Institute of Plant Diseases and Plant Protection (IPP)
Department of Horticulture - University of Hanover

and

International Institute of Tropical Agriculture (IITA)
Cotonou - Benin (West Africa)

Population structure and within-field variability of *Aspergillus flavus* field populations of a single small-scale field in West African maize production systems

by

A. Michael Klüken

October, 2003

Supervisors:
PD Dr. C. Borgemeister
Prof. Dr. B. Hau, both Institute of Plant Diseases and Plant Protection (IPP)

Declaration

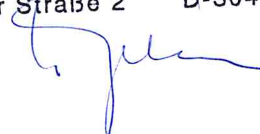
I, the undersigned, declare that this diploma thesis, to the best of my knowledge does not incorporate without acknowledgement any material previously published or written by another person except where reference is made.

It is an original piece of work conducted by myself and has never been submitted or published elsewhere.

Hannover, October 2003

A. Michael Klüken

Abteilung Angewandte Genetik - Universität Hannover
Institut für Zierpflanzenbau, Baumschule und Pflanzenzüchtung
Herrenhäuser Straße 2 D-30419 Hannover



24.10.03

Acknowledgements

I wish to express my gratitude to various persons and institutions that have contributed in one way or the other towards enabling me conduct this research.

I most sincerely thank PD Dr. Christian Borgemeister for accepting to be my academic supervisor. I highly acknowledge his academic advice and support from generating the research idea to the final thesis write up. His support in all questions and stimulating comments conducted the study towards multi faceted research work. Together with him I also thank Prof. Dr. Bernhard Hau my other academic advisor for his support.

I also thank the PhD-student Till Hoffstadt. The multitude of very detailed discussions extended several times the proposed study.

I extend my gratitude to the mycological working group at the International Institute of Tropical Agriculture in Cotonou, Benin. Their friendly 'Welcome' and their continuous support throughout the whole research period contributed to a wonderful sojourn in Benin.

Finally, I thank my lovely wife Meike and our families, whose love and support are always near.

Abstract

In Benin, West Africa, maize is the primary staple human food and it is often contaminated with aflatoxins, carcinogenic naturally occurring substances. *Aspergillus flavus,* a filamentous fungus, is the primary cause of aflatoxin contamination. The *A. flavus* population shows a high variability, which is among other things reflected in the ability of fungal strains to produce aflatoxins.

The atoxigenic *A. flavus* L-strain BN030D was applied to the soil of 5 fields planted with maize within the area of the village Djidja, in order to investigate effect on the fungal community composition residing in the fields (saprophytic fungal community, *A. flavus* community, *A. flavus* L-strain community). Samples were taken from the soil before the strain application at the beginning of the vegetation period. At harvest time samples of soil and maize cobs were taken.

The most frequent **fungal genera** within all soil samples were *Fusarium* spp. (58.9%), *Penicillium* spp. (10.6%), and *Aspergillus*, with determined species *A. flavus* (20.7%) and *A. niger* (9.7%).

Prior to the release of BN030D soil populations of *Fusarium* spp., *Penicillium* spp., and *A. niger* were significant equivalent between treatment and control plots, whereas after application the *A. flavus*, *Penicillium* spp., and *A. niger* soil communities showed significant equivalence between control and treatment (as measured in colony forming units).

No significant differences between control and treatment plots were observed in the **relationship of *A. flavus* L- and S-strains**, neither before nor after the strain release (soil samples). But in maize cob samples from field II significant more L-strains were found in the treatment plot than in the control plot (at harvest time). After the release of BN030D, the *A. flavus* community composition changed slightly towards a higher frequency of *A. flavus* L-strains in all samples from treatment plots.

Within the *A. flavus* L-strain community the results of **vegetative compatibility tests** showed a significant effect of the strain application. In crop and soil samples from treatment plots taken after strain release, 26% and, respectively, 60% of all isolates belonged to the vegetative compatibility group (VCG) of the applied, atoxigenic strain BN030D. Before strain release, less than 1% of all isolates were vegetative compatibility to BN030D. Furthermore, a dispersal of the applied strain from the treatment plot to the adjacent buffer area was observed, possibly due to strains adhering to soil or plant debris driven by the vectors.

The present study has shown that it is possible to change the composition of *A. flavus* communities with a soil application of atoxigenic *A. flavus* strain BN030D. BN030D successfully replaced (excluded) prevailing *A. flavus* strains in a competitive mode without an increase in the overall *A. flavus* infection. Moreover, no influence was shown on the *A. flavus* L- and S-strains relationship or on the prevailing fungal communities. The method of competitive replacement is of great promise in order to reduce aflatoxin contamination in

maize in Djidja, Benin. However, given the scope of this study, only a short-term effect of the strain release could be investigated.

Crucial questions on the various aspects of a competitive replacement method in order to reduce aflatoxin contamination in maize in Benin are currently being answered by the research project in which this study was integrated.

Résumé

Au Bénin (Afrique de l'ouest), le maïs, l'un des aliments de base des populations est souvent contaminé par les aflatoxines, substances naturellement cancérigènes. *Aspergillus flavus,* un champignon filamenteux, est la première source de contamination des aflatoxines. La population d'*A. flavus* montre une grande variabilité, ce qui dénote d´une grande productivité en aflatoxines.

La souche L, atoxigène BN030D de *A. flavus* a été appliquée au sol de 5 différents champs de maïs situés dans le village Djidja (centre du Bénin). Le but de cette étude est d'évaluer l'effet de BN030D sur la composition fongique du sol (c.-à-d. des communautés saprophytes fongiques, des communautés *A. flavus,* des communautés des souches L d'*A. flavus*).

Des échantillons de sols ont été prélevés avant l'application de la souche dans les champs au début de végétation. À la récolte, des échantillons de sols et d´épis de maïs ont été également prélevés.

Les **champignons les plus fréquents** dans les échantillons de sols sont du genre *Fusarium* spp. (58,9%); *Penicillium* spp. (10,6%) et *Aspergillus* spp. avec 20,7% des espèce de *A. flavus* et 9,7% de *A. niger.*

Bien avant l'application de la souche BN030D, la population du sol des souches *Fusarium* spp., *Penicillium* spp. et *A. niger* est significativement équivalente entre le traitement et le contrôle (comme indiqué par les nombres de champignons en colonies par unité formée). Après l'application on a observé une équivalence significative entre le contrôle et le traitement pour *A. flavus*, *Penicillium* spp., et *A. niger.*

Aucune différence significative n'a été observée entre le traitement et le contrôle avant et après l'application en ce qui concerne la relation entre les **souches L et S de *A. flavus***. Par contre, à la récolte la population de la souche L est significativement plus élevée dans les épis de maïs provenant de la parcelle traitée du champ II comparé au contrôle. Après l'application de BN030D, la composition en *A. flavus* a légèrement changée avec une forte proportion de la souche L de *A. flavus* dans tous les échantillons provenant de la parcelle traitée.

Les résultats des **tests de compatibilités végétatives** au sein de la communauté de la souche L de *A. flavus* ont montré un effet significatif de l'inoculation. Après l'inoculation, 60% et 26% des isolats issus respectivement des échantillons de sols et d´épis provenant de la parcelle traitée appartiennent au groupe de compatibilité végétative (GCV) de la souche BN030D. Avant l'inoculation, moins de 1% des isolats étaient végétativement compatibles à BN030D. De plus, une dispersion de la souche BN030D a été observée à partir de la parcelle traitée vers les parcelles adjacentes, à cause de l'adhérence de la souche au sol ou à des débris végétaux transportés par des vecteurs.

Le présent travail a montré qu'il est possible de changer la composition en communauté d'*A. flavus* du sol par l'application de souche atoxigène BN030D de *A. flavus.* BN030D a

remplacé (exclu) avec succès les souches prévalentes de *A. flavus* par un mode compétitif sans augmenter l'incidence de l'infection. De plus aucune influence n'a été observée sur la relation entre les souches L et S ou sur la prévalence des communautés fongiques (saprophytes).

La méthode de remplacement compétitif est prometteuse pour une réduction de la contamination du maïs par l'aflatoxine à Djidja au Bénin. Cependant, avec cette étude seul l'effet a court-terme a fait l'objet d'investigation. Divers aspects de la méthode de remplacement compétitif en vue de réduire les contaminations du maïs par les aflatoxines font actuellement l'objet d'un projet « aflatoxin réduction in maïs » au Bénin.

Zusammenfassung

Mais stellt für die Bevölkerung Benins (Westafrika) das wichtigste Grundnahrungsmittel dar, ist jedoch häufig mit krebserregenden Mykotoxinen kontaminiert, den sogenannten Aflatoxinen. Der niedere Pilz *Aspergillus flavus* ist die wichtigste Ursache für die Aflatoxinkontamination. Die überaus große Variabilität und Vielfalt einer *A. flavus* Gemeinschaft zeigt sich unter anderem in der Fähigkeit einzelner Isolate, diese Aflatoxine zu produzieren.

Der nicht-toxische L-Stamm BN030D von *A. flavus* wurde auf 5 Maisfeldern in der Gegend des Dorfes Djidja ausgebracht, um einen möglichen Einfluss auf die Zusammensetzung der Pilzpopulationen zu untersuchen. Dies schließt die sarophytischen, pilzlichen Gemeinschaften, die *A. flavus* Gemeinschaften, und die Gemeinschaften der L-Stämme von *A. flavus* ein.

Bodenproben wurden vor der Ausbringung des Stammes zu Beginn der Vegetationsperiode gesammelt. Zur Ernte wurden dagegen von den Versuchsfeldern neben den Boden- auch Maisproben entnommen.

Die **Pilze der Gattungen** *Fusarium* spp. (58.9%), *Penicillium* spp. (10.6%), und *Aspergillus* spp. waren in allen Bodenproben am häufigsten vertreten. In der Gattung *Aspergillus* wurden die Arten *A. flavus* (20.7%) and *A. niger* (9.7%) unterschieden.

Signifikante Äquivalenz (Gleichwertigkeit) zwischen Behandlungs- und Kontrollflächen bezüglich der Kolonien bildenden Einheiten (colony forming units) konnte für *Fusarium* spp., *Penicillium* spp. und *A. niger* an Bodenproben gezeigt werden, die vor der Ausbringung von BN030D den Flächen entnommen wurden. Nach dem Ausbringen des Stammes BN030D zeigten Bodenproben, dass *A. flavus*, *Penicillium* spp., and *A. niger* signifikant äquivalent vorlagen.

Für das **Verhältnis von L- und S-Stämmen** innerhalb der *A. flavus* Gemeinschaften konnten keine signifikanten Unterschiede zwischen Kontrolle und Behandlung festgestellt werden, weder in Bodenproben vor noch nach der Applikation des L-Stammes BN030D. Maisproben, die von Feld II gesammelt wurden, zeigten jedoch signifikant mehr L-Stämme in der Behandlung als in der Kontrolle. In Boden- und Maisproben der Behandlungsparzellen war ein Trend zu einer höheren Anzahl an L-Stämmen zu erkennen.

Innerhalb der *A. flavus* L-Stammpopulation zeigten die Ergebnisse der **vegetativen Kompatibilitätstests**, dass durch die Applikation eine signifikante Wirkung festgestellt worden ist. Innerhalb der Behandlungsparzellen zeigten 26% bzw. 60% aller Isolate von Maiskolben- bzw. Bodenproben, die nach der Applikation entnommen wurden, vegetative Kompatibilität mit dem ausgebrachten, atoxischen Stamm BN030D. Vor der Stammausbringung waren weniger als 1% aller *A. flavus* L- Stammisolate vegetativ kompatibel mit dem Stamm BN030D. Es konnte die Verbreitung des ausgebrachten

Stammes von der Behandlungsparzelle in eine Pufferzone festgestellt werden. Vermutlich konnten an Erd- oder Pflanzenpartikeln haftende Stämme mit Vektoren verdriftet werden.

Die vorliegende Studie zeigt, dass eine Änderung der Zusammensetzung der *A. flavus* Gemeinschaften durch die Applikation des nicht-toxischen *A. flavus* Stammes BN030D möglich ist. Es ist davon auszugehen, dass der Stamm BN030D die vorherrschende *A. flavus* Gemeinschaft erfolgreich ersetzt hat, in einer Art konkurrierendem Ausschluss. Nach der Applikation konnte in keiner Probe eine erhöhte Infektion von *A. flavus* festgestellt werden. Außerdem konnte weder ein Einfluss auf das Verhältnis der L- und S-Stämme von *A. flavus* noch auf die vorherrschenden, pilzlichen Gemeinschaften gezeigt werden. Die Methode des konkurrierenden Ausschlusses ist demnach für die Reduzierung der Maiskontamination mit Aflatoxinen in Djidja, Benin, sehr vielversprechend. Vor dem Hintergrund des Umfangs der Studie muss festgehalten werden, dass nur eine kurzzeitige Wirkung mit der Stammapplikation untersucht werden konnte.

Ergänzende Fragen in bezug auf die verschiedenen Aspekte der Methode des konkurrierenden Ausschlusses werden zur Zeit in einem weiteren Projekt beantwortet. Das Ziel des gesamten Forschungsprojektes, in das die vorliegende Studie eingebettet wurde, ist die Reduzierung der Maiskontamination mit Aflatoxinen in Benin, Westafrika.

Table of contents

Title....i
Declaration....ii
Acknowledgements....iii
Abstract....iv
Résumé....vi
Zusammenfassung....viii
Table of contents....x
List of figures....xiii
List of tables....xiv
Symbols and Abbreviations....xv

1. General introduction....1
1.1. Introduction of the problem....1
1.2. Objectives of the study....3

2. *Aspergillus flavus*....4
2.1. The genus *Aspergillus*....4
2.2. Species within the genus *Aspergillus*....5
2.3. L- and S-strains of *A. flavus*....8
2.4. Characteristics of *Aspergillus* section *flavi*....10
2.5. Contamination cycle....12

3. Aflatoxin....15
3.1. Characteristics of aflatoxin....15
3.2. General effects of aflatoxin....16
3.3. Effects of aflatoxin in humans....18
3.4. Impact of aflatoxin in food production....20

4. Factors influencing Aspergillus flavus infection and aflatoxin formation in maize....22
4.1. Climatic factors....22
4.2. Biological factors....24
4.3. Agricultural factors....25

5. Competitive replacement 28
5.1. Introduction 28
5.2. History 28
5.3. Requirements 28
5.4. Engagement and constrains 30
5.5. Overview on research activities 30
5.6. Prospects of biological control strategies 31
5.7. Vegetative compatibility (VC) introduction 32
5.8. VC characteristics and application 33

6. Materials and methods 35
6.1. Selection of the study location and experimental design 35
6.2. Field preparation and strain application 35
6.3. Sampling of soil and maize 36
6.3.1. Soil collection 36
6.3.2. Maize cob collection 37
6.4. Isolation of fungi from soil and maize samples 38
6.4.1. *A. flavus* isolation form soil samples 38
6.4.2. *A. flavus* isolation form maize samples 39
6.5. Vegetative compatibility group (VCG) analysis 40
6.5.1. Generation of *nit* mutants 40
6.5.2. Complementation tests 41
6.6. Research hypothesis 43
6.7. Data analysis 43

7. Results 45
7.1. Fungal population analysis 45
7.1.1. Propagule density (CFU) in soils 45
7.1.2. Soil samples before strain application (pre-intervention) 45
7.1.3. Soil samples after strain application (post-intervention) 46
7.1.4. Fungal presence on maize cobs 47
7.1.5. Comparison of propagule densities of fungal communities in soil samples pre- and post-intervention (change over time) 47
7.2. Surface damage of insects on maize grains 49
7.3. Surface damage of fungi on maize cobs 49
7.4. *A. flavus* community composition (L- and S-strain relationship) 50

7.5. VCG tests....50
7.5.1. Frequency of VCG BN030D among *A. flavus* L-strain isolates from pre-intervention soil samples....51
7.5.2. Frequency of VCG BN030D among *A. flavus* L-strain isolates from post-intervention soil samples....51
7.5.3. Frequency of VCG BN030D among *A. flavus* L-strain isolates from post-intervention maize samples....52
7.6. Dispersal of BN030D into the buffer area (soil samples)....53
7.7. Trial design....54

8. Discussion....55
8.1. Composition of saprophytic fungal soil populations....55
8.1.1. Fungal population analysis from soil samples....55
8.1.2. Fungal population analysis from post-intervention maize samples....56
8.1.3. Changes in fungal populations over time....56
8.2. Composition of *A. flavus* soil communities....57
8.2.1. Natural changes....57
8.2.2. Effect of strain release....58
8.3. Effect of the BN030D release on the composition of L-strain field communities....58
8.3.1. In the soil....58
8.3.2. On maize cobs....59
8.3.3. Dispersal of the atoxigenic strain BN030D....59

9. Conclusion....63

10. References....64

List of figures:

Fig. 1: Habit sketches of *Aspergillus* species taken originally from culture............................5
Fig. 2: Relationship among *Aspergillus* species..6
Fig. 3: Four *A. flavus* strains growing within a Petri dish on V8 medium.............................10
Fig. 4: Chemical structure of AFB1...15
Fig. 5: The collection of soil samples in maize fields in Benin...36
Fig. 6: Scheme of one field (experimental block) 37
Fig. 7: Section of a Petri dish containing MRB medium...38
Fig. 8: Developing fungi on the surface-disinfected Petri..40
Fig. 9: Two *nit* mutants developing from one wild type isolate of *Aspergillus flavus*.............41
Fig. 10: Stabilization of the "good" *nit* mutant..41
Fig. 11: VCG test performance...42
Fig. 12: VCG test results obtained after 12 days showing positive compatibility.....................42
Fig. 13: VCG test results obtained after 19 days showing a non-respondent reaction..........42
Fig. 14: Mean propagule density in CFU g^{-1} (± SD) soil of the most frequent fungi as compared by treatment vs. control before strain application..................................46
Fig. 15: Mean propagule density in CFU g^{-1} (± SD) soil of the most frequent fungi as compared by treatment vs. control after strain application.....................................46
Fig. 16: Mean propagule densities in CFU g^{-1} (± SD) soil samples taken pre- and post-intervention of the most frequent fungi recorded in the control plots.......................48
Fig. 17: Mean propagule densities in CFU g^{-1} (± SD) soil samples taken pre- and post-intervention of the most frequent fungi recorded in the treatment plots....................48
Fig. 18: Mean numbers (± SD) of insects per maize cob in control and treatment plots........49
Fig. 19: Mean percent (± SD) decay of surface-infected maize grains per cob.....................50
Fig. 20: VCG test results of *A flavus* L-strain isolates from pre-intervention soil samples.....52
Fig. 21: VCG test results of *A flavus* L-strain isolates from post-intervention soil samples...52
Fig. 22: VCG test results of *A. flavus* L-strain isolates from post-intervention crop samples...53
Fig. 23: Proportion of isolates from pre-intervention soil samples with positive, negative or non-responding (non-respond.) compatibility to BN030D.......................................53
Fig. 24: Proportion of isolates from post-intervention soil samples with positive, negative or non-responding (non-respond.) compatibility to BN030D.......................................54

List of tables:

Tab. 1: Short classification of some *Aspergillus* species....7

Tab. 2: Mean propagule density in CFU g^{-1} soil of the most frequent fungi....45

Tab. 3: Mean percentage (± SD) of grains internally infected by different fungi....47

Tab. 4: Mean propagule density (± SD) in CFU g^{-1} soil of the most common fungi....47

Tab. 5: Relationships of L- and S-strains of *A. flavus*....51

Tab. 6: Number of *A. flavus* isolates showing positive, negative or non-respondent results in the VCG tests....52

Symbols and Abbreviations

AFB, AFG, AFM	divers metamorphoses of aflatoxins
AFPA	*Aspergillus flavus* and *parasiticus* agar
AIDS	acquired immune deficiency syndrome
BGYF	bright-green-yellow-fluorescence
C.	*Carpophilus* spp.
cf.	confer
CFU	colony forming units
CI.	confidence interval
cnx mutant	mutant in one of several genes for a molybdenum-containing cofactor
Col.	Coleoptera
Cl	*C. leucotreta*
Cq	*C. quadricollis*
CYA	Czapek yeast extract agar
CZ medium	Czapek-V-medium
DMR white-modified	a downy-mildew-resistant IITA maize variety
DNA	desoxy ribonucleic acid
e.g.	exempli gratia (lat. = for instance)
et al.	et alii (lat. = and other)
FAO	The Food and Agriculture Organisation
fig.	figure
FMS	Forest Mosaic Savanna
GTZ	(deutsche) Gesellschaft für technische Zusammenarbeit
Hem.	Hemiptera
HIV	human immunodeficiency virus
Hom.	Homoptera
hsi	heterokaryon self incompatibility
Hym.	Hymenoptera
i.a.	inter alia (lat. = among other)
i.e.	id est (lat. = that is to say)
IITA	international institute for tropical agriculture
Lep.	Lepidoptora
MEA	Malt extract agar
MIT medium	chlorate medium
Mn	*M. nigrivenella*
MRB medium	Modified Rose Bengal medium
NGS	Northern Guinea Savanna
niaD$^-$ mutant	mutant in the nitrate reductase structural locus

nit mutant	nitrate non-untilising auxotrophs (or mutants)
no.	number
NPK	nitrogen phosphate kalium (potassium) fertilizer
n.s.	not significant
dsRNA	double strand ribonucleic acid
s.	significant
SEL medium	selective medium
SD	standard deviation
SGS	Southern Guinea Savanna
SONAPRA	Société National sur la Promotion Agricole
SS	Sudan Savanna
SSA	Sub-Saharan Africa
Sz	*S. zeamais*
Tab.	table
UN	United Nations
U.S.	United States (of America)
UPGMA	unweighted paired group method with arithmetic averaging.
V8 medium	vegetable juice medium with 8 vitamins
VC	vegetative compatibility
VCG	vegetative compatibility group
vs.	versus

1. General introduction

1.1. Introduction of the problem

Aspergillus flavus Link, a world wide distributed filamentous fungus, infects maturing plants in the field. The fungus is more frequent in subtropical and tropical zones because it requires higher temperatures for its development (WILSON and ABRAMSON, 1992). *Aspergillus flavus* is usually growing as a saprophyte on dead plant debris, but if conditions are suitable (heat, drought, stressed plants) heavy infections are observed on mature crops (DOMSCH *et al.,* 1993). In the field, fungal populations show a high variability (ROSS *et al.*, 1979), which is - among other things - reflected in the ability of fungal strains to produce mycotoxins (COTTY *et al.*, 1994). Commonly *A. flavus* communities are divided into two morphs, L- and S-strains, where the latter one produces consistently higher amounts of mycotoxins (COTTY, 1989). These secondary metabolites are called aflatoxins and are produced by several species within the genus *Aspergillus*. They represent the most carcinogenic naturally occurring substances known so far. Liver cancer, the suppression of the immune system and several diseases have been linked to intake of aflatoxin-contaminated food (COTTY et al., 1994). Often, such food is consumed without knowledge of potential health impacts for children or adults because in the developing world in general, and in Sub-Saharan Africa (SSA) in particular, official monitoring of mycotoxin contamination levels is rare (ADHIKARI *et al.,* 1994). *Aspergillus flavus* is the primary cause of aflatoxin contamination in maize, cottonseed, and tree nuts. Although *A. flavus* infection and subsequent aflatoxin contamination of maize (*Zea mays* ssp. L., Gramineae) can arise during post-harvest storage and processing, infections usually commence prior to harvest during periods of late-season drought stress as maize grains are maturing. At this time the maize plant is most vulnerable to degradation by this mycotoxic fungus (COTTY *et al.,* 1994).

The aflatoxin problem is of particular concern in Benin, a coastal country in West Africa. First, in Benin maize is the most important cereal crop, produced on 517,985 ha in 1996/1997 with a total production of 504,506 to for the same year (HELL *et al.*, 2000). Nearly all maize production is used for human consumption, i.e. it is the primary staple human food. In some regions maize is consumed up to three times a day and is also used as weaning food for babies (CARDWELL and COTTY, 2001). Thus, the risk of exposure to aflatoxin in Benin is particularly high. Second, in West Africa 98% of people are serologically aflatoxin-positive (WILD *et al.,* 1991), and in Benin and Nigeria high levels of aflatoxins have been detected in pre- and post-harvest maize (SÉTAMOU *et al.,* 1997; HELL *et al.,* 2000). Third, farmers in West Africa are more aware of visible destruction and grain losses caused by insects and vertebrates (rats, birds, etc.), whereas fungal, bacterial or viral diseases are less considered. Many farmers neither know about such pathogens nor their secondary metabolites like aflatoxins (T. HOFFSTADT, pers. comm.).

The only known preventive measure to reduce pre-harvest aflatoxin contamination in maize is the complete clearing of plant debris in fields after harvest (COTTY *et al.,* 1994). During the vegetation period, no practical or reliable methods are known to entirely prevent an infection of maize plants by *A. flavus* and its subsequent aflatoxin production. Recently, however, releases of atoxigenic L-strains of *A. flavus* were shown to considerably reduce the aflatoxin contamination of cotton in the US (COTTY, 1989, 1997). For this, in field studies autoclaved wheat colonised by an atoxigenic L-strain was initially introduced into the soil of cotton plots. The overall extend of *A. flavus* infections did not decrease but the aflatoxin content in cottonseeds was significantly reduced (COTTY, 1994b). The rationale of this approach, often termed 'competitive replacement', was that the bio-competitive agent dominates the soil microflora and prevents the build-up of native, aflatoxin producing strains of *A. flavus* that usually predominate during the late-season drought. In other words, the toxigenic strains found naturally in the soil would be replaced by an atoxigenic strain added in an augmentative manner to the soil.

West Africa has a great potential for use of the competitive replacement approach, since its environment generally facilitates consistent and continuous natural infection of *A. flavus* and subsequent aflatoxin contamination.

This study was part of a larger research project which primary goal is to develop a biological control method for *A. flavus*, using the competitive replacement method, adapted to West-African small-scale maize production. Experiments were conducted on the Benin station of the International Institute of Tropical Agriculture (IITA) in Cotonou within the framework of the BMZ-funded project "Development and Evaluation of Strategies to reduce Aflatoxin Contamination in maize in Benin, West-Africa".

The village Djidja in the Borgou province of central Benin was chosen as trial location because it is situated in an aflatoxin high-risk area of Benin (HELL *et al.,* 2000). High-risk area means that the local environment of and around Djidja favours natural infections of *A. flavus* and subsequent aflatoxin contamination. Furthermore, an atoxigenic L-strain of *A. flavus* was obtained from this region and it has been shown that this atoxigenic phenotype did not change and remained stable over time (CARDWELL and COTTY, 2000).

1.2. Objectives of the study

The primary objective of this study was to detect changes in composition of fungal communities after an application of the atoxigenic *A. flavus* L-strain BN030D on five fields in Djidja. For this, we assed the influence of a strain application on the predominance of the vegetative compatibility group (VCG) of BN030D, on L- and S-strain relationships, and on the fungal communities prevailing in the soil and on the crop. Furthermore, the spread of the released strain from treatment plots into a non-treated buffer area was evaluated.

2. *Aspergillus flavus*

2.1.The genus *Aspergillus*

The genus *Aspergillus* (Aspergillaceae) belongs to the group of Deuteromycetes of the fungi imperfecti (Hymphomycetes: Monoliales). The mainstay for *Aspergillus* spp. identification is their morphology. The species are recognized when grown on special media and examined macro-morphologically for size and colour, and micro-morphologically for aspergillum characteristics and other traits useful in identification (SAMSON *et al.*, 1995). Further characteristics for grouping are metabolite production, enzyme profiles, immunoassays (ELISA) and other serological and molecular methods.

The genus *Aspergillus* is characterized by an aspergillum, consisting of a conidiospore stipe terminating in a vesicle, which bears one or two layers of synchronously formed specialized cells, the phialides (sometimes termed sterigmata), and asexually formed spores, called conidia. The radiating conidia heads are threaded like a string of pearls and their form resemble a piston or a ray of a watering can (PÜTZ and REUTER, 2002). An aspergillum with phialides borne directly on the vesicle, is referred to as an uniseriate aspergillum. In case of a second layer of specialized cells on the vesicle, the aspergillum is referred to as biseriate and these cells are called metulae, representing a layer of subtending cells and bearing small whorls of phialides (DOMSCH *et al.,* 1993). The basal portion of the stipe is usually curved and forms a "foot cell", which is often typical and useful for recognising the conidiogenous structures of the genus (Fig. 1).

Some species of *Aspergillus* that produce typical *Aspergillus* conidial states also have a telemorphic state produced by sexually formed ascosporic states and hence can be found in the Ascomycetes (SAMSON *et al.*, 1995). The mycelium of *Aspergillus* spp. usually consists of hyphae showing a characteristically Y-branching (DOMSCH *et al.,* 1993).

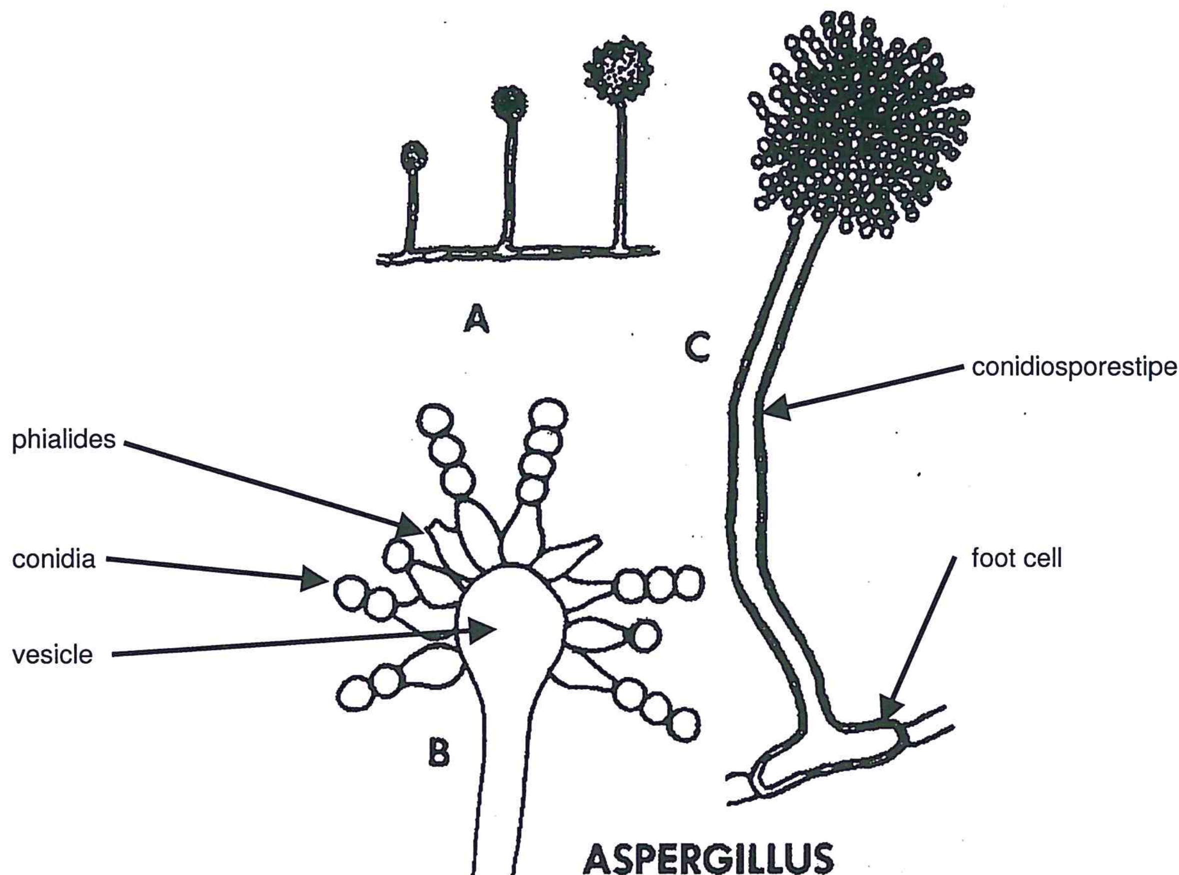


Fig. 1: *Aspergillus* species taken originally from culture (modified from BARNET and HUNTER, 1987).
A: developing conidiophore stipe
B: conidiophore with conidial heads.
C: conidiophore habit sketch.

2.2. Species within the genus *Aspergillus*

The following figure (Fig. 2) taken from EGEL *et al.* (1994) may provide a survey of the most common *Aspergillus* species occurring in natural substrates and their degree of affinity. *Aspergillus sojae* Sakag. & Yamada and *A. oryzae* (Ahlburg) Cohn possibly derived from *A. flavus* and *A. parasiticus* Speare, respectively, and were thus recently regrouped (EGEL *et al.,* 1994, ELIAS and COTTY, 1995). These authors stress that *A. parasiticus, A. flavus*, and especially *A. tamarii* Kita and *A. nomius* Kurtzman naturally occur in fields in highly divers forms. KLICH and PITT (1988) found that *A. tamarii* is closer related to *A. flavus, A. parasiticus,* and *A. nomius* than the three are to each other. Likewise, ELIAS and COTTY (1995) suggested one common ancestor based on their results using infective dsRNA (double strand ribonucleic acid) for all *Aspergillus* species. To date, the full DNA (desoxy-

ribonucleic acid) sequence of *A. flavus* is not known, but for example the DNA sequence of *A. niger* Tiegh is estimated to consists of approximately 30 million base pairs (DIJK and GRIEß, 2000).

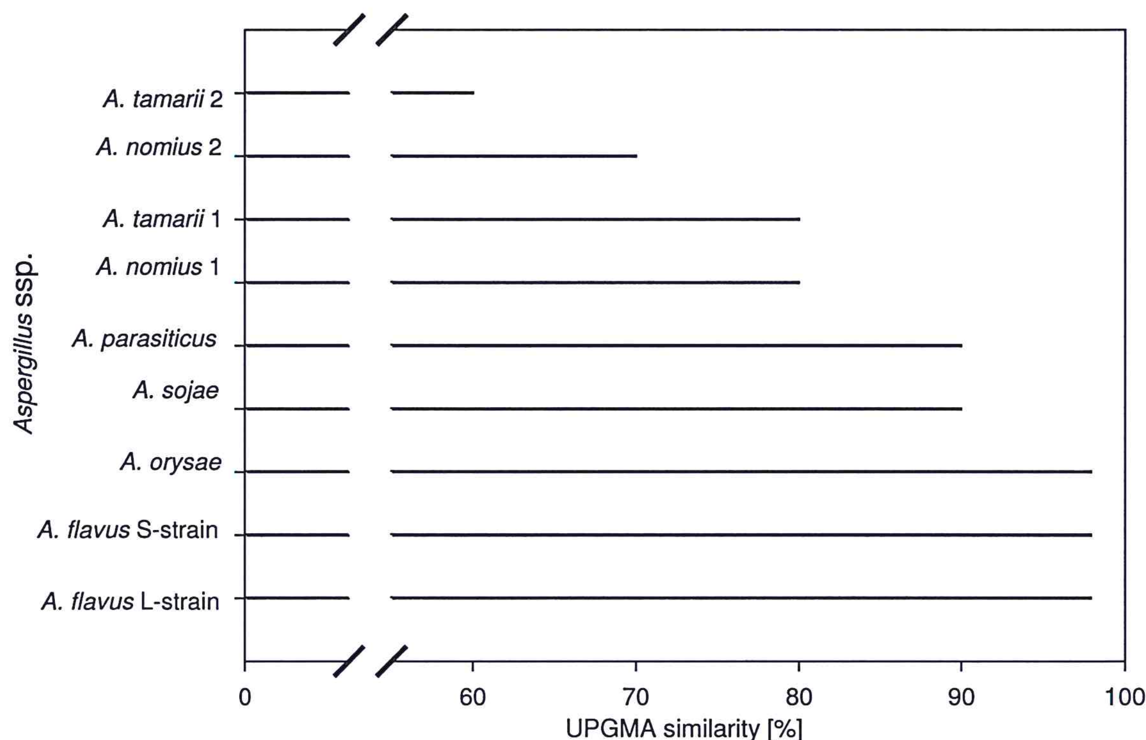


Fig. 2: Relationship among *Aspergillus* species, modified from EGEL *et al.* (1994). The spectrum of variability among *A. nomius* and *A. tamarii* isolates is reflected in numbers 1 and 2.
UPGMA = unweighted paired group method with arithmetic averaging.

Individual strains within the three aflatoxin-producing species *A. flavus, A. parasiticus,* and *A. nomius* occur in a high variability (ROSS *et al.,* 1979). Polymorphism in the Taka-amylase gene indicates, that strains may intermediate between the three species (EGEL *et al.,* 1994). The tremendous variability is reflected in genetic, physiological (production of secondary metabolites) and morphological characters (BROWN *et al.*, 1991; COTTY *et al.*, 1994).
The high variability within these three *Aspergillus* species suggests multiple adaptations and no specificity in host-parasite interactions. *Aspergillus flavus* appears to be adapted to aerial and foliar environments, whereas *A. parasiticus* seems to be better adapted to the soil environment. The latter is dominant in groundnuts, whereas the previous is more frequently found in maize crops, cottonseed and tree nuts (DIENER *et al.,* 1987). Even if the same type of a strain infects closely related crops or insects, the general interactions may reflect more an ecological adaptation and a life strategy (than specific host specific interactions). Similarly, aspergillosis-causing strains in poultry and humans are not host specific (COTTY *et al.,* 1994).

Tab. 1: Short classification of some *Aspergillus* species (modified according to KLICH and PITT, 1988; DOMSCH *et al.*, 1993; CARLSON 1996; PÜTZ and REUTER, 2002)
(AFPA = *Aspergillus flavus* and *parasiticus* agar; CYA = Czapek yeast extract agar, MEA = Malt extract agar).

Aspergillus species	Characteristics of metulae and conidiophores	Characteristics of conidia and colonies	Growth characteristics and general
A. flavus Aflatoxin production in several isolates	Metulae present in many conidiophores; sclerotia sometimes present, variable in shape and size; conidiophores hyaline, 0.4-1mm long, rough walled, uncoloured; phialides 8-12 x 3-4µm	Spreading yellow-green colonies, old colonies remaining yellow-green or greenish brown, smooth to finely rough conidia: 3-6µm, conidia globosely to finely echinulate, brown	Colonies floccose, especially centrally, bright orange on AFPA, conidial mass sometimes turning pink on anisic acid
A. parasiticus Aflatoxin production consistent in most isolates	Metulae absent, conidia heads usually radiate, conidia prominently echinulate (3-4 nucleate), uniseriate phialides, sclerotia (brown to black) mostly absent, conidiophores coarsely roughened	Even old colonies remaining dark yellow-green or greenish brown (more pronounced green tints than other members of the group); distinctly roughened, dark yellow-green conidia 3.5-6µm	AFPA – positive, mycelium usually inconspicuous, occasionally with floccose tufts, conidial mass sometimes turning pink on anisic acid, growth is restricted in presence of 10% NaCl
A. tamarii No aflatoxin production	Metulae present in many conidiophores, conidiophores generally roughened	Young colonies brown, old colonies more or less brown, conidia large thick-walled rough, coarsely tuberculate, 5-6.5 µm, conidia not prominently echinulate	AFPA – negative, conidial mass on CYA+0.5% anisic acid regularly pink; important in food fermentation in Asia
A. restrictus Aflatoxin production	Lacking metulae, conidiophores 75-200µm long, hyaline and smooth-walled with a flask-shaped or hemispherical vesicle, phialides limited to upper half of vesicle, uninucleate	Cylindrically born conidia, conidia heads strictly columnar, conidia narrowly ellipsoidal, echinulate, mostly 4.5-5 x 3-3.5µm, dark olive green	AFPA – negative, slow growth on MEA, CYA, no particular adaptation to regions with higher temperatures; colonies growing very restrictedly on CYA, but spreading broadly on MEA with 40% sucrose
A. niger No aflatoxin production	Conidiophores (1.5-3mm tall) arising from long, broad, thick-walled, mostly brownish, sometimes branched foot cells; conidia in large radiation heads, mostly globose, irregularly roughened, uninucleate; sclerotia production possible and inversely correlated to conidia production	Alimentary saprophyt; germ of Aspergillosis of the skin, lung, black conidial, 4-5µm	Serves for citron and kojic acid, Aspergillin, Penicillin, Flavacin, in industry; more a soil invader than inhabitant; low resistance to fungicide benomyl, and the fumigants allyl alcohol and formaldehyde, Sporostatic factor (nematicidal and insecticidal substance)

2.3. L- and S-strains of *A. flavus*

Due to its high variability in terms of physiology (e.g. virulence, culture characteristics, aflatoxin production), morphology (e.g. size of sclerotia) and genetics (DNA sequence), *A. flavus* is commonly divided into two morphs, i.e. the so-called L- and S-strains (RAPER and FENNELL 1965; HESSELTINE *et al.*, 1970; SAITO *et al.,* 1986; COTTY, *et al.,* 1994). However, not all researchers have agreed to this classification. Some regard the S-strains as a discrete species (T. HOFFSTADT, pers. comm.). In the present study L- and S-strains were assumed to represent at least two different morphs within *A. flavus* (Fig. 3). Both morphs are subdivided into distinct groups, so called vegetative compatibility groups (VCG) (for details see chapter 5). Gene flow within the morphs is possible but limited by the vegetative compatibility system (PAPA, 1986). Thus, communities rather than a single *A. flavus* population occupy sites (BAYMAN and COTTY, 1992; ORUM *et al.*, 1997). L- and S-strains are composed of many different VCGs (COTTY and BAYMAN, 1993) and individual fields may contain more than 50 VCGs (BAYMAN and COTTY, 1992).

Isolates of S-strains produce numerous small sclerotia (< 300 µm in diameter) and only few conidia. On average, in culture and in developing cottonseeds more aflatoxin is produced by S- than L-strains (COTTY, 1989). The latter one is producing (only) few large sclerotia (> 300 µm in diameter) and many conidia (Fig. 3). In general, L-strain isolates produce less aflatoxin, with some producing no aflatoxin at all. Both types of strains differ in several physiological traits (COTTY, 1989; COTTY *et al.,* 1994). The strong diversity in aflatoxin production is a special characteristic of *A. flavus.* For example, *A. parasiticus* produces aflatoxins rather constant and in similar amounts (DORNER *et al.*, 1995). COTTY (1989) found that on potato dextrose agar *A. flavus* S-strains produce many sclerotia greater than 50 cm^{-2}, whereas L-strains produce none to approximately 10 cm^{-2}. On V-8 medium differences in sclerotia production are even greater: S-strains produce many (10 – 50 cm^{-2}), L-strains none to only few (less than 10 cm^{-2}) (Fig. 3). Thus, in general S-strains produce more sclerotia in different media and in a greater range of temperatures. The potential to produce pectinases is consistent and variable in L- and S-strains, respectively (COTTY *et al.*, 1990). Although all *A. flavus* groups and strains occur in the soil, COTTY *et al.* (1994) hypothesised the air and soil to be the primary habitats of L- and S-strains, respectively. Among the S-strains, small sclerotia and reduced sporulation may be a better adaptation to infect and utilise resources in the soil (e.g. plant- or insect debris) and to survive. Sclerotia in fungi are elaborate chemical defence systems directed at protecting them from insect, bacteria or fungi. In *A. flavus* sclerotia with high concentrations of aflatoxins inhibit many bacteria as well as caterpillars, or they act like a shield to prevent decomposition (COTTY *et al.,* 1994).

According to BAYMAN and COTTY (1992) L- and S-strains are two different morphs, and not correlated with any culture substrate or geographical region. Their cladistic analysis of DNA

polymorphisms confirmed both the consistence of closely related groups of strains with identical loci, and the strong association between these groups and DNA polymorphisms within the two morphs.

HARPER (1977) compared the differences between L- and S-strains with those between r- and K-selected species. K-selected species are characterised by a low reproduction rate, long life cycles, and comparatively low natural mortality. Thus their population densities often correspond to the carrying capacity of a habitat. R-selected species are antonyms of K-selected species, and their population densities often exceed the carrying capacity of their habitats (HÖGGER, 1994). L-strains, which produce far more conidia, should be favoured for colonization (typical for r-selected species) whereas S-strains rely on structures like sclerotia that enable them survive unfavourable conditions, and on aflatoxins and other metabolites production (thus resembling more K-strategists). The latter strategy is more resource-intensive but may be advantageous to out-compete other microbes.

Levels of aflatoxin contamination on plants caused by *A. flavus* L- or S-strains are equal although S-strain produced higher amounts of the toxin (COTTY, 1989). This is due to the fact that L-strain isolates are generally more virulent than S-strain isolates at deteriorating the crop, i.e. they have a higher infectious potential. The pathogenic aggressiveness implies the ability of an isolate to attack another organism (e.g. a crop), to overcome its resistance and to benefit in terms of its own development.

Based on polymorphism data of the Taka-amylase A gene EGEL *et al.* (1994) developed an UPGMA-dendrogram. The dendrogram revealed 98% similarity between 20 L- and S-strains, including several atoxigenic and toxigenic isolates. S-strains might have derived from L-strains since BAYMAN and COTTY (1992) showed that in experiments with DNA polymorphisms all S-strain isolates formed a single clade. They concluded that L- and S-strains have at least diverged in evolutionary terms to the level of sister species. Consequently several authors suggest the atoxigenic forms to be wild types and the toxigenic ones mutants (BAYMAN and COTTY, 1992; COTTY *et al.,* 1994). Usually mutants have a lower selective advantage, because of their increased genetic load, possibly explaining the higher number of atoxigenic isolates in *A. flavus* (LEMKE *et al.,* 1989; BILGRAMI and CHOUDHARY, 1993).

The S-strain morphotype may be adapted to lower rainfall and higher temperatures. High incidences of S-strains are frequently found in relatively low rainfall, high temperature regions of North America (COTTY, 1993; ORUM *et al.,* 1999; CARDWELL and COTTY, 2001).

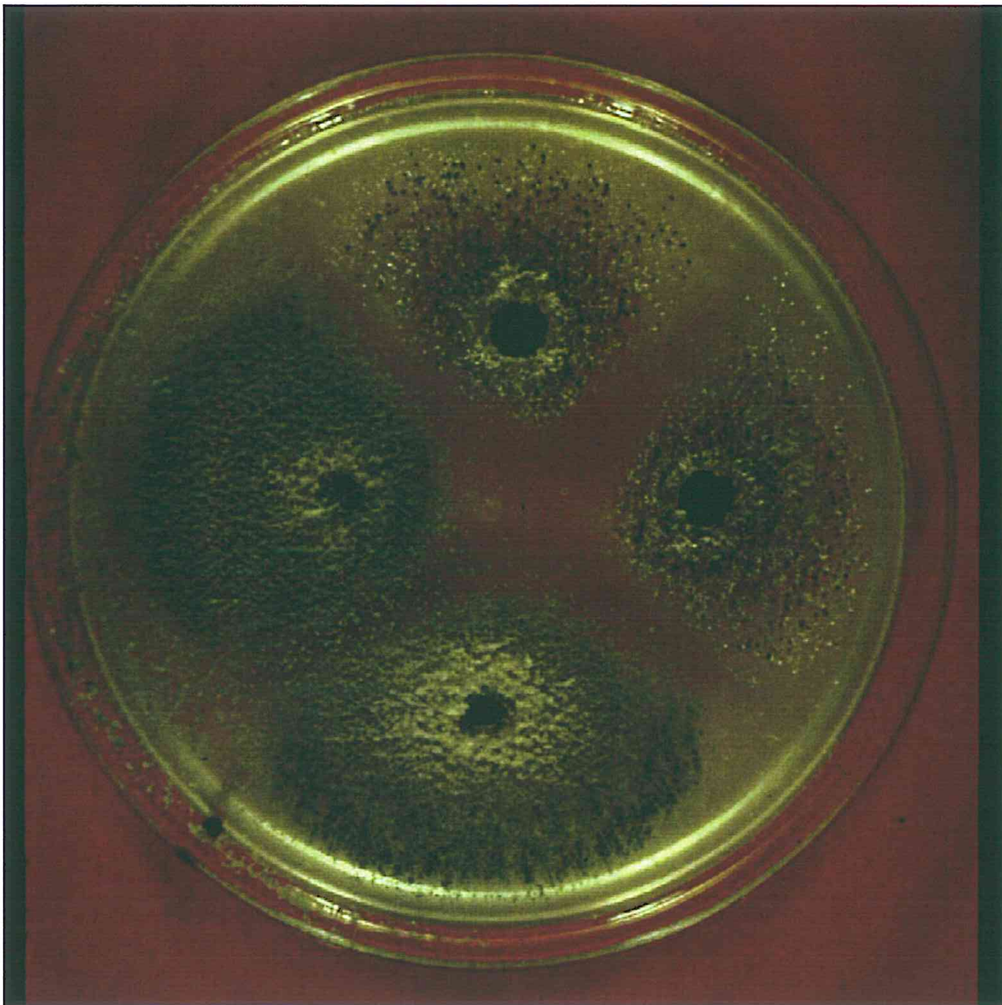


Fig. 3: Four *A. flavus* strains growing within a Petri dish on V8 medium (vegetable juice medium with 8 vitamins): L-strains on the bottom and left, S-strains on the top and right (ORUM and NELSON, 1999).

2.4. Characteristics of *Aspergillus* section *flavi*

Aspergillus spp. is found worldwide in air and soil, especially in the tropics and subtropics (PAYNE, 1983). In the Northern hemisphere the fungus increases during autumn and winter (VILAR, 2003). Originally, *Aspergillus* spp. belongs - as mould fungi - to the soil biota. The species occur in different soil types and the distribution is not limited by pH or soil depth. For instance *A. flavus* was frequently isolated from soil samples taken at 45 cm depth (DOMSCH *et al.,* 1993). Fungi within the *Aspergillus* section *flavi* are capable of utilizing many commodities as substrates and hence can grow on cotton cloth, hemp, jute, upholstery, mattress from cellular material, on wood, paper and wallpaper. The tolerance of the fungus is so high that it can even grow on gallotannin, sinigrin and *n*-alkenes (DOMSCH *et al.,* 1993). *Aspergillus flavus* is able to utilize hydrocarbon from fuel oil and *o*- and *p*-xylene, because of its large numbers of enzymes that enables the fungus to grow on many different materials (COKER *et al.*, 1984).

In plants *Aspergillus* spp. preferably colonize and contaminate maize (*Zea mays* L.), rice (*Oryza sativa* L., Gramineae), peanuts (*Arachis hypogaea* L., Leguminosae), cottonseed (*Gossypium hirsutum* L., Malvaceae), figs (*Ficus carica* L., Moraceae), pistachio nuts (*Pistácia vera* L., Anacardiaceae), tree nuts (*Prunus*, *Corylus*, *Juglans* spp.) and other nuts, residue of oilseeds and several other plants (ENIUS, 2002). Moreover, the fungus may inhibit the growth of grasses, seedlings, hypocotyls and roots of several other plants (DOMSCH *et al.,* 1993). However, in general the fungus does not seriously affect the plant growth and development because yield losses caused by an *A. flavus* infection are negligible (COTTY *et al.,* 1994).

Although *A. flavus* is often described as a storage mould, infections in maize usually commence in the field. SÉTAMOU *et al.* (2000) found in Benin that pre-harvest maize was heavily infected with *A. flavus.* According to these authors more than 35% of the maize in Benin are heavily contaminated with aflatoxins (more than 10 µg AFB_1) harvest. Moreover, during storage *A. flavus* infections never decrease but remain on the same level or even increase (COTTY *et al.,* 1994).

Aspergillus flavus is also pathogenic to many insects, causing mycosis in *Aphis* spp., *Melolontha melolontha* L. (Col.: Scarabaeidae), *Tenebrio molitor* L. (Col.: Tenebrionidae), *Laphygma exempta* (Walker) Bianchi (Lep.: Noctuidae), *Cimex lectularius* L. (Hem.: Cimicidae), *Eriosoma lanuginose* Hartig (*Schizoneura*) (Hom.: Aphididae) and *Dendroctonus frontalis* Zimmermann (Col.: Scolytidae) (Domsch *et al.,* 1993). The host range of *A. flavus* includes domesticated insects as well as their diseases, e.g. it was linked to stone-brood in honey bees (*Apis mellifica* L., Hym.: Aphidae) and to koji kabi disease in cultivated silkworm larvae (*Bombyx mori* L., Lep.: Bombicidae) (GOTO *et al.,* 1988; GILLIAM and VANDENBERG, 1990). Dead or diseased insects serve *A. flavus* for growth and multiplication (sporulation) (DIENER *et al.,* 1987). Usually, they carry strains of *A. flavus* internally and many insects represent special hosts of at least certain *Aspergillus* strains (STEPHENSON and RUSSELL, 1974).

Only few *Aspergillus* species are pathogenic to mammals. Most individuals are naturally immune and do not develop disease caused by *A. flavus.* However, when a disease does occur, it takes several forms. In mammals, diseases caused by *Aspergillus* are called aspergillosis. They vary in symptoms, extending from an "allergy"-type illness to life-threatening generalized infections. The severity of aspergillosis is determined by several factors with the condition of a person's immune system as the most important one (HENDRICKSE, 1997; VILAR, 2003). The main aspergillosis are: Allergic bronchopulmonary aspergillosis (ABPA) (an allergic reaction to the spores of *Aspergillus* moulds), Aspergilloma (the fungi grow within cavities of the lung), Aspergillus sinusitis (occurs in the sinuses, in a

similar way to aspergilloma), and invasive aspergillosis (the most severe form, with fever and symptoms in the lungs) (VILAR, 2003). Sensitive patients usually react to several enzymes of the fungus and show symptoms of a floated cold, excitation of eyes, dermatitis or baker asthma (BGFA, 2003). Adults and children can be affected and should be diagnosed and treated in the same way. However, the greatest health problem for human and livestock caused by some *Aspergillus* fungi - namely *A. flavus* - is the production of aflatoxins (see chapter 3).

The enzyme excretion by the fungus leads to very different interactions between the fungus and its environment: mutualism (insect use enzyme-degraded plant parts by the fungus), parasitism (production of potent insecticides by the fungus) as well as saprophytism (plant degradation) (COTTY *et al.,* 1994).

Despite the fact that *A. flavus* in general is toxic to mammals, insects, and many plants, if conditions are suitable, there are also potential benefits associated with the genus *Aspergillus*. Several fungi of the *Aspergillus* group are used in industrial food processing (fermented food products) and for therapeutic products (e.g. urate oxidase, lactoferrin). In food processing several *A. flavus* strains are used to increase product utility and value, namely due to their enzyme production (COTTY *et. al.,* 1994). Other benefits of *A. flavus* - more in an ecological sense - are apparent when under certain circumstances (e.g. climate) the fungus becomes dominant in the microflora. First, if under dry conditions temperatures are increasing, *A. flavus* (as a saprophyte) is present as important plant degraders and may out-compete many other colonising microbes. Even under such hostile conditions plant debris may be disintegrated and recycled by *A. flavus* and hence nutrients are made available (COTTY *et al.,* 1994). BETTS and DART (1989) reported that *A. flavus* was even capable to rot lignin. Second, due to its entomopathogenic properties, *A. flavus* may also help to limit pest populations (COTTY *et al.,* 1994).

2.5. Contamination cycle

The process how *A. flavus* infects plants is rather complex (DIENER *et al.,* 1987; COTTY *et al.,* 1994). The **primary inoculum** is often soil-born, deriving from mycelium in plant debris, insects or as sclerotia outlasting unfavourable conditions. These sporogenic sclerotia usually present the major source of primary inoculum. Exposed sclerotial surfaces produce conidiophores and conidia in spring (after unfavourable periods). Conidia from sporogenic sclerotia of *A. flavus* can be the major source of primary inoculum in maize fields. Fungal strains that produce more sclerotia (in *A. flavus* namely the S-strains) may have an important survival benefit despite their higher metabolic costs (WICKLOW, 1988). According to GARBER and COTTY (1997), S-strains rely on sclerotia production for their dispersal. Conidia produced

on sporogenic sclerotia are disseminated via air currents. Moreover, insects can also serve as vectors for dissemination of *A. flavus* spores. For example stink bugs (*Acrosternum hilare* Say, Het.: Pemtatomoidea) and lygus bugs (*Lygus hesperus* Knight, Hem.: Miridae) carry conidia internally (after feeding) and leave them via defecation. Furthermore, insects promote inoculum production by their activities on leaves (STEPHENSON and RUSSELL, 1974; DIENER *et al.,* 1987). Thoroughly removing plant debris after harvest results in low to no contamination in the following crop (i.e. in low primary inoculum). In the course of the crop development, the microflora and plants become more susceptible to the fungus (particularly during long periods of drought) and infection increase with secondary inoculum (COTTY *et al.,* 1994).

Secondary inoculum source is the production of conidia on floral parts and leaves. Most of the fungal colonies arise from the fimbriate regions of the bracts. Other sources are flowers, floral nectaries, bracts, bractioles leaves, and foliar parts damaged by leaf-feeding insects. With the occurrence of secondary inoculum, airborne conidia (e.g. from L-strains of *A. flavus*) become more frequent and important (COTTY *et al.,* 1994). Air movements disperse the airborne conidia readily, but other means of dispersal (see above) may also promote contamination (DIENER *et al.,* 1987).

Prior to harvest primary and secondary inoculum are linked to the two phases in the infection process: pre-bloom and crop maturation, respectively. Previously all *A. flavus* infections were thought to occur post-harvest. The following description is not strict and complete, but may help to point out different infection phases during the crop development (COTTY *et al.,* 1994).

The fungus can enter different parts of the plants with the aid of its enzymes (LUCAS, 1998), but usually prefers penetrating wounds, stigma and other open parts to invade the plant tissue. After the initial infection several enzymes (amylases, endo-1,3-β-xylanase, rutinase, pectineases, urate oxidase, tryptic enzymes, urease, asparaginase, lipase, phospholipase, protease, and a moderately active phytase) are important for the establishment of *A. flavus* in its host (DOMSCH *et al.,*1993). Surprisingly, unspecialised saprophytic *Aspergillus* spp. can express equally high levels of enzymes (comparable to *Erwinia* spp.), which can induce lesions in plant tissues (LUCAS, 1998). Especially the pectinases seem to play the most important role in the infection process (CLEVELAND and MCCORMICK, 1987). CLEVELAND and COTTY (1991) showed in cotton bolls that the aggressiveness of *A. flavus* isolates is correlated with their ability to secrete pectinase(s) on sterilized cotton seed, on pectin-containing liquid media, and in living host tissues. If isolates had a reduced ability to spread through boll tissues and rot them, the major endopolygalacturonase activity was lacking. Thus, fungal virulence highly depends on the presence of pectolytic enzymes produced by *A. flavus* (CLEVELAND and COTTY, 1991).

In groundnuts or maize the infection via flowers takes place as follows: Once the fungus appears on a freshly opened flower, its conidia germinate on the stigma and on the surface of the pollen adhering to the stigma. Under favourable conditions a hyphen is formed, which enters the style through the stigma, and ramifies in the stylar tissue near the stigma. After growing down the style, the fungus sometimes reaches the top of the ovary. Subsequently the stamens of the flower are colonized and two days after inoculation, the sporulation is visible on the anthers, providing optimal secondary inoculum to infect other flowers (DIENER *et al.,* 1987).

In maize the infection of *A. flavus* via developing cobs and grains is more important. The invasion usually occurs through the silks. Infection starts at the tip of the cob, where the fungus starts growing towards the base by colonising the silks first, then the glumes, and finally grains in the milky stage. Under favourable conditions (optimal temperatures and silk senescence after pollination) the fungus is able to reach the base in 4 - 13 days and starts colonising the grain surfaces but rarely penetrates the cob pith (DIENER *et al.*, 1987).

Other entry points for *A. flavus* are the scars where the silk was attached. There, in a small area of the crown bright greenish-yellow fluorescence, associated with the fungus, was observed under ultraviolet light (DIENER *et al.,* 1987; MARSH and PAYNE, 1984). MARSH and PAYNE (1984) showed that conidia on yellow-brown silks germinated in 4 - 8 hours and silks were colonized extensively, especially near the pollen, where thick hyphal mats produce numerous conidiophores. The colonization observed was mainly superficial, restricted to the parenchyma and oriented parallel to the axis of the silks. Via cracks and intercellular gaps the direct and indirect penetration of yellow-brown (pollinated) silks is ensued. Furthermore, DIENER *et al.* (1987) suggested that pollen, as a rich source of nutrients for fungus sporulation, could play an important role in establishing the fungus on the silks. When profound changes in the physiology and structural integrity of the silks are caused by pollination – the beginning of silk senescence – the growth of the saprophytic fungus may be permitted.

Mycelium was often associated with the hilar layer, covering the outer surface of the embryonic axis and the vascular tissue of the scutellum (FENNELL *et al.*, 1973). RAMBO *et al.* (1974) observed heavy growth of *A. flavus* around the pedicel region of the grains. Mycelium in the tip cap was revealed in SEM studies, where hyphae were present in the intercellular spaces (a large part of the tip cap) and across the surfaces of the tip cay parenchyma cells, sclereids and protoxylem elements (MARSH and PAYNE, 1984). Like in the infection process of other common fungi, *A. flavus* most often enters the plant via the tip cap. However, until today, the dispersal, changes in population structure over time, and natural selection in Aspergillus spp. populations are poorly understood (COTTY *et al.,* 1994).

3. Aflatoxin

3.1. Characteristics of Aflatoxin

Aflatoxins were first identified in the early 1960's with the occurrence of Turkey-X disease in England, killing 100,000 of turkeys (LANCASTER *et al.,* 1961). The turkeys had been fed with *A. flavus* infected Brazilian groundnut meal and contained a toxic substance. In 1962 the food-related poison was termed 'aflatoxin' because it was derived from *A. flavus* (*Aspergillus flavus* toxin) (BUTLER, 1974). In the following four major toxins produced by *A. flavus* and *A. parasiticus* were identified, characterised and destined: AFB_1, AFB_2 and AFG_1, AFG_2 (according to their blue and green fluorescence in ultraviolet light, respectively) (DIENER *et al.,* 1987). To date, 20 different aflatoxins have been discovered and described, and the most toxic one is AFB_1, produced by *A. flavus, A. parasiticus* and *A. nomius* (STOLOFF *et al.*, 1991). Because of their effects on agriculture, aflatoxins are often characterized as 'non-nutritional chemicals', affecting other species in the environment. The precise function of the secondary metabolites of *Aspergillus* spp. is still not fully understood (COTTY *et al.,* 1994).

AFB_1 is a blue, crystalline solid substance, with a melting point of 268-269℃ and a chemical structure of $C_{17}H_{12}O_6$ (Fig. 4). Chemically aflatoxin is a fluorescent, heterocyclic compound, consisting of a unit of dihydro- or tetrahydrofuranofuran, connected with a substituted cuman derivative (ENGELHARDT, 2002). Thus, the toxin represents a descendant from difuran (PÜTZ and REUTER, 2002). After intake of AFB_1-contaminated food AFB_1 is transferred to AFM_1 (with the "M" signifying so-called "milk aflatoxins") in mammals (hydroxy-group adding reaction) (ENGELHARDT, 2002).

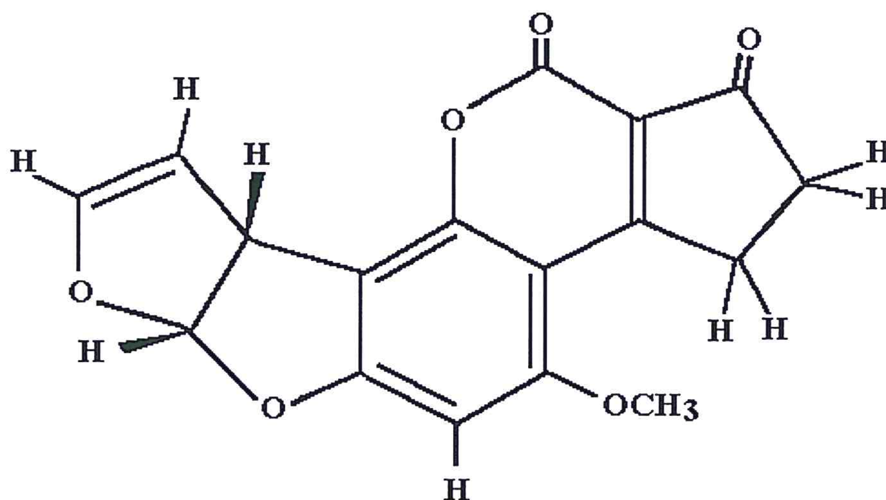


Fig 4: Chemical structure of AFB1 (Food and Agricultural Organisation (FAO) Corporate Document Repository, 1998).

In several studies the relationship between *Aspergillus* spp. strains and their associated toxigenicity has been investigated. As markers served lipid and sterol production (RAMBO and BEAN, 1974), esterase and peroxidase isoenzymes (SCHMIDT *et al.*, 1977), VCGs (PAPA, 1986, BAYMAN and COTTY, 1992), polymorphisms in mitochondrial (MOODY and TYLER, 1990a) and nuclear DNA (MOODY and TYLER, 1990b), and random amplified polymorphic DNAs (RAPD) (BAYMAN and COTTY, 1992). However, none of these markers showed a clear association between aflatoxin production and *Aspergillus* spp. strains.

L-strains produce either no aflatoxin or only one third of AFB1 compared to the S-strains (COTTY, 1997). These findings have been shown to be consistent over the years. It seems evolutionary the ability to produce aflatoxins do not present an important advantage for at least several L-strains that may be adapted to the air as ecological niche. Atoxigenic S-strain isolates are rare (COTTY, 1989). S-strains from West Africa differ from North America isolates, for instance in their phenotypes. West African isolates consist of both, the S_B and the S_{BG} phenotypes, producing only AFB, or AFB and AFG, respectively. African S_B isolates produce less AFB_1 than those reported from North America (COTTY and CARDWELL, 1999). The authors hypothesised that the geographical and physiological differences can influence aflatoxin management. However, prerequisites for a successful adaptation of aflatoxin management strategies developed for North America in Africa are their suitability for the predominant small-scale agriculture in SSA with its great diversity in cultivated crops (COTTY and CARDWELL, 1999).
VC- and RAPD tests showed no extensive variation between different isolates of the same VCG but between isolates from different VCGs, whereas aflatoxin production was similar between closely related VCGs (BAYMAN and COTTY, 1992). Hence, the ability of isolates in individual strains and groups of closely related strains to produce aflatoxins in nature is stable within the genotypes (e.g. VCGs). High concentrations of aflatoxins can be found in both conidia and sclerotia of *A. flavus*. Combined activities of aflatoxins and other metabolites, present in the sclerotia, were attributed to certain toxicities in *A. flavus* infected samples (COTTY, 1997).

3.2. General effects of aflatoxin

Aflatoxin has been detected in several plants. Even on many sterilized substrates, inoculated *A. flavus* is able to grow and produce aflatoxin (LILLEHOJ, 1983). Aflatoxins are toxic to plants, microorganisms, cells in culture, farm, domestic and laboratory animals as well as to humans. Aflatoxin affected the growth of excised, mature maize embryos. When small amounts of AFB_1 (2.0 μg ml^{-1}) were applied, shoot elongation was strongly inhibited. Furthermore,

application of 10, 20 and 25 μg ml^{-1} seriously interfered with foot elongation already seven days after toxin exposure (McLEAN *et al.*, 1992).

Whereas *Aspergillus* spp. may colonize and contaminate fruits, vegetables, and many plants (including maize) (ENIUS, 2002), additionally, aflatoxins may occur in detectable concentrations in animals (especially in the liver and kidney), which were fed with aflatoxin-contaminated food. Thus, the intake of aflatoxins in humans is usually related to the food chain and has serious health effects on animals and humans (ENGELHARDT, 2002).

Research on feed samples used in feeding dairy cows in Egypt demonstrated that rations contaminated with aflatoxins lead to changes in certain physiological blood parameters. For instance mean corpuscular haemoglobin concentration, level of red blood corpuscles and level of packed cell volume were affected. Furthermore, in the serum from aflatoxin fed cows low levels of total protein, iron, copper, magnesium, sodium and potassium, as well as high levels of asparate amino transferase (AST) and alkaline phosphatase live enzymes were detected (TOOS, 1997). The results confirm both, the topicality of aflatoxicosis problems in cows and the effects on blood parameters, proteins and irons.

In a study with experimental animals, PIER and McLOUGHLIN (1985) linked a number of biochemical effects to aflatoxin intake, such as inhibition of protein, nucleic acids (DNA, RNA) and lipid synthesis (phospholipids, free fatty acids, triglycerides and cholesterol and its esters), reduction of the activity of various enzymes and hepato-toxicity. Additionally, aflatoxins were correlated with the immunosuppression and impairment of cell-mediated immunity. Immunosuppression by aflatoxins results in reduced host resistance to a wide range of bacteria, viruses and fungi (RICHARD *et al.*, 1978). Moreover, the health effects of aflatoxins in mammals include carcinogenicity, teratogenicity and mutagenicity (ENGELHARDT, 2002).

The susceptibility in animals to aflatoxins greatly differs, e.g. ducklings are most, female rats are least sensitive to aflatoxin-contaminated food (COKER *et al.*, 1985). The mode of operation, how aflatoxins poison mammals, performs similarly: the primary target tissue of AFB_1 is the liver. There the toxin develops after activation of the cytochrome P450 enzyme in a metabolically way: formation of an epoxide and adduct creation with guanin bases in the DNA. During the replication of DNA, the DNA–adduct can result in mutation within the newly synthesized strain, e.g. by causing wrong base pairs. Mutations of DNA in key genes (protoonko gene, tumour suppressor genes) are initial events (TAUGS, 2002). In addition, aflatoxins often inactivate key enzymes in the cell metabolism, e.g. glutathion-S-transferase (TAUGS, 2002). Despite a considerable body of information on the metabolism of the toxin, the mode of operation of aflatoxins in mammals is still not fully understood (ANONYMOUS, 2002).

3.3. Effects of aflatoxin in humans

In humans the liver is also the most susceptible organ for an aflatoxin intoxication. It results in pathological changes like fatty infiltration, biliary proliferation and toxic necrosis in acute severe poisoning (DIENER *et al.*, 1987).

High incidence of liver cancer has been recorded in Nigeria (EDINGTON, 1977; NWOKOLO and OKONKWO, 1978). The occurrence of aflatoxin and its metabolites were studied in Nigerians with liver cancer incidence (BEAN *et al.,* 1989). They reported that AFB_1 and AFG_1 occurred in 3.1% and 9.9% of tested urine samples, respectively. The highest mean concentration of AFG_1 in urine was approximately 12µg $100ml^{-1}$, confirming the presence of aflatoxin metabolites in populations with a high incidence of liver cancer.

Ingesting aflatoxin-contaminated food causes liver cancer. Whereas lung cancers are triggered when spores of *A. flavus* are inhaled, especially in workers that are often exposed to airborne fungal spores (AUTRUP *et al.*, 1991). According to HOLMBERG (1984), the same risk exists for inhabitants of mould- and rot-infected houses, living conditions that are very prevalent in rural regions of West Africa. However, in general sensitivity to aflatoxins greatly varies in humans (HENDRICKSE, 1997). Yet, so far in plants and mammals no complete resistance to aflatoxins has been identified (CROKER *et al.,* 1985; HENDRICKSE, 1997; LEE, 1997).

The Kwashiorkor disease was first detected in the early 1930's and was associated with a maize diet (WILLIAMS, 1933). Initially, the most popular explanation for the disease was that Kwashiorkor is caused by a protein deficiency in the diet, alongside with an excess in carbohydrates (HENDRICKSE, 1997). However, until today the real cause remains obscure (COWARD and LUNN, 1981; HENDRICKSE, 1997). In biopsies of livers from 27 Sudanese children suffering from Kwashiorkor, AFB_1 and AFB_2 and aflatoxicol (an other metabolite of AFB_1) were detected, but not in 13 other children with miscellaneous liver (COULTER *et al.,* 1986). ADHIKARI *et al.* (1994) recorded in aflatoxin positive Kwashiorkor children common complications like diarrhoea, pneumonia, oral herpes, shock, monoliasis and candidiasis (thrush).

LAMPLUGH and HENDRICKSE (1988) were the first to detect aflatoxins in breast milk and cord blood. Thereafter, it was suggested that infants in high-risk areas might face continuing pre- and post-natal exposure to the toxins. This hypothesis was later confirmed by WILD *et al.* (1991), who assessed umbilical cord sera for the presence of aflatoxin-albumin adduct, with 70% of the cord sera being positive for this adduct. The highest levels of aflatoxins ever found in human tissue and fluid were recorded from cord bloods at birth (MAXWELL *et al.*, 1989). HENDRICKSE (1997) reported the obscure death of a Nigerian baby in the perinatal period. After autopsy large amounts of AFM_1 and AFM_2 in liver, kidney, lung and brain were found. In addition to the problems of pre-natal aflatoxin exposure, newborns in the tropics

have often a low birth weight, jaundice infections and other infections of obscure causes and in general a poor viability.

Given the high prevalence of *A. flavus* infections in maize grains produced and stored by subsistence farmers in rural regions of SSA, it is unlikely that any child from such a background can escape exposure to aflatoxins. The detection of large amounts of AFB_1 in human breast milk explains the higher incidence of Kwashiorkor in breast-fed babies (HENDRICKSE, 1997). Moreover, Kwashiorkor and overfeeding with carbohydrate (i.e. maize) rich diets (the latter often aflatoxin-contaminated) often go hand-in-hand: the more aflatoxin-contaminated food eaten, the greater the toxicity and severity of Kwashiorkor (HENDRICKSE, 1997).

Many children from rural areas in SSA are almost entirely fed on maize porridge. In West Africa, maize is often initially (i.e. directly after harvest) stored at rather high moisture content (often around 25% moisture contented), thus favouring fungal infections of the commodity (HELL *et al.,* 2000).

STORA *et al.* (1983) found a positive correlation between food-related uptake of aflatoxins and incidence of the Reye's syndrome, a disease that affects primarily children and which is characterised clinically by vomiting, hypoglycemia, and confusion. Most often the disease is believed to be associated with a protein deficiency (BOYER, 2003). However, autopsies of livers from 5 infants suffering from Reye's syndrome revealed amounts of AFB_1 ranging between 120 - 810 µg/g (HENDRICKSE, 1997). The author hypothesised that these children had experienced high levels of toxic ingestion and an acute intoxication by AFB_1. This hypothesis was later supported by food samples collected from homes of patients suffering from Reye's syndrome and alimentary toxic aleukia in Tunisia. Half of the samples contained AFB_1 and AFB_2, and 20% and 5% AFG_1 and AFG_2, respectively (HADIIDANE *et al.*, 1985). Thus, mycotoxins are probably related to the development of Reye's syndrome (HADIIDANE *et al.*, 1985).

Additionally, aflatoxins and their metabolites are positively correlated with gastritis: WILD *et al.* (1990) recorded in a Kenyan village high incidence of symptomatic gastritis in young people. Patients with gastritis had significantly higher levels of aflatoxins in their urine than healthy ones (5-338 µg ml^{-1} of urine). Finally, HENDRICKSE (1997) suggested a possible contributing role of aflatoxins in human immunodeficiency virus (HIV) infections in African children. There is evidence that acquired immune deficiency syndrome (AIDS) develops more frequently and rapidly in African children than ones in the Western world, except for infants born to HIV-positive intravenous heroin users (HENDRICKSE, 1997).

3.4. Impact of aflatoxin in food production

Until today, no direct means to reduce aflatoxin levels are available (COTTY, 1997). In the field, the development of aflatoxins or other mycotoxins are not effectively prevented with fungicides. Often repeated applications of fungicides in high dosages are carried out, with all the inherent negative effects for the environment. Moreover, fungicides like flavonoids, isoflavonoids, i.a. can combat the fungus (WEIDENBÖRNER *et al.,* 1990) but not the poison. They are ineffective after initiation of the aflatoxin biosynthesis, since aflatoxins produced prior to the fungicide application may survive even if the fungus has been killed (COTTY and BAYMAN, 1993; ANONYMOUS, 2002). The toxins have a high chemical stability and are immune to high temperatures, enabling them to survive in the field, during long transports and even in manufacturing processes (ENIUS, 2002). Highest contaminations occur in damaged crop components, and fines and dust generated during crop procession (LEE *et al.,* 1983). Consequently, all contaminated food has to be destroyed (COKER *et al.,* 1984). Aflatoxins in animal fodder reaches men through the food chain, namely through meat, especially liver meat, and milk products. In the stomach of ruminants the intake of aflatoxin-contaminated food is not reduced to non-poisonous substances but are transferred to AFM. Particularly silage and concentrated livestock fodder with a high proportion of oil-grist fraction are often contaminated with aflatoxins, especially those of tropical or subtropical origin (KOLLER, 1993).

In maize and other crops, only relatively few seeds, leafs or other plant parts may contain high concentrations of aflatoxins. In West Africa, this problem is of minor economic importance in regions where maize is grown as a subsistence crop. However, if Benine's farmers grow maize for (international) trade, the product must be in accordance with the (local) regulations of the respective trading partners. Although highly contaminated plant parts are rare, they typically account for the great majority of the toxins within a sample. This situation is all the more disadvantageous as tolerated aflatoxin-concentrations in food samples dedicated for human consumption are at very low levels. Often a shipment containing less than 1% of contaminated maize is rejected because the average of the entire crop exceeds allowable concentrations (COTTY *et al.,* 1994). That is just why aflatoxin has been a peculiar, annoying and frustrating agricultural problem during the last four decades. Even if the risks of aflatoxin-contaminated food have decreased (especially in the Western World) during the last years, mainly due to more controls under closed surveillance (ENIUS, 2002), the extremely high toxicity of aflatoxins are still of great concern (DEDDNER and WAEHNER, 1999; AHO, 2002; SCHINDLER *et al.*, 2003). The lethal dose for adults and children is $1 - 10$ mg kg^{-1} and $9 - 18$ µg d^{-1}, respectively (ENIUS, 2002). Hence, most developed countries have imposed extremely low residue limits for aflatoxins in foods and feeds (ENIUS, 2002). In the European Union the levels of AFB_1, AFB_2, AFG_1, and AFG_2 are not allowed to

exceed 0.05 µg kg^{-1} food alone or in combination/addition. With 0.01 µg kg^{-1} of food the limit for AFM_1 is even lower. These regulatory residue limits refer to processed food ready for consumption. Because of the carry-over of aflatoxins in the food chain, critical limits for animal fodder have also been established. For lactating animals the fodder must contain less than 5 µg AF kg^{-1} foodstuff, and for all other animals limits range between 20 and 50 µg kg^{-1} (ENGELHARDT, 2002). These low residue limits cause serious problems for agricultural exports from developing countries in SSA and elsewhere. This problem was addressed during the third United Nations (UN) Conference on the Least Developing Countries in Brussels 2001 by Kofi Annan saying: "...A World Bank study has calculated that the European Union regulation on aflatoxins costs Africa $750 million US each year in exports of cereals, dried fruit and nuts. And what does it achieve? It may possibly save the life of one citizen of the European Union every two years... surely a more reasonable balance can be found" (ANNAN, 2001).

The FAO of the UN assists developing countries in developing a legal framework for alimentary standards (e.g. sanitation, manufacture processing, care), which should enable these countries to meet the regulative requirements for exporting their agricultural products into the developed world. However, for food products destined for local consumption are rarely monitored for mycotoxin contamination. Among others, one reason are the high costs of such analyses (e.g. ca. $25 US per crop sample), where even 100 samples often exceed the yearly operational budget of the government agencies in charge of food security (CARDWELL *et al.*, 2003).

The situation is aggravated by the prevalence of other mycotoxins. For example carcinogenic and immunotoxic effects are known from *Fusarium* spp. toxins (fumonisins). A mixture of several mycotoxins is believed to have at least additive, if not synergistic, but never antagonistic effects (CARDWELL *et al.*, 2003).

4. Factors influencing *Aspergillus flavus* infection and aflatoxin formation in maize

Since the discovery of aflatoxin producing *Aspergillus* spp., several studies have been carried out on factors possibly influencing fungal invasion, infection, and subsequent toxin production. The process how *A. flavus* contaminates plants is rather complex, and in the field various factors influence the infection process (DIENER *et al.,* 1987; COTTY *et al.,* 1994). Multifaceted relationships among biotic (insects, fungi, maize genotypes), abiotic (climate, soil types) and anthropological (cultural practices) factors exist (BARRY *et al.,* 1992). Often, these factors act in combination and it is thus difficult to attribute effects to individual ones. For example, fungal growth and aflatoxin production at low temperatures occurs only when other factors are near their optimums (RAMBO *et al.*, 1975). Under optimum conditions, aflatoxin can be produced within 48 hours (ROSS *et al.*, 1979). In general these factors influence the development of and the toxin production by *A. flavus* in the field before (the maize) harvest. The most important factors are:

- climatic factors (temperature, moisture, humidity),
- biological factors (soil conditions, plant development, interactions between organisms, insect damage) and
- agricultural factors (plant varieties, cultural practices, fertilisation, etc.).

By definition an aflatoxin-contamination is the consequence of a previous *A. flavus* infection. In the following section factors favouring the formation of aflatoxins and an infection by *A. flavus* are described in greater detail, with a particular emphasis on the conditions in Benin.

4.1. Climatic factors

Temperature strongly influences the development of the fungus. WILSON and ABRAMSON (1992) reported growth of *A. flavus* from 11° - 43° C with temperature range of 15° - 37° C being most favourable for aflatoxin production. Even at temperatures as low as 8° C the fungus still grows, albeit slowly; however, aflatoxin production on sterilized rice and non-sterilized peanuts commences only at 11° and 14° C, respectively (DIENER and DAVIS, 1970). GOLDBLATT (1969) recorded strongest fungal growth and highest toxin production at 27° C (with 80% or 90% relative humidity). Furthermore a significant correlation was found between aflatoxin concentration and maximum and minimum daily temperatures and net daily evaporation following full silking in maize (WIDSTROM *et al.*, 1990).

In addition, *A. flavus* development and subsequent aflatoxin production is greatly affected by humidity. It is difficult to separate the effects of temperature and humidity since both factors affect each other. If relative humidity in the field is high, than local temperatures will

decreases (and vice versa). High relative humidity and temperatures of 11° - 40° C are essential for spore germination and fungus proliferation. Relative humidity in the atmosphere and water content of the soil are as important for the development of *A. flavus* as the moisture content in the maize grains.

Even a small increase in moisture in soil can significantly decrease the risks of aflatoxin contamination (SAUER and BURROUGHS, 1986). PAYNE *et al.* (1986) reported in their studies with irrigated and non-irrigated maize fields a significant reduction of aflatoxin contamination in the former. Subsequently, they suggest that reduced airborne *A. flavus* infection in the maize cultures is the reason for low aflatoxin content in the grains in irrigated field plots. Measuring the growth of *A. flavus* in fresh harvested maize CALDERWOOD and SCHROEDER (1968), recorded optimal growth of the fungus in maize with a grain moisture content of 20% - 28% at temperature between 20° - 30° C. Generally, the higher the moisture contents of maize grains, the higher the risk for aflatoxin contaminations. However, high levels of relative humidity in the atmosphere also favour the growth of other microorganisms, which can inhibit the growth of *A. flavus* (CHANG and MARKAKIS, 1981). *Aspergillus flavus* enters the grain late in its development (between 16 and 20 days after inoculation), when the grain moisture content is around 30%. Thus favourable weather conditions before harvest that allow a fast maturation of the maize crop, will greatly reduce the incidence of *A. flavus* infections (PAYNE, 1983).

Moreover, drought stress and high planting density in maize are associated with *A. flavus* infections and subsequent aflatoxin contaminations (WICKLOW, 1988). However, water- or drought stress increases the level of aflatoxin contamination. All in all, the effects of water stress, drought, temperatures and moisture are closely interrelated. For example local temperatures decrease if water is added to the soil via irrigation or by rain.

In Benin, there are no means to control one of these factors due to the subsistence farming in small-scale fields. Even irrigation plant has huge costs of investment (manpower, material), which are not redeemable (AJAYI, 2000). The factors described are highly dependent on climatic situation in the prevailing agro-ecological zone.

Benin is situated in the southern part of West Africa and is divided into four agro-ecological zones. The northern zone is called Sudan Savanna (SS), followed by the Northern Guinea Savanna (NGS) and the Southern Guinea Savanna (SGS) where the trial location of this study is situated. This zone is the main maize production zone in Benin (HELL, 1997). There are two rainy seasons where the first lasts from April to July and the second from October to November. Hence, two crops of maize are produced during one year. The annual rainfall rises from 900 up to 1300 mm and evaporation is high. Normally, dry season lasts for 4 – 6 months, but during the last years there was an increase of the durable dry season and hence, the two rainy seasons closed ranks (HELL *et al.*, 2000). The coastal agro-ecological zone in Benin is called Forest Mosaic Savanna (FMS). In several studies concerning the

aflatoxin contamination process in Benin the authors always point out the importance of climate factors within the different agro-ecological zones (HELL *et al.*, 1996; SÉTAMOU *et al.*, 1997; HELL *et al.*, 1999).

4.2. Biological factors

Soil and soil condition directly affects the state of the plant and thus indirectly its susceptibility to an *A. flavus* infection and a subsequent aflatoxin contamination. Groundnut grown on vertisols had lower concentrations of aflatoxins than cultivated on alfisols (MEHAN *et al.*, 1991). JONES *et al.* (1981) found higher concentrations of aflatoxin in maize grown on sandy soils in the coastal plains of North Carolina, U.S., than on finer textured soils in the tidewater region. Similar effects were observed in Alabama where DAVIS *et al.* (1986) found in a 6 year study that maize grown on sandy soils were more frequently contaminated with aflatoxins than those from soils of finer texture. A reduced water-holding capacity of the soil and drought-related interference with nutrient uptake by maize plants were the most important stress factors responsible for the higher extend of *A. flavus* infections on the sandier soils.

Several authors showed a positive correlation between high aflatoxin levels and **insect damage** to maize plants (SINHA and SINHA, 1991; SÉTAMOU *et al.*, 1998). According to PAYNE *et al.* (1986) insects are not important vectors of *A. flavus*. Likewise SINHA and SINHA (1992) reported no differences in the number of toxigenic *A. flavus* strains isolated in field plots with and without insects. However, if the insects damage the maize cobs or other plant parts, *A. flavus* spores find entry points for an infection and subsequent aflatoxin production. Insects in pre-harvest maize generally associated with aflatoxin contamination are *Sitophilus zeamais* Motsch. (Col.: Cucurlionidae), *Prostephanus truncatus* Horn (Col.: Bostrichidae), *Tribolium castaneum* Herbst (Col.: Nitidulidae), *Cathartus quadricolis* Guerin-Meneville (Col.: Silvanidae), *Chryptophlebia leucotreta* Merick (Lep.: Tortricidae), *Sesamia calamistris* Hampson, *Busseola fusca* Fuller, *Helicoverpa zea* Boddie, *Spodoptera exempta* Wik (all Lep.: Noctuidae), *Eldana saccharina* Walker, and *Mussidia nigrivenella* Ragonot (both Lep.: Pyralidae) (SÉTAMOU *et al.*, 1997; BORGEMEISTER, 2001). However, only *M. nigrivenella* was significantly correlated with an *A. flavus* infection and a later aflatoxin contamination (SÉTAMOU *et al.*, 1997). BARRY *et al.* (1992) found that maize varieties with a resistance to these cob-attacking insect pests showed lower aflatoxin contamination. The consistent correlation of pests with the *A. flavus* infections and hence subsequent aflatoxin contaminations stresses the importance of pests like insects, animals and birds as vectors for *A. flavus* in maize (BARRY *et al.*, 1992, MCMILLIAN, 1987, DIENER *et al.*, 1987).

Parasitic weeds e.g. the purple witch-weed, *Striga hermonthica* Benth. (Scrophulariaceae), which is very common in maize fields in Benin affect *A. flavus* infection and aflatoxin

production. In general, weeds compete with crop plants for nutrient and water. The draught stress caused by *S. hermonthica* leads to higher aflatoxin content in the crop. Moreover, debris of weeds provide optimal growing conditions for *A. flavus* and are a major source of primary inoculum level in the beginning of the vegetation period (HELL *et al.*, 1996; DIENER *et al.*, 1987).

Compared to pests or weeds, little is known on the impact of other fungi (**fungal interactions**) on *A. flavus* infections and aflatoxin contaminations. ZUMMO and SCOTT (1992) studied the interactions between infections of *Fusarium moniliforme* Sheld. and *A. flavus* on maize grains. The authors showed that *F. moniliforme* can competitively inhibit the growth of *A. flavus* via competition for nutrients and habitat, and hence, reduce the aflatoxin contamination. However, so far no techniques have been developed to use such an approach in the field.

A secondary metabolite produced by *Fusarium oxysporum* Schlechtend.: Fr. was found to influence *A. flavus* as well as the toxicity of rubratoxin B produced by *Penicillium rubrum* Stoll (DOMSCH *et al.*, 1993). Likewise, effects caused by *P. nigricans* Bainier & Thom *in situ* influenced the development of *A. flavus*. Here the conidia of *A. flavus* were not able to germinate and hyphae failed to grow in non-sterile soils (DOMSCH *et al.*, 1993).

Cotton insecticides or rodenticides are used to combat post-harvest pests in about 20% of the stored maize in Benin. Insecticides or herbicides are rarely applied in the field, whereas fungicides are generally insignificant (HELL *et al.*, 2000). In the major cotton production regions of central and northern Benin cotton insecticides are given to farmers on a credit basis by the national cotton board, Société National sur la Promotion Agricole (SONAPRA) (HELL *et al.*, 1996). Farmers who use insecticides in their maize stores usually have fewer problems with aflatoxins although a direct effect of the insecticide on *A. flavus* could not be detected (HELL *et al.*, 2000).

4.3. Agricultural factors

Several studies have addressed the effect of different maize **varieties** on *A. flavus* infections (BARRY *et al.*, 1986; KOSSOU *et al.*, 1993; LEE, 1997). The choice of the variety is one of the most important agricultural factors, influencing the crop's susceptibility to *A. flavus* infection and subsequent aflatoxin contamination. Two types of resistance exist, i.e. resistance against the fungus and the development of its toxin. In the latter case an infection of the plant by an *Aspergillus* spp. would be tolerated, but aflatoxin production would be suppressed (LEE, 1997). In maize, varieties with a tight and complete husk cover are less susceptible to an *A. flavus* infection than those with incomplete husk cover (MCMILLIAN *et al.*, 1987). Moreover, varieties with tight husk cover have significantly lower aflatoxin content than varieties with loose husk characteristics (BARRY *et al.*, 1986). In Benin, KOSSOU *et al.* (1993) compared two improved varieties, one partly improved, and a local maize variety and

showed that improved varieties had a significant higher grain yield (60%) but a significantly poorer husk cover. Consequently, these varieties had a higher percentage of damaged cobs following field drying, higher numbers of storage-pests (in general) and suffered higher grain weight loss after one month of storage than the local variety. In maize the form of the husk cover is of paramount importance for the fungus to successfully infect the plant and colonize the grains. Additionally, fast growing varieties, especially during cob formation, as well as those with a reduced susceptibility to stress factors like drought and insect attack are less likely to become infected by *A. flavus* (DIENER *et al.,* 1987). Recently in the U.S., a maize line was breed with unusually thick waxy outer-layers. The waxy coating helps to protect the plant against *Aspergillus* spp. infections (LEE, 1997). However, so far no variety has been developed with a complete resistance to *A. flavus* infection and / or to aflatoxin production (COKER *et al.,* 1985; Lee, 1997).

In Benin, farmers use both improved and local maize varieties but rarely hybrid varieties. Generally, improved and local varieties are open pollinated and farmers most often use self-propagated maize seeds. Among other reasons like taste and processing characteristics, use of improved maize variety is mainly related to higher yields (KOSSOU *et al.*, 1993) and in local varieties better storability because of the husk cover (MEIKLE *et al.*, 1998).

REDING *et al.* (1993) showed in their studies that gypsum or lime applications lead to a reduced aflatoxin production. Both agents are frequently used to increase pH levels of acid soils. This reduced the frequency of *A. flavus* (COTTY *et al.,* 1994).

Both excessive **fertilization** (especially of nitrogen) as well as under-fertilisation of soils cause stress in plants and increases the susceptibility to *A. flavus* and its metabolite production. A well-balanced nutrient management (based on the nutrient uptake of the maize plants) leads to lower aflatoxin levels in maize (JONES *et al.*, 1981). However, in Benin only cotton farmers in the central and northern parts of the country use mineral fertilisers (NPK and urea) in their cotton but also in other crops like maize (HELL *et al.,* 1996).

Agricultural practises like **crop rotation** and **intercropping** also play an important role in the *A. flavus* infection and aflatoxin contamination. Rotating maize with groundnuts, which in Africa are often heavily contaminated by aflatoxins, can lead to higher incidence of *A. flavus* infections and subsequent aflatoxin development (COLE *et al.*, 1982). COTTY (1994b) found that if maize was intercropped with an *A. flavus* susceptible crop there was an increased risk of toxin metabolism. Moreover, over time *A. flavus* populations easily built up in soils when planted with *Aspergillus* spp. susceptible crops (GRIFFIN *et al.*, 1982).

In Benin about one third of the maize is cultivated as a monocrop, whereas intercropping of maize and groundnuts is practised by 10% of Beninese farmers (HELL *et al.*, 1999). In the comparatively densely populated southern part of Benin, where land pressure is particularly high, mixed cropping of maize and cassava (*Manihot esculenta* Cranz., Euphorbiaceae) is common (HELL *et al.*, 1999). The consequences are increased soil cover, resulting in higher

soil moisture content (different microclimate), and increased intra-plant competition for water and nutrients. HELL *et al.* (1996) reported an increase in aflatoxin levels when maize was intercropped with cassava or cowpea (*Vigna unguiculata* Walp [Fabaceae]) . However, lower aflatoxin levels were found when maize was intercropped with vegetables. In monocropped maize, high **plant densities** lead to increased inter-plant competition for nutrients and water, thus increasing the susceptibility of the plants for *A. flavus* infections (LILLEHOJ, 1983).

A direct factor influencing *A. flavus* development and production of aflatoxins are **plant residues** in the fields after harvest. A thorough removal of plant residues after harvest considerably reduces the availability of suitable substrates for primary inoculum of *A. flavus* (GRIFFIN *et al.,* 1982).

5. Competitive Replacement

5.1. Introduction

To date no reliable approaches, neither to prevent crops from *A. flavus* attack nor to reduce the aflatoxin production and contamination of maize exist (COTTY and CARDWELL, 1999). Moreover, no methods have been developed to reduce metabolite production in crops that are both reliable and economical viable, except the competitive replacement approach, using atoxigenic strains of *A. flavus* (BAYMAN and COTTY, 1992; COTTY, 1997).

Biological control agents for preventing aflatoxin contamination in cotton were developed in the U.S. using atoxigenic strains of *A. flavus*, based on intra-specific interactions between atoxigenic and toxigenic *Aspergillus* spp. strains. The rationale is to replace toxigenic with atoxigenic *A. flavus* strains through augmentative field applications of the latter to the soil.

5.2. History

For disease control in plants similar approaches to a competitive replacement have been successfully used. For instance in virology, a plant is intentionally infected with a less pathogenic strain of a virus to prevent infections of more severe strains of the same virus type (cross protection) (MAISS, 2001). Similarly, in celery an infection by *Fusarium oxysporum* can be prevented by competitive exclusions with non-pathogenic strains of the same species. Likewise, (but not in a competitive replacement) frost injury can be avoided or reduced, by deliberate inoculation of plants with *Pseudomonas syringae* van Hall or *Erwinia herbicola* (Lohnis) Dye. These bacteria lack ice nucleation active strains and hence no ice crystallisation is possible (COTTY *et al.,* 1994).

5.3. Requirements

The method of competitive replacement to reduce pre-harvest aflatoxin contamination must meet the following requirements, if it should fit to the designated ecosystem (according to the background of biological control strategy) (COTTY, 1989; COTTY *et al.,* 1994; EGEL *et al.,* 1994; NELSON *et al.,* 2000):

1. The fungus has to have a high phenotypic diversity in the release area.
2. The atoxigenic strains need to occur naturally in the trial area, e.g. occupy similar niches.
3. Aflatoxins and other metabolites are not responsible for the aggressiveness of the fungus and hence are not necessary for an infection of the host-plant.
4. The application of atoxigenic strains must be feasible during cultivation periods under field conditions.
5. The application of atoxigenic strains has to have no negative impacts on the environment.

1. High phenotypic diversity in *A. flavus* populations has been previously demonstrated (ROSS *et al.,* 1979; COTTY, 1989). The composition of fungal communities in agricultural fields varies widely in terms of aflatoxin-producing ability, sclerotial size, and virulence. The ability of atoxigenic *A. flavus* strains to prevent aflatoxin contamination of cottonseeds underlines the high variability of this species (COTTY, 1990).

2. S-strain isolates of *A. flavus* are mutants derived from the L-strains (BAYMAN and COTTY, 1992; EGEL *et al.,* 1994). As previously shown, S-strain isolates produce consistently higher levels of aflatoxins whereas L-strains produce in general little or no aflatoxins at all. Atoxigenic strains occur naturally in *A. flavus* communities (COTTY, 1989; 1997). Atoxigenic strains of *A. flavus* occupy almost the same ecological niche as toxigenic strains, which is a prerequisite for such an approach, as they must be able to compete under the environmental conditions that lead to aflatoxin contaminations (ORUM *et al.,* 1999).

3. A superior competitiveness of the released strain, which is not related to aflatoxin production, reduces the amount of fungal inoculum that needs to be applied. Hence, atoxigenic strains need to be at least as virulent as the strains they are intended to displace. However, this does not apply if strains are applied during periods in which *A. flavus* is primarily saprophytic (ORUM *et al.,* 1997). COTTY (1989) found in about 56 isolates of *A. flavus* (including L- and S-strains) that pathogenic aggressiveness does not correlate with the ability to produce aflatoxin. One atoxigenic strain (AF36) showed a high virulence and retained this ability for several years after the initial releases (COTTY, 1989; COTTY *et al.,* 1994; COTTY and CARDWELL, 1999).

4. DAIGLE and COTTY (1995) investigated formulation and the application methods of atoxigenic *A. flavus* strains. The optimal encapsulation of mycelia was achieved in a formulation consisting of 1% sodium alginate, 5% wheat gluten and 5% maize cob grits, a by-product of the maize industry. On a per gram basis, this formulation yielded more spores than after colonisation on wheat seeds. However, until today the use of colonised wheat seed as release material, is widespread in the U.S. (COTTY *et al.,* 1994; ORUM *et al.,* 1997), because the wheat seed are easy to release (e.g. with gritter) (COTTY *et al.,* 1994).

5. So far, negative impacts of this approach on the environment have not been revealed: neither in the fields, nor in adjacent habitats (COTTY *et al.,* 1994; COTTY and CARDWELL, 1999). However, the evaluation of the possible side effects of an application of atoxigenic strains is very important. Currently, in U.S. cotton where atoxigenic *A. flavus* strains are applied to large areas, strain variability and potential possible side effects are closely monitored (BOCK and COTTY, 1999; NELSON *et al.,* 2000).

These prerequisites have been met in certain regions in the U.S., where the method of competitive exclusion was first developed (COTTY, 1994b; COTTY *et al.,* 1994; COTTY, 1997; ORUM *et al.,* 1999).

5.4. Engagement and constrains

The method of competitive replacement has so far only been developed for control *A. flavus* because under field conditions it is the most important aflatoxin-producing species. The other aflatoxin-producing *Aspergillus* spp., i.e. *A. parasiticus* and *A. nomius,* usually occur in very low densities, often comprising less than 1% of all isolated *Aspergillus* species (COTTY, 1997). *Aspergillus parasiticus* produces consistently high amounts of aflatoxins, whereas *A. nomius* is not known to do so. Furthermore, *A. nomius* is of minor importance, especially in soils of West Africa. However, some atoxigenic strains have also been detected in *A. parasiticus* and thus releases of atoxigenic strains may be extended - if available - to all aflatoxin-producing *Aspergillus* spp. (CARDWELL *et al.,* 1999).

Because of their high economic importance and likewise high susceptibility to *A. flavus* infections and subsequent aflatoxin contamination, research has so far focussed on cotton and groundnuts. Aflatoxins limit both domestic use and exports of these crops. All in all, the aflatoxin contaminations by *Aspergillus* species in U.S. agriculture cause losses of tens of millions of dollars annually (CARDWELL and COTTY, 2000).

5.5. Overview on research activities

The common approach in studies using a competitive replacement approach comprised of a threefold analysis of soil and crop samples:

1. For the total *A. flavus* propagules density and an analysis of the prevailing fungal communities (saprophytes),
2. for the incidence and relationship of the *A. flavus* L- and S-strains, and
3. for the incidence of the released atoxigenic strain of *A. flavus* (COTTY *et al.,* 1994; NELSON *et al.,* 2000).

So far, researchers have not (yet) gone into much detail about the first two points, which are necessary according to the ecology. After a number of laboratory experiments, greenhouse tests and field tests were carried out.

Under controlled conditions in greenhouses atoxigenic strains of *A. flavus* significantly reduced the AFB_1 content in cottonseeds co-inoculated with toxigenic strains of the same species (COTTY *et al.,* 1994). BROWN *et al.* (1991) first reported successful interference of an atoxigenic *A. flavus* strain in pre-harvest maize plants under field conditions in the U.S. The production of aflatoxin was reduced by 80% – 90% when the atoxigenic strain was applied simultaneously together with, or one day prior to a toxigenic strain. Low aflatoxin levels were obtained *in vitro*, after an atoxigenic strain was applied to cotton balls previously infected during a 48 h fermentation period with toxigenic strains (COTTY and BAYMAN, 1993). The atoxigenic excluded toxigenic strains both physically and via competition for nutrients. The latter is particularly important in interactions with S-strains and their resource intensive

aflatoxin biosynthesis (COTTY *et al.,* 1994). However, not all atoxigenic *A. flavus* strains competitively replace aflatoxin-producing strains in the same extend (COTTY *et al.,* 1994).
In a more extended two-year field study in cotton, COTTY (1994b) could demonstrate that a soil application of an atoxigenic strain leads to a competitive exclusion of aflatoxin-producing strains throughout the entire crop development. Consequently, the quantity of aflatoxin was significantly reduced by competitive exclusion. The proportion of *A. flavus* infected plants did not increase in the release plots, though the frequency of infected locules (1% and 25%) differed between both years. No differences in total quantity of *A. flavus* were recorded between treated and untreated plots as measured by bright-green-yellow-fluoreszence (BGYF), suggesting that the applied atoxigenic strain retained its atoxigenic phenotype. COTTY (1994b) hypothesised that the detectable rates of AFB_1 in the release plots were produced by toxigenic *A. flavus* strains belonging to other VCGs than the applied atoxigenic strain (COTTY, 1994b).

5.6. Prospects of biological control strategies

To date, the method of competitive replacement is well documented and extensively applied in the U.S. in the field and post-harvest (COTTY, 1994a; DORNER *et al,* 1995; COTTY, 1997). Formulation and application of atoxigenic *A. flavus* strains is an on-the-shelf technology for U.S. cotton (GARBER and COTTY, 1997; BOCK and COTTY, 1999; NELSON *et al.,* 2000). For a better understanding of regional and spatial patterns of *A. flavus* strain composition as a key component for managing aflatoxins in cottonseed, geographic information systems and geo-statistics are currently used in the U.S. High consistency in spatial variability of *A. flavus* community structures was recorded (ORUM *et al.*, 1999). Furthermore, predictive maps will be developed to monitor trends in *A. flavus* strain composition, particularly with regard to toxigenic S-strains (NELSON *et al.,* 2000). In the U.S., research projects are conducted, which investigate the slug tuning in composition and ratio of divers toxigenic and atoxigenic fungal strains prevailing in the fields. Moreover, atoxigenic *A. niger* and several *Trichoderma harzianum* Rifai isolates are tested in groundnuts alongside atoxigenic *A. flavus* strains. *In vitro*, hemmhof-formation of *T. harzianum* has a promising inhibitory effect on *A. flavus* and *A. niger* (OKKY, 2001). *Aspergillus niger* can possibly directly reduce aflatoxin contamination through a detoxification reaction and through inhibition of aflatoxin production (DOMSCH *et al.,* 1993). Research also continues on assessing the potential for crop colonization by atoxigenic *A. flavus* strains and their long-term influence in irrigated and non-irrigated fields (BOCK and COTTY, 1999).

5.7. Vegetative compatibility (VC) introduction

In the current study, the method of competitive replacement is used. For the post-release-monitoring markers are needed. For example, atoxigenic colour mutants of *A. flavus* and *A. parasiticus* have been used for biological control (method of competitive replacement) of aflatoxin contamination in peanuts. The colour mutants served as markers to monitor the atoxigenic strains in the soil and on the crop (DORNER *et al.,* 1995). As mentioned above, VCGs are often used as markers for the method of competitive replacement, but VCGs represent well-established tools for other kinds of studies, too.

Although no sexual stages are known in *Aspergillus* spp., gene flow within the species is possible (PAPA, 1984; BAYMAN and COTTY, 1991). As known in many filamentous fungi, physiologically distinct individuals of the same species can fuse asexually. This fusion includes plasmogamy, nuclear exchange and heterokaryon formation. When heterokaryon formation is stable, gene flow occurs and then the members are said to be vegetatively compatible, belonging to the same VCG (LESLIE, 1993). The VC has a multilocus genetic basis, called vegetative incompatibility (vic) loci. One, several or all of these vic loci, spread throughout the genome, determine both differences between VCGs and the VCG phenotype (LESLIE, 1996). These loci are analogous to histo-compatibility loci in vertebrates (BAYMAN and COTTY, 1992). In 1985, PUHALLA proposed a model suggesting that VC could be used to subdivide fungal populations into different VCGs and that these subdivisions were correlated with pathogenicity (PUHALLA, 1985). One assumption of this model is that individuals rarely, if ever, participate in recombination events, which could lead to re-assortment of the vic alleles, thereby creating new VCG phenotypes. In this model each VCG is a clone. VCG and pathogenicity are more exactly coincidentally correlated than an association in a cause-and-effect relation (PUHALLA, 1985). The model has been extensively applied to differentiate pathogenic forms of *Fusarium* spp. Most members were confined to one or a few VCGs, but some belong to a relatively large number of VCGs (PUHALLA, 1985). Many strains derived from isolated saprophytes behaved in a similar way (PUHALLA, 1985). The underlying mechanism of VC is not fully understood (LESLIE, 1996). In general two classes of mechanisms seem to be important for heterokaryosis: firstly, those genes, which are responsible for the establishment of the heterokaryon, i.e. the heterokaryon self incompatibility (hsi) loci, and secondly, genes required for heterokaryon maintenance after establishment (vic loci). Therefore heterokaryon formation is not a random effect but governed by the participating loci. In recent years, research has focused on these loci, with the overall goal to understand the network how loci function (LESLIE, 1996).

5.8. VC characteristics and application

According to PUHALLA (1985) the primary use of VCG in pathogenic fungi is for population studies. It is most useful in small scale, local populations to draw conclusions of the fate of an individual strain. The VCG technique requires considerably less technical equipment and laboratory input compared to other multilocus methods like DNA fingerprinting (CORRELL *et al.,* 1987). VCGs are well-suited for measuring genotypic diversity and to determine if two strains belong to the same VCG (multi-genetic assessment within fungal populations). Moreover, the VC may be used as a model for self-nonself recognition or as a tool for constructing genetic maps and rapid isogenisation of strains (BÉGUERET *et al.,* 1994). Finally, if the interaction mechanism is completely understood, the VC may serve as a novel target for antifungal agents (LESLIE, 1996). However, VCG analyses are not appropriate to determine isolates above the species level or to conclude the degree of relatedness between isolates (LESLIE, 1996). Furthermore, even if the isolates are vegetatively compatible it is difficult to detect the heterokaryon formation, especially when inhibitory alleles prevent its formation, e.g. mutants at hsi loci (CORRELL *et al.,* 1989). In the most complementation studies the VCG works as a polymorphic marker. Markers like RAPD may be more helpful in estimating the distance between distantly related individuals. Furthermore, the labour input per individual does not increase with sample size (BAYMAN and COTTY, 1992).

During the last 40 years research on VCG has focused on *Aspergillus, Fusarium, Neurospora,* and *Podospora* spp. (LESLIE, 1993; BEGUERET *et al.*, 1994).

In several studies with different fungi the VCGs were correlated with host range, locality, crossing ability, enzyme and nucleic acid polymorphisms, secondary metabolite production, and morphology (PUHALLA 1985; BAYMAN and COTTY, 1992). However, the VCG composition of an *A. flavus* population infecting a crop is not necessarily reflecting the *A. flavus* VCG composition in the soil, where the crop is grown (COTTY, 1992). Moreover, during crop development new resources for exploitation by *A. flavus* become available, thereby leading to rapid changes in the prevailing VCGs (BAYMAN and COTTY, 1992; COTTY, 1992).

In the current study, the VCG of the atoxigenic strain BN030D was determined and hence the VCG served as marker for the post-release-monitoring of isolates within the *A. flavus* L-strain community. If two strains of *A. flavus* meet, usually no clear signs of anastomosis or antagonism are detectable (BAYMAN and COTTY, 1992). For studying the vegetative compatibility between *A. flavus* strains, mutants are required. In *A. flavus* one type of such growth forms are nitrate-non-utilizing auxotrophs (so-called *nit* mutants) (PAPA, 1986; CORRELL *et al.,* 1987). *Nit* mutants of two isolates of *A. flavus* have identical alleles (loci) at each of several loci and so heterokaryon formation is possible (CROFT, 1987). These mutants were developed from each *A. flavus* L-strain isolate obtained from soil and crop isolations.

The subsequent VCG tests were performed in order to detect changes in the *A. flavus* L-strain population in the trial location after the strain release.

6. Materials and methods

6.1. Selection of the study location and experimental design

The area surrounding the village of Djidja (SGS), Benin, West Africa, was selected as the location for the present study. This area was previously identified as an aflatoxin high-risk area (HELL, 1997). The five fields chosen for this study were previously selected for baseline studies on species and strain distribution of *A. flavus* group fungi as well as for studies on VCG distribution (T. HOFFSTADT, pers. comm.).

Within each of the five fields, treatments were arranged in a completely randomised block design, and each field represented an experimental block with both a treatment and a control plot (Fig. 6). The size of the fields was 40 x 30 m and the treatment and control plots 10 x 30 m. A buffer area of 20 x 30 m separated the two plots within each field. In order to prevent disturbing influences by strong winds, the plot direction (i.e. the width of a field) was positioned parallel to the prevailing south-north wind direction. In each field the treatment plot was marked with a wooden peg and with an orange plastic ribbon in order to allow rapid identification of the treatment plots during field operations.

6.2. Field preparation and strain application

Most of the agronomic practices were carried out according to farmers' practices in the study area. Prior to sowing the soil was manually prepared with a cleaver (hack) to a depth of about 15 cm. The maize variety "DMR white-modified" (a downy-mildew-resistant IITA variety) was used in the experiments because of its high level of adoption by farming communities across several agro-ecological zones in West Africa (HELL, 1997, CARDWELL and COTTY, 2000). Maize was hand-sown at the beginning of the rainy season in April of 2001 at a rate of 2 - 4 seeds per hill and 20 - 30 cm and 30 - 50 cm within and between rows distance, respectively (HELL *et al.,* 2000). NPK fertilizer (15:5:20) was applied at a rate 100 kg ha^{-1} 20 days after sowing. The atoxigenic L-strain of *A. flavus* BN030D was isolated in the Djidja area in 1998 (CARDWELL and COTTY, 2000). Its great capacity for aflatoxin reduction and competition with other *A. flavus* strains had been demonstrated in several *in vitro* tests (CARDWELL and COTTY, 2000). BN030D was mass-produced in the IITA laboratory at Cotonou, Benin, and inoculated on autoclaved rice grains (BOCK and COTTY, 1999). The BN030D-inoculated rice grains were applied (plain) in the treatment plots at a rate of 0.5 g m^{-2} two days after sowing the maize seeds at the beginning of the growing season in April. The buffer area and the control plots were treated with autoclaved, un-inoculated rice grains. No additional toxigenic *A. flavus* strains were introduced into the soil. The owners of the fields carried out the common cultivation practices, whereas additional work like labelling the field plots, applications of treatment, soil or crop sampling was done by IITA personnel. All field operations were carried out uniformly block-by-block, starting in the

control plot, followed by the buffer area and finally in the treatment plot. Each single operation had to be completed within at least one day. Throughout the study, farmers watched the fields carefully in order to prevent children or animals from entering the field experiments. These arrangements were made, in order to avoid errors within a block, i.e. to prevent the removal of bits of soil or plant debris from one plot into another. Thus, conditions within the field trials were kept as equal as possible.

Fig. 5: The collection of soil samples in maize fields in Benin (the authors' photo).

6.3. Sampling of soil and maize

Samples were taken in the second week of April 2001, i.e. one day before BN030D release and in the third week of July, i.e. 12 weeks after the treatment. At the second sampling date, the maize was ready for harvest.

6.3.1. Soil collection

Soil samples were collected at two different times, i.e. prior to treatment and at harvest time. In each field, 8 composite soil samples of 200 – 300 g were taken by scrapings from the top 2 cm of the soil surface (Fig. 5). Each sample was composed of about 20 sub-samples of approximately 10 g taken along a line (Fig. 6). The first line was randomly chosen in the control plot and subsequent lines were positioned at 5 m intervals, thus covering the whole

experimental block (field). Composite soil samples no. 1 and 2 were obtained from the control plot, no. 3 - 6 from the buffer area and samples no. 7 and 8 from the treatment plot (Fig. 6). The 8 composite soil samples were kept separately in paper bags labelled with the field and sample numbers. In some cases the soils were completely dried in an oven. The paper bags were then placed in plastic bags to prevent cross contamination. In the laboratory the paperbags were subsequently stored at room temperature (23 - 27°C) until subsequent analyses.

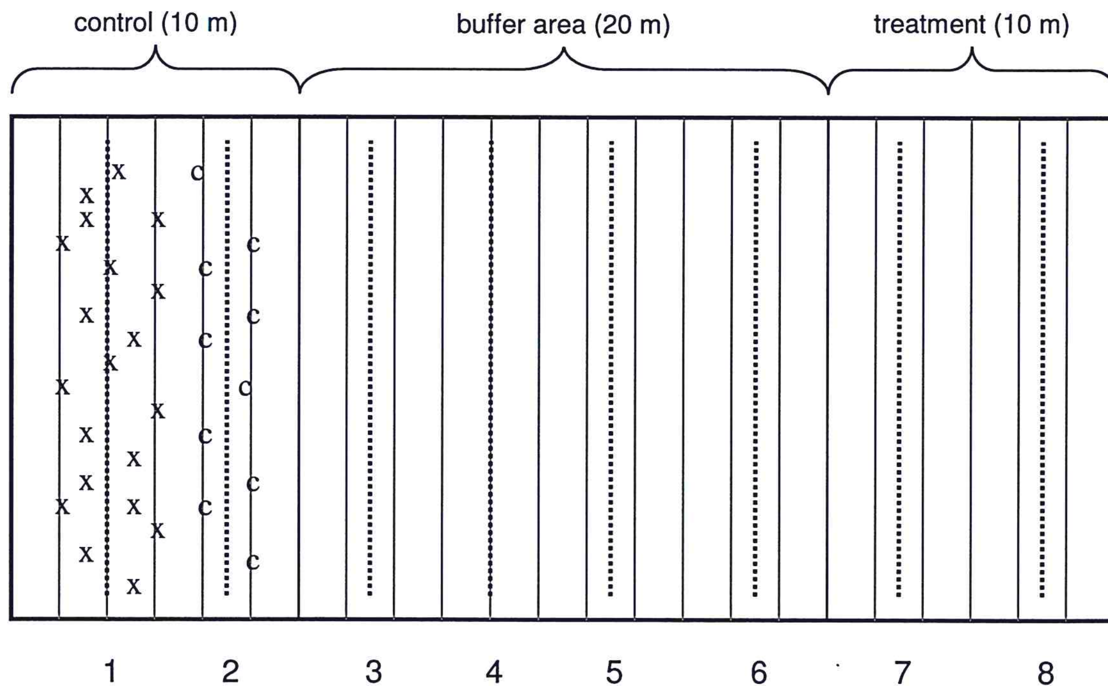


Fig. 6: Scheme of one field (experimental block). The numbers 1 - 8 and the corresponding dotted lines signify the different samples. "x" are possible sub-samples of the soil and "c" possible sub-samples of the maize cobs. Soil sub samples "x" were taken alongside each sampling line (1 - 8), whereas maize cob samples "c" were only taken from sampling lines 1 / 2 and 7 / 8. Grey lines symbolise the direction of the plant rows.

6.3.2. Maize cob collection

At harvest, maize cobs were sampled from treatment and control plots (not from the buffer area) of each field. Ten maize cobs per sampling line (as previously defined for the soil collections) were randomly collected from plants growing along the sampling line (Fig. 6). For each sample the 10 cobs were pooled and put into sampling bags, labelled according to field and sampling line.

6.4. Isolation of fungi from soil and maize samples

6.4.1. *A. flavus* isolation from soil samples

From each of the pooled composite samples 5 – 10 g of soil were randomly taken. The exact weight was measured for later calculation of colony forming units (CFU). The soil was then suspended in 50 ml of sterile, demineralised water. The soil-water-blend was stirred rapidly for 10 minutes and then 0.1 ml of the suspension was spread on 3 Petri dishes containing a Modified Rose Bengal medium (MRB medium) (COTTY, 1994b). Subsequently plates were incubated at ambient conditions (27 - 30 °C) in the dark for a period of 48 - 72 h.

Fig. 7: Section of a Petri dish containing MRB medium. Several colonies of blue-green *Penicillium* spp. and yellow-green *Aspergillus* spp. are visible (KENDRICK, 2003).

The developing fungal colonies were detected both qualitatively and quantitatively, focussing on the three genera *Penicillium* spp., *Fusarium* spp. and *Aspergillus* spp. (Fig. 7). *Aspergillus* spp. colonies were identified to the species-level both macroscopically and microscopically using a binocular and a microscope, respectively (KLICH and PITT, 1988). The fungal propagule density was determined by colony counts and expressed in CFU per gram soil or per 10 mm^3. The number of CFU was calculated using the following equation (SINGLETON *et al.*, 1992):

$$CFU/[g]soil = CFU/10[mm^3]soil = \frac{\left(\frac{50[ml]water}{0.1[ml]/plate}\right) * \#\,colonies/plate}{[g]soil * 1 * 10^{-3}}$$

From MRB medium Petri dishes, 15 - 25 *A. flavus* group colonies per sample were transferred onto Petri dishes containing V8 medium, using a sterile toothpick and working outward from the centre to prevent cross contamination. After 5 days of incubation at 27° - 30° C in the dark, species and strains were classified.
Classification of L- or S-strains of *A. flavus* was based on sclerotial formation on the V8 medium. S-strain isolates produce numerous black sclerotia (<300 µm in diameter) and only few conidia, whereas L-strain isolates produce few large sclerotia (>300 µm in diameter) and many conidia (Fig. 3) (COTTY, 1989; ORUM *et al.*, 1997).
The dilution plating was continued until 15 L-strain isolates per sample were obtained. Several 3 mm agar plugs with sporulating L-strain colonies were transferred to 4-dram vials, containing 4 ml of sterile distilled water. These conidial suspensions were then stored at room temperature for further use (COTTY, 1994b; CARDWELL *et al.*, 1999).

6.4.2. *A. flavus* isolation from maize samples

The maize samples were inspected immediately after harvest and the number of insects feeding on the maize cobs and the percentage of fungal surface-damaged cobs were recorded. To determine the different fungi present within a grain (i.e. invisible internal hyphal growth), all maize grains per composite sample were uniformly mixed within the plastic bags and 100 grains were randomly selected. Grains were surface disinfected by placing 100 grains into a Pyrex bottle filled with a 10% Eau de Javel solution (1,000 ml distilled water with 10 ml concentrate of Eau de Javel 3.5% NaOCl) and stirred for 1 min before pouring off the disinfection's broth. Then, distilled water was repeatedly added, mixed and poured off. Sterile glass Petri dishes, containing a sterile filter paper, were damped with distilled, autoclaved water, including 5 – 8 drops of lactic acid per litre. Then five maize grains were plated at equal distance next to the edge (Fig. 8). Twenty replicates per treatment were continuously incubated at 25° C with uninterrupted light for 5 - 7 days (SÉTAMOU *et al.*, 1997). All fungi that developed from the grains were identified based on macroscopical characteristics directly on the plate. The percentages of fungal decay relative to all 5 grains was estimated based on the total number of 100 grains per field. All *A. flavus* colonies were transferred to V8 medium, and classified to species and strain levels. Thirty L-strain isolates per treatment were randomly selected and later stored in 4-dram vials as previously described.
Finally, the conidial suspensions originating from both soil and maize samples were retained for the subsequent VCG analyses.

Fig. 8: Developing fungi on the surface-disinfected Petri dish with one germinating grain (the authors' photo).

6.5. Vegetative compatibility group (VCG) analysis

6.5.1. Generation of *nit* mutants

For generating nitrate-non-utilizing mutants of each wild type isolate, 0.1 ml of the above-mentioned conidial suspension of each *A. flavus* L-strain was spread into a well in the centre of a Petri dish, containing a selective medium (so-called SEL medium) (COTTY, 1994b). Petri dishes were incubated at 27° - 30° C in the dark for 1 - 4 weeks. The Growth of wild type strains is restricted on chlorate, presumably because chlorate is reduced by nitrate reductase to highly toxic chlorite. Nitrate non-utilizing mutants are unable to reduce chlorate to chlorite and hence are chlorate-resistant (PAPA, 1986). Most fungal isolates spontaneously sectored into *nit* auxotrophs from the initially restricted colonies. Sectors, which grow as thin expansive colonies with no aerial mycelium, were considered as *nit* mutants. Their occurrence on the SEL medium was examined periodically within the 4 weeks period (Fig. 9). *Nit* mutants were circled on the SEL medium Petri dish; then the mycelium block was punched out and transferred to another chlorate medium (so-called MIT medium) to stabilize the mutants (Fig. 10) (COTTY, 1994b).

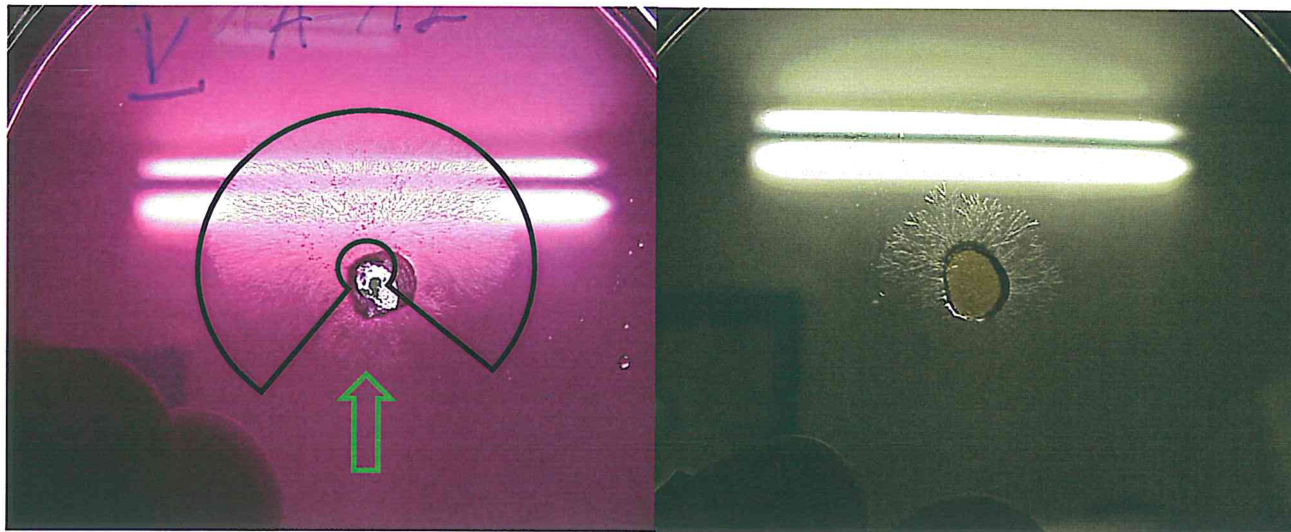


Fig. 9 (left): Two *nit* mutants developing from one wild type isolate of *Aspergillus flavus*: dense aerial-mycelial growth ("bad" *nit* mutant in the black circled area) and thin, expansive mycelium growth ("good" *nit* mutant marked with green arrow) (the authors' photo).
Fig. 10 (right): Stabilization of the "good" *nit* mutant: SEL medium plug placed on MIT medium (the authors' photo).

After 3 to 5 days of incubation in the dark at 27 - 30°C stabilized *nit* mutants on MIT medium were transferred to both V8 medium and Czapek-V-medium (CZ medium) (COTTY, 1994b). On the V8 medium the mutants showed wild type growth. This medium was appropriate to generate spore suspensions for subsequent storage in 4-dram vials until further use (as previously described). CZ medium served as test medium for the complementation tests (COTTY, 1994b).

6.5.2. Complementation tests

For the complementation tests, tester pairs were available (T. HOFFSTADT, pers. comm.). In the present study, each complementation test performed consisted of pairing an unknown isolate with two complementary testers of VCG BN30D, one *niaD*$^-$ mutant (tester 1) and one *cnx* mutant (tester 2). A *niaD*$^-$ mutant is a mutant in the nitrate reductase structural locus, whereas a *cnx* mutant represents a mutant in one of several genes for a molybdenum-containing cofactor necessary for nitrate reductase activity (Cove, 1976). Three wells (each 3 mm in diameter, 2 cm apart) were cut out in a triangular pattern in the centre of the medium contained in a 9 cm CZ medium Petri dish. For each complementation test, one well was seeded with BN030D *niaD*$^-$ mutant, one with BN030D *cnx* mutant and the third well received the conidial suspension of an unknown isolate (*nit* mutant) (Fig. 11) (COTTY, 1994b).

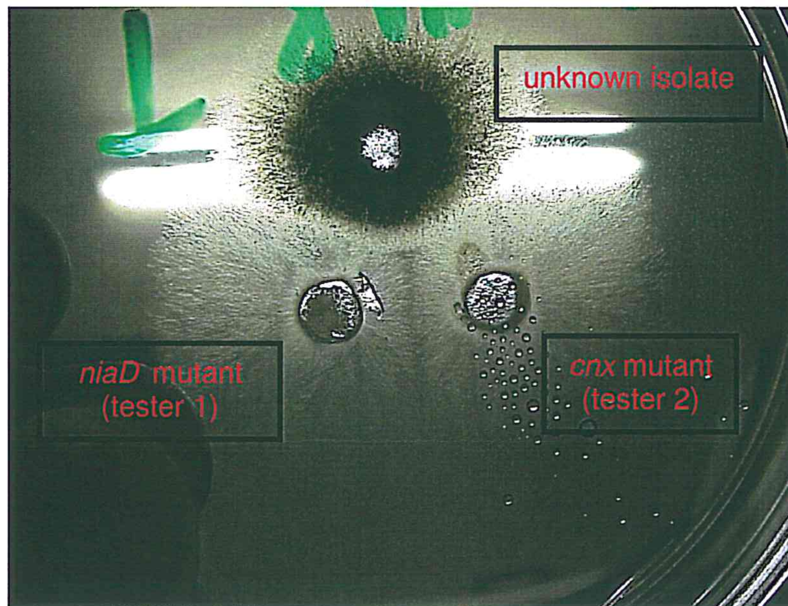


Fig. 11: VCG test performance: The unknown isolate can be assigned to a VCG if a heterokaryon formation occurs either with tester 1 (*niaD*⁻ mutant) or with tester 2 (*cnx* mutant) or with both of them. The figure shows the complementation test after 3 days of incubation (the authors' photo).

Due to difficulties caused by the conidial nature of *A. flavus* and due to instability of some mutants, only one complementation test was performed on each plate (COTTY, 1994b). In later tests, the conidial suspension of the two *cnx* / *niaD*⁻ mutants were seeded into wells two days prior to the introduction a plug of the MIT mycelial block containing the unknown *nit* mutant isolate. The growth rates of the three *A. flavus* mutants within the CZ medium Petri dish were adjusted with this alteration and some time was saved. In case of heterokaryon formation, the unknown *nit* mutant isolate could be assigned to the VCG of BN030D (Fig. 12) (COTTY, 1994b).

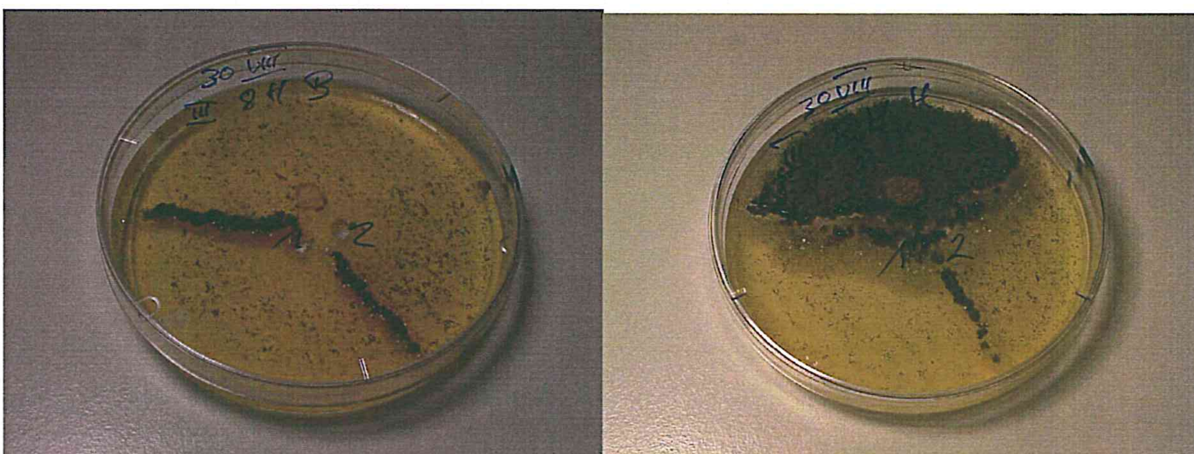


Fig. 12 (left): VCG test results obtained after 12 days showing positive compatibility to the VCG of BN030D. Two heterokaryon formations are visible: between the unknown isolate and tester 1 and between the both tester pairs (the authors' photo).
Fig. 13 (right): VCG test results obtained after 19 days showing a non-respondent reaction, i.e. no distinct compatibility (no heterokaryon formation) to the VCG of BN030D (the authors' photo).

6.6. Research hypothesis

In this study the following 3 research hypotheses were tested:

1. The composition of prevailing fungal populations (biodiversity) will not be affected by the application of the atoxigenic *A. flavus* strain BN030D.

$$H_{11}: \mu_c \neq \mu_t$$

$$H_{12}: \mu_c = \mu_t$$

   The number of CFUs and the percent decay (μ) in soil and maize samples, respectively, is different or equivalent in control (c) and treatment (t) plots.

2. The frequency of *A. flavus* L- and S-strains in soil and maize samples will be affected by the application of the atoxigenic *A. flavus* L-strain BN030D.

$$H_{21}: \mu_c = \mu_t$$

$$H_{22}: \mu_c < \mu_t$$

   In control (c) and treatment (t) plots the relationships of L- and S- strains (μ) is not affected by the BN030D release. Or, the relationship is affected and hence more L-strains are present in the treatment plots.

3. The application of the atoxigenic strain BN030D will change the VCG composition of *A. flavus* communities in the soil and on the crop towards a higher number of isolates compatible to BN030D.

$$H_{31}: \mu_c = \mu_t$$

$$H_{32}: \mu_c < \mu_t$$

   No differences between control (c) and treatment (t) plots in terms of numbers of isolates (μ) belonging to the VCG of strain BN030D are found, or more isolates belonging to the same VCG as BN030D are detected.

All three hypotheses will be verified by comparing post-release soil and maize samples with the corresponding pre-release samples.

6.6. Data analysis

The relationship of the different **fungal communities** (hypothesis 1) was compared excluding the effect of the block (block alignment). The problem requires, as test theory, the problem of equivalence. Significant equivalence between two groups exists if the quotient is located within the equivalence threshold. In safety studies, as it is supposed in this case, the threshold is usually defined from 0.8 for the lower to 1.25 for the upper limits (F. BRETZ, pers. comm.). Due to the obtained data, Gauss distribution was not assumed and therefore a non-

parametric test on quotients according to Hodges-Lehmann served to test on equivalence (HOTHORN and MUNZEL, 2001). It was computed (program R) with Wilcoxon Rank Sum test.
In order to show independence from the initial composition of fungal populations residing in the soil before the strain application, the paired Wilcoxon Rank Sum test (program R) was performed for each fungus on both treatment replicates (block alignment). If the confidence interval excludes zero, significant differences exist between the two sampling times within one plot.
The relationship of **strain composition**, e.g. L- and S-strain incidence of *Aspergillus flavus* communities (hypothesis 2), was demonstrated using Pearson χ^2-test (program StatXact) for each experimental block, because each single field was considered as independent locations.
Similarly, the frequency of isolates with complementation to the **VCG** of the applied atoxigenic strain BN030D (hypothesis 3) was analysed using Pearson's χ^2-test (program StatXact).
The proc glm (program SAS) was used on fungal population data from soil samples to determine whether the fields (each experimental block) differed significantly.

7. Results:

The results are presented in the following order: (1) Pre- and post-intervention propagule density of fungal communities in soil and cob samples, as expressed in mean CFU g^{-1} of soil and in percent decay on maize grains, respectively. (2) Post-intervention occurrence of and damage by insects and fungal diseases on maize cob surfaces, respectively. (3) Pre- and post-intervention composition of *A. flavus* L- and S-strains in soil and cob samples. (4) Pre- and post-intervention frequency of BN030D within *A. flavus* L-strain communities (VCG tests) in soil and cob samples. (5) Dispersion of BN030D from treatment plots into the buffer area with soil samples. For soil samples pre- and post-intervention data were compared whereas for maize cob samples only post-intervention data were taken.

7.1. Fungal population analysis

7.1.1. Propagule density (CFU) in soils

The predominant fungal genera in soil samples were *Fusarium* spp. (58.9%), *Penicillium* spp. (10.6%), and *Aspergillus* spp. Within the *Aspergillus* genus, fungi of the *A. flavus* group (20.7%) and *A. niger* (9.7%) were most frequent. The majority of the fungi were *Fusarium* spp. (Tab. 2).

7.1.2. Soil samples before strain application (pre-intervention)

In the pre-intervention soil samples significant equivalence in propagule density of *A. niger, Fusarium* spp. and *Penicillium* spp. between the control and the subsequent treatment plots were recorded. A slight decrease in CFU g^{-1} soil within the treatment plots was observed. However, no significant equivalence between control and treatment was detected for *A. flavus* communities, with lower amounts of CFU g^{-1} soil observed in treatment plots (Tab. 2; Fig. 14).

Tab. 2: Mean (±SD, standard deviation) propagule density in CFU g^{-1} soil of the most frequent fungi for both sampling times (pre- and post- intervention) and treatments (control and treatment). CFU (in absolute numbers) per row followed by the same letter are significantly equivalent (Hodges-Lehmann tests with Bonferroni α-adjustment for each sampling time).

Sampling time	Fungal communities	Control [CFU]	Treatment [CFU]	Lower level (0.80)	Upper level (1.25)
Pre-intervention	*A. flavus*	1.30^{a} (±0.61)	1.14^{b} (±1.31)	0.73	0.84
	Fusarium spp.	2.75^{a} (±0.64)	1.64^{a} (±0.53)	0.98	1.10
	Penicillium spp.	0.36^{a} (±0.31)	0.28^{a} (±0.31)	0.85	1.01
	A. niger	0.26^{a} (±0.34)	0.23^{a} (±0.35)	0.87	1.04
Post-intervention	*A. flavus*	0.75^{a} (±1.09)	1.03^{a} (±0.46)	1.01	1.24
	Fusarium spp.	2.51^{a} (±0.64)	2.39^{b} (±0.66)	1.27	1.51
	Penicillium spp.	0.46^{a} (±0.55)	0.87^{a} (±1.34)	0.98	1.11
	A. niger	0.31^{a} (±0.39)	0.50^{a} (±0.64)	0.94	1.06

7.1.3. Soil samples after strain application (post-intervention)

In the post-intervention soil samples taken at harvest time, results of the Hodges-Lehmann tests revealed significant equivalence between control and treatment plots for propagule density of *A. flavus, A. niger,* and *Penicillium* spp., but not for *Fusarium* spp. Here lower CFU densities were observed in treatment plots. In the other fungi slightly higher CFU numbers were recorded in treatment plots (Tab. 2; Fig. 15).

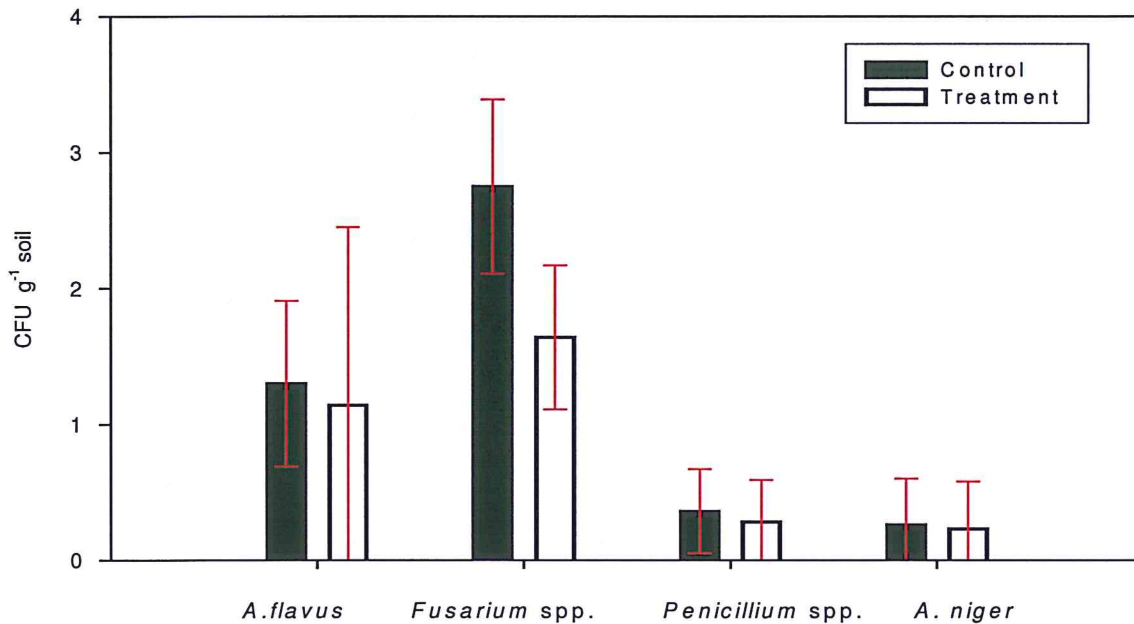


Fig. 14: Mean propagule density in CFU g^{-1} (± SD) soil of the most frequent fungi as compared by treatment vs. control before strain application (**pre-intervention**).

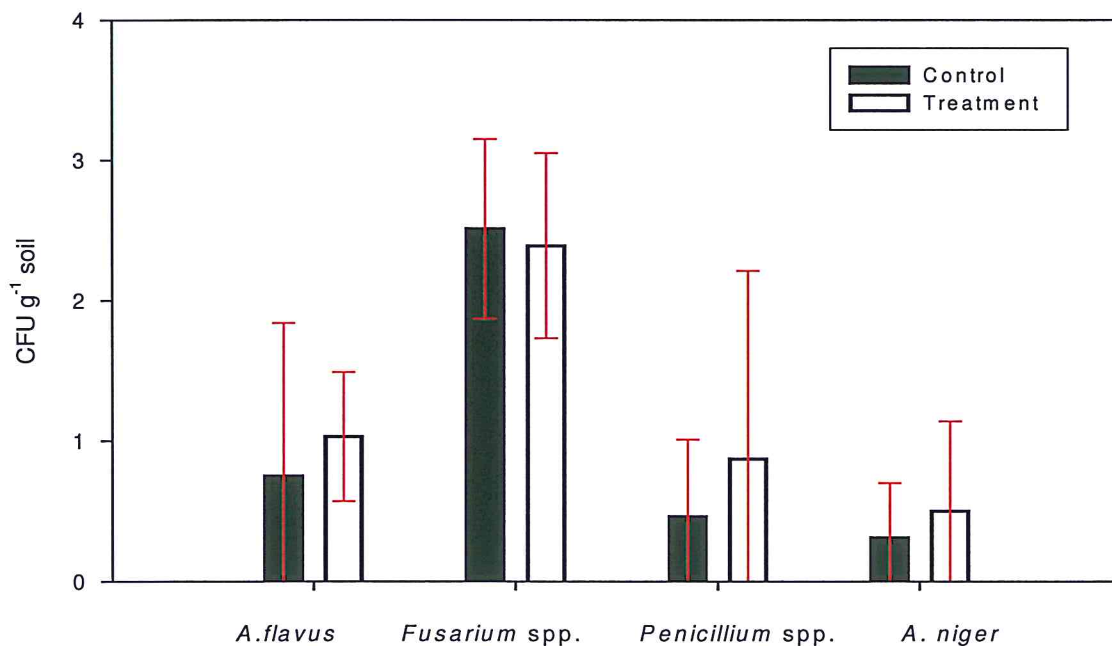


Fig. 15: Mean propagule density in CFU g^{-1} (± SD) soil of the most frequent fungi as compared by treatment vs. control after strain application (**post-intervention**).

7.1.4. Fungal presence on maize cobs

In post-intervention samples taken from the crop at harvest time *A. niger* was not particularly observed, but was determined with other fungi in the group "others" (e.g. Tab. 3). In cob samples the proportion of maize grains infected by *Fusarium* spp., *Penicillium* spp., and "other" fungi were significantly equivalent between control and treatment plots. For *A. flavus* no significant equivalence was detected, and a higher percentage of infected grains were recorded in the control compared to the treatment plots (Tab. 3).

Tab. 3: Mean percentage (± SD) of grains internally infected by different fungi in control and treatment plots at harvest. For each fungal community lower and upper limits were computed using the Hodges-Lehmann test on equivalence between control and treatment (with Bonferroni α-adjustment). Percentage values for each fungal species followed by the same letter are significantly equivalent.

Fungal Community	Control [%]	Treatment [%]	Lower limit 0.80	Upper limit 1.25
A. flavus	42.2[a] (±24.0)	31.3[b] (±14.7)	0.79	0.93
Fusarium spp.	66.9[a] (±27.6)	65.8[a] (±23.5)	0.84	1.00
Penicillium spp.	13.6[a] (±17.8)	12.7[a] (±15.2)	0.99	1.06
"others"	18.7[a] (±20.5)	28.5[a] (±22.4)	1.08	1.23

Tab. 4: Mean propagule density (± SD) in CFU g^{-1} soil of the most common fungi pre- and post-intervention in control and treatment plots. Lower and upper confidents limits were calculated using paired Wilcoxon Rank Sum test on differences between the sampling times (with Bonferroni α - adjustment according to on each field: confidence interval (CI) – level = 0.9875). For each fungal species CFU densities followed by different letters are significant different.

Field plot	Fungal communities	Pre-intervention [CFU]	Post-intervention [CFU]	Lower CI [CFU]	Upper CI [CFU]
Control	*A. flavus*	1.30[a] (±0.61)	0.75[b] (±1.09)	-0.97	-0.55
	Fusarium spp.	2.75[a] (±0.64)	2.51[a] (±0.64)	-0.45	0.21
	Penicillium spp.	0.36[a] (±0.31)	0.46[a] (±0.55)	-0.08	0.21
	A. niger	0.26[a] (±0.34)	0.31[a] (±0.39)	-0.12	0.23
Treatment	*A. flavus*	1.14[a] (±1.31)	1.03[b] (±0.46)	-0.14	-0.06
	Fusarium spp.	1.64[a] (±0.53)	2.39[b] (±0.66)	0.53	1.05
	Penicillium spp.	0.28[a] (±0.31)	0.87[b] (±1.34)	0.27	0.52
	A. niger	0.23[a] (±0.35)	0.50[b] (±0.64)	0.09	0.43

7.1.5. Comparison of propagule densities of fungal communities in soil samples pre- and post-intervention (change over time)

Only data from soil samples taken pre- and post-release could be analysed as crop samples were only collected at harvest. In both treatment and control plots propagule density of fungal communities in the soil changed over the time, though in control plots no significant differences between both sampling times were recorded for *Fusarium* spp., *Penicillium* spp., and *A. niger*. For *A. flavus*, significant lower propagule density was detected post-

intervention (Tab. 4). However, in treatment plots significant differences between the two sampling times were recorded for all fungi with lower propagule densities for *A. flavus* (Tab. 4; Fig. 16 and 17).

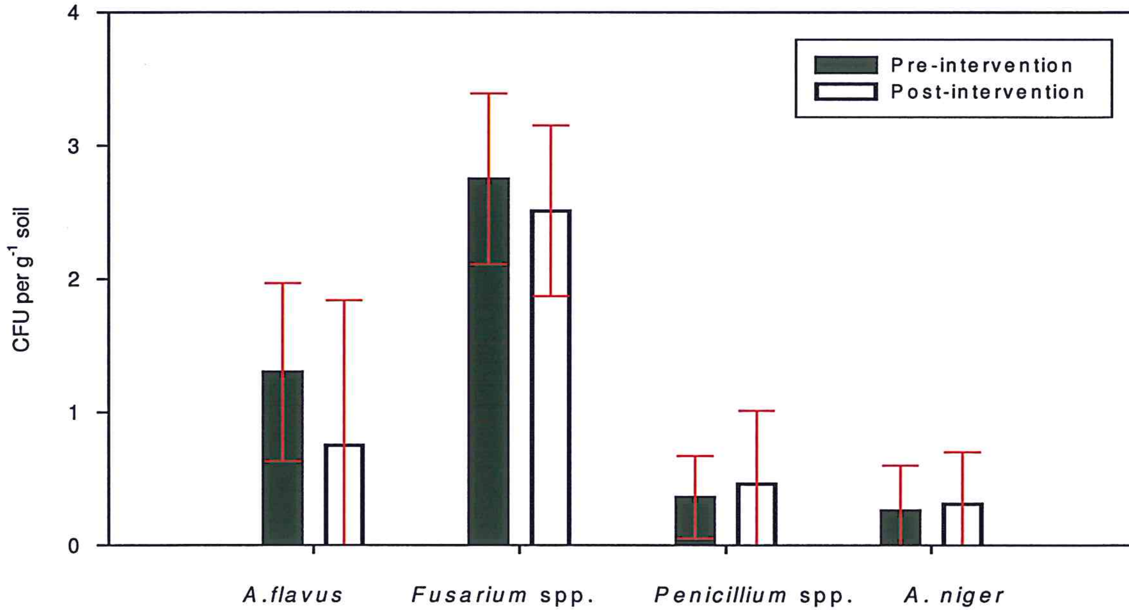


Fig. 16: Mean propagule densities in CFU g^{-1} (± SD) soil samples taken pre- and post-intervention of the most frequent fungi recorded in the **control plots**.

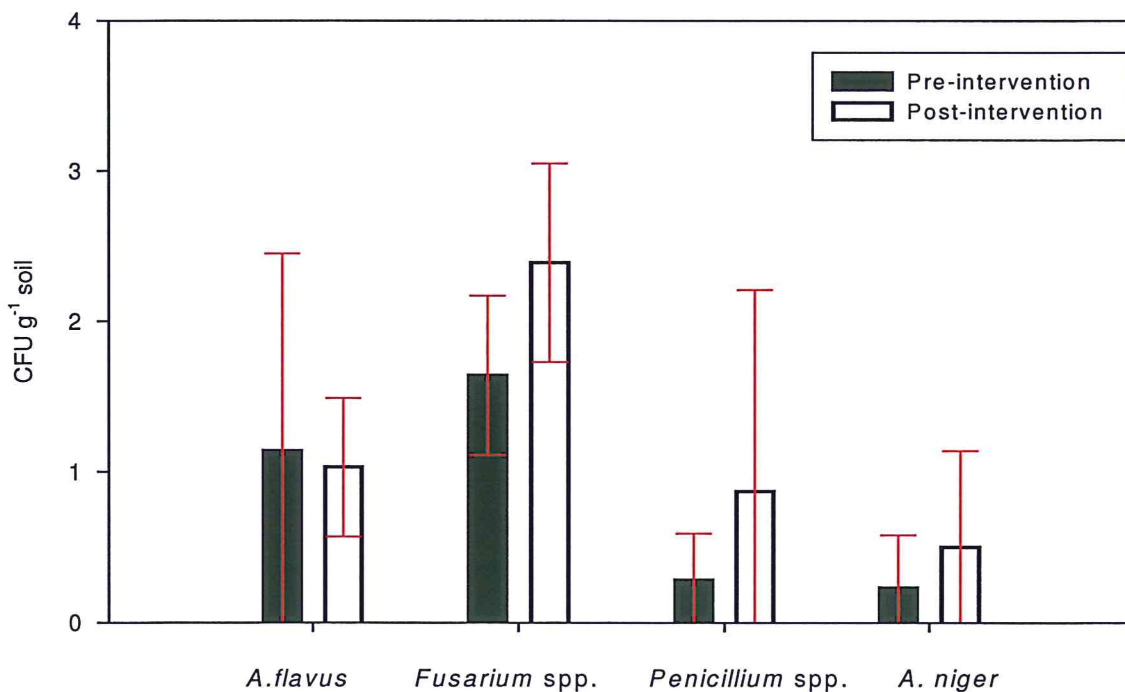


Fig. 17: Mean propagule densities in CFU g^{-1} (± SD) soil samples taken pre- and post-intervention of the most frequent fungi recorded in the **treatment plots**.

7.2. Surface damage of insects on maize cobs

In cob samples taken from post-intervention treatment and control plots at harvest the following insect pests were found on the maize cobs: *M. nigrivenella*, *C. leucotreta*, *C. quadricolis*, *S. zeamais*, and *Carpophilus* spp. Damage symptoms that could be attributed to vertebrates (birds, rats, etc.) occurred only marginally. No distinct differences between control and treatment plots were recorded. In treatment plots slightly more damage causing individuals of *C. leucotreta*, *S. zeamais*, and *Carpophilus* spp. were found, whereas fewer numbers of *M. nigrivenella*, and *C. quadricolis* were observed (Fig. 18).

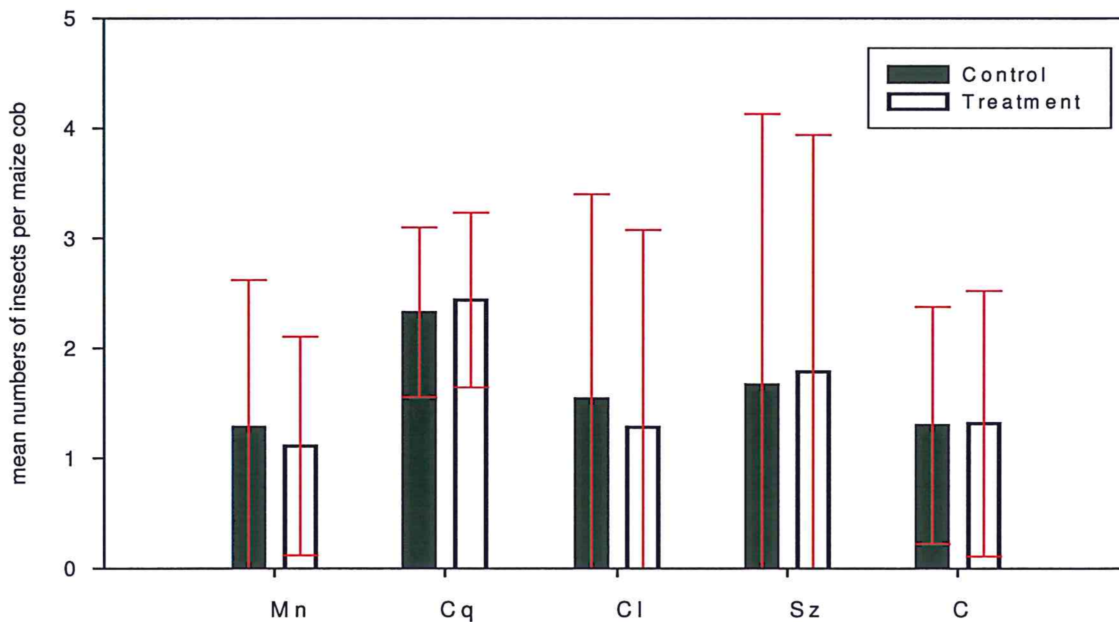


Fig. 18: Mean numbers (± SD) of insects per maize cob in post-intervention control and treatment plots (Mn = *M. nigrivenella*, Cq = *C. quadricollis*, Cl = *C. leucotreta*, Sz = *S. zeamais*, C = *Carpophilus* spp.).

7.3. Surface infection of fungi on maize cobs

The most predominant fungi externally infecting the post-intervention maize cobs were *Fusarium* spp., *Penicillium* spp., *A. flavus* and other fungi, with *Fusarium* spp. the most frequently ones recorded. In samples from treatment plots slightly more *A. flavus* and other fungi (group "others") were observed, whereas incidences of *Fusarium* spp. *and Penicillium* spp. infections were lower in treatment than control plots (Fig. 19).

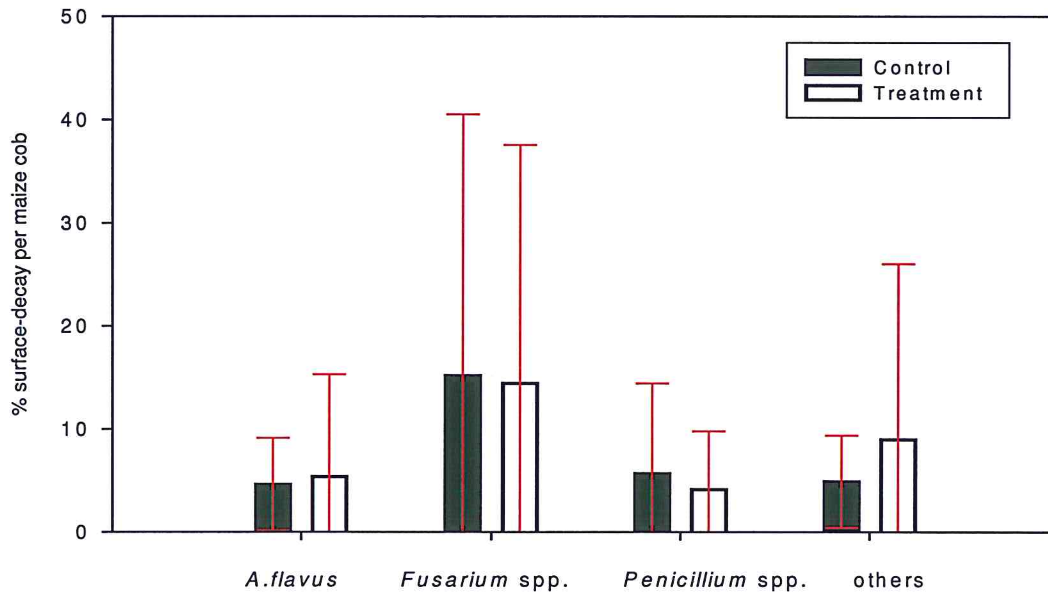


Fig. 19: Mean percent (± SD) decay of surface-infected maize cobs relevant to the different fungal communities (post-intervention control and treatment plots).

7.4 *A. flavus* soil community composition (L- and S-strain relationship)

The composition of *A. flavus* communities varied within and between the fields. In general more L- than S-strains were recorded in soil (pre- and post-intervention) and maize samples (post-intervention only). In all pre-release soil samples from (subsequent) treatment plots no significant differences were observed between the replicates. At harvest time (post-intervention), the number of L-strains in control and treatment plots de- and increased, respectively (Tab. 5). In both treatment and control plots higher numbers of S-strains were isolated in post- compared to pre-intervention soil samples. In maize samples at harvest in treatment plots slightly higher numbers of L-strains than in the control and no S-strains were recorded. However, except for one block, i.e. maize sample block II, no significant differences between control and treatment plots were found (Tab. 5).

7.5. VCG tests

From each soil sample of the corresponding sample line (pre- and post-intervention) approximately 15 *A. flavus* L-strain (wild type) isolates were collected. *Nit* mutants were generated from 84.7% of all wild-type isolates and were subsequently used in vegetative compatibility tests.

Tab. 5: Relationships of L- and S-strains of *A. flavus* (in number of isolated strains) between treatment and control in each block. Soil samples were taken pre- and post-intervention, whereas maize samples only post-intervention. Pearson's χ^2-test (with Holm α-adjustment per sampling unit) was used to test for differences between control and treatment plots.
n.s. = not significant, s. = significant.

Sampling unit	Block	Control L	Control S	Treatment L	Treatment S	χ^2-test results
Pre-intervention (soil samples)	I	49	5	26	3	n.s.
	II	47	0	35	1	n.s.
	III	44	2	28	1	n.s.
	IV	42	0	35	2	n.s.
	V	48	0	30	2	n.s.
Post-intervention (soil samples)	I	34	5	37	4	n.s.
	II	33	6	41	2	n.s.
	III	44	5	37	4	n.s.
	IV	36	8	33	1	n.s.
	V	33	1	32	1	n.s.
Post-intervention (crop samples)	I	23	1	28	0	n.s.
	II	21	7	29	0	s.
	III	29	1	29	0	n.s.
	IV	28	0	22	0	n.s.
	V	28	1	27	0	n.s.

7.5.1. Frequency of VCG BN030D among *A. flavus* L-strain isolates from pre-intervention soil samples

In pre-intervention soil samples no significant differences were found between treatment and control plots. Within one treatment and one control plot from different fields one isolate showed vegetative compatibility with BN030D. Over all blocks less than 1% of the isolates showed vegetative compatibility with the applied atoxigenic strain, whereas 75% were not. Approximately 24% of the isolates were determined as non-respondent (Tab. 6; Fig. 20).

7.5.2. Frequency of VCG BN030D among *A. flavus* L-strain isolates from post-intervention soil samples

Composition of L-strain isolates collected from post-intervention soils differed significantly in their vegetative compatibility to BN030D between all control and treatment plots (Fig. 20). Across the five fields 40% of all isolates in treatment plots could be assigned to the VCG of BN030D, whereas 26% were not compatible and 34% were non-responding (non-respond.). Whereas in the control plots 75% of all isolates were not vegetatively compatible, none was positive and 25% were non-responding (Tab. 6; Fig. 20).

Tab. 6: Number of *A. flavus* isolates showing positive, negative or non-respondent results in the VCG tests per block and sample unit. Pearson's χ^2-test (Holm α-adjustment per sampling unit) was used to compare proportions of positive and negative tested isolates between control and treatment plots. In maize samples non-respondent isolates were not counted (n.s. = not significant, s. = significant).

Sampling unit	Block	Control positive	Control negative	Control non respond.	Treatment positive	Treatment negative	Treatment non respond.	χ^2-test
Pre-Intervention (soil samples)	I	0	16	7	0	20	2	n.s.
	II	0	22	2	0	16	3	n.s.
	III	0	23	6	1	20	3	n.s.
	IV	1	15	12	0	17	2	n.s.
	V	0	17	12	0	12	10	n.s.
Post-Intervention (soil samples)	I	0	16	8	14	10	6	s.
	II	0	24	4	9	9	10	s.
	III	0	19	4	15	6	9	s.
	IV	0	15	6	16	9	5	s.
	V	0	18	6	3	8	16	s.
Post-Intervention (cob samples)	I	0	24	-	0	27	-	n.s.
	II	0	30	-	4	25	-	s.
	III	0	30	-	2	28	-	n.s.
	IV	0	28	-	8	14	-	s.
	V	0	29	-	21	6	-	s.

7.5.3. Frequency of VCG BN030D among *A. flavus* L-strain isolates from post-intervention maize samples

In control plots none of the isolated L-strains showed vegetative compatibility to BN030D. In contrast, in maize samples from treatment plots in 25.9% of all isolates positive complementation was recorded. In three out of five treatment blocks significant differences in L-strain composition compared to the corresponding control blocks were recorded (Tab. 6; Fig. 21).

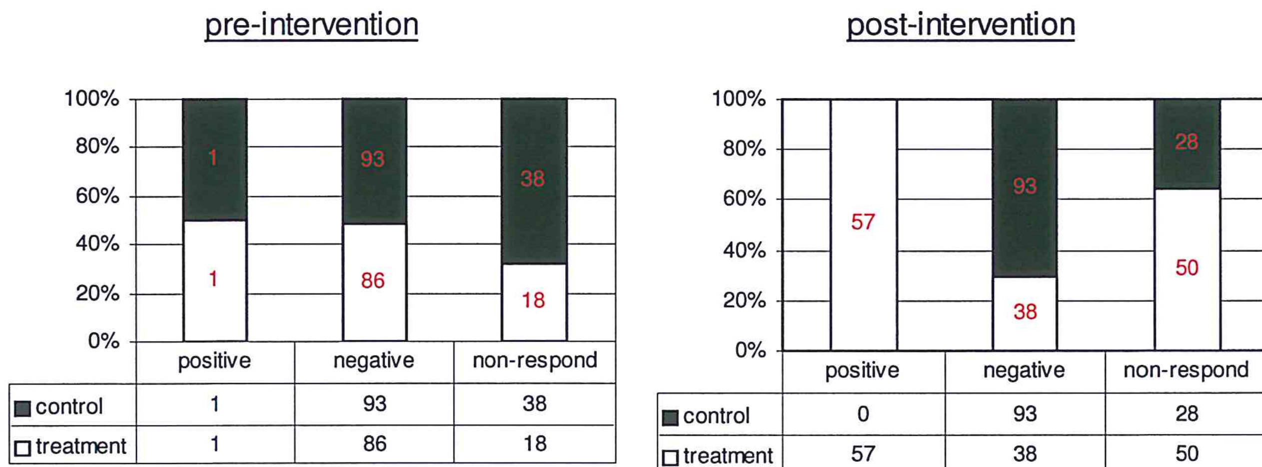
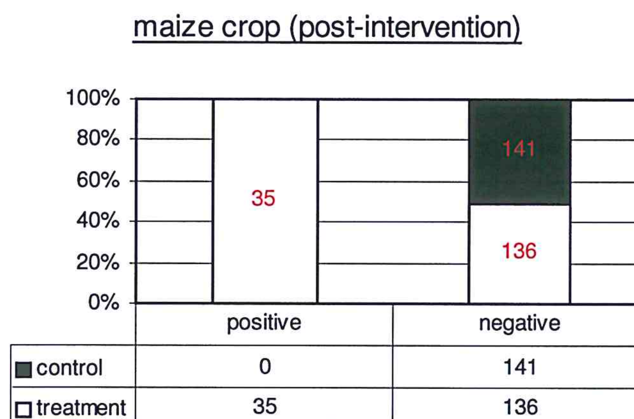


Fig. 20 (left): VCG test results of *A flavus* L-strain isolates from pre-intervention soil samples.
Fig. 21 (right): VCG test results of *A flavus* L-strain isolates from post-intervention soil samples.



	positive	negative
control	0	141
treatment	35	136

Fig. 22: VCG test results of *A. flavus* L-strain isolates from post-intervention crop samples.

7.6 Dispersal of BN030D into the buffer area (soil samples)

In pre-intervention soil samples no vegetative compatibility to BN030D was found in L-strain isolates from the buffer area. However, at harvest, 4 months after the application of the atoxigenic *A. flavus* strain in the treatment plots, several L-strain isolates from the buffer had vegetative compatibility with BN030D, indicating a spread of the strain from the original release areas (Fig. 23 and 24).

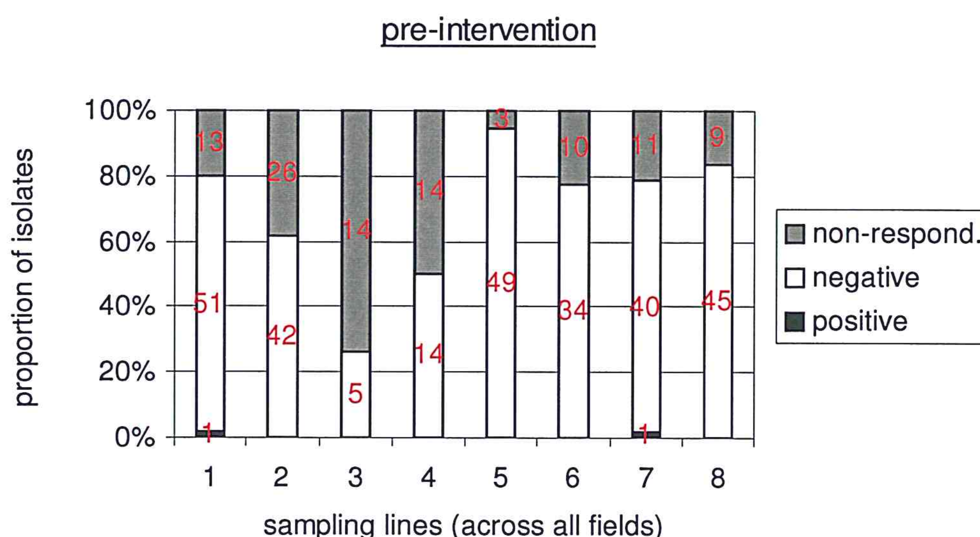


Fig. 23: Proportion of isolates from pre-intervention soil samples with positive, negative or non-responding (non-respond.) compatibility to BN030D (sampling lines 1 / 2 = control, 3 – 6 = buffer area, 7 / 8 = treatment; block structures merged). Red numerals indicate the numbers of tested isolates.

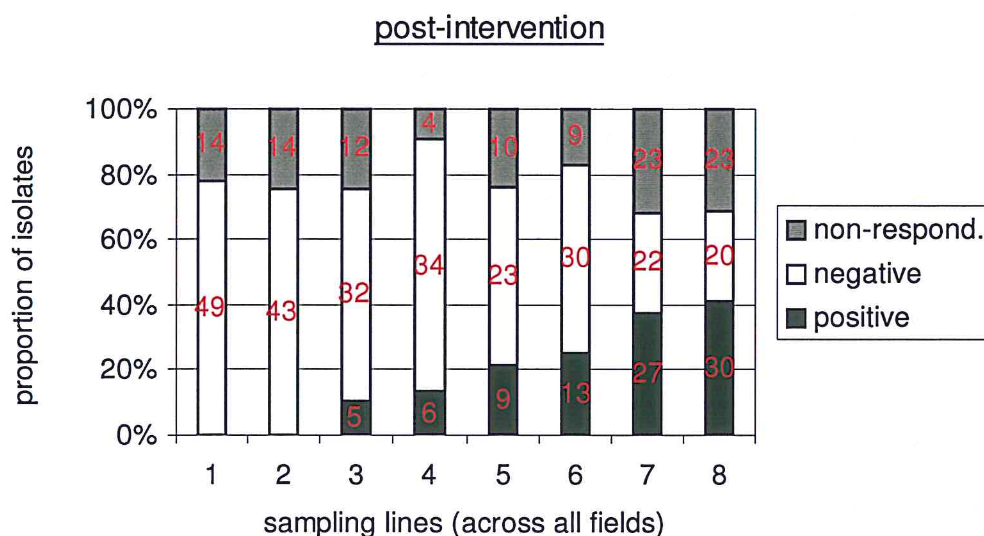


Fig. 24: Proportion of isolates from post-intervention soil samples with positive, negative or non-responding (non-respond.) compatibility to BN030D (sampling lines 1 / 2 = control, 3 – 6 = buffer area, 7 / 8 = treatment; block structures merged). Red numerals indicate the numbers of tested isolates.

7.7. Trial design

Results of analysis of variances (ANOVA), which was applied on post-intervention CFU data, detected significant differences between the experimental blocks (significant p-value ($p<0.001$) for the variable "block").

8. Discussion

8.1. Composition of saprophytic fungal soil populations

At the experimental site in Didja, Benin, the presence of three species *Aspergillus* section *flavi* was revealed, i.e. *A. parasiticus, A. tamarii* and *A. flavus.* The latter one was the most frequently found species, accounting for more than 99% of *Aspergillus* spp. recorded. Similarly, in the SGS in Benin, SÉTAMOU *et al.* (2000) reported that within the genus *Aspergillus, A. flavus* was present in 95% and 93.3% of soil samples in 1994 and 1995, respectively. In the current study, the predominant fungi in soil samples were *Fusarium* spp. (58.9%), *Penicillium* spp. (10.6%), and *Aspergillus* spp., with *A. flavus* (20.7%) and *A. niger* (9.7%) as the two most frequent species. Studies from North America showed similar frequencies for the fungal communities (DORNER *et al.,* 1992; COTTY, 1994b, 1997).

When sampling for saprophytic fungi in the soil, soil samples should be dried after collections (for 5 days at 40° C in an oven) and should remain dry during storage (ORUM *et al.*, 1997; CARDWELL and COTTY, 2001). For logistic reasons, in this study no oven was available to dry the soil samples taken at harvest (post-intervention). During the processing of the samples in the laboratory a considerable variability in soil moisture content among the samples was observed, e.g. soil samples from field IV were wetter than the ones from field II. Hence, such wet samples were left in open bags in a room without AC (temperatures ranging between 30° and 35° C) for 2 - 4 days. Thereafter, the samples were stored in non-sealed plastic bags at room temperature (23° - 27° C). In general soil samples for collections of saprophytic fungi are oven dried to assure similar treatment of all samples and to maximize stability of propagules during subsequent storage. Moreover, if the soil is not completely dry during storage, competing organisms like bacterial or yeasts may affect the composition of saprophytic fungi in the samples. The comparatively high variability in the present data and differences with results from other studies might have been caused by differences in drying and storage of pre- and post-intervention soil samples (COTTY, 1994b; ORUM *et al.*, 1997; CARDWELL and COTTY, 2001).

8.1.1. Fungal population analysis from soil samples

In this study high variability in fungal propagule density, measured in CFU per soil sample, was recorded. For instance *A. flavus* communities ranged from 0.32 - 1.64 CFU g^{-1} soil. However, high unpredictability of CFU counts is not unusual and has been described in previous studies (SÉTAMOU *et al.,* 2000; CARDWELL and COTTY, 2001).

In pre-intervention soil samples, *Fusarium* spp., *Penicillium* spp., and *A. niger* populations were significantly equivalent in samples taken in the control and subsequent treatment plots, whereas *A. flavus* communities were not. However, despite a slightly increased CFU density in post-intervention soil samples significant equivalence of *A. flavus*, *Penicillium* spp. and

A. niger between control and treatment plots were recorded. Only propagule density of *Fusarium* spp. significantly differed between control and treatment plots, with lower values obtained in the plots where BN030D had been previously applied. High variability was observed for *Fusarium* spp. propagule density and possibly this might have been the reason why no significant equivalence was detected. Another explanation might have been interactions between *A. flavus* and *Fusarium* spp. communities. ZUMMO and SCOTT (1992) showed that *F. moniliforme* competitively inhibited growth of *A. flavus*, though nothing is known about potential displacement of *Fusarium* spp. by *A. flavus*.

8.1.2. Fungal population analysis from maize samples at harvest (post-intervention)

In maize samples taken at harvest significant equivalence between control and treatment plots was found for *Fusarium* spp. and *Penicillium* spp., and "others", but not for *A. flavus*. The differences in *A. flavus* infections in grains collected in treatment and control plots are possibly linked to the sampling protocol, which was initially designed on the VCG analyses. For a thorough study on saprophytic fungal populations' compositions most likely a higher sample size would have been required as revealed by the high variability of the data. However, the significant p-value for the variable "block" in the ANOVA indicates that the trial design was appropriate and blocking the fields was meaningful. Therefore, the statistical analyses focused on the block design and the equivalence approach, i.e. the Hodges-Lehmann test. In general, no great differences between control and treatment plots in terms of *A. flavus* infections in maize grains were observed. In the SGS of Benin, SÉTAMOU *et al.* (1997) found that *A. flavus* was present on 29.2% and 20.9% of the maize grains tested in 1994 and 1995, respectively. Differences compared to the results of the present study might have been due to different climatic conditions in the different cobs, the cultivars grown, and the incidence of *M. nigrivenella* and / or other pests.

8.1.3. Changes in fungal populations over time

In the control plots, the composition of *Fusarium* spp., *Penicillium* spp. and *A. niger* did not differ significantly between both sampling times. However, significantly higher propagule densities (in CFU g^{-1} soil) were recorded for *Fusarium* spp., *Penicillium* spp. and *A. niger* in treatment plots at harvest (post-intervention), whereas numbers of *A. flavus* propagules decreased in treatment plots in the course of the vegetation period: at least 0.06 CFU g^{-1} soil and at most 0.14 CFU g^{-1} soil. Comparatively, in the control plots the decrease in propagule density of *A. flavus* within the course of vegetation period was much clearer: at most 0.55 CFU g^{-1} soil and at least 0.97 CFU g^{-1} soil.

In both post-intervention soil and maize samples in treatment plots *A. flavus* propagule density and incidence of infections of *A. flavus* in maize grains did not differ from the control, despite the previous release of the atoxigenic *A. flavus* strain BN030D (Tab. 2 and 3). However, over time (i.e. from pre- to post-intervention) *A. flavus* CFU counts in treatment plots decreased to a lesser extend than in control plots.

A methodological drawback in the analysis of saprophytic fungal population composition in this study was that all analyses were based on colony formation on MRB medium. This medium is specially designed to serve as clean-up medium for *A. flavus* strains. Consequently, the analysis of the presence of fungal species in the soil other than *A. flavus* does not necessarily reflect the real situation of compositions of fungal populations. However, it should allow detecting changes in the fungal population.

Several factors may have influenced the observed high variability of fungal propagule densities in soil samples. For instance crop residues and / or insect cadavers in the soil might have affected the composition of fungal communities in the soil. No attempt was made in this study to quantify these factors. However, the decrease in *A. flavus* propagule density within the time response is astonishing, since the climate (weather) was moister in the beginning of the vegetation period. Contrarily, at harvest time the drier climate should favour *A. flavus*. Therefore proposed higher inoculum levels of *A. flavus* were not obtained.

In summary, the results of the population analysis (soil and crop samples) confirmed the tremendous variability among fungal strains in tropical environments (CARDWELL and COTTY, 2000). All fungal genera detected are saprophytes and grow on plant debris or similar substrates. A strain application may theoretically increase the quantity of *A. flavus* inoculum, at least initially. However, it does not increase suitable substrates *A. flavus* can colonise, and the latter may have prevented an increase in proportion of *A. flavus* among the fungal communities. A major objective of the current study was to investigate whether a release of BN030D would lead to substantial shifts in the composition of fungal communities. However, results of this study indicate such risks are apparently rather low. Similarly in other studies, no increase in *A. flavus* propagule density or no negative effects were observed after release (DORNER *et al.*, 1992; COTTY and BAYMAN, 1993; COTTY, 1994b). Yet, in these studies no appropriate statistical tests had been used (e.g. equivalence tests). In general results of the present and previous studies (DORNER *et al.*, 1992; COTTY, 1994b; COTTY, *et al.*, 1994) suggest that mass releases of atoxigenic *A. flavus* strains in the field do not necessarily affect the natural balance within the eco-systems.

8.2. Composition of *A. flavus* soil communities

8.2.1. Natural changes

The results of this study indicate that the L-strains largely dominate the *A. flavus* communities in the trial area of Djidja. In all soil and crop samples S-strains occurred at very

low frequencies. Predominance of L-strains in maize fields from the same area has been previously described (CARDWELL and COTTY, 2001). There, 97.8% L- and 2.2% S-strains were detected, compared to 93% L- and 6% S-strains in the present study. However, even greater fluctuations within areas of the same ecological zone and years are common, underlining the great variability in *Aspergillus* spp. propagule densities (COTTY, 1989, 1997; ORUM *et al.*, 1997). In several U.S. cotton production areas (i.e. Arizona, Mississippi, Louisiana, Alabama) the L-strains incidence among *A. flavus* communities ranged from less than 10% to more than 95% (COTTY, 1997).

8.2.2. Effect of strain release

Post-intervention soil samples taken in treatment plots yielded no significant differences in the composition of *A. flavus* communities. Thus, the application of the atoxigenic L-strain BN030D did not significantly affect the composition of the *A. flavus* communities. However, analysis of post-intervention soil and crop samples in the treatment plots revealed a decrease in S-strains frequency (Tab. 5), underlining that the frequency of both L- and S-strains was slightly affected by the BN030D release.

In general, L- and S-strain incidence can provide a rapid, though rough estimate of the toxigenicity of an *A. flavus* community within a field: the more S-strains occur in the field, the more aflatoxin is generally produced (ORUM *et al.,* 1997). However, L-strain communities may be consistent toxin or non-toxin producers. Thus, for predictions of possible aflatoxin contaminations of a crop the relationship of the two *A. flavus* morphs do not suffice alone, but should be accompanied by other data (COTTY et al., 1994).

The classification of L- or S-strains is based on sclerotial formation on V8 medium. Typically, S-strains produce numerous small and black sclerotia. However, this is dependent on the growth medium. Any changes in the composition of ingredients may affect fungal growth. In general, fungal populations are very variable and so is the expression of L- or S-strain phenotypes. They are not always typical and may therefore be confounded. Additionally, some *Aspergillus* spp. do express sometimes similar macroscopically characteristics. Precise determination of the strain's identity therefore necessitates microscopic determination, which is not always possible due to the large number of samples that need to be analysed.

8.3. Effect of the BN030D release on the composition of L-strain field communities

8.3.1. In the soil

Two isolates (less than 1%) of the pre-intervention soil samples showed vegetative compatibility to BN030D, confirming the natural presence of BN030D in soils of the study area. In cotton in the U.S., COTTY (1994b) found in pre-intervention sampling that 6% of the isolates sampled corresponded, belonged to the released atoxigenic *A. flavus* strain AF36. After the release of BN030D, significant differences were recorded between treatment and

control plots. No isolates taken from soil samples in the control plots could be assigned to the VCG of BN030D. The results of the VCG analyses of post-intervention isolates from treatment plots are similar to results of COTTY'S (1994b) study in U.S. cotton where 69% of all isolates belonged to the applied strain AF36, compared 60% compatibility to BN030D in this study.

8.3.2. On maize cobs

In field I none and in field II and III only few *A. flavus* L-strain isolates could be assigned to the VCG of BN030D in the treatment plots (Tab. 5). On average 26% of all tested *nit* mutants generated from L-strains from the treatment plots showed vegetative compatibility to BN030D. In comparison, on crop samples in U.S. cotton, the incidence of the applied strain AF36 ranged from 67% - 100% during a two-year study (COTTY, 1994b). In both years of investigation COTTY (1994b) even found in untreated control plots that 25% and 7% could be assigned to the applied VCG of AF36. In the present study no compatibility to the VCG of BN030D was found in samples from the untreated control plots.

8.3.3. Dispersal of the atoxigenic strain BN030D

In the present study the released strain BN030D spread 15 - 20 m into the buffer area between control and treatment plots within 3 months. In U.S. cotton, the applied atoxigenic strain AF36 spread from the treatment plots to control plots. The distance between both plots were about 11 - 15 m and the rates of isolates compatible to the dispersed strain AF36 differed in both years of examination: 7% and 25% (COTTY, 1994b). Possibly BN030D spread into the buffer area, where in pre-intervention sampling no VCG compatibility to BN030D had been found, by propagules adhering to soil particles or plant parts and insect cadavers. However, the low number of samples taken in the buffer areas does not permit any definite conclusions on the means of spread of the atoxigenic strain.

With 60% and 26% in post-intervention soil and crop samples from treatment plots, respectively, the frequency of *A. flavus* L-strain isolates with VCG compatibility to BN030D in this study is comparatively low compared to previous investigations in U.S. cotton (DORNER *et al.,* 1992; COTTY, 1994b, 1997). The main reason for these discrepancies are probably the major agronomic and climatic differences between the highly mechanized cotton production systems in North America and the subsistence agriculture in tropical Benin. Moreover, some fields (e.g. field I) were situated close to farmers' houses, thus increasing the risk of livestock (chicken) entering the fields and picking up parts of the inoculated rice grains, thereby decreasing the inoculum density. The high variability in fungal population in general and rapid shifts of composition of *A. flavus* communities within areas (1 - 5 km^2) may provide a further explanation (ROSS *et al.,* 1979; ORUM *et al.,* 1999). Moreover, in several

areas in the desert valleys in the U.S., cotton crops were frequently dusted by fine soil particles (i.e. wind erosion) (COTTY, 1994b). These particles can contain large quantities of *A. flavus* inoculum (at times exceeding 5,000 propagules m^{-3} of air) (COTTY *et al.*, 1994). Wind erosion is of minor to no importance in the small-scale agriculture prevailing in West Africa. However, similar to cotton, large proportions of dead and necrotic plant and animal material become colonized by *A. flavus* during the maize crop development (STEPHENSON and RUSSELL, 1974) and these may contribute to inoculum levels.

A further explanation could be the different formulation and application methods. Colonized rice may have properties less favourable than colonized wheat, as used in most studies in the U.S. (COTTY, 1994b; DORNER *et al.*, 1995; COTTY, 1997), for both the conidia production and dispersal over longer periods of time. The application rate of rice was lower (0.5 g m^{-2}) compared to studies using wheat as carrier (COTTY, 1994b; DORNER *et al.*, 1995). Previously, atoxigenic *A. flavus* strains have been applied with rice grains and wheat seeds, in alginate pellets and in conidial suspensions. Alginate pellets, containing wheat gluten and mycelia of atoxigenic *A. flavus*, produced more conidia than wheat seeds (DORNER *et al.*, 1992; COTTY, 1994b; DAIGLE and COTTY, 1995). However, the cost of the bulk product for alginate pellets was estimated to be U.S. $ 2.53 - 5.76 kg^{-1} compared U.S. $ 0.18 - 0.26 kg^{-1} for wheat (BOCK and COTTY, 1999). Thus, the lower price, the simple inoculation technique and successes in field releases with colonized wheat and rice have favoured the use of these carrier materials. Rice production in Benin is very low, and wheat is not produced at all in the country. However, because of the high nutritional value and the comparatively high price of rice in Benin farmers might be reluctant to use rice as carrier for strain releases. Future research should focus on agricultural by-products, which - similar to rice or wheat - have optimal efficacy to release conidia over long periods of time.

In this study VCG-tests were used to determine if sampled isolates of *A. flavus* L-strains belonged to the VCG of the released strain BN030D. For this reason, *nit* mutants had to be generated from each of the collected *A. flavus* L-strain wild type isolates. The utility of VCG tests highly dependents on the quality and quantity of the generated *nit* mutants. In the present study in 84% of all sampled *A. flavus* isolates *nit* mutants were successfully generated. Similarly, in U.S. cotton COTTY (1994b) could generate *nit* mutants in 88% of all wild type isolates. It remains unclear why it was not possible to generate *nit* mutants from the remaining 16% of isolates in this study. Possibly good mutants were confounded with bad one. Another reason might have been lack of time as some isolates do sector in *nit* mutants in no earlier than one month of incubation (T. HOFFSTADT, pers. comm.) However, the most consistent problem when using the VCG technique is the accidental selection and frequent occurrence of bad mutants (Fig. 9). Moreover, it is sometimes difficult to conclude from the absence of a heterokaryon formation in a complementation test incompatibility of the tested

wild type isolate with the tested VCG. In addition, it is not clear why several isolates showed no distinct reaction ('non-respond' isolates). The reason might have been a bad *nit* mutant, which showed unspecific mycelial growth and no heterokaryon formation. Furthermore, even if the component strains are vegetatively compatible this may complicate detecting the heterokaryon formation, especially when inhibitory alleles prevent its formation, e.g. in mutants at the hsi loci (CORRELL *et al.,* 1989). Though rather labour intensive, the VCG technique was the only appropriate approach for this study, as it is not a very sophisticated method and does not require expensive instruments, which are sometimes difficult to obtain and maintain in Africa.

BN030D-compatible isolates are considered not to produce aflatoxins, because it expresses its atoxigenic phenotype consistently (CARDWELL and COTTY, 2000). In general the ability to produce aflatoxins is very stable within VCGs of *A. flavus* (COTTY, 1990). This is supported by COTTY (1994b) who found a strong negative correlation between the amount of aflatoxins produced (AFB_1 ng g^{-1} whole seed) and the percentage of isolates belonging to the applied, atoxigenic strain AF36. The more isolates showed compatibility to the VCG of AF36, the lower the aflatoxins content recorded in cottonseeds. After re-isolation of AF36 from infected crops, the atoxigenic phenotype retained the same and did not change (COTTY, 1994b). VCG analyses of *A. flavus* communities on plants and in the soil confirmed that reductions in aflatoxin content were caused by competitive displacement of prevailing *A. flavus* communities by the released atoxigenic strain (COTTY, 1994b; COTTY *et al.,* 1994). However, for logistic reasons (among others, lack of laboratory equipment and high costs of the chemicals) in this study no toxicity analyses could be carried out. Yet, toxin analyses are not absolutely indispensable, because the distinction between those isolates belonging to the applied VCG of BN030D and those of other VCG already provided clear evidence for the success of the releases as atoxigenic strains usually remain atoxigenic over longer periods of time (COTTY, 1994b; CARDWELL and COTTY, 2000). Thus results of the present study suggest that it is possible to change the community composition of *A. flavus* strains, which is a prerequisite for the successful use of the competitive replacement technique.

Due to the fact that L-strains are considered to be r-selected species (HARPER, 1977), the VCG of the atoxigenic strains will never completely occupy all destined habitats. The natural occurrence of BN030D strain was of minor importance in this and previous studies (BROWN *et al.*, 1991; COTTY, 1994b, 1997; ORUM *et al.*, 1997), suggesting that other strains may posses a superior competitiveness. Hence, a complete displacement of toxigenic strains will most likely never be realized and some more or less toxigenic *A. flavus* strains will always remain present in the field.

So far, metamorphosis of atoxigenic in toxigenic strains has not been observed in field trials but in some *in vitro* studies (COTTY, 1994b; COTTY et al., 1994). Moreover, the high recovery

rates of strains belonging to the VCG of BN030D confirm that metamorphosis is very unlikely. Furthermore, the released atoxigenic strains of *A. flavus* (AF36 in the U.S. and BN030D in Benin) exhibit the stable, atoxigenic phenotype and produced just no aflatoxins (COTTY, 1997; COTTY and CARDWELL, 1999). Nevertheless, safety studies over several years are required for a comprehensive assessment of risks possibly associated with releases of atoxigenic *A. flavus* strains.

No matter what kind of intervention strategies used, there will always be *A. flavus* fungi associated with our crops (ORUM *et al.,* 1997). Dead, weakened, and partially decaying plant tissues are readily available and there are no means to prevent utilization of these resources by the saprophytic fungi. Hence a competitive replacement approach might have great potential to substantially reduce aflatoxin contamination of maize in Benin and other countries in West Africa, thereby largely ameliorating the health of rural and urban populations. Even if aflatoxin contaminations cannot be completely eliminated by competitive exclusion, coupling this technology with others (e.g. cultural practices, improved resistant varieties, storage practice) could considerably contribute to the long-term objective of ensuring an aflatoxin-free supply in maize.

In order to achieve this objective, in future studies on competitively replacement the following aspects require further research:

- Stability of the atoxigenicity of BN030D over time
- Determination of best inoculum rates and timing
- Investigations of best and most appropriate formulation agents (e.g. agricultural by-products) and release technique.

Moreover, the potential impact of releases of atoxigenic strains on the environment, e.g. intra- (L- and S-strains) and interspecific interactions among fungal communities need to be addressed in comprehensive safety studies.

9. Conclusion

The present study has shown that it is possible to change the soil and cob composition of *A. flavus* communities with a soil application of atoxigenic *A. flavus* strain BN030D to 5 maize fields in Djidja area. BN030D successfully replaced (excluded) prevailing *A. flavus* strains in a competitive mode without an increase in the overall *A. flavus* infection. Moreover, no serious influence was shown on the *A. flavus* L- and S-strain relationship or on the prevailing fungal population (saprophytes). The method of competitive replacement is of great promise in order to reduce aflatoxin contamination in maize, because BN030D successfully interfered with the prevailing *A. flavus* community in West African maize production systems. However, given the scope of this study, only a short-term effect of the strain release could be investigated.

Many other questions concerning the strain application, its behaviour after release, and its dispersal need to be answered in order to consider that the method of competitive replacement is a successfully, sustainable control approach in the aflatoxin reduction in agricultural maize production systems in Benin.

10. References:

AHO (2002): Belgien sperrt Milchviehbetriebe wegen Aflatoxin. AHO Aktuell, Animal-Health-Online und @grar.de. Aktualisiert: 05.07.2002. http://ticker-grosstiere.animal-health-online.de/20020705-00003/.

ADHIKARI M., G. RAMJEE and P. BERJAK (1994): Aflatoxin, Kwashiorkor and Morbidity. In: Natural Toxins (1994) 2: 1 - 3.

AJAYI, O. (2000): Pesticide use practices, productivity and farmers' health: the case of cotton-rice systems in côte d'Ivoire, West Africa. Institut für Gartenbauökonomie, Universität Hannover and Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) GmbH. In: pesticide policy project publication series: special issue (2000) No.3.

ANNAN, K. (2001): UN general secretary general tells special event on poverty eradication, 'best hope' for least developed countries would be new round of global trade negotiations. Press Release G/SM/7802 Dev/2311, 14 May 2001. http://www.un.org/News/Press/docs/2001/sgsm7802.doc.htm.

ANONYMUS (2002): Aflatoxin im Essen ist noch in vielen Ländern Alltag - Stress bei Pflanzen begünstigt Schimmelpilz-Befall / Werden Körner und Nüsse vor dem Lagern gut getrocknet, haben Pilze kaum eine Chance. In: Ärzte Zeitung, Last update: 26.02.2002. http://www.arzneimittelzeitung.de/docs/2002/02/26/037a1301.asp.

AUTRUP, J.L, J.L. SCHMIDT, T. SEREMET, and H. AUTRUP (1991): Determination of exposure to aflatoxins among Danish workers in animal-feed production through the analysis of aflatoxin B1 adducts to serum albumin. In: Scandinavian Journal of Work, Environment and Health (1991) 17 (6): 436 - 440.

BARNET, H.L. and B.B. HUNTER (1987): Illustrated genera of imperfect fungi. Fourth Edition, MacMillian Publishing Company, New York, 1987.

BARRY, D., N.W. WIDSTROM, L.L. DARRAH, W.W. MCMILLAN, T.J. RILEY, G.E. SCOTT and E.B. LILLEHOJ (1992): Maize ear damage by insects in relation to genotype and aflatoxin contamination in pre-harvest maize grain. In: Journal Economic Entomologist (1992) 85: 2492 - 2495.

BAYMAN, P. and P.J. COTTY (1992): Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. In: Canadian Journal of Botany (1992) 71: 23 - 31.

BEAN, T.A., D.M. YOURTEE, B. AKANDE, and J. OGUNLEWE (1989): Aflatoxin metabolites in the urine of Nigerians: comparison of chromatographic methods. In: Journal of Toxicology, Toxin Reviews (1989) 8 (1 – 2): 43 - 52.

BÉGUERET, J., B. TURCQ and C. CLAVÉ (1994): Vegetative incompatibility in filamentous fungi: *het* genes begin to talk. In: Trends in Genetic (1994) 10 (12): 441 - 446.

BGFA (2003): Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin. http://dienste.t-online-business.de/busi/them/mein/hand/aktu/ar/0302/CP/ar-enzyme-krankheit-2802.html.

BETTS, W.B. and R.K. DART (1989): Initial reactions in degradation of tre- and tetrameric lignin–related compounds by *Aspergillus flavus* and *A. parasiticus*. In: Mycologia (1989) 71: 415 - 422.

BILGRAMI, K.S. and A.K. CHOUDHARY (1993): Impact of habitats on toxigenic potential of *Aspergillus flavus*. In: Journal of Stored Products Research (1993) 29 (4): 351 - 355.

BOCK, C.H. and P.J. COTTY (1999): Wheat seed colonized with atoxigenic *Aspergillus flavus*: Characterization and production of a bio-pesticide for aflatoxin control. In: Biocontrol, Science and Technology (1999) 9: 529 - 543.

BORGEMEISTER, C. (2001): personal communication and lecture scriptum. University of Hanover, Fachbereich Gartenbau, Institut für Pflanzenkrankheiten und Pflanzenschutz.

BOYER, C. (2003): Reyes syndrome. @health on the net Foundation - Non governmental organisation. Last update: 28.03.2003. http://www.hon.ch.

BROWN, R.L., P.J. COTTY and T.E. CLEVELAND (1991): Reduction in aflatoxin content of maize atoxigenic strains of *Aspergillus flavus*. In: Journal of Food Protection (1991) 54 (8): 623 - 626.

BUTLER, W.H. (1974): Aflatoxin. In: mycotoxins. Ed. I.F.H. Purchase, Amsterdam/New York: Elsevier: 1 - 28.

CALDERWOOD, D.L., and H.W. SCHROEDER (1968): Aflatoxin development and grade of rough rice following prolonged storage in aerated bins. US Department of Agriculture, ARS Report (1968): 52 - 56.

CARDWELL, K.F., P.J. COTTY and M. SÉTAMOU (1999): Distribution and disposition of toxic and atoxigenic isolates of *Aspergillus* sect. *flavi* from soils taken from maize fields across four agro-ecological zones of the Republic of Benin, West Africa. In: European journal of plant pathology (1999). Pre-proposal.

CARDWELL, K.F. and P.J. COTTY (2000): Aflatoxin reduction in maize through competitive exclusion by *Aspergillus flavus*. Project proposal.

CARDWELL, K.F. and P.J. COTTY (2001): Distribution of *Aspergillus* section *flavi* among field soils from the four agro-ecological zones of the republic of Benin, West Africa. In: Plant disease (2001). Pre-proposal.

CARDWELL, K.F., A. DESJARDINS, S.H. HENRY, G. MUNKVOLD and J. ROBENS (2003): Mycotoxins: the cost of achieving food security and food quality. The American phytopathological society. Feature Story August 2001. Last update: 08.07.2003. http://www.apsnet.org/online/feature/mycotoxin/.

CARLSON, N. (1996): Indoor fungi resources: Fungal Glossary. Environmental health & safety: improving the quality for our work and study environment. Last update: 1996. http://www.dehs.umn.edu/iaq/fungus/glossary.html.

CHANG, H.G. and P. MARKAKIS (1981): Effect of moisture content on aflatoxin production in barley. In: Cereal Chemistry (1981) 58: 89 - 92.

CLEVELAND, T.E. and P.J. COTTY, (1991): Invasiveness of *Aspergillus flavus* isolates in wounded cotton bolls is associated with production of a specific fungal polygalacturonase. In: Phytopathology (1991) 81: 155 - 158.

CLEVELAND, T.E. and S.P. MCCORMICK (1987): Identification of pectinases produced in cotton bolls infected with *Aspergillus flavus*. In: Phytopathology (1987) 77: 1498 - 1503.

COKER, R.D., B.D. JONES and M.J. NAGLER, (1984): Mycotoxin Training Manual. In: Tropical Development and Research Institute (1984): 109.

COKER, R.D., K. JEWERS and B.D. JONES (1985): The destruction of aflatoxin by ammonia: practical possibilities. In: Tropical Science (1985) 25: 139 - 154.

COLE, R.J., R.A. HILL, P.D. BLANKENSHIP, T.H. SANDERS and J.H. GURREN (1982): Influence of irrigation and drought stress on invasion of *Aspergillus flavus* of corn kernels and peanut pods. In: Developments in Industrial Microbiology (1982) 23: 229 - 236.

CORRELL, J.C., C.J.R. KLITTICH and J.F. LESLIE (1987): Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. In: Phytopathology (1987) 77: 1640 - 1646.

CORRELL, J.C., C.J.R. KLITTICH and J.F. LESLIE (1989): Heterokaryon self-incompatibility in *Gibberella fujikuroi* (*Fusarium moniliforme*). In: Mycology Research (1989) 93 (1): 21 - 27.

COTTY, P.J. (1989): Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. In: Phytopathology (1989) 79: 808 - 814.

COTTY, P.J. (1990): Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. In: Plant Disease (1990) 74: 233 - 235.

COTTY, P.J. (1992): Soil populations of *Aspergillus flavus* group fungi in agricultural fields in Alabama, Arizona, Louisiana, and Mississippi. In: Phytopathology (1992) 82: 1064.

COTTY, P.J. (1993): Comparison of four media for the isolation of Aspergillus flavus group fungi. In: Mycopathologia (1993) 125: 157 - 162.

COTTY, P.J. (1994a): Method for the control or prevention of aflatoxin contamination using a non-toxigenic strain of *Aspergillus flavus* (filed 1991). United States Patent, Patent Number: 5,294,442. Date of patent: 15.03.1994: 1 - 14.

COTTY, P.J. (1994b): Influence of field application of an atoxigenic strain *of Aspergillus flavus* on the population of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. In: Phytopathology (1994) 84 (11): 1270 - 1277.

COTTY, P.J. (1997): aflatoxin-producing potential of communities of *Aspergillus* section *flavi* from cotton producing areas in the United States. In: Mycology Research (1997) 101 (6): 698 - 704.

COTTY, P.J. and P. BAYMAN (1993): Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. In: Phytopathology (1993) 83: 1283 - 1287.

COTTY, P.J. and K.F. CARDWELL (1999): Divergence of West African and North American communities of *Aspergillus* section *flavi.* In: Applied and Environmental Microbiology (1999) 5: 2264 - 2266.

COTTY, P.J., P. DAYMAN and D. BHATNAGAR (1990): Two potential mechanisms by which atoxigenic strains of *Aspergillus flavus* prevent toxigenic strains from contaminating cottonseed. In: Phytopathology (1990) 80: 994.

COTTY, P.J., P. BAYMAN, D.S. EGEL and K.S. ELIAS (1994): Agriculture, aflatoxins and *Aspergillus.* In: The genus *Aspergillus.* Eds. K.A. Powell *et al.* (1994), Plenum Press New York: 1 - 27.

COTTY, P.J., T.E. CLEVELAND, R.L. BROWN and J.E. MELLON (1990): Variation in polygalacturonase production among *Aspergillus flavus* isolates. In: Applied Environmental Microbiology (1990) 56: 3885 - 3887.

COULTER, J.B.S., G.I. SULIAMAN, S.M. LAMPLUGH, B.I. MUKHTAR and R.G. HENDRICKSE (1986): Aflatoxins in liver biopsies from Sudanese children. In: American Journal of Tropical Medicine and Hygiene (1986) 35 (2): 360 - 365.

CROFT, J.H. (1987): Genetic variation and evolution in *Aspergillus.* Eds. A.D.M. Rayner, C.M. Brasier, and D. Moore: Evolutionary biology of the fungi. Cambridge University Press, Cambridge (1987): 311 - 323.

DAIGLE, D.J. and P.J. COTTY (1995): Formulation atoxigenic *Aspergillus flavus* for field release. In: Bio-control Science and Technology (1995) 5: 175 - 184.

DAVIS, N.D., C.G. CURRIER and U.L. DIENER (1986): Aflatoxin contamination of corn hybrids in Alabama. In: Cereal Chemistry (1986) 63: 467 - 470.

DEDDNER, S. and A. WAEHNER (1999): Aflatoxine. Zusammenfassung von Schadwirkungen durch Aflatoxine bei landwirtschaftlichen Nutztieren. Zusammenfassung von Schadwirkungen beim Menschen durch Aflatoxine in Lebensmitteln pflanzlicher Herkunft. Zusammenfassung von Schadwirkungen beim Menschen durch Aflatoxine in Lebensmitteln tierischer Herkunft. Hochschule Anhalt (FH). Last update: 05.07.1999. http://www-proj.loel.hs-anhalt.de/oeko/mykotoxine/afla.html.

DIENER, U.L. and N.D. DAVIS (1970): Limiting temperature and relative humidity for aflatoxin production by *Aspergillus flavus* in stored peanuts. In: Journal of the American Oil Chemists Society (1970) 47: 347 - 351.

DIENER U.L., R.J. COLE, T.H. SANDERS, G.A. PAYNE, L.S. LEE and M.A. KLICH (1987): Epidemiology of aflatoxin formation by *Aspergillus flavus.* In: Ann. Rev., Phytopathology (1987) 25: 249 - 270.

DIJK, A. and E. GRIEß (2000): Largest Industrial Genome-Sequencing Project in Europe. Last update: 2000. http://www.medigenomix.de/news/news_de_apergillus.htm.

DOMSCH, K.H., W. GAMS and T.H. ANDERSON (1993): Compendium of soil fungi. Vol. 1 (1993). IWH-Verlag, Eching: 80-124.

DORNER, J.W., R.J. COLE, and P.D. BLANKENSHIP (1992): Use of bio-competitive agent to control pre-harvest aflatoxin in drought stressed peanuts. In: Journal of Food Protection, (1992) 55 (11): 888 - 892.

DORNER, J.W., R.J. COLE, and P.D. BLANKENSHIP (1995): Biological control of aflatoxin contamination of peanuts with color-mutants of *Aspergillus flavus* and *A. parasiticus* (1995). Pre-proposal: 1-7.

EDINGTON, G. M. (1977): The pattern of cancer in the Northern Savanna of Nigerian with special reference to primary liver cell carcinoma and Burkitt's lymphoma. In: British Journal of Cancer (1977) 20: 416 - 426.

EGEL, D.S., P.J. COTTY and K.S. ELIAS (1994): Relationships among isolates of *Aspergillus* sect. *flavi* that vary in aflatoxin production. In: Phytopathology (1994) 84: 906 - 912.

ELIAS, K.S. and P.J. COTTY (1995): Incidence and stability of infection by double-stranded RNA genetic elements in *Aspergillus* section *flavi* and effects on aflatoxigenicity. In: Canadian Journal of Botany (1994) 74: 716 - 725.

ENGELHARDT, G. (2002): Aflatoxine. Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit. Last update: 2002. http://www.vis-ernaehrung.bayern.de/de/left/fachinformationene/risiken/stoffe-bio/mykotoxine/aflatoxine.htm.

ENIUS (2002): Aspergillus. Last update: 2002. http://enius.de/schadstoffe/aspergillus.html.

FAO Corporate Document Repository (1998): Bio-detioration – molds and mycotoxins: The significance of mycotoxins. Last update: 24.04.1998. http://www.fao.org/docrep/T1838E/T1838E0I.GIF.

FENNEL, D.I., R.J. BOTHAST, E.B. LILLEHOJ and R.E. PETERSON (1973): Bright greenish-yellow fluorescence and associated fungi in white corn naturally contaminated with aflatoxin. In: Cereal Chemistry (1973) 50: 404 - 414.

GARBER, R.K. and P.J. COTTY (1997): Formation of sclerotia and aflatoxins in developing cotton bolls infected by S-strain of *Aspergillus flavus* and potential for biocontrol with an atoxigenic strain. In: Phytopathology (1997) 87: 940 - 945.

GILLIAM, M. and J.D. VANDENBERG (1990): Fungi. Eds: Morse, R.A. and R. Nowogradzki: Honey Bee Pests, Predators, and Diseases. Cornell University Press, Ithaca.: 64 - 90.

GRIFFIN, G.J., K.H. GARREN and J.D. TAYLOR (1982): Influence of crop rotation and minimum tillage on the population of *Aspergillus flavus* group in peanut field soil. In: Plant Disease (1982) 66: 898 - 900.

GOLDBLATT, L.A. (1969): Aflatoxin. New York: Academic Press.

GOTO, T., K. TANAKA, O. TSURUTA and M. MANABE (1988): Presence of aflatoxin producing *Aspergillus* in Japan. In: Procedures of Japanese Association Mycotoxicology Supplement (1988) 1: 179 - 182.

HADIIDANE, R., C. ROGER-RENAULT, H. BOUATTOUR, F. ELLOUZE, H. BACHA, E.E. CREPPY and G. DIRHEIMER (1985): Correlation between alimentary mycotoxin contamination and specific diseases. In: Human Toxicology (1985) 4 (5): 491 - 501.

HARPER, J.L. (1977): Population biology of plants. Academic Press. London.

HELL, K., KF. CARDWELL, M. SÉTAMOU and H.-M. POEHLING (1996): Production practices and their influence on aflatoxin contamination in stored maize in Benin, West-Africa (draft-paper).

HELL, K. (1997): Factors contributing to the distribution and incidence of aflatoxin producing fungi in stored maize in Benin. Dissertation, University of Hanover (December, 1997).

HELL, K., K.F. CARDWELL and M. SÉTAMOU (1999): Fungal infection and aflatoxin contamination in stored maize in four agro-ecological zones in Benin. In: Plant Disease (1999) 85: 11 - 31.

HELL, K., K.F. CARDWELL, M. SÉTAMOU and H.-M. POEHLING (2000): The influence of storage practices on aflatoxin contamination in maize in four agro-ecological zones of Benin, West Africa. In: Journal of Stored Products Research (2000) 36: 365 - 382.

HENDRICKSE, R.G. (1997): Of turkeys, kwashiorkor, malaria, perinatal mortality, heroin addicts and food poisoning: research on the influence of aflatoxins on child health in the tropics. In: Annals of Tropical Medicine & Parasitology (1997) 91 (7): 787 - 793.

HESSELTINE, C.W., O.L. SHOTWELL, M. SMITH, J.J. ELLIS, E. VANDEGRAFT and G. SHANNON (1970): Production of various aflatoxins by strains of the *Aspergillus flavus* series. Ed. M. Herzberg: Proceedings of the 1st U.S.-Japan conference on toxic micro-organisms; mycotoxins and botulism, Honolulu, Hawaii, October 7-8, 1968. U.S. Department of the Interior, Washington, D.C. (1970): 201 - 211.

HÖGGER, C.H. (1994): Nematologie – Fachausdrücke. Sonderdruck der Mikroskopischen Nachrichten der Mikroskopischen Gesellschaft Zürich (1994/1). Last update: 31.01.1994. http://www.mysunrise.ch/users/choegger/Nematodes/Fachausdr.PDF.

HOLMBERG, K. (1984): Mold damage to buildings and risk to health. In: Lakartidningen (1984) 81 (38): 3327 - 3333.

HOTHORN, T. and U. MUNZEL (2001): Exact nonparametric confidence intervals for the ration of medians. Technical Report. Last update: 2001. http://www.imbe.med.uni-erlangen.de/~hothorn/hothorn.html.

JONES, R.K., H.E. DUNCAN and P.B. HAMILTON (1981): Planting date, harvest date, and irrigation effects on infection and aflatoxin production by *Aspergillus flavus*. In: Plant Disease (1981) 65: 741 - 744.

KENDRICK, B. (2003): All about fungi. The mycolog page. Mycologue Publications 1998-2003. Last update: 01.03.2003. http://www.mycolog.com

KLICH, M.A., and J.I. PITT (1988): A laboratory guide to the common *Aspergillus* species and their teleomorphs. Commonwealth Scientific and Industrial Research Organization, Division of Food Processing (1988): 1 - 16, 24 - 94.

KOLLER, U. (1993): Aflatoxine. GSF-Forschungszentrum für Umwelt und Gesundheit, Umweltberatung Bayern. Last update: 1993. http://www.gsf.de/IU/infos/ubb_aflatoxine.html.

KOSSOU D.K., J.H. MARECK and N.A. BOSQUE-PEREZ (1993): Comparison of improved and local varieties in the Republic of Benin with emphasis on susceptibility to *Sitophilus zeamais* (Motschulsky). In: Journal of Stored Products Research (1993) 29: 333 - 343.

LAMPLUGH S.M. and R.G. HENDRICKSE (1988): Aflatoxin in breast milk, neonatal cord blood and serum of pregnant women. In: British Medical Journal (1988) 296: 968.

LANCASTER, M.C., F.P. JENKINS and J.M. PHILIP (1961): Toxicity associated with certain samples of groundnuts. In: Nature (1961) 192: 1095 - 1096.

LEE, J. (1997): Corn waxes makes toxin wane. ARS (usda) – news and information. Last update: 24.02.1997. http://www.ars.usda.gov/is/pr/1997/970224.htm

LEE, L.S., S.P. KOLTUN and S. BUCO (1983): Aflatoxin distribution in fines and meats from decorticated cottonseed. In: Journal of American Oil Chemistry (1983): 1548 - 1549.

LEMKE, P.A., N.D. DAVIS and G.W. CREECH (1989): Direct visual detection of aflatoxin synthesis by mini-colonies of *Aspergillus* species. In: Applied Environnemental Microbiology (1989) 55: 1808 - 1810.

LESLIE, J.F. (1993): Fungal vegetative compatibility. In: Annual Review Phytopathology (1993) 31: 127 - 150.

LESLIE, J.F. (1996): Fungal Vegetative Compatibility – Promises and Prospects. In: Phytoparasitica (1996) 24 (1): 3 - 6.

LILLEHOJ, E.B. (1983): Effect of environmental and cultural factors on aflatoxin contamination of developing corn kernels. In: Journal of Economic Entomologist (1983) 21: 27 - 34.

LUCAS, J.A. (1998): plant pathology and pant pathogens. 3. Edition, Blackwell Science, 1998.

MAISS, E. (2001): personal communication and lecture scriptum.

MARSH, S.F., and G.A. PAYNE (1984): Scanning EM studies on the colonization of dent corn by *Aspergillus flavus*. Phytopathology (1984) 74: 557 - 561.

MAXWELL, S.M., F. APEAGYEI, H.R. DE VRIES, D.D. MWANMUT and R.G. HENDRICKSE (1989): Aflatoxin in breast milk neonatal cord blood and sera of pregnant woman. In: Toxicology (1989) 8: 19 - 29.

MCLEAN, M., P. BERJAK and M.P. WATT (1992): The influence of aflatoxin B_1 on the *in vitro* germination and growth of excised, mature zea mays embryos. In: Journal of Natural Toxins (1992) 2 (1): 13 - 26.

MCMILLIAN, W.W., N.W. WIDSTROM and D.M. WILSON (1987): Impact of husk type and species of infesting insects on aflatoxin contamination in pre-harvest corn at Tifton, Georgia. In: Journal of Entomological Science (1987) 22 (4): 307 - 310.

MEHAN, V.K., D. MCDONALD, L.J. HARAVU and S. JAYANTHI (1991): The groundnut aflatoxin problem: a review and literature database. International Crops research Institute for Semi-Arid Crops. In: India, Patanchaberu, A.P.: 32 - 35.

MEIKLE, W.G., C. ADDA, K. AZOMA, C. BORGEMEISTER, P. DEGBEY, D. DJOMAMOU and R.H. MARKHAM (1998): The effects of maize variety on the density of *P. truncatus* (Col.: Bostrichidae) and *S. zeamais* (Col.: Curculionidae) in post-harvest stores in Benin Republic. In: Journal of Stored Products Research (1998) 1: 45 - 48.

MOODY, S.F. and B.M. TYLER (1990a): Restriction enzyme analysis of mitochondrial DNA of the *Aspergillus flavus* group: *A. flavus, A. parasiticus, and A. nominus.* In: Applied Environmental Microbiology (1990) 56: 2441 - 2452.

MOODY, S.F. and B.M. TYLER (1990b): Use of nuclear DNA restriction fragment length polymorphisms to analyse the diversity of the *Aspergillus flavus* group: *A. flavus, A. parasiticus, and A. nominus.* In: Applied Environmental Microbiology (1990) 56: 2453 - 2461.

NELSON, M.R., T.V. ORUM, D.M. BIGELOW and P.J. COTTY (2000): sentinel sites for monitoring *Aspergillus flavus* community structure in southwestern desert agricultural soils. Last update: 2000. http://ag.arizona.edu/PLP/GIS.

NWOKOLO, C. and P. OKONKWO (1978): Aflatoxin load of common food in Savanna and Forest regions of Nigeria. In: Transactions of the Royal Society of Tropical Medicine and Hygiene (1978) 72 (4): 329 - 332.

OKKY, S.D. (2001): The Use of non-toxigenic *Aspergillus flavus*, *A. niger* and *Trichoderma harzianum* to control aflatoxigenic *A. flavus* in peanuts. Last update: 2001. http://www.biotrop.org/research2001/Aspergillus.htm.

ORUM, T.V., D.M. BIGELOW and M.R. NELSON (1997): Spatial and temporal patterns of *Aspergillus flavus* strain composition and propagule density in Yuma Country, Arizona, Soils. In: Plant Disease (1997) 81: 911 - 916.

ORUM, T.V. and M. NELSON (1999): Monitoring trends in *Aspergillus flavus* S-strain incidence in Arizona. The university of Arizona, department of plant pathology. http://ag.arizona.edu/PLP/GIS/SvsLfull.html.

ORUM, T.V., D.M. BIGELOW, P.J. COTTY and M.R. NELSON (1999): Using Predictions based on geostatistics to monitor trends in *Aspergillus flavus* strain composition. In: Phytopathology (1999) 89: 761 - 769.

PAPA, K.E. (1986): Heterokaryon incompatibility in *Aspergillus flavus.* In: Mycologia (1986) 78: 98 - 101.

PAYNE, G.A. (1983): Nature of field infection of corn by *Aspergillus flavus*. Eds. Diener, U.L., R.A. Asquith and J.W. Dickens: Aflatoxin and *Aspergillus flavus* in Corn. Southern Cooperative Series Bulletin 279. Alabama Agricultural Experiment Station, Auburn, Alabama, USA.: 16 - 19.

PAYNE, G.A., D.K. CASSEL and C.R. ADKINS (1986): Reduction of Aflatoxin contamination in Corn by irrigation and tillage. In: Phytopathology (1986) 76 (7): 679 - 684.

PIER, A.C. and M.E. MCLOUGHLIN (1985): Mycotoxic supression of immunity. Ed. Lacey, J.: "Trichothecesnes and Other Mycotoxins". Chichester: John Wiley &Sons: 507 - 517.

PÜTZ, M. and C. REUTER (2002): Aspergillus - Morphologie und Kultur - Feinstruktur - Krankheiten - Epidemiologie und Prophylaxe. A Med-World AG - Aktiengesellschaft zur Darstellung von Medizin und Gesundheit im Internet. http://www.m-ww.de/krankheiten/erreger/pilze/aspergillus.html.

PUHALLA, J.E. (1985): Classification of strains of *Fusarium oxysporum* on basis of vegetative compatibility. In: Canadian Journal of Botany (1985) 63: 179 - 183.

RAPER, K.B. and D.I. FENNELL (1965): The genus *Aspergillus*. Williams and Wilkins, Baltimore.

RAMBO, G.W., J. TUITE and G.L. ZACHARIAH (1975): Fluorescence associated with corn infected with *Aspergillus flavus* and *A. parasiticus* in storage. In: Cereal Chemistry (1975) 52 (7): 757 - 764.

RAMBO, G.W. and G.A. BEAN (1974): Sterols and fatty acids of aflatoxin and non-aflatoxin producing isolates of *Aspergillus*. In: Phytochemistry (1974) 13: 195 - 198.

RAMBO, G.W., J. TUITE and P. CRANEE (1974): Pre-harvest inoculation and infection of dent corn ears with *Aspergillus flavus* and *Aspergillus parasiticus*. In: Phytopathology (1974) 64: 797 - 800.

REDING, C.L.C., M.A. HARRISON and C.K. KVIEN (1993): *Aspergillus parasiticus* growth and aflatoxin synthesis on Florunner peanuts grown in gypsum-supplemented soil. In: Journal of Food Protection (1993) 56 (7): 593 - 594, 611.

RICHARD, J. L., J.R. THURSTON and A.C. PIER (1978): Effects of mycotoxins on immunity. Ed. Rosenberg, P: Toxins: Animals, Plants and Microbiology. Oxford: Pergamon Press: 801 - 817.

ROSS, I.J., J.O. LOEWER and G.M. WHITE (1979): Potential for aflatoxin development in low temperature drying systems. In: American Society of Agricultural Engineers Paper (1979) 2: 12 - 18.

SAITO, M., O. TSURUTA, P. SIRIACHA, S. KAWASUGI, M. MANABE and D. BUANGSUWON (1986): Distribution and aflatoxin productivity of the atypical strains of *Aspergillus flavus* isolated from soils in Thailand. In: Proc. Japanese Association of Mycotoxicology (1986) 24: 41 - 46.

SAMSON, R.A., E.S. HOEKSTRA, J.C. FRISVAD and O. FILTENBORG (1995): Introduction to food-borne fungi. Eds. Ponsen and Looyen (4th ed.): Centraal-Bureau voor Schimmelcultures. Wagenigen, The Netherlands: 322.

SAUER, D.B. and R. BURROUGHS (1986): Disinfections of seed surfaces with sodium hypoclorite. In: Phytopathology (1986) 34 (3): 21 - 26.

SCHINDLER, B., S. ROTH, R. MOSENTHIN and H.P. RAIBLE (2003): Muskatnüsse - Belastung mit Aflatoxinen (Erhebung zur Prüfung der Rohware). Kurzmitteilung. Last update: 22.05.2003. http://www.uni-hohenheim.de/i3ve/00068900/23391041.htm.

SCHMIDT, A.L., C.R. CURTIS and G.A. BEAN (1977): Electrophoretic comparisons of mycelial enzymes from aflatoxin-producing and non-producing strains of *Aspergillus flavus* and *Aspergillus parasiticus.* In: Canadian Journal of Microbiology (1977) 23: 60 - 67.

SÉTAMOU, M., K.F. CARDWELL, F. SCHULTHESS and K. HELL (1997): Aspergillus flavus infection and aflatoxin contamination of pre-harvest maize in Benin. In: The American Phytopathological Society (1997) (Plant Disease / November 1997): 1323 - 1327.

SÉTAMOU, M., K.F. CARDWELL, F. SCHULTHESS and K. HELL (1998): Effect of insect damage to maize ears, with special reference to *Mussidia nigrivenilla* (Lepidoptera: Pyralidae), on *Aspergillus flavus* (Deuteromycetes: Monoliales) infection and aflatoxin production in maize before harvest in the Republic of Benin. In: Journal of Economic Entomology (1998) 91: 433 - 438.

SÉTAMOU, M., F. SCHULTHESS, H-M. POEHLING and C. Borgemeister (2000): Monitoring and modelling of field infestation and damage by the maize ear borer *Mussidia nigrivenella* (Lepidoptera: Pyralidae) in Benin, West Africa. In: Journal of Economic Entomology (2000) 93 (3): 650 - 657.

SINHA, K.K. and A.K. SINHA (1991): Effect of *Sitophilus oryzae* infestation on *Aspergillus flavus* infection and aflatoxin contamination in stored wheat. In: Journal of Stored Products Research (1991) 27: 65 - 68.

SINHA, K.K. and A.K. SINHA (1992): Impact of stored grain pests on seed deterioration and aflatoxin contamination in maize. In: Journal of Stored Products Research (1992) 28: 211 - 219.

STEPHENSON, L.W. and T.E. RUSSELL (1974): The association of *Aspergillus flavus* with hemipterous and other insects infesting cotton bracts and foliage. In: Phytopathologia (1974) 64: 1502 - 1506.

STOLOFF, L., H.P. VAN EGMOND and D.L. PARK (1991): Rationales for the establishment of limits and regulations for mycotoxins. In: Food Addition Contamination (1991) 8: 213 - 222.

STORA, C., I. DVORACKOVA and N. AYRAUD (1983): Aflatoxin and Reye's syndrome. In: Journal of Medicine (1983) 14 (1): 47 - 54.

TAUGS, H. (2002): Worin liegt die kanzerogene Wirkung des Aflatoxin B1 begründet? Universität Göttingen. Last update. 12.02.02. http://regulus.pharbp.med.uni-goettingen.de/Lehre/Natwiss/Klausur2002.htm.

TOOS, A.R. (1997): Aflatoxicosis as a field problem in some cow farms in Kafr el Sheikh province. Animal Health Research Institute, Agricultural Research Centre, Giza, Egypt. In: Egypt Journal of Agricultural Research (1997) 76 (1): 198.

VILAR, J. (2003): *Aspergillus.* The Aspergillus Website. Last update: 2003: http://www.aspergillus.man.ac.uk.

WEIDENBÖRNER, M., H. HINDORF and H.C. JHA (1990): Einsatz von Flavonoiden und Isoflavonoiden zur Kontrolle von Lagerpilzen der Gattung *Aspergillus* an Leguminosen. Institut für Pflanzenkrankheiten und Institut für Physiologische Chemie der Universität Bonn. Vortrag gehalten auf der Arbeitstagung „Mykotoxine" der Vereinigung für Angewandte Botanik in Braunschweig am 05.10.1989.

WICKLOW, D.T. (1988): Epidemiology of *Aspergillus flavus* in corn. Ed. Shotwell, O.L.: Aflatoxin in corn - New Perspectives. North Central Regional Research Publication 329. Iowa State University, Ames, Iowa: 315 - 323.

WIDSTROM, N.W., W.W. MCMILLIAN, R.W. BEAVER and D.M. WILSON (1990): Weather associated changes in aflatoxin contamination of pre-harvest maize. In: Journal of Production Agriculture (1990) 3 (2): 196 - 199.

WILD, C.P., F. SITAS, G. LACHLAN, Y.Z. JIANG and D. FORMAN (1990): Correlation between aflatoxin (AF) - albumin adducts, urinary AF metabolites and gastritis in a Kenyan population. In: Proceedings Annual Meeting of the American Association for Cancer Research (1990) 31: 231.

WILD, C.P., F.N. RASHEED, M.F.B. JAWLA, A.J. HALL, L.A.M. JANSEN and R. MONTESANO (1991): In-utero exposure to aflatoxin in West Africa. In: The Lancet (1991) 337: 1602.

WILLIAMS, C.D: (1933): A nutritional disease of children associated with a maize diet. Archives of Disease. In: Childhood (1933) 8: 423.

WILSON, D.M. and D. ABRAMSON (1992): Mycotoxins. Ed. Sauer, D.B.: Storage of cereal grains and their products. American Association of Cereal Chemists, Inc. St. Paul, MN.: 341 - 366.

ZUMMO, N. and G.E. SCOTT (1992): Interaction of *Fusarium moniliforme* and *Aspergillus flavus* on kernel infection and aflatoxin contamination in maize ears. In: Plant Disease (1992) 76: 771 - 773.