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ORIGINAL ARTICLE

Control of *Aspergillus* section *Flavi* growth and aflatoxin accumulation by plant essential oils

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Abstract

Aims: The antifungal effect of *Pimpinella anisum* (anise), *Pëumus boldus* (boldus), *Mentha piperita* (peppermint), *Origanum vulgare* (oregano) and *Minthostachys verticillata* (peperina) essential oils against *Aspergillus* section *Flavi* (two isolates of *Aspergillus parasiticus* and two isolates of *Aspergillus flavus*) was evaluated in maize meal extract agar at 0·982 and 0·955 water activities, at 25°C.

Methods and Results: The percentage of germination, germ-tube elongation rate, growth rate and aflatoxin B_1 (AFB_1) accumulation at different essential oils concentrations were evaluated. Anise and boldus essential oils were the most inhibitory at 500 mg kg^{-1} to all growth parameters of the fungus. These essential oils inhibited the percentage of germination, germ-tube elongation rate and fungal growth. AFB_1 accumulation was completely inhibited by anise, boldus and oregano essential oils. Peperina and peppermint essential oils inhibited AFB_1 production by 85–90% in all concentrations assayed.

Conclusions: Anise and boldus essential oils could be considered as effective fungitoxicans for *Aspergillus* section *flavi*.

Significance and Impact of the Study: Our results suggest that these phytochemical compounds could be used alone or in conjunction with other substances to control the presence of aflatoxigenic fungi in stored maize.

Introduction

Fungi are significant destroyers of foodstuffs during storage, rendering them unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins (Mishra and Dubey 1994).

Maize (*Zea mays* L.) is widely grown in Argentina. This cereal grain is colonized by a range of spoilage fungi, including mycotoxigenic species. The dominant genera, which are considered 'storage fungi', include *Aspergillus, Fusarium* and *Penicillum* species (Gonzalez *et al.* 1995). The fungal growth and survival of these genera are markedly affected by water availability, which is one of the limiting factors in the functioning ecosystems (Ramos *et al.* 1999).

Aflatoxin, the most carcinogenic, mutagenic and teratogenic substance found naturally in foods and feeds (IARC 1993), can be produced in preharvest as well as in stored products (Wilson and Payne 1994). The species of *Aspergillus* section *Flavi* group that produce aflatoxins include *Aspergillus flavus*, *Aspergillus parasiticus*, *A. nomius*, *A. tamarii* and *A. bombycis* (Kurtzman *et al.* 1987; Wilson and Payne 1994; Goto *et al.* 1996; Peterson *et al.* 2001). These fungi can grow on a wide range of agricultural commodities, such as maize grain.

Storage fungi are commonly controlled by synthetic compounds, among them are pesticides used to protect crops, fungicides have been perceived until as relatively safe. A National Academy of Science (NAS) report (1987) about pesticide residues on food indicated that fungicides possess more carcinogenic risk than insecticides and herbicides together. Therefore, synthetic fungicides should be minimized in our food chain. Pressure is increasing to find safer alternatives. Additionally,

resistance of pathogens to fungicides has rendered certain fungicides ineffective, creating the need for new ones with alternative modes of action (Wilson *et al.* 1997).

As a result of the increasing public awareness of the pollutive, residual, carcinogenic and phytotoxic effect of many synthetic fungicides, the importance of alternative indigenous products to control mycotoxigenic fungi is gaining popularity (Gould 1996). Now-a-days, interest in the use of natural antimicrobials is growing, especially those from herbs, plants, and spices (or their components), which are traditional ingredients or flavour enhancers (Lopez-Malo *et al.* 2005).

The antimicrobial properties of the spices have been known and used for centuries. Essential oils are volatile products of plant secondary metabolism, which in many cases become biologically active because of their antimicrobial, antioxidant and bioregulatory properties (Caccioni *et al.* 1995a,b). Essential oils have long been recognized as having good fungitoxic activity (Singh *et al.* 1980, Paster *et al.* 1995; Basilico and Basilico 1999; Yin and Tsao 1999), but they have not been developed into products for postharvest treatments, as industry finds it easier to produce and patent newly synthetic compounds than natural plant product.

Pimpinella anisum L. (anise), *Pëumus boldus* Mol (boldus), *Origanum vulgare* L. (oregano) and *Mentha piperita* L. (peppermint) are widely used as medicinal herbs for the drug industry and local medicinal systems, and as flavouring agents for many kinds of food products (Gómez Sosa 1998). Some studies have shown that their essential oils can inhibit the growth of different micro-organisms (Paster *et al.* 1990; Cowan 1999; Soliman and Badeaa 2002; Burt 2004; Musa and Claude 2006). *Minthosthachys verticillata* (Griseb) Epl. known as 'peperina' is one of 12 species of aromatic plants that comprise the genus *Minthosthachys* (Lamiaceae); these plants are spread on the highlands of the Andes from Venezuela to Argentina (Epling 1937; Epling and Jativa 1963). Peperina aromatic plant is used in South-American countries as condiment food, flavouring agent (having peppermint-like odour); moreover, its infusion and essential oils are used as topical antiparasitic and antimycotic (De Feo 1992). Antibacterial activity of peperina essential oil has also been shown (De Feo *et al.* 1998).

The objectives of this study were to evaluate the effect of anise, boldus, peppermint, oregano and peperina essential oils at different concentrations on germ-tube elongation rate, growth rate and AFB_1 accumulation by *Aspergillus* section *Flavi* in maize meal extract agar (MMEA) at 0·982 and 0·955 water activity (a_W), at 25°C.

Materials and methods

Fungal isolates

Four isolates belonging to the genus *Aspergillus*: two of *A. flavus* Link (RCD65 and RCI105) and two *A. parasiticus* Speare (RCT20 and RCD106) were used in these experiments. These toxigenic isolates were all recovered from maize agroecosystem obtained from soil (*A. parasiticus* RCT20), crop residues (*A. parasiticus* RCD106 and *A. flavus RC*D65) and Insects (*A. flavus* RCI105) (Nesci and Etcheverry 2002). The isolates were maintained at 4°C on slants of malt extract agar (MEA) and 15% glycerol at −80°C.

Culture medium

The basic medium used in this study was MMEA. This medium contained 3% maize meal and 15% agar. The a_W of basic medium (0·999) was adjusted to 0·982 and 0·955 by adding nonionic solute, glycerol, according to Dallyn and Fox (1980) and Marín *et al.* (1995). The medium was autoclaved at 121°C for 20 min before cooling at 50°C, and poured into 9 cm sterile Petri dishes.

Plant materials and essential oils extractions

The plant species used in this work were: *P. anisum* L. (anise), *P. boldus* Mol (boldus), *M. piperita* L. (peppermint), *O. vulgare* L. (oregano), *M. verticillata* (Griseb) Epl. (peperina). The essential oils from those plants showed inhibitory activity against *Aspergillus* section *Flavi* growth (anise, boldus and oregano) and *Aspergillus* section *Flavi* sporulation (peppermint and peperina) (Bluma *et al.* 2005). The plants used in this study were purchased from the local market. The essential oils were extracted by the hydrodistillation method using Clevenger's apparatus (Langenau 1948). The dried plant material obtained from anise (seeds), boldus (leaves), peppermint, oregano and peperina (leaves and stems), were separately cut into small pieces and placed in a distillation apparatus with 1·3 l of double-distilled water and hydrodistilled for 1 h. After steam distillation, the extract oil samples were dried with anhydrous sodium sulfate and stored in sterilized vial at 4°C until use.

In vitro assays

Spore germination studies

Fungal isolates were grown on MEA for 5 days at 25°C to obtain heavily sporulating cultures. The spores of each fungal isolate were suspended in 10 ml of sterile glycerol/water solution with the required water availability.

This conidial suspension was poured over the solidified MMEA, which contained the appropriate concentration of each essential oil (0, 150, 300, 500 and 700 mg kg^{-1}) (Cairns and Magan 2002; Hope *et al.* 2002). To cover the entire surface, excess liquid was drained off. Petri dishes of the same a_W treatment were enclosed in polyethylene bags and incubated at 25°C. All the experiments were carried out with at least three separate replicate Petri dishes per treatment. Periodically (6, 8, 12, 19, 24, 48 and 55 h), agar discs (5 mm diameter) were aseptically removed from each treatment Petri dish and placed on a slide using a cork borer and examined microscopically. A total of 100 single spores per disc (300 spores per treatment) were examined.

The spores were considered to have germinated when the germ tube was longer than the spore diameter, and the test was considered initiated when at least 10% of the spores had germinated. Germinated spores were reported as a percentage of the total spore population and compared with the controls of the same a_W. The lag phase for germination was the number of hours needed for 10% of the spores to germinate. The germ-tube elongation was measured periodically (hours) and the germ-tube elongation rate was calculated.

Growth studies

Maize meal extract agar dishes of different a_W values, containing appropriate amount (0, 150, 300, 500 and 700 mg kg^{-1}) of each essential oil, were spot inoculated with 2 μl in the centre of each Petri dish, with spores suspended in 0·2% soft agar (Pitt 1979).

The plates were incubated at 25°C for a maximum of 11 days in polyethylene bags. Fungal growth was examined daily; the diameter of the fungal growing colonies was measured in two directions at right angles each other to obtain the mean diameter for each colony. The growth rate was calculated by linear regression of the linear phase for growth, and the time at which the line intercepted the *x*-axis was used to calculate the lag phase in relation to isolates, essential oils and a_W. In all cases, the experiments were carried out at least in three replicates per treatment. The growth of fungal cultures containing different concentrations of all essential oils was compared with that of the control culture that was grown with no essential oils. After growth was evaluated, all samples were frozen for later extraction and aflatoxin B_1 (AFB_1) quantification.

Aflatoxin B_1 analysis

A piece of MMEA with mycelium (1 cm × 1 cm), which was incubated for 11 days at 25°C, was taken from inoculated MMEA cultures (aflatoxins producers + different concentrations of EO), transferred to an Eppendorf tube, into which 500 μl of chloroform was added. The mixture was centrifuged at 850 ***g*** for 20 min. The chloroform extract was dried under nitrogen gas. The residue was redissolved in 200 μl acetonitrile/water (9 : 1) and then derivatized with 700 μl of trifluoroacetic acid/acetic acid/water (20 : 10 : 70). Fifty microlitres of derivatized solution was analysed using a reversed-phase HPLC/fluorescence detection system (Trucksess *et al.* 1994). The HPLC system consisted of a Hewlett-Packard 1100 pump (Palo Alto, CA, USA) connected to a Hewlett-Packard 1046[a] programmable fluorescence detector, interfaced to a Hewlett-Packard Chem Station. Chromatographic separations were performed on a stainless steel Supelcosil LC-ABZ C_{18} reversed-phase column (150 × 4·6 mm i.d., 5 μl particle size; Supelco, PA, USA). Water/methanol/acetonitrile (4 : 4 : 1) was used as the 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 respectively. Aflatoxin B_1 Standard (AFB_1) was obtained from Sigma Chemical (Dorset, UK) and Standard curves were constructed with different levels of AFB_1. This toxin was quantified by correlating peak height of sample extracts and those of standard curves. The analytical method detection limit was 1 ng g^{-1}.

Statistical analyses

All data analyses were performed by analysis of variance (ANOVA). F-value was found in ANOVA using SAS program 6·1. SAS institute, Cary, NC (1998). Mean values of treatment were compared with Tukey test at the $P < 0{\cdot}05$ level.

Results

Influence of essential oils on spore germination

The effect of essential oils on percentage of spore germination is shown (Table 1). At 0·982 a_W, isolates germination without essential oils (controls) was faster than at 0·955 a_W. At 0·982 a_W, a 12-h incubation period was needed to obtain 100% germination, whereas at 0·955 a_W, the same result was observed after a 17-h incubation period. The duration of the lag phase of germination was 1·6 to 3·7 h at 0·982 a_W, and 3·9 to 10 h when a_W was 0·955. As the essential oil concentration in the media increased, a reduction in the percentage of spore germination and an increase in the period of the lag phase were observed.

The greatest reduction in spore germination was observed at the highest essential oil concentration (500 mg kg^{-1}) at two water activities for all *Aspergillus* section *Flavi* isolates. Anise essential oils completely inhibited spore germination at two water activities

Table 1 Effect of different concentrations of anise and boldus essential oils on germination percentage in maize meal extract agar at two water activity (a_w) and at 25°C

	Germination (%)														
	0·982 a_w							0·955 a_w							
	Control	Anise (mg kg^{-1})			Boldus (mg kg^{-1})			Control	Anise (mg kg^{-1})			Boldus (mg kg^{-1})			
		0	150	300	500	150	300	500	0	150	300	500	150	300	500
Aspergillus parasiticus T20															
8 h*	50	0	0	0	50	0	0	15	30	0	0	20	0	0	
12 h	100	0	0	0	100	0	0	63	65	0	0	58	0	0	
19 h		57	0	0		37	0	100	84	0	0	100	10	0	
24 h		80	17	0		59	0		100	55	0		44	0	
48 h		100	45	0		100	45			100	0		56	0	
55 h			100	0			50				0		100	0	
Aspergillus flavus D65															
6 h*	19	0	0	0	19	0	0	0	0	0	0	0	0	0	
8 h	56	36	0	0	56	0	0	5	52	0	0	10	0	0	
12 h	92	48	0	0		0	0	72	99	0	0	68	33	0	
19 h	100	72	0	0	92	18	0	100	100	0	0	100	42	0	
24 h		100	0	0	100	55	0			0	0		50	0	
48 h			0	0		100	36			78	0		100	0	
55 h			0	0			50			100	0			0	
A. flavus I105															
6 h*	29	0	0	0	29	0	0	0	0	0	0	0	0	0	
8 h	40	0	0	0	40	0	0	23	0	0	0	20	0	0	
12 h	100	21	0	0	100	0	0	85	30	0	0	90	0	0	
19 h		71	13	0		20	0	100	39	0	0	100	23	0	
24 h		100	39	0		60	0		85	0	0		25	0	
48 h			100	0		100	0		100	85	0		60	0	
55 h				0			100			100	0		100	0	
A. parasiticus D106															
6 h*	16	0	0	0	16	0	0	0	0	0	0	0	0	0	
8 h	49	0	0	0	49	0	0	17	0	0	0	20	0	0	
12 h	100	21	0	0	100	0	0	76	0	0	0	69	0	0	
19 h		71	17	0		17	0	100	20	0	0	100	0	0	
24 h		100	52	0		80	0		42	0	0		0	0	
48 h			100	0		100	19		100	0	0		10	0	
55 h							60			0	0		40	0	

*Incubation hours.

assayed, whereas boldus essential oils had the same effect at the lowest a_W. The lowest concentration of boldus (150 mg kg^{-1}) did not reduce the percentage of spore germination when compared with controls at 0·982 and 0·955 a_W, whereas at the same concentration, anise essential oil reduced the conidial germination of three of the *Aspergillus* section *Flavi* isolates assayed.

When boldus EO was used, the concentration, a_W, isolates, as well as two- and three-way interactions, had a significant effect on germ-tube elongation rates of *Aspergillus* section *Flavi.* No significant differences between a_W and interaction $a_W \times$ isolates (I) were observed with anise EO (Table 2). Peppermint and peperina essential oils did not inhibit germ-tube elongation rate or percentage of spore germination compared to the controls (data not shown).

Table 3 shows percentage of germ-tube elongation rates inhibition of four *Aspergillus* section *Flavi* isolates, inoculated in MMEA with 150, 300 and 500 mg kg^{-1} of anise and boldus essential oils. In general, greater inhibition of germ-tube elongation rates was observed at the highest essential oils concentrations (500 mg kg^{-1}) at two water activities; for all *Aspergillus* section *Flavi* isolates, the percentage of inhibition was greater than 85%. At 0·982 a_W, inhibition caused by boldus and anise essential oils was proportional to the concentration used; these essential oils inhibited elongation rates from 300 and 500 mg kg^{-1} respectively.

Table 2 Analysis of variance of the effect of different concentrations (C) of anise and boldus essential oils on germ-tube elongation rates of *Aspergillus* section *Flavi* isolates (I) at different water activities (a_W)

		Anise EO		Boldus EO	
	DF†	Ms‡	*F*§	Ms	*F*
a_W	1	0·0500	0·34	11·0055	115·77*
C	3	22·9198	153·69*	18·4296	193·86*
I	3	1·17197	7·86*	1·1158	11·74*
$a_w \times C$	3	3·7252	24·98*	0·3942	4·15*
$a_W \times I$	3	0·2017	1·35	2·1115	22·21*
$C \times I$	9	0·9920	6·65*	0·3615	3·80*
$a_W \times C \times I$	9	1·5438	10·35*	0·2830	2·98*

*$P < 0·001$.
†Degree of freedom.
‡Mean square.
§*F* value.

Inhibition in elongation rate occurred at 300 and 500 mg kg^{-1} when aflatoxigenic isolates grew with boldus essential oil at 0·955 a_W. Anise was effective only at the highest concentration when a_W was 0·955.

Effects of essential oils on *Aspergillus* section *Flavi* growth

Statistical analysis showed that concentration of essential oils as well as two and three-way interactions had a significant effect on growth of *Aspergillus* section *Flavi*. No significant differences between isolates were shown when anise and peppermint essential oils were used. Water activity had no differences concerning fungal growth when oregano essential oils were used. (Table 4).

Figure 1 shows the effect of different concentrations of anise, boldus and oregano essential oils on growth rate of

Table 3 Percentage of germ-tube elongation rate inhibition of *Aspergillus* section *Flavi* produced by boldus and anise essential oils in maize meal extract agar at 0·982 and 0·955 a_w and 25°C

	A. parasiticus T20		*A. flavus* D65		*A. flavus* I105		*A. parasiticus* D106	
a_w	0·982	0·955	0·982	0·955	0·982	0·955	0·982	0·955
Boldus (mg kg^{-1})								
150	4 ± 0·4*	0	10 ± 1·4	0	0	0	0	28 ± 9·8
300	43 ± 3	81 ± 3·4	14 ± 2·7	0	15 ± 7·4	67 ± 0·4	34 ± 1·6	63 ± 4·7
500	83 ± 9·8	100	92 ± 10	100	88 ± 9·8	100	87 ± 9·5	100
Anise (mg kg^{-1})								
150	12 ± 3·9	0	0	0	33 ± 3·1	35·5 ± 0·3	71 ± 3·0	32 ± 4·6
300	80 ± 2·7	0	100	28 ± 1·3	75 ± 0·6	17 ± 1·3	80 ± 3·4	100
500	89 ± 3·8	100	100	100	89 ± 0·8	100	90 ± 0·15	100

*Mean values ± SD.

Table 4 Analysis of variance of the effect of different concentrations (C) of essential oils on growth of *Aspergillus* section *Flavi* isolates (I) at different water activities (a_W)

		Anise EO		Boldus EO		Peperina EO		Peppermint EO		Oregano EO	
	DF†	MS‡	*F*§	MS	*F*	MS	*F*	MS	*F*	MS	*F*
a_W	1	0·1206	17·50*	0·1214	30·36*	1·8249	337*	0·5899	54*	0·0075	1·5
C	3	1·2461	180·8*	1·1147	278·6*	0·1596	29·5*	0·6611	61*	0·7739	157*
I	3	0·0137	1·98	0·0287	7·18*	0·1026	18·9*	0·0167	1·5	0·0354	7·19*
$a_W \times C$	3	0·1319	19·1*	0·2254	56·36*	0·0673	12·5*	0·2157	20*	0·1524	30·9*
$a_W \times I$	3	0·0005	0·08	0·0174	4·37**	0·0291	5·4**	0·0145	1·3	0·0207	4·2**
$C \times I$	9	0·0542	7·87*	0·0461	11·53*	0·0279	5·16*	0·0541	5*	0·0248	5·0*
$a_W \times C \times I$	9	0·0276	4·01*	0·0538	13·46*	0·0219	4·05*	0·1074	9·9*	0·0181	3·7*

*$P < 0·001$.
**$P < 0·05$.
†Degree of freedom.
‡Mean square.
§*F* value.

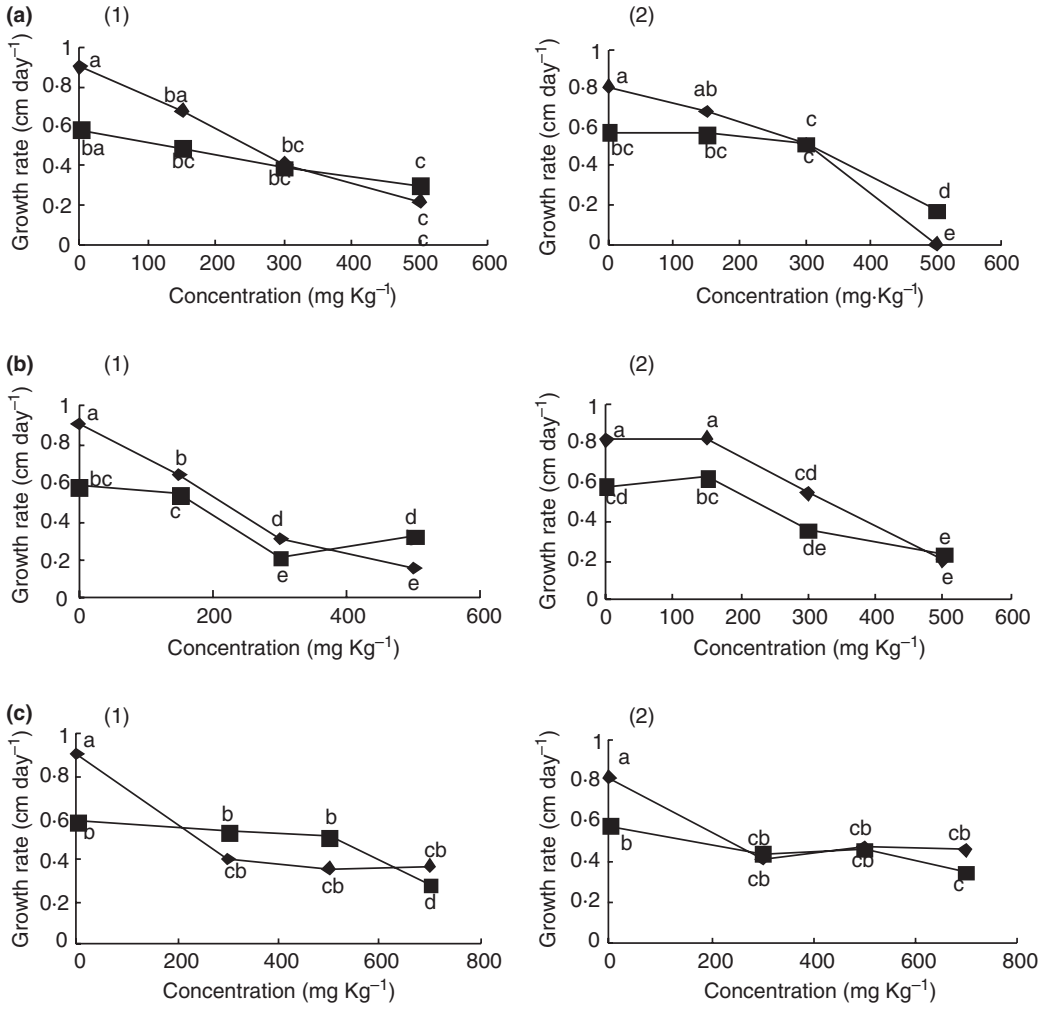

Figure 1 Effect of anise (a) boldus (b) and oregano (c) essential oils on growth rate (cm day^{-1}) of: (1) *Aspergillus parasiticus* T20 and (2) *Aspergillus flavus* I105 in maize meal extract agar under different water activity conditions; ♦, 0·982; ■, 0·955 a_w. Data with the same letter for each essential oil and isolate are not significantly different ($P < 0·05$) according to Tukey's test.

A. parasiticus T20 and *A. flavus* I105 under different a_W conditions in MMEA, at 25°C.

Mycelial growth of *Aspergillus* section *Flavi* was affected by the addition of essential oils. The inhibition in the growth rate of all *Aspergillus* section *Flavi* caused by anise and boldus essential oils was proportional to the EO concentration assayed and was greater than 80% at 500 mg kg^{-1}. The influence of oregano EO on growth rate was similar at all concentrations tested. The percentage of growth rate inhibition produced by oregano EO was not different among the concentrations assayed. Only when a_w was 0·955, oregano EO was most inhibitory at the highest concentration assayed (700 mg kg^{-1}).

Significant inhibition of growth occurred at a concentration of 150 mg kg^{-1} when aflatoxigenic isolates grew with anise EO at 0·982 a_w, but not all aflatoxigenic isolates reduced their growth rate at boldus and oregano lowest concentration. At 0·955 a_w, the lowest concentration assayed for anise, boldus or oregano was insufficient to affect growth of aflatoxigenic isolates.

Aspergillus section *Flavi* growth rate was weakly inhibited by some concentration of peppermint and peperina essential oils at all the a_w conditions tested (Fig. 2). The lowest concentration of these essential oils showed growth rate stimulation in the aflatoxigenic strains. It is important to remark that the highest concentrations of this EO were able to reduce sporulation and vegetative mycelia in aflatoxigenic growth zones, compared to *in vitro* growth controls (data not shown).

The ANOVA analysis of lag phase (Table 5) showed that concentration of essential oils, as well as two- and three-way interactions, had significant differences. Water activity had differences on lag phase when boldus, peperina

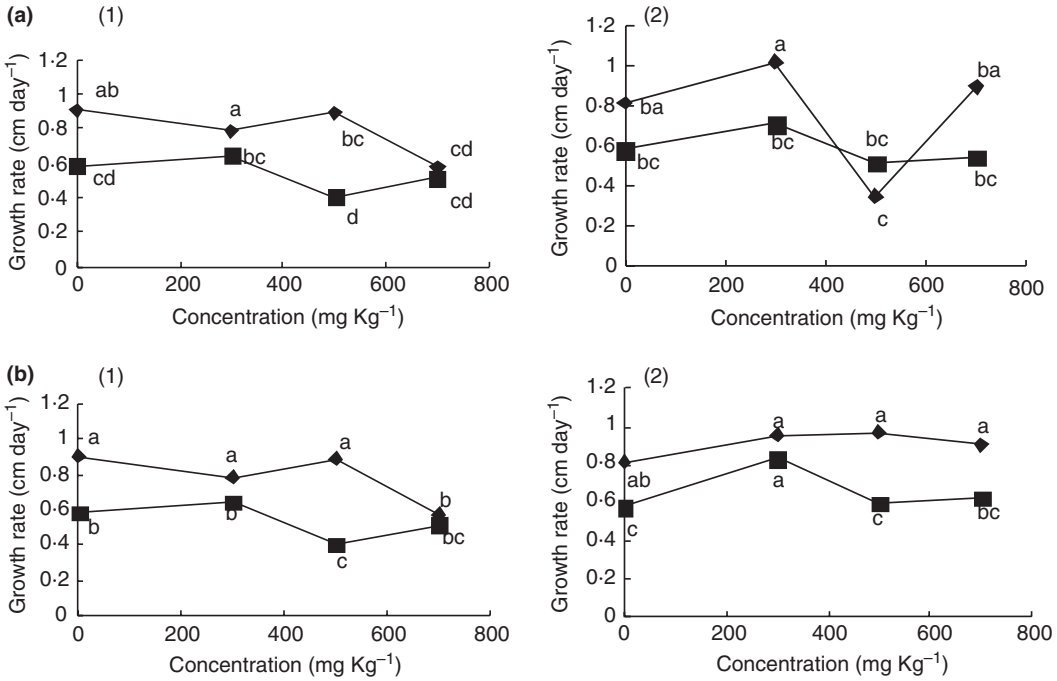

Figure 2 Effect of peppermint (a) and peperina (b) essential oils on growth rate (cm day^{-1}) of: (1) *Aspergillus parasiticus* T20 and (2) *Aspergillus flavus* I105 in maize meal extract agar under different water activity conditions; ♦, 0·982; ■, 0·955 a_w. Data with the same letter for each essential oil and isolates are not significantly different ($P < 0·05$) according to Tukey's test.

and oregano EO were studied and the isolates did not show any difference when peperina EO was used.

Lag phase increased (Table 6) following the same pattern of growth rates reduction. At a_w 0·982, the lag phase was the longest at the essential oils concentrations that were able to reduce growth rate, some isolates did not show lag phase influence at boldus and oregano concentrations, lower than 500 mg kg^{-1} of boldus and oregano. At these a_w conditions, some isolates of *Aspergillus* increased their lag phase in the presence of peperina and peppermint EO.

When a_w was 0·955, anise prolonged the lag phase at the highest concentrations, whereas some boldus EO concentration increased fungal lag phase. The lowest influences on the lag phases were shown with oregano, peppermint and peperina EO; some concentrations of these produced a significant reduction in lag phase (Table 6).

Effects of essential oils on aflatoxin B_1 accumulation by *Aspergillus* section *Flavi*

Table 7 shows AFB_1 accumulation by *Aspergillus* section *Flavi* in MMEA at different concentrations of essential oils.

Aflatoxigenic isolates growing without essential oils (controls) were able to produce higher levels of AFB_1 when a_W was 0·982 than at 0·955 a_W. *Aspergillus parasiticus* isolates were the best producers.

When different concentrations of anise, boldus, oregano, peppermint and peperina essential oils were added to the cultures, a reduction in AFB_1 synthesis was observed.

In the presence of anise essential oils, AFB_1 accumulation was inhibited at 0·982 and 0·955 a_w. When *Aspergillus* section *Flavi* grew in MMEA with different concentrations of boldus EO, AFB_1 accumulation was detected only with 500 mg kg^{-1} of boldus essential oils. At 500 mg kg^{-1} of boldus EO, AFB_1 level was reduced to values that went beyond 85% in all aflatoxigenic isolates, only when a_W was 0·982 a_w.

The AFB_1 accumulation by aflatoxigenic isolates was reduced at all oregano and peperina EO concentrations assayed the greatest inhibitions were observed at 500 and 700 mg kg^{-1}. At 0·955 a_w three aflatoxigenic isolates did not reduce AFB_1 accumulation when they grew with the lowest concentration of peperina EO.

Discussion

In this study, spore germination, mycelial growth, and AFB_1 production of four isolates of *Aspergillus* section *Flavi* were significantly influenced by anise, boldus and oregano essential oils, while the effect of peppermint and peperina essential oils on the accumulation of AFB_1 was

Table 5 Analysis of variance of the effect of different concentrations (C) of essential oils on lag phase of *Aspergillus* section *Flavi* isolates (I) at different water activities (a_W)

		Anise EO		Boldus EO		Peperina EO		Peppermint EO		Oregano EO	
	DF†	MS‡	*F*§	MS	*F*	MS	*F*	MS	*F*	MS	*F*
a_W	1	0·013	0·01	1·8984	5·37**	2·0768	9·28**	1·3689	2·34	17·1194	90·62*
C	3	215·37	86·1*	33·282	94·06*	2·1207	9·47*	5·8075	9·91*	4·3159	22·85*
I	3	22·888	9·16*	4·578	12·93*	3·9873	17·81*	0·7665	1·31	0·9807	5·19**
$a_W \times C$	3	3·214	1·29	17·787	50·27*	1·2500	5·58**	4·1123	7·02*	13·0043	68·84*
$a_W \times I$	3	9·313	3·73*	0·6074	1·72	1·8527	8·28*	4·9425	8·44*	0·3896	2·06
$C \times I$	9	31·138	12·5*	1·9076	5·39*	0·6140	2·74**	2·4326	4·15*	0·8301	4·39*
$a_W \times C \times I$	9	1·477	0·59	1·5991	4·52*	0·7515	3·36**	1·27071	2·17**	0·8293	4·39*

*$P < 0·001$.
**$P < 0·05$.
†Degree of freedom.
‡Mean square.
§*F* value.

Table 6 Lag phases (days) of *Aspergillus* section *Flavi* developed in maize meal extract agar with different concentrations of essential oils at two water activities and at 25°C

a_W	0·982				0·955			
Strains	*A. parasiticus* T20	*A. flavus* D65	*A. flavus* I105	*A. parasiticus* D106	*A. parasiticus* T20	*A. flavus* D65	*A. flavus* I105	*A. parasiticus* D106
Controls	0·29d	1·50hi	1·20cd	0·51b	1·44de	1·98ced	0·75b	0·92gfe
Anise (mg kg^{-1})								
150	1·00cd	0·62ij	1·51cd	1·07bc	2·00de	1·53ced	0·81b	1·37gfe
300	2·72bcd	2·83bcd	3·31cb	3·79ba	3·91ba	4·05b	2·39b	3·83b
500	4·34ab	4·10a	>11a	7·76a	4·79a	5·80a	>11a	5·48a
Boldus (mg kg^{-1})								
150	0·97d	0·81ij	0·67d	1·23bc	1·64de	1·45ced	0·79b	1·56gfh
300	1·86cd	2·38edf	1·64cd	2·53ba	4·06a	2·27ced	1·59b	3·98b
500	6·6a	3·49ba	3·91cb	5·75a	2·00dce	2·15ced	1·26b	2·97cb
Peperina (mg kg^{-1})								
300	1·05cd	1·70ih	0·95cd	1·89bc	2·25dc	2·50ced	1·77b	2·08cde
500	1·23cd	1·39ih	1·34cd	1·72bc	2·25dc	1·98ced	0·30bc	1·62fde
700	0·55d	0·92ih	1·40cd	2·06bc	1·11fe	2·96bc	0·41bc	1·87fde
Peppermint (mg kg^{-1})								
300	1·69cd	1·98ih	1·41cd	1·86bc	2·97dc	2·52ced	1·33b	2·69cd
500	8·75bc	1·92ih	6·46ab	1·72bc	1·53de	1·98ced	1·23b	0·88gfh
700	1·21cd	1·06ih	1·89cd	2·62bc	1·14de	1·03ced	0·77b	1·35gfe
Oregano (mg kg^{-1})								
300	2·1bcd	2·5dc	1·56cd	1·19bc	1·54de	1·50ced	1·17b	1·92fde
500	1·42cd	0·58j	1·59cd	1·05bc	1·80de	0·38fe	0·23bc	0·56gh
700	3·44gc	3·64ba	2·49cb	3·42ba	0·20f	0·20f	0·35bc	0·35h

Data with the same letter for each isolate and water activity are not significantly different ($P < 0·05$) according to Tukey's test.

irregular. Fungal growth was weakly affected by peppermint and peperina essential oils and the germination of spores was not affected by these essential oils.

Some studies have shown that specific essential oils can inhibit the growth rate and spore germination of spoilage fungi. Paster *et al.* (1995) found that oregano essential oils inhibited *Aspergillus niger*, *Aspergillus ochraceus* and *A. flavus*. Growth was completely inhibited with 400 mg kg^{-1} of oregano essential oils; these concentrations also prevented spore germination.

Our results with anise, boldus and oregano EO were similar to those of Soliman and Badeaa (2002) who tested

Table 7 Effect of different concentrations of anise, boldus, oregano, peppermint and peperina essential oils on aflatoxin B_1 accumulation (ng g^{-1}) in MMEA at two a_w and at 25°C

a_W	0·982				0·955			
Strains	*A. parasiticus* T20	*A. flavus* D65	*A. flavus* I105	*A. parasiticus* D106	*A. parasiticus* T20	*A. flavus* D65	*A. flavus* I105	*A. parasiticus* D106
Controls	22552 ± 26·3	97·45 ± 10·7	56·02 ± 31	755·84 ± 376	49·68 ± 6·65	5·513 ± 0·74	13·47 ± 5·48	12·71 ± 3·76
Anise (mg kg^{-1})								
150	ND	ND	ND	10·7 ± 10·2	ND	ND	ND	ND
300	ND	ND	ND	ND	ND	ND	ND	ND
500	ND	16·7 ± 6·7	ND	ND	ND	ND	ND	ND
Boldus (mg kg^{-1})								
150	ND	ND	ND	ND	ND	ND	ND	ND
300	ND	ND	ND	ND	ND	ND	ND	ND
500	3·46 ± 0·04	10·3 ± 0·54	7·5 ± 1·4	3·77 ± 0·12	41·7 ± 21·7	1·25 ± 1·05	56·4 ± 8·5	9·5 ± 7·6
Peperina (mg kg^{-1})								
300	105·8 ± 99·9	2·8 ± 2·58	2·85 ± 1·2	41·6 ± 3·3	16·4 ± 14·4	11·2 ± 0·2	10·99 ± 0·3	7·9 ± 3·4
500	ND	7·1 ± 2·11	ND	ND	ND	ND	ND	ND
700	1·97 ± 0·76	4·12 ± 3·28	ND	ND	ND	ND	ND	ND
Peppermint (mg kg^{-1})								
300	30·2 ± 0·57	29·2 ± 7·47	123·08 ± 21·5	19·4 ± 9·7	ND	70·7 ± 0·54	25·07 ± 23·3	1·65 ± 0·3
500	3·86 ± 1·90	3·26 ± 0·68	17·2 ± 13·8	ND	ND	56·5 ± 8·8	119·4 ± 8·0	12·07 ± 5·6
700	ND	31·1 ± 15·0	ND	ND	ND	1·29 ± 0·88	ND	ND
Oregano (mg kg^{-1})								
300	ND	0·93 ± 0·23	1·0 ± 0·85	ND	ND	ND	ND	1·27 ± 1·12
500	34 ± 19·8	62·1 ± 53·8	31·6 ± 13·2	2·0 ± 0·1	13·9 ± 1·17	1·23 ± 0·02	14·3 ± 0·65	15·20 ± 7·10
700	ND	ND	ND	ND	ND	ND	ND	1·12 ± 0·56

ND: no aflatoxin detected (detection limit: 1 ng g^{-1}).

12 essential oils of different medicinal plants for inhibitory activity against *A. flavus*, *A. parasiticus*, *A. ochraceus* and *F. verticilloides* in PDA medium. They demonstrated that the inhibitory effect of the oils was proportional to their concentration. They found that thymus and anise essential oils were more effective than cinnamon (*Cinnamomum zeylanicum* L.) and spearmint (*Mentha viridis*). Anise essential oils have more inhibitory effect than the other members of *Umbellifereae* family, like caraway and fennel. Anise completely inhibited the *A. flavus* and *A. parasiticus* strains studied at 500 mg kg^{-1}, while caraway and fennel EO had the same effect at 2000 mg kg^{-1}. Anise, fennel, and caraway EO contain anethole, its concentration being approximately 90% in anise and 60% in both fennel and caraway. The effect of anise as fungicide is much greater than the other members of the same family, the difference in anethole concentration may explain the antifungal effect observed. According to Baydar *et al.* (2004) phenolic components, such as eugenol, anethole, carvacrol, γ-terpinolene (main components of clove, anise, oregano and boldus EO respectively), ρ-cimene and thymol are chiefly responsible for the antimicrobial properties of essential oils.

In a previous work (Bluma *et al.* 2005), peperina and peppermint essential oils showed total inhibition on *Aspergillus* section *Flavi* sporulation. Chalfoun *et al.* (2004) observed that garlic, mint, thyme, oregano and bay leaf showed the highest levels of sporulation inhibition when these powdered spices were evaluated in PDA culture media and CYA20S. The inhibitory effect on sporulation increases the efficacy of treatments. If the fungus has a certain degree of mycelial growth in the presence of spice treatments, the sporulation reduction has an additional impact in relation to mycelial development of the fungus (Chalfoun *et al.* 2004).

Growth rate stimulation was observed in some cases when *Aspergillus* section *Flavi* grew with the lowest concentration of peperina and peppermint essential oils; however, this stimulation did not occur in all aflatoxigenic isolates. The stimulation on growth rate and mycotoxin production by suboptimal concentration of different essential oils was observed by Cairns and Magan (2002) and Hope *et al.* (2002). Under these conditions, active growth is likely to occur and the imposition of stress could result in a survival or defence response by enhanced mycotoxin production (Magan *et al.* 2002).

Essential oils can comprise more than 60 individual components (Russo *et al.* 1998). Major components can constitute up to 85% of the EO, whereas other components are present only as a trace (Bauer *et al.* 2001). Some

researchers reported that there is a relationship between the chemical structures of the most abundant compounds in the essential oils and the antimicrobial activity. There is evidence that minor components have a critical part to play in antimicrobial activity, possibly by producing a synergic effect between other components (Burt 2004). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antimicrobial activity is not attributable to one specific mechanism but to the existence of several targets in the cell (Carson *et al.* 2002) According to Pawar and Thaker (2007), the physical nature of essential oils, i.e. low molecular weight combined with pronounced lipophilic tendencies allow them to penetrate cell membrane more quickly than other substances. Conner and Beuchat (1984a,b) suggested that the antimicrobial activity of essential oils or their constituents such as thymol, carvacrol, vanillin could be the result of damage to the enzymatic cell system, including those associated with energy production and synthesis of structural compounds. Nychas (1995) indicated that phenolics could denature the enzymes responsible for spore germination or interfere with the aminoacid involved in germination. Rasooli and Owlia (2005) and Helal *et al.* (2007) showed irreversible damage in cell wall, cell membrane and cellular organelles when *A. parasiticus* and *A. flavus* were exposed to different essential oils. In addition to this, it is important to recognize that there are complex interactions of environmental factors, like water availability, with the efficacy of essential oils. It could be possible to use a combination of these could be used to reduce growth and aflatoxin production of *A. flavus* and *A. parasiticus.*

These findings clearly indicate that essential oils should find practical application in the inhibition of mycotoxin production in stored grain. Essential oils, such as anise and boldus, could be safely used as a preservative material on some foods because they stopped fungal growth and AFB_1 accumulation. They could also be added to grain in storage to protect it from fungal infection. These oils could be used as a substitute for chemical fungicides. They may also prove valuable as 'lead structure' for the development of synthetic compounds as they are natural and nontoxic to humans and animals alike (Soliman and Badeaa 2002).

Studies are now in progress to examine the effect on growth and AFB_1 accumulation with natural maize grain stored under different environmental regimes.

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