# 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 µl $g^{-1}$, 6.4 µl $g^{-1}$ and 4.8 µl $g^{-1}$, respectively. At the concentration of 4.8 µl/g, these oils affected, but not significantly, fumonisin levels in maize stored in closed containers. They adversely affected seed germination at 4.8 µl/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 stream 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).

Fumonisins 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 µl 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 µl $ml^{-1}$ 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 µl 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 µl $g^{-1}$ of maize grain. These concentrations were obtained by adding 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 µl of each oil to 25 g of maize grains (100 grains). Each volume of oil was diluted in 100 µl 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 µl 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 µl of 95 % ethanol, at 4.8 µl $g^{-1}$ 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 µl/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 ± 25°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 γ-terpinene (15 %), and thymol (17 %). The major compounds detected in the oil from *L. camara* were the sesquiterpenes β-caryophyllene (32 %) and α-humulene (11 %). Oil from *E. citriodora* contained mainly citronellal (66 %) along with a small amount of the alcohol citronnellol (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 µL/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 21day test period (Table 5), with minimal inhibitory concentrations (MIC) of 1.3 µl $ml^{-1}$, 1.3 µl $ml^{-1}$ and 2.0 µl $ml^{-1}$, 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 µl $ml^{-1}$ and 4.7 µl $ml^{-1}$, 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 µl $g^{-1}$, the oils significantly reduced the fungal incidence to approximately 10 %. Complete inhibition occurred at 8.0 µl $g^{-1}$ for *C. citratus*, 6.4 µl $g^{-1}$ for *O. basilicum*, and 4.8 µl $g^{-1}$ 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 µl $g^{-1}$, 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, theis reduction was quite low (Figure 3). In closed storage conditions, the oils maintained the fumonisin level in maize at very low levels (about 0.7mg $kg^{-1}$) 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 µl/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 µl $ml^{-1}$. 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 β-pinene. According to Bishop and Thornton (1997), terpinoid 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 γ-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 µl $g^{-1}$, 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 µl/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 mg $kg^{-1}$, 1.40 mg $kg^{-1}$ and 2.40 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 mg $kg^{-1}$) was not closer to the mean fumonisin level detected in the treated grains (0.57 mg $kg^{-1}$).

At the concentration of 4.8 µl $g^{-1}$, 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 µl/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 [2] | Plant parts | Essential oils yield (%) | |
|---|---|---|---|---|---|
| | | | | Range | Mean |
| *Cymbopogon citratus* | Lemongrass, citronella | AAC 173 HNB | Leaves | 0.5 – 1.3 | 0.9 |
| *Ocimum basilicum* | Basil, sweet basil | AAC 175 HNB | Leaves | 0.2 – 1.2 | 0.7 |
| *Ocimum gratissimum* | African basil | AAC 176 HNB | Leaves | 0.2 – 1.2 | 0.7 |
| *Lantana camara* | Lantana, shrub verbena | AAC 174 HNB | Leaves | 0.2 – 1.2 | 0.7 |
| *Eucalyptus citriodora* | Lemon eucalyptus | AAC 181 HNB | Leaves | 2.8 – 6.0 | 4.4 |
| *Clausena anisata* | Horsewood, Clausena | AAC 177 HNB | Leaves | 1.2 – 2.0 | 1.6 |
| *Melaleuca quinquenervia* | Niaouli | AAC 179 HNB | Leaves | 1.2 – 3.6 | 2.4 |
| *Xylopia aethiopica* | African pepper | AAC 180 HNB | Fruits (pods + seeds) | 3.0 – 6.0 | 4.5 |
| *Azadirachta indica* [1] | 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 |
|---|---|---|---|---|---|
| *Cymbopogon citratus* | 16 | 61 | Myrcene | 28 | 995 |
| | | | Neral (citral B) | 20 | 1254 |
| | | | Geranial (citral A) | 27 | 1283 |
| | | | Geraniol | 4 | 1268 |
| *Ocimum basilicum* | 24 | 87 | Linalol | 33 | 1098 |
| | | | Eugenol | 22 | 1356 |
| | | | Estragol | 20 | 1193 |
| | | | Trans-α-bergamotene | 5 | 1431 |
| | | | Terpinen-4-ol | 4 | 1176 |
| *Ocimum gratissimum* | 29 | 27 | P-cymene | 22 | 1030 |
| | | | γ-terpinene | 15 | 1063 |
| | | | Thymol | 17 | 1303 |
| | | | α-thujene | 5 | 930 |
| | | | Myrcene | 4 | 995 |
| | | | Caryophyllene Oxide | 3 | 1591 |
| | | | 1-8 cineol | 2 | 1045 |
| *Lantana camara* | 23 | 37 | β-caryophyllene | 32 | 1442 |
| | | | α-humulene | 11 | 1476 |
| | | | γ-cadinene | 7 | 1519 |
| | | | 1-8 cineol | 6 | 1051 |
| | | | spathulenol | 6 | 1580 |
| | | | γ-epi-eudesmol | 5 | 1606 |
| | | | β-eudesmol | 4 | 1647 |
| | | | α-phellandrene | 2 | 1042 |
| *Eucalyptus citriodora* | 15 | 86 | Citronnellal | 66 | 1162 |
| | | | Citronnellol | 12 | 1241 |
| | | | Citronnellyl acetate | 4 | 1362 |
| | | | Isopulegol | 3 | 1170 |
| *Clausena anisata* | 17 | 83 | Estragol | 93 | 1207 |
| *Melaleuca quinquenervia* | 26 | 77 | 1-8 cineol | 51 | 1045 |
| | | | α-terpineol | 11 | 1205 |
| | | | Viridiflorol | 10 | 1611 |
| | | | Spathulenol | 3 | 1574 |
| *Xylopia aethiopica* | 31 | 20 | Sabinene | 30 | 978 |
| | | | β-pinene | 8 | 981 |
| | | | Germacrene D | 8 | 1492 |
| | | | Terpinen-4-ol | 7 | 1191 |
| | | | 1-8 cineol | 6 | 1048 |
| | | | Linalol | 2 | 1116 |
| | | | α-terpineol | 2 | 1205 |
| *Azadirachta indica* | - | - | - | - | - |

(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 µlml$^{-1}$ conc.** | **1.3 µlml$^{-1}$ conc.** | **2.0 µlml$^{-1}$ conc.** | **2.7 µlml$^{-1}$ conc.** | **3.3 µlml$^{-1}$ conc.** | **4.0 µlml$^{-1}$ conc.** | **4.7 µlml$^{-1}$ conc.** | **5.3 µlml$^{-1}$ conc.** | **6.0 µlml$^{-1}$ conc.** | **6.7 µlml$^{-1}$ conc.** | **13.3 µlml$^{-1}$ conc.** |
| *Cymbopogon citratus* | 2 b | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Ocimum basilicum* | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Ocimum gratissimum* | 3 c | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Lantana camara* | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Eucalyptus citriodora* | 9 e | 3 b | 2 b | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Clausena anisata* | 4 d | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Melaleuca quinquenervia* | 24 f | 17 c | 11 c | 10 b | 7 b | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Xylopia aethiopica* | 39 g | 37 d | 30 d | 26 c | 22 c | 20 b | 20 b | 18 b | 15 b | 14 b | 1 b |
| *Azadirachta indica* | 75 h | 75 f | 76 f | 68 e | 62 e | 60 d | 60 d | 60 d | 60 d | 60 d | 60 d |
| Untreated control | 39 g | 39 e | 39 e | 39 d | 39 d | 39 c | 39 c | 39 c | 39 c | 39 c | 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 µlml$^{-1}$ conc.** | **1.3 µlml$^{-1}$ conc.** | **2.0 µlml$^{-1}$ conc.** | **2.7 µlml$^{-1}$ conc.** | **3.3 µlml$^{-1}$ conc.** | **4.0 µlml$^{-1}$ conc.** | **4.7 µlml$^{-1}$ conc.** | **5.3 µlml$^{-1}$ conc.** | **6.0 µlml$^{-1}$ conc.** | **6.7 µlml$^{-1}$ conc.** | **13.3 µlml$^{-1}$ conc.** |
| *Cymbopogon citratus* | 5 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Ocimum basilicum* | 34 d | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Ocimum gratissimum* | 20 b | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Lantana camara* | 33 c | 27 c | 25 c | 22 d | 20 d | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Eucalyptus citriodora* | 64 g | 22 b | 10 b | 6 b | 5 b | 5 b | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Clausena anisata* | 62 f | 36 d | 33 d | 20 c | 19 c | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Melaleuca quinquenervia* | 65 h | 55 e | 51 e | 48 e | 42 e | 29 c | 25 b | 19 b | 6 b | 0 a | 0 a |
| *Xylopia aethiopica* | 90 i | 90 g | 70 g | 65 g | 46 f | 44 d | 40 c | 37 c | 28 c | 23 b | 3 b |
| *Azadirachta indica* | 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 µlml$^{-1}$ conc.** | **1.3 µlml$^{-1}$ conc.** | **2.0 µlml$^{-1}$ conc.** | **2.7 µlml$^{-1}$ conc.** | **3.3 µlml$^{-1}$ conc.** | **4.0 µlml$^{-1}$ conc.** | **4.7 µlml$^{-1}$ conc.** | **5.3 µlml$^{-1}$ conc.** | **6.0 µlml$^{-1}$ conc.** | **6.7 µlml$^{-1}$ conc.** | **13.3 µlml$^{-1}$ conc.** |
| *Cymbopogon citratus* | 5 a | 0 a | 0 a | 0a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Ocimum basilicum* | 46 d | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Ocimum gratissimum* | 48 c | 5 b | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Lantana camara* | 45 b | 32 c | 28 c | 23 c | 21 c | 0 a | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Eucalyptus citriodora* | 90 e | 33 d | 13 b | 9 b | 4 b | 3 b | 0 a | 0 a | 0 a | 0 a | 0 a |
| *Clausena anisata* | 90 e | 77 f | 65 e | 67 e | 42 d | 25 c | 20 b | 16 b | 5 b | 0 a | 0 a |
| *Melaleuca quinquenervia* | 90 e | 75 e | 69 d | 60 d | 42 d | 36 d | 32 c | 28 c | 20 c | 0 a | 0 a |
| *Xylopia aethiopica* | 90 e | 90 g | 90 f | 90 f | 77 e | 75 e | 65 d | 60 d | 55 d | 42 b | 8 b |
| *Azadirachta indica* | 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 ($\mu l ml^{-1}$) | MIC after 14 days ($\mu l ml^{-1}$) | MIC after 21 days ($\mu l ml^{-1}$) |
|---|---|---|---|
| *Cymbopogon citratus* | 1.3 | 1.3 | 1.3 |
| *Ocimum basilicum* | 0.7 | 1.3 | 1.3 |
| *Ocimum gratissimum* | 0.7 | 1.3 | 2.0 |
| *Lantana camara* | 1.3 | 4.0 | 4.0 |
| *Eucalyptus citriodora* | 2.7 | 4.7 | 4.7 |
| *Clausena anisata* | 1.3 | 4.0 | 6.7 |
| *Melaleuca quinquenervia* | 4.0 | 6.7 | 6.7 |
| *Xylopia aethiopica* | 13.3 | > 13.3 | > 13.3 |
| *Azadirachta indica* | 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 ($\mu lg^{-1}$) | MIC after 14 days ($\mu lg^{-1}$) | MIC after 21 days ($\mu lg^{-1}$) |
|---|---|---|---|
| *Cymbopogon citratus* | 8.0 | 8.0 | > 8.0 |
| *Ocimum basilicum* | 4.8 | 4.8 | 4.8 |
| *Ocimum gratissimum* | 6.4 | 6.4 | 6.4 |

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

| Treatments | % Germinated kernels |
|---|---|
| Maize treated with *Cymbopogon citratus* | 34 a |
| Maize treated with *Ocimum basilicum* | 57 ab |
| Maize treated with *Ocimum gratissimum* | 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.

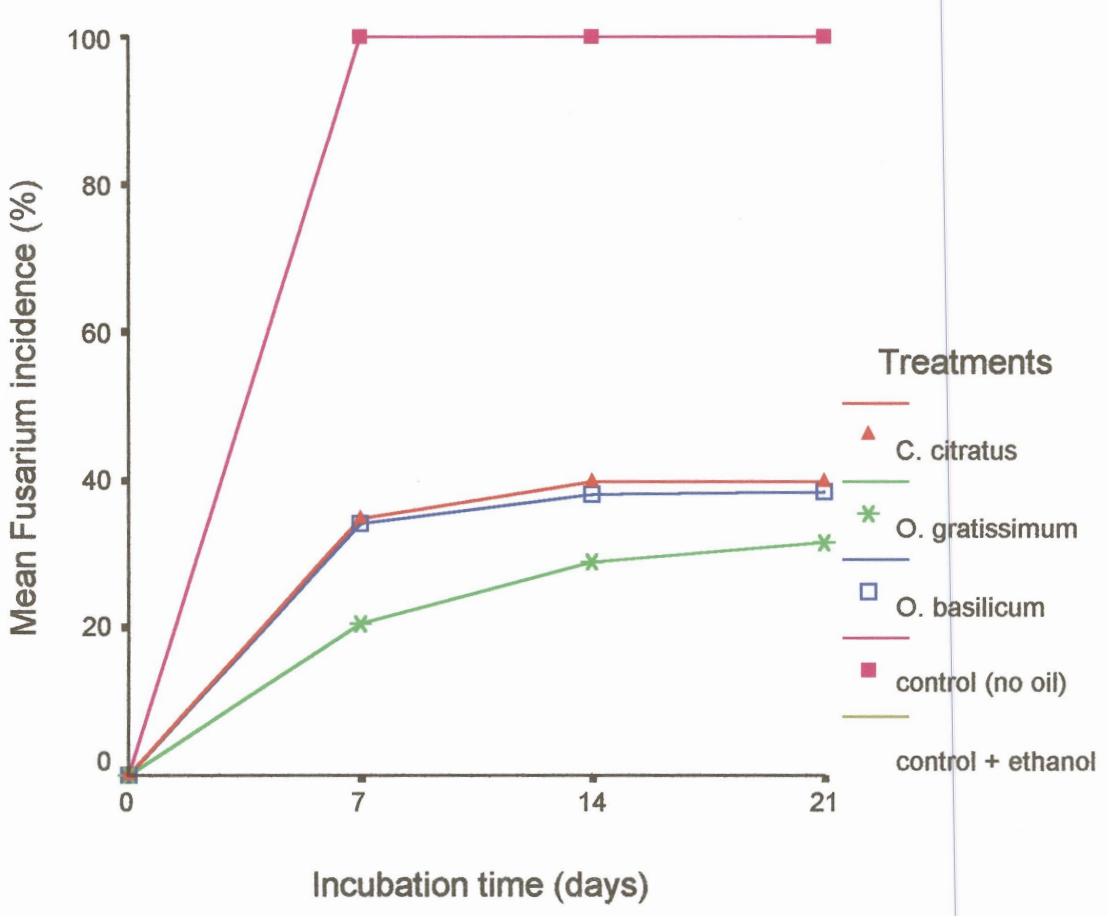



Figure 1: Effect of essential oils on incidence of *F. verticillioides* during the incubation period of artificially inoculated maize grains

| | | |
|---|---|---|
| *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.

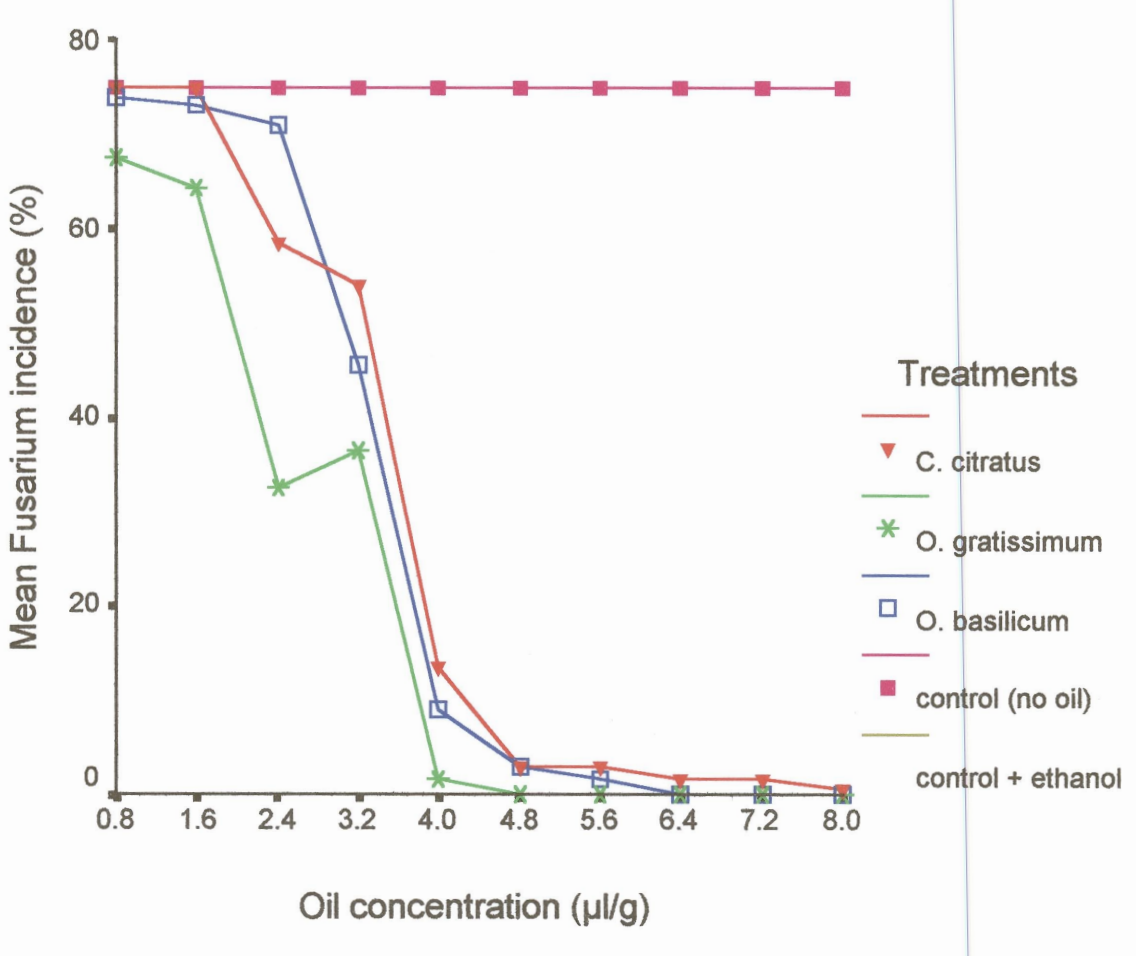



Figure 2: Mean incidence of *F. verticillioides* at different concentrations of essential oils after 21 days of incubation of artificially inoculated maize grains

| | | |
|---|---|---|
| *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.

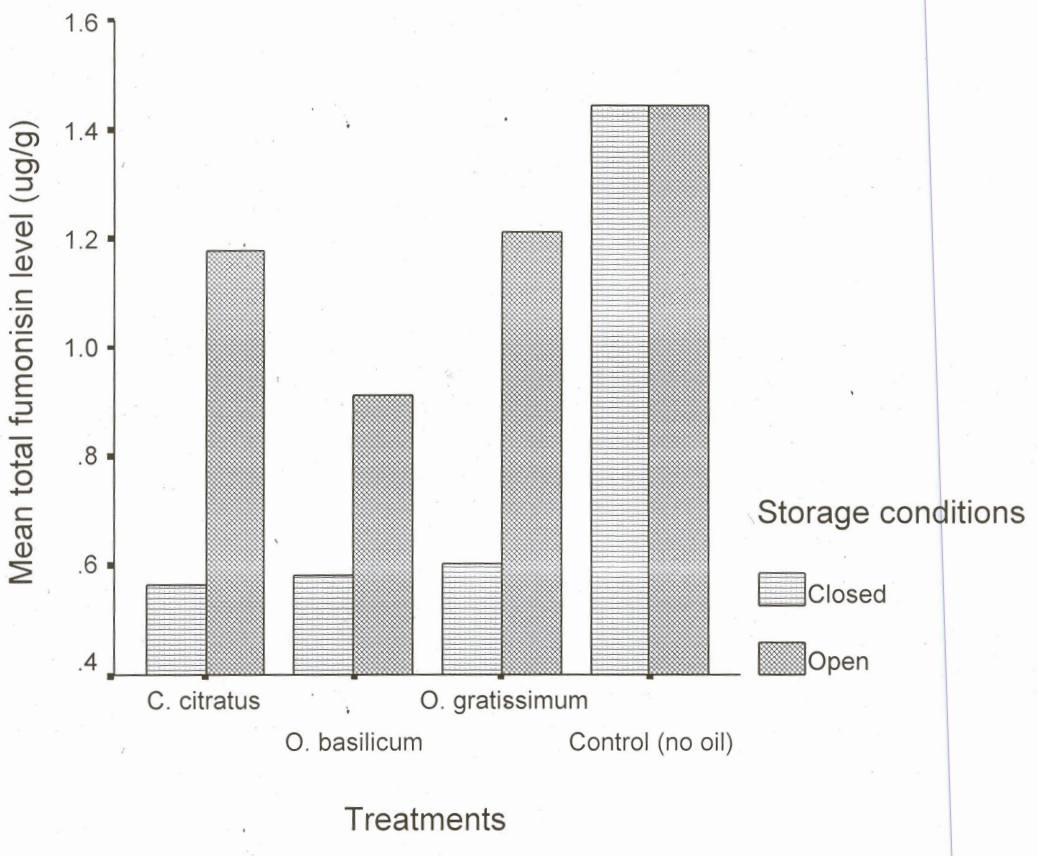
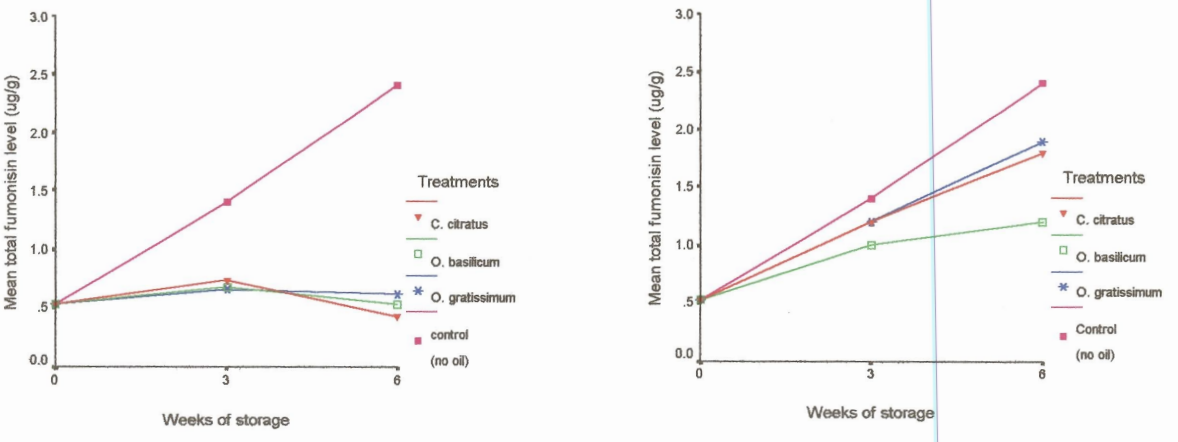



Figure 3: Effect of essential oils on fumonisin production in artificially inoculated maize grains stored in closed and open containers after six weeks of storage

| | | |
|---|---|---|
| *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 $kg^{-1}$. *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*.