colonization inside the kernel may depend on the infection pathway (Marsh & Payne, 1984a, 1984b). Our results suggest that intercropping maize with cowpea contributes towards reducing aflatoxin levels in very susceptible maize varieties.

Understanding the process involved in crop contamination by *A. flavus* is very important since aflatoxins are only produced by certain *A. flavus* strains. During the first month of storage, all treatments had almost zero aflatoxin content, but the level increased over time. This tendency has been reported in other studies (Hell et al., 2000). *Aspergillus flavus* requires favorable environmental conditions and a susceptible maize variety for colonization and toxin production. The toxin production depends on infection initiation and fungal colonization (Klich, 2007).

However, not all *A. flavus* propagules that adhere to the grain surface lead to infection.

We found significant interactions between fungi, insects, and the level of aflatoxins. *Penicillium* spp. was negatively correlated with *A. flavus* infection and level of aflatoxin. Either through competition or as a result of aflatoxin production, *A. flavus* seemed to exhibit antagonistic properties against *Penicillium* spp. during storage. Both fungi require similar conditions and substrate for growth (Marin et al., 1998). However, *Fusarium* spp. was positively correlated with *A. flavus* and aflatoxin. The assessment of *Fusarium* spp. colonization was based on visual estimation which is probably less accurate as compared to the cfu method. No evidence of competition or inhibition was observed between *A. flavus* and *Fusarium* spp.

The positive correlation between aflatoxin level and the sizes of insects' populations suggests that the activities of the insects promoted an increase in aflatoxin production. Cardwell et al. (2000) and Udoh et al. (2000) studied the effect of insects' activities and qualified them as favorable for aflatoxin contamination when they increased the level of infection of *A. flavus*. A similar trend was observed during our study. Inoculation by the three insect species in our study could have predisposed stored maize kernels to *A. flavus* infection and favour an increase in the levels of aflatoxin production. It is also possible that the level of aflatoxin production was affected by the competition for nutrients between *A. flavus* and the other fungi (Calvo et al., 2002).

In conclusion, this study demonstrates the role of primary inoculum on *A. flavus* infection and the rate of colonization of grains, and how they affect aflatoxin production in stored maize. Many studies showed that with direct inoculation of a substrate the resultant aflatoxin content depended directly on the level of the inoculum (Odamtten et al., 1987; Karunaratne & Bullerman, 1990). This study identified soil inoculation with a toxigenic strain of *A. flavus* and high maize water content level at harvest time (at the beginning of the storage) as two key factors for a successful colonization by *A. flavus* in stored maize. Further investigations are required in order to better establish whether storage fungi, other than *A. flavus*, and storage insects are of relevance for resulting levels of aflatoxin in maize requires.

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