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ORIGINAL RESEARCH

Antifungal Activity of a Chitosan and Mint Essential Oil Coating on the Development of *Colletotrichum Gloeosporioides* in Papaya Using Macroscopic and Microscopic Analysis

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Abstract
Chitosan and mint essential oil treatments have been studied as natural alternatives to chemical fungicides to control fruit diseases caused by phytopathogens, such as *Colletotrichum gloeosporioides* that causes anthracnose in papaya fruit; however, their combination has been scarcely studied and their microscopic effect on the infected papaya surface has not been reported. The aim of this investigation was to evaluate the effect of chitosan (CH), mint essential oil (MEO) or their combination (CH + MEO) on the in vitro growth of *Colletotrichum gloeosporioides*, the macroscopic and microscopic development of anthracnose and the postharvest quality of papaya. Fruit were treated by immersion in solutions of CH (1%), MEO (0.2%) or CH + MEO (1%, 0.2%) for 5 min and stored for 20 days at 12 °C. Untreated fruit were used as control. In vitro and in vivo antifungal activity and postharvest quality were assessed for all treatments, and anthracnose development was evaluated using scanning electron microscopy only for control and the treatment with the best antifungal activity. In vitro, the applied treatments exhibited an important antifungal activity due to the high inhibition of mycelial growth and conidial germination. In vivo, the lowest incidence and severity of anthracnose was obtained in fruit treated with CH, which was also observed by microscopic analysis. In addition, the treatments maintained the fruit postharvest quality. CH was effective to inhibit anthracnose development in papaya and the addition of MEO did not provide an additional effect. Chitosan coating may be a useful strategy to control anthracnose and maintain the postharvest quality of papaya fruit.

Keywords *Carica papaya* L. · Anthracnose · Postharvest quality · *Mentha piperita* L. · Essential oil · Scanning electron microscopy

Introduction

Papaya (*Carica papaya* L.) is a climacteric fruit cultivated around the world in tropical and subtropical areas, occupying México the third world-wide place in production (Ayón-Reyna, González-Robles, et al., 2017; Garcia et al., 2014). This fruit is a good source of bioactive compounds and some vitamins like B1, B2 and C (Ali et al., 2015). However, it is very susceptible to diseases caused mainly by fungi, resulting in losses of up to 50% of fresh product (Ali et al., 2016; Dotto et al., 2015).

Anthracnose is the main disease that affects papaya and is caused by the fungus *Colletotrichum gloeosporioides* (Ali et al., 2015). Its main symptoms are shown in rounded form, presenting subsidence and orange to pink mycelium (Ayón-Reyna, González-Robles, et al., 2017). Currently, synthetic fungicides are used to combat this disease; however, their use for long periods of time can cause pathogen resistance, environmental contamination and represents a risk for the health of consumers (Ali et al., 2015; Hewajulige et al., 2007).

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In recent years, the research of alternatives for the control of postharvest diseases with a non-toxic, safe and biodegradable approach, such as the use of edible coatings, has been undertaken (Ayón-Reyna, González-Robles, et al., 2017; Dos Passos Braga et al., 2019; Villegas-Rascón et al., 2018). The edible coatings form a semipermeable barrier to some gases and volatile compounds and help to prolong the fruit shelf life, by a reduction of water loss and respiration rate, maintaining the organoleptic and nutritional quality of the fruit for a longer period of time (Dotto et al., 2015). The main components used in the preparation of edible coatings are proteins, polysaccharides and lipids.

Chitosan is one of the most used polysaccharides in coating formulations due to its antimicrobial and barrier properties. Some authors report that chitosan presents antifungal activity against *Monilinia fructicola* (Li & Yu, 2001), *Colletotrichum gloeosporioides* (Ayón-Reyna, González-Robles, et al., 2017), *Fusarium oxysporum*, *Rhizopus stolonifer* and *Penicillium digitatum* (Bautista-Baños et al., 2004). Also, studies have reported chitosan is effective to control postharvest decay in several fruit, such as kiwifruit (Zheng et al., 2017), peach (Li & Yu, 2001), apple (Li et al., 2015) and papaya (Hewajulige et al., 2007). Moreover, chitosan is a good carrier of substances that help to improve the physicochemical, nutritional and microbiological quality of food, and the incorporation of essential oils may enhance these properties (Dantas-Guerra et al., 2015; Das et al., 2021; Kujur et al., 2021).

The essential oils, obtained from herbs and spices, have been widely used in the preparation of coatings with antimicrobial activity. These natural compounds are secondary metabolites produced by aromatic plants and their antimicrobial properties are due to their composition in terpenes, aldehydes and alcohols. In addition, they are approved by the FDA (Food and Drug Administration, USA) as GRAS-type compounds (Generally Recognized as Safe) (Abdolahi et al., 2010; Ali et al., 2015; de Oliveira et al., 2017). Particularly, the essential oil of mint (*Mentha piperita* L.) has shown good antifungal properties by inhibiting the development of postharvest diseases such as anthracnose produced by *Colletotrichum gloeosporioides* in papaya (Sarkhosh et al., 2017) and grey mold caused by *Botrytis cinerea* in plum fruit (Aminifard & Mohammadi, 2013).

Several studies have reported the efficacy of coatings elaborated with chitosan and essential oils for food preservation, both of animal and vegetable origin, showing additive or synergistic inhibitory effects when both compounds were combined (Alparslan & Baygar, 2017; Barreto et al., 2016; Correa-Pacheco et al., 2017; Mohammadi et al., 2016; Munhuweyi et al., 2017; Vatavali et al., 2013; Wang et al., 2017). Specifically, the application of coatings elaborated with chitosan and mint essential oil to reduce fungal development in fruits like cherry tomato (Dantas-Guerra et al., 2015), mango (de Oliveira et al., 2017) and papaya (Dos Passos Braga et al., 2019) have already been reported. However, there are no reports in the literature on the combined use of these natural compounds in papaya where microscopic tests have been performed to study its effect on microstructure of the inoculated fruit and the development of anthracnose as well as its relationship with postharvest quality. The aim of this study was to evaluate the effect of a chitosan coating combined with mint essential oil on the in vitro growth of *Colletotrichum gloeosporioides*, the macroscopic and microscopic development of anthracnose, and the postharvest quality of papaya fruit.

Materials and Methods

Materials

Papaya fruit (cv. Maradol) at maturity index 4 (slightly orange skin with green stripe, according to Santamaría-Basulto et al., 2009) and in the absence of pesticides were obtained from a local market, in the region of Culiacan, Sinaloa, Mexico. The selected fruit presented uniformity in size (900 ± 100 g) and skin color and were free of physical damage. Mint essential oil (*Mentha piperita* L.) was acquired in Aceites y Esencias, S.A., Mexico City, Mexico. Food-grade chitosan was acquired in Agrinos, S.A., Sonora, Mexico.

Inoculum Preparation

A spore suspension of *Colletotrichum gloeosporioides* was prepared with a culture of two weeks, previously isolated and identified using molecular techniques and deposited in sterile distilled water. The concentration of spores was adjusted to 1×10^6 conidia/mL using a hemacytometer (Marienfeld, Germany), and 0.5% Tween 80® was added to avoid spore agglomeration (Ayón-Reyna, González-Robles, et al., 2017).

Edible Coatings Preparation

A chitosan solution (1% w/v) was prepared following the technique of Aloui et al. (2014) with some modifications. Chitosan was dissolved in deionized water at 40 °C using glacial acetic acid (1% v/v) and shaking for 12 h. The mint oil solution was prepared at a concentration of 0.2% (v/v) as previously reported by Dantas-Guerra et al. (2016) and de Oliveira et al. (2017); the essential oil was homogenized with deionized water at 35 °C for 5 min

using a homogenizer (T18 Basic Ultra-Turrax, IKA, UK) at 13,500 rpm. A chitosan-based solution (1%, w/v) was prepared and mint oil was added to obtain a final concentration of 0.2% (v/v), homogenizing at 13,500 rpm for 4 min, as reported by Ali et al. (2015). All solutions were adjusted to pH 5.6 by adding NaOH (1 M) and were added with 1% Tween 80® to stabilize them for at least 24 h.

In Vitro Antifungal Assays

The in vitro mycelial growth was carried out using the "poison food" technique with some modifications (Aloui et al., 2014). Potato dextrose agar (PDA) was prepared and 1% (v/v) Tween 80® was added and sterilized at 121 °C for 15 min. Aseptic solutions of CH (1%), MEO (0.2%) and CH + MEO (1%, 0.2%) were added to the agar (~ 45 °C) and immediately poured into Petri dishes (9 cm diameter) and solidified at room temperature for 30 min. Untreated Petri dishes were used as control. After that, 1 µL of the spore suspension was poured into the center of the Petri dishes and incubated at 25 °C until the mycelial growth in untreated dishes reached the edge of the plate. For each treatment, three replicates were made with six repetitions. The fungitoxicity of the treatments was measured in terms of the percentage of mycelial growth inhibition (MGI), calculated by the following equation:

$$MGI(\%) = \frac{\left(d_c - d_t\right)}{d_c}$$

where d_c and d_t are the radial growth of the fungus in the control and treatment, respectively.

For the inhibition of conidial germination, Petri dishes with PDA and solutions of CH, MEO and CH + MEO were prepared in the same way as mycelial growth. The inoculation of the dishes was carried out with 100 µL of the conidial suspension (1×10^6 conidia/mL) and spread with a sterile loop and then dishes were incubated at 25 °C for 6 h. Germination was examined using an optical microscope (40X, Photoelectric microscope, Axiophot Carl Zeiss, Germany), and 100 conidia were examined per treatment. A conidium was considered germinated when the length of the germinative tube equaled or exceeded half the length of the conidium. The results were expressed as inhibition of germination (%) according to the following formula described by Ong et al. (2013):

$$IG(\%) = 1 - \frac{G_t}{G_c} \times 100\%$$

where G_t and G_c are the number of spores germinated in the treatment and control, respectively.

In Vivo Treatments Application

Papaya fruit, previously washed, disinfected (1% NaClO for 5 min) and rinsed in sterile distilled water, were inoculated by immersion during 5 min into the spore suspension of *Colletotrichum gloeosporioides* (Ayón-Reyna, González-Robles, et al., 2017). Then, the inoculated fruit were randomly divided into four lots for the application of the treatments: one lot was immersed in chitosan solution (CH, 1%), another lot in mint essential oil solution (MEO, 0.2%), while another one was immersed in the combination CH + MEO (1%, 0.2%). All immersions were carried out for 5 min at 25 °C and then the fruit were placed for 1 h at 25 °C to remove moisture excess. Fruit from the remaining lot were not treated and were used as control. Treated and untreated fruit were stored at 12 °C for 20 days. Three replicates and three repetitions were made per treatment.

In Vivo Antifungal Assays

Disease incidence was evaluated every 4 days according to the presence of anthracnose on the fruit surface following the methodology of Ayón-Reyna, González-Robles, et al. (2017). The results were expressed as the number of fruit that showed anthracnose symptoms of the total number of fruit in each treatment:

$$Disease\ incidence(\%) = \frac{Number\ of\ infected\ fruits}{Total\ number\ of\ inoculated\ fruits} x100\%$$

Anthracnose severity was visually evaluated every 4 days (Ayón-Reyna, González-Robles, et al., 2017) using a 5-point scale, where 1 represents no symptoms of anthracnose in the fruit surface (0%), 2 represents 1–25% of symptoms, a rating of 3 was scored when 26–50% of the fruit surface was rotten, 4 means that 51–75% of the fruit surface was infected by anthracnose and 5 represented ≥ 76% exhibited anthracnose symptoms. Three replicates per treatment were performed and each treatment included 12 fruits.

Physical Quality Parameters

Weight loss and firmness were evaluated after 20 days of storage. Weight loss was determined according to Ayón-Reyna, López-Valenzuela, et al. (2017). The weight of the fruit was recorded using a balance (Sartorius, model TE 4101 Goettingen, Germany). Twelve fruit of each treatment were weighed at the beginning and the end of the storage and the values were expressed as percentage of weight loss (WL), according to the following equation:

$$WL(\%) = \frac{(final\ weight - initial\ weight)}{(final\ weight)} \times 100\%$$

Firmness was evaluated according to Ali et al. (2011), with some modifications. Papaya samples of similar sizes were taken from the equatorial region of each fruit. A total of nine measurements per fruit were obtained using a penetrometer (Chatillon, DFE AMETEK, Florida, USA) equipped with a flat tip of 11 mm in diameter at constant penetration rate (50 mm/min—5 mm penetration). Results were expressed in Newtons (N).

Biochemical Quality Parameters

Total soluble solids (TSS) and ascorbic acid contents were evaluated after 20 days of storage. TSS content was determined using a manual refractometer (Atago Fisher Scientific, GA, USA) (AOAC, 2012). The evaluation was performed by placing a drop of juice from each sample directly on the refractometer. Results were reported as ºBrix.

Ascorbic acid content was determined using the method described by Dürüst et al. (1997) with some modifications. The vitamin was extracted by homogenizing 0.5 g of fresh tissue with 15 mL of oxalic acid (0.4%, w/v) and subsequently reacting with 2,6-diclorophenolindophenol (DCPI) (0.0012%, w/v). The absorbance was measured using a spectrophotometer (UNICO SQ2800, New Jersey, USA) at 520 nm. Three replicates with three repetitions per treatment were performed, and the results were expressed as mg of ascorbic acid per 100 g of fresh fruit (gff).

Scanning Electron Microscopy (SEM)

The evaluation of the microstructural changes in the papaya tissue was carried out following the methodology of Cárdenas-Pérez et al. (2017) using an environmental scanning electron microscope (Zeiss, Evo LS10, Germany). Samples of approximately 1 cm^3 were excided of equatorial region of the papaya surface at 0, 3 and 10 days of storage. The cubes were placed on double-sided carbon conductive tape and were observed directly under the electron microscope in environmental mode at 20 Pa of water vapor and 20 kV. A backscattered electron detector (NTS BSD) was used.

Statistical Analysis

A completely randomized experimental design was performed with three replicates and three repetitions, considering 72 fruit per replica. Data were analyzed through analysis of variance using Statgraphics Plus 5.1, and the means were compared using minimal significant difference (LSD) ($p \leq 0.05$) by Fisher's test.

Results and Discussion

In Vitro Antifungal Activity

The in vitro mycelial growth of *Colletotrichum gloeosporioides* was completely inhibited by the treatments CH, MEO and CH + MEO, while in control treatment a normal growth was observed (Table 1). Researchers have also reported the efficacy of chitosan to inhibit the proliferation in vitro of some fungi such as *Colletotrichum gloeosporioides* (Bautista-Baños et al., 2003), *Rhizopus stolonifer* (Hernández-Lauzardo et al., 2007) and *Penicillium digitatum* (Bautista-Baños et al., 2004). The antifungal activity of chitosan could be due to this polysaccharide causing damage in the fungal membrane by the interaction of amino groups with the phospholipids of the membrane, resulting in an increase in the permeability of the plasma membrane. In addition, it is believed that chitosan causes morphological alterations in the fungal mycelium, delaying its growth by affecting several stages of its development (Alvarado-Hernández et al., 2011; Bautista-Baños et al., 2003, 2004). On the other hand, Moreira et al. (2012) found that mint essential oil presented high inhibition in the growth of phytopathogenic fungi such as *Aspergillus flavus*, *Aspergillus glaucus* and *Aspergillus niger,* which could be due to menthol, one of the main components of this oil with antimicrobial properties. In addition, the chemical constituents of the oils are mostly hydrophobic and can accumulate in the lipid region of the cell membrane of microorganisms, causing structural and functional damage in the cell (Abdolahi et al., 2010).

Chitosan and mint essential oil had an outstanding effect on the inhibition of mycelial growth when they were applied individually; therefore, it was not possible to observe significant differences with the combined treatment. However, the study published by Munhuweyi et al. (2017) reported synergistic in vitro antifungal activity of chitosan combined with cinnamon, lemongrass or oregano essential oils against *Botrytis sp., Penicillium sp.* and *Pilidiela granati*. Also,

Table 1 In vitro antifungal activity of chitosan (CH), mint essential oil (MEO) and their combination (CH + MEO) against *Colletotrichum gloeosporioides*

Treatments	MGI (%)	IG (%)
Control	0^{b}	0^{c}
CH	100^{a}	98.4^{ab}
MEO	100^{a}	96.8^{b}
CH + MEO	100^{a}	100^{a}

Values correspond to means of data for the four treatments. Different letters in the same column indicate significant differences ($p \leq 0.05$) among treatments. Least significant difference (LSD) for MGI = 0; LSD for IG = 1.72

MGI (%) Mycelial growth inhibition, *IG* Inhibition of germination

Mohammadi et al. (2016) reported low mycelial growth diameter of *Phytophthora drechsleri* treated with chitosan in combination with *Cinnamomum zeylanicum* essential oil or *Zataria multifora* essential oil. In the same way, Bill et al. (2014) reported a better in vitro antifungal activity against *Colletotrichum gloeosporioides* when chitosan and thyme oil were combined than when they were individually applied, which could be due to the fact that the incorporation of oil into chitosan coating improved the activities of chitinase and β-1, 3-glucanase because these enzymes are related to the hydrolysis of polymers of fungal cell wall, favoring the defense mechanisms of plants against fungal pathogens.

Conidial germination of *Colletotrichum gloeosporioides* was affected by the applied treatments (Table 1). Statistical differences were observed between treatments and control. The combined treatment (CH + MEO) presented 100% of inhibition of conidial germination, followed by CH (98.4%) and MEO (96.8%), showing significant differences ($p \leq 0.05$) only between MEO and CH + MEO. Similar results were reported by Dantas-Guerra et al. (2015) who observed that the combination of chitosan and mint essential oil strongly inhibited the mycelial growth and spore germination of *Aspergillus niger*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*. According to de Oliveira et al. (2017), the improvement of the antifungal activity of chitosan and mint essential oil, due to their combination, could be related with the capacity of chitosan to alter the permeability of fungal membrane, enabling the partition of mint essential oil components into the fungal cells where they can act on target structures.

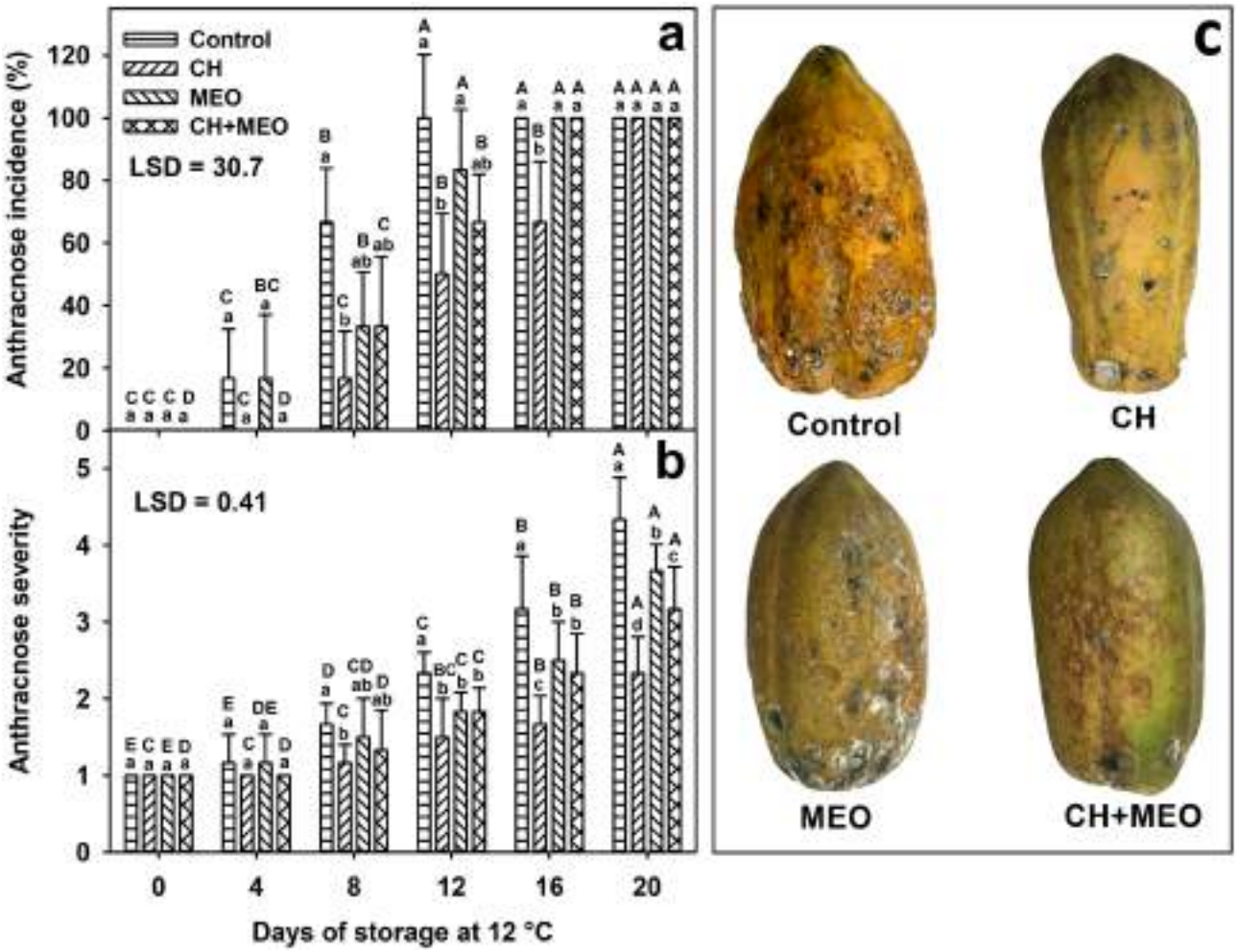

Fig. 1 Effect of chitosan (CH), mint essential oil (MEO) and their combination (CH + MEO) on anthracnose incidence (**a**) and severity (**b**) of papaya inoculated with *Colletotrichum gloeosporioides* and stored at 12 °C for 20 days. Severity index: 1 = 0%, 2 = 1–25%, 3 = 26–50%, 4 = 51–75%, 5 = 76–100%. Vertical bars on columns represent standard deviation of the means of three replicates. For the same day, different lowercase letters indicate significant differences among treatments according to Fisher´s test ($p < 0.05$). For the same treatment, different uppercase letters indicate significant differences among days of storage according to Fisher´s test ($p < 0.05$). (**c**) Representative images of papaya fruit for each treatment showing anthracnose symptoms on the surface after 20 days of storage at 12 °C

In Vivo Antifungal Activity

According to anthracnose incidence results shown in Fig. 1a, the first anthracnose symptoms appeared in the control and MEO-treated fruit after 4 days of storage, while the CH- and CH + MEO-treated fruit remained without symptoms until day 8. At day 12, all control fruit presented symptoms of anthracnose, this is, 100% of anthracnose incidence, while 83.3% of fruit treated with MEO and only 66.6% and 50% of the fruit treated with CH + MEO and CH, respectively, presented the disease. On day 16, CH-treated fruit maintained a low percentage of incidence (66.6%); meanwhile, 100% of the fruit treated with MEO and CH + MEO had anthracnose symptoms. It should be noted that CH and CH + MEO treatments delayed the appearance of anthracnose for 4 days compared to control. Similar results were reported by Li and Yu (2001) in peach fruit treated with chitosan, where the fungal rot caused by the fungus *Monilinia fructicola* was delayed for 6 days. These authors concluded that the polysaccharide delayed the development of symptoms by inducing aggregation of the mycelium, as well as protein breakdown of the fungal cells.

Untreated fruit had the highest values of anthracnose severity throughout the storage period, showing statistical differences ($p \leq 0.05$) with the CH-treated fruit from day 8 (Fig. 1b). At the end of the storage, fruit treated with CH presented the smallest affected surface area, followed by the combination (CH + MEO) and MEO (Fig. 1b, c). Chitosan-based coating could have acted as a barrier around the fruit that limited the penetration of the fungal germ tube and prevented the flow of nutrients to the outside, thus limiting the growth of pathogenic microorganism (Bill et al., 2014; Hewajulige et al., 2007). In addition, it has been reported that chitosan induces host defense mechanisms, such as physical barriers and the production of compounds such as phytoalexins (Li et al., 2015). By the other hand, the MEO has antifungal properties associated to the effect of compounds like menthol and isomenthone, which cause disorganization of cell membrane structure, physical or chemical alterations and disturbing of fungal metabolic activities (de Oliveira et al., 2017). Similar results were reported by Alvarado-Hernández et al. (2011) who observed that the application of chitosan was better to reduce the development of *Rhizopus stolonifer* in tomato than the chitosan mixture with essential oils; they suggested that chitosan might interact through hydrogen bonds with the terpenes of essential oils affecting the antifungal activity of mixtures. The antifungal activity of chitosan has been demonstrated against several phytopathogenic fungi such us *Rhizopus stonolifer* and *Mucor spp.* in tomatoes (Hernández-Lauzardo et al., 2007), *Colletotrichum gloeosporioides* in papaya (Hewajulige et al., 2007), *Penicillium expansum* in apple (Li et al., 2015) and *Monilinia fructicola* in peach (Li & Yu, 2001). On the other hand, several studies have reported the development of postharvest diseases in fruit as affected by chitosan combined with different essential oils such as thyme, *Zataria multiflora*, *Cinnamomum zeylanicum*, cinnamon, lemon and oregano essential oils (Correa-Pacheco et al., 2017; Mohammadi et al., 2016; Munhuweyi et al., 2017; Xing et al., 2015). However, there are few studies about the combination of chitosan with mint essential oil in fruit to combat postharvest diseases, such is the case of Dantas-Guerra et al., (2015, 2016) who evaluated the effect of this combination on the development of *Aspergillus niger*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* in cherry tomato and table grape fruit. Also, de Oliveira et al. (2017) reported a synergistic effect of chitosan and mint essential oil on the development of different *Colletotrichum* species when this combination was applied on mango. Nevertheless, the application of chitosan and mint essential oil to inhibit the anthracnose development in papaya fruit has not been reported.

Physical and Biochemical Parameters

Weight loss, firmness, total soluble solids and ascorbic acid are shown in Table 2. Weight loss increased during the storage period and no statistical differences were observed among the control, CH- and CH + MEO-treated fruit after

Table 2 Changes in quality parameters of papaya fruit treated with chitosan (CH), mint essential oil (MEO) and their combination (CH + MEO) after 20 days of storage at 12 °C

Treatments	Quality parameters			
	Weight loss (%)	Firmness (N)	Total soluble solids (°Brix)	Ascorbic acid (mg AA/100 gff)
Control	4.13 ± 0.56^{b}	10.86 ± 2.67^{b}	7.25 ± 0.26^{a}	38.19 ± 4.37^{b}
CH	4.21 ± 0.24^{b}	20.75 ± 2.58^{a}	6.85 ± 1.21^{b}	45.28 ± 2.84^{ab}
MEO	4.84 ± 0.27^{a}	15.77 ± 2.23^{ab}	6.72 ± 0.12^{b}	38.73 ± 2.47^{b}
CH + MEO	4.32 ± 0.13^{b}	21.54 ± 2.93^{a}	6.67 ± 0.96^{b}	47.65 ± 4.02^{a}

Values correspond to means ± standard deviation of data for the four treatments. Different letters in the same column indicate statistical differences ($p \leq 0.05$) among treatments. Initial firmness = 87 N, initial total soluble solids = 6.4 °Brix, initial ascorbic acid = 51 mg of ascorbic acid /100 g of fresh fruit

20 days of storage ($p \leq 0.05$), which indicates that CH and CH + MEO did not decrease the water vapor output and the weight loss occurred naturally in the fruit (Ali et al., 2015). Similar results were reported by Dantas-Guerra et al. (2015) who observed that cherry tomato fruit coated with chitosan combined with mint (*Mentha piperita* and *Mentha x villosa* Huds) essential oils exhibited weight loss rates similar to the control fruit. Nevertheless, Barreto et al. (2016) reported that cherry tomato fruit coated with chitosan-oregano essential oil had lower weight loss than the control fruit, and they attributed these effects to the hydrophobicity of oregano essential oil which improved the physical barrier properties of chitosan coating. On the other hand, fruit treated with MEO presented the highest weight loss percentage even greater than untreated fruit, which can be attributed to a damage in the fruit skin and the stress caused by the essential oil because the fruit can detect it as a strange agent, limiting the consumption of oxygen and thus affecting the rate of respiration (Pontigo-Suárez et al., 2015). Similar results were reported by Pontigo-Suárez et al. (2015) who found that papaya fruit treated with oregano essential oil had higher weight loss than the untreated fruit. However, contrary to our results, Abdolahi et al. (2010) reported that table grape fruit treated with thyme (*Thymus vulgaris* L.), summer savory (*Satureja hortensis* L.) and sweet basil (*Ocimum basilicum* L.) essential oils had a lower weight loss than the untreated fruit. Although the MEO treatment increased the weight loss, showing the highest value, this loss was less than 5% (4.84%). It has been reported that in uninfected papaya fruit a weight loss bigger than 5% is required to make the deterioration visible (Almeida-Castro et al., 2011).

Papaya fruit presented initial firmness values of approximately 87 N. These values decreased in all treatments during the storage, showing that after 20 days the control fruit obtained the lowest firmness values, whereas fruit covered with CH + MEO, CH and MEO had the highest (Table 2). The reduction of the firmness of the fruits is due to the degradation of the cell wall, which involves the hydrolysis of pectin and other components of the cell wall due to the action of hydrolytic enzymes. The combination chitosan and essential oil can have inhibited the hydrolytic enzymes preventing the lysis of cell wall components. In addition, the coating acted as a semipermeable film to gases causing a decrease in respiration rate and delaying the ripening process of the fruit (Olufunmilayo & Uzoma, 2016). It has been previously reported that the application of chitosan coatings combined with thyme, O*riganum vulgare* L., and mint essential oils maintained the firmness of avocado (Bill et al., 2014; Correa-Pacheco et al., 2017), cherry tomato (Barreto et al., 2016) and table grape fruit (Dantas-Guerra et al., 2016), respectively. According to Bill et al. (2014), avocado fruit treated with the combination of chitosan and thyme oil had greater firmness than fruit treated only with chitosan or thyme oil. These authors attributed the results to the formation of a semi-permeable gas barrier that decreased the respiration rate and also to the lower development of the fungus in the fruit caused by the combined treatment, which protected the fruit against fungal enzymes that degrade the cell wall and helped to maintain firmness (Barreto et al., 2016). Contrary to our results, Dantas-Guerra et al. (2015) did not observe significant differences between untreated cherry tomato

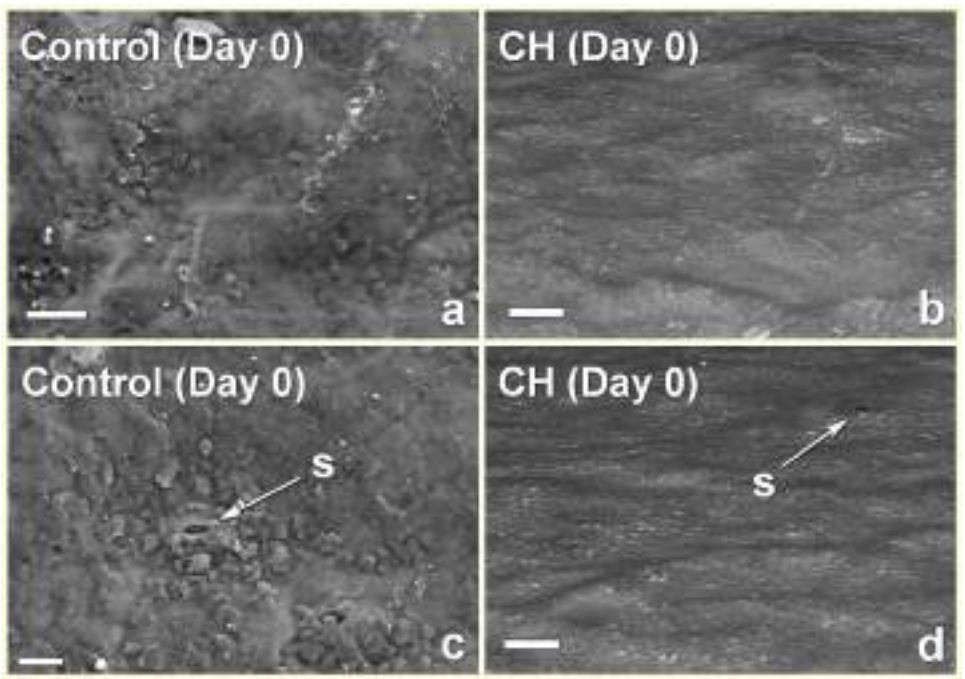

Fig. 2 Scanning electron photomicrographs of surface of untreated papaya (**a**, **c**) and treated with chitosan (**b**, **d**) at 0 days of storage. Micrographs of the surface papaya control show a normal morphology being this irregular, while CH coating gives a smoother and homogenous surface in the fruit. CH = chitosan; s = stomata. Bars = 20 μm

fruit and those treated with chitosan combined with mint essential oil.

The content of total soluble solids of the fruit at the beginning of the storage was approximately 6.4°Brix. This value increased at the end of storage in all treatments without showing statistical differences ($p \leq 0.05$) among CH-, MEO- and CH + MEO-treated fruit. The untreated fruit had a higher content (7.25%) of soluble solids compared to the treated fruit (about 6.75%) (Table 2). The behavior of the treated fruit could be related to a delay in the ripening process of the fruit by preventing the hydrolysis of carbohydrates into simple sugars and organic acids due to the treatments were able to reduce the fungal infection and thus reduce the stress of the fruit that causes an increase of respiration rate (Ali et al., 2016; Barreto et al., 2016). Similar results were reported by Barreto et al. (2016) in cherry tomato fruit, where fruit treated with chitosan, *Origanum vulgare* L. essential oil and the combination chitosan with *Origanum vulgare* L. essential oil had higher total soluble solids than the control fruit. Contrary to our results, Correa-Pacheco et al. (2017) did not observe significant differences in total soluble solids content between control fruit and fruit treated with chitosan combined with thyme essential oil. Moreover, Dantas-Guerra et al., (2015, 2016) did not find significant differences between uncoated cherry tomato and table grape fruit and fruit coated with chitosan and mint essential oil.

At the beginning of the storage, fruit presented an average ascorbic acid content of 51 mg /100 g of fresh fruit and this content decreased by day 20 (Table 2). Control and MEO-treated fruit had the lowest vitamin C contents, followed by CH and CH + MEO. This retention is attributed to a surface barrier formed by CH which limited oxidative reactions, postponing degradation of this compound (Ali et al., 2011). These results are similar to those reported by Barreto et al. (2016) in cherry tomato, where the decrease in ascorbic acid was greater in control fruit or fruit treated only with *Origanum vulgare* L. essential oil or chitosan than in fruit treated with the combination chitosan with essential oil. Moreover, Xing et al. (2015) found that the decrease rate in vitamin C was significantly lower in jujube fruit coated with both chitosan and cinnamon oil than in uncoated fruit or fruit coated with chitosan or cinnamon oil only.

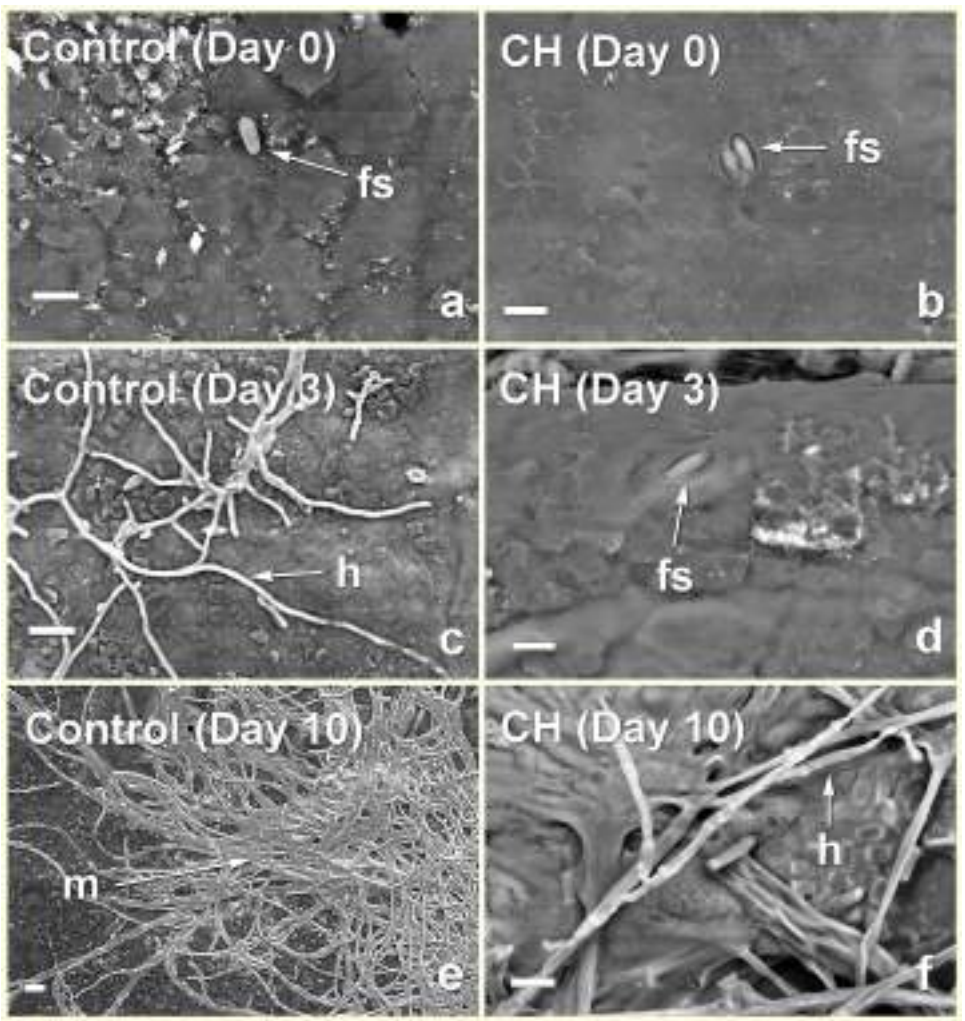

Fig. 3 Scanning electron microscopy of spores and hyphae of *Colletotrichum gloeosporioides* on papaya cv. Maradol. Fungal spores on the uncoated papaya surface (**a**) and coated with chitosan (**b**). Hyphae on the uncoated papaya surface (**c**), and spore coated with chitosan without presence of germ tube or hyphae (**d**). Abundant mycelium on the uncoated surface (**e**) and scarce presence of hyphae on the surface coated with chitosan (**f**). CH = chitosan; fs = fungal spore; h = hyphae; m = mycelium. Bars = 10 (a, b, d, f) and 20 (c, e) µm

SEM

According to the results obtained in the in vivo antifungal tests, CH was the treatment that presented the highest antifungal activity, showing the lowest anthracnose incidence and severity (Fig. 1); therefore, SEM test to analyze anthracnose development was performed only in control and CH-treated fruit. The surface of untreated fruit at the beginning of storage showed a rough appearance (Fig. 2a), which is considered as typical morphological characteristic of papaya fruit (Ayón-Reyna et al., 2017; Ong et al., 2013). Contrariwise, the papaya surface coated with CH was smoother and more homogeneous producing a uniform cover (Fig. 2b). Also, open stomata were observed in the control fruit surface (Fig. 2c), while in the CH-treated fruit natural openings of the fruit were partially or completely covered by chitosan (Fig. 2d). Ferrão et al. (2018) observed a smooth surface in grapes when a coating based on nanoparticles of chitosan was applied. In addition, Varasteh et al. (2017) found that the SEM of the peel of the treated pomegranate fruit showed that chitosan films covered pericarp surface and natural porosity on the peel. Chitosan forms a semi-permeable barrier on the surface of fruit, limiting fungi penetration and fungal rot by its direct antifungal activity (Zhou et al., 2016).

On the other hand, it was observed that the spores of the fungus *Colletotrichum gloeosporioides* were adhered to the surface of the papaya after the inoculation, which was observed in both control and CH-treated fruit (Fig. 3a, b). However, it is appreciated that the application of CH formed a layer that covered the spores that were deposited on the surface and this way limiting oxygen transfer and respiratory activity of pathogens (Zhou et al., 2016). After 3 days of storage, some hyphae were already observed in control fruit (Fig. 3c), meanwhile in CH-treated fruit spores without apparent germination were visualized, observing an important effect of chitosan upon germination and development of *Colletotrichum gloeosporiodes* (Fig. 3d). After 10 days of storage, there was an abundant mycelium on the control fruit (Fig. 3e) compared with scarce development of mycelium in the fruit treated with CH (Fig. 3f). Results suggested that chitosan layer around the spores could cause difficulty for the entry of nutrients in the fungal cells. Also, the interaction of amino groups (positive charges) of chitosan with negatively charged fungal cell membrane components can interfere with the normal growth of the fungal cells. Some authors have reported that the inhibitory effect of chitosan may be due to the direct contact of the chitosan with the pathogen entering through the fungal cell wall interacting with its DNA and modifying its configuration, inhibiting mRNA synthesis and protein synthesis. In addition, chitosan is associated with the decrease in respiratory activity and activity of some enzymes of the fungi and provides a more effective barrier between fruit and the external environment (Dotto et al., 2015). Zahid et al. (2012) reported the antifungal activity of chitosan, using SEM, in banana, papaya and pitahaya inoculated with two species of *Colletotrichum*. The authors observed an agglomeration of the conidia in the treated fruit in comparison with the untreated ones in which spores with a normal morphology were observed. Additionally, Oliveira-Junior et al. (2012) reported abnormal shapes, swelling and hyphae size reduction of *Alternaria alternata*, *Botrytis cinerea* and *Rhizopus stolonifer* treated with chitosan.

Conclusions

CH coating delayed anthracnose development in papaya and its combination with MEO had no synergistic effect. Therefore, the application of edible coatings based on CH in papaya fruit may be a good strategy to prevent the development of anthracnose caused by *Colletotrichum gloeosporioides* and to improve the shelf life of the fruit due to chitosan is not a synthetic chemical compound and recently it has been recognized as GRAS by the FDA. Also, CH treatment maintained the postharvest quality of the papaya, since it maintained the firmness, decreased the TSS content and delayed the loss of vitamin C.

Authors´ Contributions L.E. Ayón Reyna and Y.G. Uriarte Gastelum designed the experiment, conducted all experiments, interpreted the results and prepared the manuscript. B.H. Camacho Díaz, D. Tapia Maruri and M.E. López provided technical support and revised the manuscript. J.G. López Velázquez analyzed the data. M.O. Vega García managed the whole experiment in general and prepared the manuscript.

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Data Availability Data will be available in a request.

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Declarations

Conflict of Interest The authors declared that they have no conflict of interest.

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