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Antifungal Activity of Chitosan Biofilms Enriched with Stingless Bee Honey Against *Colletotrichum gloesporioides*

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ABSTRACT

Purpose: *Pathogens that affect agricultural products after harvest are controlled using several strategies during the postharvest process. To accomplish this, refrigeration, controlled atmospheres, ethylene absorbers, coatings, and biofilms are used. The antifungal activity of chitosan biofilms added to other polymers (starch, wax and bovine serum albumin) mixed with stingless bee honey (Melipona beecheii, M. solani and Scaptotrigona mexicana) was evaluated.*

Research Method: *The biofilm combinations were performed as follows; honey origin (M. beecheii, M. solani, and S. mexicana), concentration (10%, 20%, 30%), and polymers (starch, S. mexicana wax and bovine serum albumin). The overlay method against Colletotrichum gloesporioides was used to evaluate the antifungal activity, the same as the physical and mechanical characteristics.*

Findings: *Biofilms made with a) 30% of M. beecheii honey + 3g of starch, b) 30% + 20g of S. mexicana honey and wax, and c) 20% of S. mexicana honey + 20g of S. mexicana wax registered the highest growth inhibition values against C. gloesporioides (77.6%, 76.6% and 73.4%). The one made with 20% of S. mexicana honey and wax showed the highest value in thickness (103,660.57 m), as well as a greater resistance in terms of breaking strength (20,133.95 N).*

Research Limitations: *It was not easy to design biofilms with stingless bee honey, due to their humidity and pH content, until we got the exact combination.*

Value: *This research provides information and knowledge to generate better biofilms with more feasible properties that keep the development of pathogens in postharvest fruit restricted.*

Keywords: *Biopolymers, chitosan, Melipona beecheii, M. solani, Scaptotrigona Mexicana*

INTRODUCTION

The Agricultural production from Mexico places 12th worldwide, and 3rd in Latin America; with 11,812,000 tons of fruit trees produced in 2016 throughout Chiapas, Oaxaca and Colima (SAGARPA, 2017). Nonetheless, harvested fruits are affected by the great diversity of pathogens during the post-harvest stage being fungi one of the main problems (Suárez-Quiroz *et al.*, 2013). Therefore, several strategies of pathogen control are evaluated and applied; refrigeration, controlled atmospheres, ethylene absorbers and biofilms or coatings (Escobar-Hernández *et al.*, 2014).

Biofilms are continuous matrices made up of biopolymers that are applied to fresh fruits to reduce the presence and damage of microorganisms (Porta *et al.*, 2013; Salvador-Figueroa *et al.*, 2017). They are made from one or more biopolymers, such as lipids, polysaccharides, and proteins (Park and Bae, 2006; Adetunji *et al.*, 2012; Poverenov *et al.*, 2014).

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Chitosan is one of the polysaccharides which make more resistant and flexible biofilms. It also added antifungal properties (Albarracín and Valderrama, 2014). Recent studies have reported that when combining chitosan with other materials, for instance, essential oils like cinnamon (*Cinnamomum verum*), thyme (*Thymus vulgaris* L.), and Aloe vera gel the antifungal effect against *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* is increased (Manoj *et al.*, 2016; Salvador-Figueroa *et al.*, 2017; Monzón-Ortega *et al.*, 2018). In papaya fruits (*Carica papaya*), chitosan biofilms added with other molecules (Bakkali *et al.*, 2008; Wang *et al.*, 2011; Alvarado *et al.*, 2011), registered values up to 45% inhibition of C. *gloeosporioides*. Some other substances could be added to chitosan biofilms, such as honey and wax, being honey a source of antimicrobial properties due to its physicochemical characteristics; moisture content, acidity, sugars, peroxidase activity, phenol and flavonoid content, among others (Palomino *et al.*, 2010; Moussa *et al.*, 2011; Manzanares *et al.*, 2014). In the Tropics, this sweet substance called "honey" is also produced by stingless bees, whose product is different in their physicochemical composition, and that has shown an antimicrobial effect even higher than that reported by *Apis mellifera* honey (Vit *et al.*, 2004; Guerrini *et al.*, 2009; Albores-Flores *et al.*, 2018). Wax is composed of alcohol esters and fat acids including lactone, flavonoids, alcohols and free fat acid. The main physicochemical properties are solubility in lipids and solved by organic solvents. They are secreted by young stingless bees produced in abdominal glands deposited in small white lies mixed with vegetable resins and it is used to build honey pots (Shanahan and Spivak, 2021). Therefore, this study aimed to evaluate the antifungal activity of biofilms made with chitosan, and added with stingless bee honey and wax of different species of bees (*Melipona beecheii*, *Melipona solani* and *Scaptotrigona mexicana*) from the Soconusco region against *C. gloeosporioides*, which is one of the main post-harvest fruit pathogen identified in this region.

MATERIALS AND METHODS

Honey and Wax

The honey used in this study was collected in March 2017 from local stingless beekeepers from the main producer species; *Melipona beecheii*, *Melipona solani* and *Scaptotrigona mexicana*. The samples were placed in sterile amber labeled bottles and refrigerated at 4°C until use. The wax of Scaptotrigona mexicana was bought from "Asociación de Meliponicultores del Soconusco, S. C. del R. L", which was chosen due to its quantity and to evaluate its effect in biofilms.

Colletotrichum gloeosporioides Strain

Pathogens were provided by the "Instituto de Biociencias" strain repository, which was isolated from papaya fruits. The pathogen strain was seeded in the center of Petri dishes containing Potato Dextrose Agar (PDA) culture medium at a pH of 7.0 and incubated at 30 ± 2°C for mycelial growth.

Biofilms Design

We used chitosan of PM = 340.33 g mol^{-1} and 75-85% deacetylation, glacial acetic acid, Tween 20® (Sigma-Aldrich®), glycerol (Meyer®), cassava starch, bovine serum albumin (Sigma-Aldrich®), stingless bee honey, and wax. The biofilm combinations were performed under a multifactorial design 3x3, with the following variables; honey origin (M. beecheii, M. solani and S. mexicana), honey concentration (10%, 20%, 30%), and three different polymers (starch, S. mexicana wax, and bovine serum albumin) (Table 01). As a control, we made biofilms of each polymer separately without adding stingless bee honey and a control treatment only with chitosan. Biofilms were made with a stock solution of chitosan (15 gL^{-1}) dissolved in water with acetic acid (10 mL L^{-1}) where 1 mL L^{-1} of glycerol was added and kept under stirring for 24 h at 30°C. After that, the stingless bee honey was added separately (10%, 20%, or 30% (v/v)), then the polymer, starch (3 gL^{-1}) or S. mexicana wax (20 gL^{-1}) or bovine serum albumin (0.5 gL^{-1}) was added based on the treatment (n=5). In particular, for wax treatments, 1 mL of Tween was added to form the emulsion and heated at 60°C for 30 min. In all treatments after homogenization, we poured 7 mL of this solution into sterile Petri dishes (60 mm in diameter) at 30°C until biofilm formation.

Biofilms Antifungal Activity

We used the overlay method to evaluate the antifungal activity of biofilms (Mayr-Harting, 1972). In the center of Petri dishes containing PDA, a fragment (3 mm in diameter) was placed, and then on top of it, a biofilm circle (diameter of 10 mm) was placed. Subsequently, the plates were incubated for 7 days at 30 ± 2°C. The fungus growth inhibition was performed by measuring the size of the diameter of the halo around the biofilms by using a Vernier (Stainless). We measured the diameter every 24 hours for 7 days, besides a photographic recording with a high definition camera (Cannon fix 14 Mp).

Fungus growth inhibition was recorded on day 7 with the equation I = [(Ac - Ar) / Ac] x100 where I am

Table 1: Biofilms treatment; stingless bee honey (species and concentration) and biopolymer (T1 to T30). T31 was made only with chitosan (control)

Stingless bee honey	Type and amount of polymer (g L^{-1})		
Species and concentration (%)	Starch (3)	Wax (20)	Bovine serum albumin (0.5)
M. b- 10	T1	T2	T3
M. b- 20	T4	T5	T6
M. b- 30	T7	T8	T9
M. s- 10	T10	T11	T12
M. s- 20	T13	T14	T15
M. s- 30	T16	T17	T18
S. m- 10	T19	T20	T21
S. m- 20	T22	T23	T24
S. m- 30	T25	T26	T27
Control A	T28		
Control C		T29	
Control P			T30

**M. b= Melipona beecheii, M. s=Melipona solani, S. m= Scaptotrigona mexicana, 10, 20, 30 are the concentrations*

growth inhibition (%), Ac is the control growth area and Ar is the area of mycelium growth in treatment (Li *et al.*, 2015).

Fungus growth rate with the biofilms was determined with the formula μ (d-1) = (InX - InX0) / Δt based on the 7 days of inhibition, where is the growth rate, d is the halo diameter, X is the treatment growth value at a time, "7 days"; X0 is the growth at a time, "zero", In is the logarithm Neperian and Δt is the difference between time "7 days" and time "zero".

Physical and Mechanical Characteristics of Biofilms with the Best Antifungal Properties

We determined the following physical and mechanical characteristics of the biofilms with the best antifungal properties: force required to infringe rupture (FR) using a handheld penetrometer (Tr® Italy), equipped with an 8 mm diameter tip; solubility (S) according to the method proposed by Wang *et al.*, (2011); water retention (RA) and humidity (H) following the method described by Binsi *et al.*, (2013). The thickness was measured with a digital micrometer (Fowler®).

Data Analysis

The results were analyzed by an analysis of variance and a subsequent contrast of means by the Tukey test (95% confidence). The same analysis was used for the physico-mechanical characterization of biofilms with the best antifungal activity. We used the Statgraphics CenturionMR version XVII statistical program.

RESULTS AND DISCUSSION

Biofilms Antifungal Activity

The results obtained from the *C. gloeosporioides* in vitro growth with the biofilms from the different treatments are shown in Table 02.

Biofilms made of chitosan + 30% *S. mexicana* honey + wax, registered inhibition values of 77.6%, followed by chitosan treatment + 30% *M. beecheii* honey + starch (76.6% inhibition value) and chitosan + 30% *M. beecheii* honey + wax (73.4% inhibition value). These results differed from biofilms made with chitosan + starch, wax and bovine serum albumin without honey (47.9, 43.5, and 41.1% respectively). Significant differences were found between treatments ($P < 0.05$).

Fungal growth inhibition was observed from the second day of exposure to the biofilm in most of the treatments, thereby recording a decrease in the value of the growth rate (Fig.01). It is interesting to note the contrast between other studies performed with biofilms, such as Manoj *et al.*, (2016), where inhibition values ranged between 20 and 30% against phytopathogenic fungi with chitosan biofilms + A. vera as a coating on peppers. Fungal growth inhibition from the second day of exposure to the biofilm in most of the treatments could be caused by the amount of moisture content existing between the biofilm-fungus complex, as well as the starch, wax, and protein components. These

Table 2: Antifungal activity of biofilms against *Collethotricum gloeosporioides*

Treatments			Inhibition (%)
Species	Stingless bee honey concentration (%)	Biopolymer	X±EE
Melipona beecheii	10	Starch	65.5 ± 3.06 de
	10	Wax	75.8 ± 4.33 abcde
	10	Bovine serum albumin	36.4 ± 3.0 h
	20	Starch	70.8 ± 2.74 abcd
	20	Wax	33.8 ± 4.33 fgh
	20	Bovine serum albumin	47.0 ± 3.5 fgh
	30	Starch	76.6 ± 3.06 ab
	30	Wax	73.4 ± 2.74 abc
	30	Bovine serum albumin	47.8 ± 3.5 f
M. solani	10	Starch	54.3 ± 3.06 e
	10	Wax	53.4 ± 4.33 abcde
	10	Bovine serum albumin	72.6 ± 3.0 abc
	20	Starch	48.7 ± 3.06 abcde
	20	Wax	66.5 ± 3.06 abcd
	20	Bovine serum albumin	70.1 ± 3.5 abcd
	30	Starch	56.8 ± 4.33 f
	30	Wax	60.9 ± 3.54 abcde
	30	Bovine serum albumin	66.3 ± 3.0 abcd
Scaptotrigona mexicana	10	Starch	65.1 ± 2.74 cde
	10	Wax	63.5 ± 3.5 bcde
	10	Bovine serum albumin	52.4 ± 3.5 f
	20	Starch	67.1 ± 3.0 e
	20	Wax	77.6 ± 3.5 a
	20	Bovine serum albumin	39.9 ± 3.5 fgh
	30	Starch	69.5 ± 2.7 abcde
	30	Wax	72.1 ± 3.0 abcde
	30	Bovine serum albumin	38.8 ± 2.7 i
		Starch	47.9 ± 2.7 fg
		Wax	43.5 ± 2.7 fgh
		Bovine serum albumin	41.1 ± 2.7 gh

* Same letters are not significantly different (P>0.005)

compounds could regulate the transport of the bioactive molecules from the honey by convection dependent on the moisture content of the fungus mycelium at the time of contact, which is proposed by González-Brambila and López-Isunza (2007). On the other hand, the effectiveness of this complex lies in the control of the concentration of active agents that migrated in moisture conditions towards the surface of the agent in contact, generating a dosage of these.

Garde-Izquierdo (2014), indicated that the delay in the migration in these films could depend on several factors, such as; the type of molecule used as a structural matrix, the additive compound used to ensure the mobile compound and the physicochemical characteristics of the substance used as the third mobile compound (Bourtoom, 2008). Hromiš *et al.*, (2017), indicated that the bioactive components transferred through the biofilm surface are greater when hydrophilic components are used, despite the hydrophobic ones, where the migration or kinematics movement occurs 24 hours after the contact existed. In our study, we registered this activity at 48 hours, where the delay observation is due to the presence of chitosan and beeswax, which are substances with low hydrophobicity properties (Kong *et al.*, 2010; Altoik *et al.*, 2010). These substances reduced the migration movement of biomolecules with antifungal activity to the surface of the mycelium, which could influence the amount of antifungal molecules that received the fungus through honey biofilms, registering lower values in fungal colonial development (Albores-Flores *et al.*, 2018).

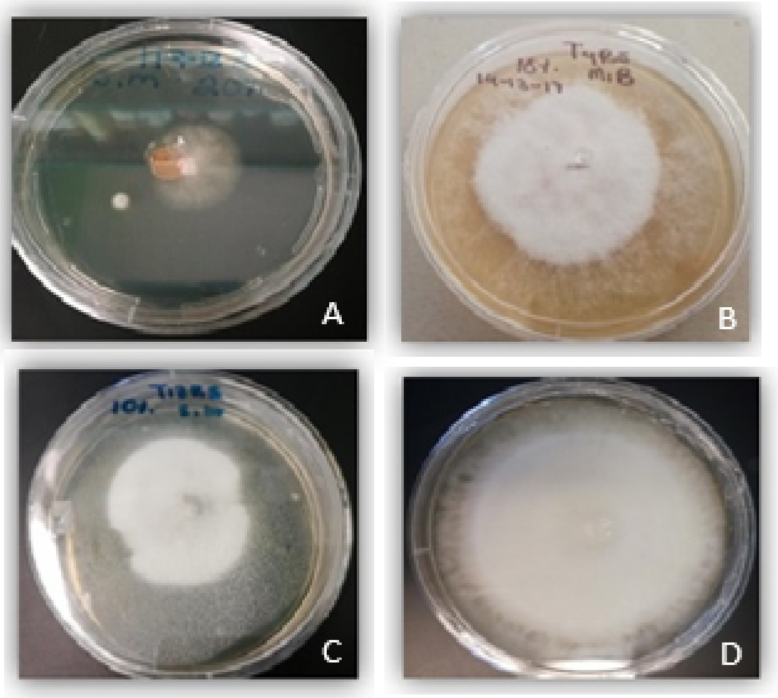

Figure 1: ***In vitro*** **activity of biofilms made with stingless bee honey + chitosan and biopolymers; A) 10%** ***S. Mexicana*** **honey+ starch, B) 10%** ***M. beecheii*** **honey + wax, C) 20%** ***S. mexicana*** **honey + wax, D) Control**

According to Hong *et al.*, (2000) and Talens *et al.*, (2005), honey properties such as acidity, hydrogen peroxide content, and phytochemicals (phenols, flavonoids and aromatic acids) can inhibit fungal growth. The low water activity (aw), the low redox potential (due to the high content of reducing sugars), and the presence of chemical agents such as benzyl alcohol, among others (Han *et al.*, 2006) are also responsible for such activity. Moreover, the stingless bee honey used in this study is reported to a 35% humidity, 24-100 meq kg^{-1} of acidity, as well as a phenol and flavonoid content greater than reported in A. mellifera honey (Espinoza-Toledo *et al.*, 2018). All of these variables are related to the antimicrobial activity reported in recent studies against C. gloesporioides and Staphylococcus aureus (Albores-Flores *et al.*, 2018).

Physical and Mechanical Characteristics of Biofilms with the Best Antifungal Properties

Biofilms with higher percentages of inhibition (77.6%, 76.6%, and 73.4%) were physical-mechanical analyzed. considering the ones with 30% *M. beecheii* honey + starch; 30% *M. beecheii* honey + wax and 20% *S. mexicana* honey + wax, which were statistically different ($P<0.05$) from the rest and showed the highest values in thickness and therefore greater measured resistance as the breaking force.

Regarding to physical and mechanical characteristics of biofilms, our results are similar to those reported by Thakhiew *et al.*, (2010). The best treatments contained the lowest volume of stingless bee honey, which could be related to the water content (<35%, Espinoza-Toledo *et al.*, 2018), because when interacting with chitosan, could allow greater plasticization of the film, and thus formed more flexible films with less mechanical resistance.

The water content in stingless bee honey had an impact on drying speed during biofilm production, where polysaccharides, such as chitosan, are hydrophilic and allow hydrogen bond formation, constituting a very efficient barrier against oxygen and promoting water entrapment (Wu *et al.*, 2015); in our study, we registered a drying time (with stingless bee honey) within 6 and 8 days, which is longer than the data observed with chitosan biofilms (3 to 4 days). Biofilms with biopolymers showed a smooth appearance, without pores or visible cracks on the surface, e.g.; 20% of *S. mexicana* honey + beeswax were thicker (Table 03), while the smallest thickness was registered by biofilms with 30% of *M. beecheii* honey + starch, with a greater solubility. These results could be related to stingless bee honey concentration, where the treatment with the highest value registered a greater solubility, while biofilms that contained wax as a polymer registered lower values in water retention capacity and solubility, therefore lipids added could reduce water vapor permeability (Guimaraes-Farias *et al.*, 2012). Thus, lipid-based biofilms are often used as a moisture barrier (Bourtoom, 2008).

Table 3: Average results ± standard deviation of biofilms; solubility, the force required to infringe rupture, thickness, and water retention

Treatments	Solubility (%)	Force required to infringe rupture (N)	Thickness (m)	Water retention (%)
30% Melipona beecheii honey + starch	24.5 ± 0.71^{ab}	13.80 ± 8.33^{c}	82.33 ± 0.57^{c}	49.66 ± 5.50^{ab}
30% M. beaches honey + wax	21.0 ± 0.0^{b}	14.33 ± 5.94^{b}	83.66 ± 0.57^{b}	32.66 ± 3.05^{b}
20% Scaptotrigona mexicana honey + wax	19.0 ± 1.41^{c}	20.13 ± 3.95^{ab}	103.66 ± 0.57^{ab}	19.33 ± 2.08^{c}

*The same letters are not significantly different (P>0.005)

Biofilms that contained the highest concentration of honey exhibited the highest values in retained water content, solubility and humidity. On the contrary, the biofilms where the honey concentration was maintained at 10% had the lowest values. The moisture content is directly associated with solubility, which is an important variable since in many cases greater solubility is desired, associated with the degradation of the biofilm. On the contrary, low solubility values are desirable when the biofilms are sought to have greater resistance and lower material transfer rate (water vapor, gases) (Wang *et al.*, 2011).

CONCLUSION

Biofilms designed with stingless bee honey showed the highest antifungal activity against *C. gloesporioides* during the 7 days of study. The use of this type of honey could be an alternative in fruit treatment against this fungus, as well as in the post-harvest stage. Therefore, we should continue with this study to focus our efforts on natural alternatives for organic post-harvest treatments.

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