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Effectiveness of Mexican oregano essential oil from the Dominican Republic (*Lippia graveolens*) against maize pests (*Sitophilus zeamais* and *Fusarium verticillioides*)

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SUMMARY

The insecticidal and antifungal properties of Mexican oregano (*Lippia graveolens*) essential oil from the Dominican Republic were investigated under laboratory conditions against two main pests of stored grains: *Sitophilus zeamais* and *Fusarium verticillioides*. Although oregano essential oil at 600 µl/l air did not result in a significant mortality of *S. zeamais* after 24 hours of exposure by fumigation, this essential oil was a powerful acetylcholinesterase (AChE) inhibitor *in vitro*. One of the main components of oregano essential oil, p-cymene, presented fumigant toxicity and AChE inhibition activity against the maize weevil. The *L. graveolens* essential oil antifungal activity against *F. verticillioides* was evaluated at 50, 100 and 200 µl/l, and it was found that growth parameters were affected by the presence of oregano essential oil in the media, whereas FB_1 production was not inhibited. The results demonstrate that oregano essential oil and p-cymene can be used as alternatives to synthetic pesticides against *F. verticillioides* and *S. zeamais*, respectively.

Key words: Acetylcholinesterase inhibition, fungal pathogen, *Lippia graveolens*, maize pest control, maize weevil.

Peschiutta, M.L.; J.S. Arena, A. Ramirez Sanchez, E. Gomez Torres, R.P. Pizzolitto, C. Merlo, M.P. Zunino, A.B. Omarini, J. S.Dambolena y J. A. Zygadlo, 2016. Efectividad del aceite esencial de orégano mexicano de República Dominicana (*Lippia graveolens*) contra plagas del maíz (*Sitophilus zeamais* y *Fusarium verticillioides*). Agriscientia 33 (2): 89-97

RESUMEN

Se estudiaron en condiciones de laboratorio las propiedades insecticidas y fungicidas del aceite esencial de orégano mexicano (*Lippia graveolens*) de

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República Dominicana contra dos plagas principales de granos almacenados: *Sitophilus zeamais* y *Fusarium verticillioides*. Aunque el aceite esencial de orégano a 600 µl/l de aire no resultó en una mortalidad significativa de *S. zeamais* después de 24 horas de exposición a la fumigación, fue un potente inhibidor de la acetilcolinesterasa (AChE) *in vitro*. El p-cimeno, uno de los principales componentes del aceite esencial, presentó actividad fumigante y de inhibición de la AChE contra el gorgojo del maíz. Además se evaluó la actividad antifúngica frente a *F. verticillioides* a 50, 100 y 200 µl/l y se encontró que los parámetros de crecimiento del hongo fueron afectados por la presencia del aceite esencial de orégano en el medio, mientras que la producción de fumonisina B_1 (FB_1) no se inhibió. Los resultados demuestran que el aceite esencial de orégano y el p-cimeno se pueden usar como alternativas a los pesticidas sintéticos contra *F. verticillioides* y *S. zeamais*, respectivamente.

Palabras clave: Inhibición de la acetilcolinesterasa, hongo patógeno, *Lippia graveolens*, control de plagas del maíz, gorgojo del maíz.

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INTRODUCTION

The maize weevil *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) is a serious pest of stored grains, especially corn. It consumes the grains and creates the conditions that favor the development of fungi, resulting in major losses, both quantitative and qualitative (Caneppele *et al*., 2003; Trematerra *et al*., 2013). *Fusarium verticillioides* (Sacc,) Nirenberg (= *F. moniliforme* Sheldon teleomorph *Giberella fujikuroi* [Sawada] Ito in Ito & Kimura) is a frequent fungal pathogen in stored maize. In addition, *F. verticillioides* is one of the most important mycotoxin fumonisin B_1 (FB_1) producers. It can be produced during maize storage and represents a major problem due to its toxicological implications for humans and farm animals (Stockmann-Juvala & Savolainen, 2008; Theumer *et al*., 2010). The use of synthetic fumigants, such as methyl bromide, phosphine and sulfuryl fluoride, is the most widespread method to control stored product pests. However, the use of these compounds has led to a variety of problems including the development of insecticide resistance (Pimentel *et al*., 2009), toxic residual effects in food grains, toxicity to non-target organisms and environmental pollution (Isman, 2006). Moreover, the increasing public awareness of pesticide safety and possible damage to the environment has resulted in greater attention being given to the control of stored food pests by means of natural products (Rajendran & Sriranjini, 2008), of which essential oils (EOs) are considered to be an interesting alternative because of their effectiveness and versatility. In fact, their volatility and chemical diversity makes them excellent fumigants, insecticides, fungicides and repellents. The use of EOs as low-risk biopesticides has increased considerably owing to their popularity with organic farmers and environmentally conscious consumers (Rajendran & Sriranjini, 2008; Gleiser & Zygadlo, 2009; Regnault-Roger *et al*., 2012; Hernández-Lambraño *et al*., 2014; Sousa *et al*., 2015), with this demand for naturally active compounds such as EOs having stimulated the search for these chemicals in biodiversity hotspots.

Lippia graveolens Kunth (Lamiales: Verbenaceae), one of the most commercial species within the genus *Lippia*, is an aromatic plant commonly known as Mexican oregano or oreganillo. Interest in this species is increasing due to its insecticidal and antimicrobial activities (Cavalcanti *et al*., 2004; Gleiser & Zygadlo, 2007; Bueno-Durán *et al*., 2013) as a result of its chemical constituents, such as thy-

mol and carvacrol. In this context, the aim of this study was to analyze the EO composition of *L. graveolens* growing in the Dominican Republic, and to evaluate the insecticidal activity and acetylcholinesterase (AChE) inhibition of *L. graveolens* EO and its main components against *S. zeamais.* The antifungal properties and FB_1 inhibition of Mexican oregano EO from the Dominican Republic against the *F. verticillioides* strain M3125 were measured.

MATERIALS AND METHODS

Insects

Adults of *S. zeamais* were obtained from Metan, Salta, Argentina, and were maintained in sealed containers (10 l) under controlled conditions (26 °C and 60% relative humidity) with a lighting regime of 12:12 hours (D:L). The insects were reared on whole maize grains, and all bioassays were carried out under the same environmental conditions and in complete darkness (FAO, 1974). The colony was kept in the laboratory for two years without being exposed to insecticides before testing. The unsexed adult weevils used in all the experiments were approximately two weeks old.

Fungal strain

Fusarium verticillioides (Sacc) Niremberg (= *F. moniliforme* Sheldon teleomorph *Giberella fujikuroi* [Sawada] Ito in Ito & Kimura (Leslie *et al*., 2006) strain M3125 (provided by Dr. Robert Proctor, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL, United States) isolated from maize in California (fumonisin-producing strain) was used in all the antifungal tests (Leslie *et al*., 1992).

Essential oil and pure compounds

Lippia graveolens Kunth samples were collected in commercial crops in Santo Domingo, Dominican Republic. The aerial parts of the plants were air dried, chopped into small pieces using a mill with rotary knives, and extracted by hydro-distillation for 2 hours in a Clevenger's apparatus in order to collect their vaporized EO. The EO obtained was stored in dark glass tubes under refrigeration (4 °C) until evaluation.

The density of EO was measured by gravimetric method and found to be 0.93 g/ml at room temperature (± 2 °C). Identification of the constituents in EO was determined using electron impact mass spectra (EI-MS) obtained from gas chromatography-mass spectrometry (GC-MS) and by co-injection of standards (Sigma Aldrich Co. Buenos Aires, Argentina), with the mass spectra libraries Adams, NIST and a homemade library being utilized. Compound concentrations were expressed as a percentage of the peaks area, and the retention index (RI) of each compound was obtained for a homologous series of n-alkanes C_9-C_{20} (Sigma Aldrich Co. Buenos Aires, Argentina). GC-MS was performed on a GC-MS Perkin Elmer 600 with the chromatography conditions being as follows: DB-5 capillary column, which was programmed: temperature profile of 60 °C for 5 minutes, ramped up to 170 °C at 4 °C/minute, and then to 240 °C at 20 °C/minute; injector temperature 250 °C; detector temperature 250 °C; carrier gas, H_2 at 45 cm/second, split into 50 ml/minute. The identification and quantification of limonene, β-phellandrene and 1,8-cineole were carried out using Carbowax capillary columns, at the same conditions mentioned above for the DB-5 column.

Fumigation toxicity assay

Insecticidal activity against *S. zeamais* was evaluated using a fumigant toxicity assay previously described by Herrera *et al*. (2015a), with modifications. Different amounts of oregano EO and its main pure compounds (p-cymene, thymol and carvacrol) were applied to Whatman filter paper disks (2 cm diameter) placed on the underside of the screw cap of fumigation chambers (30 ml-glass vials) at doses from 75 to 600 µl/l air. A piece of voile was also placed under the screw cap to avoid direct contact of the weevils with the EOs. Ten adults of *S. zeamais* and 5 g of whole maize grains were placed in each vial, with the inclusion of corn allowing the natural conditions in a silo to be mimicked. Five replicates per dose were carried out, and control treatments were performed under the same conditions without EOs or pure compounds (negative control) and with 2,2-dichlorovinyl dimethyl phosphate (DDVP) compound 0.06 µl/l air (positive control). This latter compound was used as a positive control due to its high vapor pressure and its known insecticidal activity. Insect mortality was evaluated after 24 hours, and the mortality percentages and LC_{50} and LC_{95} values were then calculated.

In vitro AChE inhibition tests

The effects of oregano EO and its main components (p-cymene, thymol and carvacrol) on ace-

tylcholinesterase (AChE) activities were examined at different concentrations (0.09, 0.19, 0.46, 1.39, 4.65, 13.95 and 23.25 mg/ml for oregano EO, and 0.025, 0.05, 0.1 and 0.5 mM for its components). Untreated whole *S. zeamais* adults (0.1 g) were homogenized with 1 ml phosphate buffer (pH 7.4), and then the homogenate was centrifuged (6500 rpm for 20 minutes at 0°C) and the supernatant was used as crude AChE. Inhibition of AChE was then determined by the colorimetric method of Ellman *et al*. (1961) using acetylthiocholine iodide (ATChI) at 2.5 mM (Sigma Aldrich Co., St. Louis, MO USA) as the substrate. Enzyme aliquots (20 µl) and 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB) (20 µl of 4 mM) were added to 0.1M phosphate buffer (pH 7.4; 120 µl), and the EO active compounds (20 µl) prepared in absolute ethanol were added to this mixture. Control treatments used the addition of absolute ethanol (20 µl) instead of an active compound. All mixtures were incubated at 35 °C for 15 minutes, and the reactions were started by adding ATChI (20 µl), with absorbance being measured at 412 nm using a spectrophotometer (Model 680 Microplate Reader, Bio-Rad).

AChE activity was examined, with each treatment being corrected by blanks for nonenzymic hydrolysis. The inhibition percentage of AChE activity was calculated as follows: AChE inhibition% = (ODC-ODT)/ODC x 100, where ODC is the optical density of control and ODT is the optical density of the treatment. Each concentration was analyzed for triplicate assays, and the mid-point inhibitive concentration (IC_{50}) values were determined graphically from the inhibition curves (log inhibitor concentration vs percentage of inhibition) (Mohammadi-Farani *et al*., 2013).

In vivo AChE inhibition tests

For *in vivo* AChE inhibition tests, *S. zeamais* adults were cultured for 24 hours in the absence or presence of Mexican oregano EO (150 µl/l air), with the experimental conditions and procedures carried out as described above. Homogenates were prepared in phosphate buffer (0.1 M, pH 7.4) and kept on ice during the homogenization, with approximately fifty insects (0.1 g) per ml of buffer being used for each sample and the activity of AChE being determined immediately after the preparation of the homogenates.

Effect of *Lippia graveolens* EO on fungal growth and fumonisin production

Fusarium verticillioides M3125 inoculum was grown on Czapek-dox agar Petri plates for 7 days at 28 ºC in the dark to allow profuse sporulation. Then, sterile distilled water was added to each plate, and a conidia suspension was obtained by scraping the colony surface with a sterile Drigalsky spatula and filtering it through a cheesecloth. The suspension was then counted using a Neubauer chamber and adjusted to 10^6 conidia/ml.

The antifungal activity was tested at different concentrations of *L. graveolens* EO using Czapek-Dox agar in Petri dishes (90 mm). The culture medium was autoclaved at 120 °C for 15 minutes, and before cooling at 45 °C, an appropriate volume of EO was added to the media to obtain concentrations of 50; 100 and 200 µl/l. Czapek-Dox agar plates were inoculated centrally with 10 µl of the conidia suspension and incubated in the dark at 28 °C. Czapek-Dox Agar plates without the addition of EO were used as control.

The amount of radial mycelial growth was determined by periodical measurement of two right-angled diameters of the colonies, until the colonies reached the edge of the plate. Colony diameters versus time were plotted, and radial growth rates (mm/day) were evaluated from the slope by linear regression. Lag phase was determined as the abscissa from these growth rate curves.

To evaluate FB_1 biosynthesis, the inoculated plates were incubated in the dark at 28 °C for 28 days. After this incubation, the agar in the experimental plates was dried for 96 hours at 60 °C in a forced-air oven before being ground to a fine dry powder. Finally, 10 ml of water was added to the dried agar, and FB_1 was extracted by shaking the dried dishes with water for 120 minutes on an orbital shaker, after which the mixture was centrifuged at 5000 rpm for 15 minutes. All experiments were performed in triplicate.

Fumonisin B_1 quantification

FB_1 quantification was carried out according to a methodology described by Shephard *et al*.(2000). Briefly, samples (1000 µl) from the FB_1 extracts were diluted with acetonitrile (1:1), and then an aliquot (50 µl) was derivatized with 200 µl of a solution prepared by adding 5 ml of 0.1 M sodium tetraborate and 50 µl of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of o-phthaldialdehyde. The analysis of the derivatized samples was performed using a Perkin Elmer HPLC equipped with a fluorescence detector with the wavelengths used for excitation and emission being found to be 335 nm and 440 nm, respectively, and with an analytical reverse phase column C_{18} (150 mm × 4.6 mm in-

ternal diameter and 5 µm particle size) connected to a precolumn C_{18} (20 mm × 4.6 mm and 5 µm particle size). Methanol and NaH_2PO_4 0.1 M (75:25) were used as the mobile phase, with the pH being set at 3.35 ± 0.2 with orthophosphoric acid and a flow rate of 1.5 ml/min. The quantification of FB_1 was carried out by comparing the peak areas obtained from the samples with those corresponding to the analytical standards of FB_1 using PROMEC (Program on mycotoxins and experimental carcinogenesis, Tygerberg, Republic of South Africa).

Statistical analysis

The concentration-mortality data were subjected to Probit analysis (Finney, 1971) to obtain the LC_{50} and LC_{95} values using the SPSS 21.0 software program. In the AChE inhibition, antifungal activity and anti-mycotoxicogenic activity assays, the data were analyzed by one-way analysis of variance (ANOVA). The mean values were compared using Fisher's LSD posteriori test.

RESULTS AND DISCUSSION

The hydro-distillated EO of *L. graveolens* from the Dominican Republic was analyzed by GC–MS, identifying thirty-seven different components in the oil (Table 1). According to this analysis, thymol (22.8%), carvacrol (22.7%) and p-cymene (18.8%) were the major volatile compounds of Mexican oregano EO, similar to the results reported by Calvo-Irabién *et al*. (2014) and Rodriguez-Garcia *et al*. (2016).

In the present study, the EO from oregano was evaluated for use as an insecticide against *S. zeamais* adults. Although pure EO obtained from *L. graveolens* leaves did not show significant mortality of *S. zeamais* after 24 hours of exposure by fumigation at 600.0 µl/l air (LC_{50}> 600.0 µl/l air), p-cymene, a principal compound of oregano EO, presented a higher insecticidal activity and had LC_{50} and LC_{95}values of 237.9 µl/l air and 390.3 µl/l air, respectively. Similarly, in another study, Lee *et al*. (2001) found that p-cymene was an important toxic fumigant against the rice weevil (LC_{50}=25.0 µl/l air). The present results revealed higher LC_{50} values, possibly due to differences in the experimental procedure causing effects such as interactions taking place between the EO and corn added in the vials. In contrast with the present findings, some authors have reported that EOs of *Lippia* species present insecticidal (Gleiser & Zygadlo, 2007; Silva *et al*., 2008), acaricidal and repellent activities (Ruffinengo *et al*., 2005; Martinez-

Table 1. Chemical composition of essential oil extracted from *Lippia graveolens* leaves. RI: identification based on Retention indices; GC-MS: identification based on mass spectra; Co: coinjection with standard. The compounds are listed by elution order in the DB-5 column. (*) Limonene, β-phellandrene and 1,8-cineole were also separated on the Carbowax column. Relative contents are expressed as percentages.

Compound names	RI	Relative content (%)[1]	Identification
α-thujene	922	1.4	GC-MS, RI
α-pinene	932	0.5	GC-MS, RI, Co
α-fenchene	950	0.1	GC-MS, RI
β-pinene	973	0.1	GC-MS, RI, Co
β-myrcene	986	3.0	GC-MS, RI, Co
α-phellandrene	997	0.1	GC-MS, RI
δ-3-carene	1001	0.2	GC-MS, RI
α-terpinene	1009	2.3	GC-MS, RI
p-cymene	**1011**	**18.8**	GC-MS, RI, Co
limonene	1019*	0.6	GC-MS, RI
β-phellandrene	1019*	0.1	GC-MS, RI
1,8-cineole	1021*	3.4	GC-MS, RI, Co
Z-β-ocimene	1028	0.1	GC-MS, RI
γ-terpinene	1050	9.6	GC-MS, RI
terpinolene	1069	0.1	GC-MS, RI
p-cymenene	1073	0.1	GC-MS, RI
linalool	1082	0.9	GC-MS, RI
4-terpineol	1158	1.8	GC-MS, RI
p-cymen-8-ol	1164	0.5	GC-MS, RI
α-terpineol	1172	0.9	GC-MS, RI
thymyl methyl ether	1214	0.3	GC-MS, RI
carvacryl methyl ether	1221	0.1	GC-MS, RI
geranial	1230	0.3	GC-MS, RI
thymol	**1268**	**22.8**	GC-MS, RI, Co
carvacrol	**1275**	**22.7**	GC-MS, RI, Co
neryl acetate	1314	0.1	GC-MS, RI
β–caryophyllene	1418	6.8	GC-MS, RI, Co
E-α-bergamotene	1437	0.4	GC-MS, RI
aromadendrene	1440	0.1	GC-MS, RI
α–humulene	1451	0.7	GC-MS, RI
alloaromadendrene	1462	0.3	GC-MS, RI
γ–gurjunene	1471	0.1	GC-MS, RI
α–muurolene	1480	0.1	GC-MS, RI
β–bisabolene	1488	0.1	GC-MS, RI
γ–cadinene	1498	0.1	GC-MS, RI
δ–cadinene	1508	0.2	GC-MS, RI
caryophyllene oxide	1611	0.6	GC-MS, RI

Velazquez *et al*., 2011; Gomes *et al*., 2012). These differences in biological activities, with respect to the present study, may have been due to differences in the chemical composition between the EOs of the plant species (Gomes *et al*., 2012). According to some authors, the main chemical constituents of Mexican oregano EO, thymol and carvacrol, are the ones responsible for insecticide activity (Martinez-Velazquez *et al*., 2011; Gomes *et al*., 2012), but in the present study, these compounds did not show insecticidal activity at 300.0 µl/l air (LC_{50}> 300 µl/l air). Similarly to the findings of this study, Phillips & Appel (2010) and Yeom *et al*. (2012) demonstrated that the fumigant toxicities of carvacrol and thymol were lower than those of other compounds, such as α-pinene, β-pinene and limonene against adult German cockroaches.

Inhibition of AChE activity is one of the most important mechanisms of insecticidal action. The compounds that inhibit or inactivate AChE cause acetylcholine to accumulate at the cholinergic site, which brings about continuous stimulation of the cholinergic nerve fibers throughout the central and peripheral nervous system, resulting in paralysis and death (Siramon *et al*., 2009). In this study, AChE inhibition in insects exposed to Mexican oregano EO was not observed, but enzyme inactivity was important *in vitro* (IC_{50}= 7.38 ± 3.06 mg/ml). Although carvacrol and thymol did not reveal insecticidal activity, they produced AChE inhibition. The IC_{50} of carvacrol could not be determined, but it showed the highest AChE inhibition percentages of all tested concentrations. On the other hand, p-cymene showed insecticide activity and also produced AChE inhibition (IC_{50}=0.048 ± 0.015 mg/ml) (Figure 1). Similar results to those of the present study were obtained by Herrera *et al*. (2015b) using camphor against *S. zeamais.* It was found that this compound produced a strong inhibitory activity on AChE, but did not show any insecticidal activity, probably because of its inability to reach its target site due to a hindrance caused by its topological and physicochemical properties. Related to this, several authors did not find a direct correlation between insect toxicity and AChE inhibition (Lee *et al*., 2001; Yeom *et al*., 2012; Yeom *et al*., 2013; Herrera *et al*., 2015b) suggesting that there may be different modes of action of monoterpene toxicity to the insects, and consequently, mortality may be caused by other factors (Martinez-Velazquez *et al*., 2011).

The antifungal activity of Mexican oregano EO was evaluated against *F. verticillioides* M 3125, and the 100 and 200 µl/l concentrations of EO were observed to affect the growth and lag phase, while the growth rate showed a significant difference with

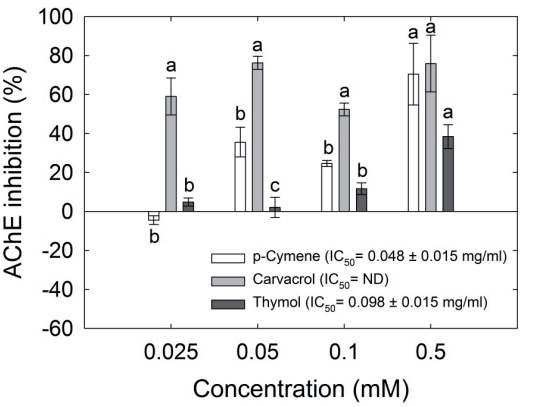

Figure 1. Dose-dependent acetylcholinesterase inhibition (AChE inhibition) *in vitro* of p-cymene (white column), carvacrol (light gray column) and thymol (gray column). Columns represent the mean value + SE (n= 3) for each compound concentration. Different letters indicate significant differences (P< 0.01) between compounds. IC_{50} values of thymol and p-cymene are shown against *Sitophilus zeamais* acetylcholinesterase activity.

the control at all the evaluated concentrations with a dose dependent response being found (Table 2). Antimicrobial properties of Mexican oregano have also been reported in other studies, and the present results are in agreement with Portillo-Ruiz *et al*. (2012), who observed that *Lippia berlandieri* EO inhibits *Aspergillus, Penicillium*, and *Rhizopus* sp. growth and with Mendez *et al*. (2012) also reporting antibacterial activity of oregano EO against *Enterobacter aerogenes*, *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus*. In the present study, although FB_1 production did not show a significant difference with the control, differences among treatments were detected at concentrations of 200 µl/l of EO (Table 2).

Several authors have attributed the antimicrobial activity of the EOs to their phenolic compound contents (Dambolena *et al*., 2012; Abbaszadeh *et al*., 2014; Gallucci *et al*., 2014). In this sense, previous studies have shown that thymol and carvacrol present antifungal activity against *F. verticillioides*, thereby reducing the radial growth, fungal sporulation and FB_1 production (Dambolena *et al*., 2011; Dambolena *et al*., 2012). Lipophilicity is the main property involved in the antifungal activity of phenolic compounds with this property indicating the possibility that the compounds reach the target site (Dambolena *et al*., 2012).

Summing up, the present results should encourage research for new active natural compounds in commercial plants, in order to offer an alternative to synthetic pesticides. Mexican oregano EO is an interesting source of compounds which has

Table 2. Antifungal and antimycotoxicogenic activities of *Lippia graveolens* essential oil on *Fusarium verticillioides* M3125

	Essential oil concentration (µl/l)			
	0	**50**	**100**	**200**
Growth inhibition (%)[1]		50.78 ± 6.29[b]	86.62 ± 4.59*[a]	93.82 ± 2.51*[a]
Growth rate (mm/day)	7.77 ± 0.75	5.79 ± 0.37*[b]	3.29 ± 0.39*[a]	3.33 ± 0.39*[a]
Lag phase (hours)	33.12 ± 1.73	43.81 ± 2.70[b]	79.56 ± 12.06*[a]	118.37 ± 17.80*[a]
FB_1 inhibition (%)		-47.87 ± 25.75 [b]	-83.97 ± 53.24 [b]	50.62 ± 10.71[a]

Values are expressed as means ± SE; [1]Inhibition of fungal growth was determined after 5 days of incubation. *Indicates significant difference with the control, and values having different letters are significantly different for each treatment. The experiments were performed twice in triplicate ($P < 0.05$).

grain-protectant properties against *F. verticillioides* and *S. zeamais*, and should be analyzed in future studies in order to develop new biopesticides to be used in integrated pest management plans of stored grains. Due to the practical use of biopesticides developed from EOs further studies are necessary to elucidate their mode of action, side effects, and formulation development in order to improve their efficacy and stability. Moreover, toxicity and *in vivo* efficacy studies using formulated products are clearly required, and the economic feasibility of these products developed from EOs has still to be demonstrated.

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