

REGULAR ARTICLE

# Chitosan-propolis combination inhibits anthracnose in 'Hass' avocados

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## ABSTRACT

The effect of chitosan-propolis combination on the control of *in vitro* and *in vivo* growth of *Colletotrichum gloeosporioides*, a causal agent of anthracnose, as well as on the quality of avocado of Hass cultivar has been evaluated. Chitosan and propolis were added to the culture medium separately and in combinations to verify the efficacy of inhibition of *C. gloeosporioides* mycelium *in vitro*. Avocados were immersed for 1 minute in the treatments that best inhibited the mycelium growth (*in vitro*) with the aim of testing the fungus control *in vivo*. The tested treatments were 1.5% chitosan and combinations of propolis (1.0%, 1.5%, 2.0%) with 1.5% chitosan and then inoculated with *C. gloeosporioides*. Control fruits were dipped in distilled water only. In the second experiment, avocados were immersed for 1 minute in the treatments that best inhibited the mycelium growth (*in vivo*) with the aim of testing the fruits quality. The tested treatments were 1.5% chitosan isolated and in the combination of 1.5% chitosan with 1.5% propolis. The control treatment were immersed only in distilled water. Avocados were stored at 22 ± 0.3 °C and 90 ± 3% RH for 7 days. The use of 1.5% chitosan in combination with 2.0% propolis controlled the mycelial growth of *Colletotrichum gloeosporioides in vitro*. In addition, coating with only 1.5% chitosan provided the best results in avocados, reducing the severity and incidence of anthracnose and maintaining the fruit quality.

**Keywords:** *Colletotrichum Gloeosporioides*; Natural Products; *Persea Americana*; Post-Harvest; Quality

## INTRODUCTION

The increasing demand for fruits by consumers relies on the fact that they are sources of vitamins, minerals and can help in prevention of numerous diseases. Avocado (*Persea americana* Mill.) stands out because it has high nutritional value, which explains its economic importance. It has fibers, proteins, minerals and vitamins, mainly A and B (Francisco and Baptistella, 2005; Nieto-Angel et al., 2006; Oliveira, 2000). It also contains a significant amount of unsaturated fatty acids that have beneficial effects in the prevention of cardiovascular diseases (Tango et al., 2004).

The avocados production in Brazil is around 195,000 tons, according to FAO (2016), where Hass and Fuerte cultivars are the most commercialized in the external market. Characteristics of these varieties, such as higher lipid content, smaller diameter, thicker bark and lower water content, in relation to the 'Manteiga' cultivar, makes the pulp more consistent and possible to use in salty dishes. The

fruit is the 25<sup>th</sup> best selling product in CEAGESP, Brazilian main public market, with 46,455.10 tons of avocado sold in 2016 (CEAGESP, 2018).

Post-harvest fungal diseases represent one of the main causes of tropical fruit losses during the commercialization period (Dantas et al., 2003). Among the diseases, anthracnose caused by the fungus *Colletotrichum gloeosporioides*, is considered one of the most important diseases of tropical fruits and affects all the avocado producer countries (Avila-Quezada, 2007; Pegg et al., 2002).

Natural products has been shown as an efficient alternative for use in the control of fungal diseases because they have an antimicrobial effect and because they are considered a natural alternative to synthetic fungicides (Combrinck et al., 2011; Kouassia et al., 2012). In this context, we can highlight the propolis that presents chemical compounds with antimicrobial properties (Mattiuz et al., 2015).

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Chitosan, a natural and biodegradable compound, has been used as film or edible coating, also acting to reduce microbial deterioration, proving to be a potential product in the protection of plants against pathogens (Bautista-Baños et al., 2006). This polymer has an effect on the control of post-harvest pathogens when applied alone or in combination with other natural products (Ramos-García et al., 2012).

The aim of this study was to evaluate the chitosan associated with propolis in the control of the *in vitro* and *in vivo* growth of *Colletotrichum gloeosporioides*, causal agent of anthracnose, and its effects on the quality of 'Hass' avocados.

## MATERIAL AND METHODS

*C. gloeosporioides* was isolated from naturally infected 'Hass' avocados. The natural products, with antifungal properties, were propolis (P) from aqueous extract of Propomax®, and chitosan (C) with low molecular weight and degree of deacetylation of 75-85% (Sigma-Aldrich Co). The chitosan was diluted in distilled water with addition of 1% acetic acid in the volume and the pH corrected to 5.6 with 0.1 M NaOH as described by Jiang and Li (2001).

### *In vitro* experiment

In a total, the effect of 11 treatments was investigated. The treatments included the incorporation of propolis and chitosan to the culture medium separately or in the combinations such as:

[1] Control (PDA), [2] C1.5%, [3] P1.0%, [4] P1.5%, [5] P2.0%, [6] C1.0% + P1.0%, [7] C1.0% + P1.5%, [8] C1.0% + P2.0%, [9] C1.5% + P1.0%, [10] C1.5% + P1.5%, [11] C1.5% + P2.0%

Six replicates were performed per treatment. The propolis at different concentrations was incorporated into the culture medium (PDA) at 50-55 °C after autoclaving. The chitosan solutions were obtained by mixing part of the dissolved product with the PDA culture medium until the desired concentration. In all treatments 0.01% Tween 80 was added.

The warmed culture media were poured under aseptic conditions (laminar flow chamber), into sterile Petri dishes 9 cm in diameter. After solidification of the medium, 5 mm diameter mycelium discs were transferred from the edge of the colonies to the center of the plates containing the treatments. The plates, containing the fungus, were maintained at  $27 \pm 1$  °C and photoperiod of 12h.

The mycelial growth of the fungus was measured daily with the aid of a digital caliper (Mitutoyo, model CD-6), until

the mycelium of one of the treatments reached the edge of the Petri dish. The percentage inhibition of mycelial growth was calculated by the formula of Yang et al. (2011):

$$IM = \frac{dc - dt}{dc - di}$$

Where: IM = percentage of mycelial inhibition; dc = is the mean diameter of the control colony; dt = is the mean diameter of the treatment colony; di = is the initial diameter of the fungal colony on the plates.

### *In vivo* experiment - effect on fungus control in avocados

'Hass' avocados were harvested at Jaguacy company located in the municipality of Bauru (22°14'7" S and 49°8'27" W, 532 m altitude), at the stage of physiological maturation. The fruits were washed with neutral detergent, rinsed in running water and sanitized in Sumaveg® solution (Sodium Dichloroisocyanurate Dihydrate) at 0.33%, for 5 minutes.

The suspension of conidia of *Colletotrichum gloeosporioides* was obtained by surface scraping of the colonies in the presence of distilled and sterilized water, and 0.01% Tween 80, and after that, the filtration was done on sterilized Whatman filter paper, obtaining a conidial suspension of the pathogen in the concentration of  $2 \times 10^5$  conidia mL<sup>-1</sup>.

The fruits were inoculated through wounding with chromatography syringe at two opposite points in the equatorial region. The inoculated volume was 25 µL of the conidial suspension of *C. gloeosporioides* ( $2 \times 10^5$  conidia mL<sup>-1</sup>), and then stored for 10 h at 22 °C, for fungus colonization. After this period, the fruits were immersed for 1 minute in the combinations of propolis and chitosan that best inhibited fungus mycelial growth *in vitro*: [1] Control – water dipped, [2] C1.5%, [3] C1.5% + P1.0%, [4] C1.5% + P1.5%, [5] C1.5% + P2.0%.

After drying and coating, the fruits were stored at  $22 \pm 0.3$  °C and  $94 \pm 5.5\%$  RH.

The experiment was conducted in a completely randomized design, consisting of five treatments (4 concentrations + control). Four replicates were used with 5 fruits each.

After 7 days, the disease severity analyzes were performed by measuring the size of the lesion in two perpendicular directions on the fruit; and the incidence of the disease was determined according to Xing et al. (2010) using the following equation:

$$\text{Incidence of disease (\%)} = \frac{\text{Number of infected wounds}}{\text{Total number of inoculated fruits}} \times 100$$

### **In vivo experiment - effect of treatments on fruit quality**

Avocados were immersed for 1 minute in the treatments (C1.5% and C1.5% + P1.5%). These treatments are chosen based on results of second experiment and chosen treatments showed best performances in fungus control in comparison to other treatments. In the control treatment, fruits were immersed only in water.

The experiment was conducted in a completely randomized design, consisting of 3 treatments (2 concentrations + control). Four replicates were used with 5 fruits each.

The fruits respiration rate and the ethylene production were evaluated according to the methodology of Marques (2015).

The content of acetaldehyde and ethanol were determined according to methodology adapted from Davis (1969). Samples of 1g of crushed pulp were transferred to 50 mL flasks, sealed and stored frozen until analysis. Prepared samples were incubated in a water bath on a 50 °C for 30 minutes. After 30 minutes, 1.0 mL of headspace air of the flask was collected and injected into Trace GC Ultra, Thermo Scientific, equipped with (FID) and Porapack N column separation. The acetaldehyde and ethanol contents were calculated by correlating the respective chromatographic areas with those obtained in the standard curves. Results were expressed as µg acetaldehyde or ethanol per gram of plant material.

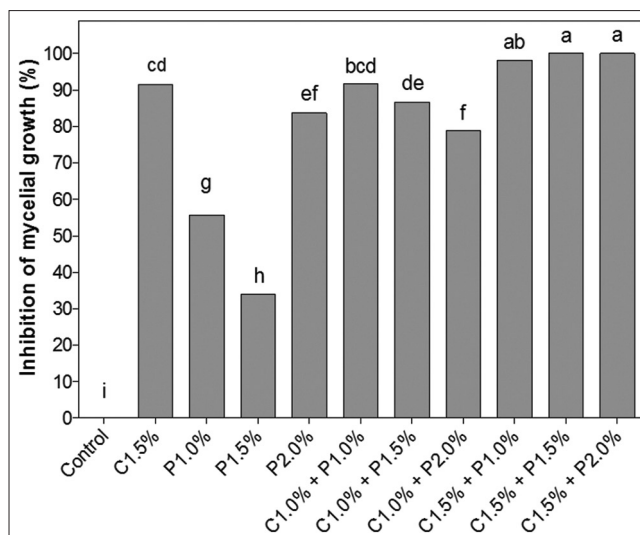
### **Data analysis**

Data were subjected to analysis of variance (ANOVA) to examine the effects of treatments and their interactions. The means values of treatments and interactions with significant effect were compared by the Tukey test ( $P \leq 0.05$ ). Statistical analyzes were performed using Sisvar software, version 5.6 and graphs were made using GraphPad Prism software, version 6.0.

## **RESULTS AND DISCUSSION**

### **Effect of propolis and chitosan on the mycelial growth of *Colletotrichum gloeosporioides***

The isolated effect of the natural products in the inhibition of mycelial growth *in vitro* showed that for propolis, the concentration of 2.0% obtained the highest percentage of inhibition (83%) (Fig. 1). Mattiuz et al. (2015) demonstrated that the concentration of 2.5% of ethanolic propolis extract (EPE) controlled in 100% the *in vitro* mycelial growth of *C. gloeosporioides*. Similarly, EPE of Chinese origin prevented the *in vitro* mycelial growth of *Penicillium italicum* (Yang et al., 2011). Propolis has demonstrated broad spectrum antimicrobial activity



**Fig 1.** Effect of propolis and chitosan on the mycelial growth of *Colletotrichum gloeosporioides* after 7 days at 27 °C. Means with equal lowercase letters do not differ from each other, by the Tukey test ( $P < 0.05$ ) ( $n = 6$ ). C = chitosan; P = propolis.

against bacterial species, yeast, and various fungal species including phytopathogenic fungi such as *Botrytis*, *Aspergillus*, *Alternaria* Nees (Campana et al., 2009; Silici et al., 2005; Umthong et al., 2009). Propolis has chemical compounds such as flavonoids, phenolic acids and esters, quinones, coumarins, sesquiterpenes, and steroids, providing antibiotic properties on it (Markham et al., 1996). According to Castro et al. (2009), flavonoids and phenols are the main natural antibiotic compounds of this product, for example Artepillin-C, present in EPE used by Mattiuz et al. (2005), which has proven antimicrobial activity (Kimoto et al., 2001). These antimicrobial properties described for propolis may explain the favorable results observed in the present study, as well for its action associated with chitosan in the control of the pathogen.

Although propolis at 2.0% presented high mycelial control (83%), treatment with 1.5% chitosan was superior (91%) and differed significantly from propolis (Fig. 1). *In vitro* studies using chitosan at concentrations of 2.5% and 3.0% also demonstrated that this product had a fungicidal effect against *C. gloeosporioides*, controlling mycelial growth, also causing changes in the morphology of the conidia of the pathogen (Bautista-Baños et al., 2003; Bautista-Baños et al., 2006). In addition to this pathogen, scientific studies prove the antimicrobial properties of chitosan in the control of fungi *in vitro* and *in vivo*, especially *Rizopus Stolonifer*, *Penicillium digitatum* and *Fusarium oxysporum* (Palou et al., 2016; Romanazzi et al., 2017).

The combination of chitosan at 1% with increasing concentrations of propolis reduces the inhibition of

mycelial growth of the fungus. When the concentration of 1.5% of chitosan is used, there is an inverse effect, that is, the percentage of inhibition increases with the concentration of the associated propolis (Fig. 1).

Chitosan at 1.5% and propolis at 2.0% inhibit *in vitro* growth of the pathogen, with about 91% and 84%, respectively. However, when we combine chitosan 1.5% and propolis, we achieved significantly higher results, reaching the control (100%) of mycelial growth, in the highest concentrations (C1.5% + P1.5% and C1.5% + P2.0%) (Figure 1).

#### Effect of propolis and chitosan on 'Hass' avocados inoculated with *Colletotrichum gloeosporioides*

The severity of the anthracnose was higher in the fruits of the control (31 mm), which differed significantly from the other treatments (Figure 2A). The best results were verified with the application of chitosan and its combination with the concentrations of 1.5 and 2.0% of propolis. Comparing with control fruits, the application of chitosan allowed a reduction of 67% in disease severity in avocados.

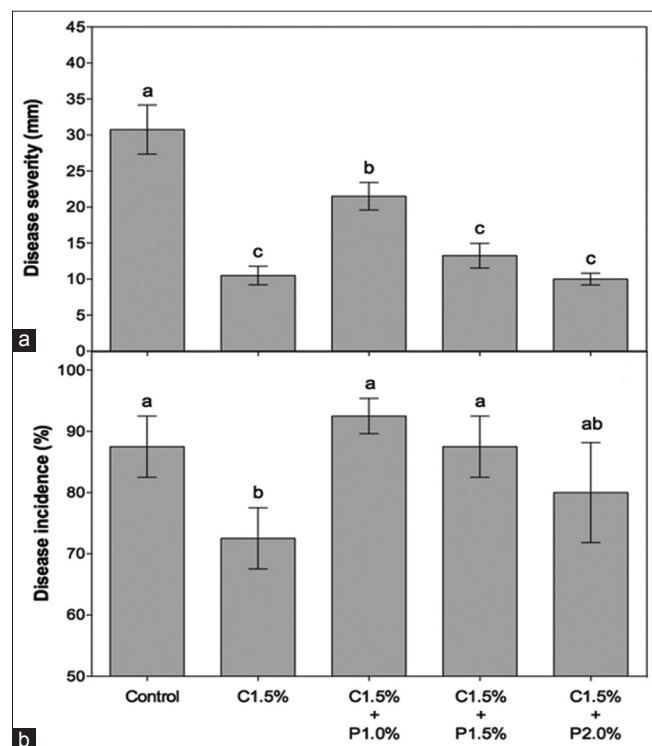
Although the incidence of *C. gloeosporioides* in avocados was high, chitosan application at 1.5% was the treatment that most reduced the incidence of the disease when compared to the fruits of the control (Figure 2B). Many studies have

demonstrated that chitosan, isolated or associated with other compounds, is effective in the control of post-harvest pathogens, such as anthracnose caused by *C. gloeosporioides* in oranges (Deng et al., 2015) and in mangos (Mattiuz et al. 2015), *Penicillium digitatum* or *Penicillium italicum* in grapefruit (Zeng et al. 2010), *Botrytis cinerea* in table grapes (Romanazzi et al., 2012).

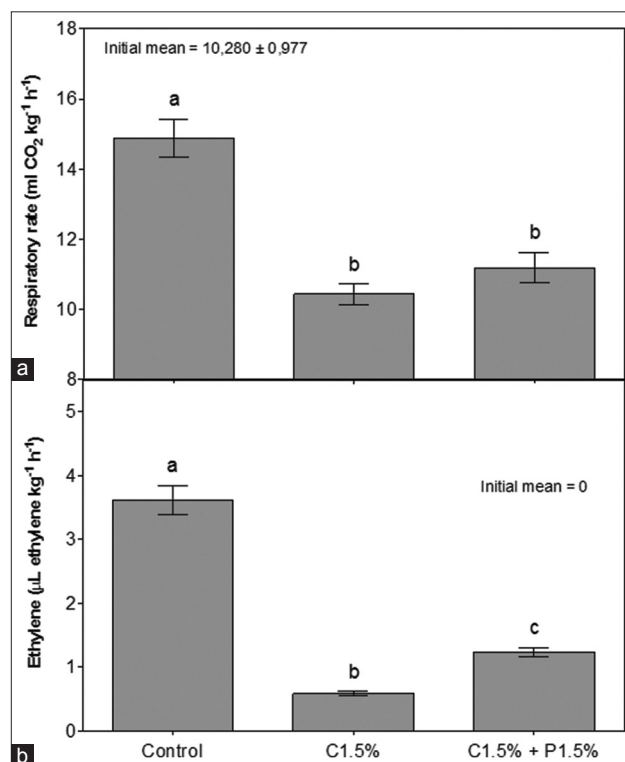
There is a considerable interest in the use of chitosan in agriculture because its triple action makes it an ideal coat for post-harvest use of fruits and vegetables: antimicrobial activity in plant pathogens, filmogenic activity that acts as a barrier and ability to develop elicitors that are plant defense mechanisms (Romanazzi et al., 2017).

As for the combination of chitosan with propolis, there is a reduction in both the severity and the incidence of the disease in proportion to the propolis concentration (Fig. 2), similar effect as *in vitro* growth results (Fig. 1). However, the best result of the combination (C1.5% + P2.0%) did not differ significantly from chitosan at 1.5%, demonstrating that, in the case of mycelial growth in fruits, the application of 1.5% chitosan would be enough to control the disease.

#### Effect of the combination of propolis and chitosan on the quality of avocados 'Hass'



**Fig 2.** Effect of chitosan and propolis on severity (A) and incidence (B) of anthracnose on 'Hass' avocados inoculated with *Colletotrichum gloeosporioides* and stored at  $22 \pm 0.3$  °C and  $94 \pm 5.5\%$  RH for 7 days. Averages  $\pm$  standard error with equal lowercase letters do not differ by Tukey test ( $P < 0.05$ ) ( $n = 4$ ). C = chitosan; P = propolis.



**Fig 3.** Effect of propolis and chitosan on respiratory rate (A) and ethylene (B) production of 'Hass' avocados stored at  $22 \pm 0.3$  °C and  $84 \pm 4.2\%$  RH for 7 days. Averages  $\pm$  standard error with equal lowercase letters do not differ by Tukey test ( $P < 0.05$ ) ( $n = 4$ ). C = chitosan; P = propolis.



The respiratory rate of control fruit increased at the end of the storage time ( $15 \text{ mL CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ), differing significantly from treatments C1.5% and C1.5% + P1.5% (Fig. 3A). Although not significantly different from the C1.5% + P1.5% treatment, the treatment with 1.5% chitosan had the lowest respiratory rate ( $10.4 \text{ mL CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ), with similar means to that of beginning of the evaluation. Similar behavior was observed for the ethylene production of avocados after 7 days of inoculation (Fig. 3B), where the 1.5% chitosan treatment had the lowest averages ( $0.5 \mu\text{L ethylene kg}^{-1} \text{ h}^{-1}$ ), significantly different from controls ( $3.6 \mu\text{L ethylene kg}^{-1} \text{ h}^{-1}$ ) and treatment with propolis ( $1.2 \mu\text{L ethylene kg}^{-1} \text{ h}^{-1}$ ).

At ambient temperature, the avocados present a maturation characterized by a rapid increase in the respiratory rate with consequent increase in the ethylene production, characterizing a very fast climacteric peak (Chitarra and Chitarra, 2005; Kosiyachinda and Young, 1975). The results observed in this work show the effect of the coating formed by the natural products used, in the modification of the atmosphere around the fruits, that reduced the respiration rate and the ethylene production, thus increasing the shelf life of the avocados and the maintenance of fruit quality.

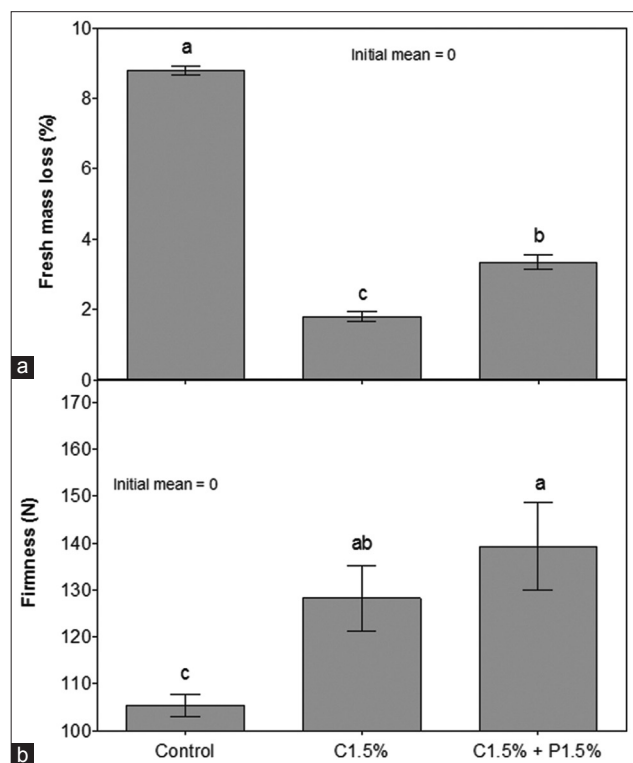
The lowest loss of fresh weight occurred in fruits treated with chitosan 1.5% (1.8%), followed by the combination

of chitosan with propolis, which differed significantly from the control (8.8%) (Fig. 4A). The fruits of the control lost 3.9 times more fresh mass than the avocados coating with chitosan. Research has shown that the film formed by chitosan forms a barrier against the diffusion of water vapor, thus reducing the loss of water by the vegetable and consequently its weight loss (Ali et al., 2011; Bautista-Baños et al. 2006).

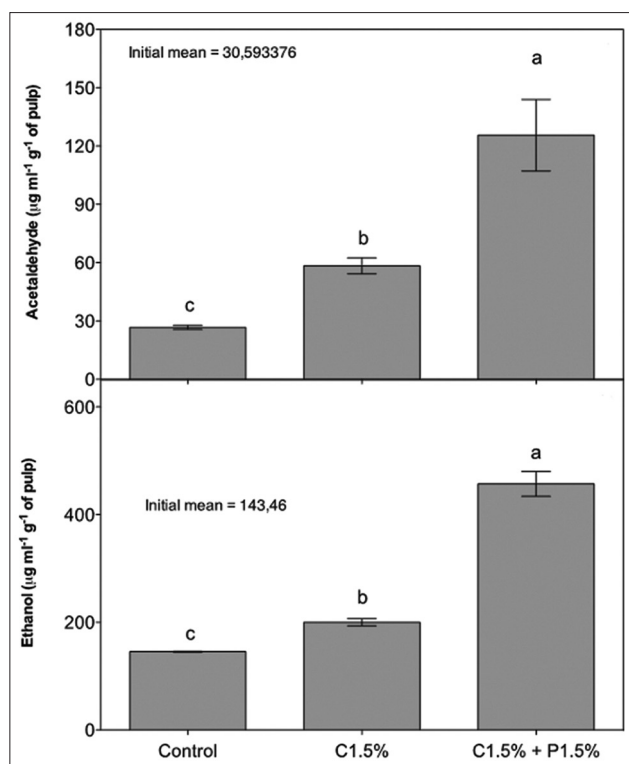
The firmness of the fruit pulp was reduced at the end of the 7 days of storage, and the fruits treated with chitosan at 1.5% and with propolis associated with chitosan at concentrations of 1.5% presented higher firmness values when compared to fruits (Fig. 4B). Firmness is a very important qualitative attribute for the consumer, associated with the useful life of the fruits. The maintenance of the firmness of the treated fruits is related to the smaller loss of fresh mass that occurred in these treatments because the fruits with greater cellular turgescence present, consequently, greater resistance to the pressure. Tissue softening (loss of firmness) in fruits is also due to the action of enzymes responsible for the degradation and solubilization of pectins, and by modifications of the cell wall composition (Toivonen and Brummell, 2008). As observed in this work, the formation of a semipermeable barrier in the chitosan avocados allowed the maintenance of firmness both by reducing the loss of fresh mass and by the delay of fruit ripening processes, as observed by several authors (Ali et al., 2011, Silva et al., 2018).

Regarding the content of acetaldehyde produced by avocados, after 7 days of storage, treatments with coating (C1.5% and C1.5% + P1.5%) presented the highest averages when compared to the control treatment (Figure 5A and 5B). These results were expected since, in fruits coated with chitosan, active modified atmosphere conditions occur (Palou et al., 2016). This condition can become beneficial when the  $\text{O}_2$  content decreases and  $\text{CO}_2$  levels increase, to the point of creating an ideal modified atmosphere around the fruit, causing a decrease in the metabolic process and, consequently, the extension of the useful life of the fruit or vegetable. However, depending on the type of coating, concentration, application and additives incorporated, the concentration limits of these two gases can be overcome and trigger the anaerobic (fermentative) breathing process, culminating in the development of acetaldehyde and ethanol, which are toxic to cells.

Acetaldehyde is naturally formed during fruit maturation (Pesis, 2005), however, according to Hribar et al. (2000), when the concentration of this compound in the fruits is high, may be toxic to the plant cells and, as a defense mechanism of the fruit, acetaldehyde is quickly converted to ethanol, considered a less toxic substance.



**Fig 4.** Effect of propolis and chitosan on fresh mass loss (A) and firmness (B) of 'Hass' avocados stored at  $22 \pm 0.3 \text{ }^\circ\text{C}$  and  $84 \pm 4.2\%$  RH for 7 days. Averages  $\pm$  standard error with equal lowercase letters do not differ by Tukey test ( $P < 0.05$ ) ( $n = 4$ ). C = chitosan; P = propolis.



**Fig 5.** Effect of propolis and chitosan on acetaldehyde (A) and ethanol (B) content of 'Hass' avocados stored at  $22 \pm 0.3$  °C and  $84 \pm 4.2$ % RH for 7 days. Averages  $\pm$  standard error with equal lowercase letters do not differ by Tukey test ( $P < 0.05$ ) ( $n = 4$ ). C = chitosan; P = propolis.

**Table 1: ANOVA analyses of results of inhibition of mycelial growth (IMG) in vitro, Disease severity (DS), Disease incidence (DI), Respiratory rate (RR), Fresh mass loss (FML), Firmness (Firm), ACE=Acetaldehyde (ACE), Ethanol (ETA) in "Hass" avocados**

Sources of variation	Probability >F <sup>1</sup>									
	IMG	DS	DI	RR	Ethylene	FML	Firm	ACE	ETA	
Treatment (A)	**	**	**	**	**	**	**	**	**	**
Time (B)	-	**	**	**	-	-	**	-	-	-
Interaction (A x B)	-	**	**	**	-	-	**	-	-	-

IMG=Inhibition of mycelial growth; DS=Disease severity; DI=Disease incidence; RR=Respiratory rate; FML=Fresh mass loss; Firm=Firmness; ACE=Acetaldehyde; ETA=Ethanol. \*\*\*  $P < 0.01$

Although no changes were observed in the aroma and fruit flavor that indicated the evolution of the fermentation process, which could compromise its organoleptic characteristics, it is expected that a good coating will prevent fruits to produce high levels of acetaldehyde and ethanol. In this study, the coating using 1.5% chitosan resulted in levels of these compounds closer to the control fruit.

These results showed the positive effect of chitosan and its combination with propolis on fungus control. This polymer may act as a barrier to the flow of nutrients present in fruits

and reduce the availability of nutrients for the growth of the pathogen, since chitosan-exposed fungal cells often exhibit signs of nutrient deficiency (Ait Barka et al., 2004, El Ghaouth et al., 2000).

The ANOVA analyses of the experimental results can be found on the Table 1.

## CONCLUSIONS

The chitosan, at a concentration of 1.5%, associated with propolis at 2.0%, promoted the control of the mycelial growth of *Colletotrichum gloeosporioides* in vitro; while the coating with only 1.5% chitosan, provided the best results in avocados, reducing the severity and incidence of anthracnose, maintaining fruit quality.

## Authors contributions

A.K.M.: experiments, data analyses, writing of manuscript. J.S.P.J.: writing of manuscript. K.M.M.: experiments, data analysis, writing of manuscript. B.H.M.: Proposal of the research topic, design of experiments, critical revision of manuscript.

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