Factors influencing *Aspergillus flavus* strains and aflatoxins expression in maize in Benin, West Africa

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Abstract

Aspergillus flavus, a soil-borne fungus, is the major responsible for aflatoxin contamination in maize in tropical area. In soil samples from different parts of Benin, the incidence of *A. flavus* and the percentage of L-strain isolates were high in the Costal Savanna (CS) and Southern Guinean Savanna (SGS) zones. In contrast, the S-strain isolates were more represented in the Northern Guinean Savanna (NGS) and Sudan Savanna (SS) zones. Atoxigenic isolates were evenly distributed throughout all four zones. Also toxigenic isolates were almost homogenously represented, only SS had more toxigenic isolates than NGS. The incidence of *A. flavus* in maize followed the pattern of soil incidence. SGS and NGS differed in aflatoxin content in maize with higher values in SGS. The site latitude and height above sea level were highly negatively correlated with the incidence of *A. flavus* in the soil, the percentage of L-strain isolates, and the *A. flavus* incidence in maize and positively correlated with the percentage of S-strain isolates. Regarding the soil texture, there were positive correlations between the sand percentage and the toxigenic isolates percentage, between the silt and the S-strain isolates percentages, and between the clay and atoxigenic isolates percentages. Negative correlations were found between the sand and the atoxigenic isolate percentages, between the silt percentages and the L-strain isolates and *A. flavus* incidence in the maize. The soil content of calcium, potassium and sodium were all three in positive correlation with the percentages of L-strain isolates and of atoxigenic isolates. Moreover, the sodium content in the soil was positively correlated with *A. flavus* incidence in the soil and negatively with the toxigenic isolates percentages. The level of aflatoxin in maize depended directly on the soil organic carbon, soil incidence of *A. flavus*, L-strain isolate percentage, S-strain isolates percentage and *A. flavus* incidence in maize. The conclusions in this first study led to a field experiment to investigate additional factors in details. In this study on the effects of the soil inoculation, maize variety and cropping system on the level of aflatoxin in stored maize in Benin, the concentration of aflatoxin B_1 and B_2 increased during storage. Variety and inoculation with *A. flavus* were the main factors influencing the production of aflatoxins in stored maize. The improved maize variety had higher levels of aflatoxin B_1 and B_2 compared to the local variety. Intercropping with cowpea (*Vigna unguiculata* (L.) Walp.) decreased aflatoxin concentration in the improved maize variety but not in the local maize variety. On the local maize variety, higher levels of *Penicillium* spp. and lower levels of *Fusarium* spp. were observed than on the improved maize variety. Neither the variety, nor the soil inoculation with an atoxigenic strain of *A. flavus* or the cropping system had an effect on the populations of major storage insects, but their numbers in the stored maize were positively correlated with aflatoxin. The initial level of fungal inoculum and the water content of the maize kernels after harvest played a significant role in the initiation and development of *A. flavus* infections. Further to assess biotic factors, maize maturating in the field at milky stage and already harvested maize kernels were inoculated with *A. flavus* spores alone or in combination with *Fusarium* spp. and/or *Penicillium* spp. In both experiments, the grains were stored in an incubator and sampled weekly. In the preharvest experiment, the incidence of *A. flavus* increased linearly during seven weeks of storage with the same slope in all treatments, but with a slightly higher level in treatments in which *Fusarium* spp. was inoculated too. In all treatments, the incidence of *Fusarium* spp. decreased initially and became larger again after four weeks of storage. The level of *Fusarium* spp. incidence was higher when *Fusarium* was co-inoculated. *Penicillium* spp. incidence had generally a slightly increasing linear trend. In the presence of *Fusarium* spp., the incidence of *Penicillium* spp. was reduced. During storage, *A. flavus* inoculation

led to an increase in aflatoxin. In the postharvest experiment, the incidence of *A. flavus* increased linearly in all treatments, including the control, starting from a low level. Compared to the control, the slope was higher after *A. flavus* inoculation and even higher when *Penicillium* was co-inoculated. The incidence of *Fusarium* spp. decreased linearly in all treatments, although the initial incidence was high. The incidence of *Penicillium* spp. varied over time without showing a uniform trend. The aflatoxin concentration in the postharvest experiment was lower than in the preharvest experiment and increased continuously and uniformly in all treatments. The final part concentrated on *A. flavus* itself and its classification subdivision. Six isolates were investigated for their growth and four for aflatoxin production. The Gompertz function described very well the colony growth of most of the isolates. The monomolecular model was good for aflatoxin production simulation. Generally, the water activity had more effect than temperature on the growth in the ranges studied in this paper. In all cases with high aflatoxin production, a degradation of the toxin followed. A water activity of 0.90 was the least efficient level while 0.96 was the most efficient one. At the latter level of water activity, the effect of the temperature was weak. Depending on the isolate, the optimal temperatures varied between 31, 33 and 35°C while the optimum water activity for all isolates remained 0.96. Concerning the aflatoxin production, the optimum water activity varied between 0.96 and 0.99 but the optimum temperatures were the two lowest in this study (26 and 28°C). The L-strain isolates also produced aflatoxin G but at lower levels of water activity (0.90 and 0.93) than the S-strains isolates (0.96 and 0.99). The highest rates of growth were recorded for isolates Z34A, Z117B and Z1TS, all being L-strain isolates. The best aflatoxin B producer was isolate Z213D that was also the best producer of aflatoxin G. Isolate Z1TS followed but only for aflatoxin B production. Z213D is an S-strain isolate and good producer of aflatoxin but had a very low growth rate. The lowest aflatoxin production rate was recorded for isolate Z34A that is an L-strain isolate characterized by very high growth rates.

When all factors important for *A. flavus* aflatoxin production in maize were quantified, they could be utilized to develop a model to predict aflatoxin occurrence in maize.

Keywords: Aflatoxin, *Aspergillus flavus*, sclerotial strains, toxinogenecity

Zusammenfassung

Aspergillus flavus, ein bodenbürtiger Pilz, ist der Hauptverursacher der Kontaminierung des Maises mit Aflatoxin in tropischen Ländern. In Bodenproben aus verschiedenen Gegenden Benins waren sowohl der Befall mit *A. flavus* als auch der Anteil der Isolate des L-Stamms in der Küstensavanne (CS) und der Südlichen Guinea-Savanne (SGS) hoch, während Isolate des S-Stamms stärker in der Nördlichen Guinea-Savanne (NGS) und der Sudan-Savanne (SS) vertreten waren. Nicht-toxigene Isolate waren gleichmäßig über die vier Zonen verteilt. Die toxigenen Isolate waren ebenfalls fast homogen auf die vier Zonen verteilt, wobei aber in SS mehr toxigene Isolate gefunden wurden als in NGS. Die Häufigkeiten von *A. flavus* im geernteten Mais und in den Bodenproben folgten dem gleichen Muster. Der Aflatoxingehalt des Maises war in SGS höher als in NGS. Die geographische Breite und die Höhe über NN der Felder waren mit der Häufigkeit von *A. flavus* im Boden sowie im Mais als auch mit dem Anteil der Isolate des L-Stamms stark negativ, mit dem Anteil des S-Stamms aber positiv korreliert. Bei den Bodeneigenschaften gab es positive Korrelationen zwischen dem Sandanteil und dem Anteil toxigener Isolate, zwischen dem Schluffanteil und dem Anteil der Isolate des S-Stamms sowie zwischen dem Lehmanteil und dem Anteil nicht-toxigener Isolate. Negativ waren dagegen korreliert: der Sandanteil mit dem Anteil der nicht-toxigenen Isolate und der Schluffanteil sowohl mit dem Anteil der Isolate des L-Stamms als auch mit der Häufigkeit von *A. flavus* im Mais. Die Calcium-, Kalium- und Natrium-Gehalte des Bodens waren mit den Anteilen der Isolate des L-Stamms bzw. der nicht-toxigenen Isolate korreliert. Darüber hinaus bestand zwischen dem Natriumgehalt des Bodens und der Häufigkeit von *A. flavus* im Boden eine positive Korrelation, eine negative Korrelation aber zu dem Anteil der toxigenen Isolate. Der Alfatoxingehalt des Maises hing von dem organischen Kohlenstoff des Bodens, der Häufigkeit von *A. flavus* im Boden und im Mais sowie von den Anteilen der Isolate der L- bzw. S-Stämme ab.

Die Schlussfolgerungen aus diesem ersten Teil der Arbeit veranlassten ein Feldexperiment, in dem der Einfluss zusätzlicher Faktoren auf den Aflatoxingehalt des gelagerten Maises genauer untersucht werden sollte. In diesem Experiment zur Wirkung der Inokulation des Bodens mit *A. flavus*, der Maissorte und des Anbausystems auf Aflatoxin stieg die Konzentration von Aflatoxin B_1 und B_2 während der Lagerung an. Die Sorte und die Inokulation mit *A. flavus* waren die wichtigsten Faktoren, die die Bildung von Aflatoxin im gelagerten Mais beeinflussten. Die verbesserte Maissorte enthielt höhere Gehalte an Aflatoxin B_1 und B_2 als die lokale Sorte. Der Mischanbau mit der Augenbohne (*Vigna unguiculata* (L.) Walp.) verminderte die Aflatoxinkonzentration in der verbesserten, nicht aber in der lokalen Maissorte. Auf der lokalen Maissorte wurde mehr *Penicillium* spp., aber weniger *Fusarium* spp. als auf der verbesserten Sorte festgestellt. Weder die Sorte, noch die Inokulation des Bodens mit einem nicht-toxigenen Isolat, und auch nicht das Anbausystem hatten einen Einfluss auf die Populationen der wichtigsten Lagerinsekten, deren Populationsgröße aber mit dem Aflatoxingehalt korreliert war. Das Ausgangsniveau des pilzlichen Inokulums und der Wassergehalt der Maiskörner nach der Ernte spielten eine wichtige Rolle für den Beginn und die weitere Entwicklung der *A. flavus* Infektionen.

Um die biologischen Faktoren näher zu untersuchen, wurde Mais im Feld zum Stadium der Milchreife und bereits geerntete Maiskörner mit *A. flavus* Sporen inokuliert, und zwar allein und in Kombination mit *Fusarium* spp. und/oder *Penicillium* spp. In beiden Experimenten wurden die Körner in einem Inkubator gelagert und wöchentlich Stichproben entnommen. In dem Experiment mit Inokulationen im Feld stieg die Häufigkeit von *A. flavus* linear während der sieben Lagerungswochen an, wobei die

Steigung in allen Varianten gleich war, aber ein leicht erhöhtes Niveau in der Variante erreicht wurde, in der auch *Fusarium* spp. inokuliert wurde. In allen Varianten fiel die Häufigkeit von *Fusarium* spp. anfänglich, stieg aber nach vier Wochen wieder an. Der Befall mit *Fusarium* spp. war stärker nach einer *Fusarium* –Inokulation als ohne. Der Befall mit *Penicillium* spp. zeigte einen generellen leicht ansteigenden Trend. In Gegenwart von *Fusarium* spp. war der Befall durch *Penicillium* spp. vermindert. Der Aflatoxingehalt nahm im Lager nach einer Inokulation von *A. flavus* zu. Im Nachernteexperiment stieg der Befall mit *A. flavus* ausgehend von einem geringen Niveau linear in allen Varianten, einschließlich der Kontrolle, an. Im Vergleich zur Kontrolle war die Steigung nach eine Inokulation von *A. flavus* höher, und noch weiter erhöht, wenn *Penicillium* ebenfalls inokuliert wurde. Der Befall mit *Fusarium* spp. verminderte sich linear in allen Varianten, obwohl der Ausgangsbefall hoch war. Der Befall mit *Penicillium* spp. variierte stark, ohne dass ein einheitlicher Trend über die Zeit erkennbar war. Die Aflatoxinkonzentration in dem Experiment mit Inokulation nach der Ernte war niedriger als in dem vor der Ernte, allerdings nahm die Konzentration in allen Varianten stetig zu.
Der letzte Teil der Arbeit beschäftigte sich mit *A. flavus* selbst und seiner Untergliederung. Dazu wurden sechs Isolate hinsichtlich des Koloniewachstums und vier Isolate im Hinblick auf die Aflatoxinproduktion untersucht. Das Koloniewachstum der meisten Isolate konnte mit einer Gompertz-Funktion sehr gut beschrieben werden, während die Toxinproduktion mit einer monomolekularen Funktion modelliert werden konnte. Generell hatte in den hier betrachteten Bereichen die Wasseraktivität einen größeren Einfluss auf das Wachstum als die Temperatur. In allen Fällen, in denen viel Aflatoxin produziert wurde, erfolgte auch ein Toxinabbau. Die Wasseraktivität von 0,90 war am wenigsten effizient, die von 0,96 am effizientesten. Bei der zuletzt genannten Wasseraktivität war der Temperatureinfluss schwach. Die optimale Temperatur variierte in Abhängigkeit des Isolats zwischen 31, 33 und 35°C, während die optimale Wasseraktivität für alle Isolate bei 0,96 lag. Für die Aflatoxinproduktion schwankte das Optimum der Wasseraktivität zwischen 0,96 und 0,99, die optimale Temperatur lag bei 26 bzw. 28°C, den niedrigsten Temperaturen des Experiments. Die Isolate des L-Stamms produzierten Aflatoxin G, allerdings bei niedrigeren Wasseraktivitäten (0,90 und 0,93) als die des S-Stamms (0,96 und 0,99). Die höchsten Wachstumsraten wurden für die Isolate Z34A, Z117B und Z1TS gemessen, die alle drei zum L-Stamm gehören. Der beste Aflatoxinproduzent war das Isolat Z213D, das auch die höchste Menge an Aflatoxin G bildete. Das Isolat Z1TS produzierte etwas weniger, allerdings nur Aflatoxin B. Z213D, ein Isolat des S-Stamms, das reichlich Aflatoxin bildete, hatte nur eine sehr kleine Wachstumsrate. Die kleinste Rate der Aflatoxinbildung wurde für Z34A festgestellt, einem Isolat des L-Stamms, das sehr hohe Wachstumsraten aufwies.
Wenn alle wichtigen Faktoren für die Aflatoxinbildung im Mais, die hier angesprochen wurden, quantifiziert worden sind, können diese benutzt werden, um ein Modell zur Vorhersage des Auftretens von Aflatoxin zu entwickeln.

Stichwörter: Aflatoxin, *Aspergillus flavus*, Skelotienstämme, Toxinbildung

Table of contents

List of Tables

List of figures

Abbreviations

°C	Degree Celsius
5/2 medium	5% V8 juice and 2% agar medium
A	Treatment: all maize plants were inoculated only with *A. flavus* spore suspension
AF	Treatment: inoculation with *A. flavus* and *Fusarium* spp.
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AP	Treatment: inoculation with *A. flavus* and *Penicillium* spp. spore suspensions
APF	Treatment: inoculation with *A. flavus* and *Penicillium* spp. spore suspensions plus *Fusarium* spp. inoculated in the stems
$ASPERG_M$	Incidence of *A. flavus* in the maize
$ASPERG_S$	Incidence of *A. flavus* in the soil
$ATOXIN_M$	Aflatoxin content in the maize
a_W	water activity: measurement of water content (*aw*=vapor pressure of the water in the substance (in Pascal) divided by vapor pressure of pure water (in Pascal) at same temperature)
Ca	Calcium
cfu/g	Colony forming units per gram of substrate
CLAY	Clay fraction of the soil
cm	Centimetre
CS	Costal Savanna zone
DIAM	Colony diameter
ESCa	Exchangeable soil Ca cations
ESK	Exchangeable soil K cations
ESNa	Exchangeable soil Na cations

et al.	*et alii* (and other)
Fig.	Figure
GLM	General Linear Model
HEIGHT	Height above sea level of the field site
HIV	Human Immunodeficiency Virus
HPLC	High-performance liquid chromatography
IV0	Treatment: maize improved variety with soil inoculation
IV1	Treatment: maize improved variety with soil inoculation
IVCP0	Treatment: maize improved variety intercropped with cowpea without soil inoculation
IVCP1	Treatment: maize improved variety intercropped with cowpea with soil inoculation
K	Potassium
LAG	time lags
LATITU	Latitude of the field site
LD_{50}	Lethal dose 50
LSD	Least Significant Difference
L-strain	*A. flavus* large sclerotia strain
L-STRAIN	Percentage of L-strain isolates of *A. flavus* in the soil
LV0	Treatment: maize local variety without soil inoculation
LV1	Treatment: maize local variety with soil inoculation
LVCP0	Treatment: maize local variety intercropped with cowpea without soil inoculation
LVCP1	Treatment: maize local variety intercropped with cowpea with soil inoculation
Min.	Minimum
Na	Sodium
NGS	Northern Guinean Savanna zone
NPK	Compound fertilizer Nitrogen-Phosphorus-Potassium
N-STRAIN	Percentage of non-toxigenic isolates of *A. flavus*
Opt.	Optimum
PDA	Potato Dextrose Agar

PDACD	Potato Dextrose Agar plus methylated ß-cyclodextrin derivative
pH	Potential of hydrogen
PHV	pH-value of the soil
ppb	parts per billions (litre per kilogram)
SAND	Sand fraction of the soil
SAS	Statistical Analysis System
SGS	Southern Guinean Savanna zone
SILT	Silt fraction of the soil
SOC	Soil Organic Carbon
SS	Sudan Savanna zone
S-strain	*A. flavus* small sclerotia strain
S-STRAIN	Percentage of S-strain isolates of *A. flavus* in the soil
TLC	Thin layer chromatography
T-STRAIN	Percentage of toxigenic isolates of *A. flavus*
TZSR-W	Tropical *Zea mays* Streak Resistant White
UV	Ultra Violet
VCG	Vegetative Compatibility Groups

1 General Introduction

Maize is one of the most grown cereals in Africa, preceding sorghum, millet and rice (FAO, 2010). Since the 90's, the maize adoption zones became wider every year (Byerlee et al., 1996). Maize is the most important food staple in the South Saharan Africa where maize constitutes about 50% of calorie intake (Byerlee et al., 1996). Maize production in Africa has undergone an important increase mainly because some new drought-tolerant and early-harvest varieties are now available that are adapted to new and more arid climate. The harvested areas and the yields kept increasing since 2001 (FAO, 2010). Maize is an important commodity of regional or international trade. Therefore it is a considerable economical factor, firstly for the producers because it can be used as cash crop (Smith et al., 1994) and secondly for political authorities because in some West African countries the price and the availability of maize on the national market are used as indicator for eventual advent of famine. Also from the consumer point of view, it is a very highly manageable crop that can be processed in different ways.

In West Africa, especially in the zone of Sahel, sorghum and millet are serious substitutes of maize. However, in the more costal countries of that zone, maize constitutes the major crop (Hell et al., 2000; Udoh et al., 2000) and makes up the main compound of the three daily meals but also it is the main weaning food in the area and enters in the composition of most snacks (Egal et al., 2005). In countries such as Benin, Ghana, Nigeria and Togo, maize is gaining over sorghum and millet toward the north in terms of exploited area. The conditions that slow down maize progress in South Saharan Africa are numerous. Besides the most common such as the more and more unpredictable climate and the archaic cultural techniques, there are many pests and diseases on maize that decrease its yield and weaken its storage. Some of these unfavourable factors are obvious and their damages easy to detect but some damages remain hidden although they are dangerous for consumers.

In a recent review of crop losses due to pests, Oerke (2006) enumerated weeds, animal pests, pathogens and viruses as the most important constraints of maize. The most common and visible constraints are insects such as Lepidoptera stem or cobs borers, or Coleoptera mostly found on mature and stored maize. Their impact on maize is a strong decrease of yield and a depreciation of the nutritional or commercial value of the harvested or stored maize.

These insects can also act as disease vectors and mould dispersers within grain bulk (Munkvold et al., 2000). Besides the insects there are weeds that compete with maize for water, nutrients, and light or sometimes hack into the maize root or vascular system (Lopez-Garcia et al., 1998). In the climatic and cultural conditions of maize production in tropical areas, fungal pathogens have very important negative impacts on the maize crop from the sowing to the end of transport and storage process, but the most insidious impact is the production of mycotoxins that are toxic for humans and animals. The production of mycotoxins is an attribute of some strains within some species. One of the most common fungal pathogen of maize is *Fusarium* spp. that is able to produce a harmful secondary metabolite named fumonisin within the maize grain (Bankole et al., 2003). Another fungus that is able to contaminate maize in the field but more often the already ripen grains is *Aspergillus flavus* that produces aflatoxin (Hesseltine, 1986), also a harmful secondary metabolite that can be dangerous for consumers of contaminated maize (IARC, 2002). It is acknowledged that for aflatoxin contamination the process starts when maize is still in the field. It is also well documented that the main part of toxin production happens during the storage period if some precautions are not taken into account during maize growth, its harvest, before and during its storage (Dorner et al., 2002; Wagacha et al., 2008). In case the necessary precautions are not observed, the maize proposed to the consumers can contain up to 1800 µg/kg of aflatoxin (Lewis et al., 2005) and that can occur on more than 30% of maize proposed to the consumers (Egal et al., 2005; Udoh et al., 2000). Once ingested, highly contaminated maize can cause an acute crisis that could be lethal (Groopmann et al., 1999), in case of continuous supply of lowly contaminated maize a chronic aflatoxicosis can occur that can be expressed as liver cancer, immunodeficiency, and some other health impairments for adults and children (Turner et al., 2003; Gong et al., 2003).

The aflatoxins found in maize are secondary metabolites produced by a number of fungi belonging to the genus *Aspergillu*s, including *A. flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. pseudotamarii*, *A. ochraceoroseus*, *A. rambelli etc.* Only *A. flavus* and *A. parasiticus* have an important impact in agriculture, and *A. flavus* is by far the most critical species in the case of maize contamination (Klich, 2006). Besides maize, other susceptible crops are cotton seeds, peanuts and tree nuts (Klich, 2007). There are four majors aflatoxins encountered in maize: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2) and less often aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Wogan, 1966). They are potentially carcinogen, mutagenic

and immunosuppressive agents. Chronic poisonings by aflatoxins can lead to liver cancer and can promote some other diseases such as hepatitis, HIV, Kwashiorkor, and other nutrition and growth impairments (IARC, 1993; Turner et al., 2000; Gong et al., 2002). In case contaminated maize is used to feed domestic animals, their productivity decreases. In the specific case of milk producing animals, their metabolism can transform AFB_1 and ABF_2 in aflatoxin M_1 and M_2 that are also toxic and are excreted in the milk (Zarba et al., 1992). According to a review of Wogan (1966), the acute poisoning by aflatoxin can also be lethal for animals but the LD_{50} varies with species. In the same review, the aflatoxins are chemically classified in the group of difuranocoumarins with the sub-group of difurocoumarocyclopentenone for aflatoxin B_1, B_2, M_1 and M_2 etc. and the sub-group of the difurocoumarolactone for aflatoxin G_1 and G_2 etc. The toxicity of the aflatoxins decreases from AFB_1 to AFG_1, to AFB_2, and to AFG_2. AFB_2 and AFG_2 are dihydroderivative of AFB_1 and AFG_1 successively and in maize their apparition follows chronologically the ones of B_1 and G_1 (Chang et al., 1963; Van Dorp et al., 1963). The examples of aflatoxins toxicity appeared in the public opinion firstly in 1960 after the death of about hundred thousands turkeys that were fed with contaminated peanut meal (Blount, 1961). Since then, research has focused on these metabolites and helped later to recognize many epidemics of aflatoxin poisoning in Asian and African countries that resulted in dozens of casualties (Krishnamachari et al., 1975; Azzi-Baumgartner et al., 2005). This situation raised the curiosity of the researchers about the health impact in countries having the most susceptible crops to aflatoxins as staple diet. It turned out that aflatoxin-albumin was detected in the blood sera of more than 99% of surveyed children in Togo, Benin, Ghana and Gambia (Gong et al., 2003). The presence of aflatoxin-albumin in the children blood was positively correlated to other affections such as growth impairment and Kwashiorkor incidence (Wild et al., 1996; Gong et al., 2002). These results should draw the attention of these countries' authorities to maize production monitoring and to aflatoxin early detection before contaminated maize is consumed. The first step to undertake is to start information campaigns in rural and urban areas of the concerned countries because the humans are not aware of aflatoxin existence and therefore do not link the mould caused by *A. flavus* to the worsening of their health condition (Cardwell, 2000). Indeed, once the existence and the toxicity of aflatoxin have been explained to the local population, primordial control measures can be taken, which are rather simple and consist in a better management of the growing maize in the

field. This includes a better planning of the harvest to avoid too wet maize ready for harvest, a better harvest management to prevent grain contact with soil that is the primary inoculum source of *A. flavus*, and finally a better condition of storage (Cardwell et al., 2004; Wagacha et al., 2007). However, these primordial control measures are often difficult to fulfil because in tropical areas where less industrialised maize production systems exist with, for instance, sun-drying or the lack of facilities for fields irrigation, maize is still exposed to natural unpredictable climatic conditions and therefore the essential precautions to prevent *A. flavus* infection of maize cannot be granted. Then, other alternatives for aflatoxin control in maize are already available or still under research. One is the biological control of aflatoxin by implementing non-toxigenic *A. flavus* strains in the field to outcompete the toxigenic ones, a method studied for adaptation in some parts of West Africa (IITA, 2003). There is also continuous search for resistant maize varieties.

In areas spotted as suitable for *A. flavus* development, mostly tropical hot areas, the composition of the *A. flavus* population is not uniform. Within the species *A. flavus*, there are some strains with morphological and physiological differences such as "L" strains and "S" strains (Cotty, 1989). Isolates of the "L" strains are only able to produce aflatoxins B_1 and B_2 and also isolates exist that cannot produce aflatoxins at all. The isolates of "S" strains are always (under adequate environmental conditions) able to produce aflatoxin B_1 and B_2 but their ability to produce aflatoxins G_1 and G_2 depends on the geographic zone. For instance, those from the United States are unable to produce aflatoxin G but those from West Africa can easily produce this kind of aflatoxin (Cotty et al., 1999). In a specific study area it would be interesting to know how these different groups of *A. flavus* strains are related and how the composition of the population depends on geographic or local climate conditions. Local agricultural practices and processes should also be taken into account (Hell et al., 2000; Udoh et al., 2000 and Lopez-Garcia et al., 1998).

In West Africa, maize is mostly intercropped with other crops such as cowpea, peanut, cassava, etc. (Hell et al., 2003). Besides the intercrops, the variety grown is important in the cropping system (Zuber et al., 1983). Some farmers are early adopters of new varieties, others are not, mainly because improved varieties are not available on the market. Research is needed to examine how the combination of the cropping system, the varieties used and the heavy presence of toxigenic *A. flavus* propagules in the soil impact the harvested and stored maize in terms of aflatoxin contamination.

On ripening, harvested or even stored maize grains, *A. flavus* is not the only pest present. There are many other insects or moulds that coexist with this major aflatoxin producer in maize (Wicklow, 1988). The two other important storage moulds are *Fusarium* spp. that is an early contaminant of maize grains in the field and *Penicillium* spp. that is much more specific for storage conditions (Kamphuis et al., 1992). Research results indicated that the presence of other moulds on maize could be detrimental to aflatoxin production by *A. flavus* (Widstrom et al., 1994; Wicklow et al., 1980). More research is needed to determine the impact of the co-presence of these three major maize storage moulds on the final aflatoxin level in maize.

Knowing that a population of *A. flavus* comprises different isolates (Klich, 2006) it would be of interest to find out if all isolates react in the same way to the main environmental factors, such as temperature or water availability, with respect to their morphological or physiological characteristics. After the study of all these aspects of aflatoxin contamination in maize grains, a work of synthesis could be undertaken to improve the level of prediction and anticipation of aflatoxin contamination in maize, especially under West African conditions.

The objectives of this study are therefore: (i) to make an inventory of the composition of *A. flavus* populations in the different study zones, to characterize these populations with respect to soil properties and climate, and finally to establish a list of the most important parameters to be taken into account for aflatoxin occurrence in maize produced in a specific zone after a certain storage period, (ii) to analyze the effects of the maize variety, of the cropping system and of the size of the primary inoculum on the production of aflatoxins by *A. flavus* and to determine the relationships between the contamination by aflatoxins and other biotic factors such as storage insects and moulds, (iii) to investigate the effects of co-inoculation of *A. flavus* with *Fusarium* spp., *Penicillium* spp. or with both species on *A. flavus* development and aflatoxin production in stored maize when maize is inoculated while maturing in the field or after harvest, and (iv) to study the growth and aflatoxin production of selected isolates of *A. flavus* on artificial media in relation to the most important environmental parameters, temperature and the water activity. Finally, the information gained from all the previous studies should allow establishing a prediction system of aflatoxin advent in maize as function of the most important parameters in the specific case of Benin.

Chapter 2. Factors determining the distribution and population composition of *Aspergillus flavus* strains in soils and the subsequent aflatoxin contamination of cultivated maize in the four agro-ecological zones of Benin

2.1 Abstract

During a survey in Benin, soil and maize samples were collected from 100 fields and analyzed to determine on one side the main properties of the soil and its different components and on the other side the *Aspergillus flavus* incidence in the soil and maize samples, the *A. flavus* population composition in the soil and the aflatoxin content in maize. The incidence of *A. flavus* and the percentage of L-strain isolates were high in the Costal Savanna (CS) and Southern Guinean Savanna (SGS) zones. In contrast, the S-strain isolates were more represented in the Northern Guinean Savanna (NGS) and Sudan Savanna (SS) zones. Atoxigenic isolates were evenly distributed throughout all four zones. Also toxigenic isolates were almost homogenously represented, only SS had more toxigenic isolates than NGS. The incidence of *A. flavus* in maize followed the pattern of soil incidence while only SGS and NGS showed differences in aflatoxin content in maize with the advantage for the SGS. The site latitude and height above sea level were highly negatively correlated with the incidence of *A. flavus* in soil, L-strain isolates percentage, *A. flavus* incidence in maize and positively with the S-strain isolates percentages. Regarding the soil texture, there were positive correlations between the sand percentage and the toxigenic isolates percentage, between the silt and the S-strain isolates percentages, between the clay and atoxigenic isolates percentages. Negative correlations were found between the sand and the atoxigenic isolate percentages, between the silt percentages and the L-strain isolates and *A. flavus* incidence in the maize. The soil exchangeable cations showed some significant correlations. The soil content of calcium, potassium and sodium were all three in positive correlation with L-strain isolates percentages on one side and atoxigenic isolates percentages on the other side. Moreover, the sodium content in the soil was positively correlated with *A. flavus* incidence in the soil and negatively with the toxigenic isolates percentages. Finally by the stepwise multiple regression analysis it came out that the level of aflatoxin in the maize depended directly on the soil organic carbon,

soil incidence of *A. flavus*, L-strain isolate percentages, S-strain isolates percentages and *A. flavus* incidence in the maize.

2.2 Introduction

Aspergillus flavus is a very common fungus and normal inhabitant of tropical and subtropical agricultural or non-agricultural soils (Klich, 2002). It is one of the soil microorganisms involved in the recycling of soil organic matter and agricultural residues (Cotty et al., 1994). From that perspective, it is a rather beneficial microorganism for quality conservation of agricultural soils. The negative aspect is due to the ability of many strains of this fungus to produce toxic metabolites, called aflatoxins, in crops that they have invaded (Geiser et al., 1998). Indeed, *A. flavus* is the main responsible for aflatoxin contaminations of maize, peanut, cotton seeds and tree-nut (Lillehoj et al., 1980; Horn et al., 1995). There are other species of *Aspergillus* belonging to the section Flavi such as *A. parasiticus* or *A. nomius* that are also able to produce aflatoxins (Horn, 2003) but *A. flavus* is by far the most prevalent species in the contaminations of crops especially of those having aerial products like maize or cotton seed (Klich, 2007). In peanut, the impact of *A. flavus* on aflatoxin contamination is less exclusive since *A. parasiticus* is more involved due to the underground crop specificity of peanut. The most important impact of *A. flavus* on public health issue is through maize especially in countries having that cereal as staple diet component (Gong et al., 2003; Hell et al., 2003). Aflatoxin is classified as a very dangerous food contaminant for human and animal health (Cast, 2003; Payne, 1992).

A. flavus is present on all continents as its occurrence follows roughly the one of the crops it colonizes naturally. The population composition of *A. flavus* communities varies with geography in terms of morphological properties or aflatoxins production abilities (Cotty and Cardwell, 1999). Besides geography, other parameters such as the agricultural techniques or crops sequences on a field can have an influence on the *A. flavus* population composition in an area (Horn et al, 1995; Jaime-Garcia and Cotty, 2004). An *A. flavus* population can comprise isolates different in morphology or different in their ability to produce aflatoxins or specific types of it. Morphologically there are mainly two strains of *A. flavus* (Cotty, 1989). The most common L-strain contains isolates that are able to produce only aflatoxin B_1 and B_2

or possibly no aflatoxin at all. These isolates form no sclerotia or only a low number of relatively large sclerotia (diameter larger than 400 μm) and have an abundant production of conidia. The S-strain includes isolates that are able to produce only aflatoxins B (B_1 and B_2) or that can produce both aflatoxins B and G (B_1, B_2, G_1, and G_2). Those isolates have numerous small sclerotia (diameter smaller than 400 μm) and produce less conidia than their counterparts (Cotty, 1989). The second way to group *A. flavus* isolates in a population is their capability of aflatoxin production. Some are able to produce aflatoxin B and G and then morphologically they are essentially assigned to the S-strain. Other isolates can produce only aflatoxin B or none and belong to the S- or L-strain (Geiser et al., 1998 and 2000). S-strain isolates that are able to produce aflatoxin G are not present in the United States but have been isolated from West Africa, Argentina, Australia and Southeast Asia (Cotty and Cardwell, 1999; Geiser et al, 2000; Hesseltine et al., 1970; Nova and Cabal, 2002; Saito and Tsuruta, 1993). Geography and latitude are acknowledged as important factors for *A. flavus* population compositions and characteristics (Klich, 2002; Cotty and Cardwell, 1999). Out of the many parameters that are relevant, the most obvious are the climate (Cotty and Jaime-Garcia, 2007), the type and characteristics of the soil (Jaime-Garcia and Cotty, 2006; Zablotowicz et al., 2007), field managements and cultural techniques (Munkvold, 2003; Jaime-Garcia and Cotty, 2006).

It seems important to weigh the impact of each of these parameters on the resulting maize contamination. To reach this goal, a first step would be to study, characterize and determine the variation of the involved *A. flavus* populations. Once the population structure is understood, the mechanism of the crop colonization has also to be taken into account, i.e. how the infectious propagules are transported from the soil, that is supposed as the primary source of inoculum (Payne, 1998), to susceptible parts of maize. Insects transported propagules (Widstrom et al., 2002) and airborne propagules (Bock et al., 2004) modulated by climate and human activity are proven to be involved. Propagules distributed by insect vectors or air can travel a relatively long distance from the inoculum source to maize grains so that the study of *A. flavus* population should cover a rather larger area than a single field. Nevertheless the impact of the local inoculum sources cannot be neglected but it is still difficult to define the area to be involved if a soil *A. flavus* population is compared with the population on harvested maize. From its growth in the field to the consumers poisoning the last opportunity for

aflatoxin assessment on maize is when it is ready for the final process before consumers' acquisition. The successive processes involve the harvest technology, crop harvested handling and storage. Many authors have shown that these steps of maize production could help to minimize aflatoxin production in maize or in contrary to increase the risks of contamination (Hell et al., 2000; Udoh et al., 2000; Kaaya et al., 2006).

Benin in the tropical zone of West Africa,, gathers all factors for aflatoxin contamination in maize that is the major cereal in the country (Adebayo et al., 1994). Preliminary investigations at different levels of maize production have been conducted in this country that has an ideal geographic disposition across four agro-ecological zones with different kinds of climates (Setamou et al, 1997; Hell et al, 2000; Cardwell and Cotty, 2002). Apart from the climate, the soils and the ethnical populations vary from the south costal zone to the north near Sahara zone. With the population also the cultural and storage techniques vary.

To have an accurate prediction of the risks to end up with highly contaminated maize in a specific agro-ecological zone, it is important to monitor all production compartments from the soil of the field used for maize cropping to the storage of such harvested maize and to weigh all the factors in relation to climate, soil and geography. The objectives of this study are therefore to make an inventory of different *A. flavus* populations of the different studied zones, to characterize them in relation to the soil, and to establish a list of important parameters to be taken into account for predicting aflatoxin occurrence in maize in a specific area after a short time of storage.

2.3 Materials and Methods

2.3.1 Fields localization and soil sampling

The Republic of Benin is located on the West African Coast between Togo in the west and Nigeria in the east. The primary subdivision of the country considered for this study followed the four agro-ecological zones (Cardwell et al., 2002) from the South to North: the Costal Savanna (CS), the Southern Guinean Savanna (SGS), the Northern Guinean Savanna (NGS), and the Sudan Savanna (SS). Within each agro-ecological zone, five villages and five

fields per village were randomly selected for soil sampling. In each field, 20 soil sub-samples from the 5-cm top layer were taken along diagonals and median and then pooled. The final sample was collected in a paper bag and transported to the laboratory where the water content was immediately determined from a homogenized sub-sample of each field sample. From each remaining sample, one part was dried at about 45°C for two days and then stored at 4°C till *A. flavus* isolation. The second part was directly stored at 4°C in tightly closed plastic bag till the determination of the soil organic and mineral nutrients, pH and particles composition.

2.3.2 Analyses of the soil samples

2.3.2.1 Soil isolation of *A. flavus*

To determine the incidence of *A. flavus* (cfu/g), about 10 g of soil were weighed in 100-ml flask containing 50 ml of sterile distilled water. The flask was closed and shaken for 30 min. Then 100 µl of the resulting soil suspension were spread on modified Rose Bengal agar medium (Cotty, 1989) in a Petri dish; for each sample three replications were prepared. The Petri dishes were then incubated at 31°C for three days in darkness. The number of colonies per Petri dish was counted and recorded. For Petri dishes containing less than ten colonies of *A. flavus*, each colony was picked up on 5/2 (5% V8 juice and 2% agar) medium (Cotty, 1989). For Petri dishes containing more than 10 colonies, a re-plating was performed after dilution. Seven days later, up to eight isolates per soil sample were collected and stocked in flasks on distilled sterile water. The flasks were stored at 4°C.

2.3.2.2 Determination of strains from soil isolates

The determination of L/S-strains was carried out on 5/2 medium. After seven days of incubation at 31 °C, the L-strain isolates had abundant green conidia and few large sclerotia or no sclerotia at all whereas the S-strain isolates were characterized by few yellowish conidia and plentiful small sclerotia.

The identification of toxigenic strains was made on PDACD (Potato Dextrose Agar plus methylated ß-cyclodextrin derivative) (Ordaz et al., 2002). After four days of incubation

at 31 °C, the reverse of colonies of toxigenic isolates looked fluorescent under UV light (365 nm). As this method is rather qualitative and not very sensitive for isolates that are only able to produce a small amount of aflatoxin, those isolates were subjected to further fermentation and aflatoxins extraction. For that, 70 ml of A&M medium (Mateles and Adye, 1965) and 100 micro liters of spore suspension containing about 4.72×10^{6} spores were mixed and shaken at 150 rpm at 31 °C for five days. Then 70 ml of acetone were added to the fermented suspension. One hour later the extraction of aflatoxins was carried out.

2.3.2.3 Aflatoxin extraction

Approximately 140 ml of the fermented suspension plus acetone were filtered through filter paper in a beaker. To 100 ml of the filtered solution, an equal volume of distilled water was added and extracted two times with 25 ml of methylene chloride. The methylene chloride plus acetone solution was filtered through anhydrous sulfate sodium and dried at room temperature. The extract was later dissolved with 1 ml of methylene chloride and spotted on TLC plates which were developed with diethyl ether-methanol-water (96:3:1, v/v/v) and observed under UV light (365 nm). The positive isolates were recorded.

2.3.2.4 Characterization of the soils

For each soil sample, the following variables were determined in the laboratory (Table 2.1): the pH-value, the soil organic carbon content (in %), the composition of the soil particles in sand, silt and clay (all in %), and finally the exchangeable cations Ca, K and Na (in cmol/kg).

Table 2.1: List of variables determined for each of the 100 fields

Variable	Units	Definition	Transformation
LATITU	° north	Latitude of the field site	
HEIGHT	m	Height above sea level of the field site	
PHV		pH-value of the soil	
SOC	%	Soil organic carbon content	$\arcsin\sqrt{x}$
SAND	%	Sand fraction of the soil	$\arcsin\sqrt{x}$
SILT	%	Silt fraction of the soil	$\arcsin\sqrt{x}$
CLAY	%	Clay fraction of the soil	$\arcsin\sqrt{x}$
ESCa	cmol/kg	Exchangeable soil Ca cations	
ESK	cmol/kg	Exchangeable soil K cations	
ESNa	cmol/kg	Exchangeable soil Na cations	
$ASPERG_S$	cfu/g soil	Incidence of *A. flavus* in the soil	ln(x+1)
L-STRAIN	%	Percentage of L-strain isolates of *A. flavus* in the soil	$\arcsin\sqrt{x}$
S-STRAIN	%	Percentage of S-strain isolates of *A. flavus* in the soil	$\arcsin\sqrt{x}$
T-STRAIN	%	Percentage of toxigenic isolates of *A. flavus* in the soil	$\arcsin\sqrt{x}$
N-STRAIN	%	Percentage of non-toxigenic isolates of *A. flavus* in the soil	$\arcsin\sqrt{x}$
$ATOXIN_S$	ppb	Concentration of aflatoxin in the soil	ln(x+1)
$ATOXIN_M$	ppb	Concentration of aflatoxin in maize kernels	ln(x+1)
$ASPERG_M$	cfu/g maize	Incidence of *A. flavus* in maize kernels	ln(x+1)

2.3.3 Analyses of the maize samples

Maize samples were taken from the same fields previously selected for soil samples. If possible, maize samples were collected three times in two-month intervals, but not all farmers were able to provide the three samplings. For each sampling, 30 cobs or an equivalent amount of maize grain, if shelled, were collected from the storage structure of each farmer. As first operation *in situ*, the percentage of each single cob area covered by molds especially by *A. flavus*, *Fusarium spp.* and *Penicillium spp.* was estimated. If shelled, all affected grains were counted. All other molds were recorded as "other fungi". Maize grains were collected in paper bags and transported to the laboratory to determine the moisture content, and the *A. flavus* incidence (cfu/g) and to extract and quantify aflatoxins.

2.3.3.1 Maize moisture content

The moisture content was determined by weighing a specified amount of ground maize (Tekmar IKA-A10, Analytical Mill), before and after drying for two hours at 130°C, and calculating the weight differences (I.S.O. 1979).

2.3.3.2 Determination of the *A. flavus* incidence in maize samples

To isolate and identify *A. flavus*, the same protocol was used as the one for soil isolation, but here the colonies were just counted and their number recorded.

2.3.3.3 Aflatoxin extraction from maize samples and thin layer chromatography

The aflatoxin from ground maize was determined as described by Thomas et al. (1975). For each sample, 50 g of ground maize were weighed in a 500-ml Erlenmeyer flask. Then 250 ml of methanol and water (60:40/vol/vol) were added and 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. 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 in a beaker and allowed to evaporate. The extract was dissolved with about 1 ml of chloroform, transferred into a small container and stored in the refrigerator for aflatoxin quantification.

Aflatoxin was quantified by thin layer chromatography method. 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 with win-CATS 1.4.2 software (Camag AG, Muttenz, Switzerland). 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 compared with the standard were diluted, spotted and chromatographed again. The concentration of different aflatoxins in maize samples were then calculated using the formula: $(S \times Y \times V)/(X \times W)$ where S = AFB_1 standard equal to unknown, µl; Y = concentration of AFB_1 standard, $\mu g\, ml^{-1}$; V = the volume in which the sample extract is dissolved, µl; X = µl sample spotted giving fluorescent intensity equal to S (AFB_1 standard); W = quantity of sample, g.

2.3.4 Data analyses

All variables used are listed in Table 2.1. For the statistical analyses, variables given in percentage were transformed with the arcsine square root transformation. The incidences of *A. flavus* in soil and maize and the concentration of aflatoxin in maize were logarithmically transformed ($\ln(x+1)$) in order to get more normalized data. Analyses of variance were carried out with the General Linear Model (GLM) of SAS. During the multifactorial analysis, the source of variation were zones (four zones), villages (5 per zone), and samplings dates (three samplings dates). The multiple comparisons of means and separation were computed by including the option LSD of the t-test in the GLM procedure. The Pearson correlations were computed between all variables.

The main goal of the survey was to determine the major factors that increase the risk of maize contamination by aflatoxin. To complete these analyses, stepwise multiple regression analyses were used by fitting the data to the equation 2.1. Variables that have proven to be directly or indirectly related to the aflatoxin contamination were used as dependent variables: the *A. flavus* incidence of the soil ($ASPERG_S$), the percentage of L-strain isolates in the soil population of *A. flavus* (*L-STRAIN*), the percentage of S-strain isolates in the soil population (*S-STRAIN*), the percentage of toxigenic strains in the soil population of *A. flavus* (*T-STRAIN*), the percentage of atoxigenic strains in the soil population (*N-STRAIN*), the *A. flavus* incidence of maize ($ASPERG_M$) and the content of aflatoxin in maize ($ATOXIN_M$). The independent variables X_i in equation 2.1 were: the height of the sampled field, the latitude of the sampled field, the pH of the sampled field soil, the soil organic carbon content, the sand, silt and clay percentages, the major significant exchangeable cations content.

$$Y = \beta_0 + \beta_1 \times X_1 + \beta_2 \times X_2 + ... + \beta_{(i-1)} \times X_{(i-1)} + \beta_i \times X_i \quad (2.1)$$

The parameter β_0 represents the common intercept and β_i are the slopes translating the effect of the independent variable *i*. In the first step, all independent variables X_i were used in equation 2.1. Then by stepwise procedure all non-significant variables were removed.

Further, to check the relationships of the *A. flavus* incidence and aflatoxin content in maize, the soil variables such as the incidence of *A. flavus*, the percentages of L- and S-strain isolates, and the percentages of toxigenic and atoxigenic isolates were used as X_i and the dependent variables were $ASPERG_M$ and $ATOXIN_M$. For all regression analyses SigmaPlot 10 was used.

2.4 Results

After the multifactorial analyses of variance, the comparisons of means between zones revealed a similar trend for soil and maize incidence of *A. flavus*. For both variables, the values of the costal savanna (CS) and the South Guinean Savanna (SGS) were significantly higher than those of the Northern Guinean Savanna (NGS) and Sudan Savanna (SS) (Table 2.2), while no differences between CS and SGS on one hand and NGS and SS on the other hand were detected. The same trend was determined for the percentage of L-strain isolates in

the soil population of *A. flavus*, which was significantly higher in the two southern zones CS and SGS. Consequently, the percentage of S-strain isolates was significantly lower in these zones compared to the northern zones NGS and SS (Table 2.2). It seems that the incidence of *A. flavus* propagules in the soil was related to the one in the maize. Also one may conclude that the percentage of L-strain isolates increased with higher number of *A. flavus* propagules in the soil. This is confirmed by the Pearson correlation analyses on field level with a positive correlation between the $ASPERG_S$ and *L-STRAIN* (r = 0.38) (Table 2.3). A strong negative correlation (r = -0.65) between the percentages of soil L- and S-strain isolates was found. Theoretically that correlation coefficient should be -1, but it was not always possible to assign all isolates to the L- or S-strain because of atypical morphology such as having few conidia like the S-strain isolates but having no sclerotia like some isolates of the L-strain. The percentages of isolates that were not classified as L- or S-strain were from the south to the north 5.28 % (CS), 6.82 % (SGS), 9.82 % (NGS) and 19 % (SS).

The second variable series that are expected to have opposite trends are the percentages of toxigenic and atoxigenic strains, *T-STRAIN* and *N-STRAIN*. The percentage of atoxigenic strains was roughly 25% and did not differ in the four zones. In contrast, the percentage of toxigenic strains was about 55% and was significantly lower in NGS (46%) than in SS (64%). The percentage of toxigenic isolates was in a positive correlation with the soil *A. flavus* incidence and with the percentage of the S-strain isolates (Table 2.3). Logically, the percentage of atoxigenic isolates was positively correlated with the percentage of L-strain isolates but negatively with the percentage of toxigenic isolates. Again, it was not always possible to determine accurately the toxigenic group of some isolates due to the non-correspondence between the morphologic and the toxigenic status. Then only isolates with a reliable status were recorded as toxigenic or atoxigenic. The percentages of isolates with unclear toxigenic status were 4.69 % in CS, 4.84 % in SGS, 9.82 % in SS and 12.47 % in NGS.

Table 2.2: Comparison of characteristics (see Table 2.1) of the *A. flavus* population in the soil and of the infestation and aflatoxin production in maize between four agro-ecological zones: CS = Costal Savanna, SGS = Southern Guinean Savanna, NGS = Northern Guinean Savanna, SS = Sudan Savanna

Zones	$ASPERG_S$	*L-STRAIN*	*S-STRAIN*	*T-STRAIN*	*N-STRAIN*	$ASPERG_M$	$ATOXIN_M$
CS	4.94±1.65 a[1)]	81.08±25.11 a	0.00±0.00 b	58.66±34.77 ab	23.31±31.20 a	3.16±1.30 a	0.79±0.69 ab
SGS	5.05±1.04 a	74.28±22.90 a	5.16±12.68 b	56.63±25.67 ab	25.79±23.94 a	3.24±1.24 a	1.13±1.13 a
NGS	3.91±1.13 b	46.76±37.21 b	28.29±32.29 a	46.33±35.17 b	27.84±32.26 a	1.97±1.24 b	0.44±0.68 b
SS	4.20±1.05 b	50.38±39.13 b	22.82±33.80 a	63.87±34.60 a	17.21±26.27 a	2.05±1.38 b	0.82±1.10 ab

[1)] Values in a column followed by the same letter are not significantly different (P = 0.05). $ASPERG_M$ and $ATOXIN_M$ are averages of three maize samplings. $ASPERG_S$, $ASPERG_M$ and $ATOXIN_M$ were ln(x+1) transformed prior to analysis, the other variables in percent were arcsine square root transformed.

Finally, the average concentration of aflatoxin from the three maize samplings showed a significant difference only between the SGS ($ATOXIN_M$ = 1.13 ppb) and NGS ($ATOXIN_M$ = 0.44 ppb) zones (Table 2.2). As expected, the concentration of aflatoxin in maize was positively (r = 0.38 and P < 0.0001) correlated with the incidence of *A. flavus* in maize ($ASPERG_M$) but also with the one in soil ($ASPERG_S$) though with a weaker coefficient of correlation (r = 0.20, P = 0.05).

Table 2.3: Correlation coefficients between geographic, soil and *A. flavus* population characteristics (see Table 2.1) of 100 fields

Variables	Correlation coefficients[1)]						
	ASPERG$_S$	*L-STRAIN*	*S-STRAIN*	*T-STRAIN*	*N-STRAIN*	*ASPERG$_M$*	*ATOXIN$_M$*
LATITU	-0.29**	-0.39***	0.41***	-0.03	-0.00	-0.39***	-0.11
HEIGHT	-0.38**	-0.45***	0.46***	-0.11	0.02	-0.44***	-0.10
PHV	0.13	0.35**	-0.27*	-0.02	0.22*	0.03	-0.17
SOC	0.09	0.17	-0.13	-0.13	0.19*	0.01	-0.20*
SAND	-0.09	0.01	-0.05	0.20*	-0.27*	0.06	0.11
SILT	-0.07	-0.23*	0.19*	-0.18	0.15	-0.21*	-0.15
CLAY	0.16	0.13	-0.05	-0.16	0.27*	0.04	-0.08
ESCa	0.18	0.21*	-0.12	-0.06	0.23*	0.06	-0.05
ESK	0.13	0.20*	-0.18	-0.17	0.26*	-0.18	-0.16
ESNa	0.22*	0.21*	-0.17	-0.24*	0.34**	0.10	0.03
ASPERG$_S$	---	0.38**	-0.14	0.19*	0.08	0.05	0.20*
L-STRAIN		---	-0.65***	0.09	0.39***	0.11	-0.09
S-STRAIN			---	0.23*	-0.22*	-0.17	-0.06
T-STRAIN				---	-0.70***	-0.00	0.05
N-STRAIN					---	-0.07	-0.16
ASPERG$_M$						---	0.38***

[1)] Significance of correlation coefficients: *** $P < 0.0001$; ** $0.001 \leq P \leq 0.01$; * $0.01 \leq P \leq 0.05$. Percentage data were arcsine square root transformed prior to analysis. *ASPERG$_S$*, *ASPERG$_M$* and *ATOXIN$_M$* were ln(x+1) transformed prior to analysis

The geographical and soil variables (Table 2.4) showed various trends over the four zones. As expected, the four zones were clearly separated from each other by the latitude (from the South of the country to the North). For the site height, the NGS zone had the significantly highest sites followed in decreasing order by SS, SGS and CS. The soil characteristics in the different zones were not so clearly different. The average pH was significantly higher in the two southern zones CS and SGS than in the SS in the north. The soil organic carbone (*SOC*) of the CS zone was higher than those of the zones SGS and SS, and the one of the NGS was significantly more important than the one of the SGS zone. The soil texture was considered from three perspectives. The first perspective was the percentage of sand in the soil that was more important in the zones in the middle and incidentally in the costal zones. The silt percentage, the second perspective, decreased from the North to the South, and finally the percentage of the clay was lower in the SGS and NGS zones. The significantly highest percentage of clay was found in the soil from the CS zone. The free cations of the soils in the four zones were very similar. There were no differences for calcium between zones, while for potassium only the content of the NGS zone exceeded those of the CS and SS zones. For sodium, the soils in both southern zones had more than both northern zones but only the SGS zone had content significantly higher than those of both north zones.

Table 2.4: Comparison of the geographic and soil characteristics (Table 2.1) between the four agro-ecological zones: CS = Costal Savanna, SGS = Southern Guinean Savanna, NGS = Northern Guinean Savanna, SS = Sudan Savanna

Zones	*LATITU*	*HEIGHT*	*PHV*	*SOC*	*SAND*	*SILT*	*CLAY*	*ESCa*	*ESK*	*ESNa*
CS	6.79±0.07 d [2)]	74.48±48.16 d	6.83±0.30 a	6.50±1.69 a	55.41±14.61 b	17.87±5.66 c	27.46±11.79 a	4.90±1.58 a	0.15±0.04 b	0.18±0.06 ab
SGS	7.48±0.33 c	134.44±69.16 c	6.80±0.60 a	5.42±1.23 c	59.33±4.56 a	20.51±4.06 b	21.57±2.79 c	4.69±0.79 a	0.17±0.03 ab	0.20±0.04 a
NGS	9.56±0.67 b	370.32±49.77 a	6.68±0.56 ab	6.16±1.83 ab	57.67±5.74 ab	21.28±4.02 b	22.93±3.74 c	4.47±1.43 a	0.18±0.08 a	0.17±0.05 b
SS	11.17±0.50 a	291.08±74.66 b	6.53±0.46 b	5.63±1.09 bc	50.74±5.72 c	27.26±4.26 a	25.59±4.07 b	4.68±0.70 a	0.14±0.03 b	0.16±0.03 b

[1)] Values in a column followed by same the lowercase letter are not significantly different (P = 0.05). Percentage data (*SOC*, *SAND*, *SILT*, *CLAY*) were arcsine square root transformed prior to analysis.

More interesting for this study were the correlations between these geographic/soil variables and those of the *A. flavus* populations in the soil and in the maize grains and finally the aflatoxin produced in maize. The coefficients of correlation, all based on the data of the 100 fields, are summarized in Table 2.3. The latitude and the height were positively correlated with the percentage of S-strain isolates and negatively with the *A. flavus* incidence in the soil and in maize as well as with the percentage of L-strain isolates in the soil. The soil pH was in positive correlation with the percentages of L-strain isolates and of atoxigenic strains and in negative correlation with the percentage of S-strain isolates. The organic carbon content was positively correlated with the percentage of atoxigenic strains (r = 0.19), but negatively with the aflatoxin content (r = -0.20). The sand was positively correlated with the toxigenic strains percentage and negatively with the atoxigenic ones. The soil percentage of silt was in negative correlation with the percentage of L-strain isolates and with the incidence of *A. flavus* in maize, but in positive correlation with the percentage of S-strain isolates. Finally, the clay was favorably correlated with the atoxigenic strains percentage (Table 2.3). Generally, the soil free cations were positively correlated with the percentages of L-strain isolates and of atoxigenic strains. Moreover, sodium was in positive correlation with the soil incidence of *A. flavus* and L-strain percentage and N-strain (atoxigenic strain percentages) but in negative correlation with the toxigenic strain percentage.

Maize samples collected in all villages at three times allowed to determine that whatever the sampling date, the incidence of *A. flavus* in maize grains ($ASPERG_M$) was always higher in the CS and SGS zones, but without differences between these two zones. There was also a similarity between the incidence of maize in the NGS and SS zones. In contrast, the aflatoxin concentration in maize did not differ statistically between the four zones at the first sampling date; at the second sampling date, only the aflatoxin contents in maize from SGS and NGS zones were different and at the third sampling date, the aflatoxin concentration of maize in the SGS zone was significantly higher than in the northern zones. Finally, the last two samplings of maize grains from the CS and SGS zones contained more propagules of *A. flavus* than the first sampling and that trend was also observed for aflatoxin concentration in maize (Table 2.5). In the NGS and SS zones, neither the incidence of *A. flavus* in maize nor the concentration of aflatoxins changed with time.

Table 2.5: Comparison of the *A. flavus* density ($ASPERG_M$) and aflatoxin concentration ($ATOXIN_M$) in maize between four agroecological zones (CS = Costal Savanna, SGS = Southern Guinean Savanna, NGS = Northern Guinean Savanna, SS = Sudan Savanna) at three samplings dates.

Zones	Logarithm of *Aspergillus flavus* density ($ASPERG_M$) and aflatoxin concentration ($ATOXIN_M$) in the maize					
	First Sampling		Second Sampling		Third Sampling	
	$ASPERG_M$	$ATOXIN_M$	$ASPERG_M$	$ATOXIN_M$	$ASPERG_M$	$ATOXIN_M$
CS	2.73±1.44 a B[2)]	0.52±0.60 a B	3.37±1.13 a A	0.86±0.74 ab AB	3.60±1.13 a A	1.16±0.52 ab A
SGS	2.86±1.32 a B	0.53±0.87 a B	3.27±1.10 a AB	1.28±1.00 a A	3.80±1.13 a A	1.83±1.22 a A
NGS	1.79±1.44 b A	0.25±0.55 a A	1.94±1.13 b A	0.49±0.60 b A	2.21±1.13 b A	0.63±0.86 b A
SS	1.89±1.46 b A	0.53±1.04 a A	2.07±1.33 b A	0.89±1.13 ab A	2.20±1.39 b A	1.04±1.09 b A

[1)] Values in a column followed by the same lowercase letter are not significantly different ($P = 0.05$) and values of the same variable in a row followed by the same uppercase letter are not significantly different ($P = 0.05$). Both variables were $\ln(x+1)$ transformed prior to analysis.

The stepwise multiple regression analyses, considering the characteristics of the *A. flavus* population in the soil in relation to the geographic and soil variables, resulted in equations with only a few significant independent variables.

The incidence of *A. flavus* in the soil decreases with the site height according to the equation 2.2.

$$ASPERG_S = 5.34 - 0.003 \times HEIGHT \quad \text{with } R^2 = 0.14 \quad (2.2)$$

In case of the percentage of L-strains in the soil population, four significant independent variables were identified: the site height, soil pH, sand and clay percentages. Only the height had a slightly negative impact on the L-strain percentage, the other variable had a positive impact (equation 2.3).

$$L-STRAIN = -203.39 - 0.08 \times HEIGHT + 17.23 \times PHV + 1.92 \times SAND + 2.52 \times CLAY \quad \text{with} \quad R^2 = 0.30 \quad (2.3)$$

As expected from the earlier statements, the percentage of S-strains in the soil was positively affected by the site height (equation 2.4).

$$S-STRAIN = -6.01 + 0.09 \times HEIGHT \quad \text{with} \quad R^2 = 0.21 \quad (2.4)$$

The percentage of toxigenic strains was positively influenced by the sand, the silt and the clay percentages in the soil, but also by the calcium content. On the other hand, the potassium content had a high negative impact (equation 2.5).

$$T-STRAIN = -1620.20 + 18.12 \times SAND + 11.67 \times SILT + 16.04 \times CLAY + 11.80 \times ESCa - 213.03 \times ESK \quad \text{with} \quad R^2 = 0.16 \quad (2.5)$$

The percentage of atoxigenic strains was positively influenced by the soil pH and the sodium content (equation 2.6).

$$N-STRAIN = -96.05 + 12.48 \times PHV + 199.34 \times ESNa \quad \text{with} \quad R^2 = 0.17 \quad (2.6)$$

The incidence of *A. flavus* in the harvested maize decreased with higher latitude and the potassium content (equation 2.7).

$$ASPERG_M = 7.04 - 0.39 \times LATITU - 6.05 \times ESK \quad \text{with} \quad R^2 = 0.24 \quad (2.7)$$

And finally only the soil organic carbon content had a negative effect on the aflatoxin concentration in maize:

$$ATOXIN_M = 2.01 - 0.13 \times SOC \quad \text{with} \quad R^2 = 0.04 \quad (2.8)$$

Multiple regression analyses were also used to describe the *A. flavus* incidence and its aflatoxin production in maize in relation to soil population characteristics and to find a relationship between aflatoxin content and *A. flavus* incidence in the same maize sample. It turned out that aflatoxin concentration in maize was influenced by *A. flavus* soil incidence and the percentages of L- and S-strains in the soil (equation 2.9).

$$ATOXIN_M = 1.04 + 0.24 \times ASPERG_S - 0.01 \times L - STRAIN - 0.01 \times S - STRAIN \quad \text{with} \quad R^2 = 0.11 \qquad (2.9)$$

Finally, the relationship between aflatoxin content and *A. flavus* incidence in maize was calculated as equation 2.10.

$$ATOXIN_M = 0.54 + 0.26 \times ASPERG_M \quad \text{with} \quad R^2 = 0.15 \qquad (2.10)$$

2.5 Discussion

Many studies describing the composition of *A. flavus* communities in relation to certain factors such as climate (Orum et al, 1997; Cardwell et al., 2002) and soil (Jaime-Garcia et al., 2006) have been undertaken especially in the United States of America and in Benin. The main interest of these studies was to determine the principal components of the soil or the climate characteristics that have the determinant impact on *A. flavus* incidence and its population composition, in order to forecast *A. flavus* incidence or aflatoxin contamination in a particular zone over a certain period of time. Though in a more simplified way, the same methodology was used here with emphasis on the soil and geographic parameters responsible for *A. flavus* incidence and aflatoxin production in maize in the Republic of Benin.

The positive correlation between the incidence of *A. flavus* (cfu/g) in the soil and the percentage of L-strain isolates in this study is fairly logical since even in zones where high percentages of S-strain isolates were found, the L-strain isolates are still dominant in percentage (NGS zones and SS zones). In this study, there was no correlation between the percentage of S-strain isolates and the density of *A. flavus* propagules. But there was a positive and significant correlation between *A. flavus* incidence and L-strain percentages. This result is comparable to a previous study of Orum et al. (1997) who found a correlation between the *A. flavus* incidence in the soil and the composition of its community. Moreover, the *A. flavus* incidence, in this study, was slightly positive correlated with the percentage of

the toxigenic isolates. The lack of correlation between *A. flavus* incidence and on one hand the percentage of S-strain isolates and on the other hand the percentage of atoxigenic isolates may be due to the fact that these two kinds of *A. flavus* isolates were low in number and hardly representative of the population. This was especially true for the S-strain isolates for which the percentage in certain villages was zero. However, in the specific case of the S-strain isolates in Benin, there were strong positive correlations with the latitude and the site height above sea level of the studied fields and these correlations were negative in relation to the L-strain percentage. This is also not a new finding since Cardwell et al. (2002) already noticed this fact and linked it to the climate that is getting wetter toward the south of Benin while the S-strain isolates are getting rarer. In our study and a similar study of Cardwell et al. (2002), the climatic effect can be suggested though in Benin climatic characteristics change in the same North-South direction with the driest area in the North. That hypothesis was first made by Orum et al. (1997) based on different seasons in the same area with higher incidence of S-strain isolates in the driest season. The question that remains to be confirmed is the high correlation of the site height and L/S-strains composition of the population. This could be also due to the climate change with height since the highest percentages of S-strain isolates were recorded in the highest site zone (NGS). But in the specific case of this study this cannot be definitively conclusive for at least one reason. It is not easy to conclude that the change of the climate due to the height is more important than the one due to the latitude. It would be more accurate to consider a synergistic effect of the height and the dryness to promote the proliferation of S-strain isolates.

The incidence of *A. flavus* in the soil was not significantly correlated with the one in maize. Thought unexpected, the fact could be explained by the variability of the virulent isolates on the maize varieties used. Only the virulent isolates can be infective. A zone with a weak incidence of *A. flavus* could nevertheless contain more virulent isolates for the maize variety cultivated there. Cotty (1989) showed that on cotton *A. flavus* strains had variable virulence. This possible explanation was also raised in the study of Cardwell et al. (2002). Among 227 maize samples that were aflatoxin positive, only 7 were aflatoxin G positive and they assumed that the fraction of S-strain isolates in the population has produced this aflatoxin G but they recognized that the virulence of S-strain isolates in normal cropping system on maize was not yet fully studied.

Since it is known that the S-strain isolates produce generally a higher level of aflatoxin than L-strain isolates, it is understandable that the percentage of S-strain isolates is positively correlated with the percentage of toxigenic isolates, but negatively correlated with that of the atoxigenic isolates. Thus it is obvious that a high incidence of S-strain isolates in a population increases the potential of the population to produce aflatoxin. From this same assertion it is normal to expect that the high frequency of the L-strain isolates in a population increases the chance to have atoxigenic isolates in the involved population since it is well known that only L-strain isolates could be atoxigenic. This conclusion is supported by similar results of Cotty (1997) in an earlier study in the United States. The correlation coefficient and even the probability of the correlation between the aflatoxin content in maize and *A. flavus* incidence in maize were higher when compared to *A. flavus* incidence in the soil. This is surely due to the fact that only isolates that successfully infect maize are able to produce aflatoxin in it. The maize infection by isolates from the soil depends on numerous factors such as their own virulence on cultivated maize, the environmental and cultural conditions to infect and colonize maize. There is no certainty that the actual isolates present on maize kernels are all directly related to those in the soil beneath the maize. Indeed, Wicklow et al. (1998) attributed a high role to the wind and insects that can carry foreign isolates to the observed field.

Pearson correlations did not conclusively help to describe the effect of the soil texture on *A. flavus* incidence and its community's composition. In most of the cases, the correlations were not significant and when they were, both the coefficient and the probability of the correlation were rather weak. Jaime Garcia et al. (2006) attributed the within region (distance > 25km) variation of the percentage of S-strain isolates to the soil type especially to the clay percentage in the soil. But in the current study the S-strain isolates were correlated neither with the clay nor with the sand percentage in soil. But there was a slightly positive correlation between the silt content and the S-strain isolates (r = 0.19, P = 0.05) and a negative correlation between the L-strain isolates and the silt content (r = -0.23, P = 0.02). The specific properties that favor S-strain but not L-strain isolates remain obscure and could only be explained if it could be proven that both strain isolates have different behavior in relation to environmental factors including soil properties and contents. The same authors attributed the interregional variation of S-strains to crop variation especially with predominance of cotton and sorghum. Moreover, Orum et al. (1997) and later Cardwell et al. (2002) identified the dry

climate as main factor for an increase of S-strain percentage. Both reasons, in the case of Benin, could be taken into account and even their interdependency should be considered since with the agricultural technology level of Benin, crops such as sorghum are only cultivated in the north while maize is cultivated in all zones. Cotton is commonly grown in the SS, NGS and SGS zones. Then only tolerant crop for a zone climate could be grown in that zone and even if maize cultivation is expanding in terms of area exploited through the North since some decades because of more tolerant varieties promotion it should be remembered that that crop is relatively new in these regions (northern zones) where the traditional cereals were millet and sorghum. Then the climate variation from the South to the North with the additional influence of the cropping systems or crops grown in each specific zone could explain the variation of S-strain isolates frequency in the soil. Some uncertainties apply to the soil availability of cations action on the *A. flavus* population composition. It is interesting to notice the positive correlation between the sodium content and the percentage of atoxigenic strains. In this study, there was no correlation between S-strain isolates and any of the studied soil cations. The L-strain isolates were positively correlated with calcium, potassium and sodium contents and the latter is also in positive correlation with the soil incidence of *A. flavus* and the percentage of atoxigenic isolates and in negative correlation with the percentage of toxigenic isolates in the soil. This effect of sodium could be linked to its properties to decrease the availability of other cations. It is known that an excess of sodium cations in a soil tends to make the other cations less available for the plant. A hypothesis could be that the presence of sodium leads to the lack of some other cations needed by some microorganisms that compete in the soil with *A. flavus*. If there is no competition then there is no need for aflatoxin that is sometimes described as competition tools of *A. flavus* (Lillehoj, 1980; Wicklow, 1981). Then the capability of *A. flavus* strains to produce aflatoxins is lowered and the number of atoxigenic isolates is increased. It could also be inferred from this study that only the L-strain toxigenic isolates are then involved since there is no correlation with the S-strain isolates that are almost always toxigenic.

Concerning the incidence of *A. flavus* in maize, it was observed in this study that the average incidences in the southern zones were significantly higher than those in the north while the corresponding aflatoxin content did not follow the same pattern and in this case only the SGS zone had significantly higher average aflatoxin content than the NGS zone. Once

again it is hard to explain this discordance between the two variables but as it is known and said earlier in this paper the aflatoxin production in maize depends of course on the presence of toxigenic isolates but also on many other parameters that have to be taken into account. These parameters include the cultural methods (rotation) (Jaime-Garcia et al., 2006), the actual climatic condition (Cotty et al., 1994) and the population composition of *A. flavus* (Cotty, 1997), even some random effects could also be involved since the level of insect infestation and wind parameters should be part of the equation. Another remark is the temporal progress of the *A. flavus* incidence and aflatoxin content in maize in both southern zones compared to the northern zones. Only the climate could be used to explain it. The country subdivision in zones has as basis the climatic characteristics. Both northern zones have a more similar climate than both southern zones if the number of seasons and other climatic parameters such as rainfall and the length of the growing seasons are considered (Cardwell et al., 2002). The climatic conditions for stored products as maize appear more favorable in the North than in the South. In the southern part of the country, the frequency of rain is higher and consequently the favorable conditions for mould growth closer to the optimum.

The stepwise multiple regression analysis allowed to notice that the height of the sample sites was the only factor that had an impact on *A. flavus* incidence in the context of this study. That impact has been rarely reported in the previous studies. Even if this influence is relatively weak, with a coefficient of regression of -0.003, it would be very interesting to discover the main component that change with the height since the climate that would be a more plausible component changes also with the latitude. However, the multiple regression analysis did not show the latitude as determinant factor for the incidence of *A. flavus*. It showed that the height of a site should be taken into account while evaluating the incidence of *A. flavu*s in the soil. Accordingly, the soil composition in S and L-strain isolates also depended on the height that seemed to have a negative impact on the L-strain and a positive one on the S-strain isolates. In the case of the incidence of L-strain isolates, in addition to the negative effect of the height, there were positive effects of the pH, and of the sand and clay percentage of the soil. If the S-strain can be proven as virulent on maize then the site height above sea level could be used as a parameter to evaluate the risk of aflatoxin production in maize.

The toxigenic strains are promoted by sand, silt, clay and calcium content. In the case of the main components of the soil texture, it is difficult to find all of them acting in the advantage to the toxigenic strains. The possible way to explain it is to consider their respective fractions in the soil. In equation 2.5, their coefficients are close enough to think that about one third of each of them makes up the best soil texture allowing, maybe indirectly, the promotion of toxigenic strains. Besides the soil texture, the calcium content is also favorable to toxigenic strain isolates and only the potassium is strongly detrimental to these isolates. In the case of atoxigenic strain isolates, the pH and the sodium content are the only favorable factors for their increase in the soil. To understand how these different parameters promote or hinder toxigenic strains, it seems important to know the soil microbiology and dynamics that could have an indirect effect on the potential of *A. flavus* to produce aflatoxin.

A. flavus incidence in maize is negatively impacted by the latitude and the potassium content. If it is clear that the impact of the latitude would be through the climate, it is more complex to determine how potassium content could have an impact on the incidence of *A. flavus* in maize. The only possible link is found in its effects on growing maize plants. One of the roles of potassium on growing plants is its property to increase the strength of the plant and its resistance to diseases and insects (Pidwirny, 2006). Finally, the equations 2.9 and 2.10 showed clearly that the production of aflatoxin in maize depended on the incidence of *A. flavus* in the soil and also in the maize. This is rather a confirmation than a new finding if the previous comments of this study are considered. But a confusing fact is that both percentages of the L- and S-strain isolates in the soil have an impact of the same intensity (equation 2.9) and negative on the aflatoxin production in maize even if this impact is rather light (with -0.01 as coefficient). But this could be easily explained by the fact that only the L-strain isolates could provide atoxigenic strains in an *A. flavus* population. Thus a high percentage of L-strain isolates increases the chance to have less aflatoxin. The virulence could also help to explain why the percentage of S-strain isolates has a negative impact on the aflatoxin production in maize because the question about their virulence on maize is still raised. Even though they are more toxigenic, if they are unable to contaminate maize, they will reduce the number of virulent toxigenic isolates that would infect maize.

From the list of equations and tables 2.2 and 2.4, it may be possible to conclude that aflatoxin concentration in maize in a site depends on the soil organic carbon content, on the *A.*

flavus incidence in the soil, on the soil composition of *A. flavus* population (L-strain isolate percentages) and on the *A. flavus* incidence in maize. From that first step only the soil organic carbon content can be directly measured from the soil. In a second step, the estimation of the soil incidence of *A. flavus* from the site height together with the estimation of the percentage of L-strain isolates from the height, the soil pH, the sand and the clay percentages in the soil can help to refine aflatoxin risk assessment. Also in parallel with the S-strain isolates estimated from the height or the *A. flavus* incidence in maize estimated from the latitude and potassium content in the soil.

This survey has helped to have rough ideas about the major factors leading to aflatoxin contamination in cultivated maize in Benin. But further work is needed to show more accurately the relevance of these factors. First the same kind of work should be repeated over more seasons and more places to make sure that the factors are stabilized, but also more fundamental research is needed, for instance on the variability of the virulence of isolates within a population of *A. flavus*.

Chapter 3. Effects of variety, cropping system and soil inoculation with *Aspergillus flavus* on aflatoxin contamination of maize

3.1 Abstract

Effects of soil inoculation with *A. flavus*, variety and cropping system on the level of aflatoxin in stored maize in Benin were investigated. Generally, the concentration of aflatoxin B_1 and B_2 increased during storage. Variety and inoculation with *A. flavus* were the main factors influencing the production of aflatoxins in stored maize. The improved maize variety had higher levels of aflatoxin B_1 and B_2 compared to the local variety. A similar trend was observed for the number of colony forming units of *A. flavus*. Intercropping with cowpea (*Vigna unguiculata* (L.) Walp.) decreased aflatoxin concentration in the improved maize variety but not in the local maize variety. On the local maize variety, higher levels of *Penicillium* spp. and lower levels of *Fusarium* spp. were observed than on the improved maize variety. Neither the variety nor the soil inoculation with a toxigenic strain of *A. flavus* or the cropping system had an effect on the populations of major storage insects, but their numbers in the stored maize were positively correlated with aflatoxin. The initial fungal inoculum level and the water content of the maize kernels after harvest played a significant role in *A. flavus* infection initiation and development. These factors could be utilized in forecasting the rate of aflatoxin production and contamination levels during maize storage.

3.2 Introduction

In tropical Africa, maize is a staple diet of local 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 in periods when prices have increased. Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* that affect many crops, including peanut, maize (corn), cottonseed, rice, spices and other crops (Bennett & Klich, 2003). *A. flavus* is more common than *A. parasiticus* in African soils (Cardwell & Cotty, 2002) and on maize (Atehnkeng et al., 2008). Among the factors contributing to aflatoxin production in crops are genotype (Mehan et al.,

1986), environmental factors (Sander et al., 1993), inadequate agronomics practices, storage techniques (Jacques, 1988; Hell et al., 2003), the presence of insects (Dowd et al., 2005), and other fungal organisms (Hill et al., 1985). In tropical regions, the threat of aflatoxin contamination is high, since most of the factors that favor *A. flavus* and toxin development are prevalent in these agricultural systems. The combination of some or all of these factors ultimately results in favorable condition for aflatoxins development in field crops, stored food and feed (Cardwell & Henry, 2004). Aflatoxin contamination of maize is a serious public health issue that has been studied extensively, particularly in West Africa (Gong et al., 2002; Williams et al., 2004).

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. The more probable the presence of *A. flavus* inoculum in a field is, the higher is the risk of contaminated maize (Horn 2003; Jaime-Garcia & Cotty, 2004).

The impact of other biological factors affecting maize in Africa during different processes from the field to the storage structure has been studied extensively (Setamou et al., 1997; Hell et al., 2000). Insects are one of the principal factors that increase aflatoxin development, as they cause wounds on grains and cobs during their feeding by removing the natural barriers that protect maize grains, and subsequently increase the accessible area for fungi colonization. The activities of insects also raise the moisture level in the storage due to their metabolism, thereby changing environmental conditions (Beti et al., 1995). Insects can also transport fungal propagules during their movement between grains (Beti et al., 1995). Thus, a strong insect presence 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 under field conditions in West Africa. Most studies have concentrated on the correlation between aflatoxin and fumonisin contamination (Abbas *et al*., 2005).

Previous studies have shown that aflatoxin resistant maize genotypes exist (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 collected diverse 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). Knowing that quantitative genetics is highly influenced by environmental factors, they have to be integrated into an eventual explanation of the variable susceptibility of a given genotype during maize cropping.

Maize intercropping with another crop was identified as one of the possible favorable factors for aflatoxin accumulation in maize in West Africa (Hell et al., 2003). The most prevalent cropping systems in Benin are maize/cowpea, maize/cassava, and maize/peanut. 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 (Hell et al., 2003). In a previous study, Cardwell et al. (2000) found that maize intercropping with others crops such as cotton and sorghum also increased the risks of aflatoxin contamination in maize. This led to the hypothesis that maize intercropped with some crops increases the risk of aflatoxin contamination in maize kernels. Similarly, maize rotation with susceptible crops or monocropping of maize on the same plot can lead to a high level of aflatoxin contamination (Bruns, 2003).

In order to develop effective and efficient control methods, a good understanding of the effects of different abiotic and biotic factors that influence *Aspergillus* development and aflatoxin production is important. The objectives of this study were, therefore, to investigate the impact of maize variety, cropping system and primary inoculum levels on aflatoxin production by *A. flavus*, and to determine the relationships between aflatoxin contamination and other biotic factors such as storage insects and molds. Understanding these interactions could be useful in forecasting the expected level of aflatoxin contamination in pre- or post-

harvest maize, where a timely application of suitable methods for aflatoxin control is critical, especially in zones with limited production resources.

3.3 Materials and Methods

The two maize varieties (*Zea mays*) used in the experiment were “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 be less affected by aflatoxins because of the hard envelopes of its kernels that could prevent the penetration of *A. flavus*. Besides the pure stands of the two maize varieties, their intercropping with a local 90 day variety of cowpea called “Kpodjiguégué” was tested. The cowpea was sown alternating in every row. Half of the experiment was inoculated with a toxigenic *A. flavus* strain that was isolated from maize collected in the coastal zone (South) of the Republic of Benin. 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 (Table 3.1). 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 used at a rate of 160 kg per ha. At the beginning of grain formation, urea was applied at a rate of 50 kg per ha. Weeding was carried out before each fertilizer application.

Table 3.1 - Factors combinations and treatments references 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

For inoculation, the culture of the isolated strain was purified by the single spore sub-culture method and tested by spore suspension, fermentation followed by aflatoxins extraction and quantification. The purified strain was prepared on Petri dishes containing 5/2 medium (5% V8 juice and 2% agar) (Cotty, 1989) for conidia production used for soil inoculation. The Petri dishes were incubated at 31^{o}C in the dark for 7 days. The conidia were collected and suspended in sterile distilled water to make a spore suspension. Approximately 100 µl of Tween 80 were added per liter of water and the concentration of the suspension was determined using a hemacytometer. The original suspension was diluted to obtain a concentration of 2.7 x 10^{7} conidia per ml used for inoculating autoclaved rice paddy at the rate of 100 ml spore suspension per 1000 g of autoclaved paddy. The inoculated paddy was mixed by shaking, stored for 5 days at 31^{o}C in the dark in an incubator (Percival (Model I-35LL), Boone, Iowa), dried in the open air and later transferred to the field for broadcasting. The inoculum (paddy-spore mix) was propagated 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 used that was spread uniformly by hand over the whole area of each plot.

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 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/g of soil) 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 micro-liter of the resulting suspension were used to inoculate a Petri dish containing a Modified Rose Bengal Agar (Cotty, 1994). The Petri dish was then incubated at 31^{o} C in the dark for three days and all isolates belonging to the genus *Aspergillus* were subculture 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.

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 infestation 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 severity of cobs was determined by visually estimating the percentage of the cob area covered by this fungus.

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 in a 500 ml Erlenmeyer flask, 250 ml of methanol and water (60/40 vol/vol) were added and 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 small 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 canned with a densitometer, CAMAG TLC Scanner 3 with win-CATS 1.4.2 software (Camag AG, Muttenz, Switzerland). 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 compared with the standard were diluted, spotted and chromatographed again.

The multi-factorial analysis of variance was performed using the general linear model (GLM) 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 concentration c (ppb) of both aflatoxins on maize kernels increased exponentially during the four months of storage in all treatments. As the variability also increased with time t, the values of the concentration were transformed with $c' = \ln(c + 1)$ 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:

$$c'(t) = \ln(c(t) + 1) = (a + d \cdot \Delta a) + (b + d \cdot \Delta b) \cdot t \qquad (3.1)$$

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:

$$c'(t) = \ln(c(t)+1) = (a + d \cdot \Delta a) + b \cdot t \qquad \text{if } \Delta b = 0 \qquad (3.2a)$$

$$c'(t) = \ln(c(t)+1) = a + (b + d \cdot \Delta b) \cdot t \qquad \text{if } \Delta a = 0 \qquad (3.2b)$$

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. 3.2a or 3.2b) 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. 3.1 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:

$$c'(t) = \ln(c(t)+1) = (a + d_i \cdot \Delta a_i + d_c \cdot \Delta a_c) + (b + d_i \cdot \Delta b_i + d_c \cdot \Delta b_c) \cdot t \qquad (3.3)$$

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-inoculated ($d_i = 0$) pure stand ($d_c = 0$). As described above, first the full model (eq. 3.3) 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.

3.4 Results

There were high variations of the different variables especially over the sampling period. Except for water content, the highest differences between all other effects were mainly observed at the end of the trial.

3.4.1 *A. flavus* propagules in the soil

All plots had relatively high natural levels of *A. flavus* propagules 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* propagules by 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* propagules than non-inoculated ones (Fig. 3.1).

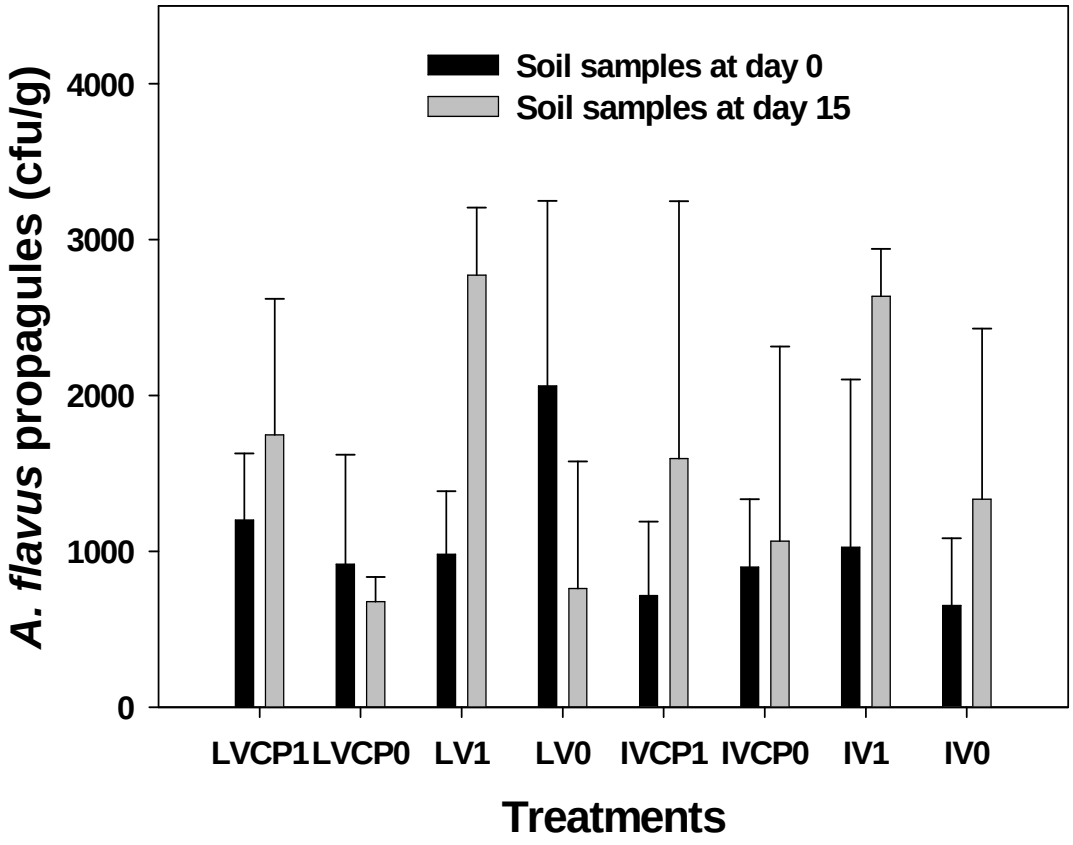

Fig. 3.1: Number of *A. flavus* propagules (cfu/g) in the soil of the eight treatments (Table 3.1) before (on day 0) and after inoculation (on day 15). Only the treatments LVCP1, LV1, IVCP1 and IV1 were inoculated.

3.4.2 Water content of stored maize cobs

The water content of the two maize varieties was significantly different during the first month in storage (Fig. 3.2); the improved variety had significantly higher water content ($P < 0.001$) than the local variety. In general, the water content during the first month of storage was higher than in later months irrespective of treatment.

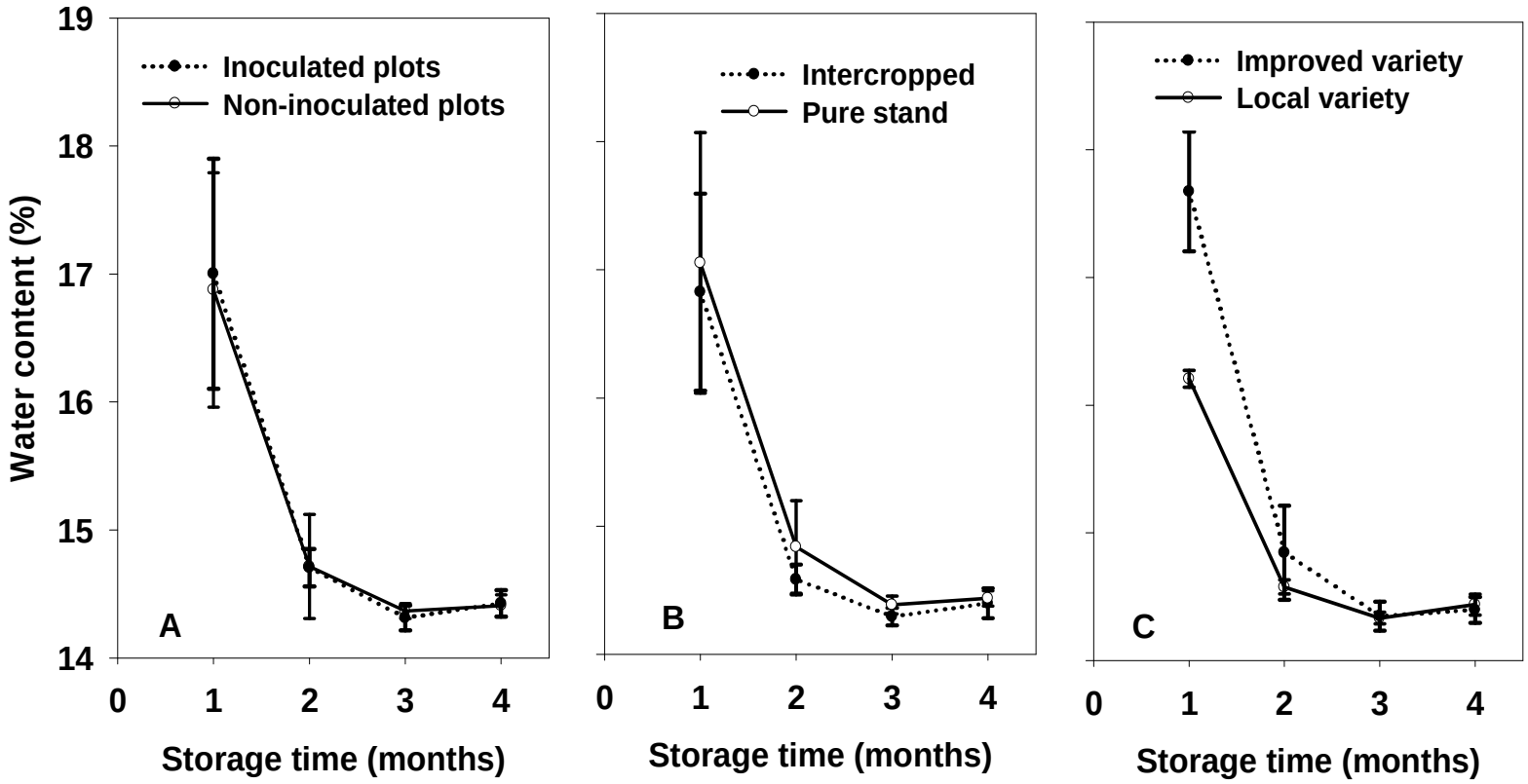

Fig. 3.2: Temporal progress of the moisture content (%) of the maize kernels during four months of storage depending on inoculation (A), cropping system (B) and variety (C)

3.4.3 *A. flavus* propagules in stored maize cobs

The number of cfu/g of *A. flavus* propagules of the different treatments varied with sampling month. During the first month, the amount of propagules did not differ between the two varieties, but in the subsequent months the improved variety consistently had a 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 (Fig. 3.3). Furthermore, there were no significant interactions between the three treatments (i.e. inoculation, varieties and cropping system).

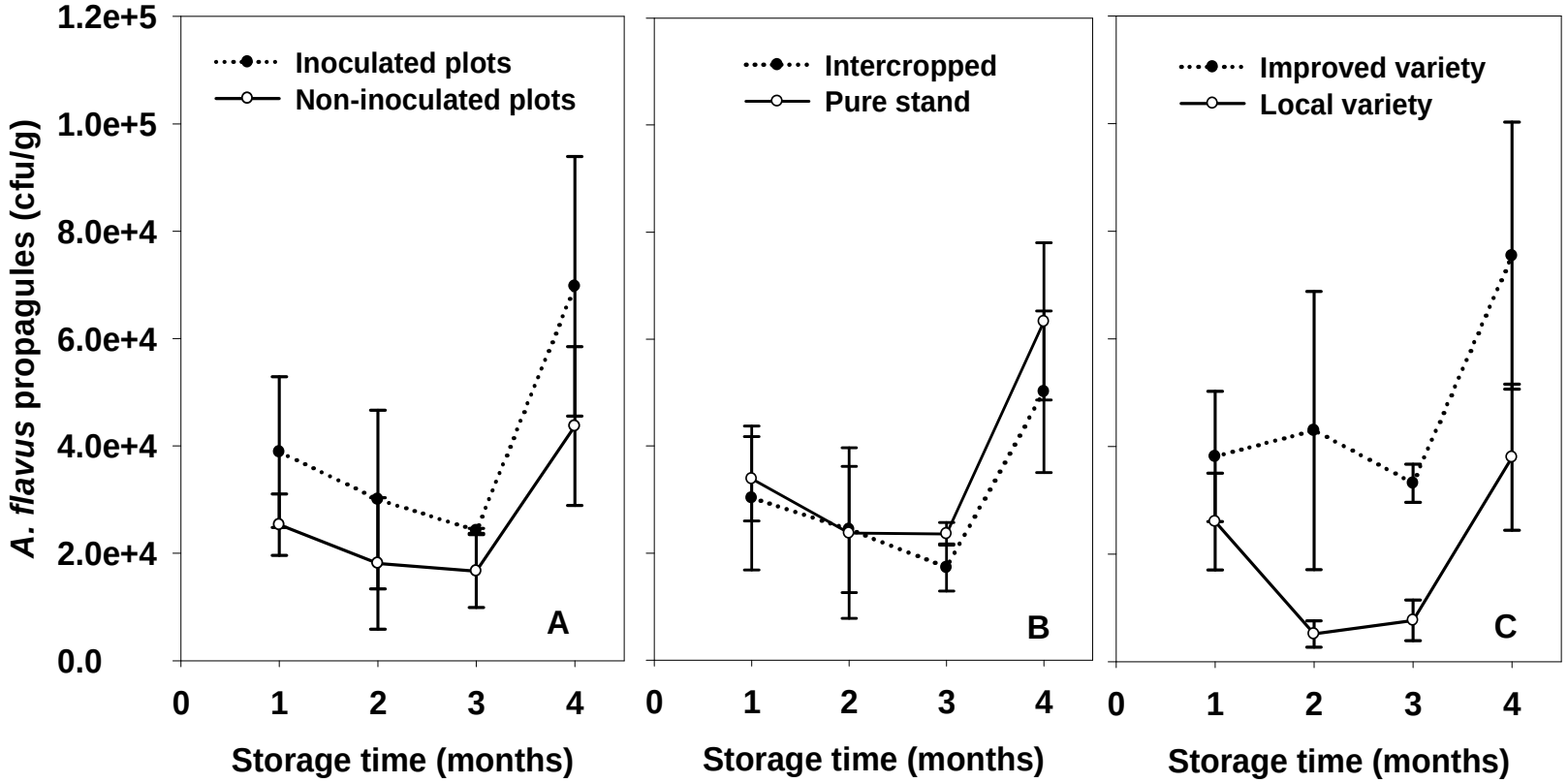

Fig. 3.3: Dynamics of *A. flavus* propagules (cfu/g) of the maize kernels during four months of storage depending on inoculation (A), cropping system (B) and variety (C).

3.4.4 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 (Fig. 3.4). When eq. 3.3 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 (Fig. 3.4 A, B). The final equation for the dynamics of the aflatoxins concentration on the local variety was (R^2 = 0.65):

$$c'(t) = -0.78 + (0.99 + d_i \cdot 0.43) \cdot t \qquad (3.4)$$

In the joint analysis of the four treatments (with eq. 3.3) 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):

$$c'(t) = (-0.58 + d_i \cdot 1.28 + d_c \cdot 3.13) + (1.48 - d_c \cdot 0.70) \cdot t \qquad (3.5)$$

The intercept and the slope of the reference treatment, i.e. of the non-inoculated pure stand, were -0.58 and 1.48, respectively (Fig. 3.4 C, 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.

Obviously the two cultivars behaved 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. 3.1 for all four cases, the differences between slopes were not statistically significant. 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. 3.2a).

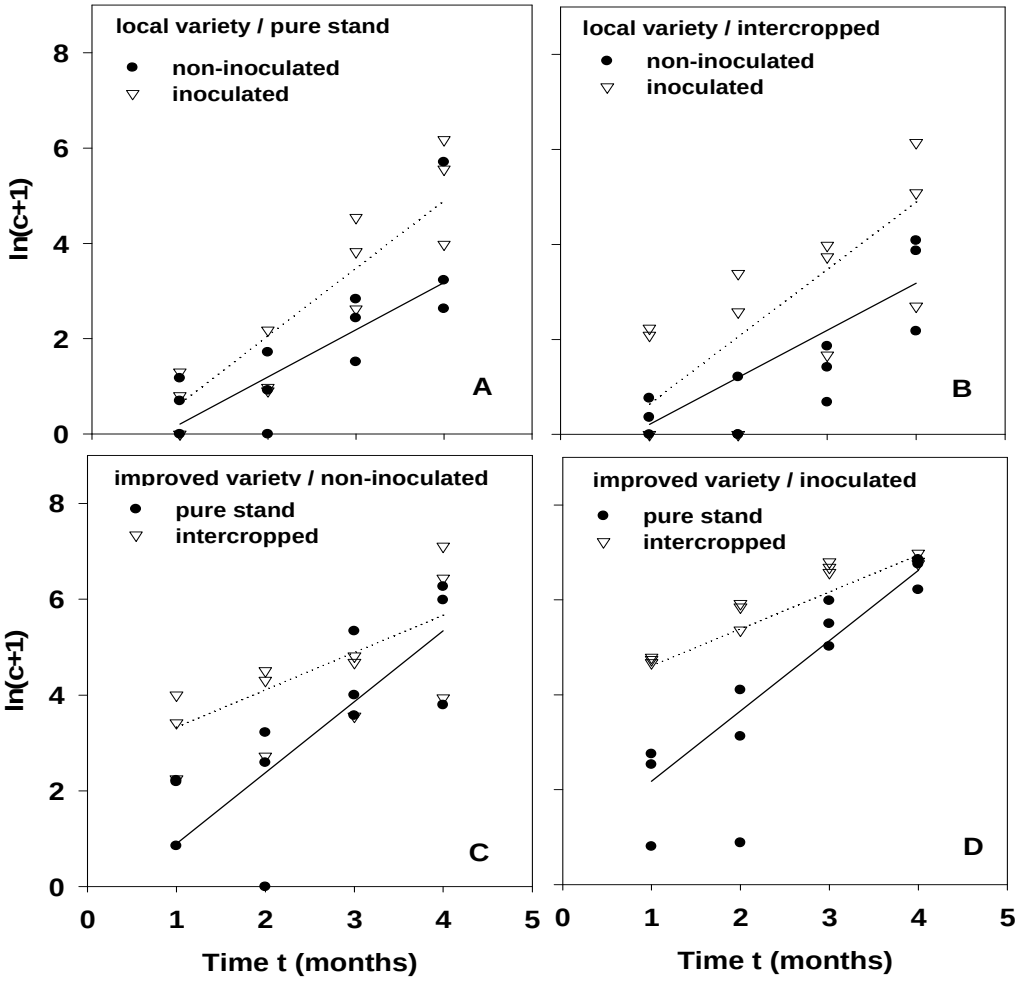

Fig. 3.4: Progress of total aflatoxin of the maize kernels during the four months of storage for the two varieties and four treatments (see Table 3.1 for treatment definitions). The original concentrations *c* (ppb) of the three samples in each treatment per observation date were log-transformed with ln(*c* + 1). The lines are the regression lines resulting from the simultaneous fitting of eq. 3.3 to the data of the four treatments for each cultivar.

The aflatoxin B_2 concentrations were clearly lower than the aflatoxin B_1 concentrations. In many samples, no aflatoxin B_2 was detected at the first and second sampling date. The aflatoxin B_2 concentrations in the different treatments (data not shown) also increased exponentially, but at 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 (Fig. 3.5). 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 (Fig. 3.5). On average, the concentration of aflatoxin B_2 in a sample was, therefore, only one fourth of the concentration of B_1.

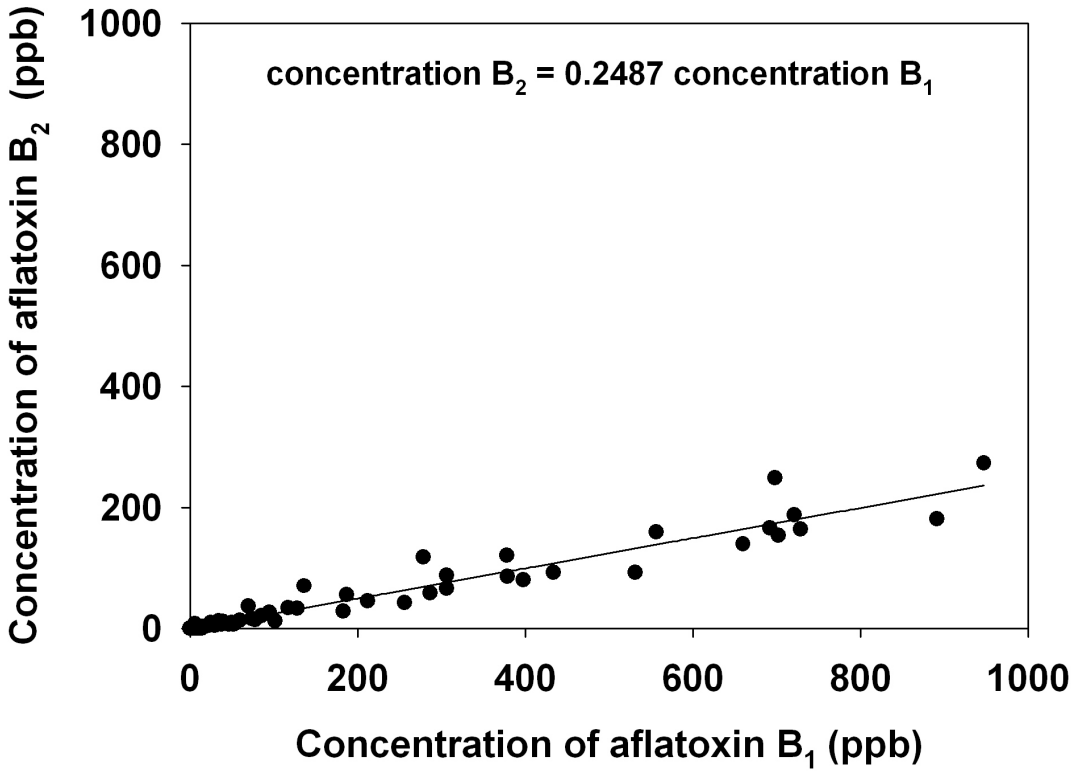

Fig. 3.5: Correlation between the aflatoxin B_1 and B_2 concentrations of the 96 maize samples that were stored for four months.

3.4.5 *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 (Fig. 3.6). Neither inoculation, nor intercropping had a significant impact on the 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 local variety was significantly more susceptible to *Penicillium* spp. than the improved variety during the first month in storage. 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.

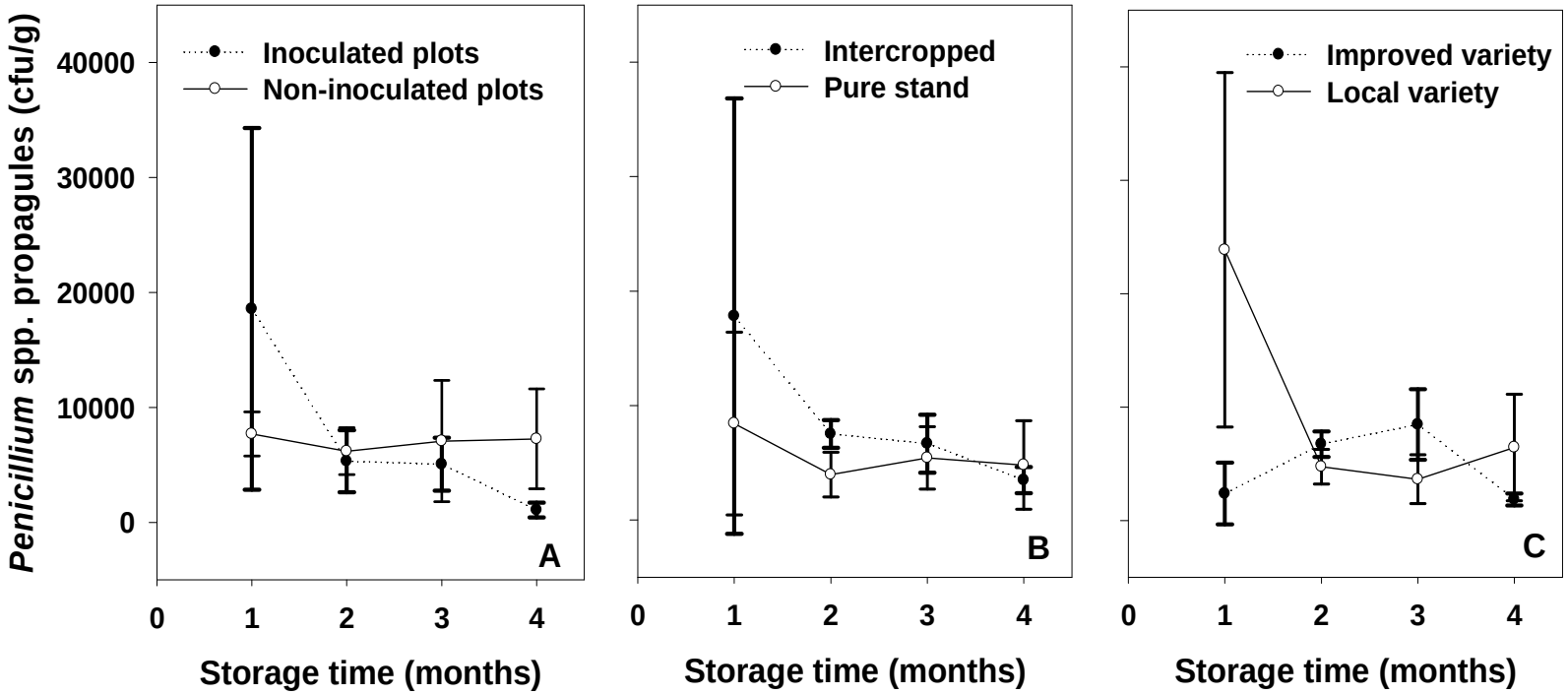

Fig. 3.6: Dynamics of *Penicillium* spp. propagules (cfu/g) of maize kernels during four months of storage depending on inoculation (A), cropping system (B) and variety (C).

3.4.6 *Fusarium* spp. severity of stored cobs

A. flavus inoculation did not significantly affect the severity of *Fusarium* spp. of the stored cobs. The only measurable impact on *Fusarium* spp. severity was variety, with the improved variety having a significantly higher disease level during the storage period (Fig. 3.7). In general, the severity of *Fusarium* spp. increased significantly during storage of maize cobs.

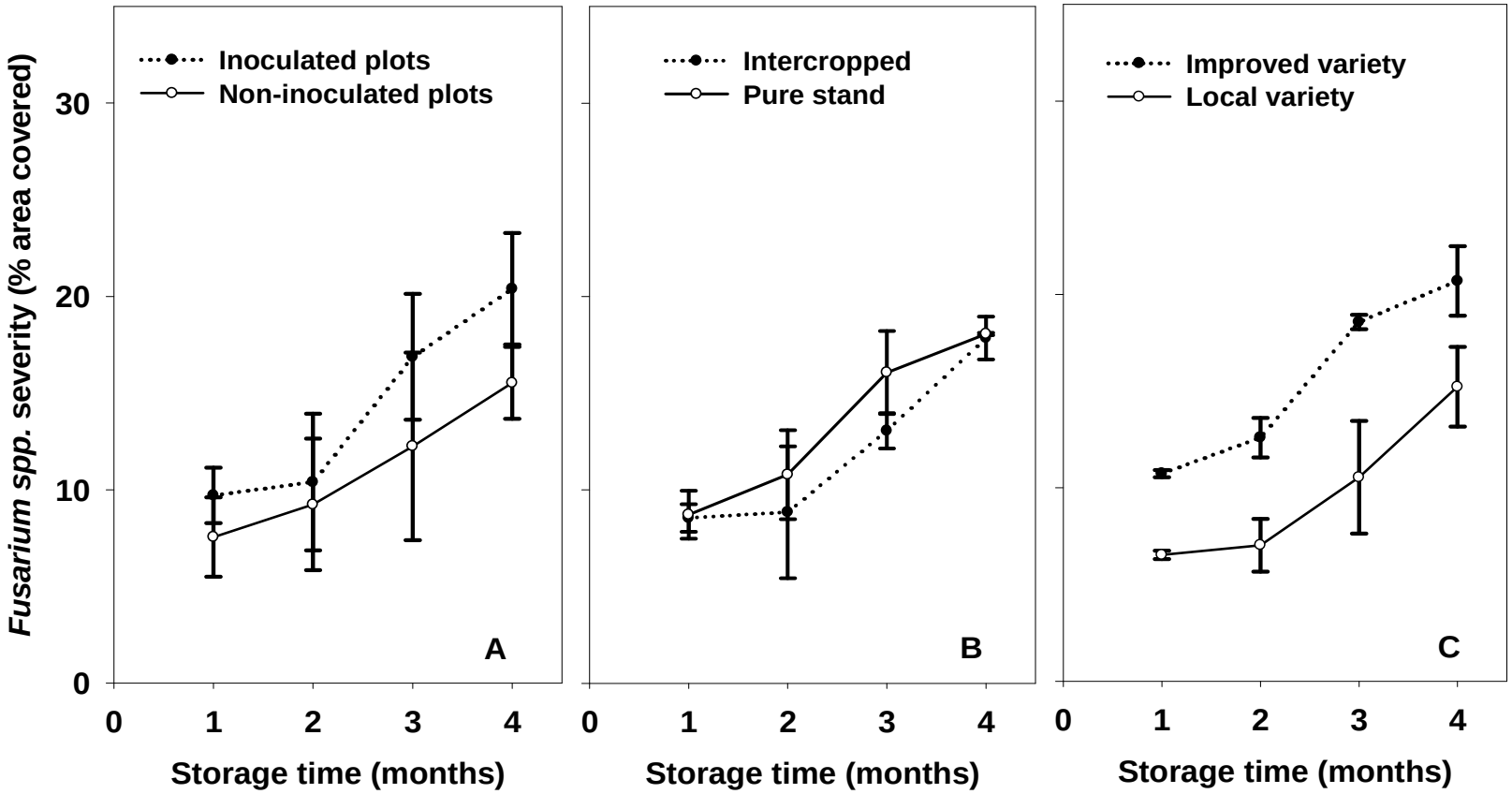

Fig. 3.7: Progress curves of *Fusarium* spp. severity of maize cobs (% area covered) during four months of storage depending on inoculation (A), cropping system (B) and variety (C).

3.4.7 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 *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) population 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 (Coleoptera: Cucujidae) increased significantly between the third and the fourth month of storage irrespective of treatment. However, there were no significant differences among the inoculation, cropping system and varietal treatments (Fig. 3.8).

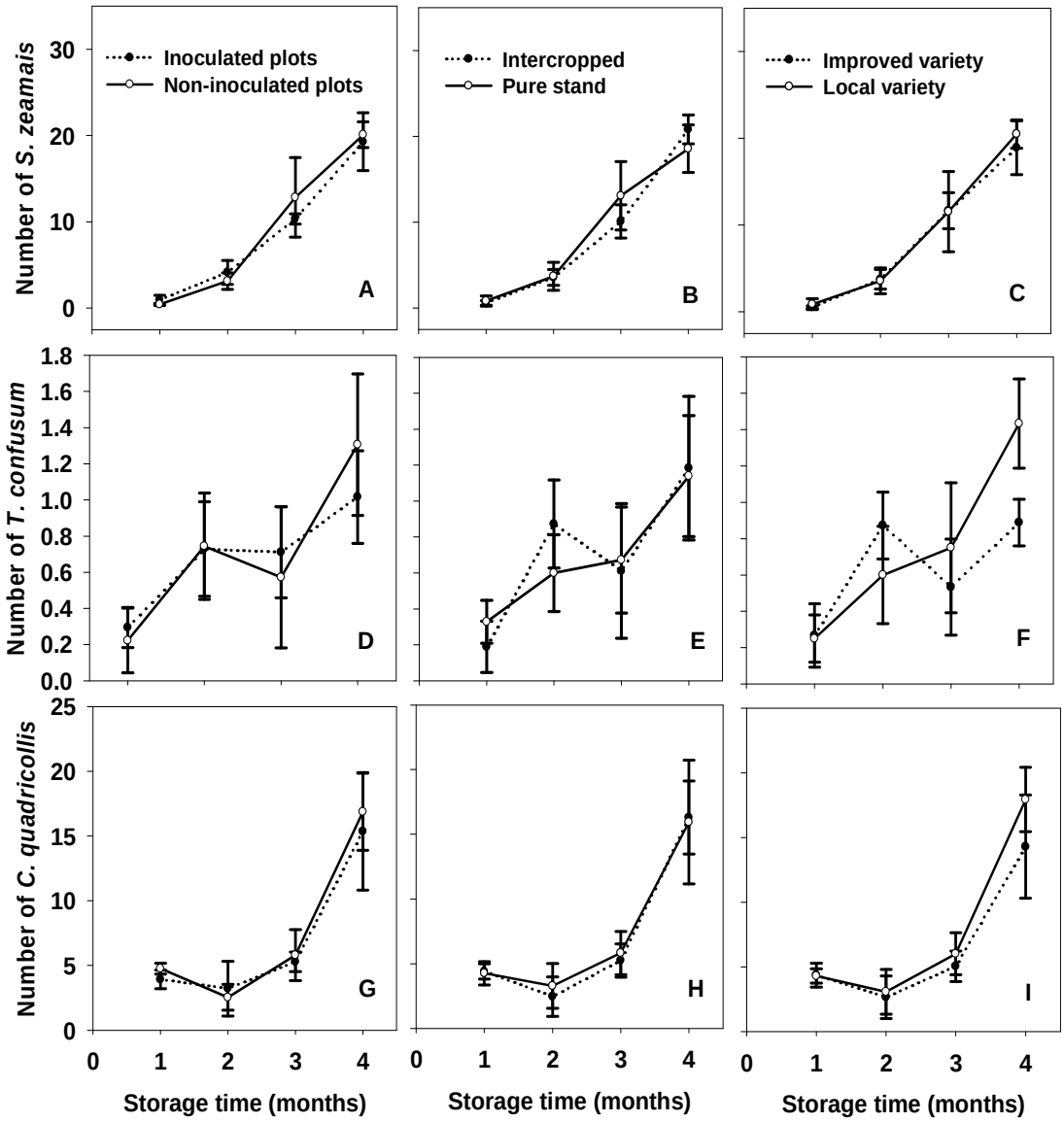

Fig. 3.8: Population dynamics of *Sitophilus zeamais* (A, B, C), *Tribolium confusum* (D, E, F) and *Cathartus quadricollis* (G, H, I) on maize cobs during four months of storage depending on inoculation (A, D, G), cropping system (B, E, H) and variety (C, F, I).

3.4.8 Correlations among characteristics of stored maize cobs

A significant and positive correlation was observed between *A. flavus* cfu and aflatoxin B_1 (C = 0.54, P < 0.0001), and between *A. flavus* cfu and aflatoxin B_2 (C = 0.53, P < 0.0001) of the stored maize cobs (Table 3.2). There was a negative correlation (C = -0.34, P = 0.0008) between *A. flavus* cfu and *Penicillium* spp. cfu (Table 3.2) and, between *Penicillium* spp. cfu and aflatoxin B_1 (C = -0.21, P = 0.05). On the stored maize cobs, the severity of *Fusarium* spp. was positively correlated with *A. flavus* cfu (C = 0.49, P < 0.0001), with aflatoxin B_1 (C = 0.53, P < 0.0001), and with aflatoxin B_2 (C = 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.

Table 3.2 - 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

3.5 Discussion

3.5.1 Effect of soil inoculation.

In this study, a relatively high level of cfu of *A. flavus* was observed in the soil of non-inoculated plots, probably because the experimental plots were intensively cultivated with maize during the previous years of the trial. In general in West Africa, the soils of farmers' fields commonly have a high level of *A. flavus* propagules, easily causing *A. flavus* infection and aflatoxin contamination. A survey conducted by Cardwell & Cotty (2002) in Benin showed that all of the 88 fields that were sampled were infested with *A. flavus* propagules and some of the soils in these fields exceeded 5000 cfu/g, while the average 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.

The results also indicated that rice paddy carrying toxigenic *A. flavus* was an effective method of inoculation. However, this methodology to inoculate fields with *A. flavus* has been rarely used in previous studies. Other studies successfully used wheat and artificial support (alginate pellets) for *A. flavus* inoculum production and inoculation (Daigle & Cotty, 1995; Bock & Cotty, 1999). The application of *A. flavus* established a toxigenic isolate 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 the levels.

3.5.2 *Aspergillus flavus* in maize

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 during the first month indicated that *A. flavus* infection of maize already started in the field prior to or during harvest, which was similar to the observations of Setamou et al. (1997) and Bankole & Mabekoje (2003). The high number of cfu observed at the beginning of the first month could have been influenced by the high water content observed in the maize kernels at the beginning of 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 and development. However, not all propagules present colonized the maize kernels effectively. 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 that were inoculated with *A. flavus* had a higher level of *A. flavus* infection than the non-inoculated and local maize variety plots.

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 insects' activities, probably as a result of favorable temperatures and the multiplication of *Cathartus quadricollis* and *Tribolium confusum* which were recorded during this period. Furthermore, biological activities of insects might have created a microenvironment that allowed *A. flavus* and other fungal growth (Picco et al., 1999). Insects can also spread *A. flavus* propagules throughout the maize bulk leading to new infections.

One of the hypotheses why cowpea intercropping leads to lower toxin levels is that the direct spread of *A. flavus* propagules from soil to maize cobs is prevented. For the local variety, neither inoculation, nor cropping system affected the values of aflatoxin concentrations at 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 prevent the infection of the maize plant. The results are different for the improved variety, in which intercropping and inoculation both led to higher toxin levels 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 reasons for this result. First, because of the cowpea covering the soil under maize plants, the strains that infected the maize in intercropping plots are most probably airborne and not directly transported from the plot soil and their behavior differed once in storage condition. This implies that there was transport or movement of *A. flavus* propagules from elsewhere to the maize plants of the intercropped plots. Many studies have shown the presence of *A. flavus* propagule in the air around maize fields (Ilag, 1975; Abdalla, 1988). The second hypothesis could be that the mechanism of infection of the cobs itself was different. There could be many ways for natural *A. flavus* infection in maize cobs. Infection can occur through the silk and in this case *A. flavus* propagule 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), or transported by cob borers and other insects (Drepper & Renfro, 1990; Beti et al., 1995). The timing of the penetration and development inside the kernel depend on the kind of penetration (Marsh & Payne, 1984a, 1984b). It appears that intercropping maize with cowpea could protect the harvested maize, except for few cases. According to these results this type of control could be positive for varieties very susceptible to aflatoxins.

3.5.3 Maize, *Aspergillus flavus* and aflatoxins

Understanding the process involved in crop contamination by *A. flavus* is very important since aflatoxins are only produced by certain *A. flavus* strains. The results from this study showed a correlation between *A. flavus* propagules and the level of aflatoxin. During the first month of storage, all treatments had almost zero aflatoxin content, but the level increased over time. This has been reported by most studies where the toxin content increased over time (Hell et al., 2000). *A. flavus* requires favorable environmental conditions and a susceptible maize variety for colonization and toxin production. The toxin production depends on infection initiation and colonization (Klich, 2007). However, not all *A. flavus* propagules that adhere to the grain surface lead to infection. The level of aflatoxin B_1 in maize was four times higher than the level of aflatoxin B_2 although both increased over time in stored maize.

3.5.4 Interactions between fungi, insects and 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. 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 three insect species observed during the experiments were *Sitophilus zeamais*, *Cathartus quadricollis* and *Tribolium confusum*. There was no treatment effect on the dynamics of the different insect species. All three insect populations increased during storage, although the progress curves were different. However, the level of aflatoxin was positively correlated with the population size of the three species. It is possible that activities of the insects played some role in the 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. Infestation by the three insect species in our study could have predisposed stored maize kernels to *A. flavus* infection and increased the level of aflatoxin production. It is possible that the level of aflatoxin production was directly or indirectly affected by the competition for nutrients between *A. flavus* and the other fungi (Calvo et al., 2002).

Overall, the improved variety had a higher level of aflatoxin B_1 and B_2 contamination compared to the local variety. In this study, the varietal effect and the *A. flavus* toxigenic strain inoculation were the main factors in the production of aflatoxin in stored maize. The effect of intercropping with cowpea was not significant. The rate of the contamination by *A. flavus* was very important for the resultant aflatoxin production. Treatments with low levels by *A. flavus* colonization showed low levels of aflatoxin contamination in maize. In conclusion, this study demonstrates the augmentative effect of primary inoculum on *A. flavus* contamination 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 the two key factors that are critical for a successful colonization by *A. flavus* in stored maize. To understand the exact effects of other storage fungi and storage insects requires further investigations. With respect to recommendations, farmers should avoid continuous cropping of susceptible crops, especially maize, peanut, cotton, in the same field. In addition, the use of maize varieties with a short maturation period is advisable.

Chapter 4. Effects of the co-inoculation of *Aspergillus flavus* with *Fusarium* spp. and *Penicillium* spp. on the growth of *Aspergillus flavus* and aflatoxins production in maize.

4.1 Abstract

Maize maturating in the field at milky stage (preharvest experiment) and already harvested maize kernels (postharvest experiment) were inoculated with *Aspergillus flavus* spores alone or in combination with *Fusarium* spp. and/or *Penicillium* spp. In both experiments, the grains were stored in an incubator and sampled weekly. In the preharvest experiment, the incidence of *A. flavus* increased linearly during seven weeks of storage with the same slope in all treatments, but with a slightly higher level in treatments in which *Fusarium* spp. was inoculated too. In all treatments, the incidence of *Fusarium* spp. decreased initially and became larger again after four weeks of storage. The level of *Fusarium* spp. incidence was higher when *Fusarium* was co-inoculated. *Penicillium* spp. incidence had generally a slightly increasing linear trend. In the presence of *Fusarium* spp., the incidence of *Penicillium* spp. was reduced. During storage, the aflatoxin concentration remained constant in the control, but increased in all other treatments with the same rate, when inoculated with *A. flavus* either alone or in combination with other fungi. In the postharvest experiment the incidence of *A. flavus* increased linearly in all treatments, including the control, starting from a low level. Compared to the control, the slope was higher after *A. flavus* inoculation and even higher when *Penicillium* was co-inoculated. The incidence of *Fusarium* spp. decreased linearly in all treatments, although the initial incidence was high. The incidence of *Penicillium* spp. varied over time without showing a uniform trend. The aflatoxin concentration in the postharvest experiment was lower than in the preharvest experiment and increased continuously and uniformly in all treatments.

4.2 Introduction

Maize is an important cereal in West Africa. The annual production is increasing since 2000 and has reached approximately 15 millions tons in 2008 (FAOSTAT, 2010). Maize is mainly used for human diet, with an increasing per capita consumption of 24, 25 and 26 kg per capita per year in 2003, 2004 and 2005 respectively (FAOSTAT, 2010). Maize is stored for periods from 3 up to 12 months depending on the climatic zone, mostly in local storage structures that are managed by farmers (Hell et al., 2000a; Udoh et al., 2000). During storage, a high percentage of maize in Africa is infested by insects and moulds (Lillehoj, 1987; Cardwell et al., 2000), resulting in quantitative and qualitative losses including losses of nutritional values (Filtenborg et al., 1996).

Mould infestation is an important problem for the still widely artisanal agriculture of the West African region, particularly in the presence of insect pests (Dowd 2003; Hell et al., 2000b). Most infections of maize grains by fungal pathogens occur early in the field (Lillehoj, 1987; Wicklow, 1994) and progress later during harvest, transportation and in the storage facilities (Hell et al., 2000a; Udoh et al., 2000). The most common storage moulds observed in West Africa are caused by *Aspergillus*, *Fusarium* and *Penicillium* spp. (Hell et al., 2003). Some of these species produce secondary metabolites that are highly toxic to humans and animals (Peraica et al., 1999; Yiannikouris and Jouany, 2002). Calvo et al. (2002) explained that the secondary metabolism is associated with fungi's developmental processes, whereas Rohlfs et al. (2007) suggested that toxins could be favourable factors for the evolution of the fungal population since fungivory predators preferably feed on atoxigenic isolates.

Aspergillus flavus Link is the most common *Aspergillus* spp. on maize (Klich, 2007; Calvert et al., 1978). It can infect maize from ripening in the field to storage, depending on agronomic and environmental conditions and management practices (Payne et al., 1989; Hell et al., 2000a). *A. flavus* contamination of maize, its development and subsequent aflatoxin production are strongly dependent on the prevailing environmental and biotic conditions (Wilson and Payne, 1994). In West Africa, Hell et al. (2003) found that up to 65% of the storage facilities were contaminated with aflatoxin.

Another very common mould in West Africa is caused by *Fusarium* spp. which can be isolated from maize plants both in the field and in stores (Cardwell et al., 2000; Adejumo et

al., 2007). *Fusarium* spp. are endophytes of cultivated maize and can also infect maize grains during their growth (Bacon et al., 2001; Yates and Sparks, 2007). Under cool to warm temperatures (15 to 30°C) and in high water activity (a_w = 0.98 Pa/Pa), toxigenic strains of these fungi can produce a toxic metabolite that impacts both humans and animals (Doohan et al., 2003). The two *Fusarium* species most commonly isolated from maize in Benin were *Fusarium verticillioides* (68%) and *Fusarium proliferatum* (31%) that are mainly responsible for fumonisin contamination of maize. Most of the maize samples collected were found to be positive for fumonisin with levels ranging from not detected to 12 mg/kg in 1999–2000, 6.7 mg/kg in 2000-2001 and 6.1 mg/kg in 2002–2003 and significantly higher levels in the two Southern Costal high humidity zones of Benin (Fandohan et al., 2005).

Penicillium spp. are by far the least studied pathogens of the three major storage moulds of maize. *Penicillium* is a very complex genus in terms of number of species and range of habitats (Logrieco et al., 2003). Some species are able to colonize crops and to produce toxic metabolites such as patulin, citrinin, penicillic acid, ochratoxin etc. (Logrieco et al., 2003; Bennett and Klich, 2003). These fungi can infect maize in the field and throughout storage.

Taking into account that very often fungal infections occur simultaneously on maize, especially in tropical regions, research has focused on studying the mutual interactions of different species of the fungal community regarding growth, development and possible consequences for mycotoxins, especially aflatoxin production (Wicklow et al., 1998; Marin et al., 1998a; 1998b; Widstrom et al., 1994). Wicklow et al. (1998) reported that there was an impact of *F. moniliforme* on the growth of *A. flavus* when developing together on a substrate. Marin et al. (1998a, 1998b) confirmed and extended these studies to *Penicillium*, *Eurotium* and *Trichoderma* and determined that besides biotic factors, environmental factors such as water activity and/or temperature are also determinants of the predominance of one or the other species. However, during their trials *Penicillium* spp. was relatively unaffected by competition.

Growth of *A. flavus* can significantly reduce the production of aflatoxin, as has been reported for *A. niger* (Wicklow et al., 1980) and *F. moniliforme* (Widstrom et al., 1994). As all these experiments were mainly focused on other moulds not *A. flavus*, it would be very instructive to understand how the latter fungus evolves in competition with the two most

commonly encountered moulds of a different genus in storage in West Africa. The resulting information could help to take into account not only the environmental parameters, but also the mycological flora, for risk assessment and for forecasting of potential aflatoxin contamination.

The objective of this work is therefore to determine the effect of co-inoculating *A. flavus* with *Fusarium* spp. or *Penicillium* spp. or with a mixture of the two fungal species on the development of *A. flavus* and aflatoxin production in maize produced and stored in West Africa (Benin). The inoculation was carried out on maize plants in the field and on already harvested maize grains in order to determine if infection in the field or infection during harvest and storage will have an important impact on further *A. flavus* development and aflatoxin production in maize.

4.3 Materials and Methods

4.3.1 Fungal isolates

All strains were isolated from maize grown in the Northern Guinea Savanna (NGS) in the Republic of Benin. *Fusarium* spp. and *Penicillium* spp. were grown on PDA (Potato Dextrose Agar) medium, the *A. flavus* strain was grown on 5/2 medium (5% V8 juice and 2% agar) (Cotty, 1989). The latter isolate was proven toxigenic after five days of fermentation in A&M liquid medium (Adye and Mateles, 1964) followed by aflatoxin extraction and quantification. For aflatoxin determination, to 70 ml of A&M medium, 100 micro liters of spore suspension containing about 4.72×10^{6} spores were added and shaken at 150 rpm at 31 °C for five days. To the fermented suspension, 70 ml of acetone were added. One hour later, approximately 140 ml of the fermented suspension plus acetone were filtered through filter paper in a beaker. An equal volume of distilled water was added to 100 ml of the filtered solution and extracted two times with 25 ml of methylene chloride. The methylene chloride plus acetone solution was filtered through anhydrous sulfate sodium and dried at room temperature. The extract was later dissolved with 1 ml of methylene chloride and spotted on a TLC plate for aflatoxin determination as described below.

4.3.2 Spore suspension preparation

Fusarium spp. and *Penicillium* spp. were grown on PDA medium for 7 days at 26 °C, the *A. flavus* strain on 5/2 medium for 7 days at 31°C. The suspensions were made using distilled and sterilized water containing 100 µl per litre of Tween 80. The spore concentrations were determined by counting the spores using a hemacytometer with a microscope. The spore concentrations of *A. flavus* and *Penicillium* spp. were $4.72 \cdot 10^7$ spores/ml and of *Fusarium* spp. $1.7 \cdot 10^7$ spores /ml.

4.3.3 Field experiment.

The field consisted of three completely randomized blocks (plots) with five treatments. Each plot had a dimension of 3.2 m × 3.2 m contained 32 maize plants planted at a distance of 0.40 m within a row and 0.80 m between rows. Each plot received a dose of 160 kg of NPK fertilizer (15-15-15) per ha, two weeks after planting and 50 kg of urea per ha at milky stage. The treatments in the field were: treatment A = all maize plants were inoculated only with *A. flavus* spore suspension; treatment AP = inoculation with *A. flavus* and *Penicillium* spp. spore suspensions; treatment APF = inoculation with *A. flavus* and *Penicillium* spp. spore suspensions plus *Fusarium* spp. inoculated in the stems; treatment AF = inoculation with *A. flavus* and *Fusarium* spp.; treatment control = no inoculation (distilled sterile water). The *Fusarium* spp. suspension was prepared 15 days prior to inoculation and was used to inoculate sterile toothpicks incubated at 26°C. At flowering stage, toothpicks with *Fusarium* spp. spores were introduced into the maize stems between two internodes just under the node supporting the main cob. The *A. flavus* and *Penicillium* spp. inocula were prepared as spore suspensions the day before field inoculation. The maize plants were inoculated with *A. flavus* and *Penicillium* spp. suspensions at the milky stage of the grains. Five ml of each suspension were injected with a syringe inside an incision made at the inferior part of the cob with a sterilized knife. After this cobs were left to maturate without further intervention till harvest. After harvest, maize cobs were collected in paper bags and transported to the laboratory where they were shelled and about 2 kg of kernels were stored in jars at 31° in an incubator. About 150 g

of kernels were sampled weekly until 7 to 8 weeks after harvest to determine water content, to assess moulds and to analyze aflatoxin as described below.

4.3.4 Laboratory Experiment

There were five treatments with three replications: treatment A = 30 ml of *A. flavus* spore suspension + 60 ml of sterile distilled water; treatment AP = 30 ml of *A. flavus* spore suspension + 30 ml of *Penicillium* spp. spore suspension + 30 ml of sterile distilled water; treatment APF= 30 ml of *A. flavus* spore suspension + 30 ml of *Penicillium* spp. spore suspension + 30 ml of *Fusarium* spp. spore suspension; treatment AF = 30 ml of *A. flavus* spore suspension + 30 ml of *Fusarium* spp. spore suspension + 30 ml of sterile distilled water; treatment control = 90 ml of sterile distilled water. For inoculation, the prepared spore suspensions were poured on 2 kg of maize kernels previously harvested and left overnight under the laminar flow hood. The following day, the so treated kernels were transferred into jars separated for each treatment and put in an incubator at 31°C. Weekly samples of about 150 g were taken and moisture content, mould species, and aflatoxin content were determined with the methods described below.

4.3.5 Mould assessment on maize kernels

Grains were washed with NaOCl (3.5%) for one minute and rinsed twice with sterile distilled water. Five kernels were placed on wetted filter paper (Whatman No. 15) in Petri dishes, with twenty dishes (100 kernels) per sample and incubated at 26°C (Percival Model I-35LL, Boone, Iowa, U.S.) for 7 days with 12 hours of light and 12 hours of dark. Moulds growing from the kernel were identified (Klich et al., 1998; Raper et al., 1949 and Burgess et al., 1994) and their incidences recorded.

4.3.6 Aflatoxins extraction

The protocol of Thomas et al. (1975) was used for aflatoxin extraction. For each sample, 50 g of ground maize grains were weighed in a 500 ml Erlenmeyer flask, 250 ml of

methanol and water (60:40/vol/vol) were added and shaken at 150 rpm for 30 min (Lab-Line Multi-Wrist Shaker®, Melrose, U.S.A.). The suspension was filtered and separated with a mixture of saturated sodium chloride (30 ml) and hexane (50 ml). The methanol water layer was collected in a separatory funnel with 50 ml of chloroform and shaken. The chloroform binds with toxins and the mixture was released into a flask 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 about 1 ml of chloroform, transferred into a small container and stored in the refrigerator at 4°C.

4.3.7 Aflatoxins quantification

Aflatoxin quantification was done by thin layer chromatography. Each sample was diluted with 1 ml of chloroform and spotted (10 µl Syringe, Hamilton, Bonaduz, Switzerland) at 2 cm from the base on pre-coated silica gel TLC plates (Sigma Chemical, St. Louis, U.S.A) 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 with win-CATS 1.4.2 software (Camag AG, Muttenz, Switzerland). Concentrated ethyl alcohol (95%) and hydrochloride were mixed 90:10 (v/v) and sprayed on the dried TLC-plates. The plates were observed under 365 nm light and those spots that gave off a yellowish-green fluorescence confirmed aflatoxin presence.

4.3.8 Moisture content determination

The moisture content was determined by weighing a specified amount of ground maize (Tekmar IKA-A10, Analytical Mill) before and after drying for two hours at 130°C and calculating the weight difference (I.S.O., 1979).

4.3.9 Data analysis.

Data were analyzed using SAS (SAS Institute, Inc., Cary NC, USA) software. Multiple regression analyses ($\alpha = 0.05$) were conducted for a linear mixed model using PROC MIXED of SAS (Littell et al., 1996). For both experiments, one analysis was carried out for each of the response variables: incidence of *A. flavus*, *Fusarium* spp. and *Penicillium* spp., moisture content and aflatoxin concentration. Prevalence and moisture content were arcsine square root-transformed before analysis, and aflatoxin concentration c (ppb) by the function $c' = \ln(c+1)$. The homogeneity of residuals was checked visually. The models included two fixed effects (treatment and time, encoded as week number) and their interaction, and replicate was a random effect. An autoregressive model was specified to account for autocorrelation through time, option *arh(1)* of SAS. The degrees of freedom were calculated using the Satterthwaite method.

For the analysis of the dynamics of the three fungal incidences and of aflatoxin concentration during seven weeks of storage in the maize, equation (4.1) or (4.2) was fitted to the joint data of the five treatments in each experiment:

$$f_1(t) = (a + d_A \cdot \Delta a_A + d_P \cdot \Delta a_P + d_F \cdot \Delta a_F) + (b + d_A \cdot \Delta b_A + d_P \cdot \Delta b_P + d_F \cdot \Delta b_F) \cdot t \quad (4.1)$$

$$f_2(t) = (a + d_A \cdot \Delta a_A + d_P \cdot \Delta a_P + d_F \cdot \Delta a_F) + (b + d_A \cdot \Delta b_A + d_P \cdot \Delta b_P + d_F \cdot \Delta b_F) \cdot t + (c + d_A \cdot \Delta c_A + d_p \cdot \Delta c_P + d_F \cdot \Delta c_F) \cdot t^2 \quad (4.2)$$

The linear function $f_1(t)$ and the quadratic function $f_2(t)$ represent the possible dynamics of the three fungal incidences (arcsine square root-transformed) or the progress of total aflatoxin (B_1 + B_2) concentration (log-transformed) over time t (measured in weeks). In the regression analyses, the data of the three replications were used for the fungal incidences, while for the aflatoxin concentration the mean value of three replicates was used because of the high variability among recorded aflatoxin levels. The dummy variable d_i, with i representing the inoculation of the different fungi (A: *A. flavus*, P: *Penicillium* spp., F: *Fusarium* spp.) were set to 1 if the corresponding inoculation was carried out and to 0 without corresponding inoculation. The parameter a is the intercept of the control, b its slope and c its vertical stretch factor for equation (4.2). The parameters Δa_i, Δb_i and Δc_i reflect the effects of fungal inoculation i on the intercept, the slope and the stretch factor (in eq. (4.2)). In the analyses, first the full model (equation (4.1) or (4.2)) was fitted simultaneously to the data of the five

treatments, and then parameters with values not significantly different from 0 were stepwise eliminated till all parameter values were significantly different from zero. The regression analyses were carried out with SigmaPlot 10.

4.4 Results

4.4.1 Moisture content

Changes in moisture content of grains were small during the experiments, yet statistically significant ($P < 0.0001$ for both), decreasing from 17 % to 16 % in the preharvest and from 15 % to 14% in the postharvest experiment (Fig. 1).

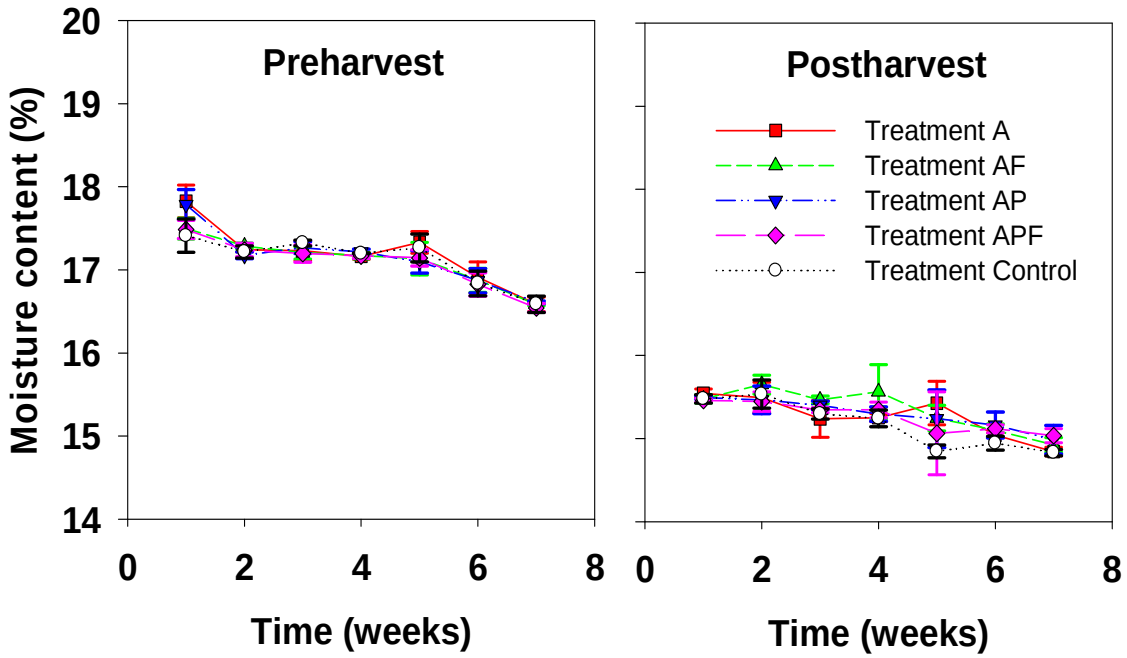

Fig. 4.1: Moisture content (%) of maize grains during 7 weeks of storage in the five treatments of the preharvest and postharvest experiment. Treatments: A – inoculation of *A. flavus*, AF – inoculation of *A. flavus* and *Fusarium* spp., AP - inoculation of *A. flavus* and *Penicillium* spp.; APF – inoculation of *A. flavus*, *Fusarium* and *Penicillium* spp., control – no inoculation.

4.4.2 *A. flavus* incidence

Overall, field inoculation of maize did not cause any difference in *A. flavus* contamination among treatments (P = 0.27) but there was a significant difference in fungal levels between weeks (P = 0.0017). The dynamics of *A. flavus* incidence after different inoculations led to equation 4.3:

$$f_1(t) = 33.96 + 3.36 \cdot d_F + 0.94 \cdot t \qquad \text{with } R^2 = 0.12 \qquad (4.3)$$

In all five treatments, including the control, the *A. flavus* incidence increased slowly, but with the same slope (0.94 %/week). Only the inoculation with *Fusarium* spp. (in treatments AF and APF) increased the intercept from 33.96 to 37.32 %, while the other inoculations had no effects on the initial level of *A. flavus* incidence (Fig. 4.2).

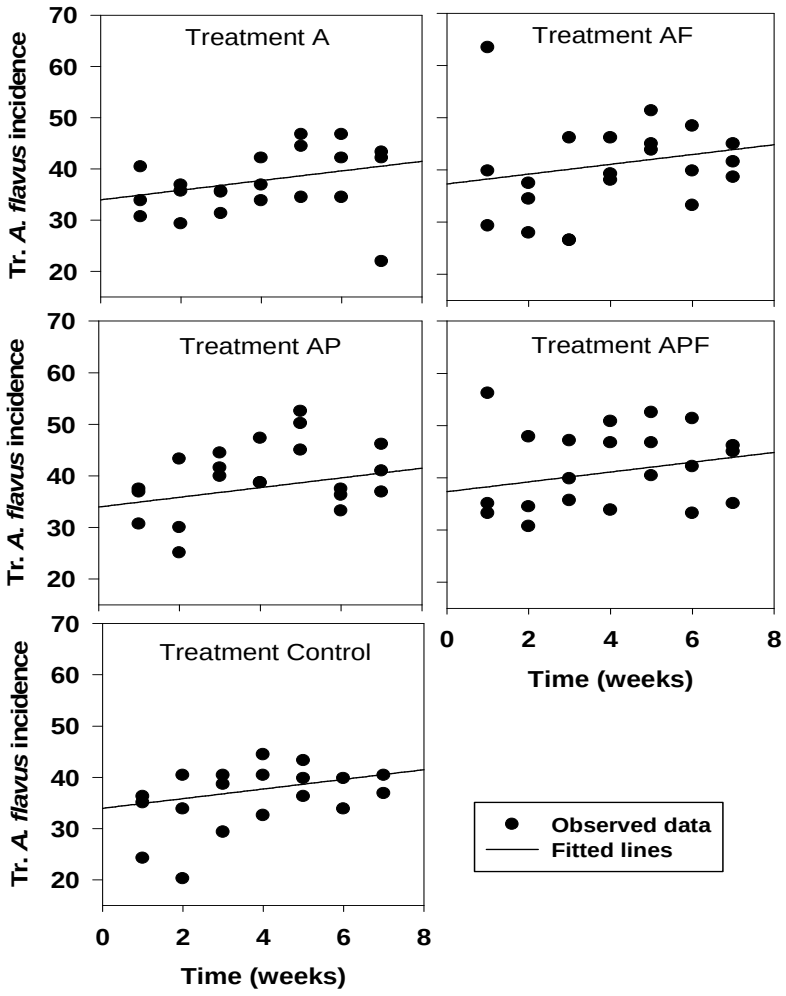

Fig. 4.2: Observed data and fitted regression lines of the progress of *Aspergillus flavus* incidence (%) on maize kernels during seven weeks of storage in the five treatments of the **preharvest** experiment. Treatments: see Fig. 4.1.

After postharvest inoculation, there were different responses between treatments ($P < 0.0001$), between weeks ($P < 0.0001$) and for the time×treatment interaction ($P = 0.001$). The resulting dynamic equation was:

$$f_1(t) = 12.28 + (1.29 + 5.58 \cdot d_A + 1.72 \cdot d_P) \cdot t \qquad \text{with } R^2 = 0.85 \qquad (4.4)$$

In this experiment, the different inoculations did not affect the initial level of *A. flavus* incidence.

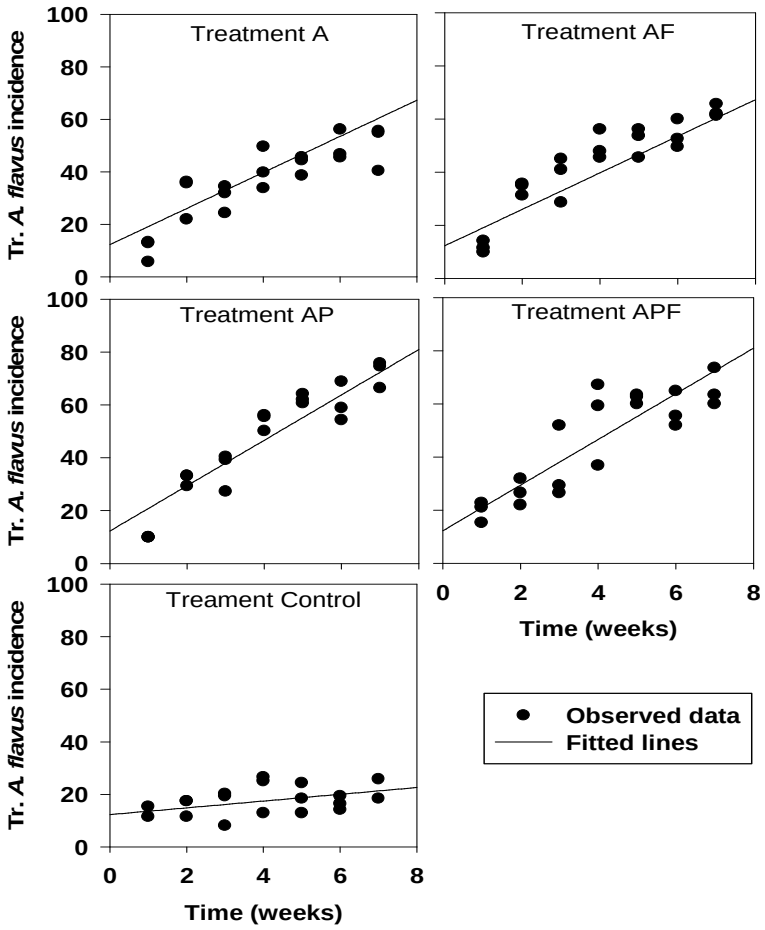

Fig. 4.3: Observed data and fitted regression lines of the progress of *Aspergillus flavus* incidence (%) on maize kernels during seven weeks of storage in the five treatments of the **postharvest** experiment. Treatments: see Fig. 4.1.

In all treatments, including the control, the fungal incidence increased linearly with time. When compared with the control, the slope was higher after the inoculation with *A. flavus* (+5.58). If *Penicillium* was inoculated in addition to *A. flavus* (treatments AP and APF), the slope increased further by +1.72, while the additional inoculation of *Fusarium* spp. had no effect on the slope (Fig. 4.3).

4.4.3 *Fusarium* spp. incidence

In the preharvest experiment, *Fusarium* spp. contamination differed between treatments (P = 0.0012). In the two treatments in which *Fusarium* was inoculated, the incidence was higher compared to the control and the *A. flavus* + *Penicillium* treatment, while the treatment with only *A. flavus* inoculation had an intermediate *Fusarium* spp. level.

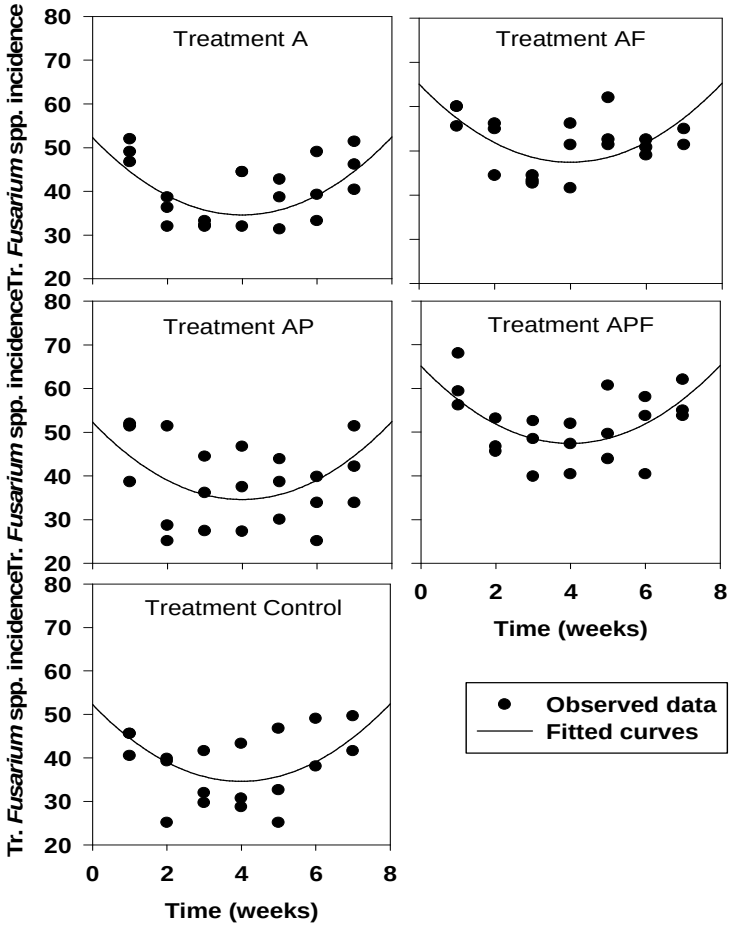

Fig. 4.4: Observed data and fitted regression lines of the progress of *Fusarium* spp. incidence (%) on maize kernels during seven weeks of storage in the five treatments of the **preharvest** experiment. Treatments: see Fig. 4.1.

The time trends were significant ($P < 0.0001$), showing an initial decrease followed by a later increase. Because of the parabolic time trend of *Fusarium* spp. incidence (Fig. 4.4), equation 4.2 was fitted to the data resulting in equation (4.5):

$$f_2(t) = 52.28 + 12.87 \cdot d_F - 8.86 \cdot t + 1.11 \cdot t^2 \qquad \text{with } R^2 = 0.57 \qquad (4.5)$$

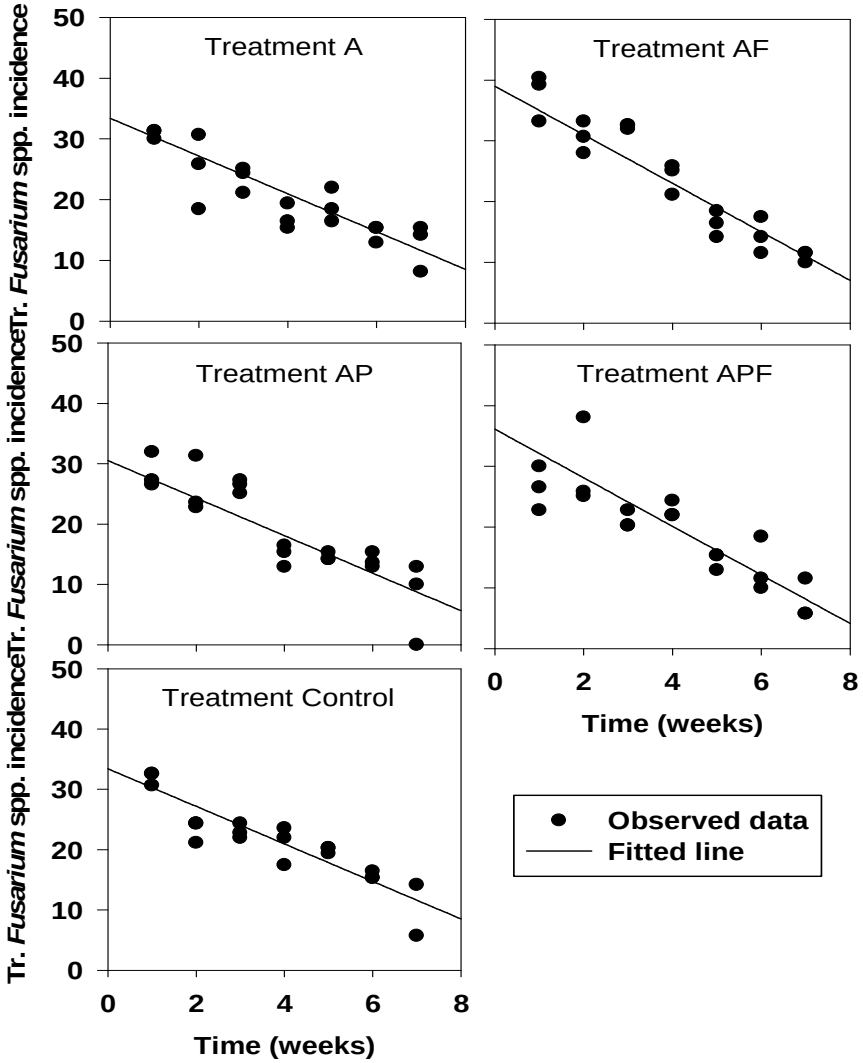

Fig. 4.5: Observed data and fitted regression lines of the progress of *Fusarium* spp. incidence (%) on maize kernels during seven weeks of storage in the five treatments of the **postharvest** experiment. Treatments: see Fig. 4.1.

The time course of *Fusarium* spp. incidence during storage was identical in all treatments (Fig. 4.4). Only the inoculation of *Fusarium* spp. caused a general shift of the *Fusarium* spp. incidence curve by 12.87 %. In the postharvest experiment, the overall levels of *Fusarium* spp. were lower than in the field experiment. Even so, a low variance enabled the differentiation of small differences between treatments. The general time trend ($P < 0.0001$) was a decrease during incubation. Both treatment ($P = 0.02$) and their time×treatment interaction ($P = 0.0002$) were significant. In contrast to the preharvest experiment, the time trend of the incidence was again a linear decrease (Fig. 4.5) resulting in the following equation 4.6:

$$f_1(t) = 33.40 - 2.88 \cdot d_P + 5.60 \cdot d_F + (-3.11 - 0.89 \cdot d_F) \cdot t \qquad \text{with } R^2 = 0.81 \qquad (4.6)$$

A. flavus inoculations had no effect on the dynamics *Fusarium* spp. incidence compared to the control. As expected, *Fusarium* spp. inoculation resulted in a higher intercept, but surprisingly in a steeper slope, while *Penicillium* spp. inoculation led to the lowest intercept, but no change in the slope.

4.4.4 *Penicillium* spp. incidence

Field inoculation led only to slight differences between treatments ($P = 0.005$) in *Penicillium* contamination. The AP treatment (one of the two treatments including *Penicillium*) showed higher levels than the AF treatment ($P = 0.003$) while other treatments were intermediate. The time trend was significant ($P < 0.0001$). The dynamics of *Penicillium* spp. was described by equation (4.7):

$$f_1(t) = 16.11 + 5.45 \cdot d_P - 6.67 \cdot d_F + 0.59 \cdot t \qquad \text{with } R^2 = 0.32 \qquad (4.7)$$

As expected, the inoculation of *Penicillium* spp. increased the initial incidence of *Penicillium* spp. by 5.45 while the inoculation of *Fusarium* spp. decreased it by 6.67. There was no effect of the different inoculations on the slope which resulted in a slow increase of the incidence over time ($P = 0.049$) (Fig. 4.6).

Postharvest inoculation led to complex dynamics that was similar for all treatments ($P < 0.0001$ for time). The fitting of function (4.1) resulted in a linear decrease of the incidence that was not affected by the inoculations:

$$f_1(t) = 31.86 - 0.95 \cdot t \qquad \text{with } R^2 = 0.03 \qquad (4.8)$$

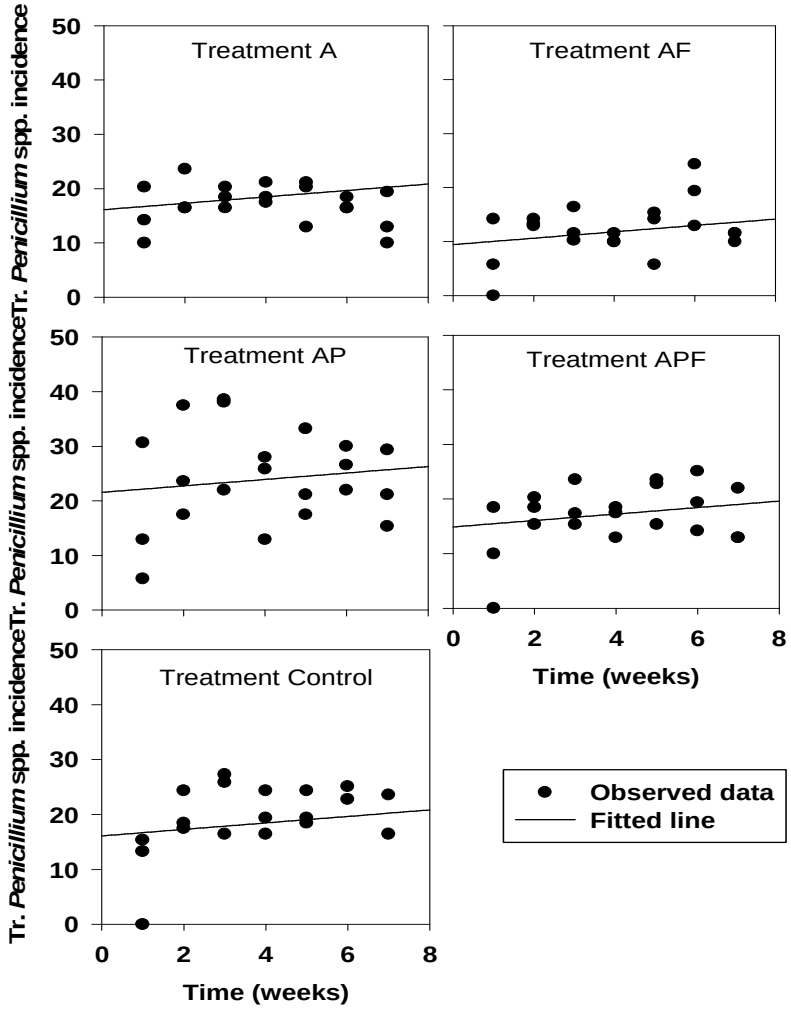

Fig. 4.6: Observed data and fitted regression lines of the progress of *Penicillium* spp. incidence (%) on maize kernels during seven weeks of storage in the five treatments of the **preharvest** experiment. Treatments: see Fig. 4.1.

However, as the slope was not significantly different from 0 (P = 0.0835), it can be concluded that there was no temporal linear trend of the *Penicillium* spp. incidence during storage. Nevertheless it seemed that the incidence decreased initially, followed by an increase and a second reduction in the 7th week (Fig. 4.7). However, if the data of week 7 are left out, because they are much lower than the data of week 6, the *Penicillium* incidences could be analysed with function (4.2):

$$f_2(t) = (64.40 - 25.21 \cdot d_F) + (-24.31 + 14.31 \cdot d_F) \cdot t + (3.32 - 1.71 \cdot d_F) \cdot t^2$$

with R^2=0.41 (4.9)

According to equation 4.9, only the inoculation of *Fusarium* spp. had a significant influence on the dynamics of *Penicillium* spp., while the inoculation of *A. flavus* or *Penicillium* spp. did not influence the dynamics. The inoculation of *Fusarium* spp. reduced the intercept and stretch factor so that the incidence was lower at the beginning and at the end of the storage period compared to the control.

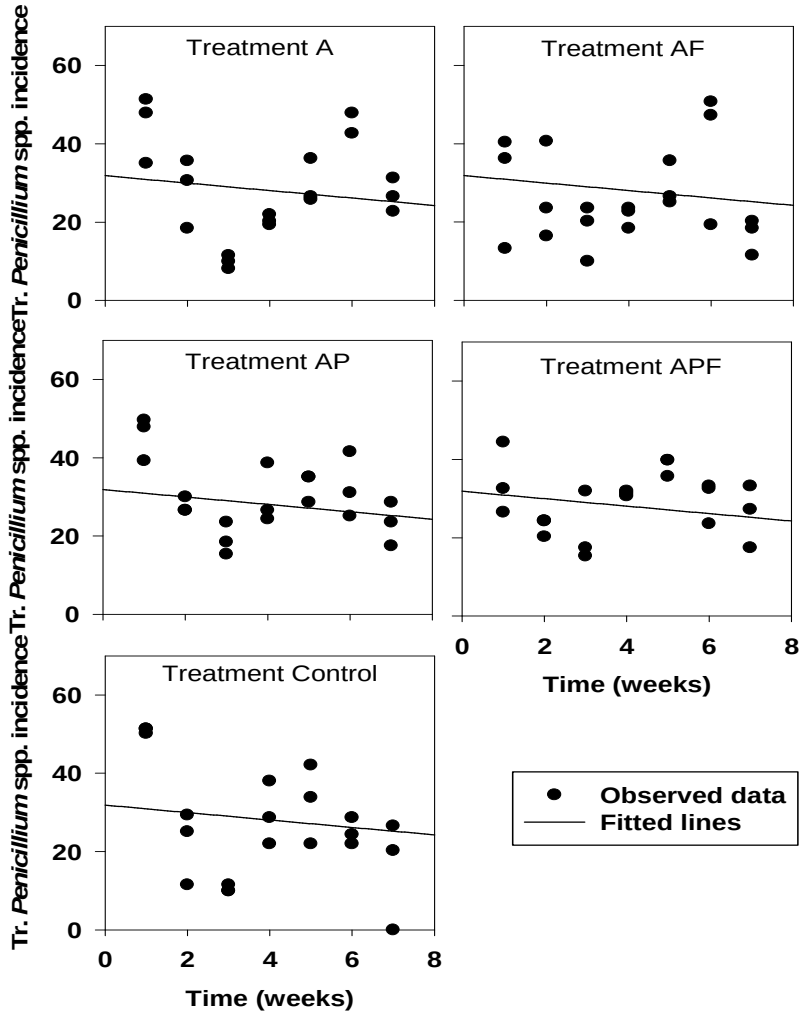

Fig. 4.7: Observed data and fitted regression lines of the progress of *Penicillium* spp. incidence (%) on maize kernels during seven weeks of storage in the five treatments of the **postharvest** experiment. Treatments: see Fig. 4.1.

4.4.5 Aflatoxin concentration.

In all samples, the concentration of aflatoxin B_1 was much higher than that of B_2 that was present only in traces. In the statistical analyses, the sum of both concentrations was used. In the preharvest experiment, the high variation between samples did not allow to detect differences between treatments, although the *A. flavus*-alone treatment (A) seemed to have the

highest aflatoxin production. The dynamic analysis using dummy variables on the average concentration of aflatoxins resulted in equation 4.10:

$$f_1(t) = 1.397 + 0.093 \cdot d_A \cdot t \qquad \text{with } R^2 = 0.19 \qquad (4.10)$$

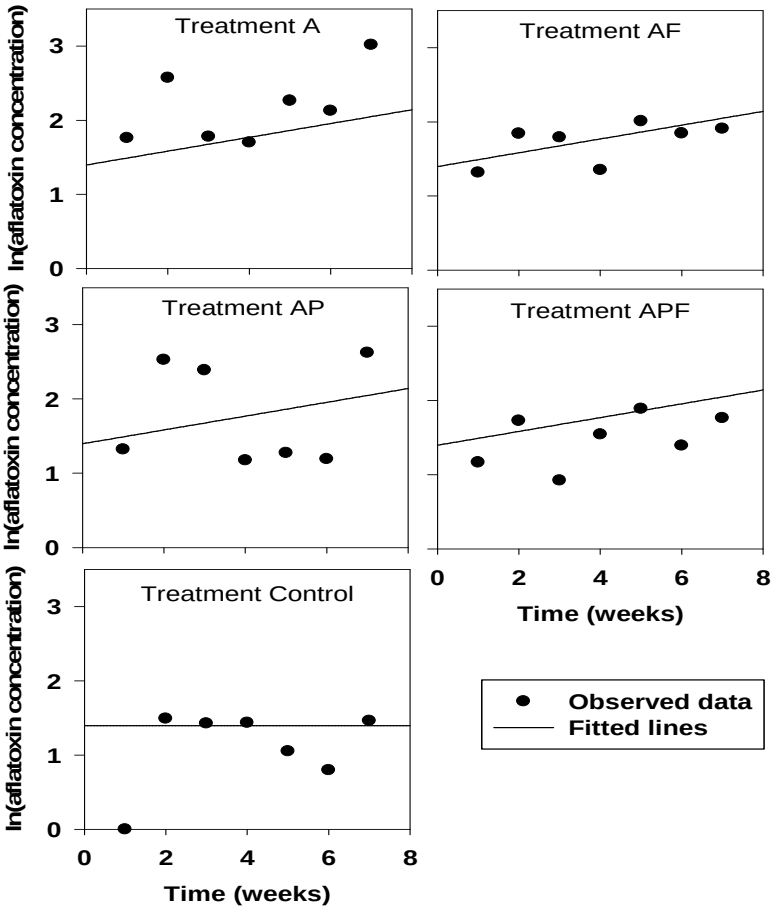

Fig. 4.8: Observed data (mean of three replicates) and fitted regression lines of the progress of the log-transformed aflatoxin concentration (ppb) of maize kernels during seven weeks of storage in the five treatments of the **preharvest** experiment. Treatments: see Fig. 4.1.

Accordingly, the aflatoxin concentration remained constant in the control, but increased in the other treatments which were inoculated with *A. flavus*. Additional inoculations with *Penicillium* or *Fusarium* spp. had no effect (Fig. 4.8).

In the postharvest experiment, the aflatoxin concentrations in all treatments were very low and showed a slight increase over time, but with high variability. Overall, there was no effect of the treatments on the progress of the aflatoxin concentration:

$$f_1(t) = 0.57 + 0.06 \cdot t \qquad \text{with } R^2 = 0.13 \qquad (4.11)$$

The initial concentration was significantly different from zero ($P < 0.0001$) and also the slope ($P = 0.03$) (Fig 4.9).

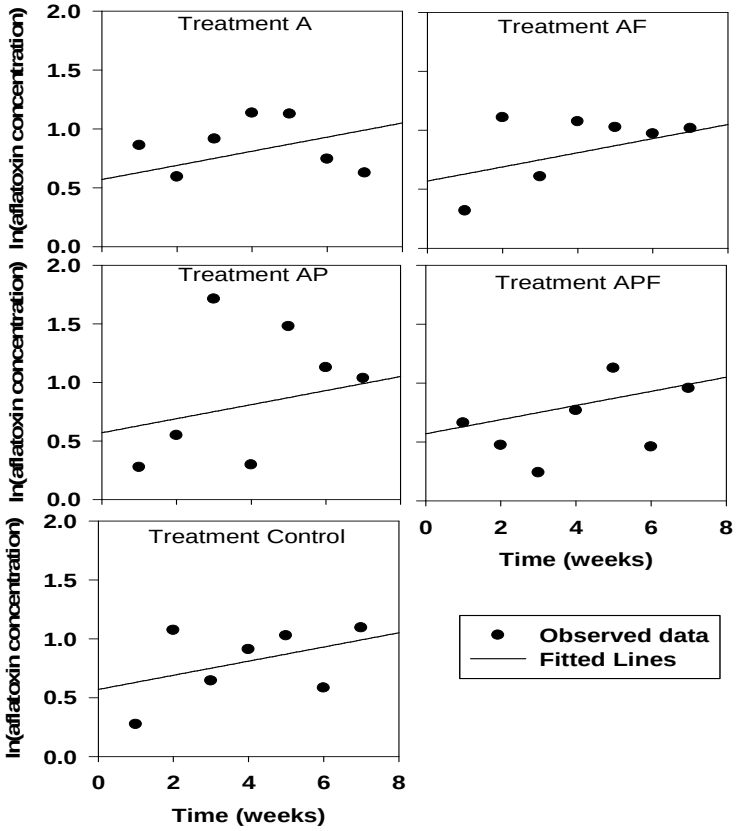

Fig. 4.9: Observed data (mean of three replicates) and fitted regression lines of the progress of the log-transformed aflatoxin concentration (ppb) of maize kernels during seven weeks of storage in the five treatments of the **postharvest** experiment. Treatments: see Fig. 4.1.

4.5. Discussion

4.5.1 General dynamics of the incidences

The dynamics of the incidences during the seven weeks of storage differed between the three fungal species and varied also with respect to the time of inoculation (preharvest or postharvest). For *A. flavus*, the incidences increased linearly in both experiments, but stronger after postharvest inoculation. The temporal trend of the *Fusarium* incidences was parabolic in the preharvest experiment with lowest values around week three, but linearly decreasing in the postharvest one. For *Penicillium* spp. incidence, the inoculation before harvest led to a slight increase during storage while the postharvest inoculation showed no significant linear change over time. The inoculation time caused clear differences with respect to the initial level of the fungal incidences: For *A. flavus* and *Fusarium* spp., the initial incidences were lower in the postharvest experiment compared to the preharvest one, while for *Penicillium* spp., is was the other way round.

4.5.2 Incidence of *A. flavus*

In all treatments of both experiments, *A. flavus* incidence increased linearly over time, in the preharvest experiment at the same rate for all treatments and in the postharvest experiment at various rates. In the preharvest experiment, *Fusarium* spp. co-inoculation increased the initial incidence of *A. flavus* during storage. Thus there was a positive effect of the presence of this species on the contamination of *A. flavus* in the growing maize kernel while the effect of *Penicillium* spp. was neutral. A study involving co-inoculation of *A. flavus* and *Fusarium* on maize in the field (Zorzete et al., 2008) showed also that *A. flavus* and *Fusarium* spp. have an increasing frequency with time with a slight advantage to *Fusarium* because of its endophytic properties (Saunder and Kohn, 2008). In the same study, Zorzete et al. (2008) found that there was no competition between *A. flavus* and *Penicillium* spp., but they did not artificially inoculate *Penicillium* spp. in their experiments. The mechanisms involved in the relationship between *A. flavus* and *Fusarium* spp. are not clearly understood.

One explanation could be that *Fusarium spp.* are able to detoxify some secondary substances produced by maize plants for their defence against fungi and by this way they allow an easier establishment of *A. flavus* (Saunder and Kohn, 2008). Another explanation could be that *A. flavus*, being rather saprophytic than parasitic, takes advantage of some "pre-processing" of the nutrients by *Fusarium* spp. more aggressive on living plant. It is well known that *A. flavus* needs weak host organisms to establish itself. This could explain the initial high levels of *A. flavus* incidence in the preharvest experiment. During storage, the environmental conditions were more favourable for *A. flavus* than for *Penicillium* spp. and *Fusarium* spp., regarding their needs in terms of moisture content and temperature, allowing a continuous and similar increase of its incidence for all treatments**.** In this case it seems that whatever the field conditions were, *A. flavus* development is not influenced by the presence of *Fusarium* spp. or *Penicillium* spp. once in the storage structure if its optimal conditions for development are met.

In the postharvest experiment, the initial rate of contamination by *A. flavus* was low in all treatments. This result was expected in the control because *A. flavus* contamination of grains should be very low without artificial inoculation in the laboratory. On the other hand, the corresponding rates of contamination increased very quickly after *A. flavus* inoculation and even stronger in the treatments when *Penicillium* spp. were co-inoculated. Once again *A. flavus* took advantage on the other species to grow but this time on already harvested grains, where this species usually finds ideal development conditions. The three species are fighting to survive and to occupy the grains but *Fusarium* spp. and *Penicillium* spp. are confronted with less favourable conditions regarding temperature and water activity than *A. flavus*. As exposed by Cooke and Whipps (1993), the competitive reaction of *A. flavus* allowed it to grow faster, decreasing the chance for other fungi to grow. *Penicillium* spp. impacted favourably the development of *A. flavus* during storage. *Penicillium* spp. and *A. flavus* are not cited as antagonistic fungi during storage and also it could be that *A. flavus* recognized *Penicillium* spp. as another storage specialized fungi (Kamphuis et al. 1992) and then set up a race for niche occupation and succeeded since conditions are optimal for this specie (Cook and Whipps, 1993). This observation differed from the conclusion of Marin et al. (1998a) but their experiments were set up at lower temperature levels (15° and 25° C) and these temperatures were below the optimum temperature for *A. flavus* specified as about 31° C by

Sautour et al. (2002). Also in the here presented experiment, the water content was favourable for *A. flavus* that is more xerophilic than other fungi especially *Fusarium* spp. (Pitt and Hocking, 1977). In their study on the competitiveness of *Aspergillus* and *Penicillium* in the presence of *Fusarium moniliforme* and *Fusarium proliferatum*, Marin et al. (1998a) observed that in most of the cases where *Fusarium* spp. outcompeted *A. flavus* this was under high water content condition (a_w = 0.98) and relatively low temperature (15° C). The incidence of *A. flavus* in the treatment "control" was observed to be very low, but still significant, indicating the presence of *A. flavus* contamination in the kernels before inoculation (Marsh and Payne, 1984). In the postharvest experiment, the domination of *A. flavus* could be imputed to environmental conditions and to the presence of *Penicillium* spp.

The high initial levels of *A. flavus* contamination in the preharvest experiment highlight the importance of field contamination. The rates of increase in both experiments were high, but more pronounced in the postharvest experiment. This shows that maize kernels that are not well handled during harvest could result in grains with high *A. flavus* contamination as highlighted by Kaaya et al. (2006), even though the infection level in the field was low. This scenario could take place especially if the storage conditions are close to the optimal requirements for *A. flavus* growth, and then a positive impact of other storage moulds could aggravate the spoiling of stored maize.

4.5.3 Incidence of *Fusarium* spp.

While the incidence of *A. flavus* increased in both experiments, the incidence of *Fusarium* spp. differed markedly between the pre- and postharvest experiment. In the postharvest experiment, the incidence decreased linearly in all treatments while in the preharvest experiment an initial decrease was followed by a final increase with a minimum disease level after four weeks. In both experiments, *Fusarium* spp. was prevalent whether there was an artificial inoculation or not. Indeed it is known that the *Fusarium* spp. is endemic on maize (Munkvold and Carlton, 1997). It is always present on maize grown in the field (Zorzete et al., 2008) and could be an active pathogen on living plants, colonizing the grains with contamination, or just be present inside the maize plant as an endophyte (Saunders and Kohn, 2008; Marasas et al., 1979; Cawood et al., 1991 and Fisher et al., 1992). It is even

thought that maize and many *Fusarium* species have co-evolved (Glenn et al., 2001). Therefore non-sterilized maize is certainly the carrier of some forms of propagules of *Fusarium* spp. among other possible fungal propagules. In the preharvest experiment, *Fusarium* spp. was well established within maize kernels before the storage period (Bacon et al., 2001; Kamphuis et al., 1992). However, in the postharvest experiment, the higher the initial *Fusarium* spp. incidence was, the more rapidly their level decreased during storage. Despite the presence of *A. flavus* and *Penicillium* spp., *Fusarium* spp. had higher initial incidence, when inoculated for instance in the treatments AF and APF, but the trend of evolution during the storage did not change for the field inoculated maize, while in laboratory inoculated maize the presence of *Fusarium* increased the rate of reduction of *Fusarium* spp. The causes could be that the environmental conditions in terms of temperature and water content during storage were far from ideal for *Fusarium* spp. growth (Marin et al., 1998b). It is clear that the highest decrease of *Fusarium* incidence was due to the presence of the inoculated strain. The naturally infested strains decreased more slowly which is shown by the fact that the decrease was less steep when *Fusarium* spp. was not inoculated. This shows again the importance of field infection of *Fusarium* spp. on maize. Initial levels of *Fusarium* spp. infestation were quite high in the postharvest experiment prior to inoculation which did not allow effective *Fusarium* spp. infections from inoculated strains on the maize kernels and their spores died slowly during storage. Widstrom et al. (1994) demonstrated that a previous infection of others strains on maize grains weakened the survival of spores from an artificial consecutive inoculation. Therefore the rate of germination of *Fusarium* propagule decreased over time while those of *A. flavus* increased.

4.5.4 Incidence of *Penicillium* spp.

The incidence of *Penicillium* spp. increased slowly in all treatments of the preharvest experiment, but the rate of increase was not significantly different between treatments. There was no effect of the *A. flavus* inoculation on *Penicillium* incidence. As expected, the incidence level in the treatment in which *Penicillium* spp. was inoculated was higher than in those without inoculation. However, the co-inoculation with *Fusarium* spp. reduced the incidence of *Penicillium* propagules at the beginning of the storage period. *Fusarium* spp. seemed to be

an antagonist of *Penicillium* spp. under field conditions. The antagonism between *Fusarium* spp. and *Penicillium* spp. was shown by Marin et al. (1998a) but in vitro. However in this study, in both treatments and those involving a sole inoculation of *A. flavus* or a co-inoculation of *A. flavus* and *Fusarium* spp., the rate of increase of *Penicillium* spp. incidence was similar showing no effect of the biotic environment during storage on *Penicillium* spp.

In the post-harvest experiment, the incidence showed a high variation over time but without a significant linear temporal trend. However, it seemed that the *Penicillium* spp. incidence decreased initially, followed by an increase and a second reduction in the 7th week of the storage period. Neglecting the data of week seven, the progress of *Penicillium* spp. during the first six weeks could be described by a quadratic function. It is possible during that seventh week that *Penicillium* spp. was outgrown by *A. flavus* that decreased its germination. For the six first weeks, the incidence of *A. flavus* was low enough to allow *Penicillium* spp. germination but apparently in the seventh week a threshold was exceeded and *Penicillium* spp. did not grown optimally. The dynamic analysis of the six first weeks allowed then to see that only the presence of *Fusarium* spp. decreased the initial incidence of *Penicillium* spp. and even its rate at the beginning and at the end of storage. This may be due to an antagonism between both fungi as already invoked earlier.

4.5.5 *A. flavus, Fusarium* spp. and *Penicillium* spp. conclusion

Nevertheless, the conclusions drawn from *Fusarium* spp. and *Penicillium* spp. dynamics during this experiment should be taken with caution since the main objective of the study was to observe the behaviour of *A. flavus*. A more accurate study of these very important maize colonists would need another experimental set-up.

The presence of *Fusarium* spp. had a positive effect on *A. flavus* incidence, while the effect of *Penicillium* spp. was rather neutral. In storage conditions with favourable environmental conditions for *A. flavus*, the increase of *A. flavus* incidence was not influenced by any of the fungi species in co-habitation situation. In case of fungi inoculation after harvest, the presence of *Penicillium* spp. boosted *A. flavus* development. These two observations showed that the presence of other fungi frequently encountered with *A. flavus* in the storage systems could allow an increase of *A. flavus* incidence with an effect of *Fusarium*

spp. restricted to the field and of *Penicillium* spp. to the storage. The field inoculation of *Penicillium* spp. had a positive effect on its own incidence at the harvest mostly when *Fusarium* spp. was not inoculated. But during storage the so contaminated maize was not influenced by the biotic conditions. In case of *Penicillium* spp. inoculation of already harvested maize, the presence of *Fusarium* spp. had a clearly negative effect on *Penicillium* spp. Whether the contamination took place in the field during maize maturation or after harvest, there was a high risk of *A. flavus* contamination of the maize but if field contamination was low, good harvesting and storage techniques can lower the risk of *A. flavus* colonization on stored maize and consequently, low initial *A. flavus* infestation would lead to a lower risk of aflatoxin contamination. From this study, it appeared that if maize colonization by toxigenic *A. flavus* strains happened in the field during maize grains maturation there was a very high probability to end up with high aflatoxin contamination, however, this can be very variable due to biotic factors.

4.5.6 Aflatoxin concentration

The aflatoxin concentrations in corresponding treatments were higher in the preharvest than in the postharvest experiment. In both experiments, the concentrations showed a high variability over time so that nearly no significant effects could be determined. The concentration of maize kernels inoculated in the field increased in all treatments with the same rate. Only in the control, the level remained unchanged over time. In the post-harvest experiment, though the general concentration of aflatoxin was relatively low, a significant slight increase was observed during storage with a common rate for all treatments. In the preharvest experiment, the rate of increase of aflatoxin was not significantly different from zero. The intercept that was common in treatments with *A. flavus* inoculation and in the treatment control showed that the registered incidence of *A. flavus* was not only due to the inoculation but also natural *A. flavus* infections must have occurred in the field. The only explanation is that the not inoculated *A. flavus* strains that are able to produce aflatoxin on maize in the field have not the same capability for aflatoxin production under storage condition of this experiment since the availability of carbon source may differ (Luchese and Harrigan, 1993).

It is obvious that the aflatoxin recorded on preharvest maize developed mainly in the field during maize maturation. In the postharvest experiment, the concentration of aflatoxin was lower but increased with time irrespectively of the treatments. The increase of aflatoxin is probably due to the few isolates that had infected the maize in the field, because it was reported previously that aflatoxin production was significantly reduced in case the substrate was already infested with other fungi before *A. flavus* was introduced (Shotwell et al., 1975).

Chapter 5. Effect of the temperature and water activity on the growth of some isolates of *A. flavus* and on their aflatoxin production

5.1 Abstract

To characterize the effects of temperature and water activity on different *A. flavus* isolates, six isolates from Benin were investigated for the colony growth and four for aflatoxin production. The Gompertz function described very well the colony growth of most of the isolates. The monomolecular model was good for aflatoxin production simulation. Generally, the water activity had a stronger effect than temperature on the growth in the ranges studied in this paper. For aflatoxin production, the interaction between temperature and water activity was most important. In all cases with high aflatoxin production, a degradation of the toxin followed. A water activity level of 0.90 was the least efficient while 0.96 was the most efficient one. At the latter level of water activity, the effect of the temperature was weak. Depending on the isolate, the optimal temperatures varied between 31, 33 and 35°C while the optimum water activity for all isolates remained 0.96. Concerning the aflatoxin production, the optimum water activity varied between 0.96 and 0.99 but the optimum temperatures were the two lowest of this study (26 and 28°C). The L-strain isolates also produced aflatoxin G but at a lower level of water activity (0.90 and 0.93) than the S-strains isolates (0.96 and 0.99). The highest rates of growth were recorded for isolates Z34A, Z117B and Z1TS all being L-strain isolates. The differences between optimum and minimum growth rates were high for the L-strain isolates. The best aflatoxin B producer was isolate Z213D that was also the best producer of aflatoxin G. Isolate Z1TS followed but only for aflatoxin B production. Z213D is an S-strain isolates and a good producer of aflatoxin, but had a very low growth rate. The lowest aflatoxin production rate was recorded for isolate Z34A that is an L-strain isolate characterized by a very high rate of growth.

5.2 Introduction

Studies of *Aspergillus flavus* and its capacity to produce aflatoxin in the products it colonizes have led to different ways to classify *A. flavus* isolates. Possible means to group *A. flavus* isolates are: firstly by the isolates capability to produce aflatoxins (Klich, 2007; Cardwell and Cotty, 2002); secondly by the kinds of aflatoxins they produce (Hesseltine et

al., 1970; Cotty and Cardwell, 1999); and thirdly by the quantity of conidia or sclerotia they can develop at a certain moment of their growth (Cotty, 1989). Other more complex methods of classification are linked to the ability of some isolates to cross in non-sexual way making up different kinds of vegetative compatibility groups (VCG) (Horn, 2005). Even though the capability to produce aflatoxin varied more between individuals of different VCG than between individuals of the same VCG (Bayman and Cotty, 1991), it is not easy to reach a systematic characterization of a region's *A. flavus* population using VCG criteria because VCGs are so variable and so numerous that one field can contain dozens of them. Therefore the population characterization based on morphological and physiological characteristics seems more appropriate.

The capability to produce aflatoxin is a key criterion for physiological classification. It is known that an important percentage of *A. flavus* in a population is unable to produce aflatoxin and these atoxigenic *A. flavus* isolates belong almost exclusively to the morphological group "L-strain" (Cardwell and Cotty, 2002). The L-strain group contains also isolates able to produce aflatoxin, specifically aflatoxin B_1 and B_2. Isolates of this group are characterized by an abundant production of conidia and few large sclerotia or no sclerotia at all, at least on most artificial media (Cotty, 1989). The other group "S-strain" includes isolates that are also able to produce aflatoxins B_1 and B_2 and sometimes G_1 and G_2, the latter two depending on the geographic (continental) situation (Cotty and Cardwell, 1999). Therefore, two subgroups of the S-strain exist: The first subgroup S-strain (S_B) contains isolates able to produce only aflatoxin B_1 and B_2 and the second subgroup S-strain (S_{BG}) comprises isolates able to produce aflatoxins B and G. The high toxicity associated with the fact that S-strain isolates always produce small and numerous sclerotia have led to their description as an evolution of *A. flavus* for resistance and persistence in difficult environment. This is reinforced by the fact that isolates of the S-strain S_{BG} occur in Benin more frequently in the most arid zones of the country in the north (Cardwell and Cotty, 2002). The consideration of these physiological and morphological characteristics of *A. flavus* populations leads to questions about possible consequences on the risk of contamination of a susceptible crop. But before any conclusions can be drawn, it is needed to know, if the different physiological and morphological groups of Benin's *A. flavus* population react similarly or differently to environmental conditions.

The environmental variables that directly affect *A. flavus* colonization and aflatoxin production in maize are the water content and temperature (Ayerst, 1969).

Knowing that the ability to produce aflatoxin may vary within an *A. Flavus* population, it would be very interesting to check the possible variation of these characteristics in relation to temperature and water activity and to determine if some major variability could be extrapolated from this study.

One of the first studies on the effects of environmental factors by Agnihotri (1964) identified temperature as the main factor of *Aspergilli* growth. Later Schindler et al. (1967) studied the effect of temperature on the production of aflatoxins by *A. flavus* and confirmed that the optimum temperature for *A. flavus* growth was higher than for aflatoxin production. Other researchers checked other environmental factors such as the relative humidity (Diener et al., 1967), the atmospheric gas (Landers et al., 1967) and the carbon dioxide (Sanders et al., 1968). However, all these early studies were confined to the growth of *A. flavus* and aflatoxin production in peanut. Trenk and Harman (1970) investigated the production of aflatoxin in maize, but they did not inoculate *A. flavus* and described just the natural occurrence of *A. flavus* and the aflatoxin production under different environmental conditions. Nevertheless their results allowed to confirm that *A. flavus* and to a lesser extend *A. parasiticus* were responsible for the aflatoxin production in maize, but not to characterize the effects of environmental factors especially of the temperature and water content on aflatoxin production. Later these aspects were taken into account by Gqaleni et al. (1997). Finally, it is well known that *A. flavus* growth and its ability to produce aflatoxin in different commodities are in the first place dependent on temperature, water activity and their interactions. This brief review shows that *A. flavus* and its ability to produce aflatoxin were well studied in relation to the environmental factors and even in relation to the geographical location (Orum et al., 1999; Cotty et al., 1999). However, even though it is known that *A. flavus* strains vary in their reaction to environmental factors, comparative studies on the reaction of different strains to main environmental factors are scarce.

The objective of this study is to determine if atoxigenic L-strain isolates, toxigenic L-strain isolates and S-strain isolates, all isolated from Benin, behave differently in growth and aflatoxin production on artificial media under the influence of various water activity and temperature levels.

5.3 Material and Methods

From a screening test of *A. flavus* isolates collected from 100 fields in the four agroecological zones of Benin, one non-toxigenic L-strain isolate, two S-strain isolates and three toxigenic L-strain isolates were selected to experimentally check growth and aflatoxin production. These selected isolates and the quantity of aflatoxin they are able to produce are represented in Table 5.1. In the growth experiment all 6 isolates were used, in the test on aflatoxin production only 4 isolates. In the latter case, the isolates Z46A and Z117B were left out because they produced no aflatoxin at all or only an extremely low amount.

Table 5.1: Characteristics of the six selected *A. flavus* isolates

Zone	Name	Toxigenic statuts	Morphological group	Aflatoxin B_1 (ppb)	Aflatoxin B_2 (ppb)	Aflatoxin G_1 (ppb)	Aflatoxin G_2 (ppb)
CS	Z1TS	T	L	1939.9±8.5	188.8±3.2	0.0±0.0	0.0±0.0
CS	Z117B	T	L	1.5±0.1	0.0±0.0	0.0±0.0	0.0±0.0
SGS	Z213D	T	S	2983.4±12.8	307.5±19.9	535.5±3.4	23.6±1.0
NGS	Z34A	T	L	94.3±0.8	5.9±0.3	0.0±0.0	0.0±0.0
SS	Z44A	T	S	1174.1±5.6	141.9±0.3	151.6±1.0	18.6±0.0
SS	Z46A	NT	L	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

T=Toxigenic, NT=Non-toxigenic, L=L-strain, S=S-strain

5.3.1 Water activity and temperature levels in the experiments

The water activity in the artificial media was controlled using the gravimetric method (Esteban et al., 1989; Fernandez-Salguero et al., 1989; Esteban et al., 1990). They have used different salt solutions to get a calibration curve to determine the corresponding water activity by calculating the water weight absorbed by a Whatman paper within 24 hours. In this study that method was applied to two artificial growth media, each containing different amounts of glycerol. In the experiments, four different amounts of glycerol were chosen corresponding to water activity values of 0.90, 0.93, 0.96 and 0.99. Five levels of temperature were used: 26°C, 28°C, 31°C, 33°C and 35°C.

5.3.2 Artificial media

Two artificial media were used in this experiment, the first one was the 5/2 medium (5% of V8 juice and 2% of agar) (Cotty, 1988) applied to investigate colony growth. That medium was chosen because it is easy to get and is perfect for *A. flavus* strains recognition. The second medium used was the Adye and Mateles (A&M) medium amended with 2% of agar (Cotty, 1988) on which the aflatoxin production was measured.

5.3.3 Inoculum preparation

The isolates were incubated in an incubator at 31°C on Petri dishes (9 cm diameter) containing about 25 ml of the 5/2 medium. After six days, the conidia of each isolate were suspended in distilled and sterile water. These suspensions were diluted to get suspensions of about 10^5 spores per milliliter and stored for further inoculation of culture media.

5.3.4 Growth media inoculation and measurements

For the growth experiment, the medium in the center of each Petri dish was punch with a 0.5-cm diameter perforator. Ten microliters of *A. flavus* suspension were poured in the small hole. For each experimental unit, three plates were enveloped in closed plastic bags before incubation. After 48 hours of incubation, two measures of the colony diameter of each plate were taken perpendicularly. These measurements continued for 8 days or until the Petri dish was fully occupied. The two measurements of the colony diameter were averaged and then 0.5 cm for the initial hole was subtracted.

5.3.5 Aflatoxin production and aflatoxin quantification

About 6 ml of the A&M medium were poured into test tubes with caps and 10 microliters of the fungal suspension were added in each treatment. To avoid any loss of water, the test tubes were enveloped in plastic bags before incubation. After 48 hours of incubation, the first series of test tubes was selected for aflatoxin extraction. Aflatoxin was determined on six consecutive day, so that for each isolate up to 120 test tubes were tested for the combination of the five temperatures and four water activity levels and six days.

The extraction followed the procedure described by Cotty (1988). In each tube, 6 ml of acetone were added and the agar crushed with a glass rode. The acetone was poured in a beaker and the remaining agar was extracted 3 times with 10 milliliters of methylene chloride. The methylene chloride and the acetone were then filtrated through 25g of sulfate sodium anhydrous and the filtrate was let to dry at room temperature. The residue was recuperated with a mixture of 50% methanol and 50% distilled water in small flask and brought to quantification by an HPLC device.

5.3.6 Data analysis

For the data analysis, the colony diameters of each isolate were used without transformation. The maximum diameter recorded was 8 cm reflecting the size of the Petri dish used in the experiments. The temporal data were described by Gompertz growth functions that resulted in a better fit than the logistic functions in some preliminary analyses. The Gompertz function used (equation 5.1) included dummy variables (d_i) to allow an accurate comparison of the 6 growth rates (b_i) by choosing the same initial value (D_0).

$$D(t) = 8 \times e^{\ln(D_0) \times e^{-(d_1 \times b_1 + d_2 \times b_2 + d_3 \times b_3 + d_4 \times b_4 + d_5 \times b_5 + d_6 \times b_6) \times t}} \quad (5.1)$$

$D(t)$ represents the diameter as function of time t measured in days after inoculation, D_0 represents the common intercept for all isolates growth curves, b_i are the relative growth rates of the 6 isolates and d_i are dummy variables that have the value 1 for the corresponding isolate and 0 for all other isolates. For instance for the isolate i = 1, d_1=1 and d_2=d_3=d_4=d_5=d_6=0. The growth rates of the isolates in the different combinations of temperature and water activity were then compared to assess the difference between isolates at different environmental conditions.

The aflatoxin concentrations c (in ppb) of test tubes were logarithmically with c' = $ln(c+1)$ transformed. In most cases the aflatoxin concentrations increased continuously with time, but in a few examples with extreme conditions, also a degradation of aflatoxins was observed after a maximum value had been reached. Nevertheless, monomolecular growth functions were fitted to the transformed progress data. The modified monomolecular model (equation 5.2) was fitted to the transformed aflatoxin B (B_1+B_2) and G (G_1+G_2) concentrations of each isolate.

$$c'(t) = 11 \times \left(1 - \left[1 - \frac{c'_0}{11} \right] \times e^{-(d_1 \times b_1 + d_2 \times b_2 + d_3 \times b_3 + d_4 \times b_4) \times t} \right) \quad (5.2)$$

The maximum level of aflatoxin after transformation was 11. Like in equation 5.1, c'_0 represents the common initial value for the aflatoxin production curves of the 4 isolates. The dummy variables d_i is again equal to 1 for the corresponding isolate i and 0 for all other isolates.

5.4 Results

5.4.1 Growth of *A. flavus* isolates

A typical example of the colony growth is given in Fig. 5.1 for the isolate Z34A at a temperature of 33°C. For a_W = 0.90, the growth beyond the initial hole started on day 3, while for a_W = 0.96 a remarkable growth was observed already on day 1. At the latter water activity level, the whole Petri-dish was covered after 6 days, in contrast to the lowest activity level in which after 6 days the diameter just reached 2 cm. In both cases, the fitted function describes the colony growth very well with a clear difference in the growth rate, 0.84 to 0.24 cm/day.

The Gompertz function (equation 5.1) described the colony growth of the 6 isolates in all 20 temperature-water activity combinations very well with the R^2 varying from 0.84 to 0.99 and all regression probabilities $P < 0.0001$. Different initial values D_0 were estimated for the 20 situations. The estimated values were always less than 0.031 cm.

For the water activity of 0.90, the time lags till the growth started ranged from 3 to 5 days, while for the higher activity levels of 0.96 and 0.99, this time lag was only 1 day (Table 5.2). The observed colony diameter after 1 day (Table. 5.2) for a_W = 0.96 and a_W = 0.99 clearly reflects the effect of temperature on colony growth. For all isolates tested, the diameter increased with temperature and reached a maximum value at 35°C, with a few exceptions.

Looking at the diameter reached after 1 day at 35°C allows to compare the growth of the isolates. For a_W = 0.93 to 0.99, the highest diameter was always reached by isolate Z34A. At a_W = 0.96 the observed diameter of isolates Z117B and Z46A were clearly lower than those of the other 4 isolates, while for a_W = 0.99, the differences among the isolates were small.

For a_W = 0.90, the growth rates of the 6 isolates at the three low temperatures (26, 28 and 31°C) varied only slightly within a temperature level, while for the two high temperatures (33 and 35°C) clear differences were observed (Table 5.3). At 33°C and 35°C, isolate Z46A had always the lowest rates. With respect to the highest rates within a temperature level, no clear superiority of a specific isolate could be identified (Table 5.3). A comparison of the rates across temperature levels is doubtful because different D_0 were determined with equation 5.1 for each temperature so that the growth rates are influenced by the known correlation between D_0 and the growth rate. At the water activity of 0.93, the growth rates were generally higher than at a_W = 0.90 isolates.

Table 5.2: The time lags *LAG* (in days) till the colony grows beyond the initial hole and the corresponding first measured colony diameter *DIAM* (in cm) of six isolates at five temperature (*T*) and four water activity (a_W) levels

a_w	*T* (°C)	Z117B		Z1TS		Z213D		Z34A		Z44A		Z46A	
		LAG (days)	*DIAM* (cm)	*LAG* (days)	*DIAM* (cm)	*LAG* (days)	*DIAM* (cm)	*LAG* (days)	*DIAM* (cm)	*LAG* (days)	*DIAM* (cm)	*LAG* (days)	*DIAM* (cm)
0.90	26	4	0.53	4	0.60	4	0.40	4	0.47	4	0.62	4	0.43
	28	4	0.25	4	0.10	4	0.07	4	0.12	5	0.47	5	0.47
	31	3	0.62	3	0.25	3	0.20	3	0.40	3	0.55	3	0.57
	33	3	0.82	3	0.32	4	0.50	3	0.67	3	0.48	4	0.17
	35	3	0.60	3	0.57	3	0.50	3	0.58	3	0.60	3	0.10
0.93	26	2	0.95	2	0.53	2	0.67	2	0.65	2	0.72	2	0.78
	28	2	0.78	2	0.80	2	0.75	2	0.78	2	1.08	2	0.83
	31	1	0.42	1	0.40	1	0.30	1	0.38	1	0.48	1	0.58
	33	1	0.53	1	0.33	1	0.07	1	0.70	1	0.58	2	0.62
	35	1	0.80	1	0.52	1	0.62	1	0.83	1	0.78	2	0.70
0.96	26	1	0.20	1	0.12	1	0.33	1	0.17	1	0.30	1	0.47
	28	1	0.50	1	0.38	1	0.72	1	0.48	1	0.55	1	0.72
	31	1	1.32	1	1.25	1	1.17	1	1.28	1	1.18	1	1.33
	33	1	1.48	1	1.48	1	1.40	1	1.67	1	1.47	1	0.72
	35	1	1.16	1	1.53	1	1.68	1	1.80	1	1.57	1	0.80
0.99	26	1	0.40	1	0.33	1	0.47	1	0.30	1	0.48	1	0.53
	28	1	0.50	1	0.50	1	0.53	1	0.48	1	0.63	1	0.50
	31	1	0.90	1	0.97	1	0.85	1	0.90	1	0.88	1	0.90
	33	1	1.02	1	1.00	1	1.02	1	1.02	1	0.87	1	0.92
	35	1	1.20	1	1.22	1	1.18	1	1.25	1	1.18	1	1.12

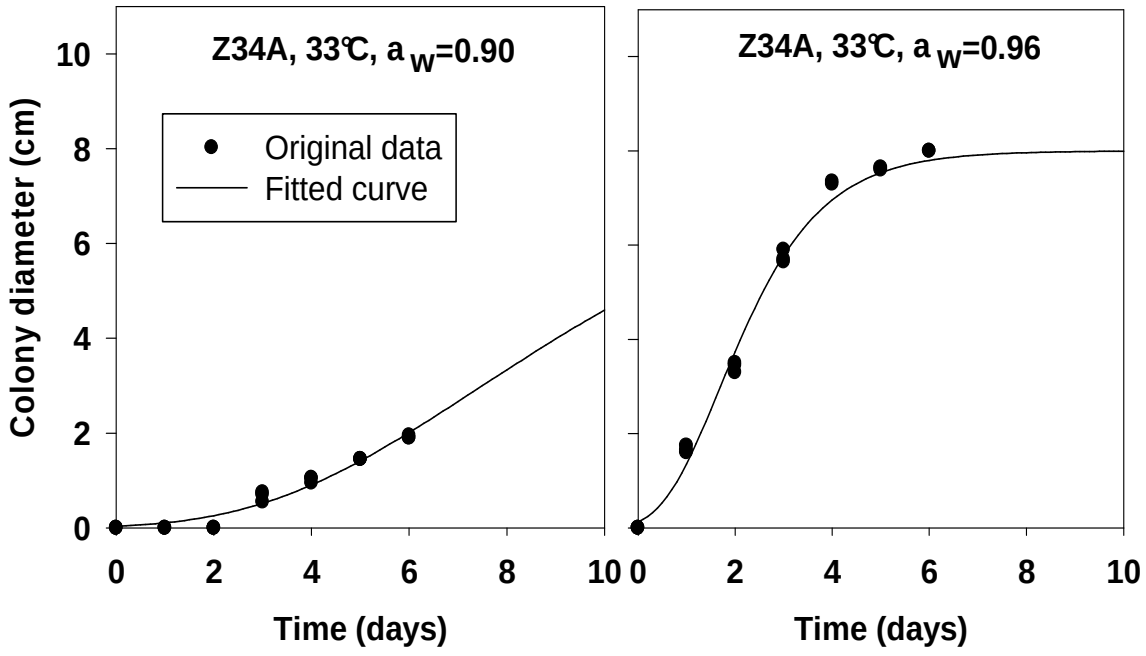

Fig. 5.1: Progress curves of the observed colony diameter (dots), measured in cm, of the isolate Z34A and fitted Gompertz curves (solid lines) at 33°C for a water activity of 0.90 (left) and 0.96 (right).

Table 5.3: Estimated growth rates (in cm/day) of the six isolates resulting from regression analysis with equation 5.1 for five temperature and four water activity (a_W) levels

Isolates	a_w = 0.90					a_w = 0.93					a_w = 0.96					a_w 0.99				
	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C
Z117B	0.23	0.27	0.17	0.23	0.26	0.52	0.45	0.40	0.50	0.56	0.73	0.68	0.75	0.75	0.80	0.49	0.48	0.52	0.46	0.53
Z1TS	0.22	0.27	0.16	0.22	0.27	0.55	0.46	0.42	0.53	0.60	0.73	0.68	0.80	0.77	0.78	0.51	0.47	0.59	0.53	0.57
Z213D	0.24	0.25	0.15	0.17	0.24	0.56	0.48	0.43	0.51	0.59	0.74	0.70	0.69	0.71	0.75	0.36	0.43	0.50	0.43	0.46
Z34A	0.22	0.26	0.16	0.24	0.26	0.55	0.48	0.46	0.56	0.66	0.77	0.65	0.77	0.84	0.81	0.49	0.44	0.48	0.49	0.53
Z44A	0.22	0.27	0.17	0.23	0.26	0.48	0.49	0.47	0.52	0.61	0.54	0.55	0.75	0.66	0.64	0.33	0.39	0.35	0.28	0.43
Z46A	0.23	0.26	0.16	0.13	0.14	0.56	0.47	0.43	0.39	0.46	0.70	0.63	0.79	0.68	0.64	0.53	0.47	0.60	0.52	0.58

For a_w = 0.96, the highest growth rates were estimated for all isolates so that this water activity level seems to be the optimal level. The growth rates were fairly close for the isolates between temperature levels. The slowest isolate was Z44A for most of the temperature levels (26, 28, 33, and 35°C), only at 31°C isolate Z213D was slower. Most of the isolates reached the maximum colony size (8 cm diameter) at this level of water activity.

For a water activity of 0.99, the growth rates were similar to those of a_W = 0.93. Isolate Z44A had again the lowest growth rates in all temperature levels. Isolate Z213D was only slightly better. The rates of isolate Z46A which were low compared to the rates of the other isolates at the lower water activity level reached now the highest values or nearly the highest values.

5.4.2 Aflatoxin B production

A typical example of the aflatoxin B production is shown in Fig. 5.2 for the isolate Z213D at a temperature of 28°C for a_W = 0.93 and 0.96. Already after one day, the production of aflatoxin B started, a little bit stronger in the higher water activity level, reaching an asymptotic phase after three days. In both cases, the fitted monomolecular function describes the aflatoxin B production very well with a slightly higher rate (0.73) for a_W = 0.96 than for a_W = 0.93 (0.62).

Generally, the monomolecular function (equation 5.2) reasonably described the temporal dynamics of aflatoxin B production of the four studied isolates in the 20 temperature-water activity combinations. In Table 5.4, the common parameter values from the regression analyses of the 20 combinations are summarized. In some cases, the coefficients of determination are very low due to the fact that aflatoxin production was extremely low or did not exist for some isolates. Anyway, it should be clear that the aflatoxin degradation, observed in a few cases, cannot be reflected by the monomolecular model.

The monomolecular rates of aflatoxin B production are represented in the Table 5.5. For the water activity of 0.90, the four tested isolates significantly produced aflatoxin B at 26, 28 and 31°C. At 33°C, only isolate Z44A produced a significant quantity of aflatoxin B and at 35°C only isolate Z1TS produced detectable levels of aflatoxin B. For the temperature levels 26°C to 31°C, isolate Z213D had the highest rate of aflatoxin B

production compared to the other three isolates. Isolate Z34A produced the lowest level of aflatoxin B at 26 and 28°C but not at 31°C where isolate Z1TS had the lowest rate.

The water activity of 0.93 led to higher aflatoxin B production rates than a_W = 0.90 for all isolates and at all temperatures levels except 33°C. Isolate Z213D was again the only isolate that produced detectable levels of aflatoxin B at all studied temperatures. At 26, 28 and 31 °C, isolate Z213D had the highest production rate, but at 35°C the lowest. At 26, 28 and 31°C, the isolates Z1TS and Z44A showed similar capabilities while Z34A produced less aflatoxin B than all other three isolates.

Table 5.4: Common statistical parameter values for aflatoxin B production of four isolates resulting from regression analyses with equation 5.2 at five temperature and four water activity (a_W) levels

Temperature (°C)	Common parameters	a_w = 0.90	a_w = 0.93	a_w = 0.96	a_w = 0.99
26	C_0'	-1.38	-1.51	-1.31	-0.48
	R^2	0.85	0.86	0.85	0.80
	P	<0.0001	<0.0001	<0.0001	<0.0001
28	C_0'	-0.99	-1.16	-1.01	0.40
	R^2	0.80	0.87	0.85	0.66
	P	<0.0001	<0.0001	<0.0001	<0.0001
31	C_0'	-0.42	0.21	0.35	0.77
	R^2	0.93	0.88	0.69	0.70
	P	<0.0001	<0.0001	<0.0001	<0.0001
33	C_0'	0.18	0.18	-0.33	0.71
	R^2	0.13	0.13	0.60	0.46
	P	0.0071	0.0071	<0.0001	<0.0001
35	C_0'	-0.01	-1.47	-1.35	0.93
	R^2	0.13	0.57	0.78	0.43
	P	<0.0001	<0.0001	<0.0001	<0.0001

Table 5.5: Individual aflatoxin B production rates for four isolates resulting from regression analyses with equation 5.2 at five temperature and four water activity (a_W) levels

Isolates	a_w = 0.90					a_w = 0.93					a_w = 0.96					a_w = 0.99				
	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C
Z1TS	0.12	0.10	0.06	NS	0.00	0.36	0.29	0.13	NS	0.22	0.51	0.37	0.09	0.09	0.27	0.35	0.29	0.03	NS	0.22
Z213D	0.37	0.36	0.47	NS	NS	0.62	0.62	0.50	0.02	0.12	0.58	0.73	0.33	0.21	0.31	0.49	0.34	0.10	0.07	0.17
Z34A	0.08	0.08	0.12	NS	NS	0.19	0.16	0.08	NS	0.19	0.34	0.26	0.04	0.04	0.25	0.26	0.23	NS	NS	0.11
Z44A	0.22	0.18	0.23	0.02	NS	0.32	0.29	0.17	NS	0.19	0.33	0.32	0.12	0.06	0.32	0.39	0.25	0.07	0.06	0.17

From the point of view of the production of aflatoxins, the water activity of 0.96 was the most efficient water activity for all isolates at all studied temperature levels. At all temperatures, the aflatoxin B production rates of all isolates were significant. As in the previous cases, isolate Z213D had the highest rate of aflatoxin B production at temperatures from 26 to 33°C. At 35°C, its rate just followed the one of isolate Z44A with both rates almost equal (0.31 and 0.32). From 28 to 35°C, isolate Z34A produced the lowest level of aflatoxin. The differences between the rates of aflatoxin B production of different isolates were low at 26, 28 and 35°C.

At the water activity of 0.99, all five temperatures allowed aflatoxin B production but not of all isolates. At this water activity, none of the isolates could reach the maximum production of 11. Especially at T = 35°C, aflatoxin was degraded after reaching a maximum value of roughly 9 at day 2. At 31°C, isolate Z34A produced aflatoxin B only in traces. The same was observed for isolates Z1TS and Z34A at 33°C. For the first four levels of temperature, isolate Z213D had the highest production rates while Z34A at temperatures 26, 28, and 35°C had the lowest rate.

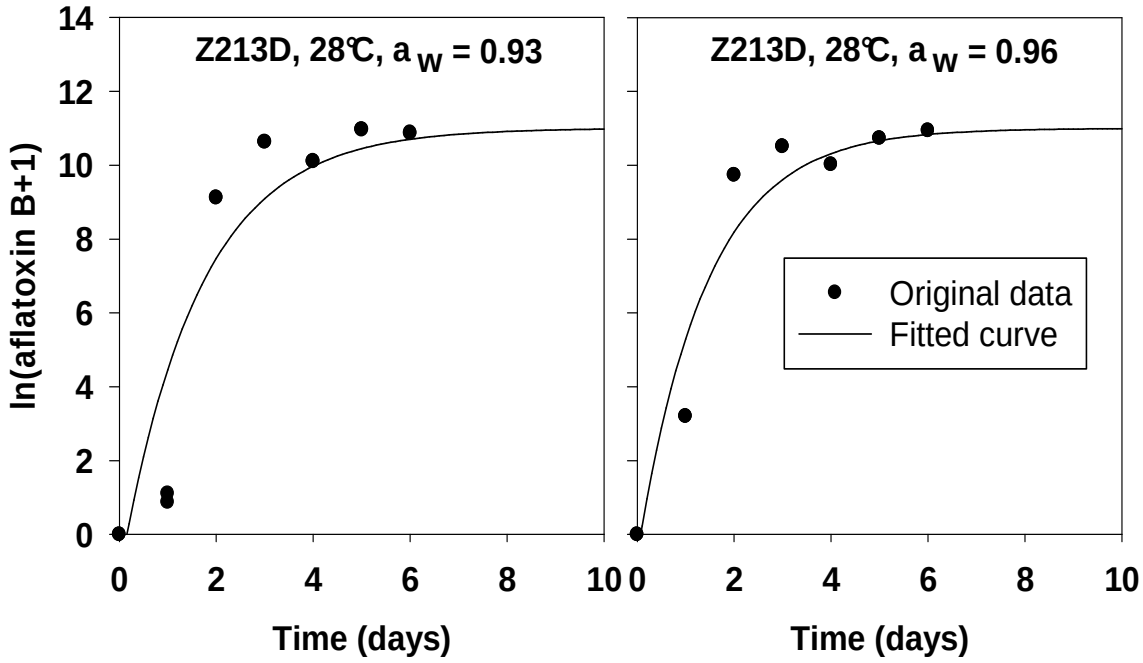

Fig 5.2: Progress of the ln-transformed aflatoxin B concentration (ppb) of the isolate Z213D (dots) and the fitted monomolecular curves (solid lines) at 28°C for a water activity of 0.93 (left) and 0.96 (right)

5.4.3 Aflatoxin G production

A typical example of the aflatoxin G production is shown in Fig. 5.3A for the isolate Z213D at a temperature of 28°C for a_W = 0.96. The production of aflatoxin G started after one day and reached the asymptotic phase already after three days. The fitted monomolecular function describes the aflatoxin G production well. In Fig. 5.3B, the production of the same isolate at the same temperature but a water activity of 0.99 is presented. The production remained 0 at day 1, but jumped to 8 at day 2, followed by a slight decrease in the next days. Therefore in this case, a monomolecular function is not a good representation of the real progress.

Except of the extreme cases, the monomolecular function (equation 5.2) reasonably described the temporal dynamics of aflatoxin G production of the four studied isolates in

the 20 temperature-water activity combinations. In Table 5.6, the common parameter values from the regression analyses for the 20 combinations are summarized. Like for aflatoxin B (Table 5.4), the coefficients of determination are very low for some cases or even not significantly different from 0. This is again due to the fact that aflatoxin production was extremely low or did not exist for some isolates.

For the water activity of 0.90, four of the five temperature levels were efficient for aflatoxin G production. No aflatoxin G was produced at 35°C, not even as trace (Table 5.7). The L-strain isolates (Z1TS and Z34A) unexpectedly produced, though low, significant levels of aflatoxin G at 26, 28 and 31°C. At these temperature levels, isolate Z213D had the highest rates of aflatoxin G production. Only isolate Z44A had a significant level of aflatoxin G at 33°C (Table 5.7).

All temperature levels were efficient for aflatoxin G production at a_w = 0.93 but not for all isolates. At 26 and 28°C, the same pattern as for a_w = 0.90 was reproduced. Isolate Z213D had again the highest rate of aflatoxin G production, isolates Z34A and Z1TS the lowest. At the temperatures of 31 and 35°C, only isolates Z213D and Z44A produced aflatoxin G and at 33°C, only isolate Z213D (Table 5.7).

For the water activity of 0.96, the isolates Z213D and Z44A produced aflatoxin G at all temperatures (except for Z44A at 33°C) whereby the production rate of isolate Z213D was higher. Isolates Z34A and Z1TS had again very low production rates at 26 and 28 °C, but no production at all at higher temperatures (except for Z34A at 35°C).

Table 5.6: Common statistical parameter values for aflatoxin G production of four isolates resulting from regression analyses with equation 5.2 at five temperature and four water activity (a_W) levels

Temperature (°C)	Isolates common parameters	a_w = 0.90	a_w = 0.93	a_w = 0.96	a_w 0.99
26	C_0'	-1.15	-0.77	-0.67	0.11
	R^2	0.85	0.90	0.88	0.90
	P	<0.0001	<0.0001	<0.0001	<0.0001
28	C_0'	-0.74	-0.56	-0.35	0.23
	R^2	0.80	0.91	0.91	0.78
	P	<0.0001	<0.0001	<0.0001	<0.0001
31	C_0'	-0.47	0.77	0.49	-0.03
	R^2	0.92	0.82	0.52	0.69
	P	<0.0001	<0.0001	<0.0001	<0.0001
33	C_0'	0.01	0.16	0.18	0.05
	R^2	0.15	0.54	0.58	0.23
	P	0.0036	<0.0001	<0.0001	0.0011
35	C_0'	NS	-0.00	-0.26	0.27
	R^2	NS	0.42	0.87	0.51
	P	NS	<0.0001	<0.0001	<0.0001

For the highest water activity level (0.99), significant aflatoxin G production rates were found only for the isolates Z213D and Z44A. The rates of both isolates were vey similar. Having in mind that the rate of the monomolecular function is correlated with the initial value, one may, nevertheless, conclude from Tab. 5.7 that the production rate at all water activity levels decreases with increasing temperature.

Table 5.7: Individual aflatoxin G production rates of four isolates resulting from regression analyses with equation 5.2 at five temperature and four water activity (a_W) levels

Isolates	a_W = 0.90					a_W = 0.93					a_W = 0.96					a_W = 0.99				
	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C	26°C	28°C	31°C	33°C	35°C
Z1TS	0.08	0.04	0.04	NS	NS	0.04	0.02	NS	NS	NS	0.05	0.03	NS	NS	NS	NS	NS	NS	NS	NS
Z213D	0.35	0.34	0.39	NS	NS	0.53	0.52	0.27	0.11	0.06	0.52	0.59	0.14	0.12	0.22	0.34	0.26	0.04	0.01	0.09
Z34A	0.06	0.07	0.07	NS	NS	0.07	0.07	NS	NS	NS	0.06	0.06	NS	NS	0.02	NS	NS	NS	NS	NS
Z44A	0.20	0.16	0.16	0.01	NS	0.25	0.24	0.07	NS	0.12	0.25	0.24	0.05	NS	0.18	0.28	0.20	0.03	0.02	0.09

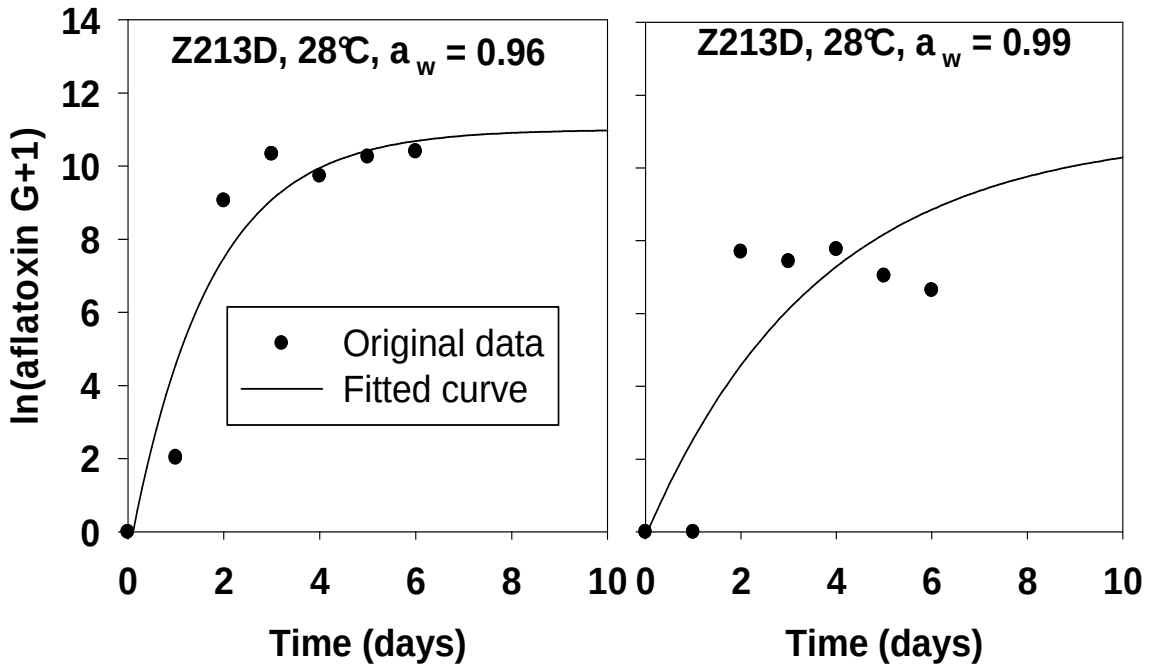

Fig 5.3: Progress of the ln-transformed aflatoxin G concentration (ppb) of the isolate Z213D (dots) and the fitted monomolecular curves (solid lines) at 28°C for a water activity of 0.96 (left) and 0.99 (right).

5.5 Discussion

In this study, the water activity level had a stronger effect on the colony growth of *A. flavus* isolates than temperature level. The time lag was long for the water activity of 0.90 and decreased with the increasing level of water activity. The temperature had a more subtle effect on growth and affected mainly the rate of increase but its effect on the time lag or the maximum diameter of the colonies was less significant. Sautour et al. (2002) showed that the influence of temperature was overlocked by water activity at non-optimum conditions. In their experiment at 31°C, the variation of the water activity from 0.97 to 0.99 increased the *A. flavus* growth rate. All isolates had a very slow growth at the water activity of 0.90, but maximum diameter of colonies at a_w = 0.96. According to Sampundo et al. (2007), data regarding the optimum water activity are limited, but, depending on the study conditions such as media used, some other authors have set it to 0.994 (Marin et al., 1998), 0.97, 0.974, 0.980 to 0.994 (Sautour et al., 2002; Gibson et al., 1994). The variation of the aflatoxin production is more subtle. For many reasons, the rate of aflatoxin production is affected by water activity and temperature even if it is clear that for all levels of water activity, the lower temperatures (26 and 28°C) are more efficient for aflatoxin production. This range of optimum temperature for aflatoxin B_1 production is included in the temperature interval from 24 to 35°C stated by Gqaleni et al. (1997). Their range was explained by the variation of water activity, isolates and media of culture. The optimum of aflatoxin production may differ from one isolate of the same species to another isolate depending on the substrate (Northolt et al., 1977). But it is also obvious that in this study the highest rates of aflatoxin production are underestimated because in the cases where the rates were very high, a systematic degradation of the produced aflatoxin followed and the rate of degradation seemed proportional to the rate of production. The aflatoxin degradation phenomenon is known since a long time (Schindler et al., 1967; Ciegler et al., 1966; Doyle et al., 1978), only the destruction mechanism remained under investigation because its explanation varied with the time.

Regarding the variations among the different water activity levels, in the case of the growth, a_w = 0.90 seemed the least efficient producing maximum time lags, weakest rates of growth and shortest maximum diameters of colonies. The real minimum a_w for *A. flavus*

growth is difficult to determine since it depends on the solute used to control it and on other parameters like pH and temperature. Rosso et al. (2001), using a mixture of glucose and fructose to control a_w, have found that the minimum water activity for *A. flavus* was between 0.797 and 0.773 for a pH of 6.5 and a range of temperature from 25 to 37°C. The increase of the temperature for a_W = 0.90 shortened the time lag and reduced the growth rate of some of the isolates. Globally, the growth parameters were relatively weak at that a_W. In contrast to the water activity of 0.90, the one of 0.96 was the most efficient with the shortest time lags, highest growth rates and the maximum possible diameter reached very quickly. The effect of temperature had less influence than in the case of a_W = 0.90. The highest temperatures (31, 33 and 35°C) allowed the best growth rates observed in this study. The optimum temperature for *A. flavus* growth varied according to authors. Samapundo et al. (2007) assumed a temperature optimum between 28 and 30°C, earlier Pitt and Hocking (1997) indicated 32 to 33°C, a range that was also different from the previous indication of Schindler et al. (1967) or Trenk and Hartman (1970) of 29 to 35°C. However, it is not surprising to have so various or so spread optimum temperature ranges for *A. flavus* growth since it depends on the media used in the studies. There were more significant variations between the rates of aflatoxin production of the isolates than between the growth rates. The most efficient water activity levels were 0.96 for 3 isolates and 0.99 for one isolate. Also the most efficient temperatures for all isolates were in the lower range (26 and 28°C). The aflatoxin G production was rarer than B production. For the isolate with the highest production of aflatoxin G, the high water activity levels (0.96, 0.99) were the most efficient while for the low producers of aflatoxin G the low level of water activity (0.90, 0.93) were the most efficient. The optimal temperature for aflatoxin G production was between 26 and 28°C. Schindler et al. (1967) who investigated growth and aflatoxin B and G production found that the two isolates of *A. flavus* they used produced both kinds of aflatoxin (B and G). In contrast, a similar study including the water content on harvested maize, scarcely found aflatoxin G (Trenk et al., 1970). But none of these two authors referred to the sclerotical classification of the isolates they observed.

The isolates Z34A, Z117B and Z1TS were those with the highest growth rates at all conditions. From these three isolates, Z117B was atoxigenic and was therefore not checked for aflatoxin production. Isolates Z1TS and Z34A were not the best producers of aflatoxins.

Their rates of aflatoxin G production were very low compared to those of other isolates. In opposite to aflatoxin B, a degradation of aflatoxin G was not observed for these two isolates. Isolate Z1TS had its growth optimum at a_W = 0.96 and T = 31°C and its minimum at 0.90 and 31°C. Isolate Z34A had the same parameters for its growth minimum but concerning the optimal growth, the water activity was 0.96 and the temperature 33°C. From the point of view of sclerotical classification, both are L-strain isolates and therefore no aflatoxin G production in their cultures was expected, but comparatively very low concentrations were observed. It should be noticed that these two L-strain isolates have produced aflatoxin G at low water activities and none at a_W = 0.99 . In the previous screening operation the temperature had been set to 31°C and the water activity to 0.99.

The three lowest growth rates (all parameters combinations considered) were observed for the isolates Z46A (r_G = 0.13 for a_w = 0.90 and T = 33°C), Z213D (r_G = 0.15 for a_w = 0.90 and T = 31°C) and finally Z1TS and Z34A (r_G = 0.16 for a_w = 0.90 and T = 31°C). From the four isolates tested, Z1TS and Z34A were also among those with the highest growth rate (in more efficient condition). But in absolute way, the smallest overall growth rates were recorded for isolate Z46A that was not checked for aflatoxin because it was considered as atoxigenic in the screening conditions, and for isolate Z213A that belongs to the sclerotical S-strain group and from this fact is supposed to produce high levels of aflatoxin B and G. In the scope of this experiment, this isolate is the best aflatoxins producer and probably because of that its aflatoxin was frequently and highly degraded.

From the four isolates analyzed for aflatoxin production, isolate Z213D was the one that had the highest production rate of aflatoxin B and also of aflatoxin G. It was followed by isolate Z1TS for the aflatoxin B. The highest rate of aflatoxin G production by Z1TS was very weak. The optimal conditions for aflatoxin B production by isolate Z213D were encountered at a_W = 0.96 and T = 28°C while for isolate Z1TS they were 0.96 and 26°C. Both other isolates, Z44A and Z34A, had their optima for aflatoxin B production at 26°C and at water activity levels of 0.96 (Z34A) and 0.99 (Z44A). The minimal conditions for aflatoxin B production were more variable. The water activity levels were 0.90 for Z1TS and Z44A and 0.96 for Z213D and 96 for Z34A. High water activity was favorable for aflatoxin B production, but in general at the lowest range of temperatures (26 and 28°C), at least in the scope of this study. That range of temperature was also more favorable for aflatoxin B

production of all four studied isolates than the high temperatures. According to Schindler et al. (1967), the maximum amount of aflatoxin B or G were registered between 24 and 29°C. On the other hand, Koehler et al. (1985) found that the maximum aflatoxin level was recovered between 0.95 and 0.96 water activity for 20 and 30°C and at 0.89 for 37°C. However, they did not distinguish both kinds of aflatoxin.

The scheme was different in the case of the production of aflatoxin G. For the S-strain isolates, the maximum rates of production of aflatoxin G were observed at the highest water activities (0.96 and 0.99). But for the isolates Z1TS and Z34A that were not supposed to produce aflatoxin G in the normal environmental condition, aflatoxin G had the best production rate at the lowest water activities (0.90 and 0.93) while for all types of isolates the temperature were close and rather lower than 26 or 28°C. Gqaleni et al. (1997) studied the effect of temperature, water activity and incubation time on the production of aflatoxins and Cyclopiazonic acid and noticed that even if two different isolates were able to produce the same kind of mycotoxin, they may have different optimum temperatures or water activity levels. The substrate composition could also play a determinant role. Isolate Z213D is the isolates that had the highest rate of aflatoxin B and G production; it is an S-strain isolate with a low maximum growth rate. The worst aflatoxin producers, for instance isolate Z34A that is an L-strain, had at the opposite the highest maximum growth rates. It is also important to notice that the growth and aflatoxins production have 0.96 as optimal water activity. The most favorable temperatures for growth are rather high (31-35°C) while those for aflatoxin production are rather low (26-28°C). According to Schindler et al. (1967), the maximum growth does not coincide with maximum aflatoxin production. And optimum temperatures for aflatoxin production are lower than those for growth.

As already mentioned, the aflatoxin G production by the isolates Z1TS and Z44A described as L-strain isolates was not expected. In this study and in contrast to the S-strains isolates, their aflatoxin G production was very low and appeared at lower water activities (0.90 and 0.93). Two parameters more or less linked could help to explain this result: 1) The water activity: it is easy to see that the more the water activity increases, the less we have aflatoxin G in the L-strain cultures. 2) The glycerol: indeed glycerol was added to the media to decrease its water activity. Only the medium with water activity 0.99 did not receive the glycerol as additive and it appeared that only at this water activity level there was no aflatoxin

G produced by the L-strain isolates. Schindler et al. (1967) attributed the change in the ratio of aflatoxin B to G to the influence of temperature on the biosynthesis of the metabolites since they did not include the factor water activity in their study. In any case, the conclusion would be that in certain conditions (remaining to be precisely defined) the L-strain isolates could produce some other secondary metabolites than in normal conditions. In this specific case, if it can be proven that glycerol was the factor that led to the aflatoxin G production by L-strain isolates, then the further direction to investigate could be the form and availability of carbon impact on the aflatoxigenesis.

Another phenomenon irregularly distributed but not really unexpected was the aflatoxin B and G degradation after a certain number of days of their production. Schindler et al. (1967) noticed the decrease of aflatoxin concentration after a certain time and considered it as a remetabolization of aflatoxin by one of the isolates as source of energy for instance. But Ciegler et al. (1966) refuted that theory and supposed that the degradation of produced aflatoxin is due to the lysis of *A. flavus* mycelium under the effect of high temperature and/or a too high agitation during fermentation. According to this theory, the lysis of the mycelium releases some “aflatoxinase” in the medium. They, however, invalidated this hypothesis themselves because they thought that in the affirmative case, aflatoxin that was also present within the mycelium but not yet lysed could not co-inhabit with the “aflatoxinase”. They also did not find any correlation between aflatoxin concentration and the rate of degradation etc. but they lacked to provide convincing explanation. Doyle et al. (1978) retained the role of the mycelium in the degradation but added that the acidification of the medium would also be a source of aflatoxin degradation. This degradation seemed directly linked to the quantity of toxin produced. In this study, the aflatoxin degradation was correlated with the level of aflatoxin produced. The isolate that produced the highest level of aflatoxin frequently degraded its aflatoxin. Also for the other isolates, the aflatoxin was degraded whenever they produced it at a higher rate. It appeared that the aflatoxin production followed the monomolecular increase as far as the concentration remained under a certain value in the medium but as soon as the curve reached a maximum possible level, the degradation process started like if the fungus started transforming the toxin because it was too much and became harmful to the isolate itself. Then one could conclude that aflatoxin is rather a competitive tool for *A. flavus*. If the environmental conditions can sustain *A. flavus* development at a

minimal rate and if in these minimal conditions the isolates cannot have high growth rates to quickly colonize the medium, then they produce aflatoxin to avoid the media encroachment by other species (Ciegler, 1982). But they are able to degrade their own secondary metabolites as soon as they need to grow. In this experiment, the isolate with the most constant aflatoxin production is also the one having degraded its aflatoxin most frequently. Now the mechanism of aflatoxin level control by the isolate could be the release of "aflatoxigenase" specifically produced at the beginning of the aflatoxin toxicity for the isolate.

Concerning the growth of the different isolates it appeared that the S-strain isolates had the slowest growth at the highest water activities. At all water activity levels, the toxigenic L-strain isolates have most of the time similar growth rates not particularly high nor low. The rate of aflatoxin production did not depend on the growth rate. There were no correlations between the growth and the aflatoxin production in previous studies (Rabie et al., 1965; Schindler et al. 1967). It was more depending on the water activity and on the temperature, but to state it once again the optimal temperatures for both physiological processes are not similar. The optimum for the growth starts at 31°C while the optimum for aflatoxin production is 28°C. The S-strain isolates produced always more aflatoxin G than L-strain isolates but in the case of aflatoxin B production it happened that an L-strain isolate (Z1TS) had a higher rate of aflatoxin B production than one S-strain isolate (Z44A).

6 General conclusions

This study allowed for the first time to link the presence of aflatoxins in cultivated maize to certain characteristics of the soil. It appeared that the content of aflatoxin in maize is directly dependent on the soil content of organic carbon, on the incidence of *A. flavus* in the soil, on the proportion of L-strain isolates in the soil *A. flavus* population and also on the presence of *A. flavus* in the cultivated maize. These four characteristics constitute the primary factors that directly influence the aflatoxin content in maize. In the study, other factors qualified as secondary were detected that influence the primary factors. The latitude of the field location and its height above sea level, the soil pH, the soil texture, the fraction of S-strain isolates in the *A. flavus* population of the soil, and also certain soil cations such as potassium could have an impact on the aflatoxin contamination of maize through the primary factors. In previous studies (Orum et al., 1997; Jaime-Garcia et al., 2006; Cardwell et al., 2002), the primary or the secondary factors as presented here have been involved in the variation of the risk of maize contamination by aflatoxin. Their effects could be focused on the dynamics of *A. flavus* population in the soil or on the *A. flavus* infection of cultivated maize. They can be involved through different mechanisms that could be related to the ecological evolution, the migration or simply by the coincidence of favorable conditions. These conditions could be the growth of a susceptible crop for *A. flavus* infection and aflatoxin contamination and the occurrence of favorable climatic conditions aggravated by bad handling of the harvest. The precise knowledge of these factors and of the magnitude of their impacts is a prerequisite for the design of forecast methods that could allow reliably predicting the risk of maize contamination in Benin. All preliminary works have shown that the soil is the primary reservoir of *A. flavus* propagules (Cotty et al., 1994). The importance of the climate or of some of its components was proven especially in relation with the population characterization of *A. flavus* in the soil (Cardwell et al., 2002; Orum et al., 1997). It was decisive to determine with maximum accuracy the components of the climate or the soil properties that could be important in assessing the risk of aflatoxin contamination in Benin.

To solve that first problem, a survey was carried out in Benin as described in the first chapter. The findings confirmed some results of Cardwell and Cotty (2002) and highlighted the most important aspects of the problem under the specific conditions of Benin. Correlations between some variables were expected, for instance between the incidence of *A. flavus* in the soil or in maize and the aflatoxin content in maize. Other

expected correlations were not proven asking for possible reasons. For instance, despite the strong correlation between the latitude or the sampled field height on one side and the incidence of *A. flavus* in the soil or the maize or the sclerotical composition of *A. flavus* in the soil on the other side, there was no correlation between the field latitude or height, both mediators of the climate, and the content of aflatoxin in maize itself. Yet this last variable is the target variable and the most important to control. It was then clear that the soil and climate conditions alone could not explain the high variability of aflatoxin contamination in the studied area, but that other influencing factors exist. These additional factors that may contribute to an increase or decrease of the risk of maize contamination by aflatoxin are for example the variety of cultivated maize (Zuber et al., 1983), the characteristics of the local population of *A. flavus* under the cultivated maize (Cole et al., 1982) that can be modified by field preparation (Jaime-Garcia et al., 2004), the most common cultural methods (Hell et al., 2003) such as intercropping and rotation. Thus, a station experiment was undertaken to examine new relevant factors such as the importance of the influence of the local virulent and toxigenic population of *A. flavus* in the cultivated field, the maize variety grown and the production system with respect to the build-up of aflatoxin content during storage.

The second chapter was an experimental study demonstrating the impact of the variety on the risk of aflatoxin contamination, the influence of an increase of toxigenic isolates in the field and also the effect of some cropping factors involved in most of the situations leading to high commodity contamination with aflatoxin. The study showed that varieties were a source of variation of the risk of maize contamination by aflatoxin. In this specific case, the length of the cropping cycle of the variety increased the risk. The soil inoculation with toxigenic and virulent *A. flavus* isolates increased the contamination risks (Cardwell et al., 2002; Cotty, 1989). In contrast, the intercropping of maize with cowpea had a noticed effect on the final aflatoxin content only in the improved variety during storage. In this study, strong correlations were observed between the detected aflatoxin levels and other natural living co-inhabitants of *A. flavus* on maize during storage. The most important ones are other microscopic storage fungi but also major storage insect species. As the relationships between aflatoxin concentration and the incidence of major insect pests has been abundantly discussed and their effects on *A. flavus* and aflatoxin well explained (Hell et al., 2000; Setamou et al., 1997, Widstrom et al., 1992), the effects of other main storage fungi, especially of *Fusarium* spp. and *Penicillium* spp., which can contaminate maize in the field or during the storage (Atehnkeng et al., 2008) remained to

be investigated more closely. Therefore chapter three dealt with the co-inoculation of *A. flavus* with other important maize moulds trying to determine how they interact, if they increase or decrease the aflatoxin risk depending on the time when they infect the maize and on the duration of the storage in favorable conditions for *A. flavus* growth and consequently for aflatoxins production.

To understand the effects of the co-inhabitation of *A. flavus* with two of the most encountered moulds on stored maize in the tropical zones, experiments in the field and in the laboratory were set up, in which *A. flavus* was inoculated either on still ripening maize in the field or on maize already harvested. The results varied strongly between the inoculation in the field or in the laboratory. After field inoculation followed by 7 weeks of storage in an incubator set at 31°C, i.e. conditions favorable for *A. flavus*, *Fusarium* spp. had a positive effect on the infection of *A. flavus* but without further consequences during storage. Indeed during storage, the rate of *A. flavus* growth did not vary. The aflatoxin production in maize was not influenced by the inoculation of other non-aflatoxigenic fungi. Only the inoculation of *A. flavus* increased the rate of aflatoxin production. After inoculation in the laboratory, the presence of *Penicillium* spp. allowed a better growth of *A. flavus* during storage, but did not affect the aflatoxin production.

Some observations during these experiments showed that besides artificially inoculated isolates, natural contaminations and infections by other isolates of *A. flavus* occurred, and these infections complicated the understanding of the dynamics of the inoculated isolates in some treatments. Moreover, knowing from the first chapter that a variability in morphological or physiological characteristics (Cotty, 1989; Cotty et al., 1999; Klich, 2007) of the different isolates exists, the idea was raised to check the reaction of some different isolates to the main environmental factors. It became then interesting to see if the common isolates encountered on maize could have different optima in water activity and temperature for their growth or aflatoxin production.

In the corresponding tests, six isolates were checked for the growth and four isolates for aflatoxin production. It was demonstrated that the growth rate of the different isolates varied on the artificial medium. However, the more important conclusion was about the production of aflatoxins, because all four tested isolates were able to produce both kinds of aflatoxin. The conditions for aflatoxin B production were similar for all tested S or L-strain isolates. These conditions were also similar for aflatoxin G production by S-strain isolates. Unexpectedly, the L-strain isolates produced aflatoxin G at lower water activity levels compared to S-strain isolates. It was further shown that the production

of aflatoxin and fungal growth had similar water activity optima, but the optimum temperature for fungi growth was higher than for aflatoxin production. The detected difference in optimal temperatures between growth and aflatoxin production supported previous results by Schindler et al. (1967). From this finding, it can be concluded that maize grains apparently free from *A. flavus* mycelium will be considerably contaminated by aflatoxin anyway. Then it should be taken into account in predicting aflatoxin concentrations that there is no necessary linear link between the presence or absence of mould and the contamination by aflatoxin.

To get reliable data for a model predicting aflatoxin, all four experiments should be repeated at least twice over more seasons and more locations. Further steps should be field experiments in real conditions with thorough observations of all climatic, geographic, and soil parameters and their effects on aflatoxin contamination of maize and other susceptible cultivated crops. Once a model has been constructed and tested, it could be generalized in many other countries in West Africa that have a similar climatic pattern.

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