Kritzinger, Q., Aveling, T.A.S. and Marasas, W.F.O. (2002), *Seed Sci. & Technol.*, **30**, 609-619

Effect of essential plant oils on storage fungi, germination and emergence of cowpea seeds

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(Accepted October 2001)

Summary

The antifungal activity of plant essential oils on storage fungi commonly associated with cowpea (*Vigna unguiculata* (L.) Walp) seed was investigated. The antifungal activity of the essential oils of thyme, clove, peppermint, soybean and peanut were tested against *Aspergillus flavus, A. niger, Fusarium oxysporum, F. equiseti* and *Penicillium chrysogenum* in vitro. Only thyme and clove significantly inhibited growth of all five fungi at 500 and 1000 ppm whereas peppermint oil successfully inhibited growth at 2000 ppm. Thyme, clove and peppermint were tested in vivo against storage fungi on naturally infected (cultivars PAN 325 and PAN 311) and artificially infected (cultivar CH 14) cowpea seed. Only thyme oil (1000 ppm) showed antifungal activity against storage fungi associated with PAN 325 cowpea seeds. In the PAN 311 cultivar, clove and thyme at 1000 ppm and peppermint at 2000 ppm exhibited antifungal activity against the storage fungi. In cultivar CH 14, thyme, clove and peppermint significantly reduced growth of *P. chrysogenum* whilst thyme and peppermint inhibited growth of *F. oxysporum*. Only thyme had an antifungal effect on *F. equiseti*. No treatment showed antifungal activity against *A. flavus* and *A. niger*. None of the oils showed harmful effects on the germination and emergence of cowpea seeds. The storage fungi significantly reduced percentage germination and emergence of the white (IT 93K452-1) seed but had little or no effect on the brown (CH 14) seed. Furthermore, all three oils significantly inhibited the storage fungi on the white seed thereby increasing the percentage germination and emergence.

Introduction

Cowpea seeds (*Vigna unguiculata* (L.) Walp) are susceptible to fungal contamination especially when stored under poor conditions together with high relative humidities and high temperatures (Esurouso, 1975; Seenappa *et al.*, 1983). It is well known that storage fungi are one of the several causes leading to a decrease in germination of seeds (Neergaard, 1977). Fields and King (1962) reported that pea seed kept at 85% relative humidity and 30°C lost germination capacity within six months when invaded by *Aspergillus* spp. Uninfected pea seed maintained a germination percentage of 95%.

Control measures to prevent fungal contamination and post-harvest losses include the control of environmental factors (temperature and moisture) (Mislivec, 1998) and chemical control (Bauer, 1994; Codifier *et al.*, 1976; Hasan, 1998; Megella and Hafez, 1982) including ammonia, which has been exploited commercially (Bauer, 1994; Coker, 1994).

Since chemicals have potential harmful effects on the environment and the community, investigation into the antifungal properties of essential plant oils has attracted wide interest. There have been many reports regarding the antifungal properties of plant essential oils. Some of these oils include thyme (*Thymus vulgaris* L.) (Thompson and Cannon, 1986; Zambonelli *et al.,* 1996); cinnamon (*Cinnamomum zeylanicum* Blume) (Thompson and Cannon, 1986), clove (*Syzygium aromaticum* (L.) Merr. and Perry) (Thompson and Cannon, 1986; Chatterjee, 1990); pimenta (*Capsicum anuum* L.) (Thompson and Cannon, 1986), basil (*Ocimum basilicum* L.) (Chatterjee, 1990; Basílico and Basílico, 1999), citronella (*Cymbopogon nardus* (L.) W. Watson) (de Billerbeck *et al.* 2001) and extracts from garlic (*Allium sativum* L.) bulbs, green garlic and green onions (*Allium fistulosum* L. var. *caespitosum*, scallion) (Yin and Cheng, 1998). Neem (*Azadirachta indica* A. Juss) leaf extract has been reported to protect chickpea (*Cicer arietinum* L.) seeds against serious fungal diseases (*Rhizoctonia solani* (Ehrenb.:Fr) Vuill., *Sclerotium rolfsii* Sacc.), to slow down growth of *Fusarium oxysporum* Schecht ex. Fries and completely stopped *Aspergillus flavus* Link ex. Fries from producing aflatoxin (National Research Council, 1992). Furthermore, Basílico and Basílico (1999) showed that essential oils of oregano (*Origanum vulgare* L.) and mint (*Mentha arvensis* L.) completely inhibited fungal growth and ochratoxin A production of *Aspergillus ochraceus* Wilhelm. Some investigations have been carried out to determine the effectiveness of various essential plant oils on the inhibition of fungi causing cowpea seed spoilage and possible mycotoxin production. Adegoke and Odesola (1996) reported that samples of cowpea seeds treated with lemon grass (*Cymbopogon citratus* (DC. ex Nees) Stapf.) powder and essential oil showed no physical deterioration or mouldiness. Furthermore, it inhibited the fungal growth of fungi like *A. flavus* and *Penicillium chrysogenum* Thom. Montes-Belmont and Carvajal (1998) recorded that thyme, clove, peppermint, basil and cinnamon oils did not inhibit germination of maize seeds or subsequent seedling growth. Similarly, Cruz and Cardona (1981) found that soybean (*Glycine max* (L.) Merrill) oil caused no reduction of germination in cowpea seeds.

This paper deals with the inhibitory effect of five essential plant oils on the growth of potentially mycotoxigenic and other storage fungi causing cowpea seed spoilage. The control of these fungi by treating cowpea seed with the essential oils of clove, thyme and peppermint is evaluated and the effect on the germination and emergence of the treated seed is investigated.

Materials and methods

1. Fungal species

The fungi used in these investigations were chosen from the range of fungi isolated from cowpea seeds during previous experiments carried out at the Department of Botany, University of Pretoria, Pretoria, South Africa (Kritzinger 2000). These fungi were found to be the predominant fungi associated with cowpea seed and could be potential mycotoxic species. The fungi chosen were *Aspergillus flavus*, *A. niger* van Tieghem, *Penicillium chrysogenum*, *Fusarium equiseti* (Corda) Sacc and *F. oxysporum*. These fungal cultures were maintained on potato dextrose agar (PDA) (Biolab) at ± 25°C under constant light.

2. Seed samples

Two cowpea cultivars, PAN 311 (brown seed) and PAN 325 (white seed), were obtained from the Vegetable and Ornamental Plant Institute (VOPI), Roodeplaat, Pretoria, South Africa. The cultivars, CH 14 (brown seed) and IT 93K452-1 (white seed), were obtained from the Agricultural Research Council (ARC) - Grain Crops Institute, Potchefstroom, South Africa.

3. Essential plant oils

The essential oils used during these investigations were clove (obtained from buds), thyme, peppermint (*Mentha piperita* L.) (both from young shoots), peanut (*Arachis hypogaea* L.) and soybean (both from seeds). The oils were 100% pure according to the manufacturers and were purchased at The Health Shop, Atterbury Value Mart, Pretoria, South Africa. The thyme, clove and peppermint oils were Escentia products and the peanut and soybean oils, Natures Choice products.

4. Effect of oils on seed-borne and storage fungi

a. In vitro *tests*

The *in vitro* tests were based on the method described by Zambonelli *et al.* (1996). The oils were dissolved in 25 µl 100% ethanol and then immediately added to autoclaved PDA, before being poured into sterile Petri dishes (9 cm diameter.). The oils were initially tested at concentrations of 100 and 1000 ppm. Based on these results, the thyme and clove oils were further tested at 500 ppm and peppermint, peanut and soybean, at 2000 ppm. Fifty µl, 250 µl, 500 µl and 1000 µl essential oils was added to 500 ml melted PDA to yield oil concentrations of 100 ppm, 500 ppm, 1000 ppm and 2000 ppm respectively. There were two controls used during this investigation. The one received the same quantity of ethanol used to dissolve the oils, mixed with the PDA. In the second control, no ethanol was mixed with the PDA. There were four replicates for each oil concentration. Inoculation of the prepared Petri dishes was carried out by placing a disk (5 mm diameter) from the actively growing zone of a 7-day-old culture in the centre of the Petri dish. The Petri dishes were then incubated at ± 25°C. Fungal growth was measured on two preset diametral lines after three, six and nine days. The 6-day growth measurements (mm) were statistically analysed.

b. In vivo *tests*

Naturally infected cowpea seed

Based on the results from the *in vitro* tests, it was decided to test the thyme, clove and peppermint oil *in vivo*. Four replicates of 25 seeds were surface disinfected for 1 min using 1% sodium hypochlorite, rinsed three times with sterile distilled water and allowed to dry. Seeds of PAN 311 and PAN 325 were treated with the thyme and clove oils at a concentration 1000 ppm and peppermint at a concentration of 2000 ppm. The required volume of oil (25 µl thyme and clove oil and 50 µl peppermint oil dissolved in 25 µl 100% ethanol) was added to 25 ml sterile distilled water and the seeds were allowed to soak for approximately 5 min. The control received the same amount of 100% ethanol. After a brief drying period (± 5 min), the seeds were plated directly onto PDA plates (five seeds per plate and six plates per replicate). After five days of incubation at ± 25°C, the seed-borne fungi present were recorded.

Artificially infected seed

Four replicates of 25 seeds (cultivar CH 14) were surface disinfected as described above. Seeds were inoculated with each of the five test fungi as follows: for each fungus, a spore suspension of 1×10^6 spores ml^{-1} was prepared by adding 20 ml sterile distilled water to a 7-day-old culture. The surface of the culture was scraped to free the spores. The spore suspensions were poured through muslim cloth into flasks. The seeds were added to the suspensions and mixed thoroughly. Thereafter, the seeds were allowed to dry for ± 5 min. The seeds were treated with thyme and clove oils at a concentration of 1000 ppm and peppermint at 2000 ppm according to the procedure for naturally infected cowpea seed. The control was also inoculated and was treated with the same amount of ethanol used to dissolve the oils. After a brief drying period, the seeds were plated directly onto PDA plates (five seeds per plate with twenty plates per replicate). The fungal colonies were counted after five days of incubation at ± 25°C.

5. Effect of seed-borne and storage fungi and oils on seed germination

Seeds of CH 14 and IT 93K452-1 were surface disinfected as previously described. After a short drying period, seeds were inoculated with a 1×10^6 spore suspension of a mixture of the five test fungi in equal proportions. Thereafter, the seeds were dried and treated with 25 µl thyme or clove oils and 50 µl peppermint oil to give a concentration of 1000 ppm and 2000 ppm respectively. The method used for seed inoculation and oil treatment of the seeds was as for the *in vivo* tests on artificially inoculated seeds. The controls included inoculated seed without oil treatment (simulating seed naturally infected with storage fungi) and the uninoculated control. Uninoculated seeds were also treated with each of the three oils. Percentage germination was determined by placing four replicates of 100 seeds each between moist paper towels which were rolled up and placed individually in polythene bags, held upright in plastic beakers and maintained at ± 25°C in an incubator. Percentage germination was determined after 4 and 8 days according to the International Seed Testing Association (ISTA) rules (International Seed Testing Association 1999).

6. Effect of seed-borne and storage fungi oils on seed emergence

Seeds of CH 14 and IT 93K452-1 were surface disinfected as previously described. Seed inoculation and oil treatments were carried out according to the method used in the germination tests. The two controls included inoculated seed without oil treatment and uninoculated seeds. Uninoculated seeds were treated with each of the three oils as described above. Potting soil was used to fill 128-cell plastic seedling trays (10 cm × 3 cm × 3 cm). A single seed was planted at a depth of 2 cm in each cell. There were four replicates of 25 seeds per treatment. The seedling trays were placed in a controlled environment room at 25°C with a 12h light/dark regime. Seedling trays were watered daily. After 10 d the emerged seedlings were counted. Thereafter, the seedlings were cut at soil level and the wet and dry mass of the upper parts (stem and leaves) of seedlings within each replicate were determined and the percentage dry mass calculated.

7. Statistical analysis

Two-way analysis of variance (ANOVA) was performed on all the data and least significant differences ($P = 0.05$) were determined according to the student's t test.

Results

The results of the effect of essential oils on the five fungi *in vitro* are shown in table 1. The essential oil of thyme significantly inhibited the growth of *A. flavus*, *P. chrysogenum*, *F. oxysporum* and *F.equiseti* at 100, 500 and 1000 ppm when compared with the controls. Thyme reduced growth of *A. niger* significantly at 500 and 1000 ppm but not at 100 ppm. Clove oil significantly inhibited growth of *P. chrysogenum*, *F. oxysporum* and *F. equiseti* at all three concentrations tested. Only concentrations of 500 and 1000 ppm significantly reduced growth of *A. flavus* and *A. niger*. The essential oil of peppermint only showed a successful inhibitory effect on all the fungi at a concentration of 2000 ppm when compared with the controls. On the contrary, growth of *F. equiseti* was stimulated by this oil at 100 ppm. The variable results obtained at 100 ppm could possibly be due to the fact that since the volume of oil used was small, the oil dispersed unevenly through the plate. The oils of both peanut and soybean had no inhibitory effect on the growth of the five fungi tested.

The results of the effect of thyme, clove and peppermint oils on the five tested fungi and other storage fungi present on naturally infected cowpea seed are represented in table 2. There was no significant inhibition of any of the five fungi tested by the three oils in both cultivars when compared with the control. Thyme oil did, however, reduce the total incidence of fungi significantly on both cultivars and was especially effective on PAN 311. Peppermint and clove oil also significantly reduced the total incidence of fungi in PAN 311. The other seed-borne fungi included *Rhizopus*, *Phoma*, *Alternaria* and other *Fusarium* and *Aspergillus* species.

The results of the percentage of fungi isolated from artificially inoculated cowpea seed are presented in table 3. There was no inhibitory effect by any of the essential oils on the growth of *A. flavus* and *A. niger* when compared with the control. Clove, peppermint and thyme did, however, significantly inhibit the growth of *P. chrysogenum* and growth of *F. oxysporum* and *F. equiseti* was significantly inhibited on seeds treated with thyme oil. Peppermint and thyme oil significantly inhibited the growth of *F. oxysporum* when compared with the control.

There were no significant differences noted in percentage germination of the brown cowpea seeds (CH 14) when compared to the controls (table 4). In the white cultivar (IT 93K452-1), the percentage germination of the uninoculated seeds treated with the oils (thyme, peppermint and clove) did not differ significantly from the uninoculated control but was significantly higher than that of all the inoculated treatments. The percentage germination of the inoculated brown seeds was significantly higher than that of the inoculated white seeds. In the uninoculated treatments, brown seeds treated with thyme and clove had a significant higher percentage germination than those of the white seeds. The latter had a higher percentage germination when treated with peppermint and clove.

The percentage of diseased seeds was generally lower in the brown seeds than in the white seeds. No significant difference in diseased seeds is shown in the brown seeds whereas a significant increase is shown in all the treatments of the white seeds when compared with both the controls. The percentage diseased seeds was significantly lower in the brown seed when compared with the white seed, except in the case of the uninoculated control and the uninoculated seeds treated with clove and peppermint oils.

Table 1. Effect of plant essential oils on the growth of selected fungi *in vitro*.

Treatments	Fungi (colony diameter in cm)																			
	A. flavus				*A. niger*				*P. chrysogenum*				*F. oxysporum*				*F. equiseti*			
	100[1]	500[1]	1000[1]	2000[1]	100	500	1000	2000	100	500	1000	2000	100	500	1000	2000	100	500	1000	2000
Control 1	8.0*^{B}	7.9^{C}	8.0^{C}	7.9^{B}	7.9^{A}	8.0^{C}	7.9^{CB}	8.0^{C}	3.5^{CD}	2.9^{C}	3.5^{D}	2.9^{CB}	8.0^{D}	6.5^{C}	8.0^{D}	6.5^{CB}	4.4^{C}	4.5^{B}	4.4^{C}	4.5^{B}
Control 2	8.0^{B}	7.9^{C}	8.0^{C}	7.9^{B}	8.0^{A}	6.9^{B}	8.0^{C}	6.9^{BC}	3.3^{C}	3.2^{D}	3.3^{CD}	3.2^{D}	6.2^{C}	8.0^{D}	6.2^{C}	8.0^{D}	4.7^{C}	4.6^{B}	4.7^{CD}	4.6^{B}
Clove	8.0^{B}	2.7^{B}	0.0^{A}	nt	7.5^{A}	1.3^{A}	0.0^{A}	nt	2.4^{B}	0.7^{B}	0.0^{A}	nt	4.0^{B}	0.9^{B}	0.0^{A}	nt	3.6^{B}	0.0^{A}	0.0^{A}	nt
Peanut	8.0^{B}	nt	8.0^{C}	7.9^{B}	8.0^{A}	nt	7.4^{B}	8.0^{C}	4.0^{D}	nt	2.9^{C}	2.7^{B}	6.5^{C}	nt	7.7^{D}	6.8^{C}	5.4^{D}	nt	4.5^{CD}	7.4^{D}
Peppermint	8.0^{B}	nt	7.6^{B}	0.0^{A}	7.9^{A}	nt	7.9^{CB}	4.7^{A}	3.1^{C}	nt	2.3^{B}	0.0^{A}	6.6^{C}	nt	4.0^{B}	0.00^{A}	5.7^{D}	nt	2.2^{B}	0.0^{A}
Soybean	8.0^{B}	nt	8.0^{C}	7.9^{B}	8.0^{A}	nt	8.0^{C}	5.6^{AB}	3.7^{CD}	nt	3.4^{D}	3.0^{CD}	8.0^{D}	nt	5.7^{C}	6.3^{B}	5.8^{D}	nt	5.2^{D}	6.5^{C}
Thyme	6.9^{A}	1.4^{A}	0.0^{A}	nt	7.6^{A}	0.7^{A}	0.00^{A}	nt	1.5^{A}	0.0^{A}	0.0^{A}	nt	3.5^{A}	0.0^{A}	0.0^{A}	nt	2.7^{A}	0.0^{A}	0.0^{A}	nt

* Each value is a mean of 4 replicates after 6 d of growth. Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to the student's t test.
[1] Concentration in ppm
Control 1 - PDA unamended
Control 2 - PDA amended with ethanol
nt = not tested

Table 2. Effect of essential oils on all the storage fungi isolated from naturally infected cowpea seed (cultivars PAN 311 and PAN 325).

Fungi	Cultivar	Treatments			
		Control	Clove 1000 ppm	Peppermint 2000 ppm	Thyme 1000 ppm
A. flavus	PAN 311	0.0*^{A}	0.0^{A}	0.0^{A}	0.0^{A}
	PAN 325	17.3^{A}	13.3^{A}	14.7^{A}	12.0^{A}
A. niger	PAN 311	0.7^{A}	0.0^{A}	0.0^{A}	0.0^{A}
	PAN 325	0.0^{A}	0.0^{A}	0.7^{A}	0.0^{A}
P. chrysogenum	PAN 311	0.7^{A}	0.0^{A}	0.7^{A}	0.0^{A}
	PAN 325	0.7^{AB}	0.7^{AB}	2.0^{B}	0.0^{A}
F. oxysporum	PAN 311	2.0^{A}	0.0^{A}	2.0^{A}	0.0^{A}
	PAN 325	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}
F. equiseti	PAN 311	0.0^{A}	0.0^{A}	0.7^{A}	0.0^{A}
	PAN 325	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}
Total[1]	PAN 311	24.0^{C}	14.0^{B}	14.0^{B}	5.3^{A}
	PAN 325	30.0^{B}	26.7^{AB}	26.0^{AB}	18.7^{A}

* Each value is a mean of 3 replicates of 30 seeds. Values within a row not followed by the same letter are significantly different ($P = 0.05$) according to student's t test.

[1] Represents all the storage fungi isolated from the cultivars, including the five fungi tested.

Table 3. Percentage of storage fungi isolated from artificially inoculated cowpea (cultivar CH 14) seeds.

Fungi	Treatments			
	Control**	Clove 1000 ppm	Peppermint 2000 ppm	Thyme 1000 ppm
A. flavus	100*^{A}	100^{A}	100^{A}	100^{A}
A. niger	100^{A}	100^{A}	100^{A}	100^{A}
P. chrysogenum	100^{D}	0^{A}	93^{C}	85^{B}
F. oxysporum	81^{B}	74^{B}	39^{A}	55^{A}
F. equiseti	14^{B}	10^{AB}	5^{AB}	3^{A}

* Each value is a mean percentage of 4 replicates of 25 seeds. Values within a row not followed by the same letter are significantly different ($P = 0.05$) according to student's t test.

** The control was also inoculated with the fungi.

Table 4. Effect of storage fungi and essential oils on germination and emergence of cowpea seeds (cultivars CH 14 and IT 93K452-1).

	% Germination		% Diseased seeds		% Emergence		% Dry mass	
Treatment	CH-14	IT 93K452-1	CH-14	IT 93K452-1	CH-14	IT 93K452-1	CH-14	IT 93K452-1
Inoculated Control***	72.0^{*AB**Y}	1.0^{AX}	19.0^{*AB**X}	95.0^{DY}	$86^{\dagger BC\dagger\dagger Y}$	16^{AX}	$12.13^{A\dagger X\dagger\dagger}$	11.67^{ABX}
Clove	71.8^{ABY}	32.0^{BX}	21.3^{BX}	68.0^{CY}	75^{AY}	36^{BX}	19.47^{ABX}	8.88^{AX}
Peppermint	72.0^{ABY}	28.0^{BX}	18.3^{ABX}	72.0^{CY}	78^{ABY}	40^{CBX}	25.33^{ABX}	12.73^{ABX}
Tyyme	69.0^{ABY}	23.5^{BX}	21.8^{BX}	76.8^{CY}	79^{ABY}	35^{BX}	20.92^{ABX}	10.07^{ABX}
Uninoculated Control***	73.0^{ABX}	77.0^{CDX}	10.8^{AX}	18.3^{ABX}	93.0^{CX}	90.0^{DX}	26.1^{ABX}	17.1^{AB}
Clove	76.0^{BX}	80.8^{DX}	11.3^{AX}	19.0^{ABX}	91^{CX}	83^{DX}	18.77^{ABX}	15.65^{ABX}
Peppermint	64.8^{AX}	75.3^{CDY}	14.0^{ABX}	15.8^{AX}	92^{CY}	52^{CX}	28.10^{BX}	16.25^{ABX}
Thyme	70.3^{ABX}	68.3^{CX}	11.8^{AX}	27.0^{BY}	88^{BCX}	90^{DX}	16.45^{ABX}	20.43^{BX}

* Each value is a mean percentage of 4 replicates of 100 seeds. Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to the student's t test.

** Each value is a mean percentage of 4 replicates of 100 seeds. Values within a row not followed by the same letter are significantly different ($P = 0.05$) according to the student's t test.

† Each value is a mean percentage of 4 replicates of 25 seeds. Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to the student's t test.

†† Each value is a mean percentage of 4 replicates of 25 seeds. Values within a row not followed by the same letter are significantly different ($P = 0.05$) according to the student's t test.

***The controls included inoculated seed without oil treatment (simulating seed naturally infected with storage fungi) and the uninoculated control. Uninoculated seeds were also treated with each of the three oils.

Only inoculated brown seed treated with clove showed a significant decrease in emergence when compared with the other treatments (table 4). All inoculated treatments in the white seed showed a significant increase of percentage emergence when compared with the inoculated control. There was no significant differences in percentage emergence of both the brown and white seed in the uninoculated treatments when compared to the uninoculated controls. In the white seed, percentage emergence of inoculated treatments was, however, severalfold more than that of the inoculated control. The essential oils had no effect on the uninoculated treatments. No significant differences are shown in the percentage dry mass between the treatments of both cultivars when compared with the uninoculated control and between the two cultivars (table 4).

Discussion

The trend shown in the results of the effect of plant essential oils on the growth of fungi *in vitro*, was that at all the concentrations tested in the present study, thyme oil significantly suppressed fungal growth. It was reported by Zambonelli *et al.* (1996) that thyme was the most effective oil against the inhibition of fungal growth of pathogenic fungi. Its fungicidal activity was attributed to the compound thymol, which was found at 50.06% in the oil tested (Zambonelli *et al.*, 1996). In the present study, clove oil also significantly inhibited the fungal growth of all the fungi tested except for *A. flavus* and *A. niger* at a concentration of 100 ppm. These results of inhibition by thyme and clove oils confirm those of Thompson and Cannon (1986) who found that these two oils inhibited mycelial growth of *A. flavus* at both 500 and 1000 ppm.

In the present study, peppermint oil appeared to have a significant inhibitory effect at the higher concentrations (1000 and 2000 ppm) tested, with the exception of *A. niger* at 1000 ppm. Zambonelli *et al.* (1996) documented that mint oil was more effective at higher concentrations (1600 ppm). During the present investigation, it was evident that the oils of both peanut and soybean were generally not effective at inhibiting fungal growth, which contradicts McGee and Misra's (1988) findings that soybean oil decreased the incidence of storage fungi in maize (*Zea mays* L.) and soybean.

In the *in vivo* tests on naturally infected cowpea seeds, the essential oils of thyme, clove and peppermint showed significant antifungal activity on PAN 311 seeds, while only thyme oil showed significant antifungal activity on the PAN 325 seeds. Although these oils did not significantly inhibit growth of the five fungi tested for in this test, they did reduce the total amount of fungi in both cultivars. In artificially inoculated seeds *P. chrysogenum* incidence was reduced by clove, peppermint and thyme whereas no inhibition of the incidence of *A. flavus* and *A. niger* occurred. Thyme and peppermint showed antifungal activity against *F. oxysporum* and with the exception of peppermint oil, *F. equiseti*. Chatterjee (1990) found that clove oil (30 µl g^{-1} grain and above) inhibited the *in vivo* growth of *A. flavus*, *Curvularia pallescens* Boedijn and *Chaetomium indicum* Corda on maize grains during storage. Furthermore, Montes-Belmont and Carvajal (1998) reported that cinnamon, peppermint, basil, oregano, clove and thyme oils caused total inhibition of *A. flavus* in maize.

It is evident from the results of the percentage germination and emergence of the two cultivars that the oils did not reduce or adversely affect the germination or emergence of seeds, supporting the findings of by Montes-Belmont and Carvajal (1998). These authors found that clove, thyme and peppermint oils did not have an inhibitory effect on maize seed germination at a dosage of 10%. In the white cultivar, the percentage germination in uninoculated seeds was higher than the treatments that were inoculated. Since the invasion of storage fungi in seeds causes a decrease in seed germination (Neergaard, 1977), storage fungi could have been responsible for the severe decrease in germination and impaired emergence of the white seed. Treating the inoculated white seed with the oils appeared to greatly reduce the effect of the storage fungi on the seeds, resulting in an increase in the percentage emergence. The lower percentage germination than seeds that actually emerged in the greenhouse experiments was probably due to the warm moist conditions in the paper towels, which favoured seed deterioration by the storage fungi.

It was also noted that PAN 311 (brown seeds) had a lower incidence of fungi than the white seeds, PAN 325. The percentage diseased seeds of CH 14 (brown seeds) was also generally lower than the white seeds (IT 93K452-1). Legesse and Powell (1992) found that cream/beige seeds of cowpea were more susceptible to infection by *Pythium* sp. than coloured (brown) seed. This could be attributed to the higher tannin content in dark seeds (Morrison *et al.*, 1995) since Mixon and Sanders (1979) reported that seed coat tannins of *Arachis hypogaea* inhibited growth of *Aspergillus parasiticus* Speare. Legesse and Powell (1992) also reported that differences in the rate of water uptake and incidence of imbibition damage in cowpea cultivars was associated with testa colour, cream/beige seeds being more prone to imbibition damage than dark seeds. Imbibition damage enhances the predisposition of cowpea to pre-emergence mortality in unsterilised soil (Legesse and Powell, 1992).

Although the essential oils of clove and peppermint controlled the storage fungi in one of the cultivars tested in naturally infected seed and inhibited growth of *P. chrysogenum*, thyme oil seemed more effective in controlling storage fungi and more specifically, *P. chrysogenum*, *F. oxysporum* and *F. equiseti*. Since thyme also does not show any harmful effects on the germination and emergence of the seed, it has potential as a natural treatment for the control of fungi on cowpea seed.

References

Adegoke, G.O. and Odesola, B.A. (1996). Storage of maize and cowpea and inhibition of microbial agents of biodeterioration using the powder and essential oil of lemon grass (*Cymbogon citratus*). *International Biodeterioration and Biodegradation*, **37**, 81–84.

Basílico, M.Z. and Basílico, J.C. (1999). Inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production. *Letters in Applied Microbiology*, **29**, 238–241.

Bauer, J. (1994). Möglichkeiten zur Entgiftung mykotoxinhaltiger Futtermittel. *Monatshefte fur Veterinamedizin*, **49**, 175–181.

Chatterjee, D. (1990). Inhibition of fungal growth and infection in maize grains by spice oils. *Letters in Applied Microbiology*, **11**, 148–151.

Codifier, L.P., Mann, G.E. and Dollear, F.G. (1976). Aflatoxin inactivation: Treatment of peanut meal with formaldehyde and calcium hydroxide. *Journal of the American Oil Chemists Society*, **53**, 204–206.

Coker, N.R.I. (1994). Biodeterioration of grain and the risk of mycotoxins. In *Grain storage techniques: Evolution and trends in developing countries*, (ed. D.L. Proctor), pp 27–38, FAO Agricultural Services Bulletin 109, Rome.

Cruz, C. and Cardona, E. (1981). Control of dry seed weavils with cooking oil. *Journal of Agriculture of the University of Puerto Rico*, **65**, 295–298.

De Billerbeck, V.G., Roques, C.G., Bessière, J-M., Fonvieille, J-L. and Dargent, R. (2001). Effects of *Cymbopogon nardus* (L.) W. Watson essential oil on the growth an morphogenesis of *Aspergillus niger*. *Canadian Journal of Microbiology*, **47**, 9–17.

Esuruoso, O.F. (1975). Seed-borne fungi of cowpea (*Vigna unguiculata*) in Western Nigeria. *Nigerian Journal of Plant Protection*, **2**, 87–90.

Fields, R.W. and King, T.H. (1962). Influence of storage fungi on the deterioration of stored pea seed. *Phytopathology*, **52**, 336–339.

Hasan, H.A.H. (1998). Studies on toxigenic fungi in roasted foodstuff (salted seed) and halotolerant activity of emodin-producing *Aspergillus wentii*. *Folia microbiologica*, **43**, 383–391.

International Seed Testing Association (1999). International Rules for Seed Testing. *Seed Science and Technology*, **27**, **Supplement, 333 pp.**

Kritzinger, Q. (2000). *Storage fungi and mycotoxins associated with cowpea*, pp 45–64, MSc thesis, University of Pretoria, Pretoria.

Legesse, N. and Powell, A.A. (1992). Comparisons of water uptake and imbibition damage in eleven cowpea cultivars. *Seed Science and Technology*, **20**, 173–180.

McGee, D.C. and Misra, M.K. (1988). Soybean oil application for dust suppression and control of storage molds in corn and soybeans. *Iowa Seed Science*, **10**, 6–7.

Megella, S.E. and Hafez, A.H. (1982). Detoxification of aflatoxin B_1 by acidogenous yoghurt. *Mycopathologia*, **77**, 89–91.

Mislivec, P.B. (1998). Training in moulds isolation, identification, handling and evaluation of conditions leading to mycotoxin production. In *Mycotoxin prevention and control in foodgrains*, (eds. R.L. Semple, A.S. Frio, P.A. Hicks and J.V. Lozare), Food and Agriculture Organisation of the United Nations (FAO) and the Information Network on Post-Harvest Operations (INPhO). Bangkok, Thailand. http://www.fao.org/inpho/vlibrary/x0036e/x0036e00.html

Mixon, A.C. and Sanders, T.H. (1979). Seedcoat and tannin influence on the mycelial growth of *Aspergillus parasiticus* for peanuts varying in fungal infection. *Agronomy abstracts*, 70.

Montes-Belmont, R. and Carvajal, M. (1998). Control of *Aspergillus flavus* in maize with plant essential oils and their components. *Journal of Food Protection*, **61**, 616–619.

Morrison, I.M., Asiedu, E.A., Stuchbury, T. and Powell, A.A. (1995). Determination of lignin and tannin contents of cowpea seedcoats. *Annals of Botany*, **76**, 287–290.

National Research Council. (1992). *Neem: A tree for solving global problems*, pp 53–55, National Academy Press, Washington, D.C.

Neergaard, P. (1977). *Seed pathology*, Vol. I, ch. 7, pp. 287-288, The MacMillan Press Ltd., London.

Seenappa, M., Keswani, C.L. and Kundya, T.M. (1983). Aspergillus infection and aflatoxin production in some cowpea (*Vigna unguiculata* (L.) Walp) lines in Tanzania. *Mycopathologia*, **83**, 103–106.

Thompson, D.P. and Cannon, C. (1986). Toxicity of essential oils on toxigenic and nontoxigenic fungi. *Bulletin of Environmental Contamination and Toxicology*, **36**, 527–532.

Yin, M-C. and Cheng, W-S. (1998). Inhibition of *Aspergillus niger* and *Aspergillus flavus* by some herbs and spices. *Journal of Food Protection*, **61**, 123–12.

Zambonelli, A., Zechini D'Aulerio, A., Bianchi, A. and Albasini, A. (1996). Effects of essential oils on phytopathogenic fungi *in vitro*. *Journal of Phytopathology*, **144**, 491–494.