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Effect of chitosan with different molecular weights on the antifungal activity against *Colletotrichum gloeosporioides* and activation of the non-enzymatic antioxidant system on infected papaya

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Abstract: Chitosan (Ch) is a biopolymer with excellent antimicrobial and antioxidant properties, capable of maintaining the organoleptic quality of fruits. These properties have been related to its molecular weight. However, the effect of different molecular weights on the non-enzymatic antioxidant system and postharvest quality of anthracnose-infected papaya is still unknown. Therefore, this study evaluated the effect of different Ch molecular weights (13, 25, and 55 kDa) on anthracnose development, antioxidant capacity, and postharvest quality of papaya. The Ch-55, Ch-25, and 13 kDa inhibited the development of *Colletotrichum gloeosporioides*. In comparison with thiabendazole, Ch with different molecular weights showed fungicidal effects. Ch-55 kDa reduced anthracnose incidence and severity and maintained postharvest quality parameters of papaya. In addition, Ch-55 and Ch-13 kDa favored synthesis of ascorbic acid, total phenolics, and antioxidant capacity. Therefore, Ch-55 kDa is an alternative to the protection against *C. gloeosporioides* and a stimulant of the non-enzymatic antioxidant system in papaya fruits during its postharvest storage.

KEYWORDS
anthracnose, antioxidant, biopolymer, *Carica papaya*, molecular weights

Practical Application: Ch with different molecular weights have different effects over anthracnose control and postharvest quality in papaya. In addition, they have different stimulation levels on the non-enzymatic antioxidant system of papaya fruits, in a higher level than the chemical fungicide TBZ, by regulating the growth of the fungus and the degradative processes that are generated in fungal infection. In this sense, we believe that our findings could be of interest for producers and farmers to take advantage of this biopolymer to control

anthracnose, preserve the postharvest quality, and stimulate the non-enzymatic antioxidant system of papaya fruit.

1 | INTRODUCTION

Papaya is a popular fruit due to its attractive taste and high content of bioactive compounds, which can reduce the risk of degenerative diseases (Ayón-Reyna et al., 2018). Around 13 million tons of papayas are currently produced worldwide; however, this fruit is susceptible to anthracnose, a devastating postharvest fungal disease caused by the fungus *Colletotrichum gloeosporioides* Penz, which leads to a loss of up to 40% of the production (Madani et al., 2016). During infection, anthracnose provokes deterioration of the cell membrane structure causing accelerated senescence, which triggers oxidative stress and catabolism of bioactive compounds (Gomes et al., 2020). Generally, the infection by *C. gloeosporioides* occurs before harvest; however, symptoms manifest until favorable conditions for fungus growth appear; therefore, the disease becomes difficult to control. Although plants produce some bioactive compounds (e.g., phenols and carotenoids) by action of enzymes (e.g., phenylalanine ammonia lyase) to protect themselves against a fungal attack, these mechanisms are not sufficient to control the disease (Ayón-Reyna et al., 2018). Synthetic chemical fungicides are commonly used to control anthracnose in papaya. Nevertheless, its application for a long time can induce resistance in the pathogen and a reduction in the content of nutrients in the fruits (Vilaplana et al., 2020). These concerns have resulted in a greater effort to explore alternative approaches to control postharvest spoilage, accelerate senescence, and conserve nutrients and other bioactive compounds in harvested papaya (Ayón-Reyna et al., 2017).

Chitosan (Ch) is a biopolymer of amino polysaccharides composed of D-glucosamine units, and it is obtained mainly from exoskeletons of crustaceans such as shrimp (Petriccione et al., 2018). Ch presents antimicrobial and antioxidant properties, extends the shelf life of fruits, and is a promising stimulant of the defense system in fruits tissue (Zhang et al., 2019). In previous studies, the antimicrobial and antioxidant activities of Ch have been related to its molecular weight (MW) (Lima-Oliveira et al., 2018; Vilaplana et al., 2020). Although some studies reported that lower MW produce better antifungal and antioxidant properties, there are some other studies in which this is not fulfilled, such as the case of Guo et al. (2006) who reported that Ch-200 kDa presented better antifungal activity against *Fusarium oxysporum* f. sp. *vasinfectum* than lower MW of Ch (˂200 kDa). Contrary to these results, other publications indicated that Ch-190-375 kDa had a lower inhibitory effect of *Rhizopus stolonifer* than Ch-50-190 kDa (Hernández-Lauzardo et al., 2008). Furthermore, it was reported that a lower MW (3.22 kDa) compared to a Ch-190-310 kDa presented a superior antimicrobial activity in nine pathogenic microorganisms (Kaya et al., 2016). In addition, the effect of Ch with MW ˂100 kDa has been scarcely studied in postharvest quality of fruits. In this sense, the application of Ch with two extremely different MW of 357 and 15 kDa in citrus fruits showed that Ch with the lowest MW favored a greater firmness retention than the fruits treated with Ch with the highest MW and TBZ (0.1%) according to Chien et al. (2007). Regarding the antioxidant activity, Park et al. (2003) and R. Xing et al. (2005) previously reported a greater effect on the antioxidant capacity as measured by the DPPH assay in Ch with MW of 1, 3, 5, 9 and 10 kDa in comparison with a Ch-280 kDa and Ch-760 kDa. However, despite the fact that there are several studies where different molecular weights of Ch are evaluated and classified as low, medium, and high; there is still some confusion on this issue because the classification ranges of the high, medium, and low MW have not been yet standardized. Taking this into consideration and also the reports found about Ch with low molecular weights, we decided to study the effect of different MW (55, 25, and 13 kDa) of Ch, generated by the industry of Sinaloa Mexico, on its in vitro and in vivo antifungal activity, postharvest quality and antioxidant properties in papaya fruits inoculated with *C. gloeosporioides*.

2 | MATERIALS AND METHODS

2.1 | Materials

Papaya fruits cv. Maradol were chosen based on weight (1 kg ± 100 g) and ripening index of 3 (skin slightly orange with green stripe and pulp completely orange) from a local plantation near Culiacan, Sinaloa, Mexico. All fruits were free from physical damage, microorganisms, and pesticides. Commercial Ch-55, Ch-25, and Ch-13 kDa with a degree of deacetylation of 85% were provided by Vepinsa Company (Los Mochis, Sinaloa, Mexico). *Colletotrichum gloeosporioides* (accession number

HM222960.1) was obtained from the Laboratory of Physiology and Postharvest Technology of the Autonomous University of Sinaloa, Mexico, previously isolated and identified by its macroscopic and microscopic characteristics, using molecular analysis (18S ribosomal RNA) (Ayón-Reyna et al., 2017). Thiabendazole (TBZ, Tecto®60) was obtained from Syngenta Crop Protection (Guelph, ON, Canada).

2.2 | Preparation of Ch solutions and films, commercial fungicide, and inoculum of *C. gloeosporioides*

Ch-55, Ch-25, and Ch-13 kDa solutions and films were prepared at a concentration of 1% (w/v) in sterile distilled water with acetic acid at 1% (v/v) and constant stirring for 24 h at room temperature (López-Mora et al., 2013). The solution for the film (20 mL) was poured in a uniform layer of 1 mm thickness onto a polypropylene plate. The optimum conditions for casting method were 45°C for 6 h in a drying oven at ambient relative humidity (RH), obtained by preliminary analysis based in the method reported by Calderón-Castro et al. (2018). The dried film was peeled from the plate and stored in a dust-free chamber at 25°C. TBZ at 0.5 mg/mL was prepared in sterile distilled water (Errampalli et al., 2006). The conidial suspension was prepared using the method described by Ayón-Reyna et al. (2017). Spores were scraped from a 2-week culture, placed in sterile distilled water, and added with 0.5% Tween 80® to prevent spore clumping. Conidial suspension was adjusted to 1×10^6 conidia/mL using a hemacytometer (Neubauer, Improved, Optik Labor, Lancing, UK).

2.3 | Thickness and water vapor permeability of films

Film thickness was measured using a digital micrometer (Mitutoyo Corp., Kawasaki-shi, Japan). Three measurements were taken at random locations along the length of each film, and the mean value was used for calculation. The water vapor permeability (WVP) was determined in accordance with Calderón-Castro et al. (2018). Films were placed on the top of glass flasks containing 15 g of calcium chloride (JT Baker, Center Valley, USA). Subsequently, the flasks were placed in a desiccator (Dry Keeper, Sanplatec Corp., Osaka, Japan) with a saturated solution of sodium chloride to generate a RH of 75%. The weight gain of the glass flasks with calcium chloride was registered every 12 h for 4 days. These procedures were done five times, and the data generated a graph of weight gain versus time. WVP was determined according to the following equation:

$$\mathrm{WVP} = \frac{Mp \times E}{A \times t \times \Delta p}, \quad (1)$$

where Mp is the absorbed moisture mass (g), E is the film thickness (m), A is the exposed film area (m^2), t is the time (s), and "Δ" p is the partial pressure difference through the film (Pa).

2.4 | In vitro antifungal activity

Mycelial growth inhibition (%) of *C. gloeosporioides* was evaluated using the "poisonous potion" technique reported by López-Mora et al. (2013) with some modifications. Potato dextrose agar (PDA) medium was prepared with distilled water and mixed with Ch (1% v/v) of different molecular weights (55, 25, and 13 kDa). Subsequently, it was sterilized and poured into Petri dishes to solidify. Afterward, Petri dishes were inoculated with 1 µL of spore suspension of *C. gloeosporioides* (1×10^6 conidia/mL) and incubated at 25°C. Petri dishes containing PDA and PDA plus TBZ (0.5 mg/mL) were similarly tested as negative and positive controls, respectively. Mycelial growth inhibition was measured until negative control reached the edge of the plate. The percentage of mycelial growth inhibition (%MGI) was calculated using the following formula:

$$\%\mathrm{MGI} = \frac{C - T}{C} \times 100\%, \quad (2)$$

where C is the colony diameter in the control, and T is the colony diameter in treatment.

Germination inhibition (%) was evaluated according to López-Mora et al. (2013). One hundred microliters of the conidial suspension (1×10^6 conidia/mL) was pipetted onto Petri dishes containing PDA (negative control), PDA with TBZ (positive control), and PDA with Ch-55, Ch-25, or Ch-13 kDa. Conidial suspension was spread with a sterile loop, and the dishes were incubated at 25°C for 6 h. Conidial germination was examined using an optical microscope at 40X magnification (Carl Zeiss Photoelectric Microscope, Germany). One hundred conidia were examined, and germination was established by the development of germ tubes. A conidium was considered germinated when the length of its germ tube equaled or exceeded half the length of the conidium. Results were expressed as percentage of germination inhibition (%GI) using the formula described by Ong et al. (2013).

$$\%\mathrm{GI} = 1 - \left(\frac{\text{Number of germinated spores in the treatment}}{\text{Number of germinated spores in the control}}\right) \times 100\%.$$

Fungistatic/fungicidal effect was determined as reported by Bill et al. (2014). Agar discs from the Petri dishes used in the mycelia growth assay, showing no growth of fungi, were transferred to dishes with fresh PDA and incubated for 7 days at 25°C to observe any growth recovery. Fungistatic effect was determined if growth was observed after the incubation period, and fungicidal effect if no growth was observed.

2.5 | Treatment applications for in vivo assays

A total of 125 papayas per replicate were washed, sanitized with 1% (v/v) sodium hypochlorite for 5 min and inoculated by immersion in the conidial suspension (1×10^6 conidia/mL with 0.5% Tween 80®) for 5 min. The fruits were randomly divided into five groups (25 fruits/group) and left to rest for 6 h at 25°C to allow the spores to adhere to the fruit. One group did not receive any treatment and was used as control, another group was treated with TBZ (0.05 % w/v), and the other three groups were treated with 1% (w/v) of Ch-55, Ch-25, and Ch-13 kDa. The application of the treatments was carried out by immersion for 5 min. Then, fruits were stored for 12 days at 25°C and 90%–95% relative humidity (Ayón-Reyna et al., 2015; López-Mora et al., 2013).

2.6 | In vivo antifungal activity

The effect of treatments on the disease incidence was evaluated daily in 10 fruits based on the anthracnose symptoms on epidermal surface of the fruits. It was expressed as a percentage of fruits that showed anthracnose symptoms of the total fruits of each treatment, according to Ayón-Reyna et al. (2017) using the following formula:

$$\text{Antracnose incidence } (\%) = \frac{\text{Number of infected fruits}}{\text{Total inoculated fruits}} \times 100\%.$$

Anthracnose severity was visually evaluated every 3 days according to Madani et al. (2016). It was reported as percentage of the fruits surface with anthracnose symptoms.

2.7 | Postharvest quality analysis

Weight loss was determined every 2 days by weighing 10 fruits from each treatment using a weighing scale (Sartorius TE 4101, Goettingen, Germany), and the values were expressed as percentage of weight loss in relation to the initial weight (Jongsri et al., 2016). Firmness of the pulp was evaluated using a digital penetrometer (Chatillon DFE 100; AMETEK Inc., Largo, FL, USA) by penetration of an 11 mm diameter flat tip, at a depth of 5 mm with a speed of 50 mm/min. The firmness was expressed in Newtons, and 15 fruits per treatment were used (Ayón-Reyna et al., 2015). External color was measured in the equatorial region of the fruits using a CR-200 colorimeter (Minolta Co. Ltd., Osaka, Japan), and CIELAB color parameters (L^* and angle H) were recorded as the means of nine measurements per treatment. Total soluble solids (TSS) were determined using a manual refractometer (Fisherbrand by Fisher Scientific S66366, Ltd., Nepean, Ontario, Canada) placing mesocarp juice directly in the refractometer, and the results were expressed as °Brix.

2.8 | Bioactive compounds

Ascorbic acid content was determined according to López-Valenzuela et al. (2011). For extraction, 20 g frozen papaya was homogenized with 100 mL of degassed and cold deionized water using a commercial blender (Osterizer; Jarden Corp., Rye, NY, USA) and filtered through three-organza cloth. A second filtration was done using 0.45-µm disposable filters (Pall Corp., Port Washington, NY, USA) followed by Sep-Pak C18 cartridges (Waters Corp., Milford, Mass, USA). A 1 mL aliquot was analyzed using a 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a Sphereclone ODS2 column (250 mm × 4.6 mm × 5 µm, Phenomenex, USA) operated at 16°C. The mobile phase used was monobasic potassium phosphate (25 mmol/L), and the flow rate was 0.7 mL/min. The absorbance was determined at 254 nm during 15 min with an injection of 10 µL. Ascorbic acid content was calculated using a curve obtained with a commercial standard (Sigma–Aldrich Co., St Louis, Mo, USA; CAT. A5960), and the results were expressed as milligrams of ascorbic acid per 100 g in fresh weight basis (mg AA/100 g FW).

For the determination of total phenolics, methanolic extracts (ME) were obtained following the methodology described by Moo-Huchin et al. (2014). Frozen papaya pulp (0.5 g) was mixed with 10 mL of methanol for 1 min using an Ultra-Turrax (Model IKA T18 basic), sonicated for 30 min and centrifuged at 277 × *g* for 10 min at 4°C. The supernatant was recovered, and the pellet was re-extracted with methanol as previously described. The two supernatants were mixed, evaporated, and later 4 mL of methanol was added as final volume. Later, 40 µL of ME diluted in methanol (1:10 v/v) was oxidized with 360 µL of Folin–Ciocalteu reagent (1:8 v/v) along with 100 µL Na_2CO_3 (7% w/v) for 90 min (21°C) in darkness. The absorbance was measured at 765 nm using a microplate reader (Synergy HT; BioTek Instruments, Winooski, VT,

USA). A standard calibration curve was prepared using gallic acid (GA) (50–600 μg/μL), and the results were expressed as milligrams gallic acid equivalent per 100 g FW (mg GAE/100 g FW).

2.9 | Antioxidant capacity

ABTS (acid 2,2′-azino-bis (3-ethylbenzothiazolin-6-sulfonic)) method was evaluated by $ABTS^{+}$ discoloration analysis according to Re et al. (1999) with some modifications. Stock solution of $ABTS^{\bullet+}$ was prepared by mixing 5 mL of ABTS (7 mM) with 88 μL of potassium persulfate (140 mM), which was stored for 12–16 h at 25°C in darkness. ABTS solution was diluted with 7 mM phosphate buffer solution (PBS) (pH 7.4) until an absorbance of 0.75 ± 0.02 was obtained at 734 nm. Three microliters of sample diluted in methanol (1:18 w/v) was mixed with 197 μL of ABTS solution and incubated for 30 min at 27°C in darkness, and the absorbance was measured at 734 nm in a microplate reader (SynergyTM HT Multi-Detection; Biotek, Inc., Winooski, VT, USA). Trolox was used as standard for the calibration curve (0–225 μg/m), and the antioxidant capacity was expressed in trolox equivalents (TE) (μmol TE/100 g FW).

DPPH (2,2-diphenyl-1-picrilhydrazyl) assay was performed according to Brand-Williams et al. (1995) with some modifications. An aliquot (20 μL) of the diluted ME (1:2) was mixed with 180 μL of the DPPH radical in 150 mM methanol and incubated for 30 min (27°C/darkness), and the absorbance was measured at 550 nm in a microplate reader (SynergyTM HT Multi-Detection; Biotek, Inc.). A calibration curve of Trolox (0–225 μg/mL) was used, and antioxidant activity was reported as μmol TE/100 g FW.

2.10 | Statistical analysis

Completely randomized studies of one factor (treatments) and two factors (treatments and storage time) were carried out to analyze in vitro and in vivo responses, respectively. One papaya fruit was used as experimental unit, and three replicates with three repetitions per treatment were used. Data were analyzed with ANOVA ($p \leq 0.05$) to determine significant differences between the treatments/days of storage or the samples according to the p-value ≤ 0.05, using Statgraphic Plus, version 5.1 (Manugistic Inc., Rockville, MD, USA). Mean values were compared using Fisher's least significant difference (LSD) test with $\alpha = 0.05$. Residue analysis was made to verify data normal distribution and homoscedasticity (equality of variances). Data normality was verified with the Kolmogorov–Smirnov test, while the equality of variances was verified with the Bartlett test, using $\alpha = 0.05$. Response data that did not meet the assumptions of normality and homoscedasticity were analyzed using Kruskal–Wallis/Dunn tests, with $\alpha = 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Thickness and water vapor permeability of films

The thickness of the films was similar in the different molecular weights of Ch (0.1 mm). In general, results showed that WVP increased as Ch MW decreased. In this study, the minimum value of WVP was 1.10×10^{-11} g m Pa^{-1} s^{-1} m^{-2}, which corresponded to Ch-55 kDa. Otherwise, Ch-25 and Ch-13 kDa obtained WVP values of 1.19×10^{-11} and 1.30×10^{-11} g m Pa^{-1} s^{-1} m^{-2}, respectively. Our results were similar to those reported by Liu et al. (2020) who found values of 1.46 to 2.08×10^{-12} g $cm/cm^2 \bullet sPa$ when used 7, 50 and 110 kDa molecular weights; Ch-110 kDa films exhibited lower WVP values than Ch-50 and Ch-7 kDa films. Because the Ch films with higher molecular weight present more hydrophilic domains than Ch with lower molecular weight, the water vapor is more easily absorbed, and the diffusion step is substantially improved.

3.2 | In vitro antifungal activity

Ch-55, Ch-25, and Ch-13 kDa inhibited 100% of radial mycelial growth of the fungus *C. gloeosporioides* showing greater effect than TBZ (71%) (Table 1, Figure 1). In fact, the activity of Ch against fungal mycelial growth has been widely demonstrated in previous reports under different molecular weights. Growth of *R. stolonifer* was completely inhibited by Ch-30.7, Ch-23.8, and Ch-17.4 kDa according to Guerra-Sánchez et al. (2009). In this study, *C. gloeosporioides* developed resistance to TBZ. Resistance to TBZ can be influenced by genetic factors, such as initial frequency of resistance alleles, and number and expressiveness of genes according to those reported by Bill et al. (2014). These authors mentioned resistance to the fungicide prochloraz in *C. gloeosporioides*, and similarly the application of Ch showed greater inhibition than the fungicide. In contrast, Ch forms an external barrier in microorganisms that chelate metals and cause the suppression of essential nutrients for microbial growth. The metal-binding capacity is related to the amino groups of the biopolymer, and it is likely that all events occur simultaneously in any Ch regardless of MW (Fernández-de Castro et al., 2016). Furthermore, it has also been suggested that Ch oligomers can

TABLE 1 Effect of different chitosan (Ch) molecular weights on the inhibition of radial mycelial growth and conidial germination of *Colletotrichum gloeosporioides* and fungicidal/fungistatic effect.

Treatments	Mycelial growth inhibition (%)	Conidial germination inhibition (%)	Fungicidal/fungistatic effect (cm)
Control	0[b]	0.0[b]	8[a]
TBZ	71[b]	80.3[b]	0[b]
Ch of 55 kDa	100[a]	92.1[a]	0[b]
Ch of 25 kDa	100[a]	92.2[a]	0[b]
Ch of 13 kDa	100[a]	93.1[a]	0[b]

Note: Values represent the median of nine replicates. Comparisons between treatments were carried out separately using Kruskal–Wallis/Dunn tests. Different letters indicate significant differences ($p < 0.05$). Thiabendazole (TBZ) was used as positive control.

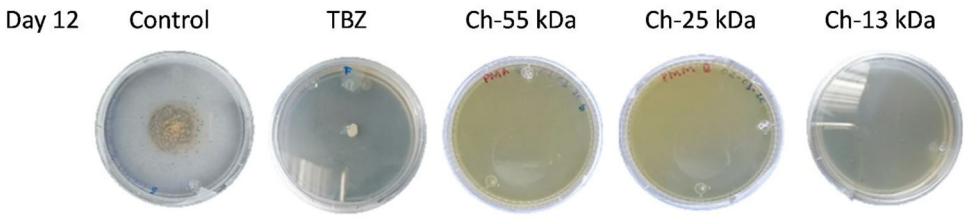

FIGURE 1 Representative images of Petri dishes inoculated with *Colletotrichum gloeosporioides* conidia treated with chitosan (Ch) of different molecular weights showing mycelial growth after 12 days of inoculation at 28°C.

penetrate microbial cells and affect RNA transcription and protein synthesis (Jovanovic et al., 2016).

Immersion in Ch had an effect on conidial germination, presenting values of about 92%–93% of inhibition in the three molecular weights, all of them with greater effect than TBZ which reached 80% (Table 1). Bill et al. (2014) previously reported a greater inhibition of conidial germination of *C. gloeosporioides* treated with Ch than those treated with the fungicide prochloraz. Likewise, when conidia of *R. stolonifer* were treated with a Ch-30.7 kDa, germination was totally inhibited in comparison with Ch-23.8 and 17.4 kDa because it caused the highest change in structure of conidia (Hernández-Lauzardo et al., 2008). Meanwhile, Badawy and Rabea (2009) evaluated four molecular weights (290, 57, 37, and 5 kDa) and found that the antifungal activity against *Botrytis cinerea* increased as the MW decreased. According to Peralta-Ruiz et al. (2020), the effect of Ch, regardless of MW, on the inhibition of the fungus development is related to structural changes of the spores, such as wilting, breakage, loss of cellular material, and deepening of the ridges. Besides, the interaction between the positive charges of the biopolymer and the negatively charged residues of the macromolecules of the fungi generates polyelectrolyte complexes that damage the function of the fungus.

Regarding the fungistatic/fungicidal effect, all Ch (55, 25, 13 kDa) showed a fungicidal effect against the development of *C. gloeosporioides*. During the incubation period, no growth was observed in the dishes, which evidenced that the treatments exerted a fungicidal effect and did not allow the development of the fungus even when the necessary conditions and nutrients were provided, even at the same level as the TBZ (Table 1). The results obtained in this investigation were similar to those reported by Sergey et al. (2014) who found that a Ch-70 kDa had antimicrobial activity toward *Candida cruisei* and *Candida glabrata*. The fungicidal effect of Ch of different molecular weights observed in this research indicates that Ch is killing the fungus. It is believed that differences in MW lead to two different mechanisms of interaction of Ch and microorganisms: a high MW of Ch can inflict at the cell wall level, covering cell walls, weakening the membrane, rupturing and allowing cell leakage, while a low MW of Ch can penetrate living cells, leading to inhibition of various enzymes and disruption of protein synthesis, interacting with mRNA synthesis (Guo et al., 2006).

3.3 | In vivo antifungal activity

First anthracnose symptoms appeared in control fruits at day 2 of storage, while they appeared in TBZ- and Ch-25 kDa-treated fruits at day 3 (Figure 2). The application of Ch-55 and Ch-13 kDa delayed the onset of anthracnose symptoms in papaya for 4 days. Anthracnose incidence increased over time for all treatments and reached a maximum of 100% in the control fruits and fruits treated with TBZ at day 7 of storage, while in fruit treated with Ch-25, Ch-13 and Ch-55 kDa, the maximum damage

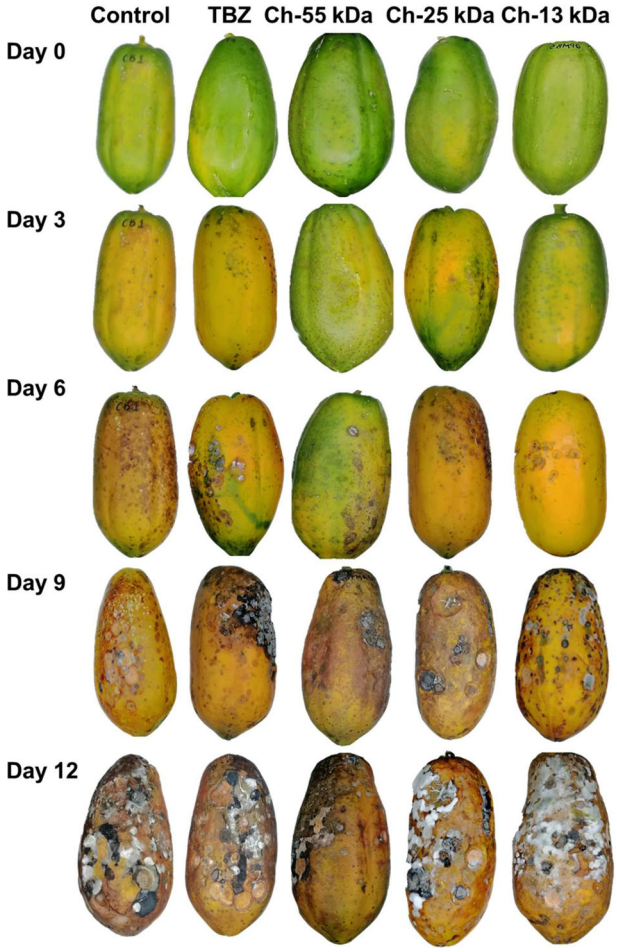

FIGURE 2 Representative images of papaya fruit treated with chitosan (Ch) of different molecular weights (55, 25, and 13 kDa) and thiabendazole (TBZ) showing anthracnose symptoms on the surface during 12 days of storage at 25°C.

percentage was reached at 8, 9, and 12 days, respectively (Figure 3a). Besides, the fruits with the largest area affected by anthracnose from the beginning to the end of storage were those corresponding to the control, while Ch-55 kDa-treated papaya presented the lowest anthracnose severity (Figure 3b). In the meantime, Ch-25- and Ch-13 kDa-treated fruits showed less damage of anthracnose than control and TBZ-treated fruits. In general, it was observed that Ch-55 kDa was the most effective in delaying the onset of symptoms and slowing down the progress of the disease in papaya. The abovementioned may be due to the fact that Ch-55 kDa had the ability to alter cell permeability that includes its deposition onto the pathogen cell surface, and consequent creation of an impermeable polymeric layer that prevents the uptake of nutrients in the cell, and in the meantime changes of the metabolite excretion in the extracellular matrix (K. Xing et al., 2015). Furthermore, in this study, fruits infected with *C. gloeosporioides* and coated with Ch revealed greater activity and antioxidant capacity (ascorbic acid, phenolic, ABTS, and DPPH) than fruits bathed in TBZ. It was evident that the application of Ch of different weights improved defense response activities. In their reports, Chien et al. (2007) and Lima-Oliveira et al. (2018) mentioned that Ch-treated papaya and citrus fruits (15 kDa) showed lower anthracnose incidence and severity caused by green, blue, gray mold in comparison with the commercial fungicides TBZ (0.1%), methyl thiophanate (10 μg/mL), and diphenoconazole (0.5 μg/mL). Meanwhile, the efficiency of Ch was evidenced by Younes et al. (2014) who mentioned that the changes in microorganisms were observed mainly in Ch-135 to Ch-42 kDa, due to a higher adsorption on the cell surface that could form a polymeric membrane, which inhibited the entry of nutrients into the cell compared to a low MW.

3.4 | Postharvest quality analysis

Weight loss increased during storage in all treatments, without showing significant differences among treatments until the end of this period, where control fruits presented the greatest loss followed by TBZ, while Ch-55 kDa presented the lowest loss (Table 2). Ch possesses barrier properties that led to a reduction in the loss of water vapor. This was in full agreement with a previous report by Chien et al. (2007) where Ch-15 kDa prevented the weight loss of citrus fruits compared to TBZ-treated fruits (0.1%). In addition, Jongsri et al. (2016) and Zhang et al. (2019) analyzed different molecular weights from 30 to 1390 kDa in some fruits observing that a lower permeability to water vapor is related to higher molecular weights; due to a more compact and homogeneous structure associated to chains with a higher degree of polymerization in the biopolymer. Likewise, changes in MW would provide changes in biofilm hydrophilicity and crystallinity, and consequently, changes in water vapor permeability and mechanical properties of the biofilm obtained from Ch (de Moura et al., 2011). It is interesting to mention that there is no information about the effect of low MW Ch in the control of water loss in fruits. So, this research suggests that, at least in papaya, molecular weights in the range of 13–55 kDa are sufficient to act as a barrier to water vapor. Our results confirmed this because the highest MW was the most effective to reduce water loss.

At the beginning of the storage, firmness values in the fruit were about 73 N, which decreased as the time progressed. At the end of the storage, firmness was higher in fruit treated with Ch-55 kDa followed by fruit treated with Ch-25, Ch-13 kDa, and TBZ; however, there were no significant differences among these last treatments, while control fruits had the greatest loss of firmness with a decrease of 11% (Table 2). This could be attributed to that Ch contributes to preserve the pectin-binding bridges and

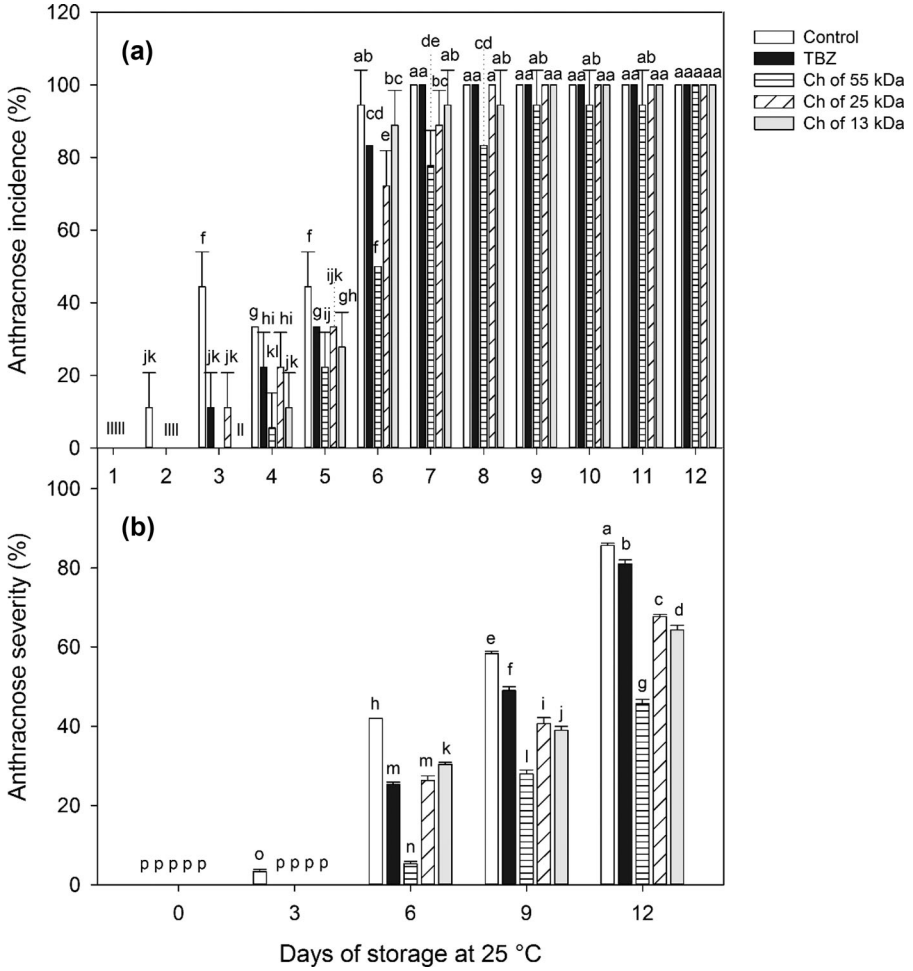

FIGURE 3 Effect of different chitosan (Ch) molecular weights on the incidence (a) and severity (b) of anthracnose of papaya infected with *Colletotrichum gloeosporioides* and stored at 25°C for 12 days. Thiabendazole (TBZ) was used as positive control. Vertical bars on columns represent the standard deviation of the means for three replicates. Different letters indicate significant differences among treatments and storage of days ($p < 0.05$). Kolmogorov–Smirnov test was used to verify data normality (p-values for incidence and severity greater than 0.05 indicated that the data follows the normal distribution), while Bartlett test was used to verify the equality of variances (p-values for incidence and severity greater than 0.05 indicated that the variances of data were equal). p-Value of analysis of variance (ANOVA) indicated the significance of the proved complete model.

cellulose distribution in the cell wall preventing its degradation, thus maintaining the turgor of the papaya fruit. These results confirm previous reports demonstrating that the highest MW preserved greater firmness compared to the lowest molecular weights of Ch in kiwi (Drevinskas et al., 2017), mango (Jongsri et al., 2016), and nectarine fruits (Zhang et al., 2019). For this part, Chien et al. (2007) observed that Ch-15 kDa in citrus fruits presented greater firmness than TBZ (0.1%).

Skin lightness (L^* value) and hue angle (H°) have been used as indicators of postharvest quality in papaya (Ayón-Reyna et al., 2015). In general, L^* values increase and H° decreases when fruit reach ripening. The immersion of papaya in any Ch treatment resulted in higher L^* values compared with the control fruits. At the end of storage at 25°C, Ch-coatings were all equally effective in preserving L^*, which indicates a lower darkening caused by senescence and the development of anthracnose in the fruit (Table 2). With respect to H°, the use of Ch-25 and Ch-13 kDa induced a minor color change compared to the control and TBZ. Nevertheless, among Ch treatments, Ch-55 kDa presented the lowest change in H° (Table 2). The implementation of Ch delayed the color changes in the rind of papaya and mango compared to untreated fruit and fruit treated with a formulation of fungicides (trifloxystrobin and tebuconazole) as reported by Jongsri et al. (2016) and Gomes et al. (2020). These authors indicated that Ch modified the internal atmosphere in the fruits, which caused a delay in the chlorophyll degradation process and a reduction in the activity of polyphenol oxidase, thus slowing down the ripening process and maintaining the color of the fruits.

TABLE 2 Effect of different chitosan (Ch) molecular weights on the weight loss, firmness, external color (L^* and Hue), and total soluble solids of papaya infected with *Colletotrichum gloeosporioides* and stored at 25°C for 12 days.

Treatments	Postharvest quality analysis				
	Weight loss (%)	Firmness (N)	External color (L^*)	External color Hue (H°)	Total soluble solids (°Brix)
		Day 0 of storage at 25°C			
Control	0.00 ± 0.0^{o}	73.23 ± 1.6^{a}	42.15 ± 1.3^{j}	131.43 ± 0.8^{a}	12.07 ± 0.6^{fgh}
TBZ	0.00 ± 0.0^{o}	73.11 ± 1.6^{a}	42.18 ± 1.1^{j}	131.99 ± 1.0^{a}	12.03 ± 0.6^{fgh}
Ch of 55 kDa	0.00 ± 0.0^{o}	72.98 ± 1.6^{a}	42.20 ± 1.3^{j}	131.23 ± 0.9^{a}	11.99 ± 0.6^{ghij}
Ch of 25 kDa	0.00 ± 0.0^{o}	73.00 ± 1.5^{a}	42.19 ± 1.2^{j}	131.96 ± 1.0^{a}	11.95 ± 0.6^{ghi}
Ch of 13 kDa	0.00 ± 0.0^{o}	73.23 ± 1.6^{a}	42.17 ± 1.3^{j}	131.32 ± 0.9^{a}	11.93 ± 0.6^{ghi}
		Day 3 of storage at 25°C			
Control	2.63 ± 0.9^{fg}	41.84 ± 1.8^{e}	55.23 ± 1.8^{f}	102.58 ± 1.8^{e}	12.40 ± 0.1^{efg}
TBZ	1.69 ± 0.6^{h}	41.76 ± 1.3^{e}	49.21 ± 1.4^{h}	112.00 ± 1.3^{c}	12.50 ± 0.1^{efg}
Ch of 55 kDa	1.33 ± 0.6^{gh}	66.07 ± 1.5^{b}	45.41 ± 0.9^{i}	124.98 ± 1.0^{b}	11.90 ± 0.1^{ghi}
Ch of 25 kDa	1.05 ± 0.4^{h}	63.54 ± 1.7^{c}	42.61 ± 1.2^{j}	125.23 ± 3.2^{b}	12.70 ± 0.1^{def}
Ch of 13 kDa	1.27 ± 1.2^{gh}	44.38 ± 1.9^{d}	51.64 ± 1.3^{g}	108.18 ± 1.1^{d}	12.85 ± 0.2^{cde}
		Day 6 of storage at 25°C			
Control	6.19 ± 1.0^{cd}	19.93 ± 1.5^{i}	61.47 ± 1.0^{d}	82.36 ± 1.1^{j}	13.33 ± 0.5^{bcd}
TBZ	5.16 ± 0.6^{de}	22.01 ± 0.9^{h}	60.87 ± 1.0^{d}	87.90 ± 1.4^{h}	13.33 ± 0.5^{bcd}
Ch of 55 kDa	5.02 ± 0.5^{e}	27.98 ± 1.0^{f}	56.33 ± 1.1^{f}	111.15 ± 1.3^{c}	11.16 ± 0.2^{j}
Ch of 25 kDa	4.84 ± 0.7^{f}	26.86 ± 2.1^{f}	57.34 ± 0.9^{e}	96.55 ± 1.0^{fg}	12.66 ± 0.7^{ef}
Ch of 13 kDa	5.03 ± 1.2^{e}	25.85 ± 1.4^{g}	56.91 ± 1.7^{f}	82.05 ± 1.0^{j}	12.83 ± 0.6^{de}
		Day 9 of storage at 25°C			
Control	8.31 ± 1.6^{b}	18.01 ± 1.3^{m}	61.89 ± 0.9^{d}	79.01 ± 0.6^{k}	13.25 ± 0.2^{bcd}
TBZ	8.10 ± 0.7^{bc}	19.61 ± 1.4^{i}	63.40 ± 0.9^{b}	81.36 ± 1.5^{j}	13.80 ± 0.6^{ab}
Ch of 55 kDa	7.72 ± 0.5^{c}	19.92 ± 0.9^{i}	65.66 ± 1.5^{a}	97.38 ± 1.4^{f}	11.33 ± 0.2^{ij}
Ch of 25 kDa	7.68 ± 0.5^{cd}	19.82 ± 2.1^{i}	64.23 ± 1.3^{a}	85.41 ± 1.0^{i}	12.83 ± 0.6^{de}
Ch of 13 kDa	7.68 ± 1.2^{c}	18.27 ± 1.4^{j}	64.49 ± 0.9^{a}	84.96 ± 0.3^{i}	14.00 ± 0.0^{a}
		Day 12 of storage at 25°C			
Control	11.80 ± 0.9^{a}	8.00 ± 0.6^{m}	61.89 ± 1.0^{c}	76.25 ± 1.5^{l}	14.00 ± 0.4^{a}
TBZ	11.24 ± 0.7^{a}	10.36 ± 0.9^{l}	64.83 ± 1.1^{a}	81.38 ± 1.5^{j}	13.75 ± 0.4^{ab}
Ch of 55 kDa	09.82 ± 0.5^{a}	13.58 ± 1.0^{k}	63.87 ± 1.5^{ab}	95.25 ± 0.7^{g}	11.50 ± 0.5^{hij}
Ch of 25 kDa	10.45 ± 0.7^{a}	10.90 ± 2.1^{l}	64.41 ± 1.3^{a}	74.91 ± 1.4^{l}	13.25 ± 0.2^{bcd}
Ch of 13 kDa	10.57 ± 1.3^{a}	10.76 ± 1.0^{l}	64.81 ± 0.7^{a}	75.10 ± 0.9^{l}	13.50 ± 0.4^{abc}
p-Value (Kolmogorov–Smirnov)	>0.150	>0.15	0.062	>0.150	>0.150
p-Value (Bartlett)	0.265	0.564	0.777	0.103	0.219
p-Value (ANOVA)	0.000	0.000	0.000	0.000	0.000

Note: Values represent the mean of three replicates ± standard deviation. Means in the same column with different letters are significantly different ($p < 0.05$). Thiabendazole (TBZ) was used as positive control. Kolmogorov–Smirnov test was used to verify data normality (*p*-value >0.05 indicated that the data follow the normal distribution), while Bartlett test was used to verify equality of variances (*p*-value >0.05 indicated that the data variances were equal). *p*-Value of analysis of variance (ANOVA) indicates the significance of the proved complete model.

The initial content of TSS was approximately 12 °Brix and increased until reach values of about 14 °Brix in control fruit at the end of the storage without showing significant differences with TBZ. Ch-25- and Ch-13 kDa-treated fruits presented a slower increase compared to control fruits, while fruits covered with Ch-55 kDa kept the TSS constant during the 12 days of storage; thus, this treatment was the one that was able to retard the synthesis of sugars in the fruit, generating significant differences with the rest of the treatments (Table 2). This effect could be due to the fact that Ch-55 kDa formed a thicker and dense film, blocking pores on the surface and reducing respiration rate. In addition, Ch-55 kDa has been shown to have high elasticity, resistance to deformation, and strong

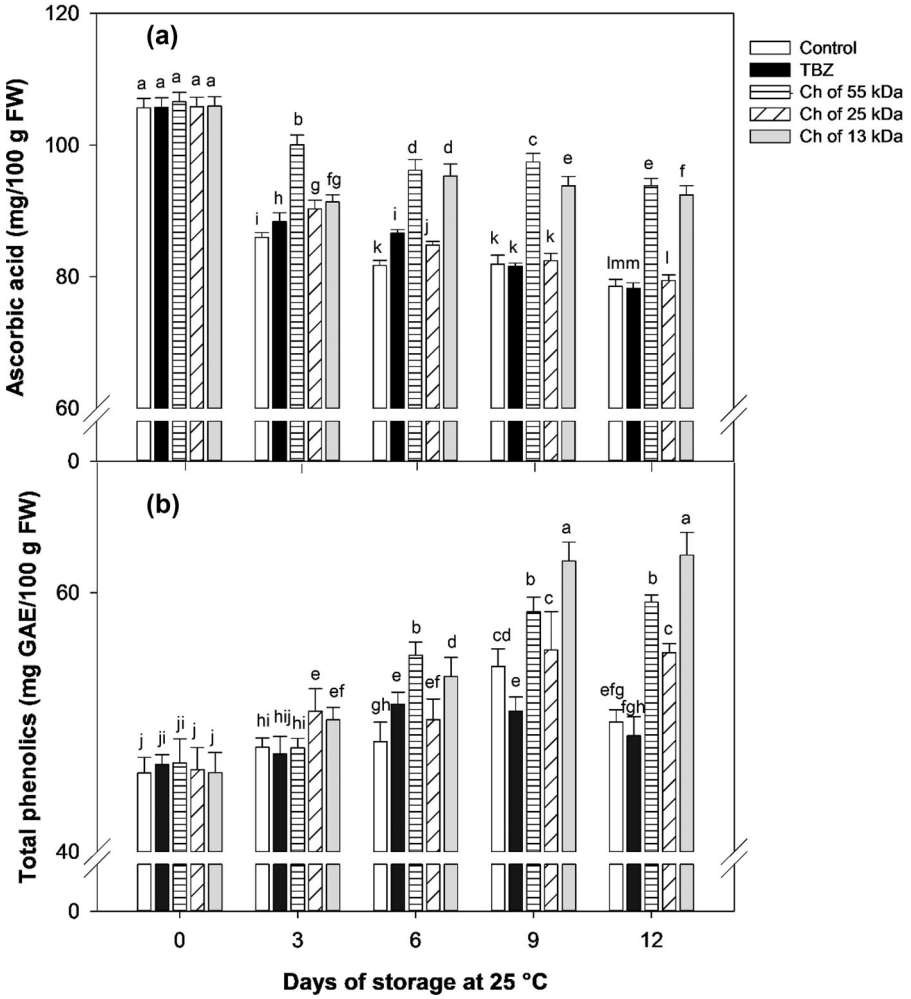

FIGURE 4 Effect of different chitosan (Ch) molecular weights on ascorbic acid content (a) and total phenolics (b) of papayas infected with *Colletotrichum gloeosporioides* and stored at 25°C for 12 days. Thiabendazole (TBZ) was used as positive control. Vertical bars on columns represent the standard deviation of the means for three replicates. Different letters indicate significant differences among treatments and storage of days ($p < 0.05$). Kolmogorov–Smirnov test was used to verify data normality (p-values for incidence and severity greater than 0.05 indicated that the data follow the normal distribution), while Bartlett test was used to verify equality of variances (p-values for incidence and severity greater than 0.05 indicated that the variances of data were equals). p-Value of analysis of variance (ANOVA) indicated the significance of the proved complete model.

matrices due to the fact that it has chains with a high degree of polymerization (Moura et al., 2011). The polymer–polymer interaction favors modifying the crystallinity of the polymer, and consequently, retaining the loss of firmness, color change, and degradation of the total content of soluble solids, which can be noted in this research. These same effects were reported by Jongsri et al. (2016) and Zhang et al. (2019) in mango and nectarine fruits treated with Ch-40, Ch-30, and 6.7 kDa, which presented low content of sugars compared to control fruit.

3.5 | Bioactive compounds

Ascorbic acid content tended to decrease during storage from 106 mg/100 g FW at the beginning until 93 mg/100 g FW for Ch-55 kDa and 78 mg/100 g FW for TBZ at the end of the storage, values which corresponded to the highest and lowest contents, respectively (Figure 4a). The Ch-coated fruits presented higher ascorbic acid content compared to untreated fruit and those treated with TBZ. Within the Ch-based treatments, Ch-55 kDa had the highest ascorbic acid content followed by Ch-13 and Ch-25 kDa. The decrease of ascorbic acid content could be due to the action of ascorbic acid oxidase, which converts ascorbic acid to dehydroascorbic acid in postharvest senescence (Suseno et al., 2014). In the study carried out by Chien et al. (2007), it was pointed out that citrus fruit treated with Ch-15 kDa presented higher content of ascorbic acid than fruit treated with Ch-357 kDa and TBZ (0.1%). Nevertheless, Jongsri et al. (2016) reported that both Ch-360 and Ch-40 kDa coating delayed the loss of the ascorbic acid content of the

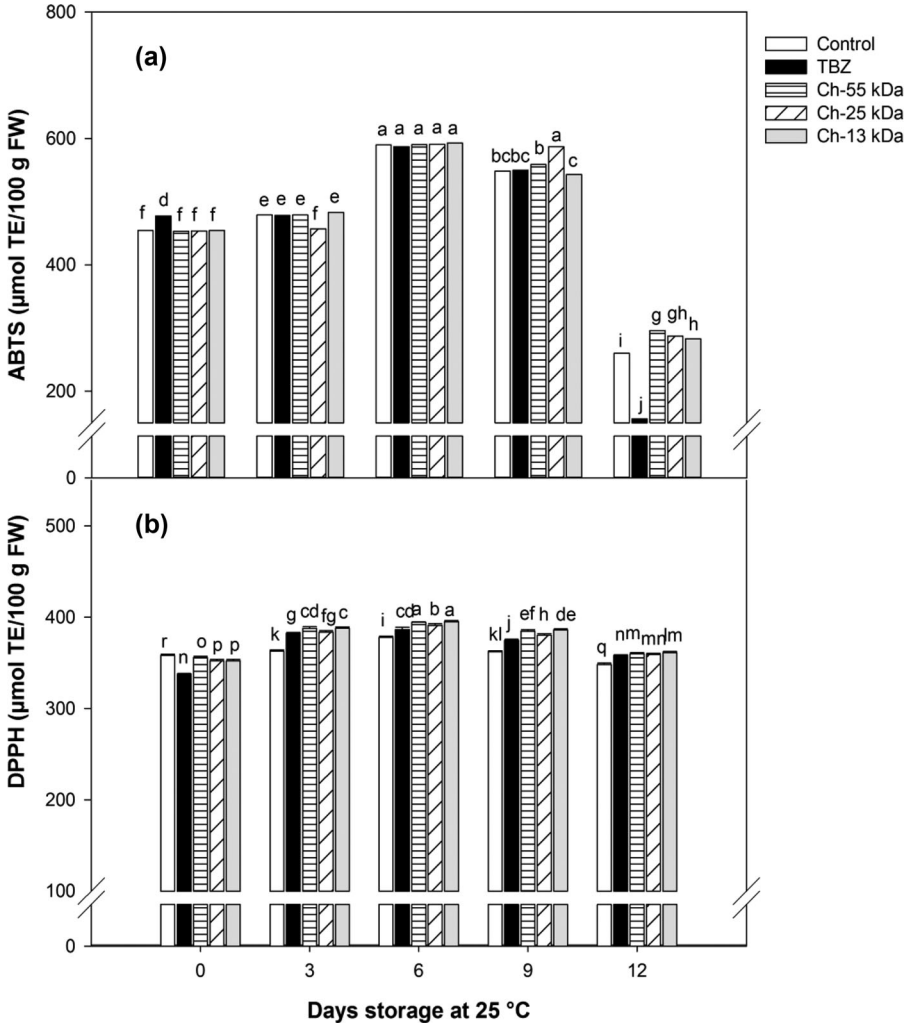

FIGURE 5 Effect of different chitosan (Ch) molecular weights on the antioxidant capacity measured by ABTS (a) and DPPH (b) methods of papayas infected with *Colletotrichum gloeosporioides* and stored at 25°C for 12 days. Thiabendazole (TBZ) was used as positive control. Vertical bars on columns represent the standard deviation of the means for three replicates. Different letters indicate significant differences among treatments and storage of days ($p < 0.05$). Kolmogorov–Smirnov test was used to verify data normality (p-values for incidence and severity greater than 0.05 indicated that the data follow the normal distribution), while Bartlett test was used to verify equality of variances (p-values for incidence and severity greater than 0.05 indicated that the variances of data were equal). p-Value of analysis of variance (ANOVA) indicated the significance of the proved complete model.

mango fruit during storage. In another study, Ch-253.9 kDa berries had a higher ascorbic acid content than Ch-6.7 kDa and uncoated berries (Drevinskas et al., 2017). The incorporation of high molecular weights Ch as a coating can reduce O_2 diffusion and slow down ripening rate and, consequently, maintain the ascorbic acid content and delay the senescence of berries favored by the presence of O_2. Yan et al. (2001) pointed that the thickness of the film has a relationship with the MW because they found that a coverage of Ch-100 kDa was less thin and transparent than those of Ch-53.3 and Ch-13 kDa. The thickness of Ch-55 kDa film could be related to a lower permeability, which could reduce the passage of oxygen and prevent the oxidation of ascorbic acid in the fruit.

During fruit storage, the content of total phenolics tended to increase, registering a range value for all the treatments of 46 to 69 mg GAE/100 g FW. At the end of the storage, fruit treated with Ch-13 kDa presented the highest total phenolics content followed by Ch-55 and Ch-25 kDa, while control fruit and those treated with TBZ had the lowest values of total phenolics (Figure 4b). The results of this research were similar to those reported by Drevinskas et al. (2017) who mentioned that Ch-13 kDa promotes the synthesis of total phenolics in berries. Badawy and Rabea (2009) observed a high content of total phenolics in treatments with Ch-290, Ch-57, and Ch-37 kDa in comparison with the lowest MW Ch of 5 kDa, which induced the smallest increase in the total content of phenolic in

tomato. On the other hand, in a previous report, it was observed that the fruit expressed a rapid accumulation of phenols as a response of the defense mechanism after infection by a pathogen (Ayón-Reyna et al., 2018). Their results coincide with the results obtained in the present study because phenolic content slightly increased at day 6 when the anthracnose symptoms intensified; and also, fruit coated with the different molecular weights Ch had lower anthracnose development and higher phenolic compounds. According to Locateli et al. (2019), the latter could be related to a double stimulus: that of the natural defense of the fruit and that induced by Ch on the production of phenols.

3.6 | Antioxidant capacity

The antioxidant capacity determined by the ABTS assay increased in all treatments until day 6 with an approximate value of 590 μmol TE/100 g FW, followed by a slight decrease at day 9 and a drastic decrease at day 12 (Figure 5a). On the last day, Ch-treated fruit of 55 kDa had a significant slightly higher value than the one of Ch-13 kDa-treated papayas. On the other hand, the TBZ-treated fruit showed the lowest values of antioxidant capacity followed by control fruit. A similar pattern of changes observed in all treatments with Ch in the ABTS assay was shown in the DPPH technique. The results indicated that the antioxidant capacity measured by the DPPH free radical scavenging test increased until day 6 (395 μmol TE/100 g FW), decreasing during the rest of the storage (Figure 5b). Likewise, there was a significant difference between the control and TBZ versus Ch of different molecular weights, where the highest values corresponded to Ch-treated fruit, without differences among them. In Ch-55- and Ch-13 kDa-treated fruit, DPPH was continuously significantly higher than the rest of the treatments on days 3, 6 and 9. These results were similar to those reported by Zhang et al. (2019) in nectarine fruit, where Ch-30 kDa was applied and promoted a greater antioxidant capacity through the ABTS and DPPH free radical elimination assays. For their part, Jung and Zhao (2012) point out that Ch-22-30 kDa was significantly higher in DPPH radical scavenging activity compared to Ch samples of 4–5 kDa. Therefore, Ch can act as an inducer molecule and interact with membrane receptors, triggering an increase in the antioxidant activity of the host (fruit) and inhibiting the growth of a series of pathogenic fungi (Benhamou, 1996). In general, in this study, it was observed that Ch-55 and Ch-13 kDa participated in the defense mechanisms because papaya fruits treated with these treatments had the lowest fungal development and the highest contents of ascorbic acid and phenolic compounds, as well as the highest ABTS and DPPH activities. In the literature, it is pointed out that Ch-coatings (300 and 30 kDa) have the potential to control disease severity in inoculated mango by inhibiting fungal decay caused by *C. gloeosporioides* conducing defense mechanisms such as H_2O_2, total phenolic content, chitinase, β 1,3-glucanase, and peroxidase activities (Jung & Zhao, 2012). Similar to our findings, there are reports that indicate Ch can act as an inducer, promoting the activation of the defense system of fruits because the biopolymer is made up of oligochitins and oligochitosans (structural components of phytopathogens), and the difference between microorganism and Ch is not recognized. Thus, the fruit defense system considers it an attack by a foreign agent triggering responses from the defense system during storage of fruits (Ngo & Kim, 2015).

4 | CONCLUSION

The three molecular weights of Ch were effective to control the development of *C. gloeosporioides*, whereas Ch-55 kDa had the highest effect delaying the incidence and severity of anthracnose in papaya. In addition, fruit treated with this Ch MW showed better postharvest quality than the others Ch molecular weights. Both molecular weights of 55 and 13 kDa increased the content of bioactive compounds and antioxidant capacity of anthracnose-infected papayas. These findings suggest that Ch-55 kDa can be applied to papayas to control the development of *C. gloeosporioides* during storage, improve postharvest quality, and act as a molecular inducer of the non-enzymatic antioxidant system, improving the redox state in the fruit. For future studies, it is suggested to evaluate other characterization parameters, such as gas (O_2, CO_2) permeability, of the film and the respiratory behavior of the covered fruit.

AUTHOR CONTRIBUTIONS

Blanca Alicia López-Zazueta: Conceptualization; Formal analysis; Investigation; Validation; Writing – original draft. **Lidia Elena Ayón-Reyna**: Conceptualization; Methodology; Resources; Writing – review & editing. **Roberto Gutiérrez-Dorado**: Supervision; Validation; Writing – review & editing. **Fernando Arturo Rodríguez-Gómez**: Resources; Validation. **Martha Edith López-López**: Resources; Validation. **Jordi Gerardo López-Velázquez**: Resources; Validation. **Denisse Aurora Díaz-Corona**: Resources; Validation. **Misael Odín Vega-García**: Funding acquisition; Project administration; Resources; Supervision; Writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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