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Chitosan and GRAS Substances: An Alternative for the Control of *Neofusicoccum parvum* In Vitro, Elicitor and Maintenance of the Postharvest Quality of Avocado Fruits

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Abstract: Postharvest avocado is susceptible to attack by *Neofusicoccum parvum*, which has been reported to cause black spot in avocado pulp. Therefore, it is necessary to look for alternatives for its control with products that are low-cost, effective and without risks to human health and the environment, and that also stimulate the defense mechanisms of the fruit. The aim was to evaluate the effect of basic and GRAS substance treatments on the in vitro control of *N. parvum*, and the induction of enzymes related to the defense mechanisms of the fruit. *N. parvum* was isolated from avocado fruit. Morphological and molecular identification was performed. In vitro and in vivo treatments were made for the control of pathogens and the induction of defense mechanisms in the fruit with basic and GRAS substance treatments based on chitosan and GRAS substances. The basic and GRAS substance treatments inhibited the development of *N. parvum* mycelium by 80–100%. In addition, they induced the activation of enzymes related to the defense mechanisms of the fruit (PAL, POD and PPO). The best basic and GRAS substance treatments, both in vitro and in vivo, were those based on chitosan (0.5%) and cinnamon essential oil (2.5%) when they were applied combined in the form of an emulsion.

Keywords: *Persea americana* Miller; avocado fruit; chitosan; diseases; quality fruit; induction of defense mechanisms

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1. Introduction

The avocado is a climacteric fruit susceptible to attack by pathogens in pre- and postharvest states. In 2021, Mexico produced 2.4 million tons of avocado (29.3% of global production), of which 1.8 million tons were produced in Michoacán, where 1.12 million tons were exported to the United States alone, making this country the main destination for export [1,2]. Therefore, postharvest diseases are inherent in the production and export of avocados to national and international markets. Although the *C. gloeosporioides* species complex has been detected as the cause of anthracnose [3] and *Lasiodiplodia theobromae* as the cause of stem-end rot [4], other pathogens are also present in the fruit at harvest time and are of great importance. *Neofusicoccum parvum* is a pathogenic fungus that has already been reported as causing fruit diseases (black spot) on avocado fruit [5] and branch dieback on avocado tree branches [6] of avocado in Mexico and stem-end rot in New Zealand [7]. *N. parvum* is a cosmopolitan fungus and has a wide geographic distribution in tropical and

subtropical areas [5]. In the same way as for *C. gloeosporioides* and *L. theobromae*, the chemical control of *N. parvum* in postharvest is carried out with synthetic fungicides (same molecules, azoxystrobin and fludioxonil) at the same concentrations [8]. Given the global need to reduce the use of synthetic fungicides, it is necessary to integrate alternative control systems constituted by low-cost, effective and generally recognized as safe (GRAS) substances that do not have a harmful or worrying effect on human health and the environment. These substances are characterized by the fact that they are not used or marketed as phytosanitary products but have a fungicidal effect or elicit natural defense mechanisms in the fruit [9,10]. One of these compounds is chitosan, a polycationic biopolymer capable of forming films and activating a defense mechanism in the fruit (elicitation effect) and fungicidal activity [11]. Organic or inorganic salts also have an elicitor and fungicidal effect [12]. In addition, other natural compounds such as salicylic acid [13], sodium silicate [14] and essential oils also have antimicrobial and defense mechanism-eliciting activity in fruits [8,15,16]. There has been little research on the use of basic and GRAS substance treatments based on chitosan and natural compounds in the control of *N. parvum*, on their application as inducers of defense mechanisms in avocado and their effect on maintaining quality until ripe for consumption. The aim of this research is to evaluate the antifungal effect of basic and GRAS substance treatments on in vitro growth of *N. parvum*, as well as their effect as an elicitor of enzymatic activity that activates defense mechanisms in avocado fruit and maintains quality until ripe for consumption.

2. Materials and Methods

2.1. Fungal Isolation

To obtain the pathogen, 'Hass' avocado fruits were sampled from an orchard in Tacámbaro, Michoacán, Mexico (19°14′33.96″ N, 101°30′17.61″ W). The fruits were transported to the LIIA-Instituto Tecnológico de Tepic's Food Biotechnology laboratory and incubated at 27 ± 1 °C and a relative humidity of 90 ± 10% until damage symptoms developed, discarding symptoms of anthracnose and stem-end rot. Black spot infections of the pulp in the peduncular area were cut and washed with 2% sodium hypochlorite and washed with distilled water, then dried and inoculated in potato dextrose agar medium, and incubated at 27 ± 1 °C until mycelium developed. Successive reseeding was carried out until isolation and purification. Pathogenicity tests were performed, and morphological identification was carried out by evaluating the shape and color of the mycelium during its development, which was observed with an optical microscope (Motic, Panthera Classic, San Antonio, TX, USA, objective 40×) and identified at the genus level according to taxonomic keys [5,17]. Molecular identification was performed at the Colegio de Postgraduados facilities at LADIFIT when the colony was 4 days old in PDA, as described by Moreno-Hernández et al. [18], using the primers ITS1las-ITS4las, EF1728Flas-EF1986Flas and BT2Alas-BT2Blas. The phylogenetic tree was built using the neighbor-joining algorithm built in MEGA v.5., to identify the haplotypes between the sequences. Sequences were compared to the NCBI sequence database using the BLAST algorithm as a rough identification for use in phylogenetic analysis [19] to compare with other fungal sequences in this database.

2.2. Preparation of Formulations

Table 1 shows the basic or GRAS substances that were mixed with chitosan, as well as the abbreviation with which they will be identified henceforth. A commercial chitosan (Zhejiang Golden-Shell Pharmaceutical Co., Ltd., Taizhou, China) with a degree of deacetylation of 89.7 ± 0.27%, a low molecular weight of 45.87 kDa, and an intrinsic viscosity (η) of 264.6 g·mol^{-1} was used. Chitosan was solubilized in water acidified with vinegar (10%), with magnetic stirring at a temperature of 50–60 °C [20]. The emulsion was prepared with cinnamon essential oil (2.5%), which was homogenized at 10,000 rpm for 5 min [21]. All formulations were mixed and homogenized (Ultra-Turrax, T18, IKA, Staufen, Germany) before application in Petri dishes. Potato dextrose agar growth medium (PDA; DIBICO,

Cuautitlán Izcalli, Mexico) was used in each formulation. Azoxystrobin 20.51% (*m*/*v*) and fludioxonil 20.51% (*m*/*v*) are included as treatment (only fungicides authorized for use in postharvest avocado).

Table 1. Basic or GRAS substances of the formulations and their abbreviations.

No.	Formulation	Code
1	Control (PDA)	Control
2	Chitosan 0.5%	Chi 0.5%
3	Chitosan 1.0%	Chi 1.0%
4	Chitosan 1.5%	Chi 1.5%
5	Chitosan 0.5% + potassium sorbate 1%	ChiSP
6	Chitosan 0.5% + Salicylic acid 2%	ChiSA
7	Chitosan 0.5% + Sodium silicate 2%	ChiSi
8	Chitosan 0.5% + sodium benzoate 2%	ChiBe
9	50/50% [Chitosan 0.5% + Emulsion (Chi 2% and cinnamon essential oil 2.5%)]	CNEO-50/50
10	75/25% [Chitosan 0.5% + Emulsion (Chi 2% and cinnamon essential oil 2.5%)]	CNEO-75/25
11	Synthetic fungicide (azoxistrobin 20.51% and fludioxonil 20.51%)	Fungicide
12	Emulsion 100% (Chi 2% and cinnamon essential oil 2.5%)	CNEO

2.3. In Vitro Evaluation

2.3.1. Inhibition of *N. parvum* with Basic or GRAS Substance Formulations

Mycelial disks (7 mm) of 4-day-old *N. parvum* were inoculated in Petri dishes with the corresponding concentration of each formulation (Table 1) and potato dextrose agar, then incubated at 27 ± 1 °C for 3 days. At the end of incubation, the diameter of the colony was measured, and the results were expressed as percentage of inhibition of the mycelium compared to the control treatment. For sporulation, 10 mL of distilled water was poured into the Petri dish, after which the surface was scraped, left to settle, and then the spore solution was poured into a test tube. The concentration of spores was determined in a cell counter (Model R1, OLYMPUS®, Waltham, MA, USA) and the results were expressed as spores per mL; in addition, spore size was also measured.

2.3.2. Scanning Electron Microscopy

Neofusicoccum parvum samples were fixed in glutaraldehyde (2.5%) and then placed under vacuum to eliminate air bubbles. They were then washed three times (20 min each wash) with Sorensen's phosphate buffer (pH 7.1, 1 M), followed by gradual washes of 30, 40, 50, 60, 70, 80 and 90% ethanol for 50 min each; at the end, three washes were performed with 100% ethanol for 20 min each. The samples were dried in the presence of CO_2 for 40 min (Sandri-780A, Rockville, ML, USA) and mounted in a sample holder before finally covering them with gold (Ion Sputter JFC-1100, Jeol, Fine Coat, Tokyo, Japan). The samples were observed in a Scanning Electron Microscope at 10 kV (JSEM 6390, JEOL, Tokyo, Japan) at the COLPOS Electron Microscopy Unit (Tokyo, Japan) [22].

2.4. In Vivo Evaluation—Application of Treatments

Fruits with a dry matter content of 36.8 ± 0.8% and a black color change of 50% were used. The fruits were washed with soap and disinfected with 2% sodium hypochlorite. The treatments in Table 1 were applied by ultra-low volume nebulization (Husky, Tecnokiller 160, Orizaba, México); the nozzle of the nebulizer was placed 50 cm from a band (10° inclination) where the fruits passed and were impregnated (2–3 mL) by the corresponding treatment. After this, the fruits were stored at a temperature of 22 ± 2 °C and 85 ± 10% relative humidity for 8 days.

2.4.1. Postharvest Quality

For the postharvest evaluations, the treatments that were applied in combination were selected, leaving out the individual treatments of chitosan, fungicide and emulsion, as well as the combination with salicylic acid. The selected treatments were ChiSP, ChiSi, ChiBe, CNEO-50/50 and H) NCEO-75/25, and a control (PDA only).

Skin Color

The change in skin color was measured on days 1, 5 and 9 at equidistant points in 10 fruits for each treatment with a colorimeter (High-Quality Colorimeter, Focus on color, Shanghai, China). Data were reported as lightness (L*), chromaticity (C*) and hue angle (h*) on the days evaluated.

Firmness

Firmness was measured on days 2, 5 and 6 of storage with a penetrometer (Stable Micro Systems, Ta XT Plus, Godalming, UK) with a 3 mm probe, penetrating 10 mm at three equidistant points on the equatorial region of the fruit. Results are reported as the average of the force required to penetrate the pulp in Newtons (N).

Weight Loss

Weight loss was measured daily for 8 days with a digital scale (Ohaus®, CS2000, Parsippany, NJ, USA). Data were reported as percent weight loss using the equation $[((\text{weight}_{\text{initial}} - \text{weight}_{\text{final}})/\text{weight}_{\text{initial}}) \times 100]$.

2.4.2. Apparent Fruit Quality

At the end of storage, 30 fruits were taken per treatment and the percentage of external rot, pulp rot, stem-end rot and shrivels was evaluated. First, the inspection was carried out on the whole fruit to evaluate possible external rots and shrivels; then, all the fruits were cut in half and the pulp and stem-end rots were inspected. Each variable was assigned a value from a subjective scale [23]. The apparent quality scale of the fruit is 4 points, where 0 = 0% damage, 1 = 10% damage, 2 = 25% damage, 3 = 50% damage and 4 => 50% damage, using decimals where required. Data were reported as the average of the values assigned to the fruits.

2.4.3. Measurement of Enzymatic Activity

For the enzymatic activity for PAL, POD and PPO, 10 g of peel were sampled every 12, 24 and 48 h. These samplings were carried out for the Control, ChiSP, ChiSi, ChiBe, CNEO-50/50 and CNEO-75/25 treatments. The collection of samples and the acetone powders that were used for this evaluation were carried out according to [24] with slight modifications. The acetone powders were obtained from a frozen sample with liquid nitrogen; 1 g of sample was homogenized at 10,000 rpm with 50 mL of acetone at −20 °C for 4 min, decanted, and if the sample had color, the step was repeated. It was then allowed to dry at room temperature. For the PAL, 0.1 g of acetone powders was taken and mixed with 1.5 mL of borate buffer, homogenized, and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was filtered (0.45 µm pore size). For the enzyme reaction, 0.3 mL of the enzyme extract, 0.6 mL of boron buffer, and 0.5 mL of 20 mM L-phenylalanine were taken. The mix was shaken for 1 min and incubated at 37 ± 1 °C for 1 h, the reaction was stopped by adding 0.3 mL of 1 M HCl, and immediately the absorbance was measured at 290 nm (Thermo Scientific, Genesys 10S UV-Vis, Madison, WI, USA). The enzymatic activity was reported as cinnamic acid $\text{nmol}\cdot\text{h}^{-1}$ [20]. For POD and PPO enzymes, 1 g of frozen sample was taken and mixed with 10 mL of phosphate buffer, homogenized at 10,000 rpm, and the supernatant was decanted and considered as the enzyme extract. For the POD activity, the methodology proposed by Sellamuthu et al. (2013) [25] was used with slight modifications. For the reaction, 0.5 mL of crude extract and 1.5 mL of guaiacol substrate (100 mM sodium phosphate, pH 6.5, and 8 mM guaiacol) were mixed. The mixture was incubated

for 30 min at 30 °C. The reaction was stopped by adding 0.25 mL of 24 mM H_2O_2, and the increase in absorbance was measured at 460 nm (Thermo Scientific, Genesys 10S UV-Vis, Madison, WI, USA). Enzyme activity was reported as ΔA460 of 0.01 per min per mg protein [25,26]. For the PPO activity, the methodology of Tian et al. [26] was used with slight modifications. A total of 0.5 mL of crude extract was mixed with 1.5 mL of substrate (100 mM sodium phosphate, pH 6.4, and 500 mM catechol) and measured at 490 nm (Thermo Scientific, Genesys 10S UV-Vis, Madison, WI, USA). The reaction mixture was incubated at 30 °C for 5 min. Activity was reported as units per micrograms. One unit was defined as ΔA398 of 0.1 per minute. The protein concentration was determined according to the Bradford method, using bovine serum albumin as a standard [27].

2.5. Statistical Analysis

A completely randomized experimental design was used, with the treatments, based on chitosan and GRAS substances (Table 1), being the variation factors. Analysis of variance was performed for all the variables evaluated, the normality of the data was verified, and Fisher LSD tests ($p \leq 0.05$) were performed for the comparison of means ± the standard error (SE = $s/\sqrt{N}$). All analyses were performed with the statistical software Statistica 64 ver. 12 [28].

3. Results and Discussion

3.1. Identification of Pathogen Causing Black Spot on Avocado Fruit

The BLAST search of the primers used, ITS1las-ITS4las, Bt2Alas-Bt2Blas, EF1-728Flas and EF1-986R, showed between 99 and 100% coverage and identity with *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips [29], and the phylogenetic tree shows the same pathogen (Figure 1A). The cottony aerial mycelium, initially white and rapidly changing to black (Figure 1B), grows at a rate of 17 $mm \cdot d^{-1}$, of non-septate mycelium (Figure 1D). The results of the pathogenicity test confirm that *N. parvum* is a postharvest pathogenic fungus of 'Hass' avocado (Figure 1C). With this information, it is confirmed that the taxonomy of the pathogen isolated from the black spot of the avocado pulp belongs to *Neofusicoccum parvum*. *N. parvum* has been identified as the cause of black spot on avocado fruit [5] and as the cause of dieback on avocado tree branches [6]. Therefore, its detection in postharvest avocado fruits when ripe for consumption confirms that it is an important fungus that can cause economic losses. In addition, its mention in research articles is increasingly frequent, being considered in a similar way to *C. gloesosporioides* and *L. theobromae*. The current large dispersion of *N. parvum* in avocado is due to the new areas where avocado plantations have been established, where the trees are more stressed (water or salinity) and are more susceptible to pathogen attack and their ability to remain in the fruit in a latent or quiescent way to colonize the fruit in postharvest [30]. In addition, a high incidence of black spot has been reported in postharvest avocado fruits from trees with diseases caused by *N. parvum* such as cancer and progressive death of branches in the avocado tree [31].

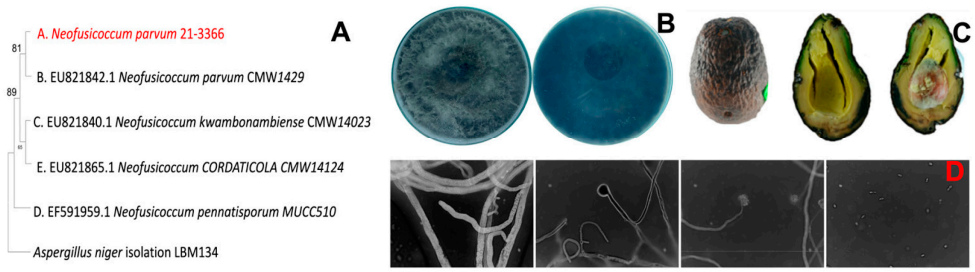

Figure 1. (**A**) Maximum likelihood tree showing relationship of *N. parvum* isolate based on ITS1las-ITS4las, Bt2Alas-Bt2Blas, EF1-728Flas and EF1-986R; (**B**) Morphological identification of *N. parvum*; (**C**) Pathogenicity test in 'Hass' avocado fruits; and (**D**) Non-septate mycelium, conidiogenous and spore of *N. parvum* (objective 40×).

3.2. In Vitro Inhibition of Non-Chemical Treatments Based on Chitosan and Natural Compounds on N. parvum

The results on the inhibition of the mycelium, the concentration and spore size are shown in Table 2. The effect of the basic and GRAS substance treatments based on chitosan on the inhibition of the *N. parvum* mycelium was different ($p < 0.05$). The control was the only one that did not present inhibition. The rest of the treatments presented inhibitions higher than 80%. The best treatments were ChiSA, CNEO-50/50, CNEO-75/25, and NCEO. The concentration of spores presented the same behavior as the inhibition of the mycelium, the control being the one with the highest production of spores compared to the rest of the basic and GRAS substance treatments, including the fungicide ($p < 0.05$). Spore size also presented significant differences ($p < 0.05$); those with the smallest size were those of ChiBe, the largest ones were those of Chi 1.0%, and the rest presented sizes like those of the control.

Table 2. Effect of basic and GRAS substance treatments based on chitosan and natural compounds on the inhibition of the mycelium, the concentration and spore size of *N. parvum*.

Treatments	Inhibition of Mycelial Growth (%)	Sporulation (1×10^6 Spores/mL)	Spore Size (µm)
1	0 ± 0 f [1]	12.8 a	4.8 ± 0.3 ab
2	90.3 ± 2.2 cd	0.171 b	4.8 ± 1.1 ab
3	89.9 ± 2.7 cd	0.0291 b	7.5 ± 2.7 a
4	90.9 ± 3.9 c	0.176 b	4.4 ± 0.9 ab
5	96.5 ± 3.9 b	0 b	ns
6	87.7 ± 1.2 e	0.148 b	5.7 ± 0.7 ab
7	100 ± 0 a	0 b	ns
8	89.4 ± 3.1 d	0.0943 b	3.8 ± 0.4 b
9	100 ± 0 a	0 b	ns
10	100 ± 0 a	0 b	-
11	95.5 ± 3.6 b	0.0352 b	5.7 ± 1.3 ab
12	100 ± 0 a	0 b	-

[1] Different letters indicate that values with the same letter in the column are not significantly different ($p \leq 0.05$) as determined using ANOVA and Fisher's LSD test (n = 6).

Micrographs of the effect of basic and GRAS substance treatments on the development of *N. parvum* are shown in Figure 2. Chitosan deposition was observed in most cases, formation of nodules (Figure 3A,B,F,G), dehydration or loss of turgor in all basic and GRAS substance treatments, along with reduced mycelium diameter (Figure 3A,B,D,E,G,H), collapsed hyphae (Figure 3G,F, treatments with essential oil), disorganized hyphae (Figure 3A,B,G,H) and little mycelium production (Figure 3F, treatment with ChBe).

Numerous works confirm the effect of retarding or completely inhibiting the growth of the mycelium with chitosan in concentrations ranging from 0.5 to 2% for pathogens isolated from avocado such as *Colletotrichum gloeosporioides* and *C. siamense* [20,32,33], which agrees with our results. This inhibitory effect of chitosan may be due to the formation of a dense film in the mycelium (deposition), limiting the excretion of metabolites and nutrient assimilation, which limits metabolic processes and signal transduction to the nucleus [20]. Although the deposition effect is better in the spore than in the mycelium, treatments with NCEO (50, 75 and 100%) managed to completely inhibit the viability of the mycelium [11]. The mechanisms of antifungal action of organic and inorganic salts (potassium sorbate, sodium benzoate, sodium silicate and salicylic acid) are the buffer action (pH modification) and the osmotic level that inhibit the action of the enzymes responsible for solubilization and expansion of the cell wall. The buffering effect alters the integrity and permeability of the membrane and the transport of nutrients, leading to cell inactivation and death, the first site of action being the wall and membrane of the fungus [34–36].

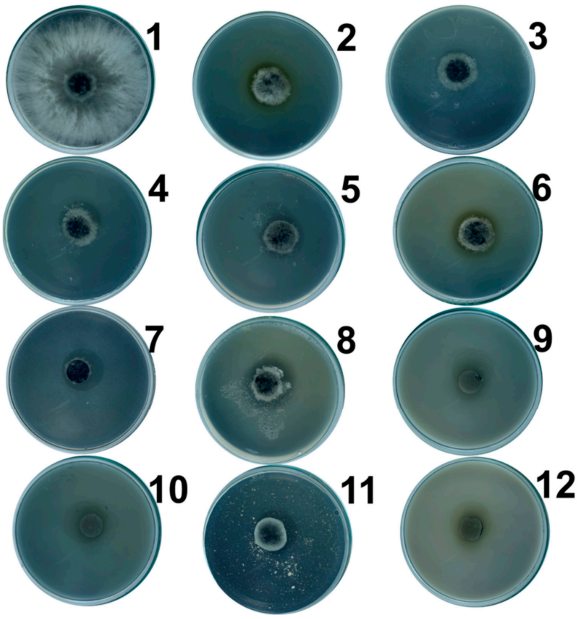

Figure 2. Effect of basic and GRAS substance treatments based on chitosan and natural compounds on the inhibition of the mycelium, the concentration and spore size of *N. parvum*. (**1**) Control, (**2**) Chi 0.5%, (**3**) Chi 1.0%, (**4**) Chi 1.5%, (**5**) ChiSP, (**6**) ChiSA, (**7**) ChiSi, (**8**) ChiBe, (**9**) CNEO-50/50, (**10**) CNEO-75/25, (**11**) Fungicide and (**12**) NCEO.

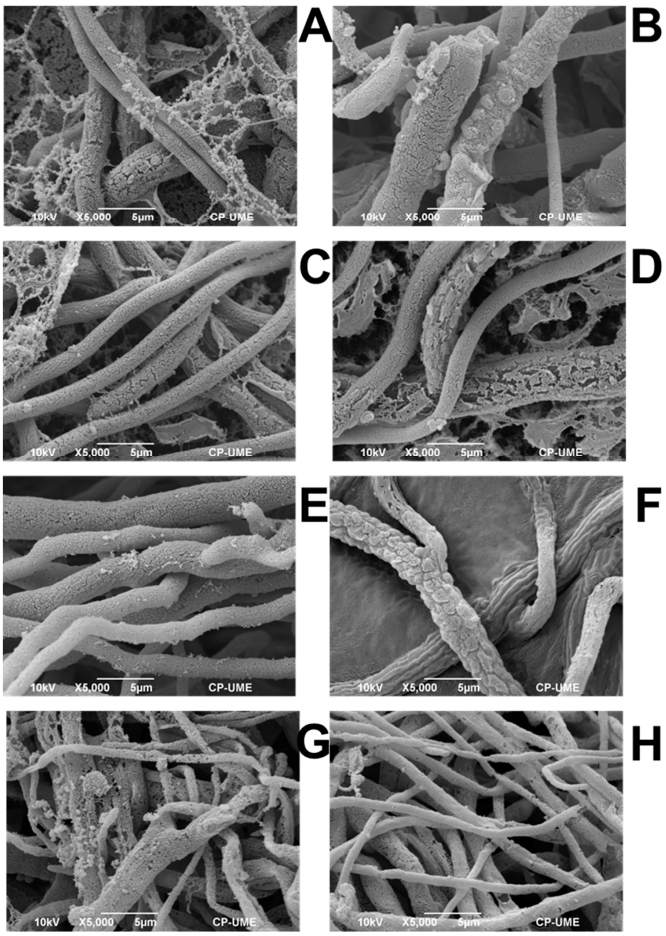

Figure 3. Scanning electron micrographs of *N. parvum* mycelium isolated from postharvest avocado, taken 5 μm. (**A**) Chi 1.0%, (**B**) Chi 1.5%, (**C**) ChiSP, (**D**) ChiSA, (**E**) ChiSi, (**F**) ChiBe, (**G**) CNEO-50/50 and (**H**) NCEO.

3.3. Postharvest Quality

3.3.1. Skin Color

The luminosity, chromaticity, and hue at the beginning of storage were different between the treatments ($p < 0.05$). However, this was not because of the treatments, but rather due to the fact that fruits harvested in Michoacán, Mexico, between April and May of each year have a dry matter content higher than 35% and a color skin change, from green to black, of between 25 and 75% [37]. On day 5 of storage, all treatments decreased in luminosity with significant differences, with ChiSi having the lowest luminosity. At the end of the storage, the luminosity was less than 27 (blacker) with differences between the treatments ($p < 0.05$); although the control had the lowest value, all the fruits already had a black color (Figure 4A). The chromaticity change (C*) during storage was different between the treatments ($p < 0.05$). However, the values were within the parameters of an avocado fruit when ripe for consumption. Finally, the change in the hue angle presented differences during storage ($p < 0.05$); although the hue values of all the treatments are in the green region, the final color of the fruit is determined by lightness and chromaticity (Figure 4).

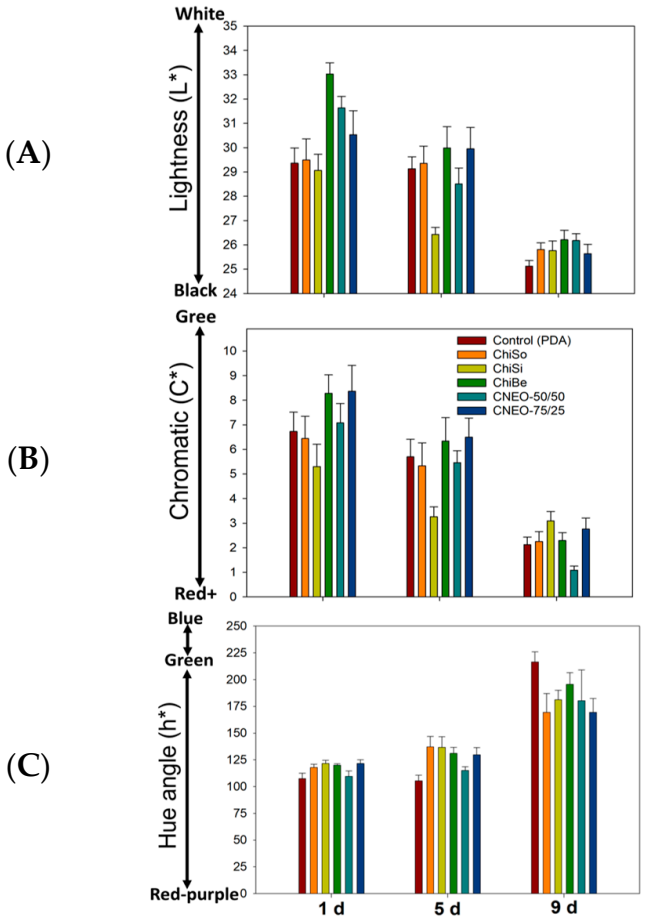

Figure 4. Effect of basic and GRAS substance treatments based on chitosan and natural compounds on the change in luminosity (**A**), chromaticity (**B**) and hue (**C**) of 'Hass' avocado fruit. Data are presented as mean ± standard error (n = 10).

3.3.2. Weight Loss

The weight loss in fruits with basic and GRAS substance treatments based on chitosan and natural compounds is shown in Figure 5. Weight loss at the end of storage was different between treatments ($p < 0.05$), the Control and ChiSP treatments being the ones with the greatest loss. The treatment with the least weight loss was ChiSi. The decrease in weight loss indicates that most of the basic and GRAS substance chitosan-based treatments are efficient in reducing gas exchange (respiration), due to the semi-permeable barrier that is reinforced

against water loss (limiting movement of the water). Treatments based on chitosan and natural compounds show that the coatings have a barrier effect, due to a modified internal atmosphere, as well as a hydrophobic activity. A lower water loss maintains high fruit quality during storage. The greater amount of water lost is given by transpiration than by respiration. However, respiration is related to metabolic processes of maturation; when respiration is limited, vital activities are reduced, maintaining quality and prolonging postharvest life. Although weight loss is a normal process during maturation, it is altered by the colonization of pathogenic fungi, which accelerate this loss. Because pathogenic fungi alter the integrity of the cuticle, increased transpiration and more water loss result. The least weight loss occurred in the mixture of chitosan and sodium silicate; the mode of action of sodium silicate is the formation of a film below the cuticle, which makes the fruit less susceptible to attack by pathogens [11]. Weight loss becomes an important parameter for consumers that determines acceptability by the consumer, since it is directly related to diseases and the apparent quality of the fruit, in addition to the fact that high weight loss reduces its commercial value. The use of chitosan in avocado postharvest decreases weight loss during storage because it forms a barrier. By slowing down the respiration process, the production of energy from carbohydrate stores is limited. In addition, the loss of water is responsible for the apparent dehydration of the fruit [21,38–40].

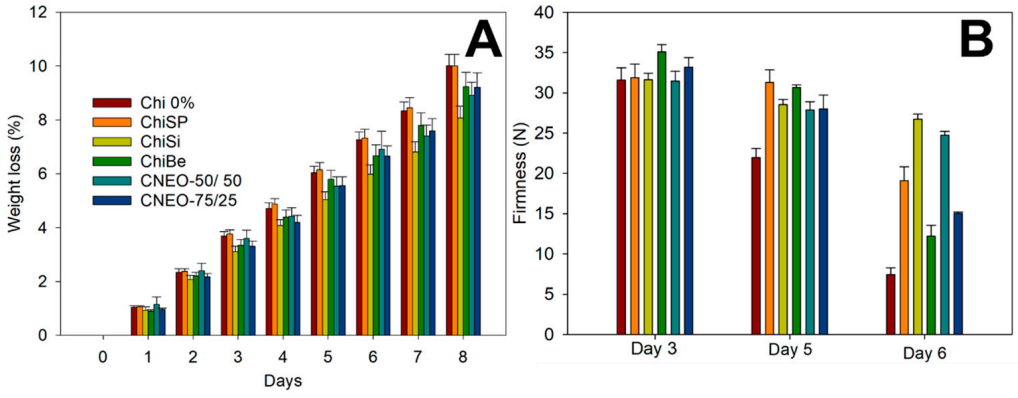

Figure 5. Effect of basic and GRAS substance treatments based on chitosan and natural compounds on (**A**) the weight loss and (**B**) firmness of 'Hass' avocado fruits stored at room temperature. Data are presented as mean ± standard error. n = 10 (**A**) and n = 3 (**B**).

3.3.3. Firmness

The firmness of the fruit between the treatments was different ($p < 0.05$, Figure 5B). The control treatment was the one that presented the lowest average firmness on day 6 of storage. The rest of the treatments presented an average firmness greater than 15 N; ChSi was the one with the greatest firmness on day 6. It has been reported that the application of chitosan reduces the changes in the ultrastructures of the cell wall of the fruit, decreasing the activity of enzymes that degrade pectin and cause cell disorder [20,41]. In addition, the formation of a film under the cuticle of the fruit by sodium silicate further decreases these degradative processes of the cell wall [11]; therefore, the high values of firmness in this study are attributed to this effect.

3.3.4. Apparent Quality of the Fruit

The effect of the treatments based on chitosan and GRAS substances was different ($p < 0.05$, Figure 6) for all the evaluated aspects. In all cases, the control was the one with the highest values: fruits with greater external rot, pulp and peduncular, as well as apparent dehydration (shrivels). In this last aspect, all the treatments presented high values, with the appearance of visible striations on the shell. This may be due to or is related to the loss of water through transpiration with the environment, rather than through fruit respiration, although it is also attributed to high ripening temperatures [23]. Storage conditions, especially relative humidity during storage, can affect this characteristic in the

present study. On the other hand, fruit rots were lower in the treated fruits than in the control. This indicates that in addition to having a fungicidal effect on the fungus, they could also activate defense mechanisms in the fruit.

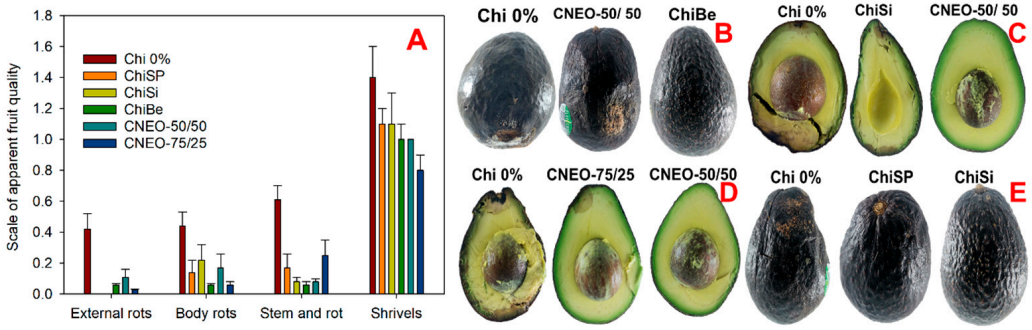

Figure 6. Effect of basic and GRAS substance treatments based on chitosan and natural compounds on the apparent quality (**A**): external rot (**B**), body rot (**C**) stem and rot (**D**) and shrivel (**E**) of 'Hass' avocado when ripe for consumption. Data are presented as mean ± standard error (n = 30).

3.3.5. Enzymatic Activity of PAL, POD and PPO

The enzymatic activity of PAL, POD and PPO was different between the treatments ($p < 0.05$) and is presented in Figure 7. For the PAL at 12 h, the activity in the treated fruits was higher than in the control, which presented its highest activity at 24 h. At 48 h, most of the treatments were superior to the control (Figure 7A). These results coincide with those reported by [20,42], who indicate that the greatest PAL activity occurs between 12 and 24 h and that this is associated with the upregulation of expression genes involved in the systemic acquired response. PAL is an enzyme considered fundamental in the synthesis of phenols, phytoalexins and lignin and is considered to be the main response against biotic or abiotic stress in plants [20]. The treatments also showed differences in the POD activity (Figure 7B), the CNEO-50/50 and CNEO-75/25 treatments, a mixture of chitosan and cinnamon essential oil, being those of gradual growth and greater than the rest up to 48 h, including all controls. The highest PPO activity occurred at 12 h and 24 h with significant differences ($p < 0.05$) (Figure 7C). The treatments with ChiSi and CNEO-50/50 were the most active. Although POD and PPO are considered enzymes that are induced by stress, since they prevent the accumulation of hydrogen peroxide (H_2O_2), oxidizing the latter's substrates in the extracellular spaces, both POD and PPO are precursors of lignin and phenols (highly toxic quinones) when attacked by a pathogen [43,44].

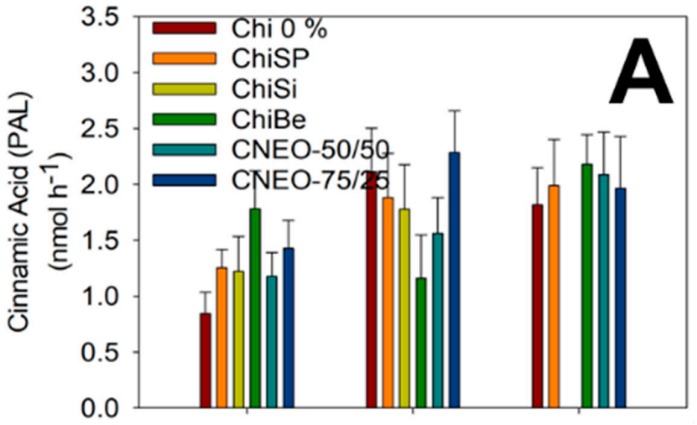

Figure 7. *Cont.*

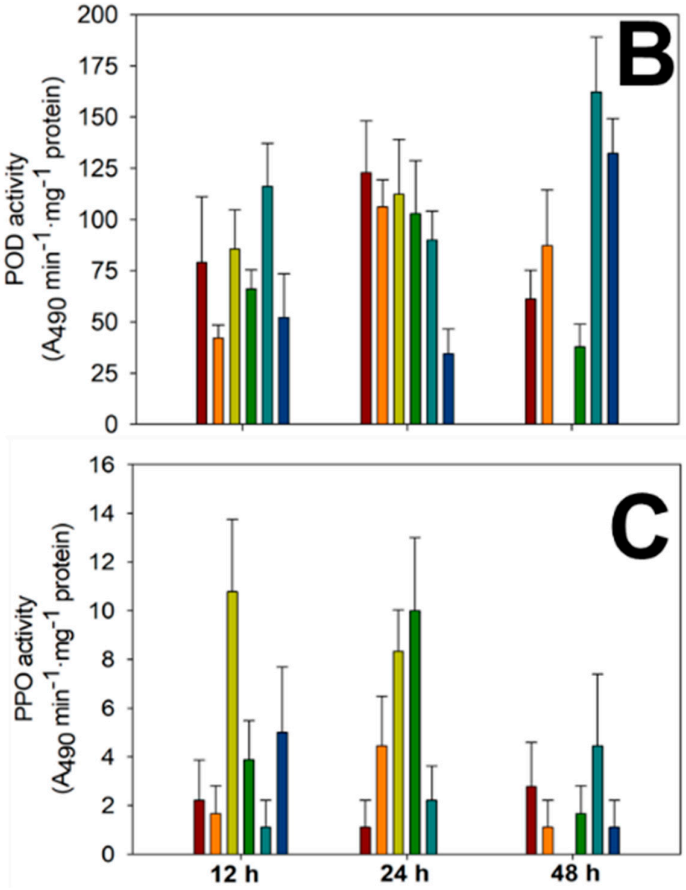

Figure 7. Effect of basic and GRAS substance treatments based on chitosan and natural compounds on the enzymatic activity of (**A**) phenylalanine ammonium lyase expressed as cinnamic acid (PAL); (**B**) peroxidase (POD); and (**C**) polyphenol oxidase (PPO) in avocado fruit peel. Data are presented as mean ± standard error (n = 3).

4. Conclusions

The basic and GRAS substance treatments, based on chitosan and GRAS substances, had a fungicidal effect against *N. parvum*, inhibiting its mycelial development and sporulation in vitro. The treatments with chitosan and cinnamon essential oil were the most efficient in the control of *N. parvum*. All the treatments induced the activity of the PAL, POD, and PPO enzymes, presenting an effect of inducing defense mechanisms in the fruit. In addition, the basic and GRAS substance treatments maintained the quality of the fruit in postharvest. Treatments with chitosan and cinnamon essential oil were the most efficient.

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