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Control of mycotoxigenic fungi with microcapsules of essential oils encapsulated in chitosan

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Abstract

Aspergillus and *Fusarium* are the fungi genera most frequently isolated from cereal grains and other commodities. They are capable of producing mycotoxins, which can affect the human and animal health. Synthetic fungicides have been used to control these fungi, nevertheless, they have acquired resistance and other alternatives are necessary since they now need higher amounts. Therefore, the aim of this study was to evaluate the efficacy of cinnamon, clove and thyme essential oils (EOs) alone and encapsulated in chitosan on the radial growth, spore germination and mycotoxin production by *Fusarium verticillioides and Aspergillus parasiticus*. The composition of the EOs was determined by gas chromatography mass spectrometry (GC-MS). EOs inhibited radial growth and spore germination of both fungal species better than when they were encapsulated, plus, they reduced mycotoxin production. The major components were eugenol in cinnamon and clove EO (70 and 63%, respectively) and 2-methyl-5-(1-methyethyl)-phenol (46.2%) in thyme EO. The microparticles with clove and thyme EO showed good surface charges, higher than +30 mV and their average size for the three types of microparticles was about 750 nm. Our findings suggest that EOs both alone and encapsulated in chitosan have a fungistatic effect on *Fusarium verticillioides* and *Aspergillus parasiticus*.

Keywords: aflatoxins; fumonisins; radial growth; spore germination.

Practical Application: Use of essential oils in chitosan microparticles to avoid fungal growth and to reduce the mycotoxin production.

1 Introduction

Pathogenic and toxigenic fungi of the *Aspergillus* and *Fusarium* genera usually contaminate cereal grains and other commodities in the field and storage. These fungi have the ability to produce mycotoxins and thus to reduce the commercial and nutritional value of the products. There are more than 300 mycotoxins, being aflatoxins and fumonisins among the most commonly isolated (Zhao et al., 2015). Synthetic fungicides are used in the field or in storage facilities mainly to control these fungi. This can be problematic due to the pollution of the environmental, the health hazards for human and animals and resistance of some strains to chemicals. Essential oils (EOs) have the potential to control these mycotoxigenic fungi due to their chemical components (Adukwu et al., 2016).

Furthermore, EOs are volatile compounds that are easily degraded when exposed to factors such as light, oxygen and temperature (Beyki et al, 2014); thus, affecting their stability, sensorial properties and functionality (de Lira-Mota et al., 2012; Soliman et al., 2013; Bakry et al., 2016). In order to maintain the EOs native structure, we have used chitosan to encapsulate it due to its chemical and physical properties. Chitosan also has antimicrobial and antifungal activity (Souza et al., 2014). For this reason, the aim of this study was to evaluate the efficacy of GRAS cinnamon, clove and thyme essential oils (EOs) alone, and encapsulated in chitosan on the radial growth and spore germination in *Fusarium verticillioides and Aspergillus parasiticus* and in the production of mycotoxin.

2 Materials and methods

2.1 Materials and preparation of chitosan microparticles

We purchased essential oils of clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum zeylanicum*) and thyme (*Thymus capitatus)* from Sigma Aldrich. We used low viscosity chitosan (CS) with a deacetylation degree of < 85% and molecular weight of 130 kDa.

For encapsulation of the EOs, we used the ionotropic gelation technique described by Cota-Arriola et al. (2013). We added 2 mL of each EO individually and sprayed 240 mL of 0.2% Sodium tripolyphosphate (TPP) in 80 mL of a 0.5% (w/v) chitosan solution in 0.1 M acetic acid. The solution was stirred for 15 min at 500 rpm and we obtained microparticles with each EOs encapsulated.

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2.2 Gas chromatography-mass spectrometry (GC-MS) analysis

Analyses of EOs were performed as described by Ayala-Zavala et al. (2008) using a Varian GC-3400 Cx with a Saturn 2100T mass-selective detector (Varian, Mexico) and a DB-5 capillary column (30 m × 0.25 mm, film thickness 0.25 μm).

2.3 Microorganisms and culture conditions

Pathogenic strains were *Aspergillus parasiticus* (ATCC 16992) and *Fusarium verticillioides* (ATCC 52539). A spore suspension was obtained from their respective 7 days old cultured in potato-dextrose agar (PDA), mixed in Tween 20 (0.1%, v/v) to obtain a homogeneous spore suspension of $1x10^5$ spores mL^{-1}.

2.4 Preparation of culture medium with EOs

Twenty-five mL of Tween 80 (1%) was deposited in a 50 mL Falcon tube and the appropriate amount of each EOs to obtain 100, 500, 750 and 1000 ppm. Then, 25 mL of Czapek medium was poured in 5-cm Petri dishes. Positive control was the commercial fungicide Terravax® (Captan 20% + Carboxin 20%) 2.5 g L^{-1}. Other two controls were Tween 80 and Czapek alone.

2.5 Preparation of culture medium with EOs-encapsulated microparticles

In other Falcon tubes were deposited 25 mL of Czapek medium and the appropriate amount of each microparticles with the EOs encapsulated to obtain 100, 500, 750 and 1000 ppm. Controls were Czapek alone, Czapek – TPP (0.2%, 7:3) and chitosan (CS) (Czapek – CS (0.5%, 7:3). All the experiment was conducted in triplicate.

2.6 Zeta potential and size of the microparticles

The zeta potential and size of the microparticles with EO was quantified using a Zetasizer Nano-25 (Malvern Instruments) at 25 °C. Each microparticles was dialyzed against 0.03 M Tris buffer, pH 5.6 for 18 h in a 12 kDa membrane (Liu & Gao, 2009).

2.7 Antifungal activity assay

For radial growth, Petri dishes of solid Czapek media containing 100, 500, 750, and 1000 ppm of each EOs, dishes with the microcapsules and the controls were centrally point-inoculated with 1 x 10^5 spores mL^{-1} from 7-day-old cultures of *F. verticillioides* (ATCC 52539) or *A. parasiticus* (ATCC 16992). The dishes were incubated at 25 °C using a 12 h light/dark cycle for *F. verticillioides* and 27 ± 2 °C for *Aspergillus*. The colony diameter was measured daily with a caliper until the control reached the plate border. The EOs concentration that delayed 50% of colony radial extension (CI_{50}) was determined at 95% of confidence intervals, using a Probit analysis with NCSS 97 statistical program (NCSS Inc., U.S.A.). All determinations were conducted in triplicate. The radial growth inhibition compared to the control was calculated as a percentage using the Equation 1:

$$\text{Radial inhibition (\%)} = \frac{(\bar{R}_c - R_i)}{\bar{R}_c} \times 100 \quad (1)$$

where $\bar{R}_c$ was the mean value of radius of colonies (mm) grown in Tween control and R_i was the colony radius of the EOs amended media.

The assay for spore germination was conducted using 2 mL Eppendorf tubes with liquid Czapek media containing 100, 500, 750, and 1000 ppm of each EOs, tubes containing the microcapsules with the CI_{50} obtained in the radial growth test and the amount of Tween 80 (1%) needed to reach 1 mL. Also, the above mentioned controls were included.

All the tubes were inoculated with 1 x 10^5 spores mL^{-1} from 7-day-old cultures of *F. verticillioides* (ATCC 52539) or *A. parasiticus* (ATCC 16992). Dishes were incubated at 25 °C using a 12 h light/dark cycle for *F. verticillioides* and 27 ± 2 °C for *A. parasiticus*. A 10 μL sample was taken at 3, 9, and 15 h after incubation from each tube, placed in a glass slide, covered and 100 spores were observed to evaluate germination by looking for the presence of the germ tube. A spore was considered germinated when its germinal tube reached one-half of the spore diameter (López-Meneses et al., 2015). Each germination assay was made in quadruplicate. The inhibition of spore germination was determined using the Equation 2:

$$Inhibition\ (\%) = \left[(\%Sc - \%St)/\%Sc\right] x\ 100 \quad (2)$$

in which S_i represents the percentage of germinated spores in the tubes treated with EOs or microcapsules, and S_c was the percentage of germinated spores in the Tween control (López-Meneses et al., 2015).

2.8 Evaluation of the effect of microparticles with EOs in mycotoxin production

Sound corn grain was used for mycotoxin production. Fifty-grams of corn were cracked and placed in 250 mL Erlenmeyer flask, adjusted to 40% moisture by adding water and sterilized for two-consecutive days in autoclave. Autoclaved corn was treated with 10 mL of sodium acetate buffer (0.03 M) and with the microcapsules with the CI_{50} of each EO. The flasks were inoculated with $1x10^5$ spores mL^{-1} of *F. verticillioides* or *A. parasiticus* and incubated for 16 days at the above mentioned conditions. Four controls were prepared following the same procedure with no microparticles with EOs added: CS, water, and TPP with the inoculum and the fourth contained just 10 mL of water without inoculum. The extraction and quantification of fumonisin and aflatoxin was carried out according to Rosas-Burgos et al. (2011) and Cota-Arriola et al. (2011), respectively. Each treatment was replicated three times.

2.9 Statistical analysis

The data analysis was conducted by ANOVA using a factorial design (type of EO, concentration, and fungus). Comparison of means in homogeneous subsets was performed using the Tukey multiple comparisons test at 95% confidence interval employing the JMP version 5.0, NCSS, SigmaPlot 10.0 (Systat Software Inc., 2006).

3 Results and discussion

3.1 Chemical composition of essential oil

The identified chemical composition and percentage composition of the EOs are shown in Table 1. The most abundant component of the thyme essential oil was 2-methyl-5-(1-methyethyl)-phenol (46.2%) whereas eugenol (70.0 and 63.0%) was the main component in cinnamon and clove, respectively.

3.2 Zeta potential of CS-TPP matrices with immobilized FA

It is reported that the zeta potential or surface charge is an essential parameter in the characteristics of a particle, mainly through its influence on stability (Du et al., 2009) and determines the antimicrobial potential on fungi and bacteria (Cai et al., 2008). The zeta potential (mV) of the chitosan microparticles with tripolyphosphate and essential oils incorporated were +38.5 ± 0.9, +38.5 ± 2.1, +29.6 ± 0.2 with size (nm) of 775.2 ± 291.6, 788.0 ± 96.8, and 735.8 ± 118.8 for thyme, clove and cinnamon, respectively. The microparticles with cinnamon oil exhibited the lowest surface charges (+29.6 ± 0.2). This may indicate that not all of the amino groups from CS were loaded and could be due to amount of sodium tripolyphosphate added for the crosslinking (Liu & Gao, 2009). The microparticles with clove and thyme EOs showed good surface charges, higher than +30 mV, which indicates their amino groups are protonated and have more opportunity to interact with the fungus membrane affecting some of their functions. In addition, it has been reported that for a physically stable nanosuspension stabilized by electrostatic repulsion a zeta potential of +30 mV is required (Müller et al., 2001). The size of the three microparticles with EOs was similar, so their distribution might be not affected by this parameter.

Inhibition of *A. parasiticus* radial growth by EOs is presented in Figure 1. Fungi in Tween control required 144 h to spread entirely in the dish. The three EOs produced total inhibition at 24 h with doses higher than 500 ppm but after this time, in treatment with 100 and 500 ppm, the effect was reduced, probably due to fungi adaptation to the EOs. There were significant differences ($p \leq 0.05$) only among the concentrations.

Figure 2 shows data for *F. verticillioides*. Inhibition of germination was observed since 48 h of incubation with the three EOs. We observed that the three EOs are effective to inhibit the radial growth. Thyme was the only EO able to cause 60% of inhibition with the lowest concentration (100 ppm) tested. Inhibition by other oils and higher concentrations were effective the three times evaluated. There were significant differences ($p \leq 0.05$) among the EOs and concentrations tested. Effects on the colonies were observed in both fungi, *A. parasiticus* and *F. verticillioides*. Their aspect and texture was irregular in color and exhibited a cottony mycelium.

3.3 Effect of the microcapsules in the radial growth

Table 2 shows radial growth of *A. parasiticus* and *F. verticillioides* in presence of chitosan microcapsules with essential oil encapsulated. Inhibition of the radial growth of *A. parasiticus* begun at 48 h of incubation whereas for *F. verticillioides* it started at 24 h. There was a statistical difference ($p \leq 0.05$) among the microcapsules with the essential oils and concentrations. Comparing the results for radial growth with EOs alone (Figures 1 and 2) and those from microcapsules, cinnamon oil caused the highest inhibition (90-100%) of *A. parasiticus* and the microcapsules with clove oil (97-98%). We were expecting high inhibition in all the treatments due to synergistic or additive effect among the antifungal activity of chitosan and the essential oils; however, inhibition by microcapsules was low compare to that caused by the essential oils alone. For *F. verticillioides*, microcapsules with thyme oil were statistically different ($p \leq 0.05$) (Table 2), they produced low effect on radial growth. Studies by Cota-Arriola et al. (2013)

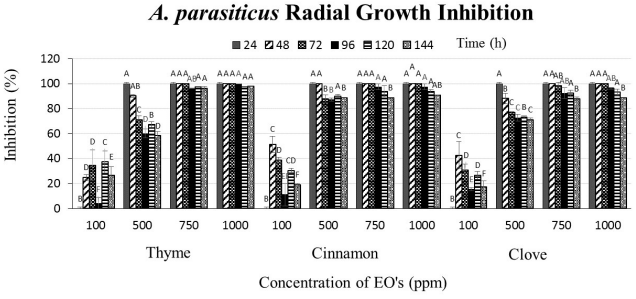

Figure 1. Inhibition of *A. parasiticus* radial growth (%) by essential oils at different incubation times. Columns having different letters are significantly different according to Tukey test ($p \leq 0.05$).

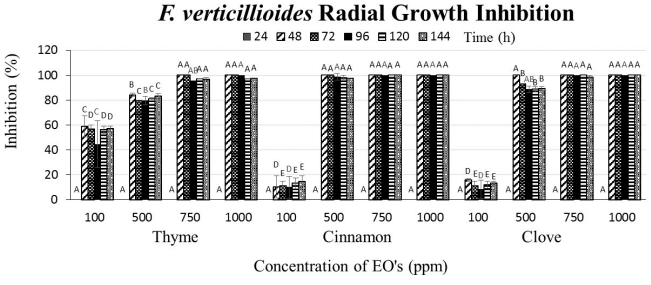

Figure 2. Inhibition of *F. verticillioides* radial growth (%) by essential oils at different incubation times. Columns having different letters are significantly different according to Tukey test ($p \leq 0.05$).

Table 1. Chemical composition and percentage of the essential oils components.

Thyme		Cinnamon leaf		Clove leaf	
Compound	%	Compound	%	Compound	%
2-methyl-5-(1-methyethyl)-phenol	46.2	Eugenol	70.0	Eugenol	63.0
Thymol	13.6	Caryophyllene	4.5	Caryophyllene	17.0
o-Cymene	12.0	Benzylbenzoate	2.8	a-Humulene	2.5
β-Pinene	6.4	*o*-Eugenol	2.2	Cadinene	1.3

Table 2. Inhibition of *A. parasiticus and F. verticillioides* radial growth (%) by chitosan microcapsules with essential oils encapsulated.

Treatment	*A. parasiticus*			*F. verticillioides*		
	Incubation time (hours)					
	48	96	144	48	96	144
C_{Tw}	0^{g}	0^{g}	0^{h}	0^{e}	0^{h}	0^{f}
Thyme						
100	14.5 ± 11.4^{efg}	24.8 ± 6.3^{e}	19.9 ± 4.3^{fg}	0^{e}	0^{h}	0^{f}
500	45.5 ± 7.0^{cde}	51.7 ± 2.9^{c}	37.9 ± 3.4^{d}	17.8 ± 14.5^{cd}	18.1 ± 5.1^{f}	0^{f}
750	63.3 ± 42.6^{bc}	68.3 ± 7.9^{b}	35.2 ± 11.5^{de}	28.9 ± 7.5^{cd}	24.0 ± 6.3^{f}	5.7 ± 6.8^{f}
1000	95.0 ± 1.1^{ab}	87.4 ± 0.1^{a}	78.7 ± 2.7^{b}	64.5 ± 3.8^{b}	51.4 ± 3.1^{d}	32.4 ± 4.7^{d}
Clove						
100	24.1 ± 14.2^{defg}	26.6 ± 4.4^{e}	25.9 ± 3.7^{efg}	14.6 ± 10.1^{de}	19.6 ± 1.3^{fg}	0^{f}
500	85.3 ± 9.9^{ab}	72.7 ± 3.7^{b}	56.1 ± 4.1^{c}	42.3 ± 8.7^{c}	55.1 ± 2.5^{cd}	39.2 ± 3.4^{cd}
750	97.6 ± 0.7^{a}	91.6 ± 0.3^{a}	83.0 ± 1.4^{ab}	64.7 ± 18.3^{b}	69.6 ± 1.7^{b}	56.3 ± 2.9^{b}
1000	98.2 ± 0.3^{a}	94.4 ± 0.5^{a}	90.7 ± 0.8^{a}	90.1 ± 3.9^{a}	84.8 ± 3.8^{a}	$76.72.9^{a}$
Cinnamon						
100	9.1 ± 10.5^{fg}	11.0 ± 4.9^{f}	15.6 ± 4.4^{g}	12.9 ± 11.2^{de}	13.0 ± 3.7^{g}	0^{f}
500	34.5 ± 12.7^{cdef}	40.4 ± 3.6^{d}	30.1 ± 3.0^{def}	32.9 ± 8.4^{cd}	35.5 ± 2.7^{e}	13.6 ± 4.5^{e}
750	50.9 ± 4.8^{cd}	49.8 ± 4.5^{c}	34.9 ± 4.0^{de}	64.3 ± 5.1^{b}	60.1 ± 2.6^{c}	44.9 ± 3.9^{c}
1000	61.0 ± 10.5^{bc}	54.3 ± 1.8^{c}	49.4 ± 5.3^{c}	86.5 ± 2.4^{a}	78.3 ± 3.8^{a}	69.3 ± 2.9^{a}

Different letters show significant differences ($P < 0.05$) between the treatments; C_{Tw} = Control Tween.

and Plascencia-Jatomea et al. (2014) mentioned that chitosan had no fungicide effect on *A. parasiticus* and *A. niger*, findings that agree to ours. Both authors mentioned that these fungi have some type of adaptation to chitosan, probably due to the physiological stress provoked by the high production of chitinases and chitosanases to degrade the chitosan, allowing the fungi to use it as a source of energy. Furthermore, microcapsules with essential oils encapsulated were less effective to inhibit radial growth in both fungi compared with essential oils alone. This could be due to the low amount of TPP (0.2%) added for preparation of chitosan microparticles. Some studies reported a higher amount of TPP and high inhibition of microorganism growth. Zhao et al. (2015) in their study to elaborate chitosan nanoparticles, added TPP (0.25%), Du et al. (2009) TPP (1%), Cota-Arriola et al. (2013) employed three amounts of TPP (2.0, 6.0 and 10.0%), and Mohammadi et al. (2015) TPP (0.3%). The IC_{50} of thyme, clove, and cinnamon EOs for *A. parasiticus* was 117.9, 111.8, and 156.1 ppm, respectively, whereas for *F. verticillioides* it was 67.7, 154.6, and 156.1 ppm, respectively. Moreover, we observed morphological changes in the colonies of both fungus developing on dishes treated with chitosan microcapsules. This agrees with the findings of Cota-Arriola et al. (2013), whose study reported changes in color of the colonies grown and mycelium formation in presence of chitosan micro and nanoparticles with TPP compared to those from the controls.

3.4 Effect of essential oils in spore germination

Figure 3 presents results on the effects of essential oils in *A. parasiticus*. The three essential oils at 500 ppm and higher inhibited the spore germination since the 3 h of incubation in comparison to the controls. We observed a high inhibition percentage was observed after 9 h of incubation with all the

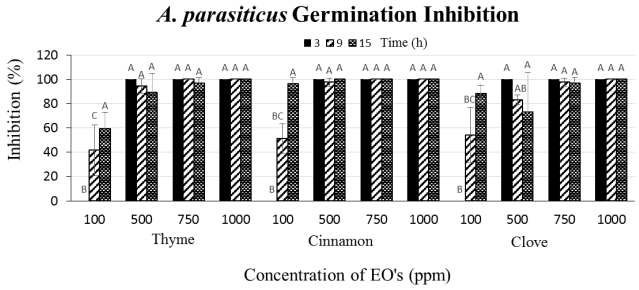

Figure 3. Inhibition of *A. parasiticus* spore germination by essential oils at different incubation times. Columns having different letters are significantly different according to Tukey test ($p \leq 0.05$).

EOs. The inhibitory effect of all EOs at 100 ppm increased proportionally to the treatment duration (from 20% at 3 h up to 75-80% at 15 h). There was statistical difference ($p \leq 0.05$) only among the concentrations.

Figure 4 shows *F. verticillioides* spore germination in presence of essential oils. Clove and cinnamon EOs caused the highest inhibition in all concentrations and incubation times. Total inhibition (100%) was reached with concentrations of 500 ppm and higher after 9 h of incubation. There were statistical differences ($p \leq 0.05$) among the concentrations and EOs. Study on essential oils in spore germination is limited, hence, the mechanism is not well known. Some authors have reported inhibitory effects and mentioned that the effect is due to inactivation of enzymes involved in the germination process and to depolarization of the mitochondrial membrane by diminishing the potential of membrane and affecting the Ca^{++} ionic channels, which are crucial for spore germination (Kocevski et al., 2013; Bakkali et al., 2008).

Table 3. Inhibition of *F. verticillioides* and *A. parasiticus* spore germination with chitosan microcapsules with DI_{50} of each essential oils encapsulated.

Essential oil DI_{50} (ppm)	*Fusarium verticillioides*				*Aspergillus flavus*		
	Incubation time (h)						
	3	9	15	DI_{50}	3	9	15
C_{Cz}	0^{B}	0^{B}	0^{B}		0^{C}	0^{C}	0^{B}
Thyme (67.7)	100.0 ± 0.0^{A}	0^{B}	0^{B}	118	100.0 ± 0.0^{A}	65.5 ± 1.7^{B}	77.6 ± 15.4^{A}
Clove (156.1)	83.3 ± 23.6^{A}	76.0 ± 1.4^{A}	0^{B}	156	75.0 ± 35.4^{AB}	73.2 ± 2.5^{A}	0^{B}
Cinnamon (154.6)	83.0 ± 23.6^{A}	0^{B}	31.9 ± 9^{A}	112	77.6 ± 15.6^{B}	0^{C}	62.5 ± 20.3^{A}

Values represent means made from three repetitions; Different letters show significant differences ($P < 0.05$) between the treatments; C_{Cz}: Control Czapek.

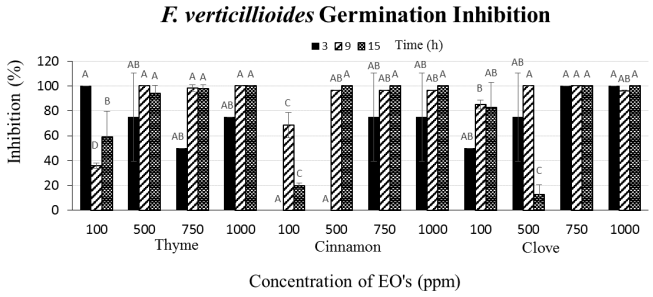

Figure 4. Inhibition of *F. verticillioides* spore germination by essential oils at different incubation times. Columns having different letters are significantly different according to Tukey test ($p \leq 0.05$).

Table 4. Effect of chitosan microcapsules with CI_{50} of essential oils in aflatoxin and fumonisin production.

Treatment	Aflatoxins (ng/g)	Fumonisins (µg/kg)
C_w	1866 ± 264^{A}	5.7 ± 0.7^{A}
C_{cs}	950 ± 476^{B}	3.3 ± 0.0^{B}
C_{TPP}	2133 ± 57^{A}	3.7 ± 0.0^{B}
Thyme	1700 ± 264^{AB}	3.5 ± 0.3^{B}
Clove	1466.6 ± 321^{AB}	3.3 ± 0.3^{B}
Cinnamon	1833.3 ± 57^{A}	3.8 ± 0.3^{B}

Data followed by the standard deviation are means from three replicates. Different letters show significant differences ($P < 0.05$) between the treatments. Cw (Control water), Ccs (Control chitosan) and C_{TPP} (Control of sodium tripolyphosphate).

3.5 Effect of microcapsules in spore germination

Results from the inhibition of *A. parasiticus* and *F. verticillioides* spore germination caused by microcapsules with the CI_{50} of each essential oil are shown in Table 3. There were statistical difference among the oils and concentrations. Spores of *A. parasiticus* were more sensitive to the different treatments than *F. verticillioides*. Plascencia-Jatomea et al. (2014) reported spores polarization in *A. niger* grown in solid and liquid Czapek medium amended with chitosan. They mentioned that the effects of chitosan varied proportionally according to the concentration and diminished with the incubation time. Additionally, they suggested that chitosan has fungistatic activity in *A. niger*, which could have happened in our study with *F. verticillioides* and *A. parasiticus*. This effect could be attributed to the chitosan chelating potential to calcium (Ca^{2+}), which is conferred by the free amino groups and hydroxyls present, or to the electrostatic interaction between the protonated amino groups (NH_3^+) with components of the cellular membrane of the fungus such as phospholipids (Ali et al., 2010; Du et al., 2009).

3.6 Effect of the microcapsules in mycotoxin production

Table 4 shows the results of aflatoxin and fumonisin production in corn grain with microcapsules of chitosan with essential oils encapsulated. Mycotoxin production was low in grain treated with the microcapsules compared to those from controls. There were no statistical differences among the oils ($p \geq 0.05$). Chitosan alone significantly reduced ($p \leq 0.05$) the aflatoxin production. In a study of Velluti et al. (2003), cinnamon, clove and thyme oils caused significant reduction of fumonisin production in three strains of *F. proliferatum*. In addition, chitosan alone has the ability to inhibit aflatoxin production due to the Ca^{2+} chelation. However, Cota-Arriola et al. (2011) found that this inhibition did not occurred in corn grain, probably because the polymer is not able to penetrate into the grain, and thus limiting the ability for calcium chelation.

4 Conclusions

Essential oils alone at doses of 500 ppm or higher are better to inhibit radial growth and spore germination of *A. parasiticus* and *F. verticillioides* than microcapsules with encapsulated EOs. We did not observe synergism or additive effect among essential oils and chitosan as we expect, even more, they showed less inhibition when encapsulated. Essential oils only have a fungistatic effect when applied pure or encapsulated in microparticles. Chitosan microcapsules with sodium tripolyphosphate reduced total production of aflatoxin and fumonisin by *Aspergillus parasiticus* and *Fusarium verticillioides* compared to control.

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