



***FUSARIUM* INFECTION AND MYCOTOXIN  
CONTAMINATION IN PREHARVEST AND STORED  
MAIZE IN BENIN, WEST AFRICA**

By

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**The light shines in the darkness and the darkness has never put it  
out**

**John 1.5**



## DECLARATION

I the undersigned hereby declare that the thesis submitted herewith is result of my own work, and has not been submitted in any form to another University.

A handwritten signature in black ink, consisting of a large, sweeping loop followed by a smaller, more intricate flourish.

Pascal Fandohan

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## PREFACE

Fumonisin are mycotoxins produced mainly in maize by some toxigenic species of the genus *Fusarium*. Since their discovery in 1988, they have become a great challenge for scientists and drawn attention of some government institutions. The interest in these toxins greatly increased since they have been found implicated in animal diseases and associated with oesophageal cancer in humans. Despite the intensive investigations implemented so far, there is still a great need to investigate further, to clarify, to confirm, and new fields remain to be studied. Many factors favouring or disadvantaging fungal infection and fumonisin contamination need to be investigated, mostly in developing countries, where maize is extensively grown and constitutes a staple food for subsistence populations. The present study is a contribution to a great deal of areas still not clarified to date, targeting Benin, a West African country, with the hope that the results provided through the six chapters of the dissertation, will be useful in solving the fumonisin problem in maize.

The first chapter is a general introduction, a review of efforts made so far by many scientists in the world to understand more about infection of maize with *Fusarium* spp. and its contamination by fumonisins. This chapter reviews information on the main species of *Fusarium* producing fumonisins in maize, toxicological effects of fumonisins, and factors (biotic and abiotic) influencing infection of maize with *Fusarium* spp. and its contamination by fumonisins. Strategies developed or still in study to control *Fusarium* infection and to minimise fumonisin contamination in maize have been also reviewed.

With respect to natural occurrence on maize of both *Fusarium* spp. and fumonisin in the world, the general observation is that data are more available for the USA and Europe. There are less for Africa, apart from South Africa. Influence of environmental and agroecological conditions on fumonisin production needs further investigations. Moreover, limited data are available on annual variation in fumonisin levels in maize in consecutive years, although it is clear that considerable variation can occur. Chapter two reports on the results of a 3-year survey of the natural occurrence of *Fusarium* spp. and subsequent fumonisin contamination in different agroecological zones of Benin.

In chapter three, results are reported of a study on the impact of indigenous storage systems on fumonisin contamination in maize. Little information is available regarding the effects of the different storage systems implemented by farmers in developing countries, which in many cases do not guarantee proper storage conditions to minimise fungal infection.

Results of the study on the impact of shelling and dehulling, on fumonisin production in maize, are reported in chapter four. Maize shelling and dehulling are two postharvest operations implemented in Africa, the former by farmers before storing maize, the latter by women as part of maize processing process. Various methods are in use in each case, sometimes involving motorised equipment. The effect of some of these methods on fumonisin production is reported.

One of the approaches explored nowadays to minimise fumonisin concentrations in maize is food processing. Research works carried out so far concern fumonisin fate during the preparation of *tortilla*, a common maize-based food of Central America. Information on Africa is almost non-existent. However, in some regions of this continent, maize undergoes long food processing. Further research, therefore, is urgently needed. With respect to this, chapter five deals with the evaluation of the fate of both aflatoxin, toxin produced by species of *Aspergillus*, and fumonisin during the preparation process of maize-based products in Benin.

Chapter six reports on the effect of essential oils extracted from local plants on *Fusarium* development and fumonisin production in maize. This study aims to propose effective essential oils for treating stored grains as an alternative control approach against *Fusarium* spp. and fumonisin contamination in maize.

All the chapters of this thesis represent interdependent entities encompassing an integrated approach to achieve a better understanding about fumonisins in maize. Consideration is given to both preharvest and postharvest factors that may reduce *Fusarium* infection and fumonisin contamination of maize in Africa.

## SUMMARY

Natural occurrence of *Fusarium* and subsequent fumonisin contamination in preharvest and stored maize were investigated through a three-year survey in four different agroecological zones of Benin, West Africa. *Fusarium* was found to be predominant in maize samples. The two *Fusarium* species most frequently isolated were *F. verticillioides* and *F. proliferatum*. Atypical isolates of *F. verticillioides* were also found. Some *F. verticillioides* strains were extremely high fumonisin producers with total fumonisin levels ranging from 8240 to 16690 mg kg<sup>-1</sup>. *Fusarium* occurrence was not significantly different from one zone to another, but varied from year to year, and significantly decreased over the six months of storage. Fumonisin occurrence in maize was widespread and levels were significantly higher in the two southern than the two northern zones. Fumonisin levels varied from one year to another, and decreased throughout the storage time, but not significantly every year.

Impact of four storage systems of maize commonly used in Benin was investigated on *Fusarium* infection and fumonisin contamination. *Fusarium* incidence was significantly higher when maize was stored on a cemented floor in a house. The lowest *Fusarium* incidence was recorded when maize was stored in a bamboo granary. In contrast, the storage systems did not have a significant effect on fumonisin contamination. Damage by lepidopterous insects was significantly and positively correlated with both *Fusarium* infection and fumonisin contamination. Conversely, damage by coleopterous insects was significantly and negatively correlated with *Fusarium* infection and fumonisin contamination.

The fate of aflatoxins and fumonisins was studied through the traditional processing of maize into maize-based foods common in Benin. Mycotoxin reduction occurred and was more significant during the preparation of *makume* and *akassa* than that of *owo*. Sorting, winnowing, washing, crushing combined with dehulling of maize grains were the unit operations that appeared very effective in achieving significant mycotoxin removal. Fermentation and cooking showed little effect.

Mechanical shelling and dehulling methods were tested to evaluate their impact on *Fusarium* infection and fumonisin contamination in maize. The mechanical shelling methods were found to damage the grains and motorised sheller type IITA caused the highest level of damage. This could be due to the operation mode of that machine. *Fusarium* populations were higher on damaged grains and highest number of colonies was recorded from grains damaged

by the IITA sheller. Total fumonisin levels were also higher in damaged grains, the highest being in maize shelled by the IITA sheller. On the other hand, the mechanical dehulling methods reduced fumonisin levels in maize.

Eight essential oils extracted from local plants in Benin and oil from seeds of the Neem tree (*Azadirachta indica*) were evaluated *in vitro* and *in vivo* for their efficacy against *F. verticillioides* infection and fumonisin contamination. Oils from *Cymbopogon citratus*, *Ocimum basilicum* and *Ocimum gratissimum* were the most effective *in vitro*. These oils totally inhibited fungal growth in stored maize and affected fumonisin levels in maize stored in closed containers. These oils also significantly reduced grain germination. The oil of Neem seeds showed no inhibitory effect but rather accelerated the growth of *F. verticillioides*.



# CHAPTER ONE: INTRODUCTION

## IMPORTANCE OF MAIZE IN BENIN

Maize (*Zea mays* L.) is a cereal crop grown throughout the world. It was introduced to Africa from South America during the 16<sup>th</sup> century (FAO 1996). Maize plays an important role in the diet of millions of African people due to its high yields per hectare, its ease of cultivation and adaptability to different agro-ecological zones, versatile food uses and storage characteristics (Asiedu 1989). The total production of Africa in 2001 was estimated to be about 42 millions tons (FAO 2002).

In Benin, maize is the most cultivated cereal, grown all over the country and the bulk of the production is by small-scale farmers, mainly for home consumption. In 2001, it occupied about 600 000 ha with a total production of 662 958 tons (FAO 2002). Today, maize has become the second cash crop after cotton and its production is increasing yearly. It is a dietary staple food for more than 70 % of the population (CIMMYT 1990, Hounhouigan 1994). It is estimated that more than 246 g are consumed per person per day in Benin (Hounhouigan *et al.* 1999). But generally, the consumption is higher in the Southern and Central parts of the country (274 – 373 g per person per day) than in the North where it is just entering into the food habits of people (Hounhouigan 1994). Maize is consumed in various and numerous traditional fermented or unfermented products including porridges, pastes, dumplings, cakes, fritters and beverages (Nago 1997). About 40 different ways of maize processing have been recorded in Benin (Nago 1997).

## IMPORTANCE OF INSECTS AND FUNGI IN MAIZE

In the field as well as in the store, many pests and parasites attack maize and during the storage period, insects are most often considered as the principal cause of grain losses (Gwinner *et al.* 1996). However, fungi are also important and rank second as the cause of deterioration and loss of maize (Ominski *et al.* 1994). Kossou and Aho (1993) reported that fungi could cause about 50 – 80 % of damage on farmers' maize during the storage period if conditions are favourable for their development. The major genera commonly encountered in maize in tropical regions are *Fusarium*, *Aspergillus* and *Penicillium* (Samson 1991, Orsi *et al.* 2000).

## **FUSARIUM SPECIES AND THEIR IMPORTANCE IN MAIZE**

*Fusarium* species are ubiquitous in soils. They are commonly considered as field fungi invading more than 50 % of maize grains before harvest (Robledo-Robledo 1991). The genus includes many phytopathogenic species that cause serious diseases in maize, consequently affect growth and yield of the crop, and lead to losses of billions dollars each year to farmers throughout the world (Doyle 1998). Several *Fusarium* species are found associated with maize including *F. verticillioides* (Sacc.) Nirenberg (previously known as *F. moniliforme* Sheldon), *F. proliferatum* (Matsushina) Nirenberg, *F. graminearum* Schwabe and *F. anthophilum* (A. Braun) Wollenweber, but *F. verticillioides* is likely to be the most common species isolated worldwide from diseased maize (Lawrence *et al.* 1981, Scott 1993, Munkvold and Desjardins 1997). Doko *et al.* (1996) reported *F. verticillioides* as the most frequently isolated fungus from maize and maize-based commodities in France, Spain and Italy. Likewise, Orsi *et al.* (2000) found in Brazil that *F. verticillioides* was the predominant *Fusarium* species on maize. In general in Africa, very little information is available on *F. verticillioides* occurrence on maize. Reports of surveys conducted in some African countries however showed it as the most prevalent fungus on maize (Marasas *et al.* 1988, Allah Fadl 1998, Baba-Moussa 1998, Kedera *et al.* 1999).

*F. verticillioides* is an endophyte of maize establishing long-term associations with the plant (Baba-Moussa 1998, Pitt and Hocking 1999). Symptomless infection can exist throughout the plant in leaves, stems, roots, grains, and the presence of the fungus is in many cases ignored because it does not cause visible damage to the plant (Munkvold and Desjardins 1997). This suggests that some strains of *F. verticillioides* produce disease in maize and others do not (Bacon and Williamson 1992).

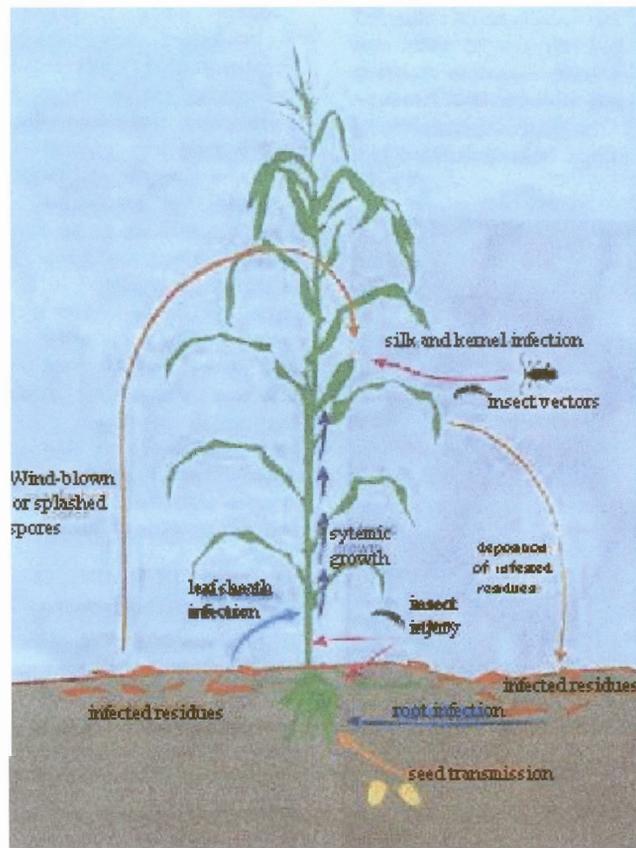
*F. verticillioides* infects maize at all stages of plant development, either via infected seeds, the silk channel or wounds, causing grain rot during both the pre- and postharvest periods (Munkvold and Desjardins 1997). Figure 1 shows *Fusarium* spp. damage on maize cob. A diagrammatic illustration of the disease cycle of *F. verticillioides* in maize is proposed on Figure 2 showing the following possible infection pathways:

- Infection from seed to cob and further to grain through systemic movement in stalk,
- Infection from root to grain through stalk and cob,
- Infection from airborne or water-splashed conidia to silk and further to grain,

- Infection through wounds caused by insects that can also act as vectors of inoculum (Munkvold and Desjardins 1997).



**Fig. 1: Apparently healthy maize cob (left) and *Fusarium* – infected maize cob (right)**



**Fig. 2: Disease cycle of *F. verticillioides* on maize showing various infection pathways**

**Source: Munkvold and Desjardins (1997)**

## FUMONISINS AND THEIR TOXICOLOGICAL EFFECTS

Maize contamination by fungi not only renders grains unfit for human consumption by discoloration and reduction of nutritional value, but can also lead to mycotoxin production. Mycotoxins are poisonous secondary metabolites produced by some fungi in staple foods and foodstuffs. Many of them are considered to be important worldwide, but the five most often reported and well documented are deoxynivalenol/nivalenol, zearalenone, ochratoxin, aflatoxins and fumonisins (Pittet 1998, Pitt 2000). There is ample evidence that mycotoxin problems affect the agricultural economies of many countries in the world, mainly the African countries. The FAO estimated that each year, between 25 % and 50 % of the world's food crops are contaminated by mycotoxins (Mannon and Johnson 1985). The direct impact of mycotoxins on the staple product quality constitutes an important danger for human health and among them fumonisins produced by some toxigenic *Fusarium* species on maize and maize-based foods and feeds increase the risk.

Fumonisin are recently discovered mycotoxins. In 1988, their chemical structure and biological activity were elucidated in South Africa (Gelderblom *et al.* 1988, Marasas 2001). Since the discovery of these toxins, numerous studies have been undertaken to investigate them further. The interesting results found so far have been thoroughly reviewed (Norred 1993, Riley *et al.* 1993, Cardwell and Miller 1996, Gelderblom *et al.* 1996, Shephard *et al.* 1996, Marasas 1996, IPCS 2000, Bolger *et al.* 2001, Marasas 2001, WHO 2002). These reviews mainly highlighted:

- Events leading to the discovery of the fumonisins,
- The toxicological effects of these toxins,
- Their worldwide occurrence in maize and maize-based foods and feeds,
- Their association with animal and human diseases,
- Their impact on animal and human health.

Fumonisin have been found as very common contaminants of maize-based foods and feeds in the United States of America, China, Europe, South America and Africa (Sydenham *et al.* 1991, Thiel *et al.* 1992, Visconti and Doko 1994, Shephard *et al.* 1996). To date, a total of 28 fumonisin analogs have been identified and characterised (Rheeder *et al.* 2002). The most abundant found in naturally contaminated foods and feeds are FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> (Shephard *et al.* 1996, Rheeder *et al.* 2002).

Fumonisin are produced by several *Fusarium* species (Marasas 2001) including:

- *F. verticillioides* (Sacc.) Nirenberg,



- *F. proliferatum* (Matsushima) Nirenberg,
- *F. nygamai* Burgess & Trimboli,
- *F. anthophilum* (A. Braun) Wollenweber,
- *F. dlamini* Marasas, Nelson & Toussoun,
- *F. napiforme* Marasas, Nelson & Rabie,
- *F. thapsinum* Klittich, Leslie, Nelson & Marasas,
- *F. globosum* Rheeder, Marasas & Nelson.

Amongst these, *F. verticillioides* and *F. proliferatum* are by far the most prolific fumonisin producers (Shephard *et al.* 1996). They produce the highest amounts of toxins: up to 17900 mg kg<sup>-1</sup> of FB<sub>1</sub> have been recorded in cultures for the former and 31000 mg kg<sup>-1</sup> FB<sub>1</sub> for the latter (Rheeder *et al.* 2002).

Maize is the product in which fumonisins are most abundant (Shephard *et al.* 1996). Fumonisin has been also detected but at lower levels in sorghum (Shetty and Bhat 1997, Leslie and Marasas 2001), rice (Abbas *et al.* 1998) and spices (Pittet 1998). Fumonisin can contaminate maize foods and feeds as a result of the *Fusarium* invasion before and after harvest (Doko *et al.* 1995).

Fumonisin has emerged as a highly visible animal and human health safety concern since they have been associated with many animal diseases such as leukoencephalomalacia (LEM) in horses (Marasas 1996), pulmonary oedema syndrome (PES) in pigs (Harrison *et al.* 1990, Colvin and Harrison 1992), and hepatocarcinogenesis in rats (Gelderblom *et al.* 2001). With respect to humans, studies on the prevalence of oesophageal cancer in regions of South Africa, China, Italy and Iran, revealed an association between this disease and the consumption of maize contaminated by *Fusarium* spp (Franceschi *et al.* 1990, Rheeder *et al.* 1992, Chu and Li 1994, Marasas 1996, Shephard *et al.* 2000, Wang *et al.* 2000). The International Agency for Research on Cancer (IARC) evaluated in 1992 the toxins derived from *F. verticillioides* as possibly carcinogenic to humans, belonging to the group 2B carcinogens (IARC 1993). More recently, based on the research results obtained so far, FB<sub>1</sub> has been evaluated as possibly carcinogenic to humans (group 2B) (IARC 2002).

Although the effects of fumonisins on humans are not yet well understood, legislation is being put in place to regulate commercial exchanges of fumonisin-contaminated maize and maize-based foods. The US Food and Drug Administration (FDA) recommended that the fumonisin levels are not higher than 4 mg kg<sup>-1</sup> in human foods, and that controlling fumonisins to that level can reduce exposure to the toxin (FDA 2000a, FDA 2000b). In Switzerland, tolerance levels for fumonisins of 1 mg kg<sup>-1</sup> in dry maize products intended for

human consumption were proposed (Marasas *et al.* 2001). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) allocated a group provisional maximum tolerable daily intake (PMTDI) of 0.002 mg kg<sup>-1</sup> body weight for FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub>, alone or in combination (WHO 2002). With respect to animals, the total fumonisin maximal tolerable levels recommended by FDA in maize-based feeds are 5 mg kg<sup>-1</sup> for horse and rabbit, 60 mg kg<sup>-1</sup> for ruminants (cattle, sheep, goat) and 100 mg kg<sup>-1</sup> for poultry (chicken, turkey, duckling) (FDA 2000c).

The mechanism of action of fumonisins in animal diseases is quite complex, but it appears that the toxins mainly cause disruption of lipid metabolism in cells, an event that can lead to cellular deregulation or toxic cell injury and finally to cell death (Wang *et al.* 1991, Riley 1998, Marasas *et al.* 2001).

Fumonisin are found phytotoxic. FB<sub>1</sub> can indeed damage a wide variety of plants including maize (Scott 1993, Lamprecht *et al.* 1994). Doehlert *et al.* (1994) showed that the presence of high levels of fumonisins in maize seeds might have deleterious effects on seedling emergence. Elongation of maize radicles was inhibited by about 75 % after 48 hours of imbibition in 100 mg kg<sup>-1</sup> of fumonisins and amylase activities in seeds significantly decreased as well.

Fumonisin are also found to be relatively stable molecules, heat stable (Alberts *et al.* 1990, Howard *et al.* 1998), light stable (IARC 1993), and stable in stored products when these are kept in airtight at very low temperatures. (Gelderblom *et al.* PROMEC Unit, Medical Research Council, Tygerberg, South Africa, 2002, unpublished data), or  $\gamma$ -irradiated (Visconti *et al.* 1996). However, instability of fumonisins in contaminated products over time has been shown (Scott *et al.* 1999, Kim *et al.* 2002). Fumonisin are also water soluble (IPCS 2000).

## **FACTORS INFLUENCING INFECTION OF MAIZE WITH *FUSARIUM* SPECIES AND FUMONISIN DEVELOPMENT**

Infection of maize with *Fusarium* species and its contamination by fumonisins are generally influenced by many factors including environmental conditions (climate, temperature and humidity), insect infestation and pre- and postharvest handling. These factors do not influence infection independently but most often there are complex interactions.

## *Influence of abiotic factors*

### *Environmental factors*

Worldwide surveys showed high levels of fumonisins associated with warmer and drier climates (Shephard *et al.* 1996) and when weather conditions are favourable for *Fusarium* infection (Marasas *et al.* 2001). At the same location, fumonisin contamination is not necessarily the same from one year to another. Hennigen *et al.* (2000) found in Argentina a marked difference in terms of fumonisin contamination for the same maize varieties during two consecutive growing seasons, due to the fact that environmental conditions may differ from one growing season to another.

Studying the effect of climatic conditions on fumonisin occurrence in freshly harvested maize in different regions of the State of Parana in Brazil, Ono *et al.* (1999) detected higher fumonisins levels in maize samples from the Northern and Central-Western regions compared to that from the South. The authors suggested that it could be due to the differences in rainfall levels during the month preceding harvest (92.8 mm in South, 202 mm in North).

Physiological stress during the period just preceding maize harvest, due to drastic oscillations in rainfall and relative humidity, is likely to create favourable conditions for fumonisin production (Visconti 1996). Shelby *et al.* (1994) suggested that dry weather at or just prior to pollination of maize might be an important factor for fumonisin production in maize. On the other hand, Doyle (1998) reported that late season rainfall increases infection of maize with *F. verticillioides*, the main fumonisin producer. All this leads to the conclusion that some climatic events such as changes in rainfall patterns or stress during the last stages of maize plant development in the field are likely to have a great influence on fumonisin production in maize before harvest.

Furthermore, temperature and moisture conditions during the growing season as well as during storage are often pointed out to affect maize infection by *Fusarium* spp. and fumonisin synthesis. In this connection, water activity ( $a_w$ ), the water available for fungal growth, depending on the relative humidity of air around maize grain, grain moisture content and temperature, plays a key role. Velluti *et al.* (2000) working *in vitro* on fungal competition on maize found that the growth rate of *F. verticillioides* was higher at a temperature of 25 °C, whereas at 15 °C, growth was much lower. These researchers also found that at a constant temperature, the growth rate of *F. verticillioides* increased with water activity.

Some authors suggest that the best temperature for production of fumonisin B<sub>1</sub> in maize is 20 °C (Scott 1993). Marin *et al.* (1999) rather found in a study on fumonisin production under different environmental conditions that the toxin was optimally produced at 30 °C and 0.98 a<sub>w</sub>. However, Alberts *et al.* (1990) showed that the mean FB<sub>1</sub> yield obtained at 25 °C (9500 mg kg<sup>-1</sup>) was significantly higher than that at 20 °C (8700 mg kg<sup>-1</sup>) and 30 °C (600 mg kg<sup>-1</sup>). Munkvold and Desjardins (1997) reported that *F. verticillioides* generally grows in grain when moisture content is more than 18 – 20 %.

### *Agricultural practices*

It has been reported that late planting of maize with harvesting in wet conditions favours disease caused by *F. verticillioides* (Bilgrami and Choudhary 1998), and the prevalence of this fungus is considerably increased with wet weather later in the season (Al-Heeti 1987). Moreover, repeated planting of maize and other cereal crops in the same or in nearby fields favours fungal infection by increasing the fungal inoculum and insect population that attack maize plants (Bilgrami and Choudhary 1998, Doyle 1998). Lipps and Deep (1991) found that the rotation maize/nonhost crop of *Fusarium* was better than maize/maize, as the former was less favourable to *Fusarium* disease outbreaks than the latter. Weed control also affects fungal infection in maize fields because it helps to eliminate nonhost weeds on which *Fusarium* can also be found (Bilgrami and Choudhary 1998).

### *Maize characteristics*

The type of maize cultivar and grain characteristics such as colour, endosperm type, chemical composition and stage of development may also influence fungal infection and subsequent fumonisin production. Late-maturing maize cultivars in which grain moisture content decreases slowly below 30 % are most susceptible to *Fusarium* disease (Manninger 1979). It is thought maize cultivars with upright cobs, tight husks (Emerson and Hunter 1980), thin grain pericarp (Riley and Norred 1999), and an increased propensity for grain splitting (Odvody *et al.* 1990) are likely to be more susceptible to *Fusarium* infection. Tight-husked varieties favour *Fusarium* problems because of slow drying (Dowd 1998).

Fumonisin are found more concentrated in the pericarp and germ of the grain than in the endosperm, so that removal of those outer parts by mechanical processes such as dehulling can significantly reduce the toxin in maize (Charmley and Prelusky 1994, Sydenham *et al.*

1995, FDA 2000b). However, influence of maize grain colour on fumonisin contamination does not seem to be clear. Shephard *et al.* (1996) reported that in some years, fumonisin levels were significantly lower in yellow than in white maize, but the reverse situation was observed in other years.

Regarding the effect of grain endosperm characteristics (dent, flint, or semi-dent) on contamination by fumonisins, Hennigen *et al.* (2000) compared contamination of maize varieties of flint endosperm to that of dent type and did not find significant differences. Shelby *et al.* (1994) tested fifteen maize hybrids and found no significant correlation between starch, lipid, fibre, and protein contents and fumonisin production in maize.

Grain age may also influence fumonisin production in maize. Warfield and Gilchrist (1999) found higher levels of fumonisins in maize grains at the dent stage and significantly lower levels in grains at the immature stage, suggesting that production of the toxin may begin early in cob development and increase as the grains reach physiological maturity. Likewise, Chulze *et al.* (1996) reported that contamination of maize by fumonisins was greater after physiological maturity.

#### *Postharvest operations*

Pre- and postharvest handling and processing (sorting, washing, dehulling, milling, fermentation, cooking) favourably or unfavourably affect fungal infection and fumonisin production in maize. Mechanical damage during and after harvest may offer entry to the fungal spores either in maize cobs or grains. Dharmaputra *et al.* (1994) found that motorised shellers can cause mechanical damage on grains providing entry points to fungal spores. Substantial amounts of fumonisins (up to 74 %) can be removed by simply washing maize grains, immersing them in water and by removing the upper floating fraction, as contaminated grains generally have a low density (Shetty and Bhat 1999). These authors also found that removal of the toxin is more significant (about 86 %) if salt is added to the water during that process. Likewise, sorting and removal of small, broken and visibly contaminated grains during processing can significantly reduce toxin levels (Charmley and Prelusky 1994, Doyle 1998). Steeping maize grains in water has also been found effective in reducing fumonisin content (Canela *et al.* 1996). In contrast, fermentation of maize does not seem to reduce fumonisin levels (Shephard *et al.* 1996, Desjardins *et al.* 2000).

As for milling, Bennett *et al.* (1996) found that by wet-milling fumonisin-contaminated maize, the toxin distribution in the different fractions is as follows: very little or no fumonisin

in the starch fraction, but detectable fumonisins in fibre, germ and steep water fractions. This indicates that maize-based foods derived from the starch fraction are likely to contain less fumonisins than that derived from the other fractions. After dry-milling contaminated maize, fumonisins levels were found lower in grits and higher in germ, bran and fines (Bolger *et al.* 2001). It has also been shown that fumonisin levels decrease as the level of refinement of maize meal during milling increases (Shephard *et al.* 1996).

Regarding cooking, it has been observed that fumonisins are fairly heat-stable and that ordinary cooking does not substantially reduce the toxin (Alberts *et al.* 1990, Scott 1993). Significant removal of fumonisins is more likely to occur only when temperature during cooking is more than 150 °C (Bolger *et al.* 2001).

Although some processing methods potentially can be selected as favourable ways to reduce fumonisin levels in maize-based products, it is important to keep in mind that their success would depend on many factors including the moisture content of the product, the degree of contamination, distribution of the toxin in the product, and the presence of additives (Charmley and Prelusky 1994, Bolger *et al.* 2001).

### ***Influence of biotic factors***

#### *Storage insects*

Insects also play an important role in infection of maize by *Fusarium* spp. They can act as wounding agents or as vectors spreading the fungus from origin of inoculum to plants (Dowd 1998). Wounding by insects may provide an opportunity for the fungus to circumvent the natural protection of the integument and establish infection sites in the vulnerable interior (Bilgrami and Choudhary 1998). Borers and insects of the family Nitidulidae are most often cited as favouring maize infection by *Fusarium* spp. They include among others the lepidopteran stem and cob borers (*Ostrinia nubilalis*, *Sesamia calamistis*, *Eldana saccharina*, *Mussidia nigrivenella* and *Busseola fusca*), thrips and sap beetles (family Nitidulidae) (Flett and Van Rensburg 1992, Munkvold and Desjardins 1997, Cardwell *et al.* 2000, Ako *et al.* 2003). Sobek and Munkvold (1995) found in the USA that damage caused by the European maize borer *Ostrinia nubilalis* increased infection by *F. verticillioides* by three- to ninefold over those with simple mechanical damage. Moreover, larvae of *O. nubilalis* can also act as vectors of *F. verticillioides* by carrying inoculum from plant surfaces into maize cobs (Munkvold *et al.* 1997). In South Africa, Flett and Van Rensburg (1992) showed that

*Busseola fusca* infestation significantly increased the incidence of *F. verticillioides*-infected maize cobs, irrespective of whether the cobs are artificially inoculated with the fungus or not. In a recent study in Benin, it has been observed that cob/stem infection by *F. verticillioides* positively correlated with infestation of *Eldana saccharina*, *Cryptophlebia leucotreta*, *Mussidia nigrivenella* and *Sesamia calamistis* (Schulthess *et al.* 2002). Regarding the beetles, it has been shown that not only nitidulid beetles are strongly implicated in *F. verticillioides* infection, but also cucurionid and silvanid species positively correlated with fungal infection (Cardwell *et al.* 2000).

All these findings pose the problem of cause and effect relationships between fungal infection and insect infestation on maize plants. It is likely that the presence of *F. verticillioides* promotes insect attacks (Schulthess *et al.* 2002) and insect infestation favours fungal infection (Dowd 1998). *F. verticillioides* may be introduced into the stem and cob via insects (Munkvold and Carlton 1997). Likewise, incidence of infection by *F. verticillioides* in maize stems is a source for cob infection by the fungus, not only through movement of the fungus, but also through increased activity of stem borers (Baba-Moussa 1998). On the other hand, *F. verticillioides* produced volatiles that are quite attractive to nitidulid beetles (Bartelt and Wicklow 1999). It has been shown that fecundity, laying of eggs and survival of larvae of *Eldana saccharina* were significantly higher on inoculated maize plants (Ako *et al.* 2003). The authors also found that development time of *Carpophilus dimidiatus* was lower and its fecundity higher on infected grain than on non-infected grain. Schulthess *et al.* (2002) suggested that keeping the plant free of the fungus could be an effective way to reduce insect damage to both stem and grain. On the other hand, any action also to avoid insect infestation is useful for reducing infection of maize by *F. verticillioides* (Riley and Norred 1999).

### *Fungal interactions*

Interactions among fungi in maize also constitute an important factor influencing fungal infection and subsequent mycotoxin production. Harvested maize grains in the tropical zones contain mycelium and spores of several fungal species including mainly *Fusarium*, *Aspergillus* and *Penicillium* that can come into contact, grow and compete for food if environmental conditions are favourable. As far as *Fusarium* species are concerned, many research reports highlighted their interaction with other fungi. Velluti *et al.* (2000) showed that populations of *F. verticillioides* and *F. proliferatum*, the most important fumonisin producers, are markedly reduced by the presence of *F. graminearum*, and that fumonisin B<sub>1</sub>

(FB<sub>1</sub>) production by them can be significantly inhibited as well in the presence of *F. graminearum*. On the other hand, Marin *et al.* (1998) found that *F. verticillioides* and *F. proliferatum* are generally very competitive and dominant against *Aspergillus flavus* and *Penicillium* spp., especially at  $a_w$  more than 0.96. This inhibition can lead to significantly reduced aflatoxin contamination in infected grains (Zummo and Scott 1992).

## ATTEMPTS TO CONTROL *F. VERTICILLIOIDES* AND TO DETOXYFY OR REDUCE FUMONISIN LEVELS IN MAIZE

There is strong evidence that due to its endophytic habit, control of *F. verticillioides* in the field is very difficult. Novel control strategies are being investigated and some reported technologies include:

- The use of an endophytic bacterium (e.g. *Bacillus mojavensis*) as a biological control agent on maize seed (Bacon and Hinton 2000).
- The use of an iodine-based product called Plantpro 45™ as a biocompatible control of the fungus. The active ingredient of that product has been used as a disinfectant in human and animal health care products (Yates *et al.* 2000).
- The use of non-producing strains of *F. verticillioides* aiming to minimise fumonisin levels in maize (Plattner *et al.* 2000).
- The use of genetic engineering approach such as engineering plants (*Bt*-maize) and *in planta* detoxification of fumonisins in maize (Munkvold and Desjardins 1997, Munkvold *et al.* 1997, Munkvold *et al.* 1998).

Additional investigations are however needed to render some of those technologies more applicable.

Decontamination of fumonisins in maize and maize-based products by means of chemical reactions is the object of many research studies. Fumonisin are quite stable molecules and their destruction is likely to be also quite difficult. Ammonisation, initially used for detoxify products from aflatoxins has been investigated for fumonisin reduction but does not always give satisfactory results. Scott (1993) reported that treatment of *F. verticillioides* culture material with 2 % of ammonium hydroxide at 50 °C decreased by 89 % fumonisin concentration, but only 32 % of toxin reduction was later measured after four days air-drying. In contrast, nixtamalisation, the alkaline cooking of maize for tortilla production in Central America, significantly reduces fumonisin concentration in maize (Dombrink-Kurtzman *et al.* 2000). However, Voss *et al.* (1996) found that nixtamalised *F. verticillioides*

culture remained toxic. This indicates that reduction in detectable fumonisins does not necessarily result in reduced toxicity.

It is therefore clear that detoxification of mycotoxins in foods is not so easy. Sinha (1998) suggested that it must be economical, simple, easy to be applied by unskilled person, not too time-consuming, capable of removing all traces of the active toxin without hazardous chemical residues in the decontaminated food, and does not impair the nutritional quality of the food.

Considering the above-mentioned review of existing findings on fumonisin contamination, several points arise and need to be emphasised:

1. Contamination of food commodities by fumonisins has become a serious food safety problem throughout the world. People are more and more aware that the fumonisins, in addition to aflatoxins, constitute a real threat to human and animal health. However, in contrast to aflatoxins, fumonisins are less documented. Indeed, they are recently discovered mycotoxins and more research studies are urgently needed in order to understand more about them.
2. Some information is available on factors contributing to fumonisin production and on those able to reduce fumonisin levels in foods. However, research results on some factors remain uncertain, or are not applicable to a developing country situation. The need for more information about environmental and agroecological influences, fumonisin toxicology in respect to human and animal health, prevention methods against fungal infection and fumonisin contamination, methods to use for reducing the toxin in foods and other aspects of fumonisins, is great enough to challenge scientists to undertake further research on these topics.
3. To date in Africa, apart from South Africa, very little information is available on the natural occurrence of both *Fusarium* and fumonisins, although this part of the world is most often suspected of having potentially higher levels of fumonisins due to its position in tropical and subtropical zones. Work undertaken so far in a few African countries basically consisted of sporadic surveys of farmers' stores and retail markets, mostly basing data measurements on a relatively small number of samples (Shephard *et al.* 1996). It is a matter of great concern that in Africa, millions of people are consuming contaminated maize and maize-based foods daily without being aware of the danger. Efforts are, however, to be saluted in investigating fumonisins contamination in maize and maize-based foods in some African

countries other than South Africa such as Benin, Cameroon, Ghana, Kenya, Zambia and Zimbabwe (Shephard *et al.* 1996, Doko *et al.* 1995, Hell *et al.* 1995, Kedera *et al.* 1999, Kpodo *et al.* 2000, Gamanya and Sibanda 2001, Ngoko *et al.* 2001). Consequently, there is great need for more investigations on the continent, mainly in the maize production and consumption zones. The present study enters this framework.

## OBJECTIVES

By targeting Benin, a West-African country, this study aims indeed to contribute to efforts of African countries to guarantee foods of good quality by specifically focusing investigations on:

- Natural occurrence of *Fusarium* and subsequent fumonisin contamination in preharvest and stored maize in the different agroecological zones of Benin,
- Some factors including insect infestation, indigenous storage systems, mechanical shelling and dehulling methods influencing the occurrence of fumonisins in maize in Benin,
- Impacts of some traditional processing techniques in use in Benin on maize contamination by fumonisins.

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## CHAPTER TWO



**NATURAL OCCURRENCE OF *FUSARIUM* AND SUBSEQUENT FUMONISIN  
CONTAMINATION IN PREHARVEST AND STORED MAIZE IN BENIN, WEST  
AFRICA**

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## ABSTRACT

Natural occurrence of *Fusarium* and fumonisin contamination were evaluated from 1999 to 2003 in both preharvest and stored maize produced by small-scale farmers in four agroecological zones of Benin. Mycological analyses revealed a predominance of both *Fusarium* and *Aspergillus* in maize samples compared to other genera. The two *Fusarium* species most commonly isolated from maize were *F. verticillioides* (68 %) and *F. proliferatum* (31 %). *F. semitectum* was also encountered but only in 2002/2003 on preharvest maize. Atypical isolates of *F. verticillioides* with some characteristics of *F. andiyazi* but apparently closer to *F. verticillioides* because the isolates were all high fumonisin producers, were also found only on preharvest maize. Study of *F. verticillioides* strains showed the presence of extremely high fumonisin producers in Benin with total fumonisin levels ranging from 8240 to 16690 mg kg<sup>-1</sup>. Apart from 2002/2003, *Fusarium* occurrence was not significantly different from one zone to another although a slight decrease was observed from south to north. *Fusarium* occurrence varied somewhat from one year to another, and significantly decreased over the six months of storage. Widespread fumonisin occurrence in maize was observed, most of the maize samples collected being found positive for fumonisin with levels ranging from not detected to 12 mg kg<sup>-1</sup> in 1999/2000, 6.7 mg kg<sup>-1</sup> in 2000/2001, and 6.1 mg kg<sup>-1</sup> in 2002/2003. In contrast to *Fusarium* occurrence, fumonisin levels in maize were found to be significantly higher in the two southern zones during all the surveys. The highest mean total fumonisin level was detected in 1999/2000 in maize samples from the southern guinea savannah (SGS) (12 mg kg<sup>-1</sup>) whereas in both 2000/2001 and 2002/2003, it was in samples from the forest mosaic savannah (FMS) (6.7 mg kg<sup>-1</sup> and 6.1 mg kg<sup>-1</sup> respectively). Fumonisin levels varied from one year to another. They also changed throughout the storage time showing a decreasing trend in each zone. However, this decrease was only significant in 1999/2000. An increasing trend was observed during some seasons in the SGS and NGS. The results of this study emphasise that farmers and consumers, not only in Benin but also in other West-African countries, should be alerted to the danger of fumonisin contamination in maize.

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**Key words:** Benin, West Africa, maize, *Fusarium*, fumonisins.

## INTRODUCTION

The increasing worldwide concern about food safety has enhanced interest in fungal infection and subsequent production of mycotoxins in food products. In this respect, attention is continuously focused on maize (*Zea mays* L.) because it is one of the most important dietary staple foods in the world (FAO 2002).

Several fungi are associated with maize during pre- and postharvest periods, of which the genus *Fusarium* contains important toxigenic species that may cause severe damage (Nelson *et al.* 1983). Amongst these, *F. verticillioides* (Sacc) Nirenberg (previously known as *F. moniliforme* Sheldon) is one of the most economically important species worldwide (Marasas *et al.* 1988, Munkvold and Desjardins 1997). *F. verticillioides* is known to produce a number of mycotoxins, primarily fumonisins (Nelson *et al.* 1993, Marasas 1996, Marasas 2001).

Fumonisin are a group of mycotoxins recently discovered (Gelderblom *et al.* 1988, Marasas 2001). Their natural occurrence in maize has become an important concern for animal and human health throughout the world (Thiel *et al.* 1992). Fumonisin have been shown to cause leukoencephalomalacia (ELEM) in horses (Marasas 1996), pulmonary oedema syndrome (PES) in pigs (Harrison *et al.* 1990, Colvin and Harrison 1992), and hepatocarcinoma in rats (Gelderblom *et al.* 2001). There is no strong evidence of adverse effects of fumonisins on human health (Shephard *et al.* 1996, FDA 2001). However, studies have reported these toxins to be associated with high incidences of oesophageal cancer in South Africa (Rheeder *et al.* 1992, Marasas 1996), China (Chu and Li 1994, Wang *et al.* 2000), Italy (Franceschi *et al.* 1990) and Iran (Shephard *et al.* 2000).

Great concern also exists that fumonisin may be spread worldwide through grain trade (Placinta *et al.* 1999). High levels of fumonisin have been detected in export maize in several maize-importing countries and consequently have marked economic implications (Shephard *et al.* 1996). This situation has already led some of these countries to propose maximum tolerated levels (MTL) of fumonisins for regulating commercial exchanges. Thus, the US Food and Drug Administration (FDA) recommend 2 – 4 mg kg<sup>-1</sup> as MTL for fumonisin in different maize-based foods (FDA 2001). The French Council of Public Hygiene advises a MTL of 3 mg kg<sup>-1</sup> (Dragacci 1999), while Switzerland has established this level not to be higher than 1 mg kg<sup>-1</sup> (Marasas *et al.* 2001a).

Many studies to evaluate the natural occurrence of *Fusarium* and fumonisin in maize have been conducted in several parts of the world, mainly in South Africa, United States of

America, South America, and Europe. Results have been thoroughly reviewed (Norred 1993, Riley *et al.* 1993, Shephard *et al.* 1996, Marasas 1996, IPCS 2000, Bolger *et al.* 2001, Marasas 2001, WHO 2002). In Africa, apart from South Africa, very little work has been undertaken on the occurrence of fumonisins in maize (Doko *et al.* 1995, Kedera *et al.* 1999, Kpodo *et al.* 2000, Gamanya and Sibanda 2001, Ngoko *et al.* 2001). There is a great need for additional investigations on the continent, at least where maize production and consumption are predominant. The aim of the present study, carried out in Benin, West Africa, was to determine the geographical distribution of *Fusarium* in this country and to evaluate the natural occurrence of both *Fusarium* and concomitant fumonisin contamination in preharvest and stored maize.

## MATERIALS AND METHODS

### *Agroecological zones*

Three national countrywide surveys were carried out from 1999 to 2003 in four agroecological zones of Benin (Fig 1), to evaluate the natural occurrence of both *Fusarium* and fumonisin in maize. Hell *et al.* (2000) described these zones as followed:

- Forest Mosaic Savannah (FMS): latitude 6°30' – 7° North. This is the southernmost zone of Benin. It is characterised by two growing seasons (April to July and September to November), with high average relative humidity (more than 90 % during almost all year) and maximum temperature ranging from 25 to 35 °C.
- Southern Guinea Savannah (SGS): latitude 7° - 8° North, considered as a transition zone between the North and the South of Benin, with the same seasonal pattern as the FMS, but less humid than the FMS zone. Relative humidity averages from 80 to 85 % during the rainy period of the year, and maximum temperature more often between 28 and 32 °C.
- Northern Guinea Savannah (NGS): latitude 8° - 11° North, in contrast, is characterised by one growing season (April to September), more or less dry climate. The relative humidity is only high (more than 70 %) during a short period running from July to September and very low during the harmattan wind (November to February), and with high maximum temperature (28 to 35 °C).
- Sudan Savannah (SS): latitude 11° - 12° North, the northernmost zone of Benin, with one growing season as well running from May to September. Climate is dry with low average

relative humidity (less than 60 %) during several months, and high maximum temperature (30 – 42 °C). This zone is at the limit of Sahel, a very dry and warm zone in West Africa covering several countries including Niger, Burkina Faso, Mali and Senegal.

### *Survey and sampling procedures*

The surveys covered the entire country during three seasons: 1999/2000, 2000/2001 and 2002/2003. They were conducted in 16 maize-growing villages (four villages per agroecological zone) (Fig 1). Ten farmers were randomly selected from a list of farmers cultivating maize in each village. The same farmers selected in the first survey were also involved in the following ones. However, replacements were selected in some cases due to death, illness, or other reasons.

During each survey, the fields of the selected farmers were sampled within the week before harvesting, and their stores at 3 and 6 months after stocking. At least 50 maize cobs were collected from each farmer at each sampling. In field (area between 0.5 and 1 ha), the cobs were collected walking along diagonals. In granary, the cobs were collected from the sides of each granary (20 cobs), at the top inside the granary (10 cobs), in centre (10 cobs) and at the bottom (10 cobs). The cobs of each sample were shelled by hand. Grains were initially sun-dried, if necessary, to moisture content less than 18 % (case of the samples collected in fields in FMS and SGS zones). The samples from the 10 farmers per village were pooled on-farm and thoroughly mixed to give one sample representative of each village. This pooled sample was then divided into 4 equal lots. The first two lots, unground, were intended for determination of grain moisture content and mycological analysis, respectively. The third lot, unground, was sent to PROMEC, Medical Research Council, Cape-Town, South Africa, for mycological and fumonisin analyses using the HPLC method. The fourth lot, ground, was intended for fumonisin analysis in Benin using the VICAM method, as there was no facility for HPLC analysis (Fig 2). Thereafter, each lot was collected in paper bag, transported to the laboratory and kept at 4 °C.

### *Determination of grain moisture content*

As the farmers' fields and stores are far from the laboratory, grain moisture content was measured on-farm immediately after sampling, using an electronic moisture meter (model HOH-EXPRESS HE 50, PFEUFFER, Germany).

### ***Mycological analyses***

Twenty-five grains from each sample collected from the fields and stores, in four replicates (100 grains), were surface sterilised in a 10 % sodium hypochlorite solution for 2 min and rinsed twice in distilled water. The grains were plated in Petri dishes containing 15 ml of potato dextrose agar (PDA) each, with five grains per Petri dish. The Petri dishes were then incubated for 5 days at 25 °C exposed to a 12:12-hour light/dark regime, after which fungal genera were identified (Singh *et al.* 1991). *Fusarium* species were isolated, transferred onto carnation leaf agar (CLA) in Petri dishes and incubated at 25 °C for 7 days exposed to a 12:12-hour light/dark regime. *Fusarium* species were identified according to Nelson *et al.* (1983). Occurrence, i.e. percentage of samples infected with fungi and incidence, i.e. percentage of infected grains in each sample were determined.

### ***Fumonisin quantification***

Fumonisin content was determined using the VICAM method (VICAM, 1998). For this purpose, a sub-sample of 300 g from each sample was finely ground. Fifty grams of the ground maize were weighed into a flask and mixed with 5 g of sodium chloride and 100 ml of methanol:water (80:20). The mixture was blended for 1 min at high speed using a blender (Waring Commercial, Torrington, USA) and filtered through a fluted filter paper. Ten millilitres of extract were diluted with 40 ml of phosphate buffered saline (PBS)/0.1 % Tween-20 wash buffer, and filtered through a 1.0 µm microfibre filter. The diluted extract was then passed through an immunoaffinity column (FumoniTest™ column, VICAM, Watertown, USA), which contains specific antibodies to fumonisins. At this stage, fumonisins are bound to the antibodies in the column. The column was then washed with 10 ml of PBS/0.1 % Tween-20 wash buffer followed by 10 ml of PBS. High performance liquid chromatography (HPLC) grade methanol (1 ml) was then passed through the column to remove the fumonisins from the antibody. A mixture of Developer A and Developer B (1 ml) was added to the eluate, collected in a cuvette that was placed in a fluorometer (VICAM Fluorometer Series 4, Watertown, USA) for fumonisin measurement.

### ***Determination of fumonisin-producing strains of F. verticillioides in maize samples***

Thirteen isolates of *F. verticillioides* were obtained from cultures of maize collected in the different agroecological zones of Benin. The isolates were grown from lyophilised cultures on maize patties at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), South Africa, for fumonisin analyses using the HPLC method (Sydenham *et al.* 1996). Results were expressed in terms of level of fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> produced by each isolate. MRC 826 a typical *F. verticillioides* isolate of PROMEC, known to be high producer of fumonisins (Alberts *et al.* 1990), served as control. This experiment was replicated once.

### ***Statistical analyses***

SPSS for Window version 10.0 (SPSS Inc., Chicago, Illinois) was used for statistical analyses. A multivariate (3-way) analysis of variance (MANOVA) was performed with Roy's Largest Root test for analysing interactions of season, zone and time of maize sampling on parameters (fungal occurrence and incidence and fumonisin levels in maize). Student-Newman-Keuls test was computed in a univariate analysis of variance to compare means of fungal occurrence and incidence and means of total fumonisin per season in the different agroecological zones and throughout the storage period. Pearson correlation test was performed to determine relationships among parameters.

## **RESULTS**

Mean percentage of grain moisture content in preharvest maize was generally high in the southern zones during all the surveys (19 % - 25 %), but lower in the northern zones (11 % - 15 %) (Fig 3). Maize harvest generally occurs in northern Benin in December- January during harmattan, which is a very dry tropical wind. Mean grain moisture content significantly decreased in maize during the 6-month storage period in farmers' stores ( $p < 0.05$ ). In contrast to the other years, mean grain moisture content in the preharvest maize in the SGS zone was low in 2001/2002 (17.2 %) (Fig 3). Maize harvest occurred that year in very dry conditions, which was uncommon compared to a normal year.

Results of mycological analyses on the maize samples collected from the agroecological zones during the surveys showed that *Fusarium* and *Aspergillus* were the predominant fungal

genera in maize during every season (Fig 4). More than 70 % of the samples were always found to be infected with species of these two genera. Their incidence, overall, was respectively about 48 % and 32 % in 1999/2000, 46 % and 38 % in 2000/2001, and 45 % and 36 % in 2002/2003 (Fig 5). The genus *Penicillium* was also detected in many samples (more than 50 %) (Fig 4), but with lower incidence, about 13 % in 1999/2000, 15 % in 2000/2001 and 12 % in 2002/2003 (Fig 5). Species of *Trichoderma* and *Mucor* were encountered but in less than 5 % of the samples (data not shown). Other non-*Fusarium* species isolated in a few samples from fields, only during the survey of 2002/2003, were *Lasiodiplodia theobromae* (Pat) Griff & Maubl, *Colletotrichum graminicola* Wilson and *Aspergillus niger* van Tiegh. The former fungus was found in all the zones whereas the latter two fungi were encountered only in the northern zones.

The two *Fusarium* species most commonly found in the maize samples were *F. verticillioides* and *F. proliferatum* (Matsushima) Nirenberg. In 1999/2000 for example, their occurrence was 68.1 % and 31.9 %, respectively (data not shown). *F. verticillioides* was present in almost all the samples whether in the south or north, whereas *F. proliferatum* was mostly encountered in the samples collected in the southern zones. This species was not detected in 2002/2003, but another *Fusarium* species, *F. semitectum* Berk. & Rav., was found during that season in some preharvest maize samples mainly in the SGS zone.

Mycological analyses also revealed the presence of atypical *F. verticillioides* isolates in preharvest maize samples in the two southern zones (11 %) and in the NGS (3 %) (data not shown). Cultures on PDA were salmon coloured with concentric purplish rings on the reverse of Petri dishes (Fig 6). On carnation leaf agar, long microconidial chains were present and polyphialides absent. Cells resembling pseudochlamydospores described by Marasas *et al.* (2001b) were observed in the carnation leaf pieces. The characteristics resemble *F. andiyazi*, recently described from sorghum (Marasas *et al.*, 2001b).

With respect to *Fusarium* occurrence, there was no significant difference from one zone to another ( $p > 0.05$ ) except in 2002/2003. A slight decrease was, however, generally observed from south to north, with higher percentage of infected maize samples in both FMS and SGS (Fig 7). *Fusarium* occurrence, however, differed significantly from one season to another ( $p < 0.05$ ). *Fusarium* occurrence decreased significantly over the 6 months of storage ( $p < 0.05$ ) from about 94 % of infected samples at the beginning to 76 % at 6 months of storage in 1999/2000, from 98 % to 55 % in 2000/2001 and from 100 % to 76 % in 2002/2003 (data not shown).

*Fusarium* incidence did not vary significantly from one zone to another in any season ( $p > 0.05$ ). Overall means of incidence were, however, slightly higher in maize in the south ( $58.1 \pm 20.9\%$  in FMS,  $51.8 \pm 18.8\%$  in SGS), and particularly lower in the SS ( $35.9 \pm 26.7\%$ ) (data not shown). No significant differences were observed in *Fusarium* incidence from one season to another ( $p > 0.05$ ). However, *Fusarium* incidence decreased significantly throughout the storage period every season ( $p < 0.01$ ), from  $70.4\%$  at harvest to  $24.6\%$  at 6 months of storage in 1999/2000, from  $75.1\%$  to  $13.9\%$  in 2000/2001 and from  $69.5\%$  to  $17.0\%$  in 2002/2003. *Fusarium* incidence was positively and significantly correlated with *Fusarium* occurrence ( $r = 0.6$ ,  $p < 0.01$ ) (data not shown).

Regarding fumonisin levels in maize samples, overall, a widespread occurrence of the toxin was observed during all seasons (Table 1). Almost all the samples collected were found to be fumonisin-positive, the levels ranging from not detected to  $12 \text{ mg kg}^{-1}$  in 1999/2000,  $6.7 \text{ mg kg}^{-1}$  in 2000/2001, and  $6.1 \text{ mg kg}^{-1}$  in 2002/2003. Fumonisin levels were higher in the two southern zones during all the seasons ( $p < 0.05$ ). The highest mean total fumonisin level was detected in 1999/2000 in the samples from the SGS ( $12 \text{ mg kg}^{-1}$ ) whereas in both 2000/2001 and 2002/2003, this was detected in the samples from the FMS ( $6.7 \text{ mg kg}^{-1}$  and  $6.1 \text{ mg kg}^{-1}$ , respectively).

Fumonisin levels detected in maize samples varied significantly from one season to another, except in the FMS ( $p < 0.05$ ) (Fig 8). They were higher in maize in 1999/2000 than in the two other seasons (Fig 8). Thirty-seven maize samples of 48 studied had a mean total fumonisin level of more than  $1 \text{ mg kg}^{-1}$  in 1999/2000, 35 in 2002/2003, and 24 samples in 2000/2001 (Table 1). Moreover, six samples out of 48 had fumonisin levels more than  $4 \text{ mg kg}^{-1}$  in 1999/2000, four in 2000/2001, and only one in 2002/2003 (Table 1). These high fumonisin containing samples were mainly from the FMS and SGS zones. The maize samples from 11 villages of the 16 visited had fumonisin levels more than  $4 \text{ mg kg}^{-1}$  in 1999/2000. There were five in 2000/2001 and only one in 2002/2003, all situated in the southern zones (data not shown). Fumonisin levels changed throughout the 6-month storage period showing a decreasing trend in each zone (Table 2). However, this decrease was not significant every season. An increasing trend was observed during some seasons in the SGS and NGS zones (Table 2). A positive and significant correlation was observed between the fumonisin level in maize and both *Fusarium* occurrence ( $r = 0.4$ ,  $p < 0.01$ ) and incidence ( $r = 0.4$ ,  $p < 0.01$ ) (data not shown).

Highly significant interactive effects of factors such as season, agroecological zone and time of maize sampling during the surveys were observed on *Fusarium* occurrence and

incidence and fumonisin level in maize (Tables 3). Roy's Largest Root test was significant for all the factors including their interactions ( $p < 0.01$ ) (Table 4). The interaction between season and time of sampling was found to be significant for all parameters, whereas the others were significant for only one or two of the parameters. The interaction between season and zone was not significant for *Fusarium* incidence, nor that between zone and time of sampling for fumonisin level in maize ( $p > 0.05$ ). The interaction between season, zone and time of sampling was significant for *Fusarium* occurrence only ( $p < 0.05$ ).

Most of the isolates (11 of 13) tested for their ability to produce FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> were found to be very high fumonisin producers with total fumonisin levels ranging from 8240 to 16690 mg kg<sup>-1</sup> (Table 5). Only 2 of 13 were low fumonisin producers (124 mg kg<sup>-1</sup> and 6 mg kg<sup>-1</sup>), and these were also the only isolates that did not produce FB<sub>3</sub>. High yielding isolates were detected in all the agroecological zones. Both the highest fumonisin producer (16690 mg kg<sup>-1</sup>) and lowest (6 mg kg<sup>-1</sup>) were isolated from maize from the SS zone. The three atypical *F. verticillioides* isolates (MRC 8263, MRC 8265 and MRC 8269) were all high producers with total fumonisin levels ranging from 9250 to 16580 mg kg<sup>-1</sup> (Table 5). This experiment was repeated with essentially the same results (Table 5).

## DISCUSSION

Analysis of fungal contamination showed that *F. verticillioides* and *F. proliferatum* were the two *Fusarium* species commonly isolated from the maize samples during the three-year survey. This is the first time *F. proliferatum* is reported on stored maize in Benin. *F. verticillioides* and *F. proliferatum* occur worldwide on maize. Several surveys carried out in many parts of the world have revealed that they are the fumonisin-producing *Fusarium* species most frequently isolated from maize in tropical and subtropical zones such as Benin (Shephard *et al.*, 1996). Studies showed that these two species often occur together on maize (Leslie *et al.*, 1990; Logrieco and Moretti, 1995). Logrieco and Moretti (1995) explained their coexistence by the fact that (a) they have similar optimum growth conditions and (b) they do not show apparent antagonism when growing together. It is also, however, common to find one without the other, as it was the case in the present study in 2002/2003. *F. semitectum* was encountered in preharvest maize samples in the south. This species is known to not produce fumonisin (Rheeder *et al.*, 2002).

Regarding the atypical *F. verticillioides* isolates from preharvest maize samples, it is uncertain at present whether these are *F. verticillioides* or *F. andiyazi*. Fumonisin analyses

indicated that they are closer to *F. verticillioides* than to *F. andiyazi* as all three of them were high fumonisin producers, whereas *F. andiyazi* produces only trace amounts (Rheeder *et al.*, 2002). Two of them were extremely high producers (up to 16580 mg kg<sup>-1</sup>), i.e. close to the highest fumonisin-producing isolate of *F. verticillioides* (16690 mg kg<sup>-1</sup>) and higher than *F. verticillioides* MRC 826, which is known as a high producer (13310 mg kg<sup>-1</sup>). Moreover, it is not certain whether the cells found in the carnation leaf pieces were actually the pseudochlamydospores characterising *F. andiyazi* (Marasas *et al.*, 2001b). They could also be thick-walled hyphae as found in some cultures of *F. verticillioides* by Klaasen and Nelson (1998), or chlamydospore-like structures similar to those that have been induced to form in *F. verticillioides* (Mandal and Chaudhuri, 1990). Further investigations are therefore being undertaken on the fumonisin producing ability and molecular characterisation of these isolates.

The presence in Benin of *F. verticillioides* strains, which are high fumonisin producers, suggests a permanent risk of marked *Fusarium* and fumonisin contamination in maize in the country. The non-significant difference between *Fusarium* occurrences in the four agroecological zones indicates that maize contamination with *Fusarium* is possible everywhere in the country. This situation, however, might change in time from one season to another if environmental conditions become favourable. *Fusarium* occurrence was also found to be higher in preharvest maize and significantly decreased gradually over the time of storage. This decrease over time may be due to unfavourable humidity conditions for fungal growth during the storage period. There is tangible evidence that growth of *Fusarium* mostly occurs in the field before harvest because these fungi have higher humidity requirements (Müller and Schwadorf, 1993). Munkvold and Desjardins (1997) stated that *F. verticillioides* has not been reported to grow in grain at moisture content below 18 to 20 %. Such humidity conditions generally occur in the field before harvest. This supports the conclusion that *Fusarium* species, field fungi, are unable to proliferate during the storage period due to the lowered grain moisture content and unfavourable storage environmental conditions (Christensen and Kaufmann, 1974; Mycock and Berjak, 1992).

This is not the first time fumonisin contamination in maize has been reported in Benin. Hell *et al.* (1995) and Doko *et al.* (1995) previously detected the toxin in maize samples collected from some farmers' stores. Doko *et al.* (1995), in their study comparing fumonisin contamination in different African countries, already noted Benin as a high occurrence area since they found high total fumonisin levels (3.3 mg kg<sup>-1</sup>) in maize samples. This level is, however, far lower than those detected in many maize samples in the present study. Up to 12

mg kg<sup>-1</sup> of total fumonisin was detected in a sample in 1999/2000. Moreover, extremely high total fumonisin levels, up to 16690 mg kg<sup>-1</sup> (12020 mg kg<sup>-1</sup> of FB<sub>1</sub>), were obtained in maize cultures from Benin. The highest FB<sub>1</sub> levels produced by isolates of *F. verticillioides* reported so far are 17900 mg kg<sup>-1</sup> from South Africa (Alberts *et al.*, 1990; Rheeder *et al.*, 2002), 10200 mg kg<sup>-1</sup> from China (Yoshizawa *et al.*, 1994), and 8160 mg kg<sup>-1</sup> from Argentina (Sydenham *et al.*, 1993), respectively. This confirms the high risk of fumonisin contamination to which the population of Benin is exposed to in the maize that is widely consumed in the country.

In terms of fumonisin contamination in each agroecological zone, levels of fumonisin were found to be significantly higher in the two southern than in the northern zones. More precisely, a decrease trend of the level was observed from south to north. The southern zones are the most humid zones of Benin with relative humidity generally higher (more than 90 %) during several months in the year, whereas in the north, this is often lower averaging 70 %. Annual rainfall patterns are characterised by two rainy periods in the south and one rainy period in the north. Temperatures in the south are high and more often vary from 25 to 35 °C. Moreover, due to the fact there are two rainy seasons in the south, farmers in that region grow two maize crops per year in contrast to the north, and production is mainly characterised by:

- Long delays in harvest of maize (generally more than one month after physiological maturity), with the consequence of considerable insect infestation and fungal infection in the field before harvest.
- Harvest of maize of the first season often takes place when late rains occur, rendering maize drying very difficult.
- Insect control is almost non-existent with considerable insect damage on maize creating entry points for fungi.
- Inadequate traditional storage facilities are very accessible to pests such as insects and rodents and do not facilitate continuous drying of maize during storage.
- Rare application of hygienic measures during storage.

Such conditions, in addition to the environmental factors, may favour fumonisin contamination. This is in agreement with the research of Hell *et al.* (1995), who previously found that in Benin fumonisin levels decrease from south to north. In Zimbabwe, Gamanya and Sibanda (2001) also found levels of fumonisin to decrease from regions with high rainfall and annual moderate temperatures to those with low rainfall.

Previous reports indicated that the highest levels of fumonisin usually occur under warm and dry conditions (Marasas *et al.*, 1979; Murphy *et al.*, 1993; Shephard *et al.*, 1996), but without specifying exactly how warm and dry these conditions are. Precision is essential for meaningful comparisons because warm and dry conditions vary in different parts of the world. Benin is situated in the tropical zone but overall environmental conditions there are less warm and dry than in Mali for example, which is much warmer and drier. Likewise, the southern part of Benin is likely to be drier than that of Ghana or Cote d'Ivoire, two other West African countries. The role of humidity in fumonisin contamination is clearly important. Shelby *et al.* (1994) who also reported high levels of fumonisin to occur with hot and dry weather qualified that this is more likely to occur when the hot and dry weather is followed by periods of high humidity. Likewise, Hennigen *et al.* (2000) found high levels of fumonisin in maize to be associated with high relative humidity in Argentina. Fumonisin contamination is likely to be strongly influenced by several environmental factors in different geo-areas and among these, temperature, humidity, drought stress and rainfall during preharvest and harvest periods are very important (FDA, 2001).

Variations of fumonisin contamination from one season to another were observed during the study. Contamination was particularly higher in maize samples in 1999/2000 than in both 2000/2001 and 2002/2003. In the United States of America, surveys over a 5-year period also showed high levels of fumonisin during the first four years followed by a drop in the fifth year (Murphy *et al.*, 1993). In Argentina, Hennigen *et al.* (2000) found fumonisin contamination to differ markedly during two consecutive growing seasons. Such yearly variations may among others be due to difference in environmental conditions. In this study for example, mean rainfall during the period of survey was higher in 1999/2000 (193.3 mm) than in both 2000/2001 and 2002/2003 (156.6 mm and 121.7 mm respectively) (data not shown).

A decreasing trend was observed in fumonisin levels detected in maize samples throughout the storage time, the highest level being recorded in the preharvest samples and the lowest in those collected six months after stocking. This decrease, however, was not significant in all seasons. An increasing trend was observed during some seasons in the SGS and NGS zones. This is in contrast with Ngoko *et al.* (2001), who found fumonisin to increase with storage time in maize collected in different zones of Cameroon. In Brazil, Ono *et al.* (2002) found fumonisin concentrations to remain unchanged in maize stored in controlled environmental conditions for 12 months.

It is possible that environmental conditions during the storage period affected fumonisin production leading to decrease of the level as observed in the present study. Munkvold and

Desjardins (1997) stated that increases of fumonisin level in farmers' stores during the storage period are unlikely as long as conditions of grain moisture content and temperature are maintained at recommended levels. Ono *et al.* (2002) found that fumonisin levels did not change during a 12-months storage period, but stressed the importance of initial *Fusarium* count that can affect fumonisin production during storage. According to these authors, a low initial *Fusarium* count is likely to have a lower risk for fumonisin production during storage of dried maize.

Results of the present study showed marked interactive effects of the various factors indicating that *Fusarium* infection may occur in any zone of Benin, but depending on seasonal and environmental conditions and on the time at which samples of maize are collected for evaluation. These results also indicate that maize contamination with fumonisin, which can occur throughout the country, is influenced by seasonal and environmental conditions. The non-statistically significant effect of the interaction between season and zone on *Fusarium* incidence is not a stable event. Although *Fusarium* appears prevalent in all parts of the country, it is likely that this also is affected by season and environmental conditions.

Information obtained from this study should result in increased awareness of farmers and consumers not only in Benin but also in other West-African countries about the danger of fumonisin contamination in maize. The risk of maize contamination by fumonisin was found to be high as many samples had fumonisin levels higher than 4 mg kg<sup>-1</sup>, the MTL for fumonisins recommended by the FDA. The presence in Benin of *F. verticillioides* strains, which are high fumonisin producers appeals for more attention and suggests that farmers should adopt adequate postharvest management procedures in order to assure good quality of stored maize. Moreover, as it has been found that fumonisin contamination was higher in preharvest maize, adequate drying before and during storage should be one of the important measures to recommend to farmers for reducing contamination with both *Fusarium* and fumonisin. Further investigations are needed for the identification of the atypical *F. verticillioides* isolates found in some maize samples from Benin.

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Table 1: Occurrence of fumonisin in maize samples collected in 1999/2000, 2000/2001 and 2002/2003 in different agroecological zones of Benin

Agroecological zones	No of samples	Total fumonisin level in maize ( $\mu\text{g/g}$ )						Number of maize samples per range of fumonisin														
		Mean			Range			0 – 1 $\mu\text{g/g}$			1 – 2 $\mu\text{g/g}$			2 – 3 $\mu\text{g/g}$			3 – 4 $\mu\text{g/g}$			> 4 $\mu\text{g/g}$		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
FMS	12	2.8 ± 1.4 ab	3.1 ± 1.6 a	2.3 ± 1.5 a	0.5 – 5.3	0.7 – 6.7	0.7 – 6.1	4	1	2	4	2	4	2	3	3	2	3	2	0	3	1
SGS	12	4.1 ± 3.6 b	1.9 ± 1.3 b	1.8 ± 0.7 a	0.7 – 12.0	0.4 – 4.6	1.1 – 3.3	2	4	0	2	3	9	4	3	2	0	1	1	4	1	0
NGS	12	2.3 ± 1.7 a	0.8 ± 0.5 c	0.9 ± 0.4 b	0.6 – 6.9	0.4 – 2.0	0.3 – 1.8	1	9	9	6	3	3	3	0	0	0	0	0	2	0	0
SS	12	1.5 ± 1.4 a	0.7 ± 0.5 c	0.5 ± 0.3 b	nd – 3.8	nd – 2.1	nd – 1.0	4	10	12	4	2	0	2	0	0	2	0	0	0	0	0
Total	48	2.7 ± 2.4	1.6 ± 1.5	1.4 ± 1.1	nd – 12.0	nd – 6.7	nd – 6.1	11	24	23	16	10	16	11	6	5	4	4	3	6	4	1

FMS = Forest Mosaic Savannah, SGS = Southern Guinea Savannah, NGS = Northern Guinea Savannah, SS = Sudan Savannah

I = season 1999/2000; II = season 2000/2001 and III = season 2002/2003

Means within a column followed by the same letter are not significantly different ( $p > 0.05$ ) (Students Newman Keuls)

nd = not detected = level  $< 0.25 \text{ mg kg}^{-1}$  for fumonisins (Vicam method).

Table 2. Mean total fumonisin levels over 6-month storage period in four different agroecological zones of Benin in 1999/2000, 2000/2001 and 2002/2003

Agroecological zones	No of samples	1999/2000			2000/2001			2002/2003		
		0 month of storage	3 months of storage	6 months of storage	0 month of storage	3 months of storage	6 months of storage	0 month of storage	3 months of storage	6 months of storage
FMS	12	4.0 ± 1.2 a	3.0 ± 1.2 ab	1.5 ± 0.7 b	4.2 ± 1.9 a	3.4 ± 0.7 a	1.7 ± 1.1 a	3.2 ± 2.2 a	1.9 ± 1.0 a	1.9 ± 0.8 a
SGS	12	7.3 ± 3.8 a	4.1 ± 2.3 ab	0.9 ± 0.2 b	1.5 ± 0.6 a	2.8 ± 1.9 a	1.4 ± 1.0 a	2.4 ± 0.8 a	1.3 ± 0.4 b	1.6 ± 0.2 ab
NGS	12	2.7 ± 2.9 a	2.8 ± 1.1 a	1.5 ± 0.4 a	0.9 ± 0.2 a	1.2 ± 0.6 a	0.4 ± 0.0 a	1.3 ± 0.4 a	0.7 ± 0.3 a	0.7 ± 0.3 a
SS	12	2.9 ± 1.0 a	1.5 ± 0.6 b	nd c	0.9 ± 0.3 a	0.6 ± 0.2 a	0.7 ± 0.9 a	0.8 ± 0.2 a	0.5 ± 0.1 b	nd b

FMS = Forest Mosaic savannah, SGS = Southern Guinea Savannah, NGS = Northern Guinea Savannah, SS = Sudan Savannah.

Values shown are mean (± Standard Deviation) total fumonisin levels in maize

Means within row followed by the same letter are not significantly different ( $p > 0.05$ ) (Students Newman Keuls).

nd = not detected = fumonisin level  $< 0.25 \text{ mg kg}^{-1}$  (Vicam method)

Table 3: Test of the interactive effects of the studied factors (season, zone and time of sampling) on *Fusarium* occurrence, *Fusarium* incidence and fumonisin levels in maize

Interactions	df <sup>(1)</sup>	Significance probability (p)
Season x zone <sup>(2)</sup>		
- <i>Fusarium</i> occurrence	6	0.005**
- <i>Fusarium</i> incidence	6	0.515
- Fumonisin level	6	0.028*
Season x time <sup>(2)</sup>		
- <i>Fusarium</i> occurrence	4	0.001**
- <i>Fusarium</i> incidence	4	0.001**
- Fumonisin level	4	0.000**
Zone x time <sup>(2)</sup>		
- <i>Fusarium</i> occurrence	6	0.000**
- <i>Fusarium</i> incidence	6	0.000**
- Fumonisin level	6	0.272
Season x zone x time <sup>(2)</sup>		
- <i>Fusarium</i> occurrence	12	0.037*
- <i>Fusarium</i> incidence	12	0.444
- Fumonisin level	12	0.064

(1): df = degree of freedom

(2): Season x zone, season x time, zone x time and season x zone x time are interactions of factors tested on *Fusarium* occurrence and incidence and fumonisin levels detected in maize

\*: Interactive effects of factors are significant on parameters with  $p < 0.05$

\*\* : Interactive effects of factors are highly significant on parameters with  $p < 0.01$

Table 4: Multivariate test of the studied factors using Roy's Largest Root

Studied factors	F value	Significance probability (p)
Season	11.303	0.000
Zone	29.034	0.000
Time of sampling	207.392	0.000
Season x zone	8.422	0.000
Season x time	12.333	0.000
Zone x time	13.392	0.000
Season x zone x time	9.262	0.000

Table 5: Fumonisin production on maize patties by fungal isolates from maize samples collected in November 2002 in different agroecological zones of Benin

<i>Fusarium</i> species	MRC Number <sup>(1)</sup>	Fumonisin content (mg kg <sup>-1</sup> ) (25-03-2003) <sup>(2)</sup>				Fumonisin content (mg kg <sup>-1</sup> ) (07-08-2003) <sup>(3)</sup>				Agroecological zone of origin <sup>(4)</sup>
		FB <sub>1</sub>	FB <sub>2</sub>	FB <sub>3</sub>	Total	FB <sub>1</sub>	FB <sub>2</sub>	FB <sub>3</sub>	Total	
<i>F. verticillioides</i>	826 (control) <sup>(5)</sup>	9200	2600	1500	13300	9050	2720	950	12720	Ex Transkei South Africa
<i>F. verticillioides</i>	8262	11590	2940	580	15100	9440	2060	1610	13110	FMS
Atypical <i>F. verticillioides</i>	8263	11140	2880	560	14560	10740	2450	810	14000	FMS
<i>F. verticillioides</i>	8264	10540	2210	560	13310	9690	1630	700	12020	NGS
Atypical <i>F. verticillioides</i>	8265	7230	1300	730	9250	5510	790	680	6980	NGS
<i>F. verticillioides</i>	8266	8030	2110	540	10670	8440	1790	820	11050	SGS
<i>F. verticillioides</i>	8267	12020	3750	910	16690	9230	2280	840	12350	SS
<i>F. verticillioides</i>	8268	10180	1940	680	12800	8000	1310	710	10020	SGS
Atypical <i>F. verticillioides</i>	8269	11750	3050	1770	16580	8400	1850	1430	11680	SGS
<i>F. verticillioides</i>	8270	9580	2930	1010	13510	14200	3360	1290	18850	NGS
<i>F. verticillioides</i>	8271	6360	1250	630	8240	3760	600	570	4930	SS
<i>F. verticillioides</i>	8272	7700	2800	620	11110	6540	1730	670	8940	NGS
<i>F. verticillioides</i>	8273	120	nd <sup>(6)</sup>	nd	120	0.2	nd	nd	0.2	SS
<i>F. verticillioides</i>	8274	9.0	1.0	nd	10	1.2	0.13	nd	1.33	SGS

(1) Accession number in the culture collection at the Medical Research Council, Tygerberg, South Africa (MRC)

(2) Date of first fumonisin measurement.

(3) Date of second fumonisin measurement (repetition) using the patties cultures

(4) FMS = Forest Mosaic savannah, SGS = Southern Guinea Savannah, NGS = Northern Guinea Savannah, SS = Sudan Savannah

(5) MRC 826 = subculture 14-09-1988, used as control

(6) nd = not detected = fumonisin level < 0.05 mg kg<sup>-1</sup> (HPLC method)

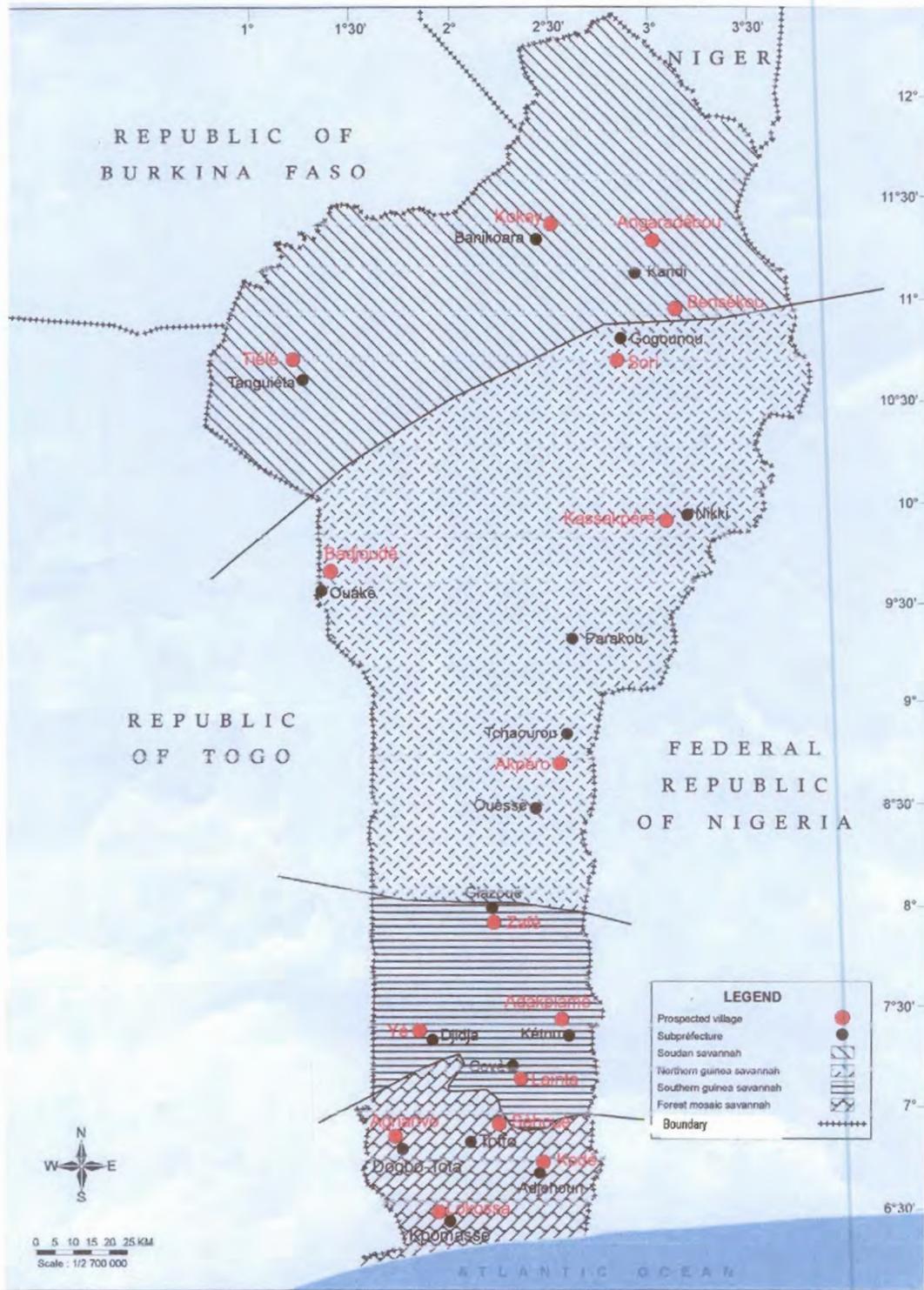


Fig 1: Map of Benin showing the different agroecological zones and villages surveyed

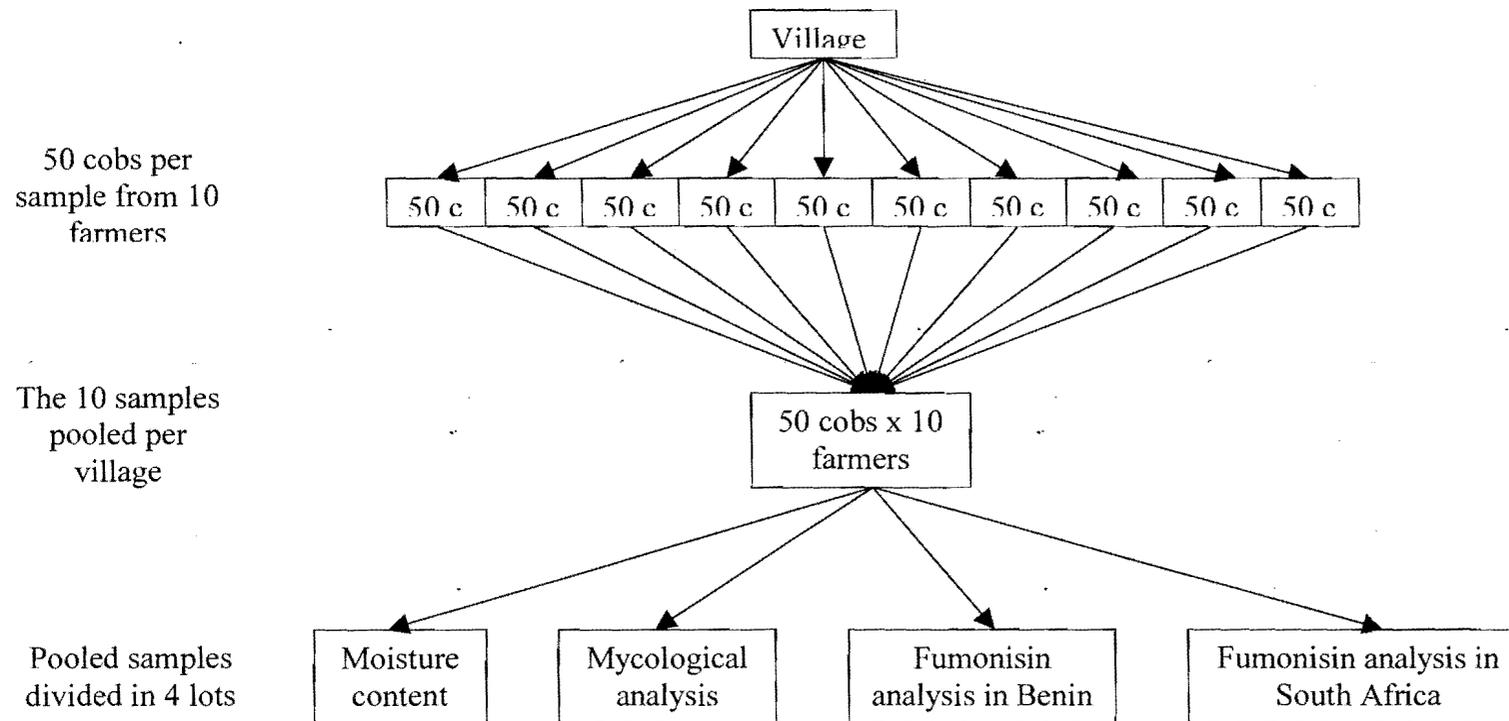


Figure 2: Diagram summarising the sampling method used during this study

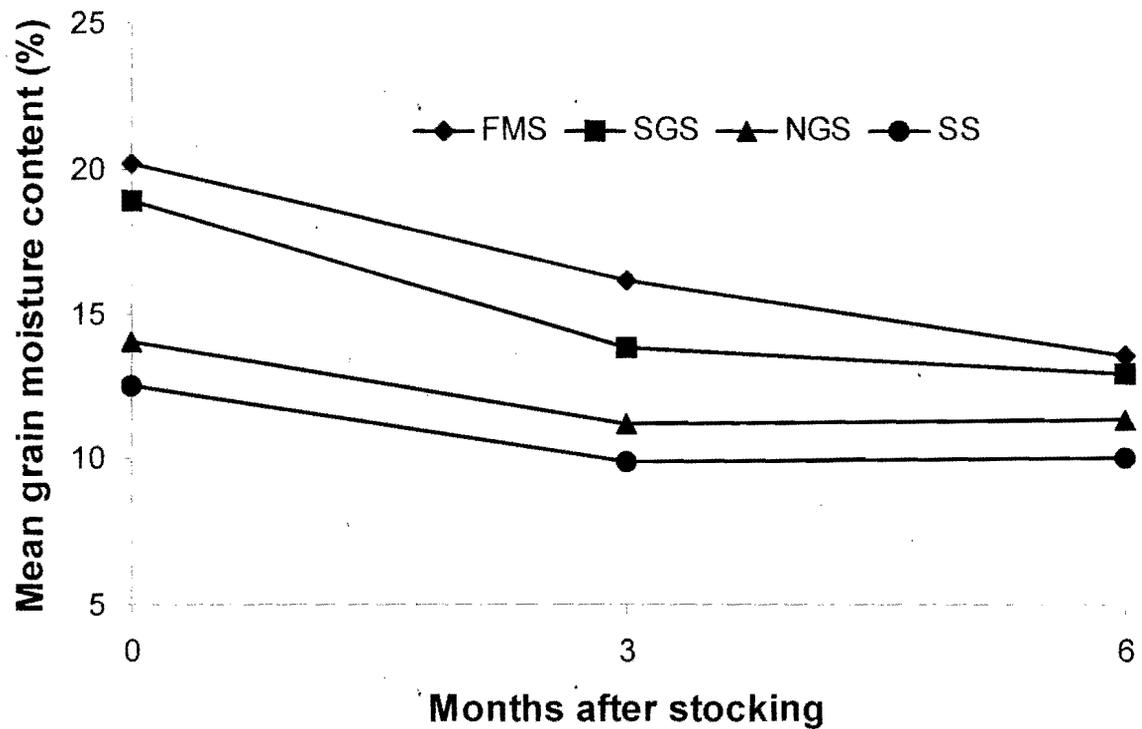


Fig 3 Mean percentage of grain moisture content over 6-month storage period in different agroecological zones of Benin in 1999/2000, 2000/2001 and 2002/2003

FMS = Forest Mosaic savannah, SGS = Southern Guinea Savannah, NGS = Northern Guinea Savannah, SS = Sudan Savannah

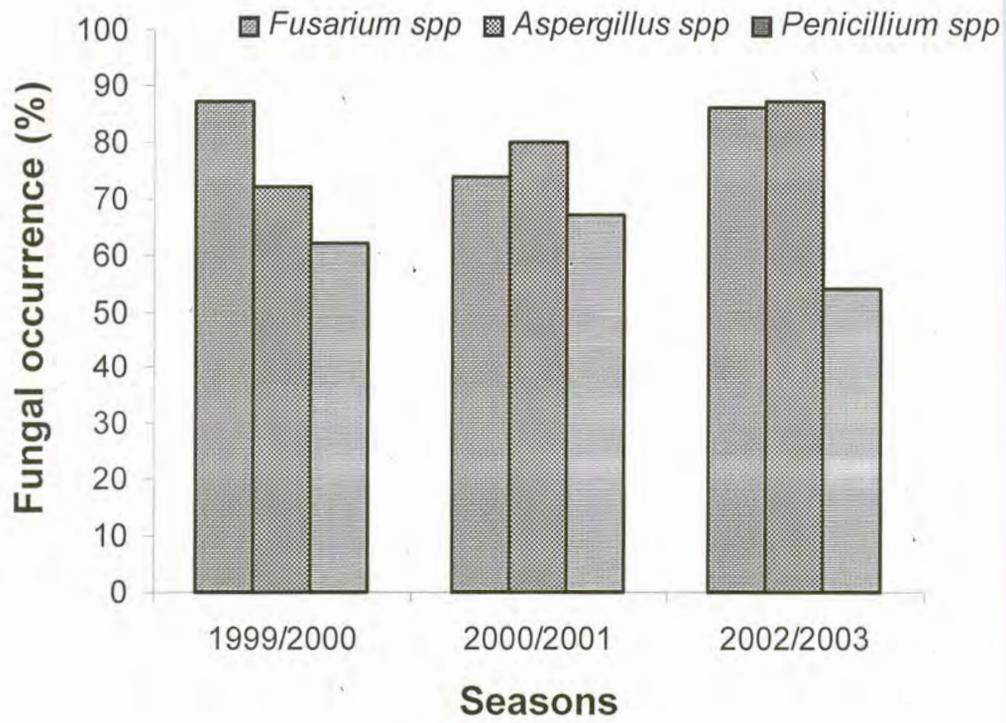


Fig 4: Fungal occurrence in Benin, in 1999/2000, 2000/2001 and 2002/2003

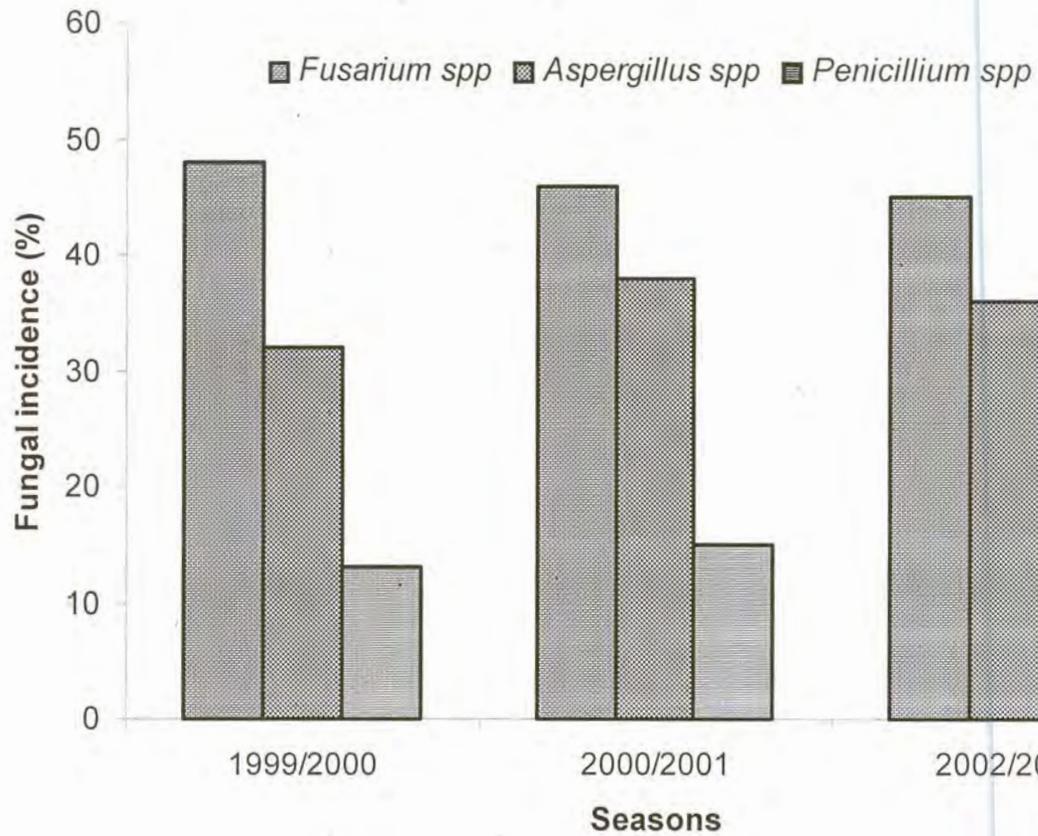


Fig 5: Incidence of fungal infection in four different agroecological zones of Benin in 1999/2000, 2000/2001 and 2002/2003



Fig 6: Atypical *F. verticillioides* isolate (MRC 8265). Reverse of culture on showing salmon-coloured colony with purplish concentric rings on PDA

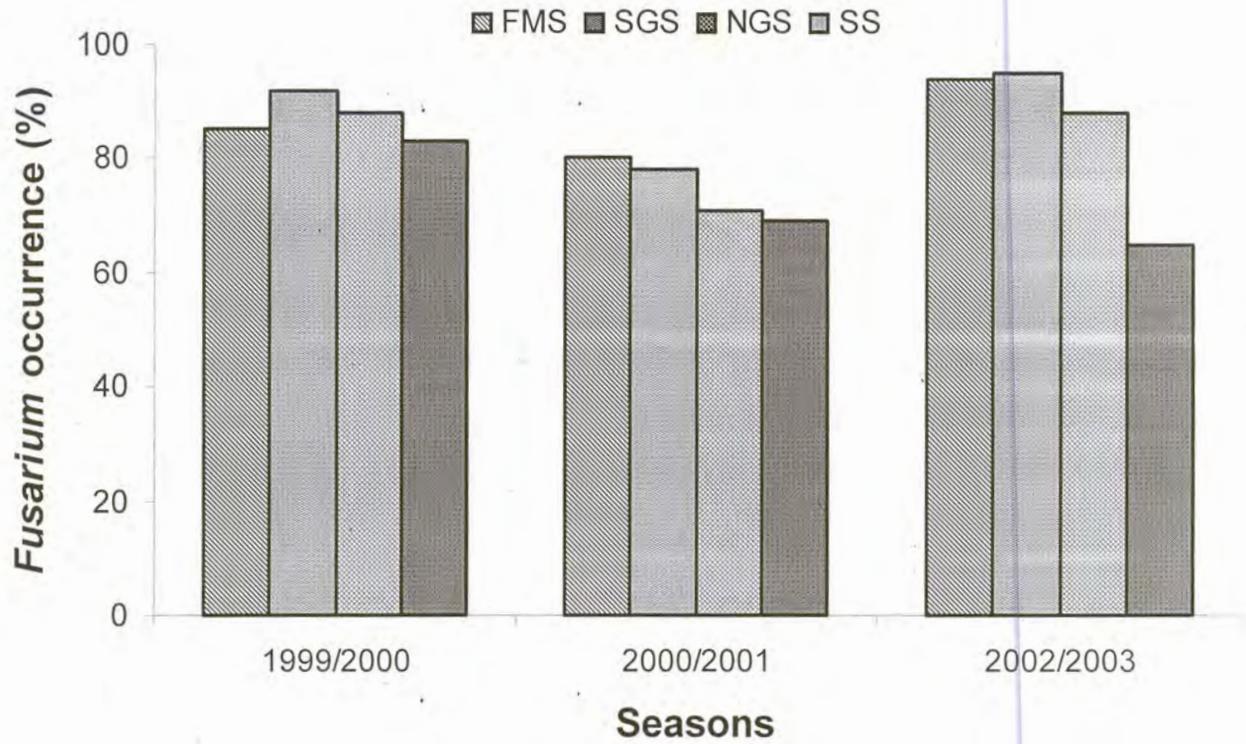


Fig 7: *Fusarium* occurrence in maize in four different agroecological zones of Benin during three seasons

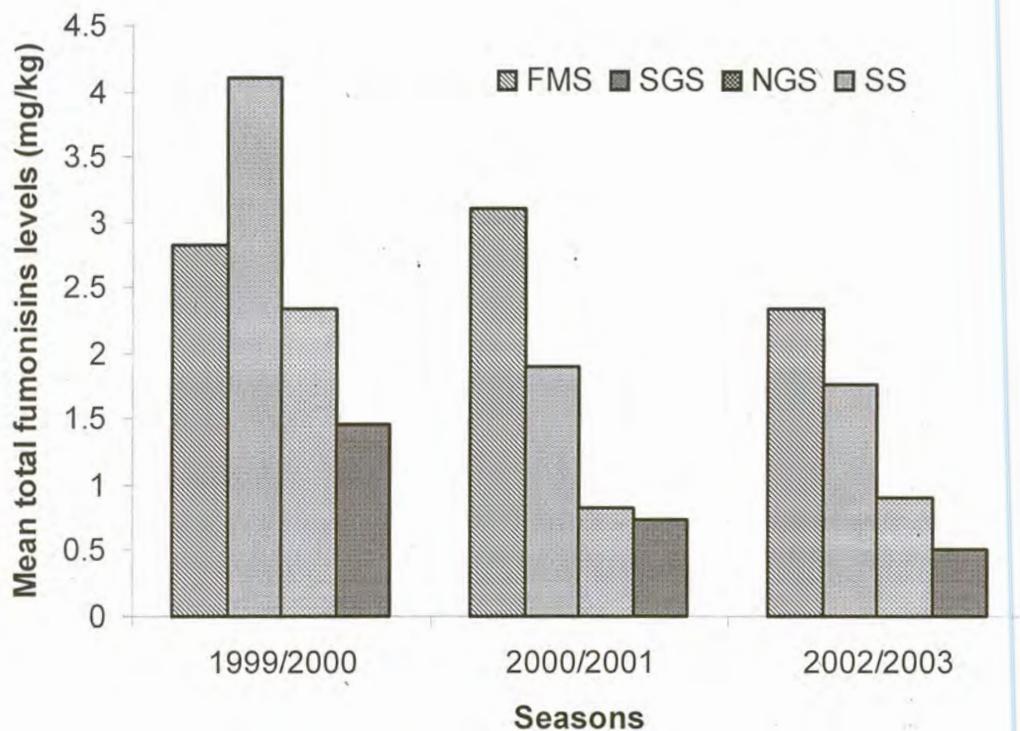


Fig 8: Mean total fumonisin level in maize in four different agroecological zones of Benin in 1999/2000, 2000/2001 and 2002/2003

FMS = Forest Mosaic savannah, SGS = Southern Guinea Savannah, NGS = Northern Guinea Savannah, SS = Sudan Savannah

Means of total fumonisin levels, except in the FMS, significantly varied in each zone from one season to another ( $p < 0.05$ ) (Students Newman Keuls).



## CHAPTER THREE

## IMPACT OF INDIGENOUS STORAGE SYSTEMS AND INSECT INFESTATION ON THE CONTAMINATION OF MAIZE WITH FUMONISINS

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## ABSTRACT

Four storage systems of maize commonly used by farmers in Benin, West Africa, were tested to determine their impact on infection of maize with *Fusarium* fungi and subsequent contamination with fumonisins. The study showed that *F. verticillioides* was the predominant *Fusarium* species found in all maize samples. *Fusarium* incidence was significantly higher when maize was stored on a cemented floor in a house ( $40.3 \pm 17.4$  %) than in the other systems. The lowest *Fusarium* incidence was recorded when maize was stored in a bamboo granary ( $25.5 \pm 13.5$  %) ( $p < 0.05$ ). This suggests that storage systems used by farmers may affect infection of maize with *Fusarium*, if these systems create conditions favourable to fungal growth. The storage systems did not have a significant effect on contamination by fumonisin, total fumonisin level being not significantly different from one system to another ( $p > 0.05$ ). This indicates that a high *Fusarium* infection level in maize in a storage structure during the storage period may not necessarily result in a high level of contamination by fumonisin. Fumonisin content significantly decreased over the storage period depending on storage systems. A 35 % decrease in fumonisin level over the storage period was observed in maize stored on the cemented floor in a house, 41 % in that stored on a platform, 57 % in maize stored in a mud silo, and 76 % in maize stored in a bamboo granary. Damage by lepidopterous pests was significantly and positively correlated with both infection of maize with *Fusarium* ( $r = 0.8$ ,  $p < 0.01$ ) and contamination by fumonisin ( $r = 0.9$ ,  $p < 0.01$ ). In general, the number and damage of coleopterous insects were significantly and negatively correlated with infection of maize with *Fusarium* and contamination by fumonisin, but one of them, *Sitophilus* spp., was found positively but not significantly related to *Fusarium* during the first month of storage ( $r = 0.2$ ,  $p > 0.05$ ).

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**Key words:** Benin, maize, *Fusarium*, fumonisins, insect infestation and storage systems,

## INTRODUCTION

In Benin like in most Sub-Sahara African countries, maize is generally harvested late and is stored in cob form in wooden granaries, under the roofs of the farmers' houses, or on cemented floors in houses (Fiagan 1994, Hell *et al.* 2000a). Maize is also stored in grain form in clay containers, mud silos, or in bags. Most of these systems create inadequate storage conditions unfavourable for good drying of maize, particularly in humid and semi-humid zones. They consequently promote the development of fungi and subsequent production of mycotoxins. There are two important mycotoxigenic fungi mostly found associated with stored maize. These are *Aspergillus flavus* that produces aflatoxins (Hell *et al.* 1995, Udoh *et al.* 2000) and *Fusarium verticillioides*, which produces fumonisins (Marasas *et al.* 1979).

Fumonisin is a recently discovered mycotoxin (Gelderblom *et al.* 1988). They cause fatal diseases in horses and swine, possess cancer-promoting activity in rats, and are associated with porcine pulmonary oedema (Nelson *et al.* 1994). Oesophageal cancer in humans has been related to consumption of maize with high concentrations of fumonisins (Marasas 1995, Wang *et al.* 2000). The International Agency for Research on Cancer (IARC) has evaluated fumonisin B<sub>1</sub> (FB<sub>1</sub>) as possibly carcinogenic to humans, belonging to the group 2B carcinogens (IARC 2002). The US Food and Drug Administration (FDA) had recommended that the fumonisin levels in human foods not exceed 4 mg kg<sup>-1</sup> (FDA 2000), whereas Switzerland proposed 1 mg kg<sup>-1</sup> as tolerance level for fumonisins in dry maize products intended for human consumption (Marasas *et al.* 2001).

It is likely that storage systems of maize with components such as time of harvest, type of storage structure, hygiene and insect infestation, interact and influence fungal infection and mycotoxin contamination. Hell *et al.* (2000a) for instance, found higher aflatoxin levels when maize was stored under or on top of the roof of farmers' houses, than in ventilated granaries. In Nigeria, Udoh *et al.* (2000) showed that insect infestation in maize stores was correlated with aflatoxins and that higher levels were found in maize stored in mud silos. Insects present in maize stores can act as wounding or spreading agents, providing an opportunity for the fungus to circumvent the natural protection of the integument and establish infection sites in vulnerable interior (Bilgrami and Choudhary 1998, Dowd 1998).

Regarding the infection with *Fusarium* and the contamination by fumonisin, a survey conducted in 2000 in Benin showed high levels of fumonisins in villages where maize cobs were stored under the roofs of farmers' houses and on the cemented floors in houses. Storage conditions were suspected to be linked to these elevated fumonisin levels (Gnonlonfin 2000).

The present study was undertaken to further consider the influence of different storage systems used in Benin on the natural occurrence of *Fusarium* and fumonisin contamination in maize. In addition, the effect of insect damage on maize infection by *Fusarium* and contamination by fumonisins was evaluated.

## **MATERIAL AND METHODS**

### ***Research site***

The experiment was conducted from August 2001 to April 2002 in Benin, West Africa, precisely in Yè, a village where a high fumonisin level (12 mg kg<sup>-1</sup>) was recorded during the national survey of 2000 (Gnonlonfin 2000). This village is situated in the Southern Guinean Savannah, a region that has been reported to have high aflatoxin contamination (Setamou *et al.* 1997, Hell *et al.* 2000b, Udoh *et al.* 2000). The annual rainfall pattern of this region is bimodal with precipitation averaging 1100 – 1500 mm, allowing for two maize growing seasons. The first and main growing season runs from April to July, whereas the second and shorter season is from September to November. Maximum temperatures range from 26 to 35°C and annual relative humidity averages 85 – 90 %.

### ***Maize cultivar***

The 90-day cultivar DMR-ESR-W, an improved IITA variety, was used. It is the improved variety most commonly recommended to farmers in Benin. DMR is resistant to downy mildew (*Peronosclerospora sorghi*) and to maize streak virus (Schulthess *et al.* 2002). Maize cobs were harvested in August according to the farmers' practice of harvesting late in order to obtain grain moisture content less than 20 %.

### ***Experimental design***

The experiment compared the development of *Fusarium* in maize during storage and contamination by fumonisins in improved and traditional storage systems. Four storage systems were assayed and there were three replicates for each system, arranged in a randomised block design. The storage systems included:

- Maize cobs with husks, harvested late (about one month after maturity), were stored for 8 months on the cemented floor in a house. This is the traditional system commonly used in the experimental area.
- Maize cobs with husks were stored for 8 months in an aerated woven bamboo granary (Fig 1).
- Maize cobs with husks were stored for 8 months on a platform (Fig 2).
- Maize cobs with husks were stored for 4 months on a platform, shelled, and grains stored, after hand sorting, in a mud silo for 4 months (Fig 3).

The latter three systems are indigenous storage systems improved by research and recommended to farmers. The storage structures (bamboo granary, platform and mud silo) were installed outside, each covered with a thatched roof. There was no insect control, but the structures were not accessible to rodents. Each wooden pole supporting the bamboo granary and platform had a simple but effective device to exclude rodents (Fig 1 and 2).

### ***Sampling method***

Fifty cobs were randomly sampled at 0, 1, 4, 5, 7 and 8 months after stocking in each storage system. The cobs were collected in each granary at top (10 cobs), in centre and on sides of the granary (30 cobs) and at bottom (10 cobs). The cobs were dehusked and manually shelled. Thereafter, a sub-sample of 1 kg of grains was taken from each sample. This sub-sample was then equally divided into two lots of 500 g. The first, unground, was intended for moisture, insect and fungal evaluations, whereas the second, ground, was intended for fumonisin analyses (Fig 4).

### ***Laboratory analyses***

Grain moisture content was determined on-farm just after sampling using an electronic moisture meter (model HOH-EXPRESS HE 50, PFEUFFER, Germany). Determination of grain moisture content was replicated three times. For insect evaluations, samples were sieved. Insects present in each sample were counted and identified in the laboratory using keys from NRI (1991) and Weidner and Rack (1984). Damage caused to grains by

lepidopterous and coleopterous insects were separately assessed on the basis of a 1000-grain sub-sample (Setamou *et al.* 1998), using the Pantenius formula as follows (Pantenius 1988):

$$\% \text{ of insect damage} = \frac{B}{A} \times 100$$

A = total number of grains in a sub-sample. In the present experiment, A = 1000

B = number of damaged grains by insects in the sub-sample

Twenty-five grains from each sample in four replicates (100 grains) were surface disinfested in a 10 % sodium hypochlorite solution for 2 min and rinsed twice in distilled water. The grains were plated in Petri dishes containing Potato Dextrose Agar (PDA) with five grains per Petri dish. The Petri dishes with grains were incubated at 25 °C with a 12:12-hour light and dark regime. After seven days of incubation, fungi were identified. *Fusarium* were isolated, transferred onto Carnation Leaf Agar (CLA) and incubated at 25 °C for seven days with a 12:12-hour light and dark regime. *Fusarium* species were identified using keys from Nelson *et al.* (1983) and Pitt and Hocking (1999). Total fumonisin content was determined at 0, 4 and 8 months after stocking using the VICAM method (VICAM 1998).

### ***Statistical analyses***

Statistical analyses were performed using SPSS for Window version 10.0 (SPSS Inc., Chicago, Illinois). Analysis of variance (ANOVA) and Tukey's HSD test were used to compare the means of fungal incidence and total fumonisins detected in each storage system and throughout the storage period. Pearson correlation test was used to assess relationships among *Fusarium* incidence, fumonisin level and damage by lepidopterous and coleopterous insects. The same test was used to assess the relationship between *Aspergillus* incidence and insect damage. Before analyses, insect numbers were transformed to log (x+ 1), but the data are presented untransformed.

## RESULTS

### *Fungal incidence, grain moisture content and fumonisin levels in the different storage systems*

The incidence of fungi in maize subjected to the different storage systems is presented in Table 1. The major genera found were *Fusarium* spp. (32 %), *Aspergillus* spp. (34 %) and *Penicillium* spp. (27 %). *F. verticillioides* was found in all samples. Total fungal incidence was significantly higher in maize stored on the cemented floor in a house than in the other storage systems ( $p < 0.05$ ). *Fusarium* incidence was also significantly higher when maize was stored on the cemented floor in a house ( $40.3 \pm 17.4$  %) and lower in maize stored in the bamboo granary ( $25.5 \pm 13.5$  %) ( $p < 0.05$ ). There was a significant decrease in *Fusarium* incidence throughout the storage period, from 48.2 % at harvest to  $11.7 \pm 3.4$  % after eight months of storage ( $p < 0.01$ ) (Fig 5). However, this trend was much lower in maize stored on the cemented floor in a house, than in the other storage systems. When maize was stored on the floor in a house, a noticeable increase of *Fusarium* incidence was observed during the first month of storage, whereas in the other systems, it steadily decreased (Fig 6). In contrast to *Fusarium*, *Aspergillus* incidence increased from 5.3 % at harvest to  $45.7 \pm 3.4$  % after eight months of storage ( $p < 0.01$ ), and this trend was markedly greater in maize stored on the floor in a house (Fig 7).

In maize stored in a mud silo following four months on a platform, there was an apparent decrease of *Fusarium* incidence from the fourth to the fifth month after storage, from  $32.1 \pm 3.1$  % to  $27.0 \pm 0.6$  % (Fig 6). This decrease, probably due to sorting after shelling the maize cobs at 4 months of storage, was not statistically significant ( $p > 0.05$ ).

There was no significant difference in maize grain moisture content (Fig 8) measured for the different storage systems ( $p > 0.05$ ). It was, however, found that at eight months of storage, overall mean of grain moisture content was about 18% in the maize stored on the cemented floor in a house and 15 % in the other storage systems. On the other hand, grain moisture content significantly decreased during the storage period in the different storage systems. However, it remained numerically but not significantly higher in maize stored on the floor in a house (Fig 8). Grain moisture content increased in all the storage systems from the fourth month of storage (Fig 8), coinciding with the beginning of the rainy season in the

experimental area. It is likely that the increase of the relative humidity during this rainy period induced the increase of the grain moisture content.

All maize samples from the different storage systems contained fumonisins with levels ranging from 0.6 to 2.3 mg kg<sup>-1</sup>, and all means higher than 0.1 mg kg<sup>-1</sup> (Table 2). There were no significant differences between the contamination levels in the storage systems ( $p > 0.05$ ), although the total fumonisin level was slightly higher when maize was stored on the floor in a house ( $1.9 \pm 0.3$  mg kg<sup>-1</sup>) and lower in maize stored in a bamboo granary ( $1.2 \pm 0.8$  mg kg<sup>-1</sup>) (Table 2). Overall, total fumonisin level was the highest in freshly harvested maize (2.3 mg kg<sup>-1</sup>), and significantly decreased during the storage period ( $p < 0.01$ ) (Fig 9). This decrease was, however, lower in maize stored on the cemented floor in a house than in the other storage systems (Fig 9). There was a 35 % decrease in fumonisin level in maize stored on the cemented floor in a house, 41 % in that stored on a platform, 57 % in maize stored in a mud silo, and 76 % in maize stored in a bamboo granary. A significant difference was found among the storage systems with respect to the percentage of decrease ( $p < 0.01$ ).

#### ***Influence of insect infestation on Fusarium infection and fumonisin contamination***

The borer *Mussidia nigrivenella* Ragonot (Lepidoptera: Pyralidae) and to a lesser extent the cob feeder *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) were the lepidopterous insects encountered on maize cobs in all storage systems. They were not very numerous but were most frequently found at harvest and during the first month of storage (Table 3). For coleoptera, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae), *Cathartus quadricollis* Guerin (Coleoptera: Cucujidae), *Tribolium* sp. and *Carpophilus* sp. were the species mostly found. They were predominant from the fourth month of storage (Table 3). *P. truncatus* was most common in the bamboo granary (318.1) and in maize stored on the platform (54.5) (data not shown).

Significant positive correlations were found between the number of lepidopterous insects and *Fusarium* infection (Table 4). *M. nigrivenella* ( $r = 0.7$ ,  $p < 0.01$ ) and *C. leucotreta* ( $r = 0.4$ ,  $p < 0.01$ ) were significantly related to *Fusarium* infection. Moreover, they were positively and significantly correlated with fumonisin contamination ( $r = 0.8$ ,  $p < 0.01$ ) and ( $r = 0.6$ ,  $p < 0.01$ ) for *M. nigrivenella* and *C. leucotreta*, respectively. There was also a significant and positive relationship between the lepidopterous insect damage and both *Fusarium* infection ( $r = 0.8$ ,  $p < 0.01$ ) and fumonisin contamination ( $r = 0.9$ ,  $p < 0.01$ ) (Table

4). Regarding the coleopterous insects, their number and damage was significantly and negatively correlated with *Fusarium* infection and fumonisin contamination (Table 4). Conversely, the lepidopterous insects *M. nigrivenella* ( $r = -0.8$ ,  $p < 0.01$ ) and *C. leucotreta* ( $r = -0.6$ ,  $p < 0.01$ ) were significantly and negatively related to *Aspergillus* infection. As for the coleopterous insects, with exception to *P. truncatus*, a positive and significant relationship existed between them and *Aspergillus* infection (Table 4).

## DISCUSSION

The results have shown that storing maize on a cemented floor in a house is not an appropriate practice. This system appears to be more favourable for fungal development, compared to the other tested systems. Grain moisture content was high (18 %) and *Fusarium* incidence was also somewhat higher under these conditions. Generally, storage conditions in farmers' houses are confined and maize cobs are usually stored in a maizeer of the house, which is not always well ventilated. Throughout the storage period, grain moisture content, therefore, diminishes slowly but remains high enough to promote fungal development. In a recent study in Benin, Hell *et al.* (2000a) found that storing maize in non-ventilated conditions such as under the roof of the farmer's house has a higher risk of aflatoxin development. The authors related these findings to the fact that this storage system is used in the humid zones of Benin, where the rainfall pattern is bimodal with averages ranging from 1200 mm to 1500 mm, the mean relative humidity of air mostly around 90 %, and part of the harvest occurring during a rainy period. Pitt and Höcking (1999) reported that high humidities are likely to create suitable conditions for fungal development. This is linked to the fact that during storage, *Fusarium* requires a substrate with high moisture content ranging from 18 to 23 % for optimal growth (Christensen and Kaufmann 1974, Bacon and Nelson 1994, Orsi *et al.* 2000).

The woven bamboo granary and platform are typically ventilated storage structures. They are potentially less favourable for fungal growth and results obtained in the present study show them to be less conducive to *Fusarium* development during maize storage. Hell *et al.* (2000a) also found bamboo granaries to be associated with lower aflatoxin levels in the humid regions. It is thought that this structure allows maize grains to dry more rapidly from 20 % of moisture content at harvest to 14 % after three months of storage, provided that its diameter is not more than 2 m (FAO 1992).

Mud silos are durable storage structures better adapted to dry regions (Fandohan 2000). They are commonly used in the northern parts of West-African countries, where

environmental conditions are less humid. Mud silos are closed and non-ventilated granaries. Therefore, they are considered as favourable for fungal development, if maize grains moisture content is above 15 % or if maize storage occurs during the rainy season, where the relative humidity of air is higher than 90 % (Fandohan 2000). Prasad *et al.* (1987) found mud silos in India associated with high aflatoxin contamination. Hell *et al.* (2000a) postulated that humidity build-up might occur through convection, permitting *Aspergillus* spores to persist for a long time in a granary leading to a high risk of aflatoxin contamination. To overcome this risk, maize must be sufficiently dried (moisture content less than 15 %) to ensure unfavourable conditions for fungal development. In this study, the prestorage of maize on a platform before its final storage in a mud silo reduced grain moisture content from 22.5 % at harvest to less than 11 % after four months, hence creating unfavourable conditions for the development of fungi.

The overall changes observed in the incidence of both *Fusarium* (a significant decrease) and *Aspergillus* (a significant increase) with the storage time is probably due to storage conditions, unfavourable to *Fusarium* development and favourable to that of *Aspergillus*. Overall grain moisture content decreased during the storage period, falling below 18 % in all storage systems before the third month of storage. Marasas and Smalley (1972) found the same changes in the incidence of these fungi in mouldy maize meal, stored for three weeks. In contrast, Ngoko *et al.* (2001) found a significant increase of the incidence of both *Fusarium* spp. and *Aspergillus* spp. in maize samples collected from humid zones of Cameroon. This indicates that fungal development in stored maize depends on many factors including the storage conditions (grain moisture content, temperature during storage, insect infestation, type of storage structure used), and climatic conditions (relative humidity, temperature).

Although the storage systems tested in this study significantly influenced *Fusarium* development in stored maize, they did not have a significant effect on fumonisin contamination. Previous studies on aflatoxins, however, found that storage systems significantly influenced aflatoxin contamination in maize (Hell *et al.* 2000a, Udoh *et al.* 2000). Whereas aflatoxins are potentially serious problems during storage, fumonisin production primarily occurs in the field before harvest as *Fusarium* species do not grow in maize at less than 18 % moisture (Munkvold and Desjardins 1997, Doyle 1998, Riley and Norred 1999, Bolger *et al.* 2001). Consequently, Pittet (1998) found that the highest concentrations of aflatoxins are associated with the postharvest growth of *Aspergillus* moulds on poorly stored products. It is likely that in the current study, storage conditions in all systems were reasonably sound and that moisture content did not reach levels conducive for

the production of fumonisins. According to Munkvold and Desjardins (1997), there is strong evidence that proper storage systems with grain moisture content maintained at 13 – 14 % would normally prevent the production of fumonisins in stored maize. But, in tropical regions, which are known for their high mean temperatures and high relative humidities in many regions, ideal storage conditions are almost impossible to achieve (Vanek and Hoberg 1992).

The decrease in fumonisin content in maize observed during storage is reassuring with respect to the stability of the toxin in contaminated stored grains. In a recent 3-year survey study in Benin, similar results were found (Chapter 2). Instability of fumonisins was also found in previous studies in naturally or artificially contaminated food products over time. Scott *et al.* (1999) found fumonisins unstable in naturally contaminated ground rough rice, maize starch and maize meal over storage time. Orsi *et al.* (2000), also found an overall decrease of fumonisin content in stored maize after 140 days of a one year-storage period in Brazil. More recently, Kim *et al.* (2002) observed FB<sub>1</sub> and FB<sub>2</sub> to disappear completely in artificially contaminated Thai white rice flour after ten hours. These authors found up to 75 % and 90 % of decrease respectively in maize meal and in the flour of another type of white rice produced in the United Kingdom, after two months of storage. About 30 % decrease of total fumonisin B was also observed in maize cultures of *F. verticillioides* kept at 4 °C over 13 - 20 years (Gelderblom *et al.* PROMEC, Medical Research Council, Tygerberg, South Africa, 2002, unpublished data). In contrast, Ngoko *et al.* (2001) found FB<sub>1</sub> to increase with storage time in maize collected in different zones of Cameroon.

No explanation has yet been found as to the fate of fumonisins in naturally contaminated food products over the storage time. However, some factors including environmental conditions, intrinsic characteristics of stored products and chemical reactions are suspected. Munkvold and Desjardins (1997) argued against the view that fumonisin concentration increases in maize stores during storage, as long as conditions of grain moisture content and temperature are maintained at recommended levels. It is also suggested that fumonisin molecules might bind with the starch of the product during storage to form a complex, which is not detectable (Kim *et al.* 2002). Scott *et al.* (1999) reported that reaction of FB<sub>1</sub> with reducing sugars such as D-glucose is likely to explain the rapid fumonisin loss observed in maize starch. Kim *et al.* (2002) suspected the moisture content of the product, its texture and metal ions present in the product to influence fumonisin loss. In the case of the present study, environmental conditions during storage are likely to have affected fumonisin content in maize. Dry season and rainy season alternated during the eight months of storage.

Moreover the type of storage system used might also play a role and the decrease in fumonisin content was higher in one than in another system.

It was always believed that fumonisins are stable molecules and that keeping contaminated products at very low temperatures or submitting them to  $\gamma$ -irradiation before storage preserves stability of the toxin in the products for a long time. Thus, Gelderblom *et al.* (2002) found that analytical standards of fumonisin FB<sub>1</sub> remained stable (94 % of recovery) when kept in airtight conditions at 4 °C during a ten-year storage period. Likewise, Visconti *et al.* (1996) found fumonisins to be stable in  $\gamma$ -irradiated maize for at least six months at 25 °C.

In this study, insects played an important role in *Fusarium* development. Miller (1995) reported that a strong relationship exists between insect damage and *Fusarium* grain rot. Indeed, injuries caused by insects are generally sites of fungal infection of maize (Munkvold and Desjardins 1997). Results of the present study show that damage caused by lepidopterous insects was significantly correlated with both *Fusarium* development and fumonisin contamination in maize (Table 4). Schulthess *et al.* (2002) reported similar results considering the effect of *F. verticillioides* on the infestation of maize by various insects. Depending on the feeding habits or preferences of the larvae, they can attack maize stems, cobs, silks or grains, spreading fungal inoculum within the plant during their movement and feeding (Dowd 1998). Studies showed that in Indiana, lepidopterous insect-damaged grains were colonised by *Fusarium* spp. up to 82 %, but only 0.03 % by *A. flavus* (Rambo 1974), and that cobworms (lepidopterous species not specified) collected from maize in Missouri, were infected by *Fusarium* spp. (63 %) and *A. flavus* (37 %) (Fennell *et al.* 1975). Dowd (1998) reported that holes created by lepidopterous larvae have the highest levels of colonisation by *Fusarium* spp.

*Mussidia nigrivenella* was the lepidopterous pest most often found on maize in the present study. Ako *et al.* (2003) found that many insects were associated with *Fusarium* in West Africa, including *M. nigrivenella*, which attacks maize, cotton and beans and causes maize yield losses ranging from 5 to 15 % (Setamou *et al.* 2000). According to Setamou *et al.* (1998), *M. nigrivenella* usually attacks maize cobs and damages grain from the tip of the cob. By boring a channel, the insect breaks the testa of grains, which constitutes a natural barrier for fungal growth, promoting easy spread of fungi. *M. nigrivenella* damage also predisposes maize to pre- and postharvest infestation with storage coleopterous insects. The latter preferentially enter the holes produced by the *M. nigrivenella* larvae, thus further enhancing in the dissemination of fungal inoculum (Sanford and Luckmann 1963, Setamou *et al.* 1998).

It would look surprising that in this study, whereas there was no significant correlation between *Fusarium* infection and fumonisin contamination, a positive correlation was observed between lepidopterous insect damage and fumonisin contamination. These two correlations would match if this study was conducted only in field. Fumonisin is more likely to be produced in field subsequently to *Fusarium* infection if the environmental conditions are favourable. Damage of lepidopterous insects also more often occurs in field, not during the storage period. In this work, *Fusarium* infection, fumonisin contamination as well as damage of lepidopterous insect were all evaluated after harvesting i.e. during the storage period. The positive correlation observed between damage of lepidopterous insects and fumonisin contamination is probably due to the fact both insect damage and fumonisin level were found to decrease throughout the storage period. This positive correlation would not be observed in other circumstances as fumonisin production is dependant of several factors including environmental conditions.

In the present study, the abundance of coleopterous insects increased only after four months of storage. Results indicated that these insects are less likely to be implicated in the infection process of maize with *Fusarium*. There is, however, ample evidence of their involvement in fungal infection in the field, and the sap beetles, *Carpophilus* spp. are the insects best known to spread *Fusarium* in maize (Dowd 1998). Fennell *et al.* (1975) found that amongst sap beetles collected in maize fields in Missouri, 7 % were contaminated with *A. flavus* and 60 % with *Fusarium* spp. According to Dowd (1998), the implication of the sap beetles in vectoring *Fusarium* spp., as is the case with the other insects, may vary from one area to another within a country. This also depends on many agronomic factors including husk coverage of maize cobs, silk channel, grain pericarp thickness, the abundance of beetles, maize variety and weather conditions (Dowd 1998). Moreover, because of their association with the damage caused by the lepidopterous pests, the presence of sap beetles may not be recognised when evaluated at harvest, unless great care is taken to look for characteristic feeding marks and the presence of frass (Dowd 1998). All this may explain the negative correlation found between *Carpophilus* spp. and *Fusarium* development in the present study.

*Sitophilus* spp. and *Cathartus* spp. were negatively and significantly correlated with *Fusarium* incidence throughout the storage period, but there was a non-significant positive relationship between *Sitophilus* spp. and *Fusarium* incidence during the first month of storage ( $r = 0.2$ ,  $p > 0.05$ ) (data not shown). In contrast, Cardwell *et al.* (2000) in a recent field study on artificially infected maize with *F. verticillioides* found significant and positive correlations between the number of these insects and the fungus. The authors artificially infected maize

cobs in the field whereas in the present study, maize cobs were left to be naturally infected and evaluated during the eight-month storage period.

Unlike with *Fusarium*, both number and damage by all coleopterous insects found in maize samples, with exception to *P. truncatus*, was positively and significantly correlated with *Aspergillus* incidence during the storage period. The coleopterous insects were predominant, their number and damage increased in the granaries during storage. Their activity is likely to promote *Aspergillus* development, which markedly increased during the storage period, in contrast to that of *Fusarium*. Riley and Norred (1999) reported that growth of *Aspergillus* species and subsequent aflatoxin production during storage are favoured, among other factors, by insect and rodent activity. Moreover, it has been found that coleopterous insects, such as the nitidulid beetles (*Carpophilus* for instance), vector *Aspergillus* spp. and easily feed on maize contaminated by *Aspergillus*, whereas the lepidopterous insects cannot. The former insects are resistant to aflatoxin, possessing enzymes capable of degrading aflatoxin, whereas the latter insects are sensitive to aflatoxin (Dowd 1992).

It can be concluded that maize storage systems used by farmers may influence *Fusarium* development and fumonisin contamination. This suggests that any system creating conditions favourable for fungal growth and fumonisin production is not recommended. High levels of *Fusarium* infection in maize in a granary may not necessarily result in fumonisin contamination. Fumonisin level decreases during storage, more in some storage systems. Insect damage, mainly that caused by lepidopterous insects occurring on maize in the field before harvest, is likely to facilitate infection of maize with *Fusarium*. This suggests that any action undertaken to reduce insect infestation before harvest and during storage could help to reduce *Fusarium* infection and subsequent fumonisin contamination. Some recommendations to reduce insect damage and resultant fungal contamination include using maize cultivars less susceptible to lepidopterous insects, harvesting without delay to avoid insect infestation in the field, sorting out damaged cobs or grains at harvest, where possible insect control, and choice of storage structure to ensure good drying.

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Table 1: Mean fungal incidence in maize in the different storage systems over all storage period

Storage systems	No. of samples	Infected maize grains (%)		
		<i>Fusarium</i>	<i>Aspergillus</i>	<i>Penicillium</i>
Storage in bamboo granary	18	25.5 ± 13.5 a	24.4 ± 11.0 a	19.9 ± 8.0 a
Storage on platform	18	31.1 ± 15.7 ab	31.6 ± 15.1 a	24.5 ± 10.1 a
Prestorage on platform + storage in mud silo	18	30.2 ± 15.8 ab	33.5 ± 16.3 ab	23.3 ± 9.7 a
Storage on cemented floor in house	18	40.5 ± 17.4 b	46.8 ± 23.6 b	40.5 ± 18.2 b
Total	72	31.8 ± 16.2	34.1 ± 18.6	27.1 ± 14.4

No. of samples: number of samples collected from each storage system

Values shown are the mean ( $\pm$  Standard Deviation) percentage of maize grains infected by the different fungi.

Means within a column followed by the same letter are not significantly different ( $p > 0.05$ )

Table 2: Mean total fumonisin level in maize samples in different storage systems over all storage period

Storage systems	No. of samples	Total fumonisin level (mg kg <sup>-1</sup> )	
		<i>Range</i>	<i>Mean</i>
Storage in bamboo granary	9	0.6 – 2.3	1.2 ± 0.8
Storage on platform	9	1 – 2.3	1.5 ± 0.6
Prestorage on platform + storage in mud silo	9	1 – 2.3	1.5 ± 0.6
Storage on cemented floor in a house	9	1.5 – 2.3	1.9 ± 0.3
Total	36	0.6 – 2.3	1.6 ± 0.6

No. of samples: number of samples collected from each storage system

Values shown are the range and the mean (± Standard Deviation) total fumonisin level



Table 3: Mean number of insects in maize (per 1 000 grains sample) during the storage period

Months after stocking	<i>Mussidia</i>	<i>Cryptophlebia</i>	<i>Sitophilus</i>	<i>Prostephanus</i>	<i>Tribolium</i>	<i>Carpophilus</i>	<i>Cathartus</i>
0	12.7	2.0	4.0	0	0.7	5.3	8.7
1	2.5	0.4	18.6	0	0.3	5.7	46.8
4	0	0	295.8	32.5	33.1	21.3	285.7
5	0	0	671.8	143.3	107.6	18.3	473.0
7	0	0	641.1	253.8	110.7	16.4	710.4
8	0	0	570.7	134.2	100.7	23.9	633.4
Overall	2.5	0.4	367.0	94.0	58.8	15.2	359.7

Table 4: Correlation among insect infestation, fungal incidences and fumonisin contamination in maize

	Lepidopterous insects			Coleopterous insects					Grain damage (%)
	No <i>M. nigrivenella</i>	No <i>C. leucotreta</i>	Grain damage (%)	No <i>Sitophilus</i>	No <i>P. truncatus</i>	No <i>Tribolium</i>	No <i>Cathartus</i>	No <i>Carpophilus</i>	
<i>Fusarium</i> incidence	+ 0.653 **	+ 0.438 **	+ 0.802 **	- 0.611 **	- 0.642 **	- 0.573 **	- 0.339 **	- 0.463 **	- 0.645 **
Fumonisin level in maize	+ 0.834 **	+ 0.631 **	+ 0.852 **	- 0.792 **	- 0.671 **	- 0.696 **	- 0.505 **	- 0.641 **	- 0.581 **
<i>Aspergillus</i> incidence	- 0.823 **	- 0.594 **	- 0.517 **	+ 0.754 **	+ 0.095	+ 0.701 **	+ 0.533 **	+ 0.349 **	+ 0.557 **

\*\* Correlation is significant at the 0.01 level (2-tailed)



Figure 1: Bamboo granary used in South-Benin for storing maize cobs



Figure 2: Platform used in Benin for storing maize cobs



Figure 3: Mud silo used in Benin for storing maize grains

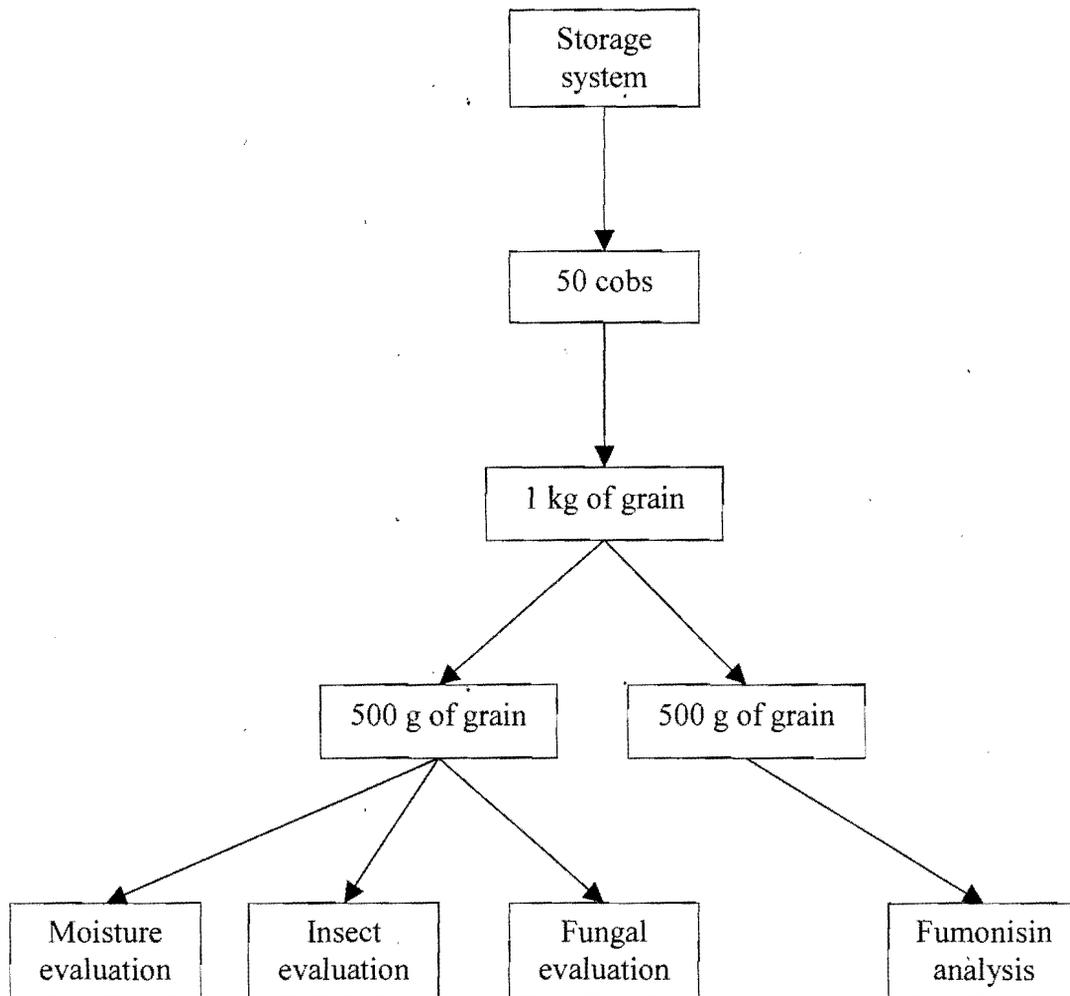


Figure 4: Diagram summarising the sampling method used during this study

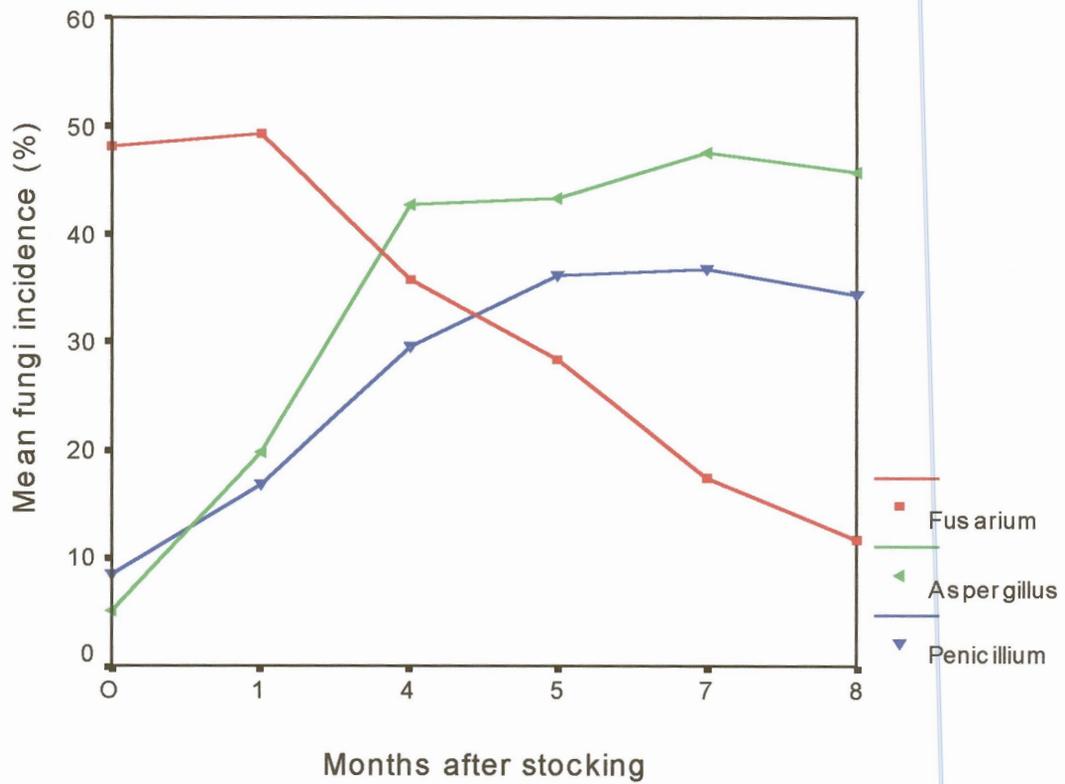


Figure 5: Changes in the mean incidences of fungi in maize during the storage period

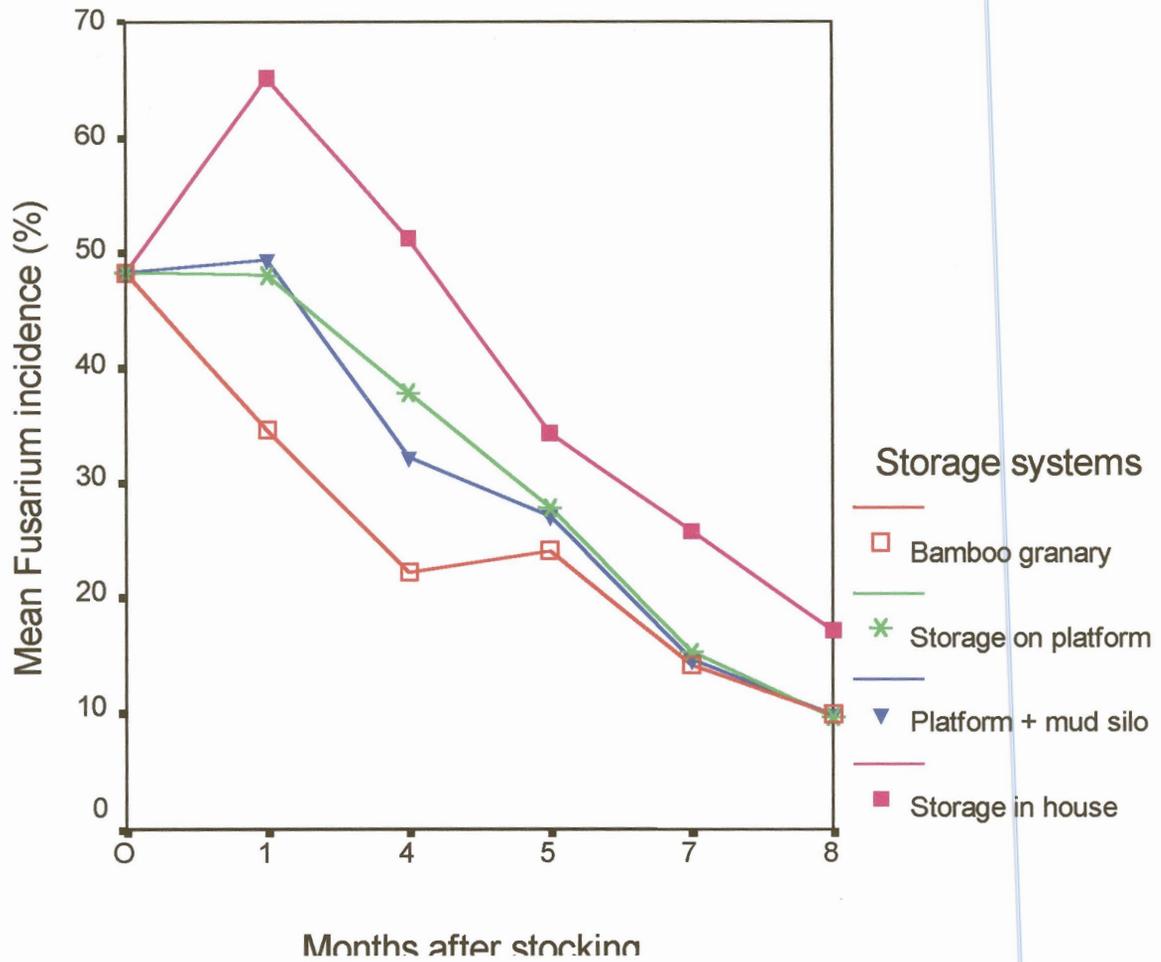


Figure 6: Changes in *Fusarium* incidences in different storage systems during the storage period

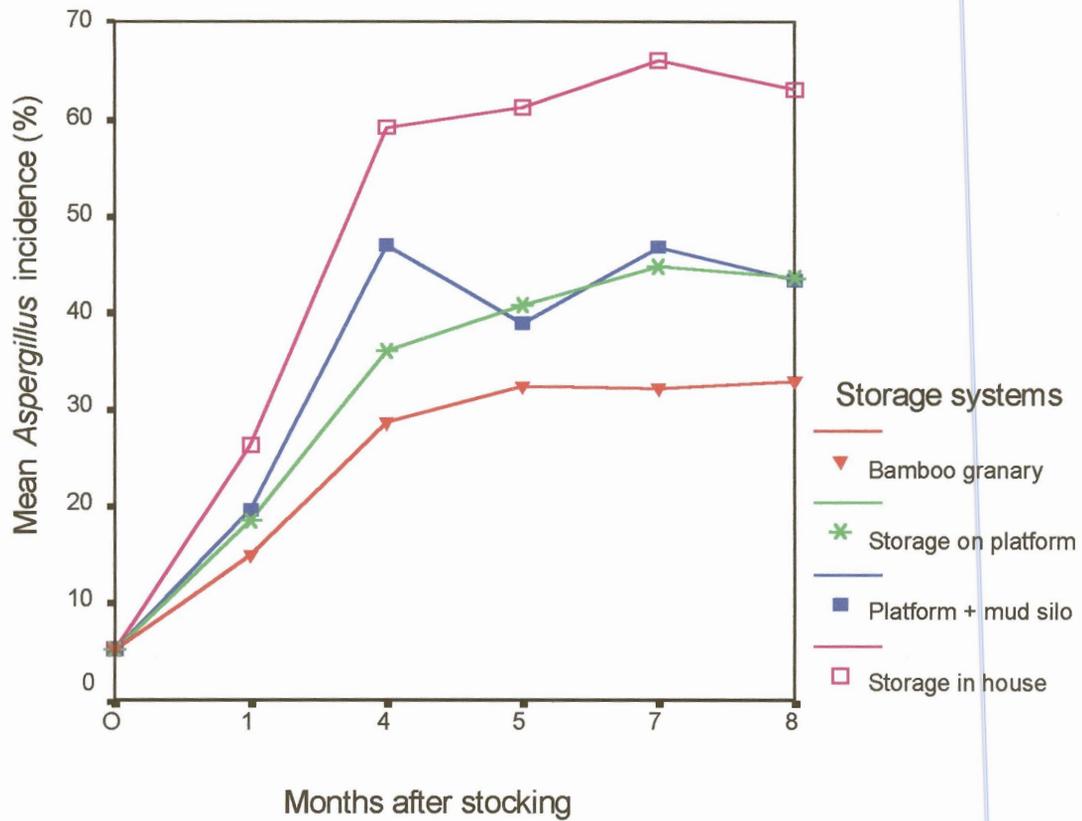


Figure 7: Changes in *Aspergillus* incidences in different storage systems during the storage period

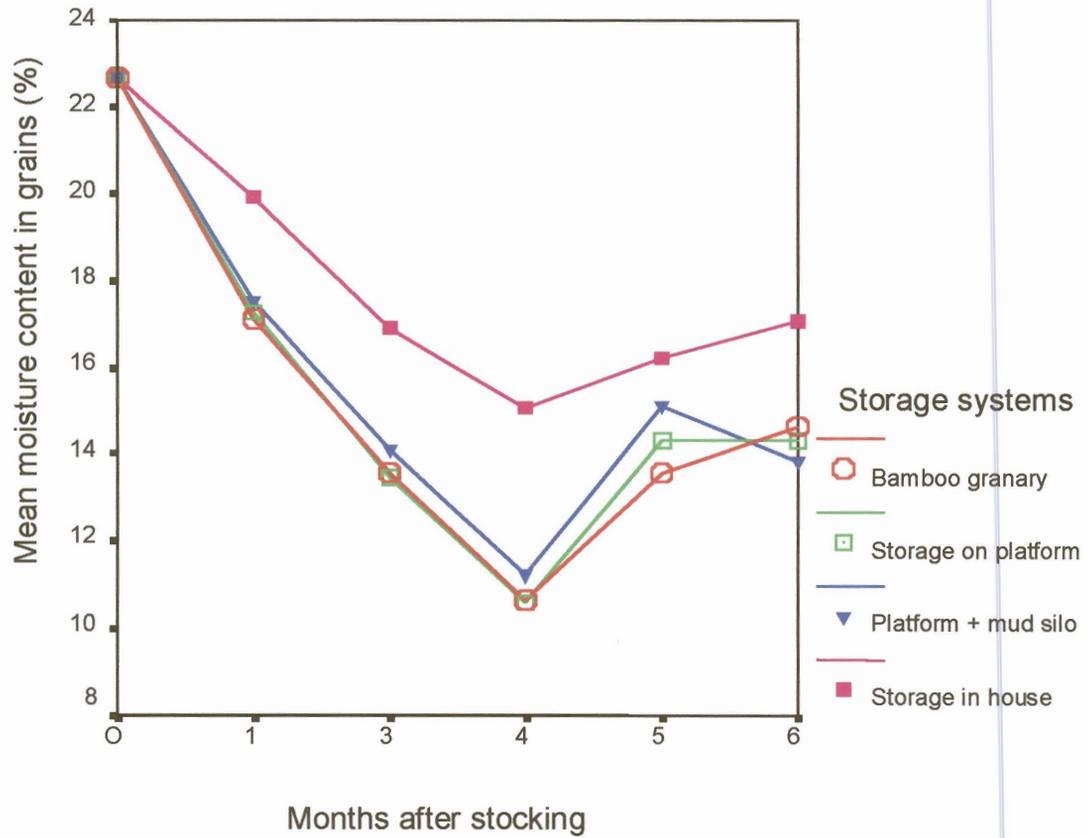


Figure 8: Changes in grain moisture content in different storage systems during the storage period

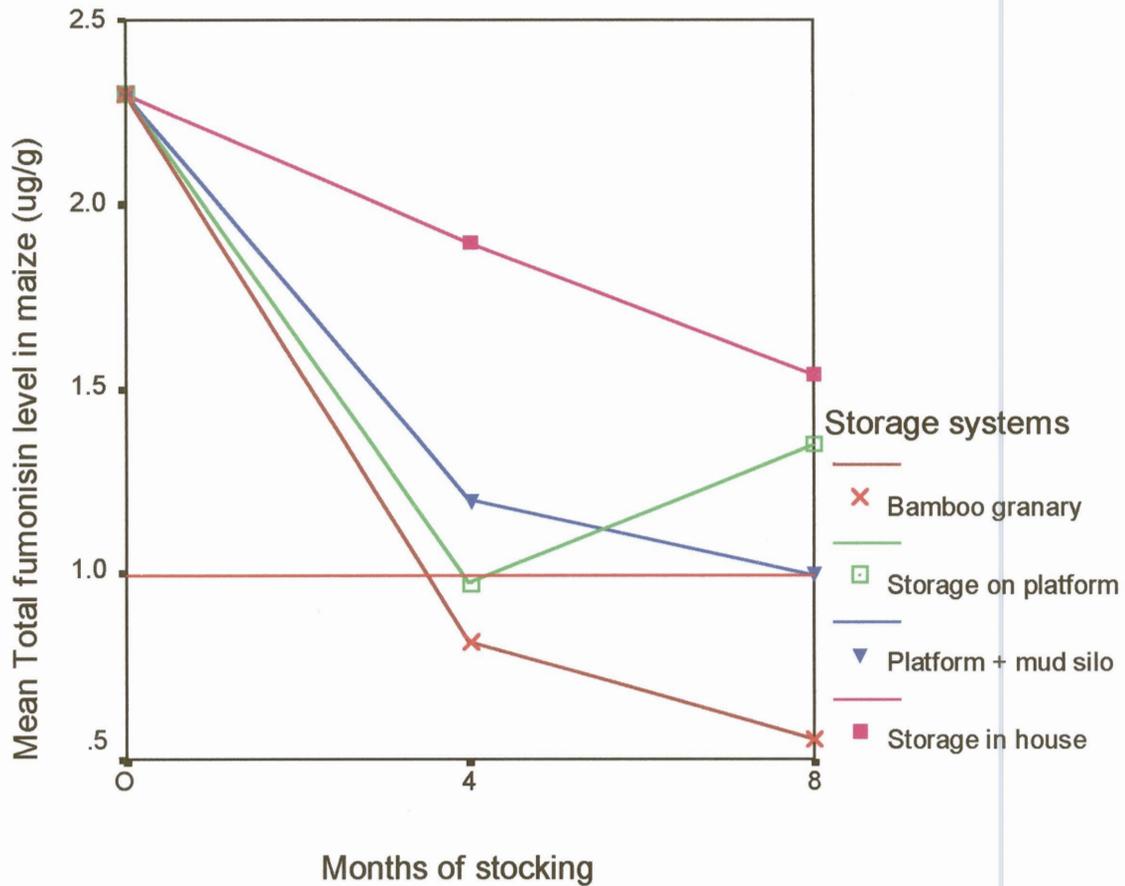


Figure 9: Changes in total fumonisin level in maize in different storage systems during the storage period

Line at  $1.0 \text{ mg kg}^{-1}$  is the maximum tolerated fumonisin level accepted in Switzerland.



## CHAPTER FOUR

**FATE OF AFLATOXINS AND FUMONISINS DURING THE PROCESSING OF  
MAIZE INTO FOOD PRODUCTS IN BENIN**

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## ABSTRACT

The fate of aflatoxins and fumonisins, two mycotoxins that co-occur in maize, was studied through the traditional processing of naturally contaminated maize in *mawe*, *makume*, *ogi*, *akassa* and *owo*, maize-based foods common in Benin, West Africa. Levels of total aflatoxin and fumonisin were measured at the main unit operations of processing and the unit operations that induce significant reduction of mycotoxin level were identified. Overall reduction of mycotoxin level was more significant during the preparation of *makume* (93 % reduction of aflatoxins, 87 % reduction of fumonisins) and *akassa* (92 % reduction of aflatoxins, 50 % reduction of fumonisins) than that of *owo* (40 % reduction of aflatoxins, 48 % reduction of fumonisins). Sorting, winnowing, washing, crushing combined with dehulling of maize grains were the unit operations that appeared very effective in achieving significant mycotoxin removal. Fermentation and cooking showed little effect. During the preparation of *ogi* and *akassa*, reduction of fumonisin levels measured in food matrix was lower (50 %) compared to *mawe* and *makume*, probably due to significant fumonisin release in *ogi* supernatant. Consequently, the use of *ogi* supernatant for preparing beverages or traditional herbal medicines could be harmful as it is likely to be contaminated with mycotoxin from the raw maize.

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**Keywords:** Maize, maize-based products, processing, aflatoxins, fumonisins, Benin.

## INTRODUCTION

Aflatoxins and fumonisins are metabolites respectively produced by toxigenic species of *Aspergillus* and *Fusarium*, of which *A. flavus* and *A. parasiticus* (Diener *et al.* 1987, Pittet 1998) and *F. verticillioides* and *F. proliferatum* (Gelderblom *et al.* 1988, Keller *et al.* 1997) are by far the most important. Attention is increasingly given to these mycotoxins for several reasons. They have been shown to be directly responsible for several animal diseases. Aflatoxins are found to be hepatotoxic and potent hepatocarcinogens in animals (Wogan 1968, Wogan 1992). Fumonisin have been shown to be the causative agents of leukoencephalomalacia in horses (Kellerman *et al.* 1990) and pulmonary oedema syndrome in pigs (Harrison *et al.* 1990).

Aflatoxins and fumonisins are known to be hazardous to the health of humans, in some cases directly causing illness and even death. Aflatoxins are implicated in liver cancer (IARC 1993, JECFA 1998, Wild and Hall 2000). Chao *et al.* (1991) reported an incident when aflatoxins present in a foodstuff consumed by people in Malaysia in 1988 were strongly implicated as the cause of death of 13 children. Aflatoxins have been reported to impair childhood growth in children from Benin and Togo (Gong *et al.* 2002). Fumonisin were reported to be associated with oesophageal cancer in rural areas in South Africa (Rheeder *et al.* 1992) and China (Chu and Li 1994), and liver cancer in China (Ueno *et al.* 1997). Consumption of mouldy sorghum and maize containing fumonisin B<sub>1</sub> has been associated with an outbreak of abdominal pain and diarrhoea in India (Bhat *et al.* 1997).

Another important reason to be concerned is that these mycotoxins occur worldwide in maize, either alone or together (IARC 1993, Chamberlain *et al.* 1993, Marasas 1996, Shephard *et al.* 1996, Marin *et al.* 1999, Kpodo *et al.* 2000, Gelderblom *et al.* 2002). Maize is a dietary staple food in many countries in the world (Thiel *et al.* 1996). In Africa, maize is the cereal with highest production and consumption (ISAAA 2002). Per capita daily consumption of maize averages more than 246 g in Benin (Hounhouigan *et al.* 1999), 342 g in Kenya (ECAMAW 2002) and 488 g in South Africa (CIMMYT 2002). Maize is consumed in different forms in the world. Nago *et al.* (1997) reviewed traditional African maize products and found various forms including porridges, pastes, dumplings, cakes, fritters and beverages.

Effects of processing on mycotoxin contamination in food products are increasingly being investigated throughout the world and this strategy is showing great promise for mycotoxin reduction. The use of physical methods including cleaning, separation of screenings, washing, aqueous extraction, dehulling and milling, have been shown to be

effective, to a certain extent, in reducing mycotoxins in cereals (Sydenham *et al.* 1994, Charmley and Prelusky 1994, Voss *et al.* 1996, Shetty and Bhat 1999). Aflatoxin and fumonisin levels in tortillas were found to be significantly reduced due to alkaline cooking (Guzman de Pena *et al.* 1995, Dombink-Kurtzman *et al.* 2000, Voss *et al.* 2001).

There is very little information concerning attempts to reduce mycotoxin contamination in maize using traditional processing methods in Africa, even though many maize-based food products exist. In Benin, about 40 different maize processing methods have been recorded (Nago 1997). The present research was carried out to determine the fate of both aflatoxins and fumonisins in naturally contaminated maize products and to identify operations that give a significant reduction in mycotoxin levels during processing.

Three traditional maize-based products commonly consumed in West Africa were used in this study. These were *mawe*, *ogi* and *owo*. *Mawe* is a solid state fermented dough used in Benin, Togo and Nigeria for cooking several dishes (Hounhouigan *et al.* 1993). One popular dish from *mawe* is *makume*, a thick paste consumed with stew. *Ogi* is a gruel obtained by fermentation of a suspension of wet-milled maize in water, processed into *akassa*, another thick paste and also consumed with stew (Hounhouigan 1994). It is also popular in West African countries. Steinkraus *et al.* (1983) reported a product similar to *ogi*, known as *uji* in Kenya. *Owo* is a non-fermented thick paste obtained from whole maize meal named *lifin*. It is a common and popular food in many African countries (Nago *et al.* 1997). A similar product to *owo* in South Africa is the stiff porridge (NIM. Somdyala, PROMEC, Tygerberg, South Africa, 2002, personal communication).

## MATERIALS AND METHODS

### *Origin of maize samples*

Maize, in three separate samples of 20 kg each collected from the same store, was obtained from the research station of Ina situated in the North of Benin. This was of the 90-day cultivar DMR-ESR-W, an improved IITA white variety. DMR-ESR-W is resistant to downy mildew (*Peronosclerospora sorghi*) and to maize streak virus (Schulthess *et al.* 2002).

### *Preparation of maize products*

Preparation of *mawe*, *ogi* and *lifin* (whole maize meal) into derived products namely *makume*, *akassa* and *owo* respectively was executed using the expertise of four women, experienced in the production of these products.

*Mawe* was prepared following the traditional procedure indicated in Fig. 1 as described by Hounhouigan *et al.* (1993). Three replicates of 5 kg of maize (total fumonisin level =  $1.99 \pm 0.06$  mg kg<sup>-1</sup> and total aflatoxin level =  $15.28 \pm 0.32$  µg kg<sup>-1</sup>) were sorted, winnowed and washed. Sorting consisted in removing visibly mouldy, insect-damaged and broken grains by hand. Winnowing, a complementary process to sorting, consisted in discarding the rest of impurities present in the sorted maize. Thus, a certain quantity of sorted maize was collected in a circular metallic tray, and thrown into the air by the operator allowing impurities and small broken grains to be blown away. Maize washing came after sorting and winnowing and included thoroughly rubbing the grains in water for a few minutes, and removing all upper floating grains and impurities.

The clean grains were crushed with a plate disc mill (AMUDA, India), passed through a 2 x 2 mm plastic sieve, and sieved with a 0.7 x 0.7 mm metallic sieve, to obtain separately grits, hulls and a fine fraction (fines). The grits were washed by rubbing them by hand for a few minutes in water and then a further soaking in the water for about 2 h. Meanwhile, the embryo and remaining hulls were discarded and added to the hulls previously collected as waste during screening. The washed grits and fines were mixed and the mixture finely ground with the plate disc mill. The resulting meal was kneaded while adding water to obtain a dough (*mawe*). The *mawe* was allowed to ferment naturally, one sub-sample for 24 h and the second one for 72 h. Fermented *mawe* was then cooked into *makume*.

*Ogi* was prepared following the traditional processing method (Fig. 2) described by Nago (1997). Three replicates of 5 kg of maize (total fumonisin level =  $3.35 \pm 0.05$  mg kg<sup>-1</sup> and total aflatoxin level  $> = 22.00 \pm 0.26$  µg kg<sup>-1</sup>) were sorted, winnowed, not washed, but precooked for 5 – 10 min. at 90 – 100 °C. Precooking consisted in initially boiling water and soaking the grains in this water not on fire but on ground for 5 – 10 min. The precooked maize was steeped in water for 24 h, milled in a plate disc mill, and sieved in water with a muslin cloth to discard hulls and embryo. The resulting dough (*ogi*) underwent a natural fermentation process, one sub-sample for 24 h and the second one for 72 h. The fermented *ogi* was then used to cook *akassa*, a fermented paste like *makume* obtained from *mawe*.

*Owo* was prepared following the traditional processing method (Fig. 3). Three replicates of maize (5 kg) (total fumonisin level =  $2.89 \pm 0.08 \text{ mg kg}^{-1}$  and total aflatoxin level  $\geq 22.00 \pm 0.26 \text{ } \mu\text{g kg}^{-1}$ ) were sorted, winnowed, not washed, and milled in a plate disc mill to obtain *lifin*. A suspension of *lifin* was cooked in water to obtain *owo*.

### **Laboratory analyses**

Moisture content of maize samples was determined at each step in the processing of each product. Three replicate samples of 5 g of each were dried to a constant weight in a forced-air oven (Memmert, Germany) at 105 °C for 24 h (AACC 1986). Fermented *mawe* and *ogi* were sampled after 0, 24 and 72 h of fermentation and pH was measured. Total fumonisin content was determined in samples collected at different steps of processing as indicated in the flow diagram for each product (Fig. 1,2 & 3), with a fluorometer using the VICAM method (VICAM 1998). Total aflatoxin content in maize was also measured in the same samples with a fluorometer using the VICAM method (VICAM 1998). Levels of both aflatoxin and fumonisin in each sample, initially measured on wet basis, were calculated on dry basis. Total aflatoxin and fumonisin measurement was performed during processing on the following intermediate products:

- Mawe production: raw maize, washed maize, washed grits, hulls, fines, *mawe*, fermented *mawe*, and *makume*.
- Ogi production: raw maize, clean maize, dough + screenings, hulls, *ogi*, fermented *ogi*, and *akassa*.
- Owo production: raw maize, clean maize, *lifin* (maize meal) and *owo*.

### **Statistical analyses**

Statistical analyses were performed using SPSS for Window version 10.0 (SPSS Inc., Chicago, Illinois). Analysis of variance (ANOVA) and Tukey's HSD test were used to compare the means of the total aflatoxin and fumonisin levels measured in samples collected at different processing steps. Mean total aflatoxin and fumonisin were transformed to  $\log(x+1)$  before analyses, but the data are presented untransformed.

## RESULTS

### *Fate of aflatoxin and fumonisin during preparation of mawe and makume*

Total aflatoxin and fumonisin levels in maize products during the preparation of *mawe* and *makume* are shown in Table 1. Both aflatoxin and fumonisin significantly decreased during the processing ( $p < 0.01$ ). Total aflatoxin level decreased from  $15.28 \mu\text{g kg}^{-1}$  in the raw maize to a non-detectable level in *mawe*. Aflatoxin was not detected in *makume*. A 91 % reduction was already observed after sorting, winnowing and washing of the raw maize. No aflatoxin was detected in discarded hulls, embryo and in fines (screenings).

Total fumonisin in maize followed the same trend as that for aflatoxin (Table 1). Levels decreased from  $1.99 \text{ mg kg}^{-1}$  in the raw maize to not being detected in *mawe*. Fumonisin was not detected in *makume* (Table 1). Initial maize cleaning induced a significant reduction of 74 % of fumonisin in maize. In contrast to aflatoxin,  $0.41 \text{ mg kg}^{-1}$  and  $0.38 \text{ mg kg}^{-1}$  of fumonisin was detected in the discarded hulls, embryo and in the fines, respectively.

Neither aflatoxin nor fumonisin were detected in the non-fermented *mawe* and consequently assessment of fermentation effect was not possible (Table 1). The assessment of cooking effect during the preparation of *makume* was not possible as well, as the toxins were at undetectable levels.

### *Fate of aflatoxin and fumonisin during the preparation of ogi and akassa*

Both aflatoxin and fumonisin levels significantly decreased ( $p < 0.01$ ) during the production of *ogi* and *akassa* (Table 2). An 80 % reduction (Table 4) of the total aflatoxin level was observed from the raw maize to *ogi* (from  $\geq 22.00$  to  $4.50 \mu\text{g kg}^{-1}$ ). Aflatoxin was detected in *akassa*, but the level was quite low ( $1.83 \mu\text{g kg}^{-1}$ ), corresponding to a significant reduction of about 92 % (Table 4). Initial maize cleaning in this case resulted in an aflatoxin reduction of 61 %. A significant level of aflatoxin ( $7.55 \mu\text{g kg}^{-1}$ ) was detected in the discarded hulls and embryo. This was about 34 % of the level found in the raw maize.

Total fumonisin level also decreased during processing, but less than in the case of aflatoxin (Table 2). A 29 % decrease (Table 4) was observed from the raw maize to *ogi* (from  $3.35 \text{ mg kg}^{-1}$  to  $2.37 \text{ mg kg}^{-1}$ ). Fumonisin level in *akassa* was  $1.74 \text{ mg kg}^{-1}$  (Table 2).

corresponding to about 48 % of the level in the raw maize (Table 4). A fumonisin level of 2.35 mg kg<sup>-1</sup> was detected in the discarded hulls and embryo.

Fermentation gave rise to significant differences in aflatoxin levels in fermented and non-fermented *ogi* ( $p < 0.05$ ), with 18 % reduction between the latter and the former product. This difference, however, was not significant in the case of fumonisin, although there was a reduction of 13 % ( $p > 0.05$ ). No significant differences were found between the aflatoxin and fumonisin levels in *ogi* whether fermentation of this product lasted 24 h or 72 h ( $p > 0.05$ ).

Cooking 24 h-fermented *ogi* to *akassa* did not significantly affect the aflatoxin or fumonisin content. In contrast, mycotoxin levels were significantly lower (1.83 µg kg<sup>-1</sup> for aflatoxins, 1.74 mg kg<sup>-1</sup> for fumonisins) in the *akassa* from 72 h-fermented *ogi* than in the *akassa* from 24 h-fermented *ogi* (3.72 µg kg<sup>-1</sup> for aflatoxins, 2.18 mg kg<sup>-1</sup> for fumonisins) ( $p < 0.05$ ).

#### ***Fate of aflatoxin and fumonisin during the preparation of owo***

The preparation of *owo* had a significant effect on mycotoxin levels (Table 3). A significant decrease in aflatoxin levels was observed from the raw maize ( $> = 22$  µg kg<sup>-1</sup>) to *lifin* (maize meal) (12.62 µg kg<sup>-1</sup>) ( $p < 0.01$ ), with a meaningful reduction of 37 % after initial maize cleaning prior to milling. No further significant reduction occurred during cooking of maize meal to *owo*.

Fumonisin decreased from 2.89 mg kg<sup>-1</sup> in raw maize to 1.45 mg kg<sup>-1</sup> in maize meal, with a 45 % reduction after initial maize cleaning. Also in this case, cooking maize meal to *owo* did not significantly reduce fumonisin level ( $p > 0.05$ ).

## **DISCUSSION**

Results of this study have shown that processing maize into traditional products can significantly reduce levels of both aflatoxin and fumonisin up to 93 %. This indicates that elimination of mycotoxins in naturally contaminated maize is, to a certain extent, possible using such food processing techniques. Reduction of mycotoxins was more substantial during the production of *makume* (*mawe*) and *akassa* (*ogi*) than in the preparation of *owo*. This might be due to the fact that techniques for making *makume* and *akassa* involve critical steps for

mycotoxin reduction. Shephard *et al.* (2002) also found a low reduction of fumonisin level (23 %) in South African stiff porridge, which has a method of processing similar to that of *owo*.

Significantly less fumonisin was removed by cleaning maize during the preparation of *ogi* and *akassa* than during the preparation of *mawe* and *makume*. This might be due to the fact the initial cleaning process during the preparation of *ogi* and *akassa* only consisted in sorting and winnowing, not washing, whereas in the case of the preparation of *mawe* and *makume*, maize was sorted, winnowed and also washed. Moreover, two different processors were used for preparing the products; the one who prepared *mawe* and *makume* was much stricter in cleaning the maize than the other who prepared *ogi* and *akassa*.

Some operations linked to the preparation of maize in Benin appeared to have been very effective in significantly reducing mycotoxins. Simple cleaning by sorting, winnowing and/or washing the contaminated maize grains reduced mycotoxin levels from 18 to 91 %. Higher levels of aflatoxin and fumonisin are generally found in visibly mouldy grains (Rheeder *et al.* 1992, Marasas 1995, Shephard *et al.* 1996, Desjardins *et al.* 1998, Sinha 1998). Systematic disposal of all visibly mouldy, insect-damaged and broken grains, and impurities could be useful in reducing toxin levels in maize. However, hand-sorting visibly mouldy grains with the aim of substantially reducing mycotoxin levels is likely to depend on the ability of the people responsible for this activity. People trained to easily recognise diseased grains are apparently more efficient at achieving this goal (Desjardins *et al.* 2000). Removal of screenings from maize bulk reduces fumonisin levels (Sydenham *et al.* 1994). Likewise, substantial amounts of fumonisin (up to 74 %) were removed by simply washing maize grains, immersing them in water and by removing the upper floating fraction, as contaminated grains generally have a low density (Shetty and Bhat 1999).

Crushing and dehulling maize during *mawe* production has been also identified as a critical operation for mycotoxin reduction, by removing the grain pericarp and embryo. In the present study, recovery of fumonisin for instance in maize after crushing and dehulling was almost negligible. This finding provides additional evidence that removal of pericarps and embryo of maize grains, mechanically or chemically, can also play an important role in the reduction of fumonisins in naturally contaminated maize (Sydenham *et al.* 1995, Canela *et al.* 1996, Katta *et al.* 1997, Bouraima 2001, FDA 2001, Voss *et al.* 2001).

The crushing and dehulling of maize did not influence aflatoxin contamination during the preparation of *mawe*, since no toxin was detected in the discarded hulls and embryo. Aflatoxin levels were already so low in the cleaned grains that levels in the hulls and embryo were below detection. In the case of *ogi*, a significant level of aflatoxin was, however,

recovered in the discarded hulls and embryo after sieving. The aflatoxin levels were still so high in the dough obtained after milling, prior to sieving, that meaningful quantities are likely to be detected in the hulls and embryo. This suggests that aflatoxin distribution in maize fractions during processing may be influenced by contamination levels (Lopez-Garcia and Park 1998).

Fermentation did not appear to have a significant impact on the levels of mycotoxins in products considered in this study. Only 18 % and 13 % reduction of aflatoxin and fumonisin levels respectively were observed during fermentation of *ogi*. Even prolonging the fermentation time from 24 h to 72 h did not significantly affect mycotoxin levels in maize products. Previous studies have presented similar results (Bothast *et al.* 1992, Kpodo *et al.* 1996, Desjardins *et al.* 2000, Kpodo *et al.* 2000). Kpodo *et al.* (1996) explained persistence of aflatoxin during the fermentation process by the presence of aflatoxin precursors in maize grains and the reduction of pH.

There was no evidence that cooking has a significant effect on the reduction of mycotoxins during production of *makume*, *akassa* or *owo*. In all cases, cooking did not last more than 30 min. and is, therefore, unlikely to significantly reduce mycotoxin levels. Kpodo *et al.* (1996) found that ordinary cooking of fermented maize dough for a three-hour period resulted in up to 80 % reduction of aflatoxin level. These authors suggested that degradation of aflatoxin during the cooking process might be favoured by moist conditions. More recently, Shephard *et al.* (2002) using commercial maize meal, found a reduction of fumonisin levels of 23 %, after only 20 min of cooking. Aflatoxin and fumonisin are usually reported to be heat-stable, and are not easily destroyed by ordinary cooking (Alberts *et al.* 1990, Scott 1993, Sinha 1998), except at high temperatures (more than 150 °C for fumonisin) (Bolger *et al.* 2001). However, aflatoxin can be partially removed by cooking, especially when this occurs under pressure in moist conditions (Sinha 1998).

Another processing step probably responsible for significant mycotoxin reduction was the way the *ogi* supernatant was used during the cooking of *akassa*. The supernatant from 72 h-fermented *ogi* was replaced with simple water and discarded or used to produce beverages when the acidity levels were inordinately high to give an acceptable *akassa*. This may explain why both aflatoxin and fumonisin contents were reduced in *akassa* prepared from 72 h-fermented *ogi*. Here, mycotoxins probably diffused in the *ogi* supernatant, which was discarded. This observation is in agreement with findings of Canela *et al.* (1996) who found fumonisin B<sub>1</sub> to migrate from contaminated maize grains to the steeping water after 48 hours. Voss *et al.* (2001) also showed that fumonisins are mostly found in the rinsing liquid during

processing of tortillas. While discarding *ogi* supernatant constitutes a significant decontamination process, its use to prepare beverages, which is a common practice in West Africa, may be harmful to consumers and should be discouraged.

Some traditional food processing techniques in Africa may potentially be useful for detoxifying foods from mycotoxin contamination. However, there is increasing evidence that mycotoxin molecules, specifically fumonisin, bind with starch to form a complex that cannot be detected (Bullerman and Tsai 1994, Kim *et al.* 2002). Alternatively, they react with reducing sugars such as D-glucose to give sugar adducts (Howard *et al.* 1998, Seefelder *et al.* 2001, Voss *et al.* 2001), or are hydrolysed to the aminopolyols AP<sub>1</sub> and AP<sub>2</sub> (Dombrink-Kurtzman *et al.* 2000, Voss *et al.* 2001). Further research is, therefore, needed to clarify whether the mycotoxins apparently lost during the preparation of foods are really destroyed, hydrolysed or bound to the food matrix to become non-recoverable.

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Table 1: Mean total aflatoxin and fumonisin concentrations in maize products at each processing stage during the preparation of *mawe* (dry basis)

Maize products	Dry matter content (%)	pH	Mean total aflatoxin ( $\mu\text{g kg}^{-1}$ )	Mean total fumonisin ( $\text{mg kg}^{-1}$ )	
<b>Raw maize</b>	84.51 $\pm$ 0.06		15.28 $\pm$ 0.32	a	1.99 $\pm$ 0.06
Washed maize	84.35 $\pm$ 0.04		1.42 $\pm$ 0.06	b	0.54 $\pm$ 0.03
Washed grit	55.90 $\pm$ 0.32		nd	c	nd
<b>Mawe</b>	54.58 $\pm$ 0.27	5.90 $\pm$ 0.14	nd	c	nd
Fermented <i>mawe</i> 24 hours	53.02 $\pm$ 0.28	4.14 $\pm$ 0.08	nd	c	nd
Fermented <i>mawe</i> 72 hours	51.01 $\pm$ 0.32	3.70 $\pm$ 0.10	nd	c	nd
<i>Makume</i> 24 hours	39.01 $\pm$ 0.24		nd	c	nd
<i>Makume</i> 72 hours	40.17 $\pm$ 0.34		nd	c	nd
Hulls + embryo	82.99 $\pm$ 0.17		nd	c	0.41 $\pm$ 0.15
Fines (screenings)	83.29 $\pm$ 0.21		nd	c	0.37 $\pm$ 0.06

Means within a column followed by the same letter are not significantly different ( $P > 0.05$ )

nd = not detected = level  $< 1 \mu\text{g kg}^{-1}$  for aflatoxins; level  $< 0.25 \text{mg kg}^{-1}$  for fumonisins.

Table 2: Mean total aflatoxin and fumonisin concentrations in maize products at each processing stage during the preparation of *ogi* (dry basis)

Maize products	Dry matter content (%)	pH	Mean total aflatoxin ( $\mu\text{g kg}^{-1}$ )	Mean total fumonisin ( $\text{mg kg}^{-1}$ )
Raw maize	84.19 $\pm$ 0.07		22.00 $\pm$ 0.26	3.35 $\pm$ 0.05
Steeped maize	84.21 $\pm$ 0.06		8.62 $\pm$ 0.09	2.76 $\pm$ 0.11
Dough + screenings	43.65 $\pm$ 0.57		7.79 $\pm$ 0.12	2.75 $\pm$ 0.09
<b>Ogi</b>	42.10 $\pm$ 0.47	6.20 $\pm$ 0.10	4.50 $\pm$ 0.20	2.37 $\pm$ 0.22
Fermented <i>ogi</i> 24 hours	40.40 $\pm$ 0.61	4.00 $\pm$ 0.10	3.69 $\pm$ 0.03	2.07 $\pm$ 0.12
Fermented <i>ogi</i> 72 hours	39.97 $\pm$ 0.48	3.05 $\pm$ 0.15	3.65 $\pm$ 0.05	2.28 $\pm$ 0.14
<i>Akassa</i> 24 hours	43.79 $\pm$ 0.32		3.72 $\pm$ 0.02	2.18 $\pm$ 0.17
<i>Akassa</i> 72 hours	36.56 $\pm$ 0.29		1.83 $\pm$ 0.02	1.74 $\pm$ 0.07
Hulls + embryo	42.19 $\pm$ 0.19		7.55 $\pm$ 0.43	2.35 $\pm$ 0.03

Means within a column followed by the same letter are not significantly different ( $P > 0.05$ )

Table 3: Mean total aflatoxin and fumonisin concentrations in maize products at each processing stage during the preparation of *owo* (dry basis)

Maize products	Dry matter content (%)	Mean total aflatoxin ( $\mu\text{g kg}^{-1}$ )		Mean total fumonisin ( $\text{mg kg}^{-1}$ )	
Raw maize	$84.67 \pm 0.02$	$22.00 \pm 0.26$	a	$2.89 \pm 0.08$	a
Clean maize	$84.62 \pm 0.04$	$13.79 \pm 0.19$	b	$1.61 \pm 0.09$	b
Maize meal	$86.15 \pm 0.10$	$12.62 \pm 0.43$	c	$1.38 \pm 0.02$	c
Owo	$70.82 \pm 0.18$	$13.13 \pm 0.25$	bc	$1.45 \pm 0.05$	c

Means within a column followed by the same letter are not significantly different ( $P > 0.05$ ).



Table 4: Mean percentage of reduction in total aflatoxin and fumonisin concentrations during maize processing

Maize processing	Mean percentage of mycotoxin reduction (%)	
	Total aflatoxin	Total fumonisin
Raw maize → <i>mawe</i>	> = 91	> = 87
Raw maize → <i>mawe</i> → <i>makume</i>	> = 93	> = 87
Raw maize → <i>ogi</i>	80	29
Raw maize → <i>ogi</i> → <i>akassa</i>	92	48
Raw maize → <i>owo</i>	40	48

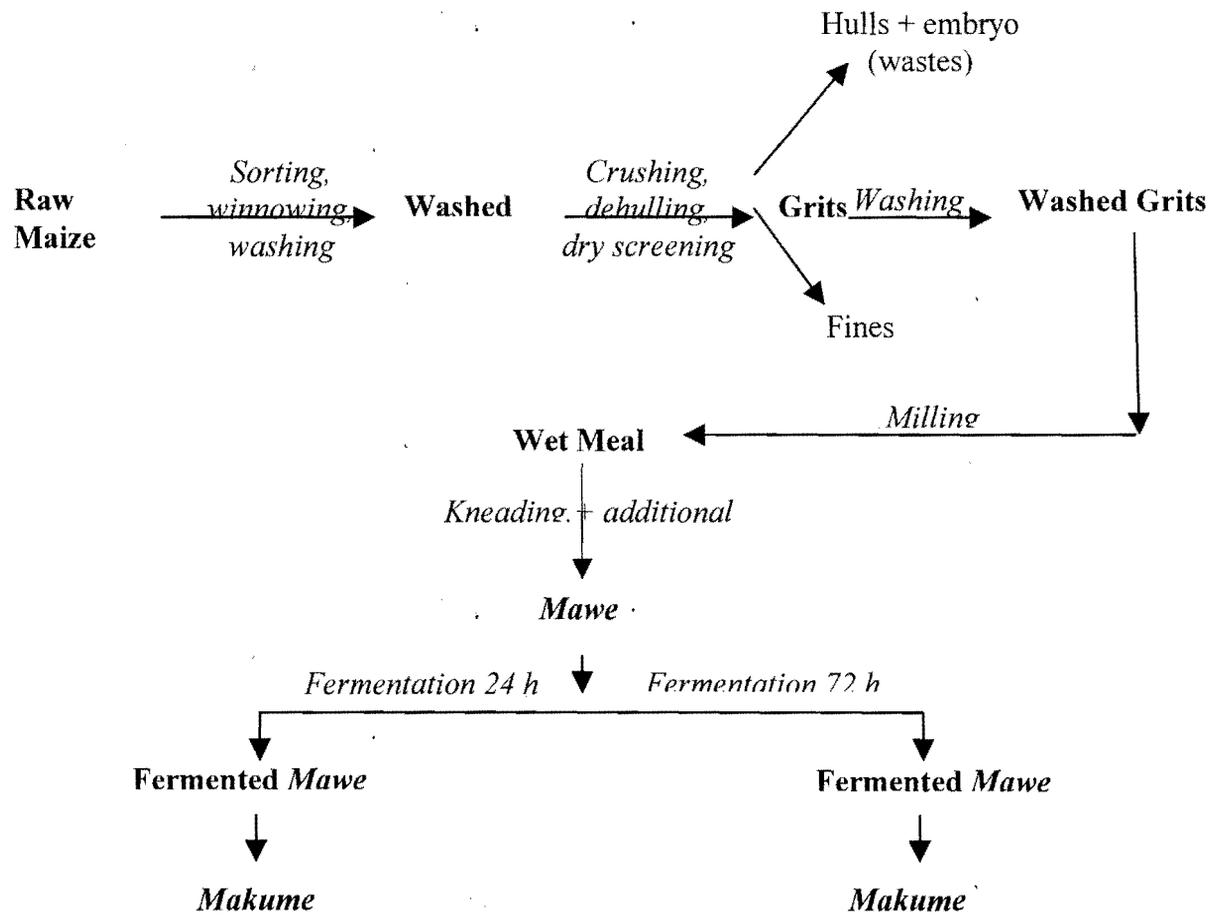


Figure 1: Flow diagram outlining the preparation of *mawe* and *makume*, two maize-based foods of Benin

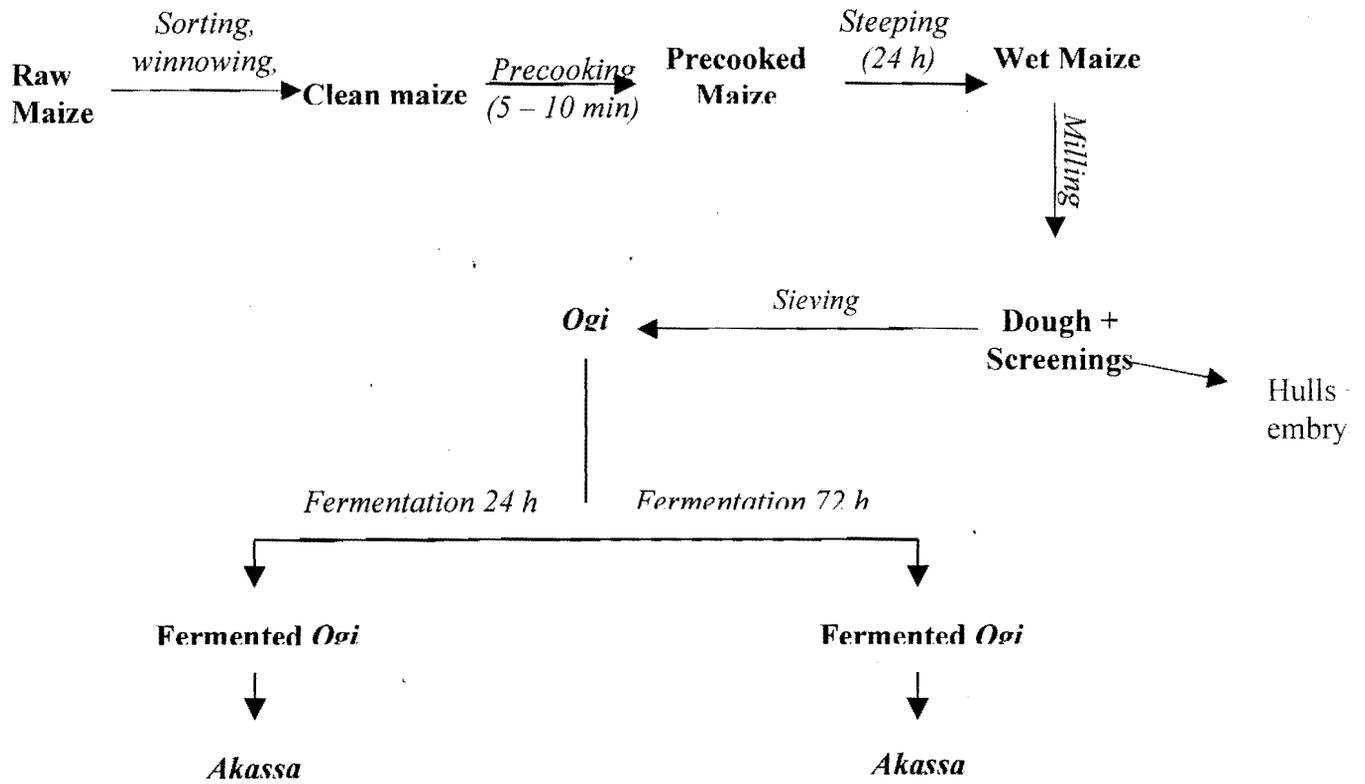


Figure 2: Flow diagram outlining the preparation of *ogi* and *akassa* in Benin

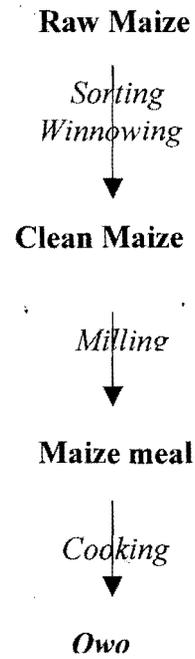


Figure 3: Flow diagram outlining the preparation of *owo* in Benin



## CHAPTER FIVE

**IMPACT OF MECHANICAL SHELLING AND DEHULLING ON *FUSARIUM*  
INFECTION AND FUMONISIN CONTAMINATION OF MAIZE**

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## ABSTRACT

Mechanical shelling and dehulling methods were tested to evaluate their impact on *Fusarium* infection and fumonisin contamination in maize. The mechanical shelling methods tested were found to damage the grains. The motorised sheller type IITA caused the highest level (up to 3.5 %) of damage. This could be due to the operation mode of that machine. *Fusarium* populations were higher on damaged grains. The highest number of colonies was recorded from grains damaged by the IITA sheller (2533.3 cfu g<sup>-1</sup>). Total fumonisin levels were also higher in damaged grains, the highest being in maize shelled by the IITA sheller (2.2 mg kg<sup>-1</sup>). Fumonisin levels were positively and significantly correlated with the percentage of damage caused by the shelling methods ( $r = + 0.6$ ,  $p < 0.01$ ), and also with the number of *Fusarium* colonies from maize ( $r = + 0.7$ ,  $p < 0.01$ ). In contrast to the other shelling methods, an increase of the fumonisin level was observed during the first month of storage in maize shelled with the IITA sheller. On the other hand, the mechanical dehulling methods reduced fumonisin levels in maize. The use of dehullers resulted in a reduction of 64 – 68 % for Mini-PRL, 62 – 67 % for Engelberg, and 56 – 62 % for the attrition disc mill. This study has clearly shown the effects of shelling and dehulling methods on fungal infection and mycotoxin contamination of maize. It is important for farmers to choose appropriate shelling methods to reduce mycotoxin contamination. Also, dehulling, which is an important step in the processing of maize in Africa should be widely promoted for the reduction of mycotoxins in maize. This is a major challenge for all agricultural institutions in Africa.

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**Key words:** Maize, mechanical shelling, dehulling, *Fusarium*, fumonisins, Benin

## INTRODUCTION

In Benin as in most West African countries, maize undergoes many postharvest operations before consumption, of which shelling and dehulling are of great importance. Shelling usually occurs prior to storage or processing and consists of separating grains from the maize cob's core. Dehulling consists of removing the pericarp from the grain. It is often accompanied by degerming (removal of the embryo).

Shelling and dehulling are generally executed by women, and are very labour intensive and time consuming (Diop *et al.* 1997). Shelling is traditionally done by hand, mortar and pestle or using a wooden stick (Houssou 2000) whereas dehulling is done by using stones or mortar and pestle (François 1988, Diop *et al.* 1997). Generally, the output of manual shelling or dehulling is very low. Hand shelling maize from one hectare (approximately 1 tonne) by a single woman requires 16 days of labour with an hourly output of 8 – 15 kg (FAO 1992). One woman can dehull approximately 10 kg of maize in one hour (François 1988, Diop *et al.* 1997).

Different types of mechanical equipment have been introduced in rural and urban areas of Africa to make shelling and dehulling of maize easier, faster and more efficient. Observations in the field indicated that some of this equipment cause damage on grains promoting serious fungal infection (Fandohan *et al.* 2002, unpublished data). Up to now, little attention has been given to the possible effects that these machines may have, not only on fungal infection but also mycotoxin contamination of maize. Kozakiewicz (1996) stressed that postharvest mechanisation in general, if not used correctly, can damage the processed products and may facilitate fungal infection. Dharmaputra *et al.* (1996) reporting results from national surveys in Indonesia, found strong evidence that maize shelling could cause mechanical damage, allowing fungi to infect grains. Some of these fungi can produce toxic substances called mycotoxins. Examples are toxigenic *Fusarium* spp. producing fumonisins in maize (Munkvold and Desjardins 1997).

Fumonisin are recently identified mycotoxins (Gelderblom *et al.* 1988) mainly produced by *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg. Since their discovery, fumonisins have attracted increasing attention because of their adverse effects on animal and human health and their negative economic impact (Bolger *et al.* 2001). They have been found to be associated with several animal diseases such as leukoencephalomalacia in horses (Kellerman *et al.* 1990) and pulmonary oedema in pigs (Harrison *et al.* 1990). Their occurrence in maize intended for human consumption has been

linked to a higher incidence of oesophageal cancer (Rheeder *et al.* 1992, Chu and Li 1994) and liver cancer (Ueno *et al.* 1997).

The present study was undertaken to elucidate the impact of automated shelling and dehulling methods currently promoted in West Africa on *Fusarium* infection and fumonisin contamination of maize. The main objective was to draw more attention to the effects that machines may have on mycotoxin contamination of maize, in order to alert agricultural institutions and farmers to take these factors into account when choosing equipment.

## MATERIAL AND METHODS

### *Maize cultivar used*

The maize used in this study was the 90-day cultivar DMR-ESR-W, which is an improved IITA (International Institute of Tropical Agriculture) white and hard grain variety. Maize cobs were obtained from the Benin Station of IITA, Abomey-Calavi, situated in the Forest Mosaic Savannah of Benin. DMR-ESR-W is known to be resistant to downy mildew (*Peronosclerospora sorghi*) and to maize streak virus (Schulthess *et al.* 2002).

### *Impact of different shelling methods on Fusarium- and fumonisin contamination*

Maize cobs were immediately dehusked after harvest and sun-dried to moisture content less than 18 %. They were divided into four lots of at least 300 cobs each. The cobs of each lot were shelled using the following four different methods with one shelling method for each lot (Fig 1, 2, 3 & 4). These methods included shelling by hand, shelling using a handle-operated sheller, and shelling using two motorised shellers type Renson, France and type IITA, Nigeria. Characteristics of the shellers are described in Table 1. Grains (10 kg) from each lot were stored in weaved polyethylene bags at room temperature (28 - 30°C) for three months. There were three bags per treatment (shelling method) as each treatment was replicated three times. Prior to storage, grains in each bag were dusted with the binary insecticide Sofagrain® (0.05 % deltamethrin and 1.5 % pirimiphos-methyl) to reduce insect damage.

A 500 g-sample was taken from each bag (Fig 5) at the beginning of the trial, and after 1 and 3 months of storage. This sample was used for determination of moisture content, percentage of damage caused by the shelling methods, *Fusarium* population and fumonisin

levels. Grain moisture content was determined just after sampling each bag using an electronic moisture meter (model HOH-EXPRESS HE 50, PFEUFFER, Germany). Percentage of grain damage caused by each shelling method was assessed after shelling at the beginning of the trial, whereas damage caused by insects was assessed before shelling (Pantenius 1988). In order to reduce the eventual influence of grain moisture content, damage on grain by insects and sheller speed, the cobs were sun-dried prior to shelling to bring the grain moisture content to a level less than 18 %. Visibly damaged and cracked grains were also carefully removed by hand. Efforts were made during the shelling operation to maintain the speed of the rotary cylinder inside the shelling chamber at 500-r min<sup>-1</sup>.

*Fusarium* species were enumerated using dilution plating at the beginning of the trial, and also at 1 and 3 months after stocking. A 10 g sub-sample of maize grains was taken from each bag, finely ground, thoroughly mixed with 90 ml of sterile 0.1 % peptone water, and serial dilutions made to 10<sup>-2</sup>. One millilitre of suspension was transferred into individual Petri dishes, mixed with potato dextrose agar (PDA) (15 ml) and the Petri dishes were incubated at 25 °C exposed to a 12:12-hour light/dark regime for 5 days. *Fusarium* colonies were isolated and transferred onto carnation leaf agar and incubated for 7 days at 25 °C exposed to a 12:12-hour light/dark regime. Colony forming units per gram of sample (cfu g<sup>-1</sup>) were enumerated. *Fusarium* species were identified according to Nelson *et al.* (1983). Fumonisin content was determined as described in Chapter 2 at the beginning of the trial, and after 1 and 3 months of storage using the VICAM method (VICAM 1998).

### ***Impact of different dehulling methods on Fusarium- and fumonisin contamination***

Grains from the bags of maize initially shelled with the two motorised shellers were thoroughly mixed after 3 months of storage and divided into three lots of approximately 7 kg each (Fig 5). Three replicates of 2 kg of maize were sampled from each lot and dehulled using one of the following three different dehulling methods, i.e. attrition disc mill type Amuda, and motorised dehullers Engelberg and Mini-PRL (Fig 6, 7 & 8). Characteristics of the dehullers are given in Table 1. To facilitate removal of pericarp and embryo, the grains were humidified to attain moisture content between 18 and 22 % in the case of the dehuller Engelberg. Grains were thoroughly washed for the attrition disc mill, but remained dry (moisture content less than 14 %) for the dehuller Mini-PRL. The grains were dehulled once for 4 - 6 min. Fumonisin content was measured as described above just before and after dehulling.

## *Statistical analyses*

SPSS program for Window version 10.0 (SPSS Inc., Chicago, Illinois) was used to test the statistical significance of differences between treatments with one-way analysis of variance (ANOVA). Tukey HSD test was performed to test differences between means of percentage damage caused on grain by each shelling method, means of *Fusarium* populations and mean levels of fumonisin in maize samples. Pearson correlation test was used to evaluate relationships among percentage damage caused by the shelling methods, *Fusarium* incidence and fumonisin level.

## RESULTS

### *Impact of different shelling methods on Fusarium- and fumonisin contamination*

Mechanical shelling methods caused damage to grain (Table 2). The percentage of damage caused by the IITA sheller was significantly higher than that of all the other methods ( $p < 0.01$ ). The handle-operated sheller and motorised Renson sheller caused significantly higher damage than hand shelling ( $p < 0.01$ ), but there was no significant difference between the percentages of damage caused by each other ( $p > 0.05$ ).

Mycological analyses showed that *F. verticillioides* and *F. proliferatum* were the *Fusarium* spp. found in the maize samples during the study. The former was encountered in all the samples whereas the latter was found only in the samples shelled by hand. The number of *Fusarium* colonies was higher in maize shelled with the mechanical shellers (Table 3). The number of colonies from maize shelled using the IITA sheller was significantly higher than in maize shelled using the other shelling methods ( $p < 0.05$ ). *Fusarium* population in maize shelled using the two other mechanical shelling methods (use of handle-operated sheller and motorised Renson sheller) was not significantly different from that found in maize shelled by hand ( $p > 0.05$ ). The number of *Fusarium* colonies found in maize was positively and significantly correlated with the percentage of damage ( $r = + 0.6$ ,  $p < 0.01$ ). *Fusarium* populations in maize changed throughout the 3-month storage period (Table 3). This change was, however, significant ( $p < 0.01$ ) only in maize shelled using the IITA sheller, *Fusarium* populations increasing from 2033.3 cfu g<sup>-1</sup> at the beginning to 3100.0 cfu g<sup>-1</sup> after 1 month, before decreasing at 3 months of storage (Table 3).

Mean fumonisin levels were found to be higher in maize shelled using the mechanical shellers (Table 4). The highest mean level was detected in maize shelled using the IITA sheller, and that was significantly different from the level found in maize shelled using other shelling methods ( $p < 0.01$ ). Fumonisin levels detected in maize shelled using the handle-operated sheller and motorised Renson sheller were not significantly different from that detected in maize shelled by hand ( $p > 0.05$ ). There was a positive and significant correlation between the fumonisin levels in maize and the percentage of damage caused by the shelling methods ( $r = + 0.6$ ,  $p < 0.01$ ). The fumonisin levels were also positively and significantly correlated with the number of *Fusarium* colonies from the maize samples ( $r = + 0.7$ ,  $p < 0.01$ ).

Changes were also observed in the fumonisin level in maize throughout the storage period, and in contrast to *Fusarium* populations, these changes were significant in all cases ( $p < 0.01$ ) (Table 4). The fumonisin level increased in maize shelled using the IITA sheller from  $1.6 \text{ mg kg}^{-1}$  at the beginning to  $3.2 \text{ mg kg}^{-1}$  after 1 month before decreasing to  $1.7 \text{ mg kg}^{-1}$  at 3 months of storage, whereas the level markedly decreased in the maize shelled using the other shelling methods (67 – 90 % of reduction of fumonisin level) (Table 4).

### ***Impact of different dehulling methods on Fusarium- and fumonisin contamination***

Fumonisin levels significantly decreased in maize after dehulling ( $p < 0.01$ ) (Fig. 9). This decrease was not, however, significantly different from one dehulling method to another ( $p > 0.05$ ). The dehuller Mini-PRL induced a reduction of 64 – 68 %, the dehuller Engelberg, 62 – 67 % and the attrition disc mill, reduced levels by 56 – 62 %.

## **DISCUSSION**

Results of this study provide firm evidence that methods of shelling can inflict damage on maize grains. Some mechanical shelling methods can be very damaging. Dharmaputra *et al.* (1994) noticed a higher percentage of damaged grains in maize shelled mechanically (5.7 %) than that in maize shelled using a nailed wood used as a sheller (2.9 %). In a previous study, Suprayitno (1980) has suggested that friction between grains and the cylinder of the sheller could cause a high number of damaged grains after mechanical shelling.

Both the handle-operated sheller and the motorised Renson sheller tested in this study function similarly to the traditional method of shelling, which consists of rubbing cobs one against each other. Separation of grains then occurs by friction. In contrast, the IITA sheller

functions similarly to the traditional method of beating cobs with a stick, after they have been placed in a bag. The cobs are beaten with beaters inside the shelling chamber. Grains are released from the cob's core due to impact between cobs and beaters, cobs and the inner surface of the shelling chamber, and the cobs against themselves while in their disordered movement inside the chamber. This may explain the high number of damaged grains found using this method.

There are some other factors that may increase the risk of grain damage. Percentage of grain damage increases if the grains are shelled at moisture levels higher than 18 % (Dharmaputra *et al.* 1994, Dharmaputra *et al.* 1996). Grains damaged by insects and those having apparent cracks probably due to stress during grain-filling period or excessive drying rates were found to be more easily damaged or broken by shellers (Ahouansou *et al.* 2002). Higher speeds of the rotary cylinder inside the sheller (more than 500 r min<sup>-1</sup>) is more likely to cause increased impact between the cobs and the shelling chamber, and between the cobs themselves, leading to more damage on grains (Ahouansou *et al.* 2002).

*Fusarium* infections were more common on damaged grains. This indicates that damage caused on grain due to mechanical shelling may serve as entry points for *Fusarium* fungi. Dharmaputra *et al.* (1994) found *Fusarium* populations on maize shelled with a mechanical sheller to be higher (7129 cfu g<sup>-1</sup>), but not significantly different to those on maize shelled with a nailed wooden instrument (5044 cfu g<sup>-1</sup>). Similarly, Douglas and Boyle (1996) reported that multistage postharvest handling of grain (including shelling) increases grain damage and cracking, providing an opportunity for fungi to develop and penetrate the grain. GASGA (1997) have, therefore, stressed that grain damage should be minimised in order to reduce fungal infection.

Levels of fumonisin were higher in maize shelled using mechanical shellers, with the highest level being detected in maize shelled using the IITA sheller. Fumonisin level was also found to significantly correlate positively with the percentage of damaged grains. This finding is in agreement with Nelson *et al.* (1993) who showed production of mycotoxin to be significantly affected by factors such as grain damage. An increase of fumonisin levels was detected in samples of maize shelled using the IITA sheller after the first month of storage. This could presumably be due to the fact that at that time, *Fusarium* infection was still very active in maize of these samples. The population of *Fusarium* was also found to be higher in these samples, and grain moisture content, still around 18 % in the first month of storage (Table 5), might allow for development of fungi and mycotoxin production. This result is

consistent with the fact that fumonisin levels were positively and significantly correlated with the number of *Fusarium* colonies found on the samples.

Mechanical dehulling significantly reduced fumonisin levels in maize. This confirms evidence that fumonisin is likely to be more concentrated in the outer parts (pericarp and embryo) of the maize grain. Removal of these parts can markedly reduce the fumonisin level in maize (Sydenham *et al.* 1994, Sydenham *et al.* 1995, Katta *et al.* 1997, FDA 2001, Voss *et al.* 2001). Trenholm *et al.* (1991) found dehulling to result in a 40 – 100 % reduction in the *Fusarium* toxins deoxynivalenol and zearalenone in contaminated barley, wheat and rye. In a more recent study in Benin, a reduction of up to 63 % of fumonisin was observed due to dehulling of maize during the preparation of maize-based foods (Chapter 4).

No significant differences in fumonisin levels were observed for the tested dehulling methods. However, both dehullers Mini-PRL and Engelberg appear to have been more efficient than the attrition disc mill in grain dehulling, inducing a numerically but not statistically significantly better reduction of fumonisin levels. The mill is commonly used in West Africa for maize milling, but it does not seem to be adapted for maize dehulling when compared to the Mini-PRL. The mill possesses two discs, one fixed and the other mobile. During the dehulling operation, the gap between these discs needs to be regularly adjusted to avoid grain breakage (François 1988).

This study has clearly shown that shelling and dehulling are important steps in the processing of maize in Africa with respect to the reduction of mycotoxin levels. In particular mechanical dehulling significantly reduced fumonisin levels and can be recommended as a decontamination method in African countries where maize is a staple diet. Much more attention should be given to this processing operation that should be widely developed in the African countries where it is still uncommon. Moreover, whereas automated shelling machines are being increasingly promoted in Africa to reduce the drudgery of food processing to farmers, mainly to women, introducing appropriate machines that are less damaging should be a great challenge for African research institutions. It is also very important to stress that efforts should be made by the farmers to always meticulously remove damaged grains from maize bulk to reduce fungal infection and mycotoxin level. The implementation of appropriate sorting, mechanical shelling and dehulling methods is therefore a major challenge for all agricultural institutions in Africa.

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Table 1: Characteristics and use conditions of the different tested shelling and dehulling methods

Characteristics	Shelling methods				Dehulling methods		
	Shelling by hand	Handle-operated sheller	Motorised sheller type Renson	Motorised sheller type IITA	Attrition disc mill	Engelberg	Mini-PRL
Manufacturer	-	Renson (France)	Renson (France)	IITA (Nigeria)	Amuda (India)	Rajan (India)	Pere et Frere (Senegal)
Type of motor used	-	-	Honda (5 HP) (Petrol)	Briggs & Stratton (Petrol)	-	-	-
Mean speed of rotary cylinder (rpm)	-	-	500	500	-	-	-
Operation mode	-	-	-	-	Continuous	Continuous	Discontinuous
Principle	Friction	Friction	Friction	Impact	Attrition	Friction	Abrasion
Hourly throughput (kg/h)	8 – 15	85	450	1600	100 - 600	100 - 600	100

Sources:

- Shelling methods: Ahouansou et al. (2002)
- Dehulling methods: François (1988), Diop et al. (1997)

Table 2: Mean percentage of damage caused to maize grains by different shelling methods

Shelling methods	n	Mean percentage of damage (%)
Shelling by hand	3	0 a
Handle-operated sheller	3	1.0 ± 0.2 b
Motorised sheller type Renson	3	0.9 ± 0.7 b
Motorised sheller type IITA	3	3.5 ± 0.8 c

n = number of maize samples on which damage caused by the shelling methods was assessed

Means within a column followed by the same letter are not significantly different ( $p > 0.05$ )

Table 3: Mean *Fusarium* population in maize samples during 3-month storage period

Shelling methods	n	Population of <i>Fusarium</i> (cfu g <sup>-1</sup> )			
		0 month after stocking	1 month after stocking	3 months after stocking	Mean over 3 months of storage
Shelling by hand	3	1766.7 ± 208.2 a	1700.0 ± 264.6 a	1466.7 ± 461.9 a	1644.4 ± 316.7 a
Handle-operated sheller	3	2066.7 ± 929.2 a	1766.7 ± 115.5 a	1533.3 ± 57.7 a	1788.9 ± 523.1 a
Motorised sheller type Renson	3	1933.3 ± 776.8 a	2000.0 ± 556.8 a	1700.0 ± 435.9 a	1877.9 ± 542.6 a
Motorised sheller type IITA	3	2033.3 ± 208.2 a	3100.0 ± 200.0 b	2466.7 ± 321.5 a	2533.3 ± 512.4 b

n = number of maize samples on which *Fusarium* population was assessed

Means within a row followed by the same letter are not significantly different ( $p > 0.05$ ) from each other.

Table 4: Mean total fumonisin level in maize samples during 3-month of storage period

Shelling methods	n	Fumonisin level in maize (mg kg <sup>-1</sup> )			
		0 month after stocking	1 month after stocking	3 months after stocking	Mean over 3 months of storage
Shelling by hand	3	1.6 ± 0.1 a	0.3 ± 0.1 a	nd a	0.7 ± 0.7 a
Handle-operated sheller	3	1.5 ± 0.2 a	1.3 ± 0.2 b	0.5 ± 0.1 a	1.1 ± 0.5 a
Motorised sheller type Renson	3	1.5 ± 0.1 a	0.9 ± 0.1 c	0.5 ± 0.2 a	1.0 ± 0.5 a
Motorised sheller type IITA	3	1.6 ± 0.1 a	3.2 ± 0.1d	1.7 ± 0.2 b	2.2 ± 0.8 b

Means in columns followed by the same letter are not significantly different ( $p > 0.05$ ) from each other.

nd = not detected = level  $< 0.25$  mg kg<sup>-1</sup> of fumonisins.

Table 5: Mean moisture content of grains during 3-month of storage period

Shelling methods	n	Grain moisture content (%)		
		0 month after stocking	1 month after stocking	3 months after stocking
Shelling by hand	3	20.4 ± 0.6	17.4 ± 0.8	14.0 ± 0.2
Handle-operated sheller	3	20.4 ± 0.5	17.2 ± 0.1	13.6 ± 0.1
Motorised sheller type Renson	3	20.7 ± 0.5	17.2 ± 0.4	14.0 ± 0.3
Motorised sheller type IITA	3	20.4 ± 0.5	17.5 ± 0.4	13.9 ± 0.9

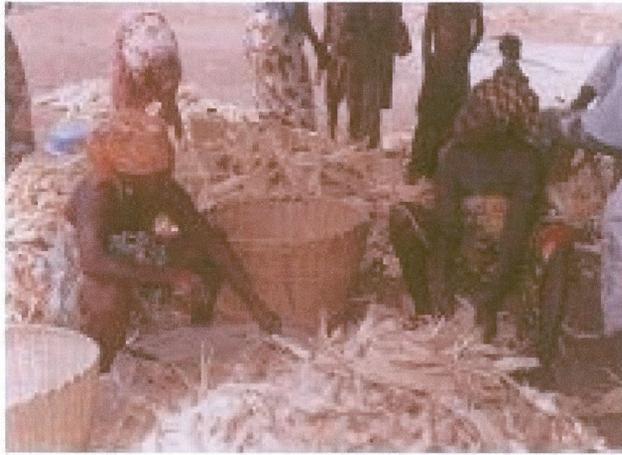


Figure 1: Manual shelling operation



Figure 2: A handle-operated sheller  
(Renson)

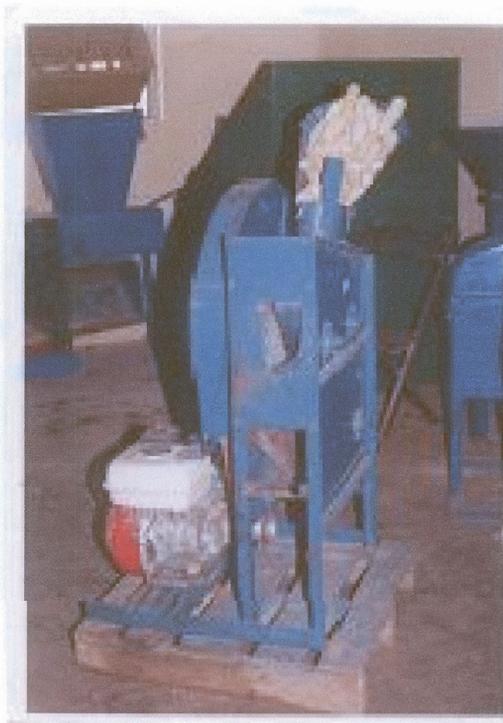


Figure 3: A motorised sheller (Renson)



Figure 4: A motorised sheller  
(IITA)

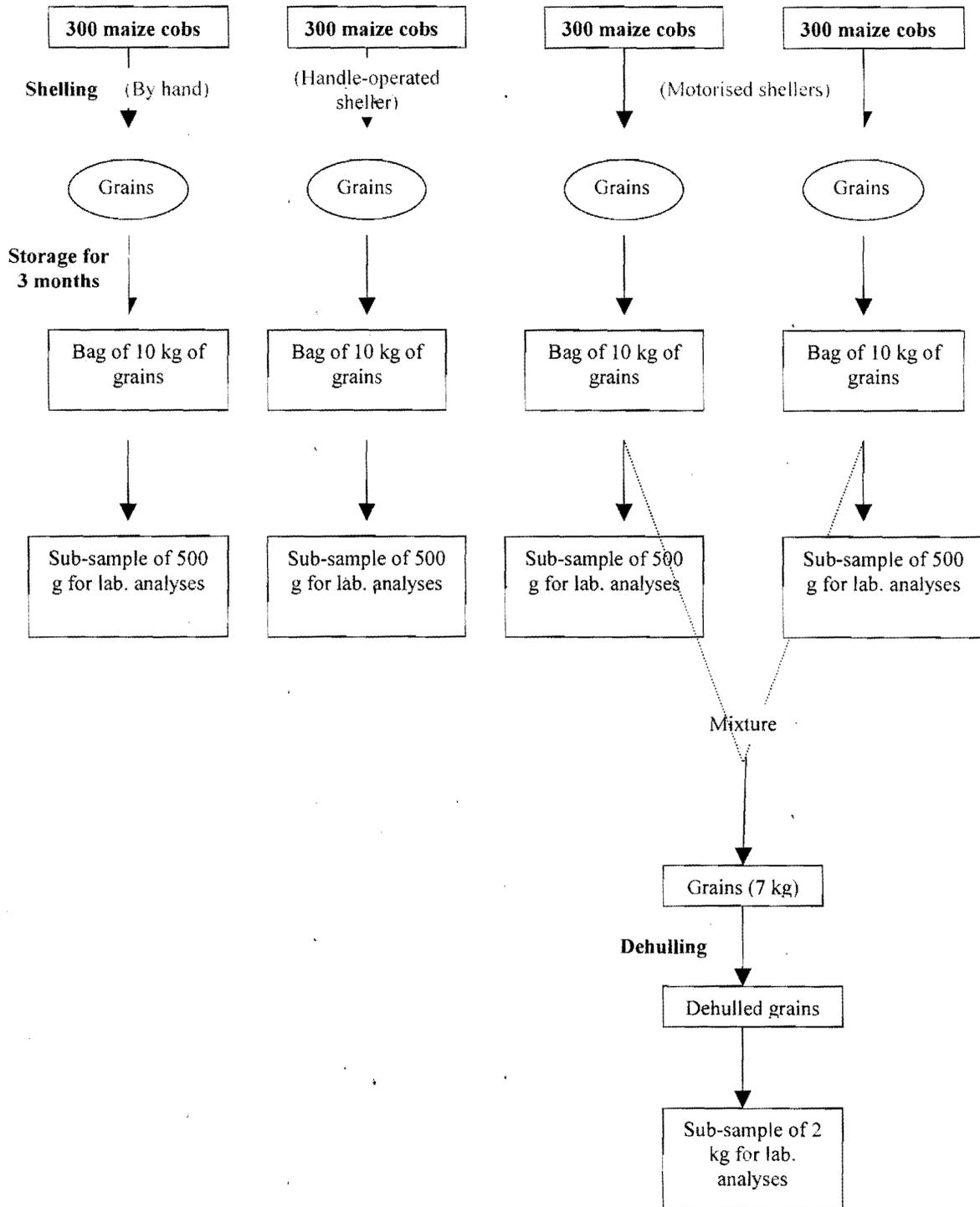


Figure 5: Diagram summarising the methods used during this study



Figure 6: Engelberg motorised dehuller



Figure 7: Mimi-PRL dehuller



Figure 8: An attrition disc mill (Amuda)

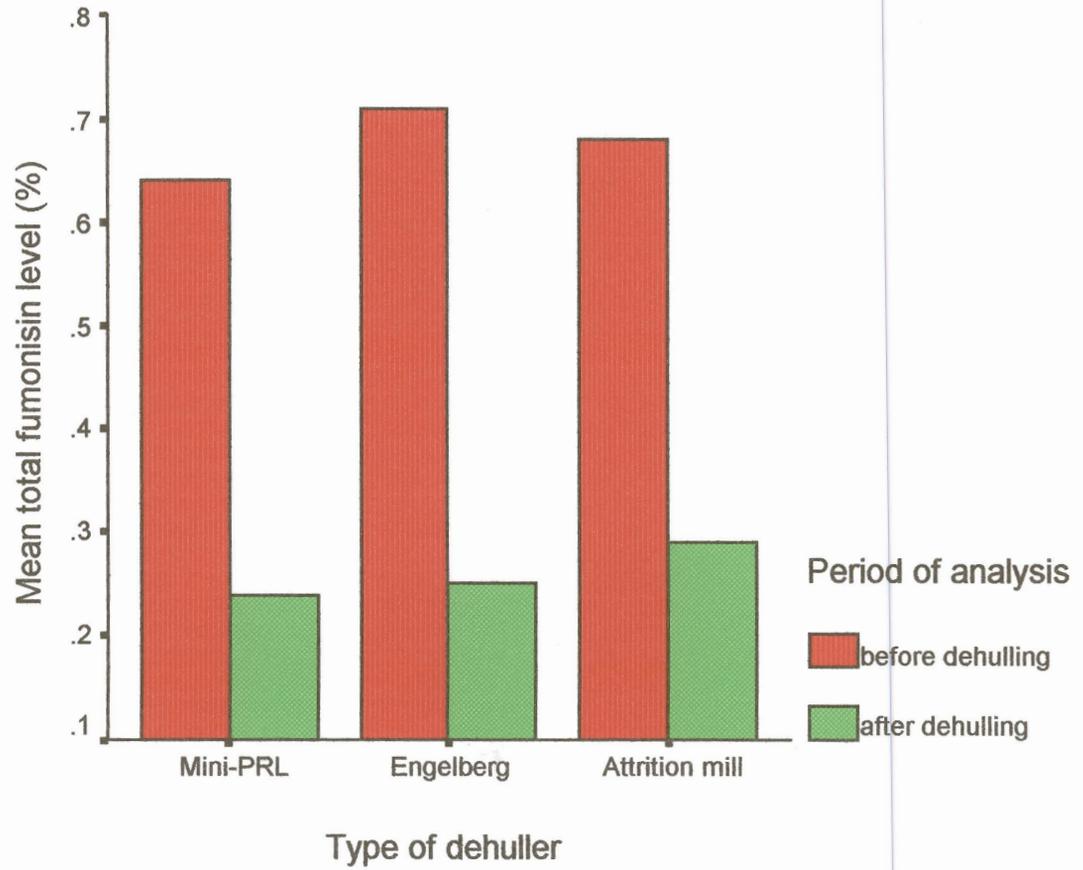


Figure 9: Mean fumonisin level in maize before and after dehulling using different types of dehulling methods



## CHAPTER SIX

**EFFECT OF ESSENTIAL OILS ON DEVELOPMENT OF *FUSARIUM VERTICILLIOIDES* AND FUMONISIN CONTAMINATION IN MAIZE**

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## ABSTRACT

Essential oils extracted by hydrodistillation from local plants in Benin and oil from seeds of the Neem tree (*Azadirachta indica*) were evaluated *in vitro* and *in vivo* for their efficacy against *Fusarium verticillioides* infection and fumonisin contamination. Fumonisin in maize was quantified using a fluorometer and the VICAM method. Oils from *Cymbopogon citratus*, *Ocimum basilicum* and *Ocimum gratissimum* were the most effective *in vitro*, completely inhibiting the growth of *F. verticillioides* at lower concentrations over 21 days of incubation. These oils reduced incidence of *F. verticillioides* by more than 50 % in maize during 21 days of incubation. They totally inhibited fungal growth in maize at concentrations of 8  $\mu\text{l g}^{-1}$ , 6.4  $\mu\text{l g}^{-1}$  and 4.8  $\mu\text{l g}^{-1}$ , respectively. At the concentration of 4.8  $\mu\text{l/g}$ , these oils affected, but not significantly, fumonisin levels in maize stored in closed containers. They adversely affected seed germination at 4.8  $\mu\text{l/g}$ , therefore cannot be recommended for controlling *F. verticillioides* on stored corn used as seeds, when used at this concentration. The oil of Neem seeds showed no inhibitory effect but rather accelerated the growth of *F. verticillioides*.

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**Key words:** Maize, *Fusarium verticillioides*, fumonisin, essential oils, *Cymbopogon citratus*, *Ocimum basilicum*, *Ocimum gratissimum*.

## INTRODUCTION

Essential oils are mixtures of different volatile aromatic compounds extracted by steam or hydrodistillation from plants. Since the discovery of their antifungal and antimicrobial properties, preparations of essential oils have been applied in pharmacology, medical microbiology, phytopathology, and food preservation (Magiatis *et al.* 2002). The use of essential oils to control postharvest fungi and pests is gaining attention because of the increasing public concern over the level of pesticide residues in food (Bishop and Thornton 1997). They are also less likely to be associated with the development of resistance than is the case with fungicides and are less hazardous to environment and human health than synthetic pesticides (Daferera *et al.* 2003).

Essential oils have been used against pre- and postharvest fungi, particularly *Aspergillus flavus* Link (Bishop and Thornton 1997, Thompson and Cannon 1986, Dube *et al.* 1990, Ansari and Shrivastava 1991, Mishra and Dubey 1994, Rahman *et al.* 1999, Soliman and Badeaa 2002, Kritzinger *et al.* 2002). On maize, Chatterjee (1990) tested the efficacy of twelve essential oils to control fungal infection during storage and found that the oils from cassia, clove, anise, geranium and basil inhibit growth of fungi including *A. flavus*. In Nigeria, Adegoke and Odesola (1996) observed that the essential oil from lemongrass (*Cymbopogon citratus* (DC.) Stapf) inhibited growth of *A. flavus*.

Very little research has been conducted on the effect of essential oils against *Fusarium verticillioides* (Sacc.) Nirenberg (previously known as *F. moniliforme* Sheldon) and subsequent fumonisin contamination (Kritzinger *et al.* 2002, Baruah *et al.* 1996, Juglal *et al.* 2002). This fungus is, however, one of the predominant pathogens associated with maize worldwide (Marasas *et al.* 1984). Not only does it cause maize cob and grain rot, it is known to be the most important producer of fumonisin mycotoxins (Munkvold and Desjardins 1997).

Fumonisin are a group of economically important mycotoxins found in maize and maize-based products (Shephard *et al.* 1996). They have been found to be associated with several animal diseases such as leukoencephalomalacia in horses (Kellerman *et al.* 1990) and pulmonary oedema in pigs (Harrison *et al.* 1990). With respect to humans, their occurrence in maize has been associated with high incidences of oesophageal cancer (Rheeder *et al.* 1992, Chu and Li 1994) and liver cancer (Ueno *et al.* 1997).

In light of the concern surrounding exposure to fumonisin, the present study was undertaken to test the antifungal activity of a range of essential oils against the development of *F. verticillioides* and subsequent fumonisin contamination in maize. These oils were

extracted from plants commonly encountered in Benin, West Africa. These plants are used worldwide for multiple purposes. Parts of lantana (*Lantana camara* L.) are commonly utilised for the treatment of itchy skin, ulcers, hepatitis and rheumatism (Bouda *et al.* 2001). The plant has been successfully used as a mosquito deterrent (Adebayo *et al.* 1999), and for the control of insect pests of tropical crops (Bouda *et al.* 2001, Ivens *et al.* 1978).

Leaves of basil (*Ocimum basilicum* L.) are used as flavouring agents in foods and beverages. The plant is traditionally used for its carminative, stimulant, and antispasmodic activity (Marotti *et al.* 1996), and possesses antimicrobial and insecticidal properties (Prasad *et al.* 1985, Keita *et al.* 2001). The African basil (*Ocimum gratissimum* L.) is commonly involved in treatment of several diseases such as respiratory infections, diarrhoea, headaches, ophthalmic and skin diseases, pneumonia, coughs, fever and conjunctivitis (Onajobi 1986, Nakamura *et al.* 1999). Dried leaves of lemongrass (*C. citratus*) are used for tea production (Plantstogo 2002) and the oil from it is an insect repellent, antiseptic, and diaphoretic and is used against cough, cuts, asthma, headache, indigestion pain, rheumatism, acne, nervousness, and stress (Tropilab 2002).

The oil of the Niaouli tree (*Melaleuca quinquenervia* Cav.) possesses disinfectant properties, is used to fortify the body against infections, to promote digestion and for skin care (Anonymous 2002). The lemon eucalyptus (*Eucalyptus citriodora*) has been involved in treatment of maladies including asthma, laryngitis, rheumatism, and possesses an excellent insect repellent property (Anonymous 2003). The African pepper (*Xylopia aethiopica* (Dunal) A. Rich.) is commonly used in West Africa to prepare soups and parts of this plant are used as a cough-medicine, calmative, purgative and in rheumatism treatment (Acquaye *et al.* 2002). Horsewood (*Clausena anisata* (Willd.) Hook. f. ex Benth.) is used as a mosquito repellent (Watt and Breyer-Brandwijk 1962) and against stored product pests (Tapondjou *et al.* 1999). Extracts from leaves of the Neem tree (*Azadirachta indica* A. Juss) have antipyretic, analgesic, anti-inflammatory and anti-aggregatory activities in malaria therapy (Iwalewa *et al.* 1999). Oil extracted from Neem seeds is used to control storage insect pests (Lale and Abdurahman 1999).

## MATERIALS AND METHODS

### *Source of oils*

Oils used in this study were extracted from parts of nine plant species collected during the dry season from different areas of Southern Benin (Table 1). Some plant materials such as dry seeds of Neem and pods of *X. aethiopica* were purchased from a market. Voucher specimens of these plants were deposited at the National Herbarium of Benin, University of Abomey-Calavi, Benin, where they were numbered (Table 1). After collection, the leaves were dried for four days in the laboratory at room temperature (25 – 30 °C) before oil extraction.

### *Oil extraction*

Essential oils were extracted by hydrodistillation for 3 h using a Clevenger apparatus (Clevenger 1928). Oil from Neem seeds was extracted using the soxhlet method, with hexane as solvent. Oil yields obtained after extraction are shown in Table 1. The extracted oils were dried with anhydrous sodium sulphate and stored at 4 °C before use.

### *Chemical analyses of the extracted oils*

The essential oils were analysed by capillary gas chromatography (GC) coupled with gas chromatography-mass spectrometry (GC-MS). GC analysis was carried out on a Trace GC ThermoQuest equipped with a DB-5 column (30 m x 0.25 mm, 0.25 µm film thickness). Oven temperature was maintained at 50 °C for 5 min and then programmed to 300 °C at a rate of 5 °C/min. Injector (splitless) and detector (Flame Ionisation Detector) were maintained at 240 °C and 250 °C respectively. The carrier gas was hydrogen with a flow rate of 35 ml/min and the combustion gas was dry air with a flow rate of 350 ml/min.

GC-MS analysis was performed using a Hewlett Packard 5970 GC fitted with a DB-1 column (25 m x 0.23 mm). An electron ionisation system was used with ionisation energy of 70 eV. The carrier gas was helium applied at a rate of 0.9 ml/min. Column temperature was initially maintained at 50 °C for 5 min, then gradually increased to 180 °C at a rate of 3 °C/min, and finally increased to 250 °C and maintained at this temperature for 5 min. Samples

of 1  $\mu\text{l}$  of oil diluted in 5 % pentane were injected. Compounds in oil were identified on the basis of both their Kovats indices and mass spectral fragmentation.

### *Fungal culture*

The culture of *F. verticillioides* used in this study was obtained from a sample of maize collected during a survey of farmers' stores in southern Benin in 2002. A culture of this fungus has been deposited in the culture collection of the PROMEC Unit of the Medical Research Council (MRC), Tygerberg, South Africa as MRC 8515.

### *Antifungal activity of oils in vitro*

A 7-day old culture of *F. verticillioides* MRC 8515 was initially prepared on potato dextrose agar (PDA) at 25 °C, exposed to a 12:12-hour light/dark regime. Oils were tested at different concentrations of 0.7, 1.3, 2.0, 2.7, 3.3, 4.0, 4.7, 5.3, 6.0, 6.7 and 13.3  $\mu\text{l ml}^{-1}$  of PDA to control growth of *F. verticillioides*. These concentrations were obtained by mixing 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 200  $\mu\text{l}$  of each oil with 15 ml of melted sterile PDA, respectively. The mixture of oil (at each concentration) and PDA was poured into ten Petri dishes (90-mm diameter). The Petri dishes were inoculated by placing a 5 mm diameter disk from a 7-day old *F. verticillioides* culture at the centre of each dish. The inoculated dishes were incubated for 21 days at 25 °C exposed to a 12:12-hour light/dark regime. Ten Petri dishes containing PDA with no oil were inoculated to serve as controls. Fungal growth was assessed by measuring colony diameters along two lines at right angles to each other after 7, 14 and 21 days.

### *Antifungal activity of oils in vivo*

A conidial suspension of *F. verticillioides* was initially prepared by adding 5 ml of sterile distilled water to a 7-day old culture. The culture was superficially scraped to free the conidia from conidiophores. The conidial suspension was filtered through a muslin cloth into 100 ml flasks. One hundred autoclaved maize grains (25 g of grain) were artificially inoculated by adding 1 ml of the conidial suspension ( $1 \times 10^6$  conidia/ml) to the grain and mixing thoroughly for 10 min. The grains were allowed to dry for 15 min.

Essential oils from *C. citratus*, *O. basilicum* and *O. gratissimum*, which were most effective against *F. verticillioides* in the *in vitro* assay, were tested at concentrations of 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6, 6.4, 7.2 and 8.0  $\mu\text{l g}^{-1}$  of maize grain. These concentrations were obtained by adding 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200  $\mu\text{l}$  of each oil to 25 g of maize grains (100 grains). Each volume of oil was diluted in 100  $\mu\text{l}$  of 95 % ethanol to enhance their solubility. Sterile distilled water (1 ml) was added to the diluted oil and mixed thoroughly. The lots of inoculated grains were treated with each oil concentration, just after drying by soaking them in the diluted oil and thoroughly mixing them for 10 min. They were again allowed to dry for approximately 15 min, and plated onto PDA in Petri dishes.

Five grains were plated on each of ten Petri dishes per oil concentration with two replications. The Petri dishes were incubated at 25 °C exposed to a 12:12-hour light/dark regime. Antifungal activity of oils was assessed by evaluating fungal incidence after 7, 14 and 21 days. This evaluation was done by counting the number of grains giving rise to *F. verticillioides* colonies in each Petri and calculating the percentage of infected grains. Two control treatments were used. The first was maize grains inoculated with *F. verticillioides* and treated with a solution of 100  $\mu\text{l}$  of 95 % ethanol + 1 ml of sterile distilled water. The second was maize grains inoculated with *F. verticillioides* with no further treatment.

#### *Effect of essential oils on fumonisin production*

Three replicate samples of 100 g maize grains, artificially inoculated with *F. verticillioides* as described above, were separately treated with the oils from *C. citratus*, *O. basilicum* and *O. gratissimum*, diluted in 100  $\mu\text{l}$  of 95 % ethanol, at 4.8  $\mu\text{l g}^{-1}$  of oil concentration. Grain treatment with oil was performed as described for the *in vivo* test. The treated grains were stored for 6 weeks in a laboratory at room temperature (25 – 30 °C), either in open 200 ml flasks or in closed 200 ml flasks with screw top lids. Total fumonisin content was assessed in grains stored in open and closed flasks at 0, 3 and 6 weeks of storage with a fluorometer using the VICAM method (VICAM 1998).

#### *Effect of essential oils on germination*

This test was performed to determine whether oils at the concentration of 4.8  $\mu\text{l/g}$  have an effect on germination of corn kernels. Four replicates of 25 kernels (25 kernels weighed approximately 6.25 g) were separately treated with the oils from *C. citratus*, *O. basilicum* and

*O. gratissimum* at the concentration of 4.8 µl/g. This concentration was obtained by using 30µl of oil for treating 25 kernels. Before treatment, oil (30 µl) was diluted in 25 µl of 95% ethanol and thoroughly mixed to 25 ml of sterile distilled water, based on the method used by Kritzinger *et al.* (2002). The treated kernels were allowed to germinate in Petri dishes on double-layered wetted Whatman No 1 filter paper. Two controls were used. The first of these consisted of sound corn kernels treated with the same quantities of 95% ethanol and sterile distilled water, whereas the second was sound kernels that received no further treatment. Petri dishes containing five kernels each were incubated at  $\pm 25^{\circ}\text{C}$ . Percentage of germinated kernels per treatment was determined after 7 days.

### *Statistical analyses*

Statistical analyses were performed using SPSS for Window version 10.0 (SPSS Inc., Chicago, Illinois). Analysis of variance (ANOVA) and Tukey HSD test were used to compare the means of the growth diameter of *F. verticillioides*, the means of fungal incidence, the means of total fumonisin levels measured in maize treated with the oils, and the means of percentage germinated grains.

## **RESULTS**

### *Chemical composition of essential oils*

GC analysis revealed that several compounds were present in the essential oils of which monoterpenes were predominant (Table 2). Oil from *C. citratus* mainly contained citral (neral and geranial) (47 %) and myrcene (28 %). That from *O. basilicum* was richer in the alcohols linalol (33 %), eugenol (22 %) and estragol (20 %). Oil from *O. gratissimum* predominantly contained p-cymene (22 %) and its precursor  $\gamma$ -terpinene (15 %), and thymol (17 %). The major compounds detected in the oil from *L. camara* were the sesquiterpenes  $\beta$ -caryophyllene (32 %) and  $\alpha$ -humulene (11 %). Oil from *E. citriodora* contained mainly citronellal (66 %) along with a small amount of the alcohol citronellol (12 %). Estragol was the major compound identified in *C. anisata* oil (up to 83 %), whereas that found in *M. quinquenervia* oil was the oxide 1-8 cineol (51 %). Oil from *X. aethiopica* mainly contained the terpinoid sabinene (30 %).

### *Antifungal activities of oils*

With the exception of *M. quinquenervia* and *X. aethiopica*, all of the essential oils tested were found to significantly affect the growth of *F. verticillioides* after 7 days of incubation ( $p < 0.01$ ) (Tables 3 and 6). They fully inhibited growth of *F. verticillioides* at concentrations of less than 2.7  $\mu\text{L/ml}$ . This inhibitory effect, however, did not last in most cases and significantly decreased with time ( $p < 0.01$ ) as the fungus started to grow after 7 days of incubation (Table 4). Only the oils from *C. citratus*, *O. basilicum* and *O. gratissimum* remained effective over the 21 day test period (Table 5), with minimal inhibitory concentrations (MIC) of 1.3  $\mu\text{l ml}^{-1}$ , 1.3  $\mu\text{l ml}^{-1}$  and 2.0  $\mu\text{l ml}^{-1}$ , respectively (Table 6).

As oil concentration increased, inhibitory effect of the oils, also, increased (Tables 3, 4 and 5). Oils from *L. camara* and *E. citriodora*, though less effective at lower concentrations, gave rise to complete inhibition of fungal growth when their concentration increased to more than 4.0  $\mu\text{l ml}^{-1}$  and 4.7  $\mu\text{l ml}^{-1}$ , respectively. The Neem oil, which is not an essential oil, showed no inhibitory effect on fungal growth. In contrast, the growth of *F. verticillioides* was significantly more rapid than in the control ( $p < 0.01$ ) at all concentrations after 7 and 14 days (Tables 3 and 4).

The *in vivo* test showed that the oils from *C. citratus*, *O. basilicum* and *O. gratissimum* all effectively reduced the incidence of *F. verticillioides* in artificially inoculated maize (Table 7, Figures 1 and 2). Over 21 days, mean fungal incidence was maintained at less than 40 % (Figure 1), ranging from 34.8 % to 39.8 %, 20.6 % to 31.6 % and 34.2 % to 38.4 % for the oils from *C. citratus*, *O. gratissimum* and *O. basilicum*, respectively. The inhibitory effect was more marked at higher concentrations (Figure 2). At 4.0  $\mu\text{l g}^{-1}$ , the oils significantly reduced the fungal incidence to approximately 10 %. Complete inhibition occurred at 8.0  $\mu\text{l g}^{-1}$  for *C. citratus*, 6.4  $\mu\text{l g}^{-1}$  for *O. basilicum*, and 4.8  $\mu\text{l g}^{-1}$  for *O. gratissimum* (Table 7). The antifungal activity of the three oils did not differ significantly ( $p > 0.05$ ), but their effect was significantly different from that of the controls ( $p < 0.01$ ) (Figure 2). The 95 % ethanol used alone as control for treating the grains did not show any antifungal activity.

### *Effect of essential oils on fumonisin production*

At 4.8  $\mu\text{l g}^{-1}$ , the three oils affected, but not statistically significantly, fumonisin production in maize ( $p > 0.05$ ). The reduction of fumonisin level observed was marked when

maize was stored in closed containers (Figure 3). In open storage conditions, however, this reduction was quite low (Figure 3). In closed storage conditions, the oils maintained the fumonisin level in maize at very low levels (about  $0.7\text{mg kg}^{-1}$ ) throughout the 6-week storage period, compared to the control in which the level gradually increased (Figure 4). In contrast, in open storage conditions, fumonisin levels were higher and significantly increased in time in all cases ( $p < 0.01$ ) (Figure 4).

#### *Effect of essential oils on germination*

At the concentration of  $4.8\ \mu\text{l/g}$ , the oils from *C. citratus*, *O. gratissimum*, and *O. basilicum* adversely affected significantly the germination of kernels ( $p < 0.01$ ) (Table 8). The percentage of germinated maize grains was low, ranging from 34 to 57 % when these grains were treated with the essential oils. *C. citratus* and *O. gratissimum* oils exhibited more harmful effects on germination than *O. basilicum* oil. All the grains germinated where they did not receive any treatment. The 95 % ethanol used for diluting the oils did not significantly affect grain germination compared to the control ( $p > 0.05$ ). Overall, there was a significant difference between the control and all other treatments ( $p < 0.05$ ) (Table 8).

## DISCUSSION

In this study, it has been shown that essential oils from plants commonly found in Benin are extremely effective in reducing the growth of *F. verticillioides*. This is one of the most important contaminants of maize in the country and is associated with high levels of the carcinogenic mycotoxin fumonisin in stored maize. Of the eight essential oils tested, only three, *C. citratus*, *O. basilicum* and *O. gratissimum*, were found to be effective for controlling *F. verticillioides*. They had a potent inhibitory effect on the growth of *F. verticillioides*, exhibiting a strong fungicidal activity over 21 days of incubation. They also markedly reduced the fungal incidence in maize inoculated with *F. verticillioides*. Adegoke and Odesola (1996) also found that the oil from *C. citratus* is very effective in controlling several fungi including *A. flavus*. Mishra and Dubey (1994) found this oil to be more effective than ten synthetic fungicides in controlling *A. flavus*, and its fungitoxic potency remained unaltered for at least 7 months of storage. The oil from *O. basilicum* has also been found to significantly reduce the growth of pathogenic fungi including *F. verticillioides* (Soliman and Badeaa 2002). Owolade *et al.* (2000) tested five aqueous plant extracts against *F. verticillioides*, and found that their

extract from *O. gratissimum* completely inhibited growth of the fungus. These results thus add further support for the view that essential oils could be effectively used for controlling pathogenic fungi.

The oils from *L. camara* and *E. citriodora* used in this study displayed only moderate inhibition of *F. verticillioides* growth, followed by those from *C. anisata* and *M. quinquenervia*. Baruah *et al.* (1996) testing four essential oils against *F. verticillioides*, observed that the oil from *E. citriodora* was less effective than the oil from *Cymbopogon* spp. As for the oil from *L. camara*, it has been reported to show strong insecticidal activities against the stored maize insect pest *Sitophilus zeamais* (Bouda *et al.* 2001) and the malaria mosquito (Adebayo *et al.* 1999). The oil from *X. aethiopica* used in this study exhibited no significant effect except at concentrations higher than 13.3  $\mu\text{l ml}^{-1}$ . Cardwell and Dongo (1994) found the aqueous plant extract from *X. aethiopica* used alone to have no fungitoxicity. However, combined with the extract from dried seeds of *Piper guineense* Schum. & Thonn, it totally inhibited growth of several fungi, even more effectively than the aqueous extracts from *O. basilicum* and *O. gratissimum*.

There is increasing evidence that specific compounds found in the oils may play an important role in their antifungal activities. This was shown with compounds such as eugenol (Juglal *et al.* 2002), p-cymene (Soliman and Badeaa 2002), thymol (Soliman and Badeaa 2002, Zambonelli *et al.* 1996), linalol (Zambonelli *et al.* 1996), and caryophyllene oxide (Magiatis *et al.* 2002). In an *in vitro* assay of the oil from *O. gratissimum* against bacteria, Nakamura *et al.* (1999) identified eugenol as the compound in the oil that was responsible for its antibacterial activity. Soliman and Badeaa (2002) related the antifungal effect of the oil from *O. basilicum* to its component  $\beta$ -pinene. According to Bishop and Thornton (1997), terpenoid phenolic and non-phenolic alcohols are the most bioactive to fungi. Similar compounds were found in significant amounts in the most effective oils tested in the present study. The oil from *O. basilicum*, used in this study contained mainly linalol (33 %) and eugenol (22 %). That from *O. gratissimum* not only was rich in the phenolic alcohol thymol (17 %), but also in its precursors p-cymene (22 %) and  $\gamma$ -terpinene (15 %).

The lower efficacy of the oils from *M. quinquenervia*, *X. aethiopica* and *L. camara* in controlling the development of *F. verticillioides* in the present study might have been due to the low activity of their main compounds against this fungus. The oil from *M. quinquenervia* for example contains 51 % of 1-8 cineol, which has been found by other researchers to be less active against fungi (Rahman *et al.* 1999, Juglal *et al.* 2002).

It is likely that the antifungal effects of the essential oils result from the synergistic action of all their components (Dubey and Kishore 1987). Although the major compounds contained in oil are mostly considered to be mainly responsible for their antifungal activity, the minor compounds may also play an important role. The synergistic or antagonistic effect of the latter may significantly influence the antifungal action of the former (Daferera *et al.* 2003). Such synergistic or antagonistic action probably occurred with the oils tested in the present study.

Regarding the oil from Neem seeds, it did not show any inhibitory effect, but rather accelerated the growth of *F. verticillioides*. Juglal *et al.* (2002) found the Neem oil did not affect the growth of *F. verticillioides in vitro*, but their observations were made only after 48 h. Oil extracted from Neem seeds is well known to be more effective in controlling storage insect pests (Lale and Abdurahman 1999). Carpinella *et al.* (2003), however, found extracts from parts of *Melia azedarach* L., a tree close to the Neem, both belonging to the Meliaceae family, to show fungicidal activity on *F. verticillioides*.

At the concentration of  $4.8 \mu\text{l g}^{-1}$ , the oils from *C. citratus*, *O. gratissimum*, and *O. basilicum* were found to reduce, but not statistically significantly, the levels of fumonisin in artificially inoculated maize during storage. However, a marked reduction of fumonisin level, due to oil treatment, was observed when the maize was stored in closed containers. These oils consist of volatile compounds that are more likely to diffuse rapidly in air when the storage container is open. In such open storage conditions therefore, they would be ineffective in controlling fungal growth and fumonisin contamination. In closed conditions, however, the compounds will not diffuse in air and would remain active. The fact the oils did not show very significant action would be due to the concentration at which they have been tested. Soliman and Badeaa (6), testing oils from thyme, anise, cinnamon and spearmint against fumonisin production, observed a gradual increase in inhibition due to increased concentration of the oils. The oils from *C. citratus*, *O. gratissimum*, and *O. basilicum* would be significantly effective on fumonisin production if they were used at concentrations higher than  $4.8 \mu\text{l/g}$ . On the other hand, surprisingly, there was no significant difference between the oil treatments and the control (no oil treatment) when the maize was stored in closed conditions. This might be because of the significant variability in the fumonisin levels measured in the three replicated controls ( $0.52 \text{ mg kg}^{-1}$ ,  $1.40 \text{ mg kg}^{-1}$  and  $2.40 \text{ mg kg}^{-1}$ ). The control would be significantly different from the oil treatments if the fumonisin level detected in one of the control ( $0.52 \text{ mg kg}^{-1}$ ) was not closer to the mean fumonisin level detected in the treated grains ( $0.57 \text{ mg kg}^{-1}$ ).

At the concentration of  $4.8 \mu\text{l g}^{-1}$ , the oils from *C. citratus*, *O. gratissimum*, and *O. basilicum* were effective against contamination of maize with both *F. verticillioides* and fumonisin, but drastically reduced germination of treated grains to a non-acceptable level (less than 60%). The oils probably killed the embryos, which consequently could not grow. These findings suggest that, at the concentration of  $4.8 \mu\text{l/g}$ , the three oils should be recommended only for treatment of grains intended for consumption and not grains to be used as seeds.

The major components in the essential oils involved in this study and their relative incidence may change depending on several factors such as region of collection, climate, soil, time of harvest, or the plant part used for extraction. For example, *O. basilicum* used in this study is different from that grown in Europe as its oil contains more eugenol (22 %) and estragol (20 %) than the European French basil, which is likely to contain rather more cineol and linalol (Katzer 2001a). Charles and Simon (1993) studied the relationship between leaf development and essential oil content and composition in *O. gratissimum*. They found that percentage of geraniol, the main component in the oil, increased from 51.6 % in the very young leaves to 73.3 % in the mature leaves and then decreased to 64.2 % in senescing leaves. The chemotype of *C. citratus* used in this study contains less of the aldehyde geranial (27 %) than that grown in India (40 – 62 %), and the fact that it is richer in myrcene (28 %) is likely to make it highly susceptible to oxidative polymerisation, therefore would shorten its shelf life (Katzer 2001b).

Comparing the effect of essential oil treatment on fumonisin production in maize to the findings reported in Chapter 3 that a decrease in fumonisin contamination was found under typical storage conditions, one might wonder whether it is necessary to use essential oil to reduce fumonisin production as the toxin level decreases during maize storage. In fact, this decrease observed in maize during storage was not significant every year as found and mentioned in Chapter 2. An increasing trend was even observed during some seasons. This phenomenon might not occur every year as it mainly depends on the environmental conditions, but further studies are needed for confirming these findings. It is believed, however, that combining these two aspects to control fumonisin contamination in maize would be very helpful for the small-scale farmers.

This study has demonstrated that essential oils, already commonly used in many parts of the world including Africa for medical purposes, can also serve as alternative means to reduce the growth of *F. verticillioides* and fumonisin contamination in stored maize. Oils from *C. citratus*, *O. basilicum* and *O. gratissimum* proved to be highly effective and can be recommended to small-scale farmers. Practically, these oils can be used on maize stored in

closed conditions, but only on stored maize intended for human or animal consumption, and not on maize to be used as seeds as they affect germination. Despite their efficacy and usefulness, the use of the essential oils is, however, limited by several factors. The plants from which they are extracted are being progressively destroyed by extensive agriculture particularly in Africa. Moreover, most of the current extraction procedures are complicated and costly, particularly to produce sufficient amounts of oil to render their utilization economical. There is an urgent need to protect the plants that produce essential oils with potent fungicidal activity and to promote their cultivation. Research must also continue to find extraction procedures, which are simple, inexpensive and easily performed on small-scale farms.

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Table 1: Plants and plant parts used for oil extraction and mean oil yield

Scientific name of plants	Common name (English)	Registration number <sup>(2)</sup>	Plant parts	Essential oils yield (%)	
				Range	Mean
<i>Cymbopogon citratus</i>	Lemongrass, citronella	AAC 173 HNB	Leaves	0.5 – 1.3	0.9
<i>Ocimum basilicum</i>	Basil, sweet basil	AAC 175 HNB	Leaves	0.2 – 1.2	0.7
<i>Ocimum gratissimum</i>	African basil	AAC 176 HNB	Leaves	0.2 – 1.2	0.7
<i>Lantana camara</i>	Lantana, shrub verbena	AAC 174 HNB	Leaves	0.2 – 1.2	0.7
<i>Eucalyptus citriodora</i>	Lemon eucalyptus	AAC 181 HNB	Leaves	2.8 – 6.0	4.4
<i>Clausena anisata</i>	Horsewood, Clausena	AAC 177 HNB	Leaves	1.2 – 2.0	1.6
<i>Melaleuca quinquenervia</i>	Niaouli	AAC 179 HNB	Leaves	1.2 – 3.6	2.4
<i>Xylopia aethiopica</i>	African pepper	AAC 180 HNB	Fruits (pods + seeds)	3.0 – 6.0	4.5
<i>Azadirachta indica</i> <sup>(1)</sup>	Neem	AAC 178 HNB	Seeds	-	-

(1) The oil of *Azadirachta indica* is not an essential oil

(2) These numbers are the registration numbers given to each specimen or part of plant when they were deposited at National Herbarium of Benin, University of Abomey – Calavi, Benin.

Table 2: Major components in tested plant oils (%)

Oils	Total number of identified compounds	% Oxygenated compounds	Major components	Percentage	Kovats retention indice
<i>Cymbopogon citratus</i>	16	61	Myrcene	28	995
			Neral (citral B)	20	1254
			Geranial (citral A)	27	1283
			Geraniol	4	1268
<i>Ocimum basilicum</i>	24	87	Linalol	33	1098
			Eugenol	22	1356
			Estragol	20	1193
			Trans- $\alpha$ -bergamotene	5	1431
			Terpinen-4-ol	4	1176
<i>Ocimum gratissimum</i>	29	27	P-cymene	22	1030
			$\gamma$ -terpinene	15	1063
			Thymol	17	1303
			$\alpha$ -thujene	5	930
			Myrcene	4	995
			Caryophyllene Oxide	3	1591
			1-8 cineol	2	1045
<i>Lantana camara</i>	23	37	$\beta$ -caryophyllene	32	1442
			$\alpha$ -humulene	11	1476
			$\gamma$ -cadinene	7	1519
			1-8 cineol	6	1051
			spathulenol	6	1580
			$\gamma$ -epi-eudesmol	5	1606
			$\beta$ -eudesmol	4	1647
			$\alpha$ -phellandrene	2	1042
			<i>Eucalyptus citriodora</i>	15	86
Citronnellol	12	1241			
Citronnelyl acetate	4	1362			
Isopulegol	3	1170			
<i>Clausena anisata</i>	17	83	Estragol	93	1207
<i>Melaleuca quinquenervia</i>	26	77	1-8 cineol	51	1045
			$\alpha$ -terpineol	11	1205
			Viridiflorol	10	1611
			Spathulenol	3	1574
<i>Xylopi aethiopica</i>	31	20	Sabinene	30	978
			$\beta$ -pinene	8	981
			Germacrene D	8	1492
			Terpinen-4-ol	7	1191
			1-8 cineol	6	1048
			Linalol	2	1116
			$\alpha$ -terpineol	2	1205
<i>Azadirachta indica</i>	-	-	-	-	-

(1) The oil of *Azadirachta indica* is not an essential oil

Table 3: Effect of plant oils on growth of *F. verticillioides* after 7 days of incubation in an *in vitro* test

Oils	Mean fungal colony diameter (mm)										
	0.7 $\mu\text{ml}^{-1}$ conc.	1.3 $\mu\text{ml}^{-1}$ conc.	2.0 $\mu\text{ml}^{-1}$ conc.	2.7 $\mu\text{ml}^{-1}$ conc.	3.3 $\mu\text{ml}^{-1}$ conc.	4.0 $\mu\text{ml}^{-1}$ conc.	4.7 $\mu\text{ml}^{-1}$ conc.	5.3 $\mu\text{ml}^{-1}$ conc.	6.0 $\mu\text{ml}^{-1}$ conc.	6.7 $\mu\text{ml}^{-1}$ conc.	13.3 $\mu\text{ml}^{-1}$ conc.
<i>Cymbopogon citratus</i>	2 b	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Ocimum basilicum</i>	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Ocimum gratissimum</i>	3 c	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Lantana camara</i>	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Eucalyptus citriodora</i>	9 e	3 b	2 b	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Clausena anisata</i>	4 d	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Melaleuca quinquenervia</i>	24 f	17 c	11 c	10 b	7 b	0 a	0 a	0 a	0 a	0 a	0 a
<i>Xylopi aethiopica</i>	39 g	37 d	30 d	26 c	22 c	20 b	20 b	18 b	15 b	14 b	1 b
<i>Azadirachta indica</i>	75 h	75 f	76 f	68 e	62 e	60 d					
Untreated control	39 g	39 e	39 e	39 d	39 d	39 c					

Means within a column followed by the same letter are not significantly different ( $P > 0.05$ )

Table 4: Effect of plant oils on growth of *F. verticillioides* after 14 days of incubation in an *in vitro* test

Oils	Mean fungal colony diameter (mm)										
	0.7 $\mu\text{ml}^{-1}$ conc.	1.3 $\mu\text{ml}^{-1}$ conc.	2.0 $\mu\text{ml}^{-1}$ conc.	2.7 $\mu\text{ml}^{-1}$ conc.	3.3 $\mu\text{ml}^{-1}$ conc.	4.0 $\mu\text{ml}^{-1}$ conc.	4.7 $\mu\text{ml}^{-1}$ conc.	5.3 $\mu\text{ml}^{-1}$ conc.	6.0 $\mu\text{ml}^{-1}$ conc.	6.7 $\mu\text{ml}^{-1}$ conc.	13.3 $\mu\text{ml}^{-1}$ conc.
<i>Cymbopogon citratus</i>	5 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Ocimum basilicum</i>	34 d	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Ocimum gratissimum</i>	20 b	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Lantana camara</i>	33 c	27 c	25 c	22 d	20 d	0 a	0 a	0 a	0 a	0 a	0 a
<i>Eucalyptus citriodora</i>	64 g	22 b	10 b	6 b	5 b	5 b	0 a	0 a	0 a	0 a	0 a
<i>Clausena anisata</i>	62 f	36 d	33 d	20 c	19 c	0 a	0 a	0 a	0 a	0 a	0 a
<i>Melaleuca quinquenervia</i>	65 h	55 e	51 e	48 e	42 e	29 c	25 b	19 b	6 b	0 a	0 a
<i>Xylopiya aethiopica</i>	90 i	90 g	70 g	65 g	46 f	44 d	40 c	37 c	28 c	23 b	3 b
<i>Azadirachta indica</i>	90 i	90 g	90 h	90 h	90 h	90 f	90 e	90 e	90 e	90 d	90 d
Untreated control	57 e	57 f	57 f	57 f	57 g	57 e	57 d	57 d	57 d	57 c	57 c

Means within a column followed by the same letter are not significantly different ( $P > 0.05$ )

Table 5: Effect of plant oils on growth of *F. verticillioides* after 21 days of incubation in an *in vitro* test

Oils	Mean fungal colony diameter (mm)										
	0.7 $\mu\text{ml}^{-1}$ conc.	1.3 $\mu\text{ml}^{-1}$ conc.	2.0 $\mu\text{ml}^{-1}$ conc.	2.7 $\mu\text{ml}^{-1}$ conc.	3.3 $\mu\text{ml}^{-1}$ conc.	4.0 $\mu\text{ml}^{-1}$ conc.	4.7 $\mu\text{ml}^{-1}$ conc.	5.3 $\mu\text{ml}^{-1}$ conc.	6.0 $\mu\text{ml}^{-1}$ conc.	6.7 $\mu\text{ml}^{-1}$ conc.	13.3 $\mu\text{ml}^{-1}$ conc.
<i>Cymbopogon citratus</i>	5 a	0 a	0 a	0a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Ocimum basilicum</i>	46 d	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Ocimum gratissimum</i>	48 c	5 b	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
<i>Lantana camara</i>	45 b	32 c	28 c	23 c	21 c	0 a	0 a	0 a	0 a	0 a	0 a
<i>Eucalyptus citriodora</i>	90 e	33 d	13 b	9 b	4 b	3 b	0 a	0 a	0 a	0 a	0 a
<i>Clausena anisata</i>	90 e	77 f	65 e	67 e	42 d	25 c	20 b	16 b	5 b	0 a	0 a
<i>Melaleuca quinquenervia</i>	90 e	75 e	69 d	60 d	42 d	36 d	32 c	28 c	20 c	0 a	0 a
<i>Xylopiya aethiopica</i>	90 e	90 g	90 f	90 f	77 e	75 e	65 d	60 d	55 d	42 b	8 b
<i>Azadirachta indica</i>	90 e	90 g	90 f	90 f	90 f	90 f	90 e	90 e	90 e	90 c	90 c
Untreated control	90 e	90 g	90 f	90 f	90 f	90 f	90 e	90 e	90 e	90 c	90 c

Means within a column followed by the same letter are not significantly different ( $P > 0.05$ )

Table 6: Minimum inhibitory concentration (MIC) of plant oils on *F. verticillioides* at 7, 14 and 21 days of incubation in an *in vitro* test

Oils	MIC after 7 days ( $\mu\text{ml}^{-1}$ )	MIC after 14 days ( $\mu\text{ml}^{-1}$ )	MIC after 21 days ( $\mu\text{ml}^{-1}$ )
<i>Cymbopogon citratus</i>	1.3	1.3	1.3
<i>Ocimum basilicum</i>	0.7	1.3	1.3
<i>Ocimum gratissimum</i>	0.7	1.3	2.0
<i>Lantana camara</i>	1.3	4.0	4.0
<i>Eucalyptus citriodora</i>	2.7	4.7	4.7
<i>Clausena anisata</i>	1.3	4.0	6.7
<i>Melaleuca quinquenervia</i>	4.0	6.7	6.7
<i>Xylopi aethiopica</i>	13.3	> 13.3	> 13.3
<i>Azadirachta indica</i>	not effective	not effective	not effective

Table 7: Minimum inhibitory concentration (MIC) of essential oils on *F. verticillioides* at 7, 14 and 21 days of incubation in an *in vivo* test

Oils	MIC after 7 days ( $\mu\text{lg}^{-1}$ )	MIC after 14 days ( $\mu\text{lg}^{-1}$ )	MIC after 21 days ( $\mu\text{lg}^{-1}$ )
<i>Cymbopogon citratus</i>	8.0	8.0	> 8.0
<i>Ocimum basilicum</i>	4.8	4.8	4.8
<i>Ocimum gratissimum</i>	6.4	6.4	6.4



Table 8: Effect of essential oils on germination of corn kernels

Treatments	% Germinated kernels
Maize treated with <i>Cymbopogon citratus</i>	34 a
Maize treated with <i>Ocimum basilicum</i>	57 ab
Maize treated with <i>Ocimum gratissimum</i>	44 b
Maize treated with ethanol 95% only	77 c
Maize not treated	92 c

Each value is a mean percentage of four replicates of 25 kernels

Means followed by the same letter are not significantly different ( $P > 0.05$ ) according to Student-Newman-Keuls test.

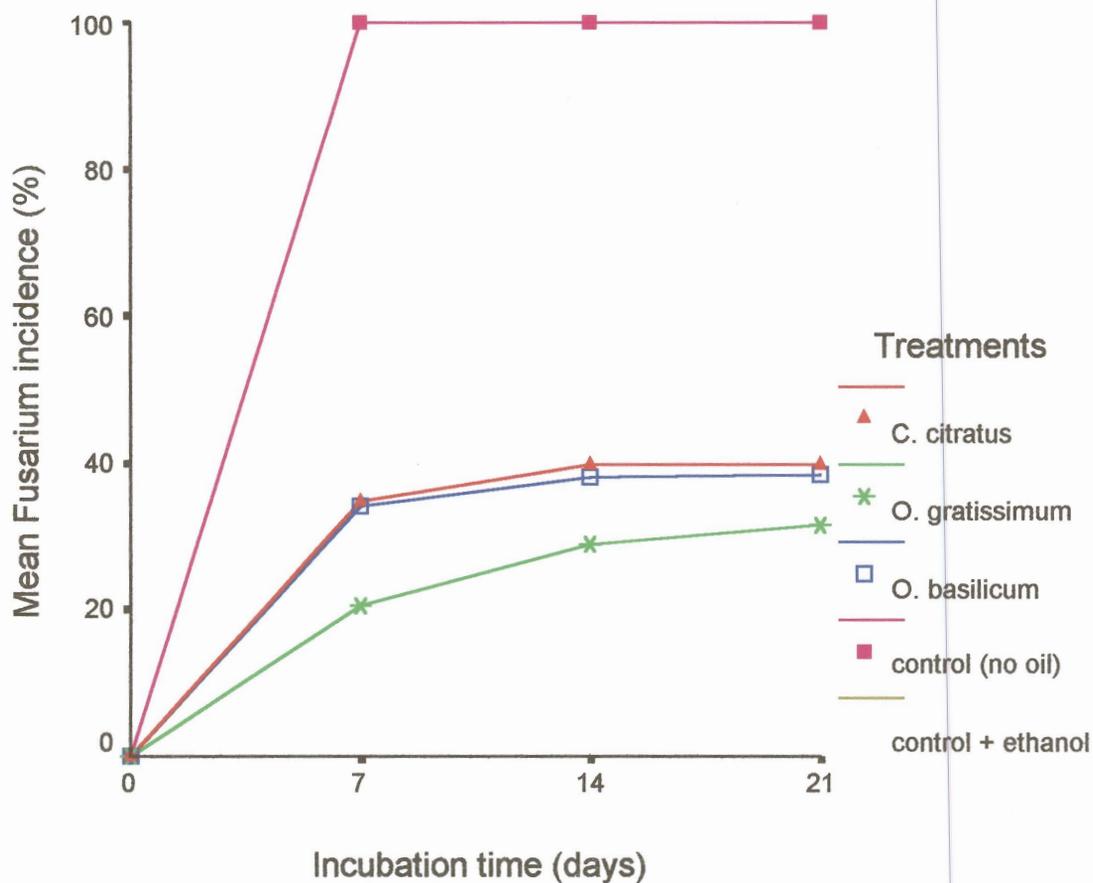


Figure 1: Effect of essential oils on incidence of *F. verticillioides* during the incubation period of artificially inoculated maize grains

*C. citratus* = *Cymbopogon citratus*

*O. gratissimum* = *Ocimum gratissimum*

*O. basilicum* = *Ocimum basilicum*

Control (no oil) = Untreated maize grains

Control + ethanol = Maize grains not treated with oils but with ethanol

The line representing the treatment control + ethanol does not appear on the figure because it is confounded with that of the control (no oil) as these two treatments gave the same results.

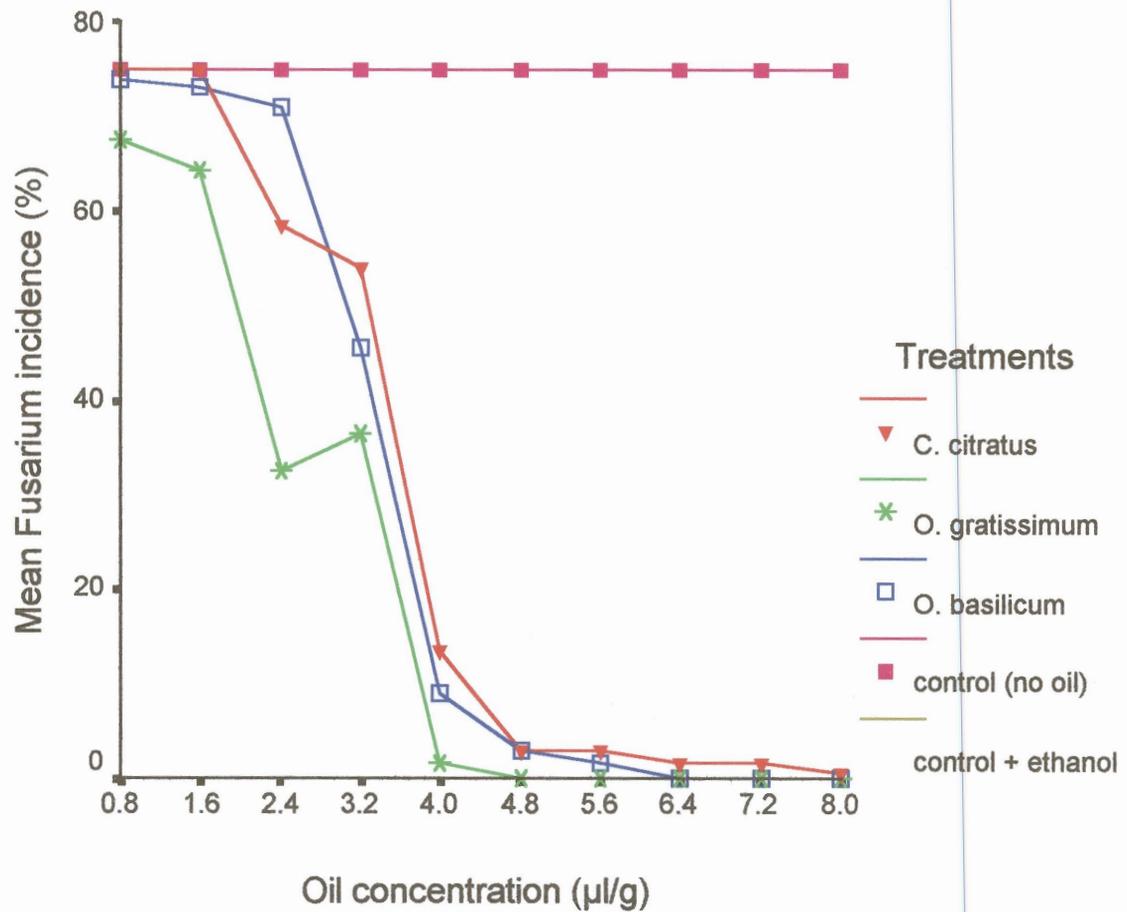


Figure 2: Mean incidence of *F. verticillioides* at different concentrations of essential oils after 21 days of incubation of artificially inoculated maize grains

*C. citratus* = *Cymbopogon citratus*

*O. gratissimum* = *Ocimum gratissimum*

*O. basilicum* = *Ocimum basilicum*

Control (no oil) = Untreated maize grains

Control + ethanol = Maize grains not treated with oils but with ethanol

The line representing the treatment control + ethanol does not appear on the figure because it is confounded with that of the control (no oil) as these two treatments gave the same results.

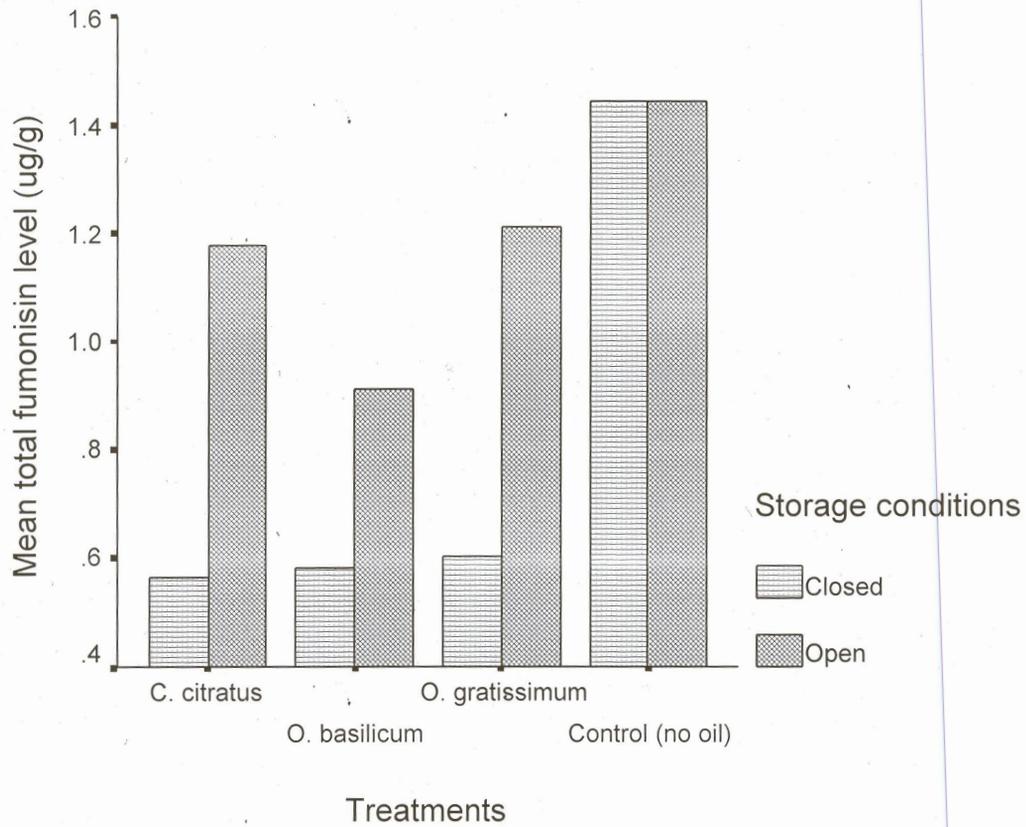


Figure 3: Effect of essential oils on fumonisin production in artificially inoculated maize grains stored in closed and open containers after six weeks of storage

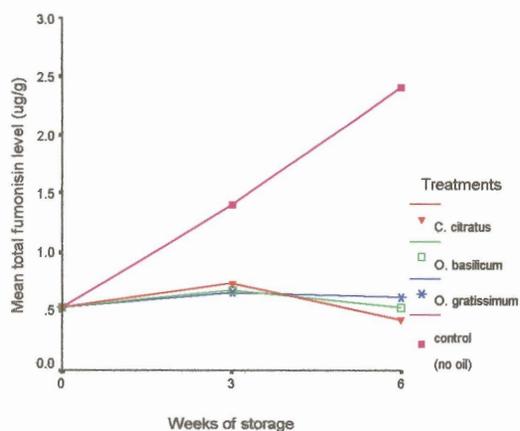
*C. citratus* = *Cymbopogon citratus*

*O. gratissimum* = *Ocimum gratissimum*

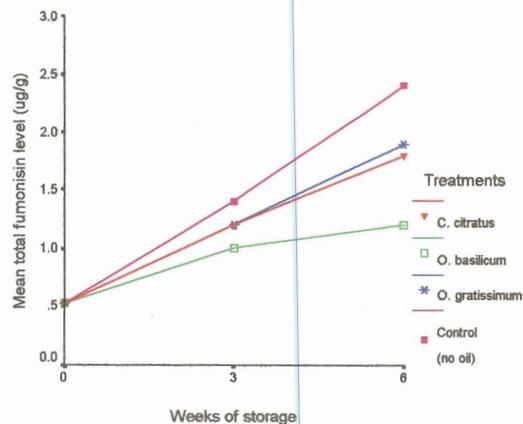
*O. basilicum* = *Ocimum basilicum*

Control (no oil) = Untreated maize grains

Control + ethanol = Maize grains not treated with oils but with ethanol



Closed storage conditions



Open storage conditions

Figure 4: Effect of essential oils on fumonisin production in artificially inoculated maize grains stored in closed and open containers at 0, 3 and 6 weeks of storage

*C. citratus* = *Cymbopogon citratus*

*O. gratissimum* = *Ocimum gratissimum*

*O. basilicum* = *Ocimum basilicum*

Control (no oil) = Untreated maize grains

Control + ethanol = Maize grains not treated with oils but with ethanol

## SUMMARY

Natural occurrence of *Fusarium* and subsequent fumonisin contamination in preharvest and stored maize were investigated through a three-year survey in four different agroecological zones of Benin, West Africa. *Fusarium* was found to be predominant in maize samples. The two *Fusarium* species most frequently isolated were *F. verticillioides* and *F. proliferatum*. Atypical isolates of *F. verticillioides* were also found. Some *F. verticillioides* strains were extremely high fumonisin producers with total fumonisin levels ranging from 8240 to 16690 mg kg<sup>-1</sup>. *Fusarium* occurrence was not significantly different from one zone to another, but varied from year to year, and significantly decreased over the six months of storage. Fumonisin occurrence in maize was widespread and levels were significantly higher in the two southern than the two northern zones. Fumonisin levels varied from one year to another, and decreased throughout the storage time, but not significantly every year.

Impact of four storage systems of maize commonly used in Benin was investigated on *Fusarium* infection and fumonisin contamination. *Fusarium* incidence was significantly higher when maize was stored on a cemented floor in a house. The lowest *Fusarium* incidence was recorded when maize was stored in a bamboo granary. In contrast, the storage systems did not have a significant effect on fumonisin contamination. Damage by lepidopterous insects was significantly and positively correlated with both *Fusarium* infection and fumonisin contamination. Conversely, damage by coleopterous insects was significantly and negatively correlated with *Fusarium* infection and fumonisin contamination.

The fate of aflatoxins and fumonisins was studied through the traditional processing of maize into maize-based foods common in Benin. Mycotoxin reduction occurred and was more significant during the preparation of *makume* and *akassa* than that of *owo*. Sorting, winnowing, washing, crushing combined with dehulling of maize grains were the unit operations that appeared very effective in achieving significant mycotoxin removal. Fermentation and cooking showed little effect.

Mechanical shelling and dehulling methods were tested to evaluate their impact on *Fusarium* infection and fumonisin contamination in maize. The mechanical shelling methods were found to damage the grains and motorised sheller type IITA caused the highest level of damage. This could be due to the operation mode of that machine. *Fusarium* populations were higher on damaged grains and highest number of colonies was recorded from grains damaged

by the IITA sheller. Total fumonisin levels were also higher in damaged grains, the highest being in maize shelled by the IITA sheller. On the other hand, the mechanical dehulling methods reduced fumonisin levels in maize.

Eight essential oils extracted from local plants in Benin and oil from seeds of the Neem tree (*Azadirachta indica*) were evaluated *in vitro* and *in vivo* for their efficacy against *F. verticillioides* infection and fumonisin contamination. Oils from *Cymbopogon citratus*, *Ocimum basilicum* and *Ocimum gratissimum* were the most effective *in vitro*. These oils totally inhibited fungal growth in stored maize and affected fumonisin levels in maize stored in closed containers. These oils also significantly reduced grain germination. The oil of Neem seeds showed no inhibitory effect but rather accelerated the growth of *F. verticillioides*.