Vol. 17, No. 1 (2018) 29-45
Revista Mexicana de Ingeniería Química

EFFECT OF CHITOSAN-PEPPER TREE (*Schinus molle*) ESSENTIAL OIL BIOCOMPOSITES ON THE GROWTH KINETICS, VIABILITY AND MEMBRANE INTEGRITY OF *Colletotrichum gloeosporioides*

EFECTO DE BIOCOMPOSITOS DE QUITOSANO-ACEITE ESENCIAL DE PIRUL (*Schinus molle*) SOBRE LA CINÉTICA DE CRECIMIENTO, VIABILIDAD E INTEGRIDAD DE LA MEMBRANA DE *Colletotrichum gloeosporioides*

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Received May 25, 2017; Accepted July 24, 2017

Abstract

Recent studies have demonstrated that the micro and nanoparticles of chitosan (CS) have an *in vitro* antifungal effect against filamentous fungus that are of importance in food. This study was made to evaluate the antifungal activity of chitosan nanoparticles, pepper tree essential oil (PEO), and chitosan biocomposites loaded with pepper tree essential oil (CS-PEO), on the *in vitro* growth of *Colletotrichum gloeosporioides*. The particles were obtained by nanoprecipitation and the size and superficial charge (Z potential) were evaluated using dynamic light dispersion analysis (DLS). The mutagenic potential and the acute toxicity were also evaluated. The effect on the radial growth, spore's germination, viability, and damage to the membrane's integrity, were determined. The effect on the kinetic growth parameters was also determined. Results showed that the CS and CS-PEO particles diameter was 341.2 ± 12.40 and 355.3 ± 25.3 mm, respectively. *In vitro* assays showed both nanoparticles with a high inhibition potential on *C. gloeosporioides*, and low mutagenicity and toxicity. At a concentration of 0.160 mg/mL, the CS-PEO biocomposite presented a greater (P <0.05) inhibitory effect on radial growth, spore germination and viability of the spores, this constitute a natural alternative to the use of fungicidal chemical agents for fungus control.

Keywords: chitosan, *Schinus molle*, biocomposites, antifungal effect, *Colletotrichum gloeosporioides*.

Resumen

Las micro y nanopartículas de quitosano (CS) poseen efecto antifúngico contra hongos de importancia en alimentos. En este estudio se evaluó la actividad antifúngica de partículas de quitosano, aceite esencial de pirul (PEO) y biocompositos de quitosano cargados con aceite esencial de pirul (CS-PEO) sobre el crecimiento de *Colletotrichum gloeosporioides*. Las partículas se obtuvieron por nanoprecipitación y se evaluó el tamaño y la carga superficial mediante análisis de dispersión de luz dinámica. Se evaluó el potencial mutagénico, la toxicidad aguda y el efecto sobre el crecimiento radial, germinación de esporas, viabilidad y daño a la integridad de la membrana. Además se determinó el efecto sobre los parámetros cinéticos de crecimiento. El diámetro de las partículas de CS y CS-PEO fue de 341.2 ± 12.40 y 355.3 ± 25.3 nm, respectivamente. Ambas partículas presentaron un alto potencial inhibitorio sobre *C. gloeosporioides*, además de mostrar una baja mutagenicidad y toxicidad. Las partículas del biocomposito de CS-PEO, a una concentración de 0.160 mg/mL, mostraron un mayor (P <0.05) efecto inhibitorio sobre el crecimiento radial, germinación de esporas y viabilidad de las esporas del hongo, por lo que constituyen una alternativa natural al uso de agentes químicos fungicidas para el control del hongo.

Palabras clave: quitosano, *Schinus molle*, biocompositos, efecto antifúngico, *Colletotrichum gloeosporioides*.

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doi: 10.24275/uam/izt/dcbi/revmexingquim/2018v17n1/Chavez
issn-e:2395-8472

1 Introduction

The *Colletotrichum gloeosporioides* phytopathogen fungus is the main cause for the anthracnose of avocado, a disease that causes losses close to 20% of the annual production. The use of synthetic fungicides is a common practice to control the deterioration of fruits post-harvest; however, despite being effective, its continued application poses a threat to human health and the environment (Paster and Bullerman, 1988). The growing demand for chemical compound free products has led to the pursuit of new secure and effective strategies for pest control in food. The use of essential oils (EO) has been considered as an alternative treatment to the use of synthetic fungicides to prevent diseases in post-harvests and prolong storage lifespan (Aloui *et al*., 2014).

There is a wide variety of EO that shows antifungal activity, including oil from thyme, clove, oregano and cinnamon, which are known to be effective against a variety of pathogens such as *Rhizopus stolonifer* and *C. gloeosporioides* (Bosquez-Molina *et al*., 2010). Pepper tree (*Schinus molle*) essential oil is mainly made of monoterpene hydrocarbons (α-pinene, β-pinene, limonene) and some sesquiterpenes, it has been demonstrated that it possess activity against filamentous fungi (Rocha *et al*., 2012, Gomes *et al*., 2013) such as *Fusarium moniliforme* and *Geotrichum candidum* (Dikshit *et al*., 1986).

Generally, even though EO have demonstrated to be good antimicrobial agents, its application is often limited due to its volatile compounds that can degrade easily with heat, pressure, light and oxygen. An alternative to obtain the maximum benefits of using the EO as antimicrobial agents, is the incorporation of these compounds in controlled release matrixes (Martín *et al*., 2010). The controlled release allows for a correct product quantity in the right place and during the convenient time. This allows for a controlled prolongation of the effectiveness time of the applied dose, avoiding the immediate release of the bioactive compound, as usually occurs. In addition, it minimizes and in some cases avoids reaching concentrations that can be toxic to plants. This is accompanied by a decrease in environmental damage by avoiding the use of high doses that may be harmful to the environment (Mozafari *et al*., 2006).

Reports have shown that the application of chitosan nanoparticles as matrixes loaded with essential oils possess an antifungal activity to control several microorganisms (Zorzi *et al*., 2015). It was demonstrated that they elevate the antifungal effect of the essential oil of mint in chitosan coatings applied in strawberries (Vu *et al*., 2011) and a greater inhibitory effect was found on *Botrytis cinerea* in treated strawberries with particles loaded with oils from *Zataria multiflora* (Mohammadi *et al*., 2015).

In comparison with micrometric materials, the nanomaterials can provide greater advantages such as solubility, improvement of the bioavailability, controlled release and targeting of the encapsulated compounds (Kato *et al*., 2007). These nanomaterials can be developed by the use of natural polysaccharides such as chitosan (CS), main deacetylated derivate of chitin (Keawchaoon and Yoksan, 2011, Luque-Alcaraz *et al*., 2016). For several years, chitosan has attracted attention in applications related to the encapsulation of bioactive compounds, because it is recognized as safe (GRAS) and due to its biological properties, such as biodegradability, biocompatibility and low or non-toxicity, and by its capacity to create films, membranes, gels, pearls, fibers and micro/nano particles (Luque-Alcaraz *et al*., 2016). The objective of the present study was to elaborate nanoparticles of chitosan and chitosan loaded with pepper tree (*Schinus molle*) essential oil using the nanoprecipitation technique, and to evaluate its biological properties on spore germination, radial growth, viability and integrity of the membrane of *C. gloeosporioides*.

2 Materials and methods

2.1 Materials

Commercial chitosan (Sigma-Aldrich, USA) with a degree of N-acetylation (DA) of 0.178 and a viscosimetric molecular weight of 1.1×10^5 kDa (estimated at 25ºC in 0.3 M acetic acid/ 0.2 M sodium acetate solution), was used. Essential oil was extracted from *Schinus molle* leaves collected in the Valle del Yaqui, Sonora, Mexico, using the hydrodistillation technique. The Biotechnology Laboratory of the Technological Institute of Tepic (ITT), Tepic, Mexico, provided the *Colletotrichum gloeosporioides* strain, which was isolated from infected Hass avocado fruits.

2.2 *Preparation of chitosan solutions*

A concentrated solution of chitosan was prepared by dissolving 0.5 g of dry flakes in 100 mL of acetic acid solution at 2% (v/v). The mixture was kept in constant magnetic stirring for 24 h at room temperature until the biopolymer was completely dissolved.

2.3 *Extraction of pepper tree essential oil*

A hydrodistillation extraction equipment was utilized. An amount of 100 g of dried pepper tree leaves were placed on a double entry reservoir beaker, which was connected to a triple entry rounded beaker with 250 mL of distilled water that was previously added. The system was kept at a constant temperature of 100°C for 4 h and the essential oil was recollected using a Clavenger trap at 4°C and shielded from the light until it was required (Luque-Alcaraz *et al*., 2016).

2.4 *Preparation of inoculum*

The strain was isolated from infected Hass avocados and kept at -80°C in a glycerol solution at 10% (v/v), then defrosted and the liquid poured on sterile filter paper inside a laminar-flux bell until the excess humidity was eliminated. Filter paper discs were used to place the fungus in Petri dishes with a PDA (Bioxon, USA) medium and were incubated at 27 ± 2°C, until they were developed. The strain was then activated in PDA agar acidified with tartaric acid (10% w/v) solution and incubated at 27°C for 5-7 days. Developed spores were re-suspended by adding sterile Tween 80 (0.05% v/v) solution and stirring with a sterile magnetic bar for 1 min. The concentration of the spore suspension was determined by count in a Neubauer chamber (Brand, Germany).

2.5 *Preparation of chitosan nanoparticles (CS) and chitosan-pepper tree essential oil (CS-PEO) biocomposites*

The CS nanoparticles (NPs) and the CS-PEO biocomposites were synthesized by nanoprecipitation technique (Luque-Alcaraz *et al*., 2016). The chitosan was dissolved in an acetic acid solution at 2% (v/v) to prepare a solution with a concentration of 0.5 mg/mL. This phase (2.5 mL) was added to the dispersing phase, consistent of 40 mL of methanol and 100 μL of pepper tree essential oil previously added, using a needle placed 2 cm above the surface at a constant flow of 0.87 mL/min controlled by a peristaltic pump (Gilson Minipuls 3, France), under moderate magnetic stirring at 500 rpm. The CS particles were prepared using the same procedure without adding PEO.

2.6 *Size and superficial load of the particles*

The size and superficial charge (Z potential) of the particles was determined by dynamic light dispersion analysis (Möbius, Wyatt Technology, Santa Barbara, CA, USA). A quantity of 90 μL of the sample was placed in the equipment cell, adapted with a vertically polarized laser with a wavelength of 488 nm (2W). The detection angle was maintained at 90° in regards to the inciting light ray and the average of 3 replicates were obtained, each one with a 60 sec lasting at room temperature.

2.7 *Antifungal activity*

2.7.1 *Radial growth*

A total of 1×10^6 spores/mL of *Colletotrichum gloeosporioides* were inoculated in the middle of Petri dishes containing Czapek agar medium previously added using different concentrations of CS, CS-PEO and PEO (0.01, 0.02, 0.04, 0.08, and 0.16 mg/mL), and incubated at 27 ± 2°C. Petri dishes with Czapek agar medium were used as control with an adjusted pH of 5.6 using acetic acid solution. The radial growth of the colonies was manually measured every 12 h and was compared with the growth of the colony in the control medium. The kinetics were performed until the colony covered the total surface of the Petri dish in the control. The radial growth inhibition percentage with respect to the average ratio in the control was determined using the equation 1:

$$\text{Radial inhibition } (\%) = 1 - \left(\frac{R_i}{R_c}\right) \times 100 \qquad (1)$$

Where R_c is the average ratio of the control medium colony and R_i is the ratio of the colony developed in different treatments (Martínez-Camacho *et al*., 2010, Plascencia-Jatomea *et al*., 2003). The experimental data was adjusted to the Gompertz modified model to estimate the kinetic parameters of radial growth (Avila-Sosa *et al*., 2012), using the equation 2:

$$\ln \frac{D_t}{D_0} = A \exp\{-\exp[(V_m \cdot e/A)(\lambda - t) + 1]\} \qquad (2)$$

Where A is the maximum growth achieved during the stationary phase, V_m is the maximum growth rate (1/0.5 days), λ is the lag phase lag (0.5 days) and e is the exp (1).

2.7.2 *Spore germination*

The liquid Czapek medium was placed in sterile microcentrifuge tubes (Eppendorf) added with different concentrations of CS, CS-PEO, and PEO. Each tube was inoculated with a spore suspension at a concentration of 1×10^6 spores/mL, incubated at 27 ± 2°C. Random samples of each treatment were taken every 4 h during 48 h and the fungus growth was observed using an optical microscope (Olympus CX311, Japan), counting a total of 200 spores (germinated and not germinated). A spore is considered as germinated when the longitude of its germinal tubules has reached double or more than the total diameter of the spore. A calculation of the inhibition percentage of the germination in respect to the control was made using the equation 3:

$$\text{Spore's germination inhibition } (\%) = 1 - \left(\frac{S_i}{S_c}\right) \times 100 \tag{3}$$

Where S_c is the percentage of germinated spores of the control and S_i is the percentage of germinated spores in different treatments (Martínez-Camacho *et al*., 2010). In order to estimate the kinetic parameters of germination, the experimental data of germinated spores (%) *versus* time was adjusted to the logistic model using the equation 4:

$$S = \frac{S_{max}}{\left[1 + \left(\frac{S_{max} - S_0}{S_0}\right)\exp^{-kt}\right]} \tag{4}$$

Where S_c is the percentage of germinated spores after a determined time (t), S_{max} is the maximum percentage of germinated spores when $t \to \infty$, S_0 is the initial percentage of germinated spores ($t = 0$), and k is the germination speed (h^{-1}) (Plascencia-Jatomea *et al*., 2003).

2.7.3 *Cellular viability*

In microplates with 96 flat-bottom wells (Costar, Corning, USA), 100 μL of the innocuous were placed by triplicate at a concentration of 1×10^6 spores/mL and were incubated for 4 h at 27 ± 2°C. Afterwards, each well was filled with 100 μL of the sample (CS, CS-PEO, and PEO) to the different concentrations and the microplates were left to incubate for 4 more h. After the time passed, 50 μL of the XTT solution (2,3-bis(2-metoxi-4-nitro-5-sulfofenil)-2-h-tetrazolium-5-carboxinilide)) (Sigma M2128) and 7 μL of menadione solution were added to obtain a final concentration of 400 μg of XTT and 25 μM of menadione. The plates were incubated again for 6 h at the same temperature and the absorbance at 490 nm was taken on a microplate reader (Bio-Rad ModeliMark). The percentage of cellular viability was determined in respect to the control (Luque-Alcaraz *et al*., 2016, Freimoser *et al*., 1999, Levitz and Diamond, 1985, Meletiadis *et al*., 2000).

2.7.4 *Damage to the membrane´s integrity*

Using microplates with 96 flat-bottom wells (Costar, Corning, USA) previously added with liquid Czapek medium, 100 μL of the inoculum was placed by triplicate at a concentration of 1×10^6 spores/mL. Each well was filled with 100 μL of the sample (CS, CS-PEO, and PEO) to the different concentrations and the microplates were incubated at 27 ± 2°C for 5 h. Afterwards, each well was filled with a propidium iodide solution (PI) 3 μM and incubated at the same temperature for 6 h before being observed in a epifluorescence microscope (Leica DM 2500, USA) (Riccardi and Nicoletti, 2006). Propidium iodide is a fluorescent stain for nucleic acids and cell membrane integrity and excludes PI from staining viable and apoptotic or damaged cells.

2.8 *Mutagenicity*

Was evaluated by the Ames assay (Maron and Ames, 1983). Samples were diluted in a serial way with sulfoxide dimethyl (DMSO) and using aflatoxin B1 (AFB1) as positive control. For the negative control, a strain of *Salmonella typhimurium* was used with dependent histidine, mixture S9 (metabolic enzymatic extract of rat liver) and DMSO. Each test tube was filled with 2 mL of histidine and biotin agar and kept at 45°C, then adding 100 μL of simple + 500 μL of the enzymatic simple S9, mixing it homogeneously before pouring the mixture on Petri dishes with minimal processed agar. The plates were incubated at 37°C for 48 h and the number of reverting colonies by plate was counted (López-Saiz *et al*., 2016).

2.9 *Acute toxicity*

Artemia salina eggs were incubated for 24 h for hatching in sterile marine water at room temperature, with controlled aeration and artificial lighting (Frías-Escalante *et al*., 2015). After its hatching, the *A. salina* nauplii were separated in groups of 10-15 specimen, transferring them with a Pasteur pipette from the hatching flask to the 18x150 mm test tubes containing

sterile marine water previously added with the (CS, CS-PEO, and PEO) treatments with suitable quantities to obtain different concentrations. The final volume was taken to 5 mL with sterile marine water and all the test tubes were kept at room temperature with illumination. After 24 h the surviving nauplii were counted with a loop and the mortality percentage was determined with respect to the quantity of organisms alive in the controls. Once the trial was done, the nauplii were sacrificed by adding 100 μL of a phenol solution at 5% (p/v).

2.10 Statistical analysis

A bifactorial design was made at significance level α = 0.05. The factors were treatments (CS, CS-PEO, and PEO) and the concentration (0.01, 0.02, 0.04, 0.08, and 0.16 mg/mL). An ANOVA varying analysis and a Fisher LSD test was used to compare averages along with the program Statistic 8.0. The values for p <0.05 were statistically significant.

3 Results and discussion

3.1 Size and superficial load of the particles

The average diameter of the NPs of CS and CS-PEO was 341.2 ± 12.4 and 355.3 ± 25.3 nm, respectively. The addition of the PEO incremented the superficial charge of the CS-PEO particles, observing values of the Z potential of +31.46 ± 2.0 and +38.85 ± 0.4 mV for the CS and CS-PEO nanoparticles, respectively. These values can be considered high, which implies a particle surface positively charged, good stability, in addition to a potential functionality. The interactions between the OH groups of the essential oil and the amino groups of the chitosan could be responsible of augmenting this value. Some authors have reported similar results for NPs of CS (420 nm) and chitosan-lemon essential oil (300 nm) (Sotelo-Boyás *et al*., 2015), while others have obtained chitosan particles with an average diameter of 177 nm and Z potential of +26.9 ± 4.51 mV (Harris *et al*., 2011).

The results obtained in this study are similar to the ones found in previous works, where values are reported of having a bigger size (124-174 nm) and superficial charge (+46-67 mV) in CS particles loaded with *Zataria multiflora* essential oil, in comparison with nanoparticles of only CS (96 nm, +53 mV) (Mohammadi *et al*., 2015). Likewise, they are similar to the ones obtained for NPs loaded with catechin obtained by tripolyphosphate ionic gelification, where particle sizes were observed of 110 and 130 nm for CS and CS-catechin particles, respectively, having found an increment in the Z potential of +5.8 mV for the CS-catechin complex particles (Dudhani and Kosaraju, 2010). However, the results do not match the ones obtained by Sotelo-Boyás *et al*., (2015), who found values for the Z potential of +20.2 and +10 mV for nanoparticles of chitosan and chitosan-essential oil of lemon, respectively, obtained by nanoprecipitation whose average size varied between 117 to 250 nm (Sotelo-Boyás *et al*., 2015). The Z potential is an important parameter to determinate the stability of the nanoparticles in aqueous media, so the nanomaterials with greater Z potential demonstrate a greater stability in a watery solution due to the electrostatic repulsion that occurs between particles.

3.2 Antifungal activity

3.2.1 Radial growth

When evaluating the effect of the NPs on the radial growth of the fungus, it was found that the biocomposites of the CS-PEO complex showed a strong inhibitory effect, significantly reducing (p < 0.05) the radial growth of *Colletotrichum gloeosporioides* with respect to control and pepper tree essential oil (Fig. 1c). This fungistatic effect was greater when the CS-PEO concentration was increased in the medium (Fig. 1c). The results are similar to the ones reported in previous studies where it has been demonstrated that the oleoyl-chitosan NPs have an inhibitory effect on the radial growth of fungi such as *Nigrospora sphaerica*, *Botryosphaeria dothidea*, *N. oryzae* and *Alternaria*, when being exposed to concentrations of 0.5, 1.0 and 2.0 mg/mL (Xing *et al*., 2016). Pepper tree essential oil did not present an effect on radial growth with respect to control after 8 d of incubation (Fig. 1a). It has been reported that the nanoparticles made from chitosan showed an antibacterial effect greater than the ones from chitosan solutions, however, most of the reports are based on the antibacterial effect of metallic ions (copper, silver, zinc, etc.) complexed to chitosan nanoparticles (Sanpo *et al*., 2009, Vareltzis *et al*., 1997).

The CS and CS-PEO particles significantly reduced the fungus radial extension, finding higher (P <0.05) inhibition percentages with respect to control (Table1). The CS-PEO particles showed a greater (P <0.05) inhibition on *Colletotrichum*

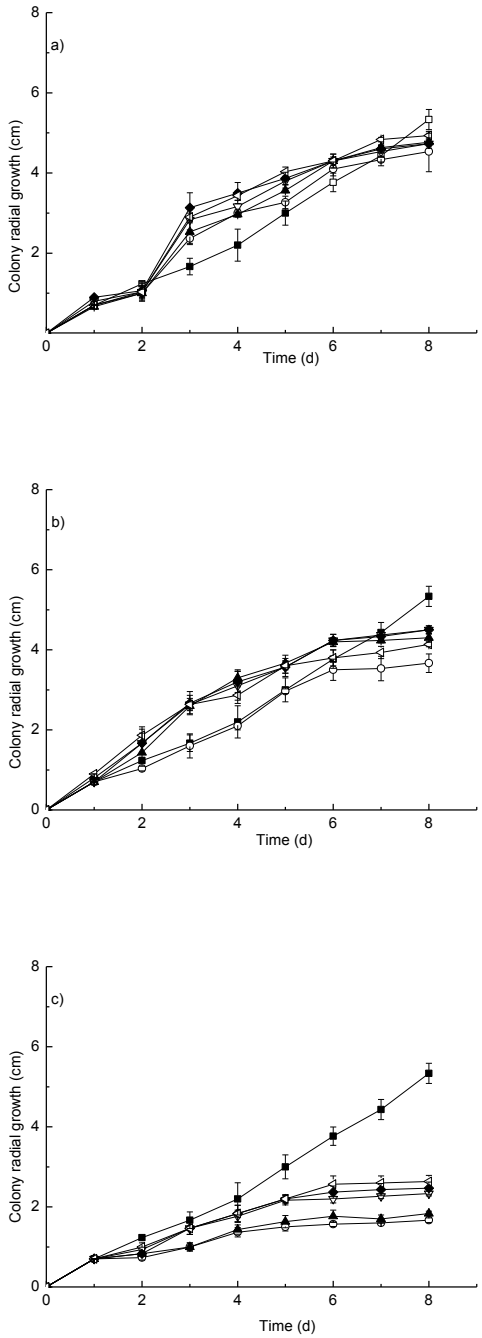

Fig. 1. Colony radial growth of *Colletotrichum gloeosporioides*, at 27ºC, in Czapek liquid media added with: a) essential oil pepper tree, b) chitosan particles, c) chitosan-pepper tree essential oil biocomposites. (■) control, (o) 0.16 mg/mL, (▲) 0.08 mg/mL, (∇) 0.04 mg/mL, (♦) 0.02 mg/mL and (◅) 0.01 mg/mL. The results represent mean value of triplicates ± standard deviation.

gloeosporioides growth stage, obtaining inhibition percentages of 65.6 and 68.7% to the concentrations of 0.08 and 0.16 mg/mL, respectively. The previous information represents an increment in the double and triple inhibitory effect compared with the values obtained for the NPs of CS, with the same concentrations (Table 1). The antifungal effect of the particles formulated with chitosan can be attributed to the interaction between the phosphate ions and divalent metals such as Ca^{2+} and Mg^{2+} which are important for fungi, as well as with positively charged components of the plasmatic membrane such as proteins or lipids (El Ghaouth *et al*., 1992, Palma-Guerrero *et al*., 2009). In this work, the effect of the CS-PEO NPs increased in a proportional way to the used concentration, which suggests that the antifungal activity can be mainly related to the amino and hydroxide groups that were left available after the electrostatic interaction between the CS and PEO.

There have been reports that the size of the particle affects the antibacterial potential. The smallest particles have a greater area/volume relation which increases the contact surface area between the particle and the bacterial membrane (Du *et al*., 2008, Palma-Guerrero *et al*., 2008). As a result they can have a greater bioactivity and the possibility to penetrate the cell faster with respect to the bigger sized NPs, possibly increasing faster its intracellular concentration (Du *et al*., 2008).

The cellular growth results from several interactions between biochemic reactions and transport events with multiple phases and multicomponent systems. In this work, the experimental data of the colony´s radial extension *versus* time were adjusted to the modified model of Gompertz and the rate of the radial extension (V_m) as a quantification parameter of the superficial growth of the colonies was determined, which allowed the estimation of the specific growth rate of fungi. By comparing the kinetic parameters of the different treatments it was found that the CS-PEO biocomposite heavily decreased the radial extension rate of the colony as the NPs concentration increased. In the media added with 0.08 and 0.16 mg/mL of CS-PEO, a decrease in the colony growth rate was observed (0.27 and 0.25 1/d, respectively), which represents nearly a three times reduction compared to the control. On the other hand, pepper tree essential oil estimulated the fungi radial growth, which can be attributed to the capability that *Colletotrichum gloeosporioides* has to use the oil as a carbon source (Table 2).

Table 1. Radial growth inhibition (%) of *Colletotrichum gloeosporioides* inoculated in Czapek agar media, at 27°C.

Conc (mg/mL)	PEO	CS particles	CS-PEO biocomposite
0.01	7.5 ± 1.0^{aA}	22.5 ± 1.0^{cB}	50.6 ± 2.8^{cC}
0.02	11.2 ± 2.1^{aA}	15.6 ± 1.8^{bB}	$53.7 \pm 2.8\text{b}^{cC}$
0.04	11.2 ± 1.0^{aA}	15.6 ± 1.8^{bB}	56.2 ± 1.0^{bC}
0.08	10.6 ± 1.0^{aA}	19.3 ± 1.8^{bcB}	65.6 ± 1.0^{aC}
0.16	15 ± 9.4^{aA}	31.2 ± 4.3^{aB}	68.7 ± 1.0^{aC}

Values represent the mean standard deviation of 3 replicates. Lowercase letters in the superscript indicate significant differences (P <0.05) between treatments (columns). Capital letters in the superscript indicate significant differences (P <0.05) between the concentrations (rows).

Table 2. Kinetic parameters of radial growth of *Colletotrichum gloeosporioides* in Czapek agar with added pepper tree essential oil (PEO), chitosan particles (CS) and chitosan-pepper tree essential oil biocomposites (CS-PEO), estimated using the modified Gompertz model.

Conc. (mg/mL)	PEO				CS particles				CS-PEO biocomposite			
	A (cm)	**Vm (1/d)**	**λ (d)**	**R**	**A (cm)**	**Vm (1/d)**	**λ (1/d)**	**R**	**A (cm)**	**Vm (1/d)**	**λ (d)**	**R**
Control	13.65	0.8	1.4	0.99	13.65	0.8	1.4	0.99	13.65	0.8	1.4	0.99
0.01	5.03	1.11	0.72	0.98	4.17	0.87	0.05	0.98	2.97	0.44	-0.37	0.98
0.02	4.71	1.19	0.73	0.97	4.66	0.91	0.2	0.99	2.68	0.46	-0.16	0.98
0.04	4.87	1.02	0.62	0.98	4.74	0.87	0.16	0.98	2.47	0.44	-0.34	0.98
0.08	7.34	0.56	-1.2	0.83	4.45	1.01	0.47	0.99	2.03	0.27	-1.2	0.96
0.16	4.91	0.84	0.48	0.98	4.32	0.63	0.32	0.98	1.83	0.25	-1.35	0.97

Values represent the mean of 4 replicates. A: maximum growth achieves during the stationary phase; V_m: maximum specific growth rate (1/0.5 d); λ: lag phase (0.5 d); R: determination coefficient.

By analysing the effect on the maximum growth (A) it was found that the PEO and CS nanoparticles reduce the mycelium production with respects to the control; however, the largest effect was found in the CS-PEO treatment, which can reduce the maximum size that the colonies can reach, besides affecting the specific growth rate (V_m) of each tested concentration (Table 2). By the estimated kinetic parameters, it is possible to predict macroscopic growth of the fungi (when $t \rightarrow \infty$), which would contribute to the establishment of "*green*" methodologies and strategies aimed at the use of chitosan biocomposites for the control of fungi in food. This is especially relevant given the increase in pre- and postharvest diseases, such as anthracnose, found in many countries.

3.2.2 Spore germination

The effect of the CS NPs and the CS-PEO complex in the germination stage of the spore is an indicative of the fungus adaptation to the biocomposites. It was found that the presence of both NPs affected the fungi germination, having lower values (p <0.05) of germinated spores with respect to the control, at 48 h (Figs 2b and 2c). The delay of the spore's germination of the threaded fungi provoked by the chitosan varies according to the physicochemical properties such as concentration, molecular weight and deacetylation degree of the biopolymer, in addition to the specie of microorganism (Quintana Obregón *et al*., 2011).

In regards to the chitosan NPs' effect upon the spore germination of fungi, there is evidence that these inhibit as far as 87.1% the *Alternaria alternata* germination when applying 0.1% of chitosan nanoparticles (Saharan *et al*., 2013). There have been reports of the chitosan sticking to the plasmatic membrane of the fungi due to the electrostatic interactions between positive charge of the biopolymer and the negative charge of the membrane's phospholipids (Palma-Guerrero *et al*.,

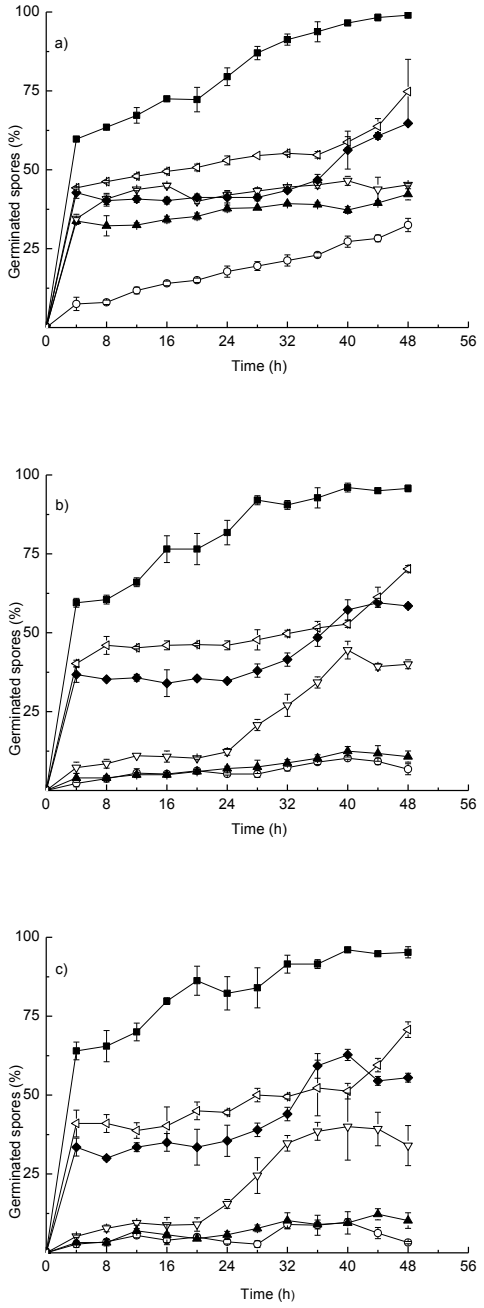

Fig. 2. Spores' germination of *Colletotrichum gloeosporioides*, at 27ºC, in Czapek liquid media added with: a) essential oil pepper tree, b) chitosan particles, c) chitosan-pepper tree essential oil biocomposites. (■) control, (o) 0.16 mg/mL, (■) 0.08 mg/mL, (∇) 0.04 mg/mL, (♦) 0.02 mg/mL and (◅) 0.01 mg/mL. The results represent mean value of triplicates ± standard deviation.

2008), mainly of the Van der Walls type, such as hydrogen bonds and London forces. It is possible that the presence of these interactions creates rigidness in the membrane with the following loss of its integrity and an impact on its cellular osmosis. Fungi normally maintain low cytosol levels of Ca^{2+} in the cytoplasm due to the barrier formed in the plasmatic membrane, which has specific regulators that prevent the free flow of Ca^{2+} gradients. By introducing chitosan to the cytosol's interior, the homeostatic mechanism is altered, since the consequent creation of canals in the membrane can allow a free flow of Ca^{2+} gradients, creating a cellular instability until its death (Honary *et al*., 2011, Palma-Guerrero *et al*., 2010, Roslev and King, 1993).

Generally, the inhibitory activity of the PEO and the CS and CS-PEO nanoparticles increased based on the increment in the concentration (Figs 2b and 2c, Table 3). The same as in the radial growth stage, the size of the particle affected the spore germination. This can be explained due to the possible agglomeration of the particles in the medium, which provide a surface where the spore can adhere and later germinate, therefore making a greater electrostatic interaction between the bioactive compounds and the fungi's plasmatic membrane. The CS-PEO biocomposite particles showed a greater (P <0.05) inhibitory activity (96.7% inhibition) against *C. gloeosporioides* compared to the CS NPs and the pepper tree essential oil. The inhibitory activity of the CS-PEO biocomposite increased in a very important manner as the concentration augmented, having a lower (P <0.05) number of germinated spores when using concentrations of 0.08 and 0.160 mg/mL (Table 3). Besides finding a larger quantity of non-germinated spores, a larger swelling was found in the inoculated spores incorporated in the medium with these NPs in regard to the control (Figs. 2a, 2b and 2c). This effect can be attributed to the chelating activity of the polymer and the PEO matrix of the biocomposite with the Ca^{2+} ion found in the medium, which is fundamental to the germination process (Bensaid *et al*., 2000, Fried *et al*., 1976).

When analysing the effect upon the kinetic of the *Colletotrichum gloeosporioides* spore germination, important differences were found in the estimated parameters of the treatments with pepper tree essential oil (PEO), the chitosan (CS) nanoparticles and chitosan-pepper tree essential oil (CS-PEO) biocomposite, with respect to control. Even though the particles effect upon the germination rate was moderated, the CS-PEO biocomposite reduced in an

Table 3. Inhibition percentage of spore's germination of *Colletotrichum gloeosporioides* in Czapek media, at 27°C, at 48 h.

Conc.	**PEO**	**CS particles**	**CS-PEO biocomposite**
mg/mL	% of Inhibition	% of Inhibition	% of Inhibition
0.01	24.5 ± 10.35^{bA}	26.6 ± 1.10^{dA}	28.5 ± 2.49^{dA}
0.02	34.6 ± 0.35^{bA}	38.9 ± 0.73^{cA}	43.9 ± 1.42^{cA}
0.04	54.3 ± 0.35^{aA}	58.2 ± 1.47^{bB}	65.5 ± 6.42^{bB}
0.08	57.3 ± 1.78^{aA}	88.8 ± 1.84^{aB}	89.6 ± 2.49^{aB}
0.16	67.1 ± 2.14^{aA}	92.9 ± 1.84^{aB}	96.7 ± 0.35^{aC}

Values represent the mean ± standard deviation of 3 replicates. Lowercase letters in the superscript indicate significant differences (P <0.05) between treatments (columns). Capital letters in the superscript indicate significant differences (P <0.05) between concentrations (rows).

Table 4. Kinetic parameters of spore's germination of *Colletotrichum gloeosporioides* in Czapek agar with added pepper tree essential oil (PEO), chitosan particles (CS) and chitosan-pepper tree essential oil biocomposites (CS-PEO), estimated using the logistic model.

	PEO				**CS particles**				**CS-PEO biocomposite**			
Conc. (mg/mL)	X_{max}	S_0	***k***	**R**	X_{max}	S_0	***k***	**R**	S_0	X_{max}	***k***	**R**
Control	100.1	56.4	0.06	0.95	100.1	56.37	0.06	0.95	56.37	100.1	0.06	0.95
0.16	51.4	6.79	0.048	0.98	9.37	2.39	0.076	0.81	2.31	7	0.083	0.48
0.08	61.5	31.46	0.013	0.87	15.38	2.75	0.058	0.91	2.86	14.6	0.055	0.77
0.04	44	21.65	0.329	0.8	53.15	2.71	0.09	0.96	0.9	41	0.154	0.94
0.02	—-	33.9	0.011	0.84	—	28.08	0.015	0.89	26.4	n.e	0.02	0.88
0.01	—-	41.45	0.01	0.88	—	38.07	0.01	0.87	34.6	—	0.01	0.88

Values represent means of 3 replicates. Confidence intervals of 95%. S_0: initial percentage of germinated spores; k: germination rate; R: determination coefficient.

important way the maximum spores' germination (Smax) of fungi (Table 4, Fig. 3). This can be attributed to the microorganism's adaptation under stress conditions tested on the highest concentrations. It is possible that the stress has caused acceleration in the germination speed in a first stage, followed by a deceleration due to the biocomposite antifungal effect giving as a result a reduction in the Smax value.

3.2.3 Cellular viability

When evaluating the effect of the chitosan-pepper tree essential oil biocomposites upon the viability of *Colletotrichum gloeosporioides* it was observed that the percentage of viable spores strongly decreased in regards to the PEO and NPs of CS treatments, finding a lower (P <0.05) viability in the CS-PEO biocomposite treatment made with the highest concentrations analysed (0.08 and 0.16 mg/mL) (Fig. 4). When analysed at the lowest concentrations (0.01 to 0.04 mg/mL), both NPs types did not show inhibitory effects on viability. By comparing the antifungal activity of the NPs of CS and the CS-PEO biocomposite, a considerable increment was noticed in the biocide potential, which can be attributed to the synergy effect of the chitosan and pepper tree essential oil when being combined as biocomposites, boosting the antifungal effect in comparison to the effect that the particles present individually.

Previous investigations have demonstrated the antifungal effect of chitosan- pepper tree essential oil upon the viability *in vitro* of *Aspergillus parasiticus*, using the XTT assay (Luque-Alcaraz *et al*., 2016). In this test, the number of viable cells is correlated with the capabilities of reducing tetrazol (XTT) to a soluble and colored product, that happens because of the mitochondrial enzymatic activity found in the

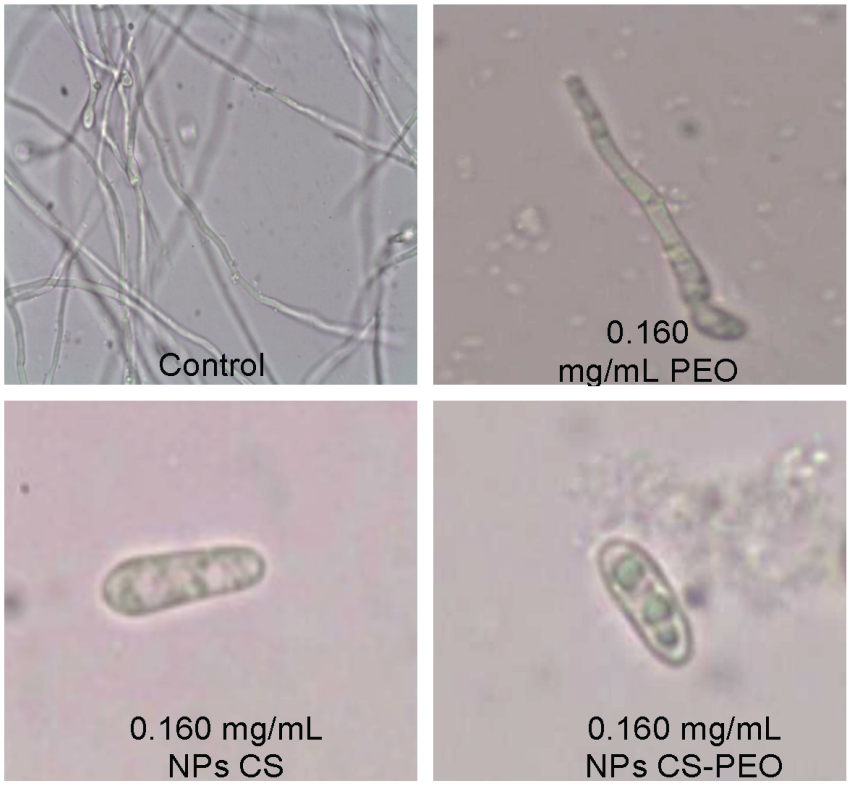

Fig. 3. Spore's germination of *Colletotrichum gloeosporioides* in Czapek media added with essential oil of pepper tree (PEO), chitosan particles (CS) and chitosan-pepper tree essential oil biocomposites (CS-PEO), at 27°C, at 48 h, at 40X.

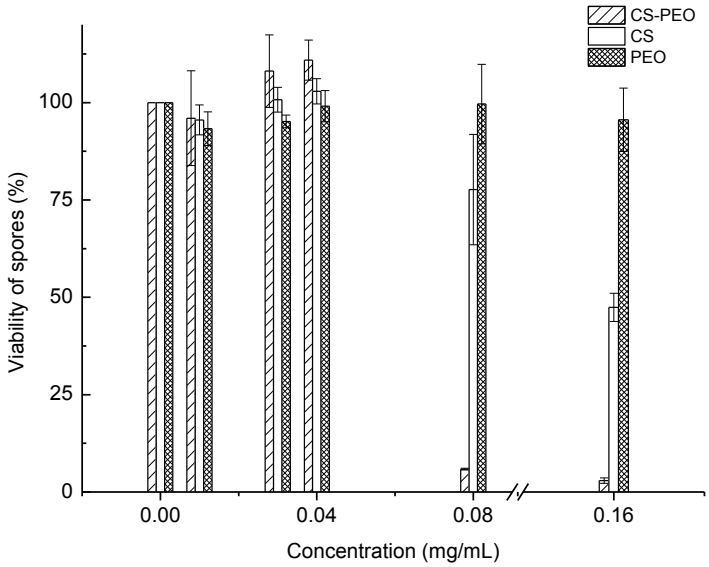

Fig. 4. Spore's viability of *Colletotrichum gloeosporioides* inoculated in Czapek media added with pepper tree essential oil (PEO), chitosan particles (CS) and chitosan-pepper tree essential oil biocomposites CS-PEO, at 27 ± 2°C. The results represent mean values of triplicates ± standard deviation.

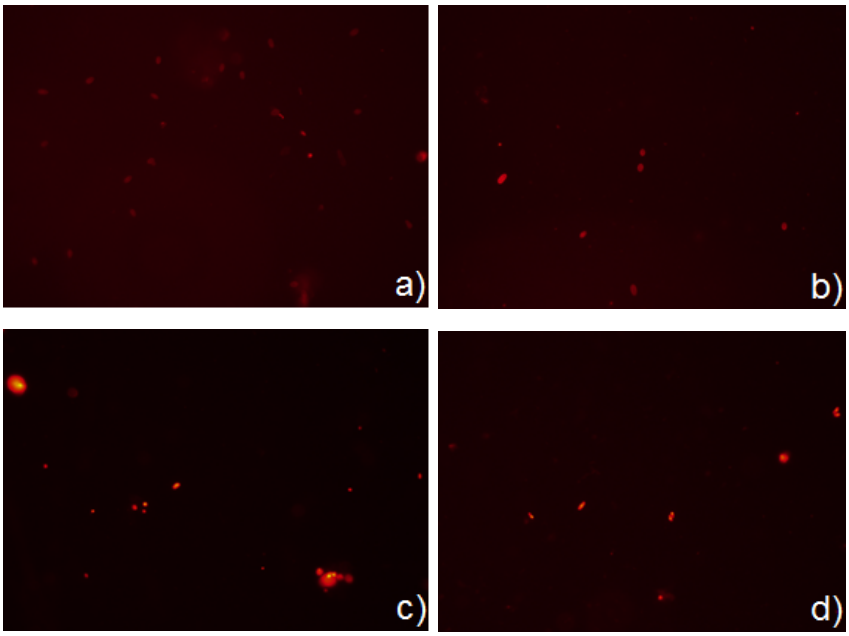

Fig. 5. Fluorescence images of spores of *Colletotrichum gloeosporioides*, at 27°C, at 8 h, indicating damage to membrane permeability: a) Control; b) Pepper tree essential oil, PEO (0.160 mg/ml); c) Particles of CS (0.160 mg/ml); d) Particles of CS-PEO (0.160 mg/ml).

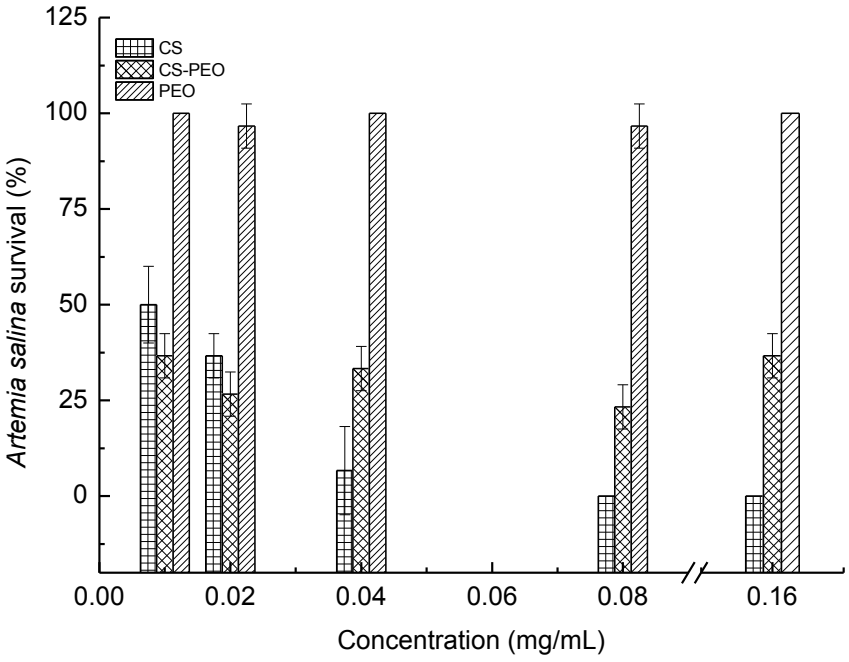

Fig. 6. Survival of *Artemia salina* exposed to different concentrations of chitosan particles (CS), chitosan-pepper tree essential oil biocomposites (CS-PEO) and pepper tree essential oil (PEO). The results represent mean values of the triplicates ± standard deviation.

living cells (Bacsó *et al*., 2000, Rieger *et al*., 2010). Biological reduction of XTT is correlated to the activity of the transporting chain of electrons and some authors have demonstrated that XTT provides direct information about this activity after 90 min of incubation (Cota-Arriola *et al*., 2011, Goodwin *et al*., 1995, Berridge *et al*., 1996).

3.2.4 Damage to the membrane integrity

Propidium iodide (PI) is a colorant for fluorescence that is impermeable to the cellular membrane. It is excluded by the viable cells, so it is commonly used for the identification of dead cells in a population. The PI joins the DNA by an intercalating between the nitrogenated DNA and RNA bases having a relation of one PI molecule per each 4-5 DNA molecules (Riccardi and Nicoletti, 2006, Ali *et al*., 2010). Once the colorant joins the nucleic acids, its fluorescence is reinforced by 20-30 times.

In this research, an observation was made by growing NPs in a medium incorporated with 0.160 mg/mL of NPs, the inoculated spores in the medium containing CS NPs and CS-PEO showed a greater fluorescence in respect to control (Fig. 5). The results indicate that the NPs create greater damage in the *Colletotrichum gloeosporioides* spores with respect to the pepper tree essential oil, having as a result the loss of the membrane´s integrity. This matches the results found when the effect on the spore viability was evaluated, confirming an increment of the CS-PEO biocomposite biocide potential. The damage to the spore´s membrane can lead to the loss of the barriers selective properties of this organelle, resulting in an intake of material from outside the medium and the output of intracellular material (i.e. ions, amino acids, fatty acids, etc.) which is vital for biosynthetic reactions of the cell (Honary *et al*., 2011).

3.3 Mutagenicity

To consider a compound as mutagenic, it is necessary to find an increment twice larger than the number of spontaneous revertants (Bensaid *et al*., 2000). In this study, the Ames test using *Salmonella typhimurium* TA98 and TA100 showed that the chitosan and essential chitosan-pepper tree essential oil particles do not induce slipping mutations in the lecturing frame (strain TA98) or mutation by substitution in the base pare (TA100) (Table 5). The number of reverting histidine colonies caused by the different treatments showed similar values to the number of spontaneous revertant colonies in presence and absence of the S9 metabolic fraction.

Several studies have highlighted the possible mutagenicity both direct and indirect, of diverse nanomaterial such as ZnO, SiO_2, CoCr, TiO_2, Fe/Pt, carbon nanotubes, SWNT, etc. (Gonzalez *et al*., 2008). Likewise, *in vitro* studies with silver nanoparticles have shown their capacity to interfere in the replication or even join DNA (Yang *et al*., 2009). This demonstrates the potential of the nanoparticulated systems based on natural polymers such as chitosan, to be applied on biological systems.

3.4 Acute toxicity

Diverse studies made previously considered *Artemia salina* larvae as biosensors that report different rates of sensibility in comparison to diverse biocide agents (Shazili and Pascoe, 1986, Williams *et al*., 1986).

Table 5. Antimutagenic potential of pepper tree essential oil (PEO), chitosan particles (CS) and chitosan-pepper tree essential oil biocomposites (CS-PEO) on *Salmonella typhimurium*, using the Ames test. Both *Salmonella* test strains TA98 and TA100, with and without bioactivation (S9).

	TA98		**TA100**	
PEO	35.6 ± 7.7	41.3 ± 14.5	329.6 ± 63	141 ± 14.4
CS particles	27 ± 8.5	21.6 ± 4.5	73.6 ± 15.5	51.3 ± 5.5
CS-PEO biocomposite	28.3 ± 27.5	20 ± 14.7	2.6 ± 4.6	47.6 ± 15.5

Results are expressed as the number of colonies Histidine+ revertants (mean ± standard deviation). Mean values of 3 independent experiments performed in triplicate. Spontaneous revertant for TA98 strain were 51 and for TA100 366. Metabolic activation was performed with rat hepatic S9 fraction.

In this study, when the effect of the chitosan NPs and CS-PEO biocomposite were evaluated, a survival rate of nearly 50% of the organisms was found at the lowest tested concentrations, while the pepper tree oil did not affect the survival of *A. salina* at any of the evaluated concentrations (Fig. 6). As the concentration of chitosan NPs was raised (0.04, 0.08, and 0.16 mg/mL) the survival of *A. salina* dropped, while in treatments made with CS-PEO it was possible to observe survival percentages of nearly 40% using the same concentrations. Pepper tree essential oil did not affect the survival, finding >85% of nauplii alive in each of the concentrations.

Different studies have evaluated the acute toxicity of vegetable extracts, individually or complexed to nanoparticles, using *Artemia salina*. While evaluating the toxicity of the *Moringa oleifera* flower extract, a survival rate of 50% was found when applying 0.2 mg/mL (Rocha-Filho *et al*., 2015). Likewise, the *A. salina* nauplii exposed to a *Bergenia ciliate* extract merged with silver nanoparticles, demonstrated a 50% survival at concentrations of 0.08 and 0.03 mg/mL of the *B. ciliata* extract and NPs of Ag-extract, respectively (Phull *et al*., 2016). An extract from *Baccaris trinervis* (0.04 mg/mL) obtained by hydrodistillation, affected 50% of the *A. salina* viability (Sobrinho *et al*., 2016) and other study demonstrated that the NPs from a *Syzygium cumini* extract, presented a toxicity of LC_{50} >1000 μg/mL (Bitencourt *et al*., 2016). Lagarto-Parra *et al*. (2001) evaluated the acute toxicity of several plants extracts in *A. salina* and reported that 0.0035, 0.0099 and 0.0039 mg/mL of *Aloe vera*, *Ocimum basilicum* L. and *Citrus aurantium* L, respectively, are necessary to produce a mortality of 50% of the organisms. This effect can be attributed to the reduction of nutrients available in the medium and the increase in the production of oxygen reactive species due to stress, as a result of a lowering of ingest by the organisms resulting in their death. The toxicity test of *A. salina* has the advantage of being really simple and cheap in regards to tests done with mice to evaluate acute toxicity.

Conclusions

The CS and CS-PEO nanoparticles obtained by nanoprecipitation have a spherical morphology and homogeneous distribution of size. In comparison with CS nanoparticles and pepper tree essential oil, the CS-PEO biocomposite nanoparticles showed an important inhibitory effect upon the *in vitro* viability of *Colletotrichum gloeosporioides*, which increased proportionally to the used concentration. Besides affecting the kinetic parameters of radial growth and spore germination, the CS-PEO biocomposite is capable to affect the integrity of the fungi's membrane. These results suggest that the antifungal activity of the CS-PEO NPs against the *C. gloeosporioides* pathogen can be associated to a synergic effect between the CS and pepper tree essential oil, constituting a potential alternative for the control of pathogens of importance in food.

Nomenclature

Rc	average radial growth of the colony in control medium, cm
Ri	radial growth of the colony in different treatments, cm
A	maximum radial growth achieved during the stationary phase
Vm	maximum growth rate (1/0.5 days)
λ	the lag phase lag (0.5 days)
e	the exp (1)
Sc	percentage of germinated spores in control medium after a determined time (t)
Si	percentage of germinated spores in different treatments
S_{max}	maximum percentage of germinated spores when $t \rightarrow \infty$
S_0	initial percentage of germinated spores ($t = 0$)
k	the germination rate (h^{-1})

Acknowledgements

Financial support of CONACyT (Project No. 219786) is gratefully acknowledged.

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