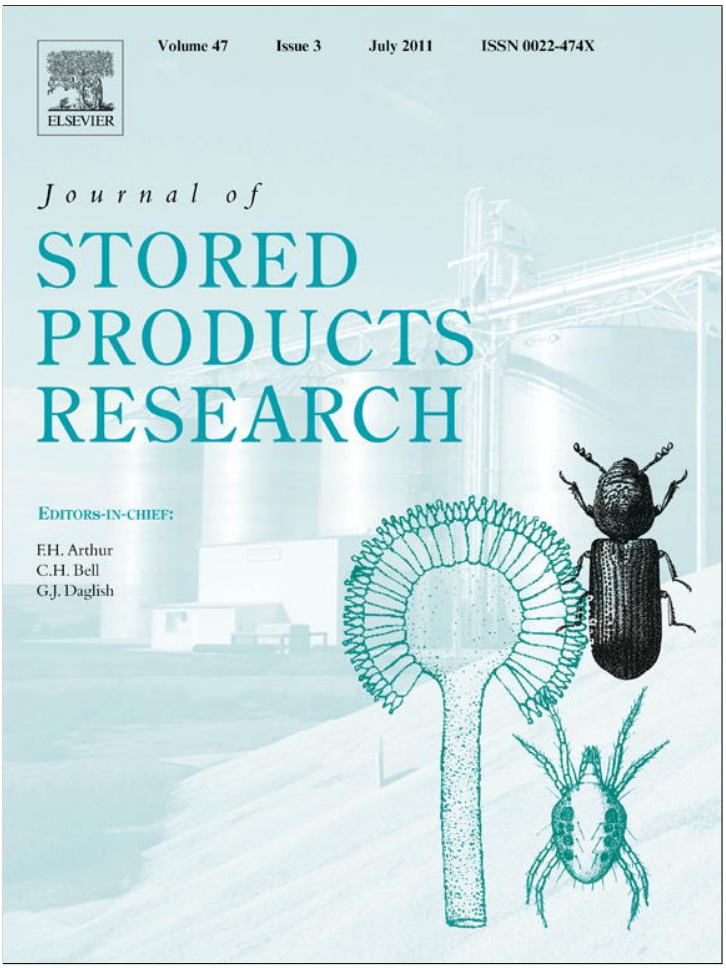

Journal of Stored Products Research 47 (2011) 231–237

Contents lists available at ScienceDirect

Journal of Stored Products Research

journal homepage: www.elsevier.com/locate/jspr

Integrated management of insect vectors of *Aspergillus flavus* in stored maize, using synthetic antioxidants and natural phytochemicals

Andrea Nesci [1], Paula Barra [2], Miriam Etcheverry*,[1]

Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 km 601 (X5806JRA), Río Cuarto, Córdoba, Argentina

ARTICLE INFO

Article history:
Accepted 16 March 2011

Keywords:
Aspergillus flavus
Sitophilus zeamais
Tribolium confusum
Rhyzopertha dominica
Insecticidal activity

ABSTRACT

The purpose of this study was to investigate the insecticidal activity of two benzoic acids 2(3)-*tert*-butyl-4 hydroxyanisole (BHA) and 2,6-di(*tert*-butyl)-*p*-cresol (BHT); two phenolic acids 3-phenyl-2-propenoic acid (CA) and *trans*-4-hydroxy-3-methoxycinnamic acid (FA) and two essential oils of *Eugenia caryophyllata* (clove tree) and *Thymus vulgaris* (thyme) against *Sitophilus zeamais*, *Tribolium confusum* and *Rhyzopertha dominica*, vector carriers of aflatoxigenic fungi in stored maize. The susceptibility of insects, the frequency of isolation of *Aspergillus* section *Flavi* in insects and maize, and the analysis of aflatoxin B_1 in maize were determined. BHA, BHT, BHA/BHT mixture and the natural phytochemicals AF and AF/AC mixture showed the highest insecticidal activity against *S. zeamais*, *T. confusum* and *R. dominica* after 120 days of incubation. The insecticidal efficacy of the volatile fraction of essential oils of clove and thyme showed less inhibition. There was no contamination of *Aspergillus* section *Flavi* in dead and live insects collected from maize treated with BHA. No aflatoxin B_1 accumulation was detected in the control and treatments. The information obtained shows that these substances have the potential to control pest insect vectors of aflatoxigenic fungi in stored maize in microcosms during 120 days.

1. Introduction

In Argentina maize is a key crop in terms of national production of grains (SAGPyA, 2009). This cereal can be colonized by fungal communities at harvest and storage (Etcheverry et al., 1999; Nesci et al., 2008), this contamination reduces the quality and quantity of grain production. Among these fungi, some species of *Aspergillus* section *Flavi* produce toxins associated with harmful effects on animal and human health (Coulombe, 1993; Eaton and Gallagher, 1994). Aflatoxins are produced by *Aspergillus flavus* Link, *Aspergillus parasiticus* Speare, *Aspergillus nomius* Kurtzman et al. (1987), *Aspergillus bombycis* Peterson et al. (2001) and *Aspergillus pseudotamari* Ito et al. (2001). The positive correlation between the consumption of food contaminated with aflatoxin B_1 (AFB_1) and increased incidence of liver cancer has led to the classification of AFB_1 in a group 1 A carcinogen by the International Agency for Research on Cancer (IARC, 1993).

Insects such as *Sitophilus zeamais* (Motschulsky), *Rhyzopertha dominica* (Fabricius) and *Tribolium confusum* (Jacquelin du Val) cause significant damage to stored maize (Mejía, 2007). During periods of postharvest, temperature, humidity and other factors play an important role in the growth of toxigenic fungi and insects in the storage ecosystem. The constant movement of insect populations within a granary ecosystem contributes the dispersal of viable spores of fungi of various species, including species of *Aspergillus* spp and *Penicillium* spp, which are carried on the body surface or deposited in insect frass (Saint Geroges-Gridelet, 1984). Insects in storage systems break the seed coat of grains, which is a natural barrier to fungus growth, which promotes easy spread of the fungus (Setamau et al., 1998).

Synthetic phenolic compounds such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been used extensively for many years as antioxidants to preserve and maintain the freshness, nutritive value, flavor and color of food and animal feed products (JECFA, 1996). Phytochemicals such as cinnamic and ferulic acids are present in cereals, which contain a wide range of phenolic compounds (White and Xing, 1997). Flavor compounds are secondary metabolites with unique properties of volatility, fat

* Corresponding author. Laboratorio Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional 36 km 601 (5800), Río Cuarto, Córdoba, Argentina. Tel./fax: +54 0358 4676231.
E-mail addresses: anesci@exa.unrc.edu.ar (A. Nesci), pbarra@exa.unrc.edu.ar (P. Barra), metcheverry@exa.unrc.edu.ar (M. Etcheverry).
[1] Members of the research career of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
[2] Doctoral fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

0022-474X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jspr.2011.03.003

solubility and low solubility in water. Because of these properties, volatile compounds are very useful in postharvest protection. Their natural occurrence as part of the human diet and its biodegradability suggest a low toxic residue problem (Tripathi and Dubey, 2004; Newberne et al., 2000).

These synthetic and natural compounds have bioactivity against fungi. To avoid contamination by mycotoxins, stored products are often treated with synthetic preservatives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Nesci et al., 2008; Passone et al., 2008), phenolic compounds (Nesci et al., 2009) and essential oils (Bluma and Etcheverry, 2008). However, little information exists on these chemicals as effective pest controls of stored maize.

We evaluated the effect of 2(3)-tert-butyl-4 hydroxyanisol (BHA), 2,6-di (tert-butyl)-p-cresol (BHT), 3-phenyl-2-propenoic acid (CA), trans-4-hydroxy-3-methoxycinnamic acid (FA), and essential oils of *Eugenica caryophyllata* (clove tree) and *Thymus vulgaris* (thyme) against *Sitophilus zeamais*, *Tribolium confusum* and *Rhyzopertha dominica* in stored maize.

2. Materials and methods

2.1. Substrate, insects and fungal culture

Maize grains collected from a commercial field located in the Department of Rio Cuarto, Cordoba Province, Argentina, with an initial water content of 0.43 a_w and free of aflatoxin B_1 (AFB_1) were used in this study. The substrate was stored at −15 °C for 10 d to ensure that all treatments were initially free of insects.

Cultures of the maize weevil *Sitophilus zeamais* (Motschulsky), the confused flour beetle *Tribolium confusum* (Jacquelin du Val) and the lesser grain borer *Rhyzopertha dominica* (Fabricius) were obtained from the Department of Agricultural Zoology, Faculty of Agronomy, University of Buenos Aires, Argentina. Mixed-sex adults 1–3 weeks old were used in the test. The *S. zeamais* were reared on a diet of seeds of wheat, *T. confusum* on a diet of wheat flour, corn starch and yeast (10:10:1,5) and *R. dominica* in broken maize, all in plastic containers. The insects were reared total darkness at 27 ± 1 °C and 70 ± 5% relative humidity (r.h.).

The fungus *A. flavus* RCM89 was used in these experiments. This strain was isolated from stored maize (Nesci et al., 2008). This strain was a producer of aflatoxin B_1 in liquid medium (110.32 ng g^{-1}), and is held in the Microbial Ecology Laboratory Collection, Department Microbiology and Immunology of the National University of Rio Cuarto, Cordoba (Argentina).

2.2. Synthetic antioxidants, phenolic acids and essential oils

Industrial grade antioxidants, obtained from Eastman Chemical Company, were benzoic acid, 2(3)-*tert*-butyl-4 hydroxyanisole (BHA) and 2,6-di(t-butyl)-p-cresol (BHT). BHA had a purity of 98.5% containing as trace elements sulfated ash <0.01%, citric acid <2500 μg g^{-1}, arsenic <3 μg g^{-1} and heavy metals <10 μg g^{-1}. BHT had a purity of 99% containing contaminants such as ash <0.02%, arsenic <3 μg g^{-1} and heavy metals <10 μg g^{-1}. Contaminants from industrial grade antioxidants do not exceed levels allowed by the JECFA (1996). Both compounds were added alone (BHA 20 mM [3.6 mg g^{-1} of maize], BHT 20 mM [4.4 mg g^{-1} of maize]) and mixed (BHA + BHT 20 + 20 mM [3.6 + 4.4 mg g^{-1} of maize]). Stock solutions of BHA (0.18 g ml^{-1}) and BHT (0.22 g ml^{-1}) were prepared in 95% ethyl alcohol, and the appropriate amount was added to maize (BHA 20 μl g^{-1} of maize and BHT 20 μl g^{-1} of maize).

The phenolic acids used were CA: trans-cinnamic acid (3-phenyl-2-propenoic acid) and FA: ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid) and were obtained from Aldrich Chemical, Steinheim, Germany. Both compounds were added to maize at different doses alone (CA 25 mM [3.7 mg g^{-1} of maize], FA 30 mM [5.8 mg g^{-1} of maize]) and mixed (CA + FA 25 + 30 mM [3.7 + 5.8 mg g^{-1} of maize]). Stock solutions of CA (0.12 g ml^{-1}) and FA (0.19 g ml^{-1}) were prepared in 80% ethyl alcohol, and the appropriate amount was added to maize (CA 25 μl g^{-1} of maize and FA 30 μl g^{-1} of maize).

The essential oils of *Eugenia caryophyllata* (Thunb) and *Thymus vulgaris* (L.) were purchased from Casa Gaia® (Buenos Aires, Argentina). Both essential oils were used at different concentrations: 2000 ppm (2000 μg g^{-1} of maize) and 3000 ppm (3000 μg g^{-1} of maize).

2.3. Insecticidal activity of synthetic antioxidants and phenolic acids, and fumigant activity of essential oils

Subsamples of maize grains of 500 g were weighed in microcosms. They consisted of plastic jars of 500 ml capacity and the solutions of synthetic antioxidants and phenolic acids were added at different doses. These substances were sprayed onto the maize grains during mixing to obtain a homogeneous distribution. They were shaken to obtain a uniform distribution of chemical compounds. Plastic jars containing maize grains of all treatments were inoculated with 1 ml of the spore suspension (10^4 spores/ml) of the *Aspergillus flavus* strain. Twenty adults of insects were placed per jar. Tests of three replicates of each insect type were performed. All jars were placed in a chamber with controlled conditions (27 ± 1 °C, 70 ± 5% r.h., in the dark). All samples for the determination of fumigant, contact and digestive activities were observed at 120 days and compared with the untreated control samples.

The method used to determine the fumigant activity of essential oils was based on that described by Prates et al. (1998) with some modifications. Maize grains subsamples of 500 g were weighed in plastic jars of 500 ml capacity. The plastic jars of all treatments were inoculated with 1 ml of the spore suspension (10^4 spores/ml) of the *Aspergillus flavus* strain. Twenty adults of insects were placed per jar. Tests of three replicates of each insect type were performed. The essential oils of *E. caryophyllata* and *T. vulgaris* at different doses were tested under controlled conditions (27 ± 1 °C, 70 ± 5% r.h., in the dark). Essential oils were applied every 30 days with a micropipette on a filter paper placed on top of plastic containers, which were then tightly sealed. All samples for the determination of fumigant activity were observed at 120 days and compared with the untreated control samples. Treatments assayed were made according to the scheme showed in Table 1.

Table 1
Treatments assayed.

	Chemical compounds
T1	–
T2	–
T3	BHA 20 mM
T4	BHT 20 mM
T5	BHA/BHT 20/20 mM
T6	AF 30 mM
T7	AC 25 mM
T8	AF/AC 30/25 mM
T9	2000 ppm essential oil of clove tree
T10	3000 ppm essential oil of clove tree
T11	2000 ppm essential oil of thyme
T12	3000 ppm essential oil of thyme

Substrate: 500 g of maize in each treatment. Insects: T1: 0 insects; T2 to T12: 20 insects in each treatment. Three replicates of each insect type were performed in each treatment. The same treatments were repeated for each insect species: *S. zeamais*, *T. confusum* and *R. dominica*. Fungal inoculum: *A. flavus* strain was inoculated in each treatment.

2.4. Determination of fungal contamination in maize and insects

The colonization of maize grains with *Aspergillus* section *Flavi* was assessed as propagules per g after 120 days of incubation. From each treatment 10 g of grains subsamples were taken, milled and homogenized with 0.1% peptone-water. Serial dilutions were performed and 0.1 ml of dilution was spread plate on DRBC (dichloran rose bengal chloramphenicol agar) media (Pitt and Hocking, 1997). Fungal populations were determined using three replicates for each experiment. The fungal count was expressed as $\log_{10}$ per g of maize. After *Aspergillus* populations had been evaluated, 50 g of all samples of incubation were frozen for later extraction and quantification of aflatoxin B_1.

After 120 days all insects found were collected and counted. The insects that survived the experiment were killed by freezing at −20 °C. All insects were plated directly on malt extract agar (MEA) with 10% NaCl media and incubated at 25 °C for 7 days, and the number of insects from which *Aspergillus* section *Flavi* colonies developed was counted. Fungal colonies isolated from insects identified as *Aspergillus* section *Flavi* were subcultured on malt extract agar (MEA) for further identification. Taxonomic identification of all colonies was achieved according schemes proposed by Pitt and Hocking (1997) and Klich and Pitt (1988).

2.5. Moisture content of maize grain

The a_w of each sample (2 g) was determined just after sampling using an equipment AquaLab (Series 4, TE, USA).

2.6. Aflatoxin B_1 analysis

Aflatoxins determination was performed according to AOAC Official Method 994.08 with modifications. Ground maize (25 g) was extracted with 100 ml of acetonitrile:water (84:16) for 30 min using an orbital shaker, and the supernatant was filtered through Whatman N° 4 filter paper. An aliquot of 5 ml of extract was applied to a multifunctional cleaned column (MycoSep® 224 AflaZon column, Romer Labs, Inc. America). The filtrate (2 ml) was evaporated to dryness and redissolved in 400 μl of mobile phase until HPLC analysis. Aflatoxin quantification was performed by high-performance liquid chromatography (HPLC) according to Trucksess et al. (1994) with some modifications. An aliquot (200 μl) was derivatized with 700 μl of trifluoroacetic acid:acetic acid:water (20:10:70). Derivatized aflatoxins (solution of 100 μl) were analyzed by reverse-phase HPLC/fluorescence detection system. The HPLC system consisted of a Hewlett–Packard workstation. Chromatographic separations were performed in a stainless steel C_{18} reversed-phase column (150 × 4.6 mm i.d. 5 μm particle size) (Luna-Phenomenex, Torrance, CA, USA). Water:methanol:acetonitrile (4:4:1) was used as mobile phase at a flow rate of 1.5 ml min^{-1}. Fluorescence of aflatoxin derivatives was recorded at excitation and emission wavelengths of 360 and 440 nm. Standard curves were constructed with different levels of aflatoxins. Aflatoxins were quantified by correlating peak height of sample extracts and calibration curves. The detection limit under these conditions was 1 ng g^{-1}.

2.7. Statistical analysis

The analysis of variance in a completely randomized design was used to compare the frequency of *Aspergillus* section *Flavi*, the number of dead and live insects and the percentage of insects contaminated with *Aspergillus* section *Flavi*. Means were compared with Tukey test ($P < 0.05$). The analysis was conducted using PROC GLM in SAS (SAS System for Windows 6.1; SAS Institute, Cary, NC, USA).

3. Results

3.1. Effects of treatments on Aspergillus section Flavi populations in maize grain

The total population of *Aspergillus* section *Flavi* in the presence of synthetic antioxidants, phenolic acids and essential oils at 27 °C are shown in Fig. 1. The untreated controls without and with insects (T1 and T2, respectively) showed no significant differences for the total population of *Aspergillus*. The mixture of AF/AC (T8) showed a reduction of 100% of the population of *Aspergillus* section *Flavi* in maize infested with *S. zeamais, T. confusum* and *R. dominica*. AF (T6) showed a total suppression of the population of *Aspergillus* in maize infested with *T. confusum* and *R. dominica*. The same effect was observed with treatments 9 and 11 of maize infested with *R. dominica*. Statistical analysis showed significant differences between treatments throughout the incubation period ($P < 0.0001$).

3.2. Insecticidal activity of chemical agents

All compounds showed statistically significant differences in the insecticidal activity after 120 days of exposure ($P < 0.0001$). BHA, BHT, the mixture of BHA/BHT, AF and the mixture of AF/AC were very effective against *S. zeamais* and *T. confusum* (Fig. 2). The mixture of BHA and BHT (T5) was as active against *S. zeamais* and *T. confusum* as synthetic antioxidants alone. The same behavior was observed with the mixture of AF/AC compared with AF alone. BHT, the mixture of BHA/BHT, AF and the mixture of AF/AC give 100% mortality of *R. dominica*. Tests for fumigant efficacy of the essential oils of clove and thyme showed higher insecticidal effect against *S. zeamais*. The essential oil of thyme at 2000 ppm (T11) was highly effective against *R. dominica*, this concentration caused 100% mortality. However, an increase of live insects was observed with treatments 9, 10 and 12.

3.3. Effect of chemical agents on infection of S. zeamais, T. confusum and R. dominica with Aspergillus section Flavi

The frequency of isolation of *Aspergillus* from insects of stored maize treated with and without chemical compounds is shown in Fig. 3. *Aspergillus* section *Flavi* was isolated from dead and live insects of different treatments. The isolation of *Aspergillus* section *Flavi* from dead and live insects expose to chemicals showed significant differences ($P < 0.0001$). The control treatment (T2) showed a high percentage of live *S. zeamais* and *R. dominica* contaminated with *Aspergillus* section *Flavi*. No *Aspergillus* section *Flavi* contamination was found in dead and live insects collected from maize grain treated with BHA (T3). The same behavior was observed in dead and live *S. zeamais* and *T. confusum* in maize grain treated with BHA/BHT mixture (T5); however live *R. dominica* showed 100% of *Aspergillus* section *Flavi* contamination in this treatment. Dead *S. zeamais*, *T. confusum* and *R. dominica* in T7 showed a low percentage of infection (2%, 12% and 7% respectively) with *Aspergillus* section *Flavi*. Also a low incidence and prevent contamination with *Aspergillus* section *Flavi* were observed in dead *S. zeamais* and *T. confusum* in treatment 8 (Fig. 3) coinciding with no isolation of *Aspergillus* section *Flavi* in maize treated with the mixture AF/AC (Fig. 1). Treatment 11 showed more *S. zeamais* and *T. confusum* live than dead in the presence of chemical compounds (Fig. 2), however, a high percentage of dead insects was contaminated with *Aspergillus* section *Flavi* compared to the low contamination percentage in live insects (Fig. 3). A similar effect was observed in *R. dominica* dead in the treatments 10 and 12.

Statistical analysis showed no significant differences in the values of water activity (Table 2). The water activities of the samples

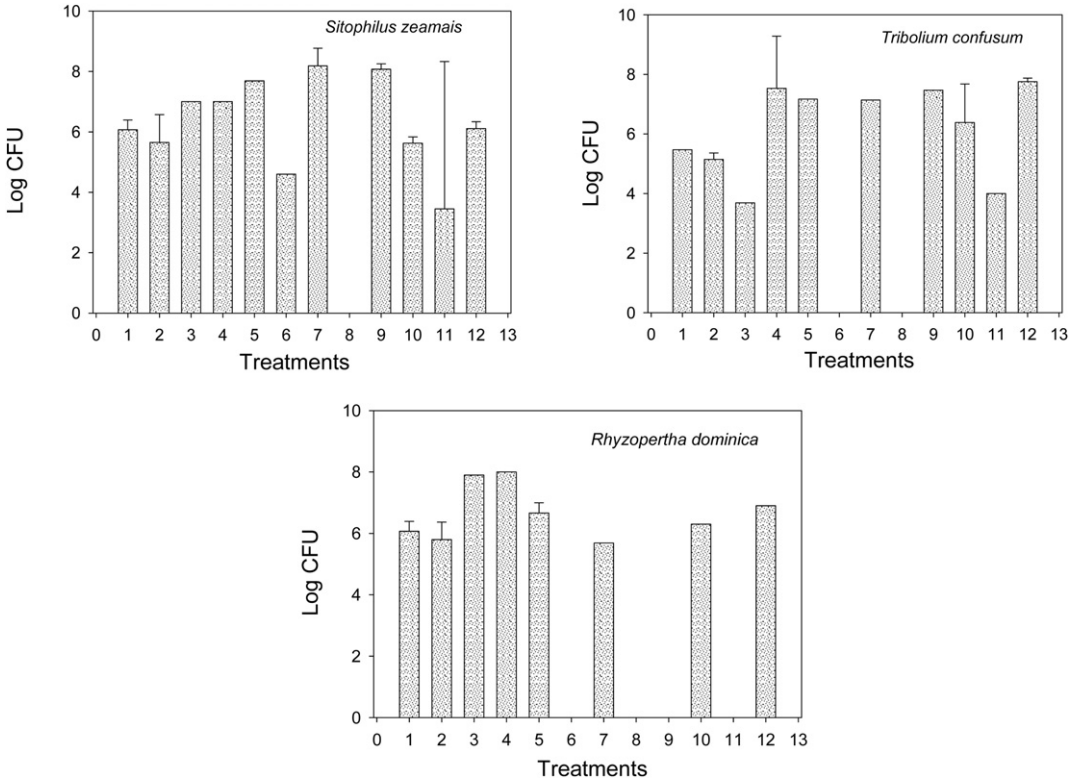

Fig. 1. *Aspergillus* section *Flavi* population (Log CFU) isolated from maize grains under different treatments. T1: control; T2: insects; T3: insects + BHA 20 mM; T4: insects + BHT 20 mM; T5: insects + BHA/BHT 20/20 mM; T6: insects + AF 30 mM; T7: insects + AC 25 mM; T8: insects + AF/AC 30/25 mM; T9: insects + 2000 ppm essential oil of clove tree; T10: insects + 3000 ppm essential oil of clove tree; T11: insects + 2000 ppm essential oil of thyme and T12: insects + 3000 ppm essential oil of thyme. Bars indicate means and standard deviation.

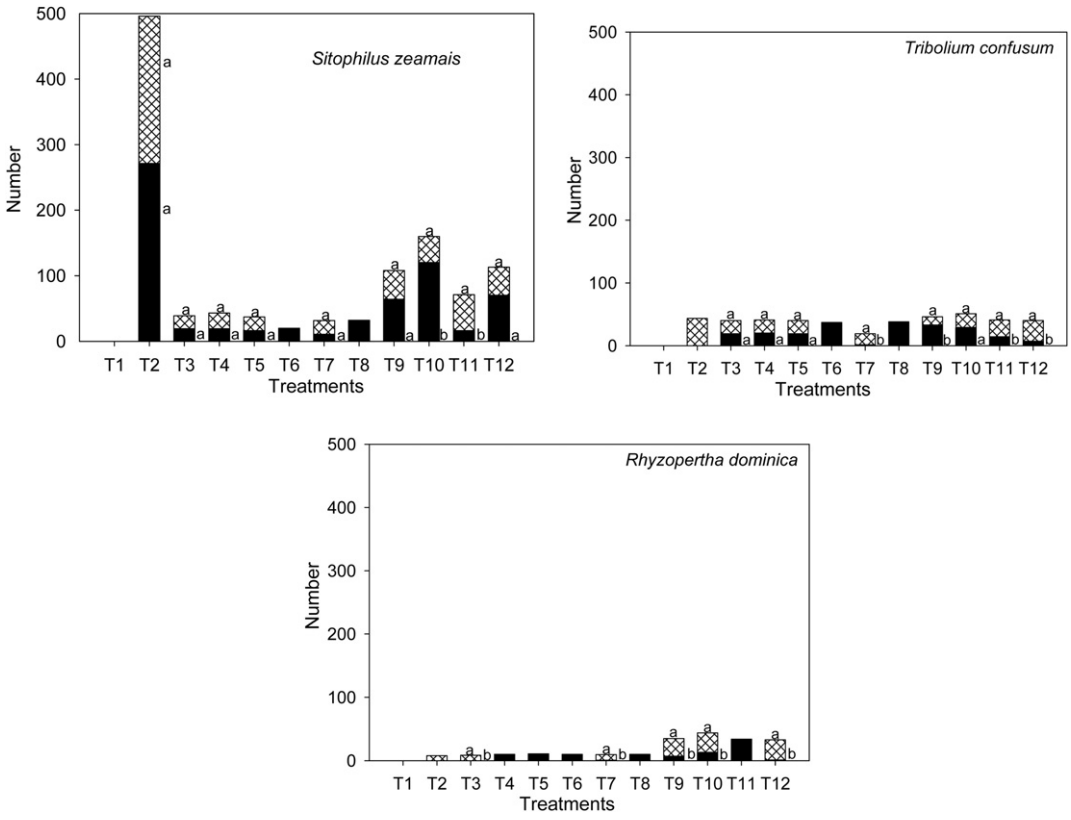

Fig. 2. Insecticidal activity of chemical agents against *Sitophilus zeamais, Tribolium confusum* and *Rhyzopertha dominica* exposed for 120 days. Black: dead insects, Rhombus: live insects, Live and dead insects for each treatment with the same letter are not significantly different according to Tukey test ($P \geq 0.05$).

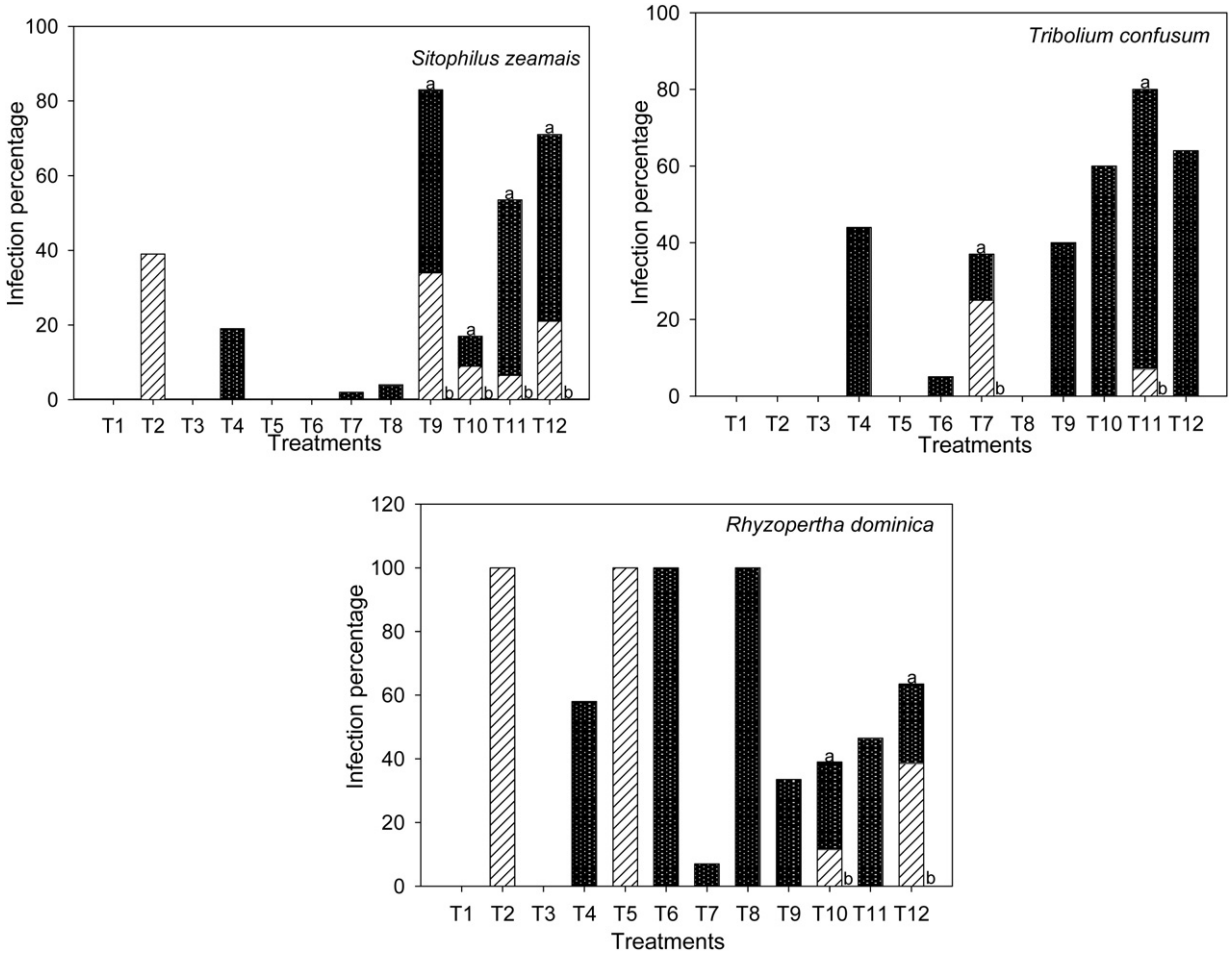

Fig. 3. Effect of chemical agents on infection (percentage) of *Sitophilus zeamais*, *Tribolium confusum* and *Rhyzopertha dominica* with *Aspergillus* section *Flavi*. Lines: live insects contaminated with *Aspergillus*, Black: dead insects contaminated with *Aspergillus*, Live and dead insects contaminated with *Aspergillus* section *Flavi* for each treatment with the same letter are not significantly different according to Tukey test ($P \geq 0.05$).

ranged from 0.424 to 0.444. No aflatoxin B_1 accumulation was detected in the control and treatments (data not shown).

4. Discussion

The application of natural and synthetic antioxidants and volatile fractions of essential oils caused an insecticidal effect and decreased the dispersion of *Aspergillus* aflatoxigenic during storage of maize *in vitro*. Food grade antioxidants BHA, BHT, and the mixture BHA/BHT and the natural phytochemicals AF and the mixture of AF/AC showed the highest insecticidal activity against *S. zeamais*, *T. confusum* and *R. dominica* after 120 days of incubation. The insecticidal efficacy of the volatile fraction of essential oils of clove and thyme showed less inhibition. Insecticidal effect of food grade antioxidants BHA, BHT and the mixture BHA/BHT were demonstrated against the saw-toothed grain beetle *Oryzaephilus surinamensis* (L.) in stored peanuts (Nesci et al., 2010a).

Table 2
Water activity determination of each treatment.

Treatments	a_w (mean ± SD)
T1	0.43 ± 0.01
T2	0.43 ± 0.00
T3	0.44 ± 0.01
T4	0.42 ± 0.01
T5	0.43 ± 0.01
T6	0.42 ± 0.12
T7	0.43 ± 0.01
T8	0.42 ± 0.01
T9	0.43 ± 0.27
T10	0.44 ± 0.27
T11	0.44 ± 0.01
T12	0.43 ± 0.01

In the present study no *Aspergillus* section *Flavi* contamination was observed in dead and live insects collected from maize treated with BHA. In previous studies, the total count of fungi and populations of *Aspergillus* section *Flavi* in maize treated with the mixture of BHA and n-propyl-p-hydroxybenzoate (PP) were significantly reduced (Nesci et al., 2008). The same behavior was observed in peanut treated with BHA, BHT and PP (Passone et al., 2008). These previous studies showed fungicidal and fungistatic activity of synthetic antioxidants against aflatoxigenic *A. flavus* and *A. parasiticus*, with consideration of the effect of the major environmental factors such as water activity. The mechanisms of action of antioxidants to inhibit the mycelial growth of toxigenic fungi are not clear. The mode of action of BHA and BHT appears to be associated with attenuation of the oxidative stress response of the fungus to organic peroxides (Deshpande et al., 1996). Khan et al. (2001) suggested that propyl paraben, and BHA seem to work primarily in the cell membrane level, eliminating the pH-related component of the proton motive force and affecting energy transduction and substrate transport. BHA has also shown a direct effect on

mitochondrial electron chain of trypanosomes, thus inhibiting respiration. BHT seems to cause a reduction in the permeability of vesicles of phospholipid bilayer membranes (Singer and Wan, 1977).

Plant phenolics may be used for insect pest management (Johnson, 2005). Some phenolic acids such as p-coumaric acid (Hattori et al., 1992) and ferulic and sinapic acids (Grant and Langevin, 2002) deter oviposition by *Etiella zinckenella* (Lepidoptera: Pyralidae) and *Choristoneura fumiferana* (Lepidoptera: Tortricidae) respectively. Cinnamic and ferulic acids showed insecticidal effect against a vector of *Aspergillus* section *Flavi* in peanut (Nesci et al., 2010a). The fungicidal and fungistatic effect of cinnamic and ferulic acids on the mycelial growth and AFB_1 production by *A. flavus* and *A. parasiticus* was demonstrated in maize meal extract agar (Nesci and Etcheverry, 2006), in irradiated maize (Nesci et al., 2007) and in natural maize grain (Nesci et al., 2009). The inhibitory effect of phenolic compounds has been attributed to the presence of OH groups able to form hydrogen bonds with the active sites of target enzymes, which was thought to increase antimicrobial activity (Farag et al., 1989). Three phenolic compounds (acetosyringone, syringaldehyde and sinapinic acid) disrupted enzyme-catalyzed reactions required to complete the synthesis of AFB_1 (Hua et al., 1999). One group, hydroxylated cinnamic acid derivatives such as ferulic acid, methyl-cis-3,4-dimethoxycinnamate, and methyl-cis ferulate are effective in inhibiting the germination of fungi (Stahmann et al., 1975; Trione, 1981; Pacifici, 2004).

Most of the active ingredients secreted in plants as chemical defense against pest organisms are secondary metabolites. Among the components of essential oil, the monoterpenoids have attracted the most attention for fumigant activity against stored-product insects (Rajendran and Sriranjini, 2008). Some studies showed the fumigant toxic activity of monoterpenoids, linalool and eugenol (Regnault-Roger and Hamraoui, 1995). These compounds had oxygenated structures or are precursors of phenolic compounds. Monoterpenoids cause insect mortality by inhibiting acetylcholinesterase enzyme (AchE) activity (Houghton et al., 2006) and may also act on other vulnerable sites as cytochrome P450-dependent monooxygenases (Ketoh et al., 2002).

In a previous study the volatile fraction of the essential oil of clove, with eugenol as the main component (Bluma and Etcheverry, 2008) showed insecticidal activity (Nesci et al., 2010a). Besides toxicity on insects, the essential oil of clove and its main component eugenol showed inhibition of growth and mycotoxin production in *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. (Cairns and Magan, 2003; Hope et al., 2003; Velluti et al., 2003, 2004; Bluma and Etcheverry, 2008). Eugenol exerts its biological effects through its antioxidant properties, including the inhibition of Cu^{2+}–H_2O_2 catalyzed lipid peroxidation (Nagashima, 1989) and non-enzymic inhibition of lipid peroxidation (Nagababu and Lakshmaiah, 1992).

Sitophilus zeamais appeared to be less involved in the distribution of *A. flavus* during the 120 days of storage, as observed in most treatments a lower rate of infection of these insects with *Aspergillus*. *Rhyzopertha dominica* was more sensitive to most chemicals tested, but showed a high percentage of these insects, both live and dead, contaminated with *A. flavus*. Perhaps the greatest susceptibility of *R. dominica* to natural and synthetic chemicals makes them more susceptible to fungal contamination. Sublethal doses of chemical insecticides can act as physiological stressors and/or behavioral modifiers and thereby predispose insects to fungal colonization (Inglis et al., 2001). On the other hand, the associated host death may benefit both saprophytic insect exploitation and movement to grain resources (Bennett, 1981).

Aspergillus section *Flavi* population was maintained in control treatments, throughout the storage period in spite of lower water activity in the substrate. These results are consistent with previous study in stored maize grain in Argentina (Nesci et al., 2008). Mixed AF/AC showed a complete reduction of *Aspergillus* section *Flavi* populations in maize infested with *S. zeamais*, *T. confusum* and *R. dominica*. AF/AC mixture (30/25 mM) was effective treatment in inhibiting the population of *Aspergillus* section *Flavi* in natural maize *in vitro* (Nesci et al., 2009). Sauer and Burroughs (1974) reported that, in yellow and white maize with low moisture content, *A. flavus* grew rapidly and produced aflatoxins. In a previous study in peanuts stored, AFB_1 was detected, despite the low water activity of the substrate (Nesci et al., 2010a). The same behavior was observed in peanuts stored in a storage company in Argentina, for five months, with the a_w values ranged between 0.43 and 0.57 (Nesci et al., 2010b). However, this did not happen in the present study where water activities ranged from 0.424 to 0.444. AFB_1 was not detected in stored maize with 0.65 a_w (Nesci et al., 2008).

Acknowledgments

This work was carried out through grants from CONICET PIP 5822/05 and SECYT-UNRC 2007 to 2008.

References

Bennett, J.W., 1981. Genetic perspective on polyketides, productivity, parasexuality, protoplasts, and plastids. In: Vezina, C., Singh, K. (Eds.), Fermentation Products. Advances in Biotechnology, vol. 3. Pergamon Press, Toronto, pp. 409–415.

Bluma, R., Etcheverry, G., 2008. Application of essential oils in maize grain: impact on *Aspergillus* section *Flavi* growth parameters and aflatoxin accumulation. Food Microbiology 25, 324–334.

Cairns, V., Magan, N., 2003. Impact of essential oils on growth and ochratoxin A production by *Penicillium verrucosum* and *Aspergillus ochraceus* on a wheat-based substrate. In: Credland, P.F., Armitage, D.M., Bell, C.H., Cogan, P.M., Highley, E. (Eds.), Proceedings of the Eighth International Working Conference on Stored Product Protection and Advances in Stored Product Protection, 22–26 July 2002, York. CAB International, Wallingford, UK, pp. 479–485.

Coulombe Jr., R.A., 1993. Biological actions of mycotoxins. Journal of Dairy Science 76, 880–891.

Deshpande, S.S., Deshpande, U.S., Salunkhe, D.K., 1996. Nutritional and health aspects of food antioxidants. In: Madhavi, D.L., Deshpande, S.S., Salunkhe, D.K. (Eds.), Food Antioxidants. Technological, Toxicological and Health Perspectives. Marcel Dekker Inc., New York, USA, pp. 159–266.

Eaton, D.L., Gallagher, E.P., 1994. Mechanisms of aflatoxin carcinogenesis. Annual Review of Pharmacology 34, 135–172.

Etcheverry, M., Nesci, A., Barros, G., Torres, A., Chulze, S., 1999. Occurrence of *Aspergillus* section *Flavi* and aflatoxin B_1 in corn genotypes and corn meal in Argentina. Mycopathologia 147, 37–41.

Farag, R.S., Daw, Z.Y., Hewedi, F.M., El-Baroty, G.S.A., 1989. Antimicrobial activity of some Egyptian spice essential oils. Journal of Food Protection 52, 665–667.

Grant, G.G., Langevin, D., 2002. Structure–activity relationships of phenolic and nonphenolic aromatic acids as oviposition stimuli for the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). IOBC/WPRS Bulletin 25, 1–10.

Hattori, M., Sakagami, Y., Marumo, S., 1992. Oviposition deterrents for the limabean pod borer, *Etiella zinckenella* (Treitschke) (Lepidoptera: Pyralidae) from *Populus nigra* L. C.v. Italica leaves. Applied Entomology and Zoology 27, 195–204.

Hope, R., Jestoi, M., Magan, N., 2003. Multitarget environmental approach for control of growth and toxin production by *Fusarium culmorum* using essential oil and antioxidant. In: Credland, P.F., Armitage, D.M., Bell, C.H., Cogan, P.M., Highley, E. (Eds.), Proceedings of the Eighth International Working Conference on Stored Product Protection and Advances in Stored Product Protection, 22–26 July 2002, York. CAB International, Wallingford, UK, pp. 486–492.

Houghton, P.J., Ren, Y., Howes, M.J., 2006. Acetylcholinesterase inhibitors from plants and fungi. Natural Product Reports 23, 181–199.

Hua, S.-S.T., Grosjean, O.-K., Baker, J.L., 1999. Inhibition of aflatoxin biosynthesis by phenolic compounds. Letters in Applied Microbiology 29, 289–291.

IARC International Agency for Research on Cancer, 1993. Monograph on the Evaluation of Carcinogenic Risk to Humans. IARC, Lyon, France.

Inglis, G.D., Goettel, M.S., Butt, T.M., Strasser, H., 2001. Use of Hyphomycetous fungi for managing insect pests. In: Butt, T.M., Jackson, C., Magan, N. (Eds.), Fungi as Biocontrol Agents. Progress, Problems and Potential. CAB Publishing, Wallingford, UK, pp. 23–69.

Ito, Y., Peterson, S.W., Wichlow, D.T., Goto, T., 2001. *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section *Flavi*. Mycological Research 105, 233–239.

JECFA, 1996. Toxicological Evaluation of Certain Food Additives and Contaminants in Food. In: WHO Food Additives Series, N°35. Joint FAO/WHO Expert Committee on Food Additives, Geneva.

Johnson, K., 2005. Plant phenolics behave as radical scavengers in the context of insect (*Manduca sexta*) hemolymph and midgut fluid. Journal of Agricultural and Food Chemistry 53, 10120–10126.

Ketoh, G.K., Glitho, A.I., Huignard, J., 2002. Susceptibility of the bruchid *Callosobruchus maculates* (Coleoptera: Bruchidae) and its parasitoid *Dinarmus basalis* (Hymenoptera: Pteromalidae) to three essential oils. Journal of Economic Entomology 95, 174–182.

Khan, S.H., Aked, J., Magan, N., 2001. *In vitro* potential for antioxidant chemicals to control the anthracnose pathogens of bananas, *Colletotrichum musae*. Plant Pathology 50, 601–608.

Klich, M.A., Pitt, J.I., 1988. Differentiation of *Aspergillus flavus* from *Aspergillus parasiticus* and other closely related species. Transaction of the British Mycology Society 91, 99–108.

Kurtzman, C.P., Horn, B.W., Hesseltine, C.W., 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie Van Leeuwenhoek 53, 147–158.

Mejía D, 2007. www.fao.org/inpho/content/compend/text/ch23_04.htm.

Nagababu, E., Lakshmaiah, N., 1992. Inhibitory effect of eugenol on non-enzymatic lipid peroxidation in rat liver mitochondria. Biochemical Pharmacology 43, 2393–2400.

Nagashima, K., 1989. Inhibitory effect of Eugenol on Cu^{2+} catalyzed lipid peroxidation in human erythrocyte membranes. International Journal of Biochemistry 21, 745–749.

Nesci, A., Etcheverry, M., 2006. Control of *Aspergillus* growth and aflatoxin production using natural maize phytochemicals under different conditions of water activity. Pest Management Science 62, 775–784.

Nesci, A., Ferrari, L., Etcheverry, M., 2008. Effect of synthetic antioxidants on stored maize grain mycoflora *in situ*. Journal of the Science of Food and Agriculture 88, 797–804.

Nesci, A., Gsponer, N., Etcheverry, M., 2007. Natural maize phenolic acids for control of aflatoxigenic fungi on maize. Journal of Food Science 72 (5), M180–M185.

Nesci, A., Marín, S., Etcheverry, M., Sanchis, V., 2009. Natural maize phytochemicals for control of maize mycoflora and aflatoxigenic fungi. World Mycotoxin Journal 3, 305–312.

Nesci, A., Montemarani, A., Etcheverry, M., 2010b. Assessment of mycoflora and infestation of insects, vector of *Aspergillus* section *Flavi*, in stored peanut from Argentina. Mycotoxin Research. doi:10.1007/212550-010-0069-0.

Nesci, A., Montemarani, A., Passone, M.A., Etcheverry, M., 2010a. Insecticidal activity of synthetic antioxidants, natural phytochemicals, and essential oils against an *Aspergillus* section *Flavi* vector (*Oryzaephilus surinamensis* L.) in microcosm. Journal of Pest Science. doi:10.1007/s10340-010-0333-2.

Newberne, P., Smith, R.L., Dull, J., Feron, V.J., 2000. GRAS flavouring substances. Food Technology 54, 66–83.

Pacifici, B., 2004. Germinazione Della Spora. URL. http://www.pacifici-net.it/biologia/Micologia/Germinazionedellaspora.htm.

Passone, M., Resnik, S., Etcheverry, M., 2008. The potential of food grade antioxidants in the control of *Aspergillus* section *Flavi*, interrelated mycoflora and aflatoxin B_1 accumulation on peanut grains. Food Control 19, 364–371.

Peterson, S.W., Ito, Y., Horn, B.W., Goto, T., 2001. *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. Mycologia 93, 689–703.

Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage. Blackie Academic and Professional, London.

Prates, H.T., Santos, J.P., Waquil, J.M., Fabris, J.D., Oliveira, A.B., 1998. The potential use of plant substances extracted from Brazilian flora to control stored grain pest. In: Zuxun, J., Quan, L., Yongsheng, L., Xianchang, T., Liangua, G. (Eds.), Proceedings of the Seventh International Conference on Stored-product Protection, Beijing, China. Sichuan Publishing House of Science and Technology, Chengdu, PR China, pp. 820–825.

Rajendran, S., Sriranjini, V., 2008. Plant products as fumigants for stored-product insect control. Journal of Stored Product Research 44, 126–135.

Regnault-Roger, C., Hamraoui, A., 1995. Fumigant toxic activity and reproductive inhibition induced by monoterpenes on *Acanthoscelides obtectus* (Say) (Coleoptera), a bruchid of kidney bean (*Phaseolus vulgaris* L). Journal of Stored Product Research 31, 291–299.

SAGPyA, 2009. Secretaría de Agricultura, Ganadería, Pesca y Alimentos. Agricultura, Estimaciones Agrícolas, Campaña 2008–2009. URL. http://www.sagpya.gov.ar.

Saint Geroges-Gridelet, D., 1984. Effects of dietary lipids on the population growth of *Dermatophagoides pteronyssinus*. In: Proceedings of 6th International Congress of Acarology, vol. 1, pp. 351–357. Chichester.

Sauer, F., Burroughs, R., 1974. Efficiency of various chemicals as grain mould inhibitors. Transaction of the American Society of Agriculture and Engineering 17, 557–559.

Setamau, M., Cardwell, K.F., Schulthess, F., Hell, K., 1998. Effect of insect damage to maize ears, with special reference to *Mussidia nigrivenella* (Lepidoptera: Pyralydae) on *Aspergillus flavus* (Deuteromycetes: Monoliales) infection and aflatoxin production in maize before harvest in the Republic of Benin. Journal of Economic Entomology 91, 433–438.

Singer, M., Wan, J., 1977. Interaction of butylated hydroxytoluene (BHT) with phospholipid bilayer membranes: effect on ^{22}Na permeability and membrane fluidity. Biochemical and Pharmacology 26, 2259–2268.

Stahmann, M.A., Abramson, P., Wu, L., 1975. A chromatographic method for estimating growth by glucosamine analysis of diseased tissues. Biochemical Physical Pflanz 168, 267–276.

Trione, E.J., 1981. Natural regulators of fungal development. In: Trione, E.J. (Ed.), Plant Disease Control: Resistance and Susceptibility. Wiley, New York, pp. 85–102.

Tripathi, P., Dubey, N.K., 2004. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetable. Postharvest Biology and Technology 32, 235–245.

Trucksess, M.W., Stack, M.E., Nesheim, S., Albert, R.H., Romer, T.R., 1994. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1, G_2 in corn, almonds, Brazil nuts, peanuts and pistachio nuts: collaborative study. Journal of AOAC 6, 1512–1521.

Velluti, A., Sanchis, V., Ramos, A., Marín, S., 2004. Effect of essential oil of cinnamon, clove, lemongrass, oregano and palmarosa on growth and fumonisin B_1 production by *Fusarium verticillioides* in maize grain. Journal of the Science of Food and Agriculture 89, 145–154.

Velluti, A., Sanchis, V., Ramos, A.J., Ergido, J., Marín, S., 2003. Inhibitory effect of cinnamon, clove, lemongrass, oregano and palmarosa essential oils on growth and fumonisin B_1 production by *Fusarium proliferatum* in maize grain. International Journal of Food Microbiology 89, 145–154.

White, P.J., Xing, Y., 1997. Antioxidants from cereals and legumes. In: Shahidi, F. (Ed.), Natural Antioxidants, Chemistry, Health Effects and Applications. AOCS Press, Champaign, IL, pp. 25–63.