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Effect of commercial chitosan on *in vitro* inhibition of *Colletotrichum siamense*, fruit quality and elicitor effect on postharvest avocado fruit

Efecto de quitosano comercial sobre la inhibición *in vitro* de *Colletotrichum siamense*, calidad de fruto y efecto inductor sobre frutos de aguacate en postcosecha

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Abstract

Anthracnose is the main disease causing postharvest losses in 'Hass' avocado. The preharvest control of anthracnose is carried out with synthetic fungicides, so its postharvest use is limited. Chitosan, a non-toxic biopolymer considered GRAS, is an alternative to synthetic fungicide. The aims of the study were to determine *in vitro* the antifungal effect of chitosan and evaluate the elicitor effect and shelf-life-extending of chitosan on postharvest 'Hass' avocado fruits. A commercial chitosan was used. Nine strains of *C. siamense* were treated with 0, 0.5, 1.0, and 1.5% chitosan. Mycelial grown, sporulation, and germination were evaluated. Avocado fruits were also treated with the same chitosan concentration and stored at room temperature until ripening, and phenylalanine ammonia-lyase (PAL) activity was evaluated daily. Finally, avocado fruits were inoculated with three strains of *C. siamense* and subsequently treated with the same chitosan concentrations. Weight loss, color, and firmness were evaluated. As a result, all strains were sensitive to increasing chitosan concentration, reaching 100% inhibition of mycelial growth at 1.5% chitosan. PAL activity was higher in the pulp than in the skin. Weight loss decreased rapidly with 0 and 0.5% chitosan, whereas 1.0 and 1.5% chitosan decreased firmness loss and color change.

Keywords: *Persea americana* Miller, mycelial growth, anthracnose, phenylalanine ammonia-lyase, postharvest quality.

Resumen

La antracnosis es la principal enfermedad causante de las pérdidas postcosecha del aguacate 'Hass'. El uso de fungicidas sintéticos en postcosecha es limitado. El quitosano (biopolímero no tóxico considerado GRAS) es una alternativa. El objetivo fue determinar *in vitro* el efecto antifúngico del quitosano y evaluar el efecto inductor en prolongar la vida de postcosecha y mantener la calidad del fruto de aguacate. Se trataron nueve cepas de *C. siamense* con 0, 0.5, 1.0 y 1.5% de quitosano. Se evaluaron el crecimiento, la esporulación y la germinación de esporas. Además, frutos de aguacates se trataron con las mismas concentraciones de quitosano y se evaluó la actividad de PAL. Finalmente, se inocularon frutos de aguacate con tres cepas de *C. siamense* y luego se trataron con de quitosano. Se evaluó la pérdida de peso, el color y la firmeza. Como resultado, todas las cepas fueron sensibles al incrementar la concentración de quitosano, alcanzando el 100% de inhibición del micelio con 1.5% de quitosano. La actividad PAL fue mayor en la pulpa que en la piel. 1.0 y 1.5% de quitosano disminuyen la pérdida de firmeza, el color cambia y la perdida fisiológica de peso.

Palabras clave: *Persea americana* Miller, crecimiento micelial, antracnosis, fenilalanina amonio-liasa, calidad postcosecha.

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1 Introduction

Anthracnose is the main disease causing postharvest losses in 'Hass' avocado. In 2020, Mexico produced 2.4 million tons, of which it exported one million tons to the United States of America alone and about 160 thousand tons to destinations such as Asia, Europe, Central America, and Canada (SIAP-Producción Agrícola, 2020). Diseases are the main cause of postharvest losses, which are potentiated by mechanical damage, inadequate storage temperatures, and physiological disorders. *Colletotrichum siamense*, the causal agent of postharvest anthracnose, has been identified in the central Pacific region of Mexico. The visible symptoms of anthracnose are circular, light brown lesions that rapidly expand and change color (Herrera-González *et al*., 2020). The control of anthracnose in preharvest is carried out with synthetic fungicides (eradication and exclusion of the inoculum), but this causes pathogen resistance. Moreover, due to their high residuality, such fungicides have effects (neurotoxic and carcinogenic) on the consumer that limit their postharvest use (González-Estrada *et al*., 2021). For this reason, it is necessary to seek alternatives to control with synthetic (chemical) fungicides that are efficient in the control of pathogens, of low cost, and carry a low risk to health. In both cases (chemical or alternative control), knowing the pathogen (genus and species) is essential for pre- and postharvest control and knowing the mode and site of action of the control agent (González-Estrada *et al*., 2020). One of these alternatives to chemical control is chitosan, a non-toxic biopolymer considered GRAS (Berumen-Guerrero *et al*., 2020; Herrera-González *et al*., 2021). Its functional properties and acetyl group (hydrophobic interactions) allow it to interact with cell wall proteins of the pathogen, its protonated amino groups (polycationic deacetylated glucosamine units) destabilize the cell wall and membrane by electrostatic interactions with phospholipid phosphates, and its non-protonated amino groups have a high affinity for metals of the pathogen cell cytoplasm. These characteristics make chitosan an efficient bio-fungicide (Bautista-Baños *et al*., 2016; Herrera-González *et al*., 2021).

Chitosan application at 1.5-2.0% has been shown to reduce mycelial growth and spore concentration and inhibit spore germination of *C. gloeosporioides* (Rodríguez-López *et al*., 2009; Correa-Pacheco *et al*., 2017; Ramos-Guerrero *et al*., 2020). It also reduces the incidence and severity of anthracnose in avocado fruits inoculated with *C. gloeosporioides*, decreases fruit weight loss, and maintains the quality of the fruit until ripening. Finally, it induces defense mechanisms in the fruit, which are precursors of salicylic acid related to the systemic response, by increasing the enzymatic activity of PAL (Kaleda-Marino *et al*., 2018; Obianom *et al*., 2019; Rajestary *et al*., 2020). The aims of this work were 1) to determine *in vitro* the antifungal effect of chitosan on the cellular viability of *C. siamense*, 2) to evaluate the elicitor effect of chitosan on the enzymatic activity in postharvest 'Hass' avocado fruits, and 3) to identify the best chitosan concentration to maintain the postharvest quality of avocado fruit.

2 Methodology

A commercial chitosan (Zhejiang Golden-Shell Pharmaceutical Co., Ltd., Zhejiang, China) of high density, low molecular weight (45,700 Mv, g·mol), chemical identity with reagent grade chitosan, acetylation grade of 10.28 ± 0.27%, and an intrinsic viscosity (η) of 264.62 mL/g was used. It was dissolved in water acidified with vinegar at 5% titratable acidity (sugar cane vinegar commercial). Potato dextrose agar (PDA; DIBICO, Mexico) was used as the growth medium.

2.1 Pathogen

Nine *C. siamense* strains, which were isolated from fruits with postharvest anthracnose symptoms, were provided by the LIIA Biotechnology Laboratory, of the TecNM-Technological Institute of Tepic. The *C. siamense* strains (municipality, state) were identified as 1. Up Cg M4 (Uruapan, Michoacán), 2. Tin Cg M13 (Tingambato, Michoacán), 4. Zira Cg M21 (Ziracuaretiro, Michoacán), 5. Tan Cg M40 (Tancítaro, Michoacán), 6. LRe Cg M46 (Los Reyes, Michoacán), 7. LRe Cg M48 (Los Reyes, Michoacán), 8. Tpc Cg N8 (Tepic, Nayarit), 9. Xal Cg N22 (Xalisco, Nayarit), and 10. CdG Cg J22 (Zapotlan el Grande, Jalisco). All strains were activated in PDA medium and incubated at 27 °C ± 2 °C for 7 d.

2.2 Chitosan preparation

Chitosan (Chi) concentrations were prepared at 0, 0.5, 1.0 and 1.5%, dissolved in water acidified with

10% vinegar. The mixture of each concentration was dissolved with magnetic stirring, at a temperature of 40-50 °C. After solubilization, it was homogenized at 13,000 rpm for 10 min (Ultra-Turrax, T18, IKA, Staufen, Germany).

2.3 Inhibition in vitro of C. siamense

2.3.1 Inhibition of mycelial growth, sporulation, conidia germination and spore viability

Every strain of *C. siamense* was subjected to treatments with chitosan. For this, PDA medium with chitosan at concentrations of 0, 0.5, 1.0, and 1.5% was prepared. Discs (7 mm) of mycelium and spores were taken from each of the strains and incubated at 27 ± 1 °C for 7 days. Mycelial diameter was measured every 24 h with a digital caliper, and the results were expressed as the percentage inhibition of mycelial growth. To determine the spore concentration, on day 7 of incubation, 10 mL of water with Tween 80 (0.05%) was added, scraped, and left to rest for 5 min. The water was filtered, the spore concentration was measured in a Neubauer chamber, and the results were expressed as spores/mL. The length (μm) and width (μm) of 200 spores were measured. For spore germination, discs (10 mm in diameter) of PDA medium with chitosan (0, 0.5, 1.0, and 1.5%) were prepared, and 30 μL of the spore suspension of each strain were added in the center of the disc and incubated at 27 °C ± 2 °C for 12 and 24 h. Germination was stopped with the application of a drop of lactophenol blue. Two hundred spores were considered per germination period, and in each hour of sampling, the percentage of germinated spores was calculated. A spore was considered germinated when the germinative was twice the length of the spore. This was observed in a microscope (Motic, Panthera Classic) with a 40× objective. For spore viability, incubated discs were taken for spore germination and were seeded in petri dishes with PDA medium and incubated at 27 °C ± 2 °C for 48 h, observing the development or inhibition of mycelium.

2.3.2 Scanning electron microscopy

Mycelium and spore discs 7 d old and 8 mm in diameter were immersed in 3% glutaraldehyde for 72 h at 4 °C, and then the samples were washed with a graded ethanol series (20%, 40%, 70%, 90%, and 100%). Afterwards, the samples were dried in refrigeration for 12 h. The samples were observed in a scanning electron microscope (SEC CO., LTD., SNE-3200M, CA, USA) operated at 20 kV.

2.4 Phenylalanine ammonia-lyase activity

The avocado fruits were collected in an export packing house in Xalisco, Nayarit. The fruits were washed with soap and immersed in 2% chlorine for 10 min, and then they were washed with distilled water and dried at room temperature (23 °C ± 2 °C). The chitosan treatments (0, 0.5, 1.0, and 1.5%) were carried out by immersion (30 s) and subsequent drying. The fruits were stored at room temperature (22 °C ± 2 °C) and relative humidity of 85% ± 10% until eating ripeness (7-10 d). Every 24 h, exocarp and mesocarp samples were taken. The enzymatic activity of phenylalanine ammonia-lyase was determined according to Obianom and Sivakumar (2018b), with slight modifications. Samples (2.5 g; exocarp or mesocarp) were placed in 30 mL of frozen acetone and homogenized at 10,000 rpm for 10 min and allowed to rest for 5 min, and the supernatant was decanted. This process was repeated until a colorless residue was obtained, which was then allowed to dry. For PAL activity determination, 0.2 g of acetone powder was weighed and mixed with 10 mL of borate buffer (0.1 M, pH 8.8) (sodium borate, 5 mmol β-metharcaptoethanol, 2 mmol EDTA, 1% polyvinyl-polypyrrolidone PVPP), which was homogenized at 15,000 rpm for 30 s. Then, the mixture was centrifuged at 6,000 rpm for 1 h at 4 °C. The supernatant was filtered on a 0.45-μm membrane. Then, 0.5 mL of L-phenylalanine (30 mmol) were added, mixed and incubated for 2 h at 25 °C. The reaction was terminated with 6 μL of HCl (6 M). The production of cinnamic acid was measured at 290 nm (Thermo scientific, Genesys 10S UV-Vis, Wisconsin, U.S.A.). The results were expressed in nmol of cinnamic acid h^{-1}·mg of $protein^{-1}$.

2.5 Postharvest quality

Avocado fruits were collected in an export packinghouse in Xalisco, Nayarit. The fruits were washed with soap, immersed in 2% chlorine for 10 min, washed with distilled water, and dried at room temperature (23 °C ± 2 °C). The fruits were inoculated as a curative treatment (inoculation and after application of treatments) with three strains (1. Up Cg M4, 7. LRe Cg M48 and 8. Tpc Cg N8), which represent the agroclimatic regions (climate and soil) that predominate in the Central Pacific Region of Mexico where avocado is produced. The fruits

were wounded with an awl (4 × 0.13 mm). Three longitudinal wounds were made (top, middle, and bottom) on two equidistant sides of fruit and 10 μL of the spore suspension of *C. siamense* (1. Up Cg M4, 7. LRe Cg M48 and 8. Tpc Cg N8) at 10^6 spores/mL. After 12 h of inoculation, the chitosan treatments (0, 0.5, 1.0, and 1.5%) were carried out by immersion (30 s) and subsequent drying. The fruits were stored at room temperature (22 °C ± 2 °C) and 85% ± 10% relative humidity until ripening (7-10 d). The color of the fruit skin was measured daily at three equidistant points on 10 fruits by treatment with a reflectance colorimeter (High-Quality Colorimeter, Focus on color, Shanghai, China), and the data were expressed as changes in luminosity, chromaticity, and hue angle. The firmness of the fruit was measured with a penetrometer (Stable Micro Systems, Ta XT Plus, Vienna Court, United Kingdom) with a 3-mm-diameter test tube, and 10 mm were penetrated at three points in the equatorial region of the fruit. The results were reported in Newtons, as averages of the force required to penetrate the pulp of the fruit.

2.6 Statistical analysis

A unifactorial experimental design was used, chitosan being the factor of variation in the *in vitro* test and in postharvest quality. For enzymatic activity, a completely randomized experimental factorial design was used with chitosan and tissue type as factor of variation. The normality of the data was verified. Analysis of variance was performed for the variables evaluated, and mean comparisons were applied using Fisher's LSD test (P ≤ 0.05). The analysis was performed with Statistica 64 software ver. 12 (StatSoft, 2014).

3 Results and discussion

3.1 Inhibition in vitro of C. siamense

The results of the mycelial inhibition of the nine evaluated strains of *C. siamense* are shown in Table 1. All strains of *C. siamense* showed sensitivity to increasing chitosan concentration ($p < 0.05$). With chitosan at 0.5%, inhibition of mycelium was around 50%; at 1.0%, close to 80% in most cases; and at a concentration of 1.5%, close to or equal to 100% in most cases. In the 5. Tan Cg M40 strain, inhibition did not reach 80%. In most chitosan treatments, the appearance of the mycelium was sparse, less dense, distorted, abnormal disorganized, reduced, and dehydrated. The acervulli and spore masses were not visible (Figure 1).

Similar results were reported by Marques *et al.* (2016), Kaleda-Marino *et al.* (2018), and Xoca-Orozco *et al.* (2018), who achieved greater than 90% inhibition with a reagent grade low-molecular-weight chitosan (75%-85% deacetylation). However, Correa-Pacheco *et al.* (2017), and Chávez-Magdaleno *et al* (2018a, 2019) achieved close to 30% inhibition with reagent grade medium-molecular-weight chitosan and found it necessary to combine it with other compounds to achieve more effective inhibition.

Table 1. Effect of chitosan concentration on the inhibition of mycelial growth of nine *C. siamense* strains incubated at 27 °C for 7 days.

Strains	Inhibition of mycelial growth			
C. siamense	Control	Chitosan 0.5%	Chitosan 1.0%	Chitosan 1.5%
1. Up Cg M4	0 ± 0^d	50 ± 7.5^c	78.3 ± 4.5^b	94.9 ± 7.4^a
2. Tin Cg M13	0 ± 0^d	48 ± 6.1^c	71.5 ± 29.4^b	85.6 ± 15^a
4. Zira Cg M21	0 ± 0^d	58.8 ± 4^c	78.9 ± 5.8^b	100 ± 0^a
5. Tan Cg M40	0 ± 0^d	47.6 ± 6.1^c	70.6 ± 1.3^b	76.0 ± 4.3^a
6. LRe Cg M46	0 ± 0^d	50 ± 5.3^c	78.3 ± 2.5^b	94.9 ± 1.7^a
7. LRe Cg M48	0 ± 0^d	49.9 ± 0.9^c	77.5 ± 1.8^b	92.9 ± 1.8^a
8. Tpc Cg N8	0 ± 0^d	72 ± 0.7^c	81.5 ± 3.2^b	96.8 ± 1.2^a
9. Xal Cg N22	0 ± 0^d	52.4 ± 0.9^c	79.7 ± 3.5^b	100 ± 0^a
10. CdG Cg J22	0 ± 0^c	49.7 ± 1.3^b	100 ± 0^a	100 ± 0^a

Superscript letters indicate that values with the same letter in the row are not significantly different; Fisher's LSD test ($p \leq 0.05$) (n = 6).

Table 2. Effect of chitosan concentration on the sporulation of nine *C. siamense* strains incubated at 27 °C for 7 days.

Strains *C. siamense*	Sporulation (1×10^6 spores/mL)			
	Control	Chitosan 0.5%	Chitosan 1.0%	Chitosan 1.5%
1. Up Cg M4	4.98 ± 0.24^{a}	1.54 ± 0.17^{b}	0.55 ± 0.6^{d}	1.07 ± 0.15^{c}
2. Tin Cg M13	-	-	-	-
4. Zira Cg M21	4.05 ± 0.206^{a}	1.67 ± 0.107^{b}	0.805 ± 0.0083^{c}	0 ± 0^{d}
5. Tan Cg M40	0.22 ± 0.1^{c}	3.24 ± 2.47^{ab}	2.55 ± 0.98^{b}	4.0 ± 1.21^{a}
6. LRe Cg M46	6.82 ± 0.22^{a}	2.38 ± 0.13^{c}	0.63 ± 0.06^{d}	2.96 ± 0.11^{b}
7. LRe Cg M48	1.5 ± 0.12^{a}	1.05 ± 0.07^{b}	0.2 ± 0.2^{d}	0.57 ± 0.9^{c}
8. Tpc Cg N8	3.54 ± 0.14^{a}	1.41 ± 0.09^{b}	1.09 ± 0.3^{b}	0 ± 0^{bc}
9. Xal Cg N22	3.41 ± 1.89^{a}	0.302 ± 0.04^{b}	0.27 ± 0.07^{bc}	0 ± 0^{c}
10. CdG Cg J22	0.33 ± 0.01^{a}	0.02 ± 0.002^{b}	0.02 ± 0.008^{b}	0 ± 0^{c}

Superscript letters indicate that values with the same letter in the row are not significantly different; Fisher's LSD test ($p \leq 0.05$) (n = 6).

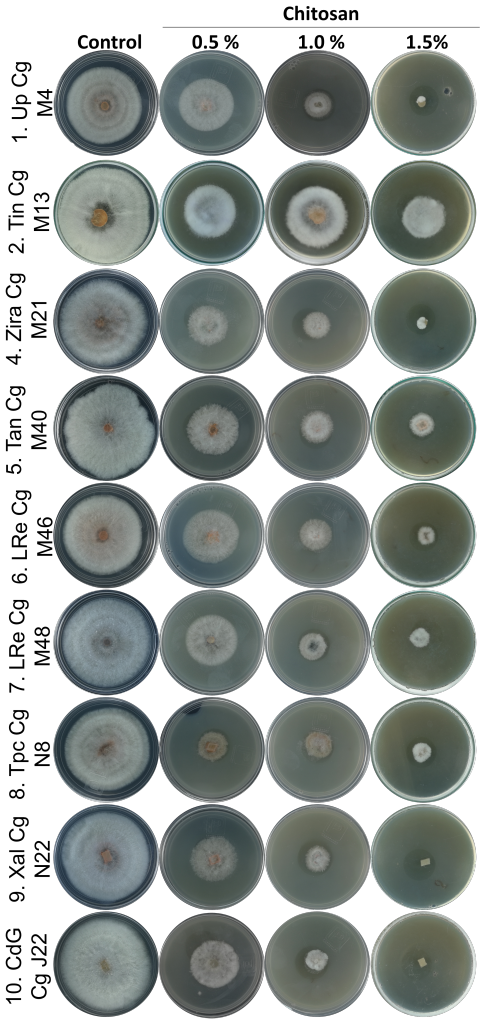

Figure 1. Effect of chitosan concentration on the inhibition of mycelial growth of nine *C. siamense* strains incubated at 27 °C for 7 days.

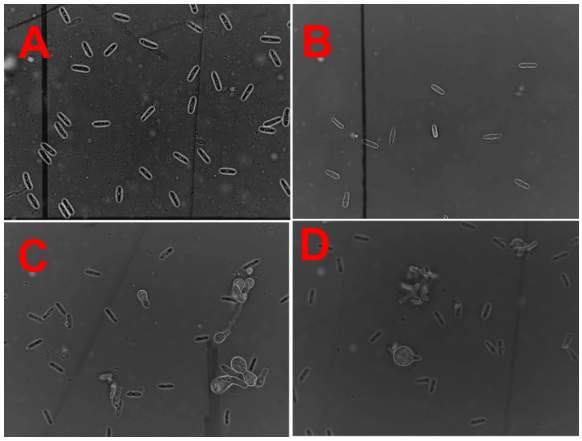

Figure 2. Effect of chitosan on spore length and width. A) Control; B) strain 4. Zira Cg M21 (chitosan 1.0%) scarcity and heterogeneous; C) Strain 5. Tan (chitosan 1.5%) deformations; and strain 6. LRe (chitosan 1.5%) deformation. Motic Microscope, 40×.

Spore production in the presence of different chitosan concentrations differed between strains ($p < 0.05$) at 7 days of incubation. The concentrations 1.0% and 1.5% were those that most decreased the production of spores of the pathogen (Table 2). The length and width of the spore produced by each strain was statistically different between the different concentrations of chitosan ($p < 0.05$). Depending on the origin of the strain, the length or width of the spore was greater or less than that of the control (Table 3 and 4; Figure 2).

Sporulation is part of the life cycle of the fungus, although it is stimulated by different environmental factors, including a lack of nutrients (Yuan-Ying *et al.*, 2012).

Table 3. Effect of chitosan concentration on spore length in nine *C. siamense* strains incubated at 27 °C for 7 days.

Strains *C. siamense*	Conidia length (μm)			
	Control	Chitosan 0.5%	Chitosan 1.0%	Chitosan 1.5%
1. Up Cg M4	47.2 ± 4.6^{a}	45.1 ± 4.3^{b}	46.4 ± 3.7^{a}	44.3 ± 4^{b}
2. Tin Cg M13	-	-	-	-
4. Zira Cg M21	41.1 ± 4.5^{c}	42.7 ± 4.9^{b}	45.6 ± 5.5^{a}	-
5. Tan Cg M40	45.4 ± 4^{bc}	44 ± 7.1^{c}	47.9 ± 4.7^{a}	46.8 ± 4.3^{b}
6. LRe Cg M46	42.8 ± 0.2^{b}	42.4 ± 0.2^{b}	44.7 ± 0.3^{a}	41.7 ± 0.3^{c}
7. LRe Cg M48	41.2 ± 0.4^{b}	45.7 ± 0.3^{a}	44.4 ± 0.5^{a}	45.2 ± 0.5^{a}
8. Tpc Cg N8	40.6 ± 0.3^{a}	38.5 ± 0.4^{b}	39.7 ± 0.3^{ab}	-
9. Xal Cg N22	38.5 ± 0.2^{c}	43.6 ± 0.5^{b}	46.4 ± 0.7^{a}	-
10. CdG Cg J22	-	-	-	-

Superscript letters indicate that values with the same letter in the row are not significantly different; Fisher's LSD test ($p \leq 0.05$) (n = 200).

Table 4. Effect of chitosan concentration on spore width of nine *C. siamense* strains incubated at 27 ° C for 7 days.

Strains *C. siamense*	Conidia width (μm)			
	Control	Chitosan 0.5%	Chitosan 1.0%	Chitosan 1.5%
1. Up Cg M4	16.3 ± 2^{a}	13.4 ± 1.8^{b}	14.6 ± 1.5^{d}	12.3 ± 1.5^{c}
2. Tin Cg M13	-	-	-	-
4. Zira Cg M21	11.3 ± 1.6^{c}	12.7 ± 1.8^{b}	13.2 ± 1.7^{a}	-
5. Tan Cg M40	12.8 ± 1.7^{b}	13.3 ± 2^{a}	12.7 ± 1.8^{b}	13.4 ± 2.1^{a}
6. LRe Cg M46	14.5 ± 0.1^{a}	13.6 ± 0.1^{b}	14.4 ± 0.2^{a}	12.4 ± 0.1^{c}
7. LRe Cg M48	13.3 ± 0.1^{b}	13.8 ± 0.2^{a}	13.9 ± 0.2^{a}	12.4 ± 0.2^{c}
8. Tpc Cg N8	12.3 ± 0.1^{c}	12.9 ± 0.1^{b}	13.3 ± 0.1^{a}	-
9. Xal Cg N22	12.7 ± 0.1^{b}	12.4 ± 0.2^{b}	14.5 ± 0.2^{a}	-
10. CdG Cg J22	-	-	-	-

Superscript letters indicate that values with the same letter in the row are not significantly different; Fisher's LSD test ($p \leq 0.05$) (n = 200).

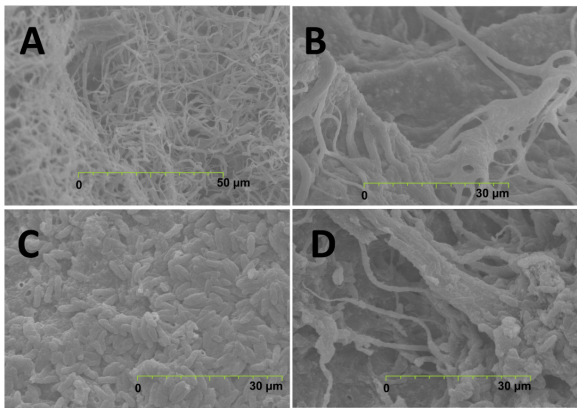

Figure 3. Scanning electron micrographs of *C. siamense* of different origins treated with 1.0% chitosan. A) Strain. 1. Up Cg M4 with overproduction of disorderly mycelia; B) Strain. 4. Zira Cg M21 with collapsed mycelia; C) Strain. 6. LRe Cg M46 with excessive spore production; D) Strain. 8. Tpc Cg N8 spores and collapsed mycelia.

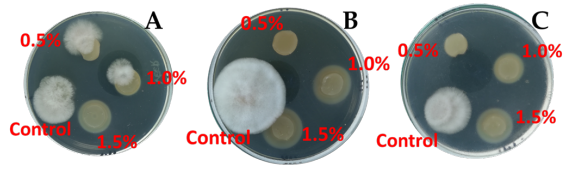

Figure 4. Spore viability after 12 and 24 hours of exposure to chitosan A) 9. Xal Cg N22 (12 h); B) 5. Tan Cg M40 (24 h); C) 1. Up Cg M4 (24 h).

However, the observed low sporulation rate was due to high mycelial inhibition and mycelial damage that cause the low assimilation of nutrients in the presence of chitosan. In addition, chitosan alters processes in the production of spores (duplication of genetic material). Sporulation is a characteristic of the aggressiveness of the pathogen: as it is diminished, its pathogenicity decreases (Oliveira-Junior, 2016). Similarly, to the inhibition of mycelium, germination was affected by the concentration of chitosan. This can be explained by the deposition effect of chitosan in the spore (formation of a dense film) that

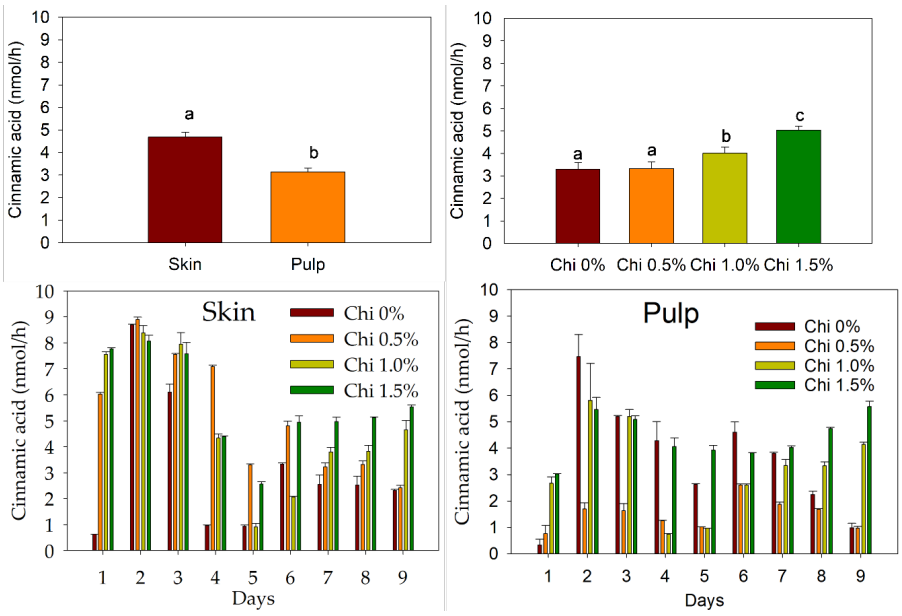

Figure 5. PAL activity as cinnamic acid (nmol/h) in avocado fruit treated with different concentrations of chitosan. Data are presented as means ± Std. Err (n = 3).

limits metabolic processes and interaction with the environment (excretion of metabolites, availability of nutrients, or change in pH) (Chávez-Magdaleno *et al*., 2018b; Herrera-González *et al*., 2021).

Figure 3 shows the micrographs of *C. siamense* with 1.0% chitosan treatment. The 1. Up Cg M4 strain showed overproduction of disorganized mycelia with reduced hyphal diameters (Figure 3A). The 4. Zira Cg M21 strain showed distorted and collapsed hyphae (Figure 3B). The 6. Lre Cg M46 strain showed excessive spore production of irregular shapes without hyphae (Figure 3C). The 8. Tpc Cg N8 strain has few spores and few mycelia arranged in a disorganized way. Table 5 shows the inhibition of spore germination of *C. siamense* of different origins treated with chitosan. The chitosan concentration affected germination at 12 and 24 h of exposure. This inhibition depended on the strain, as some were more sensitive than others. However, although some strains presented germ tubes, the majority were not viable to produce hyphae, presenting antifungal effect. Only strain 9. Xal Cg N22 (12 h) from Nayarit was able to produce mycelia at chitosan concentrations of 0.5 and 1.0%, presenting a fungistatic effect at these concentrations, but at 1.5% chitosan, the effect was fungicidal (Figure 4).

3.2 *Phenylalanine ammonia-lyase (PAL) activity*

There was a difference in PAL production between the peel and the pulp, the activity being greater in the peel than in the pulp ($p < 0.5$) (Figure 5A). The activity of the PAL enzyme differed between chitosan treatments ($p \leq 0.05$). The treatment that presented the highest PAL enzymatic activity was 1.5% chitosan (Figure 5B). The activation of the enzyme was faster in the fruits treated with chitosan than in the control (Chi 0%). The maximum activation of PAL occurred after 2 days of storage in all treatments (with and without chitosan); from day 3 to day 5, all showed a decrease in enzymatic activity, with the control and chitosan at 1.0% being the ones with the lowest activity. On day six, PAL activity increased again to values between 2 and 6 nmol (Figure 5).

Similar studies have reported that chitosan at different concentrations increases PAL activity, considering it as an elicitor of defense mechanisms in avocado fruit (Xoca-Orozco *et al*., 2017; Obianom & Sivakumar, 2018a, 2018b; and Obianom *et al*., 2019). The importance of PAL is that it can fluctuate significantly in short periods of time in response to biotic or abiotic stimuli. PAL is the precursor of cinnamon acid, the precursor of a great variety of secondary metabolites, such as the metabolites of phenyl propanoids (phenolic compounds such as

anthocyanins and flavonoids) (Zhao, 2016; Ding and Ding, 2020; Kumar Patel *et al*., 2020;).

3.3 Postharvest quality

3.3.1 Skin color

The changes in color parameters in avocado fruits inoculated with *C. siamense* strains differed between chitosan treatments ($p < 0.05$). The Chi 0% and Chi 0.5% treatments were the ones that rapidly decreased the lightness, chromatic, and hue angle values, compared with the Chi 1.0% and Chi 1.5% treatments, which took time to decrease these color parameters in the three strains inoculated in the fruits. The low final values of lightness (loss of white color), chromatic (loss of green color), and hue angle (dark colors, red/purple) indicate eating ripeness, which all the fruits reached in different periods of time and depended on the treatment and on the inoculated strain (Figure 6). Thus, a slow change in these color parameters indicates slow maturation.

The color results indicate that all treatments produced regular color changes in the fruit skins, but the rates of change differed. Lightness (from 24 to 12), chromatic (from 15 to 3), and hue angle (from 100 to 220) values were within the normal parameters to reach eating ripeness (Herrera-González *et al*., 2017). These values are due to the decrease in chlorophyll (responsible for the green color) and carotenoids (β-carotene, neoxanthin, violaxanthin, Lutein, R-carotene, zeaxanthin, and antheraxanthin, responsible for the orange and yellow color) during ripening, in addition to the accumulation of anthocyanins (cyanidin 3-O-glucoside mainly),

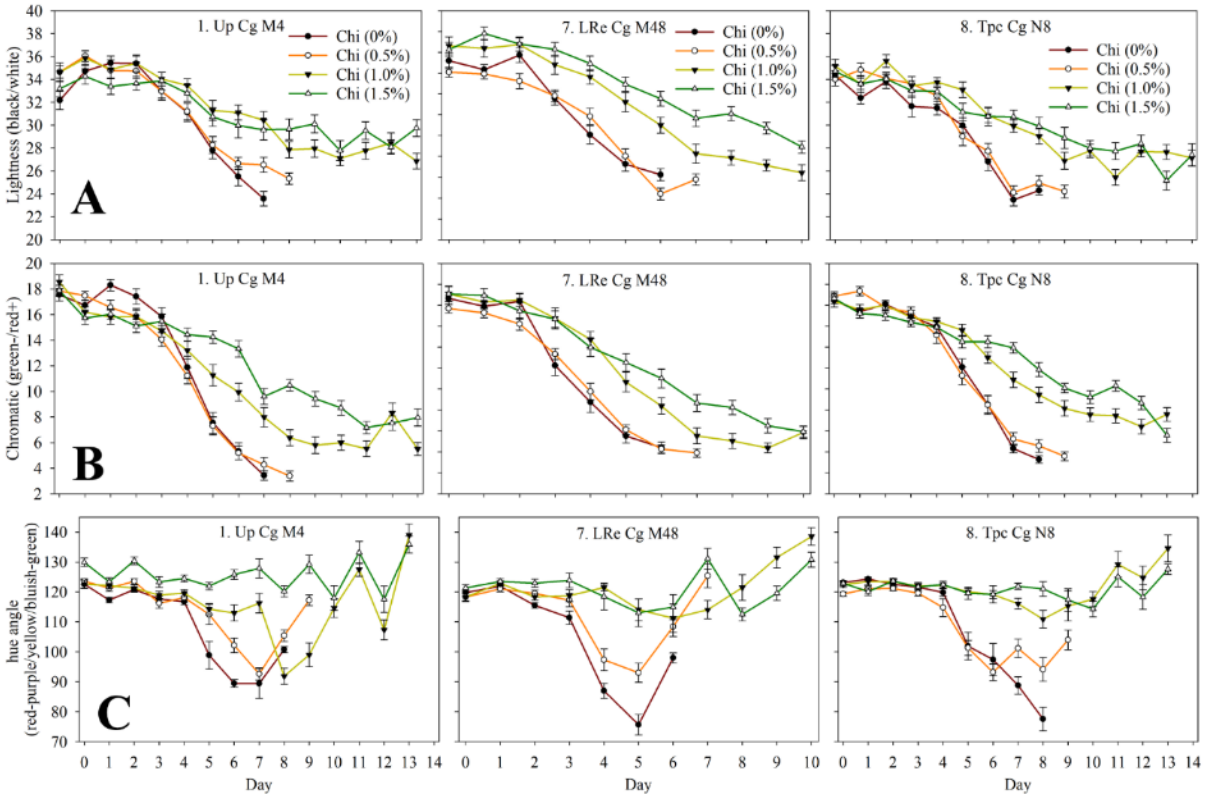

Figure 6. Color parameter changes during ambient storage until ripening of avocado fruit (skin) treated with different concentrations of chitosan and inoculated with *C. siamense* (1. Up Cg M4 Michoacán, 7. LRe Cg M48 Michoacán, and 8. Tpc Cg N8, Nayarit). A) lightness, B) chromatic, and C) hue angle. Data are presented as means ± Std. Err ($n = 30$).

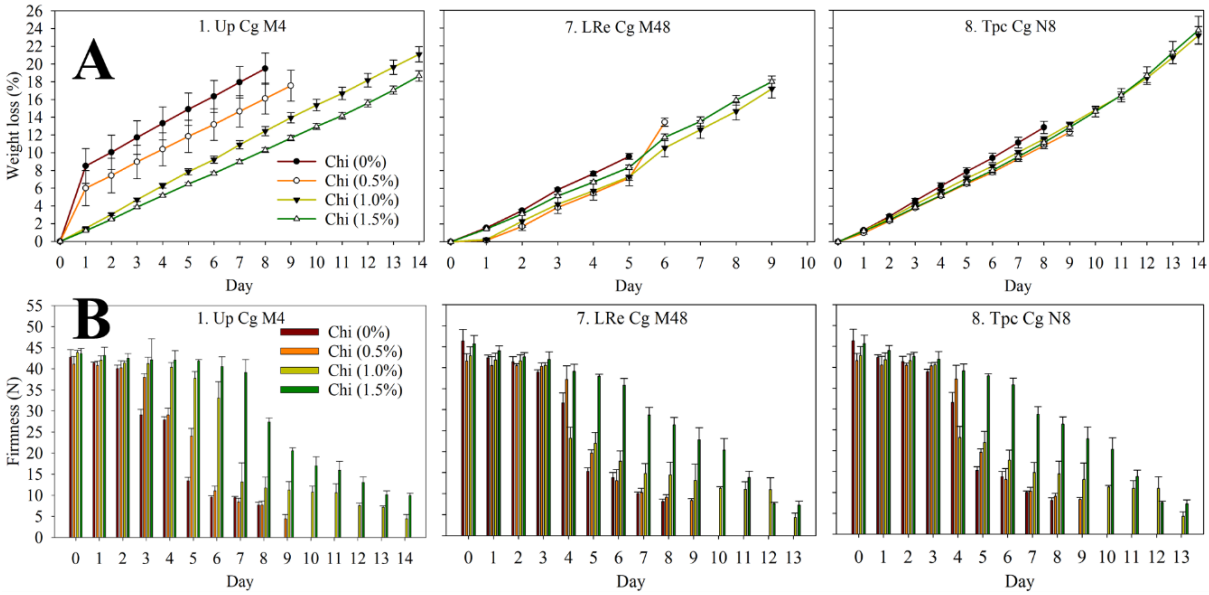

Figure 7. Effect weight loss (A) and firmness (B) changes during ambient storage until ripening of avocado fruit treated with different concentrations of chitosan and inoculated with *C. siamense* (1. Up Cg M4 Michoacán, 7. LRe Cg M48 Michoacán, and 8. Tpc Cg N8, Nayarit). Data are presented as means ± Std. Err (n = 3).

which are responsible for dark colors (red-purple) and are synthesized via phenylpropanoids under the influence of temperature, time, and water loss (McGuire, 1992; Cox *et al*., 2004; Ashton *et al*., 2006)

3.3.2 Weight loss and firmness

The effect of chitosan on weight loss in the inoculated fruit was different ($p < 0.05$). The fruit inoculated without chitosan (Chi 0%) was the one that lost the most weight when it reached eating ripeness. The rest of the treatments had a longer shelf life, so the weight loss was greater in the fruits treated with chitosan (Figure 7A). These results coincide with that reported by Chávez-Magdaleno *et al*. (2018b) and Xoca-Orozco *et al*. (2018), since they report similar loss percentages when they applied 1% chitosan. This may be due to the fact that chitosan forms a semi-permeable film that reduces the loss of water vapor due to the exchange between the fruit and the environment.

The decrease in the firmness of fruits treated with chitosan was different ($p < 0.05$). The fruits treated with Chi 0% and Chi 0.5% lost more than half of their initial firmness in the first 5 d, reaching eating ripeness at 8 and 9 days, respectively, while those treated with Chi 1.0% and Chi 1.5% reduced the initial firmness by half until days 7 and 9, respectively, and reached a firmness level corresponding to eating ripeness until days 13-14, depending on the inoculated strain (Figure 7B).

The results coincide with those reported by various authors, who establish that the fruit reaches eating ripeness at between 5 and 10N and that chitosan lengthens the postharvest life of the fruit (Correa-Pacheco *et al*., 2017; Cho *et al*., 2020). This is mainly due to the fact that chitosan reduces the rapid ultrastructural changes of the components of the cell wall, due to a low enzymatic activity of cellulase and polygalacturonase that degrades pectin, avoiding cellular disorder (Bill *et al*., 2014a, 2014b).

Conclusions

Chitosan had a fungicidal effect on the mycelial growth and spore germination of nine *C. siamense* strains at concentrations of 1.0% and 1.5% in *in vitro* application. Moreover, chitosan induced the synthesis of phenylalanine ammonia lyase in the peel of the fruit at all concentrations. Moreover, chitosan at 1.0% and 1.5% was able to delay the color change (from green to black), loss of weight, and firmness for a longer time in avocado fruits inoculated with *C. siamense*.

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