Anthracnose control by Mexican Oregano (*Lippia berlandieri* Schauer) essential oil added to edible films

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Abstract

Edible films can incorporate antimicrobial extracts to provide microbiological stability on food surfaces. The aim of this study was to evaluate chitosan or starch edible films added by selected concentrations of Mexican oregano (*Lippia berlandieri* Schauer) essential oil to control anthracnose. Chitosan and starch edible films were formulated with essential oil concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 2.00, or 4.00%. Mold radial growth was evaluated by modified Gompertz equation. A significant ($p < 0.05$) change of Gompertz parameters was observed among essential oil concentrations, increasing the lag phase and decreasing radial growth rates as oil concentration increased.

Introduction

Parasitic diseases, physiological disorders and senescence normally cause postharvest losses. Anthracnose disease caused by species of Colletotrichum is one of the most economically important plant diseases and is responsible for reducing the marketable yield of tropical fruits such as avocado, guava, papaya, mango and passion fruit. Is one of the most worldwide and devastating genus of plant pathogenic fungi specially during storage (Bajpai *et al*., 2009; Ali *et al*., 2010; Intra *et al*., 2011). *Colletotrichum gloeosporoides* inocula in the form of conidia spread from dying infected petioles of the lower leaves of the plants. Under favorable conditions, the conidia develop appressorium from which infection penetrates; stays the skin of fruits but stay quiescent until the fruit ripens (du Plooy *et al*., 2009; Saravanakumar *et al*., 2011).

Application of chemical fungicides slows the spread of anthracnose, however *C. gloeosporioides* develops resistance to fungicides. Thus recent studies have investigated naturally occurring substances able to act as safe alternative antimicrobials (Koppula *et al*., 2010; Du *et al*., 2011). In these sense essential oils (EOs) have demonstrated antimicrobial activity to different kind of microorganisms than other natural extracts (Burt, 2004; Holley and Patel, 2005). Nevertheless when EOs are directly applied on food surfaces by dipping, powdering or spraying, their highly hydrophobic and volatile active substances are bound by food components. To avoid this problem an alternative is incorporating EOs within edible films (Tunc *et al*., 2007; Avila-Sosa *et al*., 2012). The use of edible films and coatings in preservation has recently increased, for food product protection, improving quality and shelf life without impairing consumer acceptability (Du *et al*., 2012).

Interest in development of natural preservative coatings with plant essential oils to protect food against bacterial pathogens and spoilage organisms is increasing due the safety aspects of chemical additives. Several studies have shown that incorporation of essential oils into films may not only enhance the films antimicrobial properties but also reduces water vapor permeability (Fontes *et al*., 2011; Hager *et al*., 2012). Edible antimicrobial films have shown an efficient alternative in controlling food contamination. In the last years, research is concerning in the use of edible films for surface application of different antimicrobial compounds (Flores *et al*., 2007; Jiang *et al*., 2012). The aim of this study was to evaluate chitosan or starch edible films with added Mexican oregano (*Lippia berlandieri* Schauer) essential oil to control *C. gloeosporioides*.

Materials and Methods

Essential oil

Mexican oregano (*L. berlandieri* Schauer) EO was provided by CiReNA (Natural Resources Research Center of Salaices, López, Chihuahua, Mexico), which was obtained by vapor distillation for 4 h with a Cleavenger-type apparatus.

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C. gloeosporioides isolation and inoculum preparation

C. gloeosporioides was isolated from infected papaya fruits (*Carica papaya* L.). Fruits showing lesions and characteristic symptoms of fungal infection were collected at local market. These fruits were washed with tap water, and small portions (0.5 cm²) of contaminated epidermis were cut off and then disinfected with a 1% solution of sodium hypochlorite (Aldrich Chemical Co. Milwaukee, WIS) for 1 min. After disinfection, tissue portions were washed thoroughly three times with sterile distilled water to eliminate chloride residues. Each portion cut was placed in Petri dishes (100 x 15 mm) containing potato-dextrose agar plates (PDA Merk, Mexico), and the dishes were stored in the dark in an incubator at 28°C for 5-8 days until fungal growth was observed. Fungus structure (conidia and mycelia) was observed with an optical microscope (Zeiss Primo Star, Göttingen, Germany), and identification was according to published taxonomic key (Barnett and Hunter 1986). Once *C. gloeosporioides* was isolated, an aqueous solution containing 2500 spores/mL (using a hemocytometer) was prepared from the fungal growth to purify it, and by means of a capillary tube, spores were transferred to the center of another Petri dish containing PDA medium, which was incubated in the dark for 8 days at 28°C (Peraza-Sánchez *et al.*, 2005). In order to recover fungal spores, sterile physiological water was poured on growth agar plate surface, followed by a gentle scraping using a sterile rake to remove the maximum quantity of spores. After that, spore suspensions were transferred into sterile tubes. The number of spores present in the suspension was determined using a hemocytometer and an optical microscope (Zeiss Primo Star, Göttingen, Germany), and expressed as number of spores per milliliter (spores/mL) (Sebti *et al.*, 2005).

Films preparation and casting

All edible films (chitosan or starch) were made by the casting method, which consists of drying the corresponding film forming solution (FFS) that has been applied on a support. Chitosan films were formed according to Zivanovic *et al.* (2005), medium molecular weight (450 kDa) of chitosan (Aldrich Chemical Co. Milwaukee, WI) was prepared with 1.5% w/w chitosan in 1.5% v/v acetic acid; the solution was stirred overnight at room temperature. Chitosan solutions were sterilized at 121°C for 15 min. To enable film formation, Tween 20 (Aldrich Chemical Co. Milwaukee, WI) 0.5% v/v was added as plasticizer. One g of high amylose corn starch

(CPI Ingredients, Mexico) was mixed with 10 mL of previously sterilized 0.25 N sodium hydroxide and 10 mL distilled water. FFS were maintained 60 min under stirring conditions. Starch FFS was gelatinized in a shaker water bath at 78–80°C for 10 min; when the solution was near 40°C, glycerol (1.2% v/v) was added (Bertuzzi *et al.*, 2007). FFSs were mixed (IKA High Performance Disperser T18, Chicago, IL) under aseptic conditions at 20,000 rpm for 1 min at room temperature with the incorporation of EO at 0.00%, 0.25%, 0.50%, 0.75%, 1.00%, 2.00%, or 4.00% (v/v) final concentration and poured into 60 mm inner diameter sterile Petri dishes. Films were prepared with 7 mL of FFS per Petri dish (1 film), dried under 0.35 kg/cm² vacuum at 30°C for 12 h. Films were kept in sealed Petri dishes at 4°C until analysis. Films were cut in 5 mm discs under sterile conditions.

Antifungal assay

In order to evaluate the antifungal effect film discs were placed on solidified agar plates. After waiting 30 min for the edible film to adhere to the agar plate, the spore suspension was inoculated onto the discs, dishes were incubated at 25°C. A growth control was prepared in parallel, radial growth was measured every 24 h during 7 d. Every test was performed by triplicate.

Data modeling and statistical analysis

Growth data were modeled using the modified Gompertz equation (Char et al. 2007):

$$Ln\left(\frac{D_t}{D_o}\right) = Aexp\{-exp[(v_{max} \cdot \exp(1)/A)(\lambda - t) + 1]\} \quad (1)$$

where: D_t (cm) is the average colony diameter at time t (d), D_o (cm) is the average colony diameter at initial time; A is the maximum mold growth achieved during the stationary phase, v_{max} is the maximum specific growth rate (cm/d), and λ is the lag phase (d).

To compare the Gompertz parameters statistical analyses were performed with the General Linear Model procedure in Minitab 15 (LEAD Technologies Inc., N.J., U.S.A.). Significantly ($P < 0.05$) different means were separated with Tukey's test.

Results and Discussion

Table 1 shows the EO minimal inhibitory concentration of Mexican oregano (*Lippia berlandieri* Shauer) essential oil was 0.50% for chitosan and starch films. The modified Gompertz equation adequately fits the experimental data (mean coefficient of determination 0.995 ± 0.01) for the growth curves. Fungal growth is inhibited at a lower

Table 1. Modified Gompertz model parameters[*] (mean ± standard deviation) for *Colletotrichum gloeosporioides* growth curves subjected to selected concentrations of Mexican oregano (*Lippia berlandieri* Schauer) essential oil added to chitosan, or starch edible films.

Edible film	Gompertz parameters		
	A	υ_{max} (cm/d)	λ (d)
Chitosan			
0.00%	5.16±0.05[a]	6.07±0.36[a]	2.15±0.01[a]
0.25%	5.02±0.01[b]	6.23±0.18[a]	2.10±0.04[a]
0.50%	–[c]	–[b]	>10[b]
0.75%	–[c]	–[b]	>10[b]
1.00%	–[c]	–[b]	>10[b]
2.00%	–[c]	–[b]	>10[b]
4.00%	–[c]	–[b]	>10[b]
Starch			
0.00%	5.33 ± 0.01[a]	5.07 ± 1.02[a]	2.12 ± 0.03[a]
0.25%	5.26 ± 0.01[b]	4.23 ± 0.73[b]	2.59 ± 0.75[a]
0.50%	–[c]	–[c]	>10[b]
0.75%	–[c]	–[c]	>10[b]
1.00%	–[c]	–[c]	>10[b]
2.00%	–[c]	–[c]	>10[b]
4.00%	–[c]	–[c]	>10[b]

[*]A: maximum mold growth in the stationary phase; υ_{max}: maximum specific growth rate; λ: lag phase.
- No growth
Means followed by a different superscript letter within a column for each studied film (chitosan or starch) are significantly different (p > 0.05).
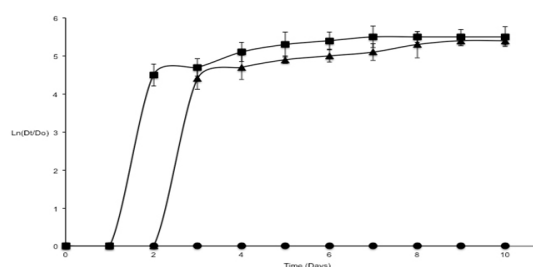

Figure 1. Effect of chitosan edible films added with Mexican oregano (*Lippia berlandieri* Schauer) essential oil at selected concentrations (0% ■, 0.25% ▲, 0.50% ●) on *Colletotrichum gloeosporioides* growth.
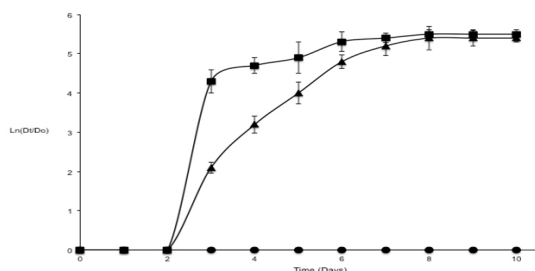

Figure 2. Effect of starch edible films added with Mexican oregano (*Lippia berlandieri* Schauer) essential oil at selected concentrations (0% ■, 0.25% ▲, 0.50% ●) on *Colletotrichum gloeosporioides* growth.

concentration of EO. Statistical analysis of Gompertz parameters showed significant differences (P < 0.05) in maximum mold growth (A) and maximum specific growth rate (υ_{max}) at lower concentrations of Mexican oregano EO added to starch films, and A value in chitosan films. No significant differences (P > 0.05) were found in lag phase for either kind of film, growth curves (Figures 1 and 2) showed that the EO concentration increases fungal radial growth decrease.

Several studies have reported the antifungal activity of different EOs on *C. gloeosporioides* growth. Somda *et al.* (2007) reported a significant inhibition of *C. gloeosporioides* and *Colletotrichum graminicola* with lemongrass, eucalyptus, and neem EOs at concentrations close to 1,000 mg/L. Barrera-Necha *et al.* (2008) reported inhibition of *C. gloeosporioides* with clove and cinnamon EOs at concentrations of 200 to 400 mg/L. The use of lemongrass and basil EOs inhibited appressorium formation, conidial germination, and disruption of conidial activity of *C. gloeosporioides* and *Colletotrichum musae* (Herath and Abeywickrama 2008). Avila-Sosa *et al.* (2011) reported antifungal activity against *C. gloeosporioides* for ethanol and chloroform extracts of Mexican oregano at 400 mg/L and 200 mg/L, respectively. Thymol and carvacrol are the major components of Mexican oregano EO. It has been reported that they are responsible not only for fungal growth inhibition but also bacterial growth inhibition (Conner and Beuchat, 1984). It is considered that thymol and carvacrol attack the cytoplasmic membrane, allowing the leakage of intracellular components; also, they can inactivate cellular enzymes, which would explain the activity against fungal development (Nychas, 1995). Likewise, low O_2 permeability of polysaccharide films including chitosan and starch, can also affect fungal growth, by producing a modified atmosphere around the inoculum, with a reduction in O_2 and an increase in CO_2 (Smith *et al.*, 1987).

Differences in antifungal activities between chitosan and starch films depend on their polymer chemical composition; there are several reports that indicate that chitosan has antimicrobial effect on different microorganisms (Zivanovic *et al.*, 2005; No *et al.*, 2007; Ponce *et al.*, 2008). On the other hand, chitosan films are able to disperse homogeneously different compounds; Suppakul *et al.* (2003) reports that the use of plasticizers is favorable for an increased solubility of EO in water and reduces the interaction with chitosan molecules. In starch films, amylose is responsible for EO retention. Flores *et al.* (2007) added potassium sorbate and mixtures of potassium sorbate-citric acid to starch films, causing a decrease in bacterial and yeast growth.

The incorporation of plasticizers in edible film preparation decreases tension forces between the polymer chains and thereby improves the release of EO (Wilhelm *et al.*, 2003). Garcia *et al.* (2000) observed the presence of pores and fissures in corn starch films with sorbitol and sunflower oil affected antimicrobial characteristics. Cagri *et al.* (2004) established that components utilized to form the edible films certainly affect film structure, which could favor the antimicrobial activity of incorporated EO. Cha and Chinnan (2004) suggested that diffusion effectiveness of an antimicrobial incorporated to an edible film depends on the polarity of the molecule,

its chemical structure and the formation of cross-links between molecules. Valencia-Chamorro *et al.* (2008) reported inhibition of *P. digitatum* and *P. italicum* by addition of several antimicrobials (parabens, potassium sorbate, or sodium benzoate) to methylcellulose edible films.

Nowadays, the incorporation of antimicrobial agents for potential use in films, coatings and packaging is under experimentation. It has been demonstrated in different food types such as meat and bakery products (Cagri *et al.*, 2004). Keeping this in mind, the results of this paper will provide enough information for applying this technology for coating tropical fruits and validate the results with other parameters like ripening and sensory evaluation.

Conclusions

Mexican oregano EO added to edible films can inhibit *C. gloeosporioides* growth by improving the release of the antimicrobial compounds of EO. These edible films could be applied on food surface and become an alternative to control anthracnose.

Acknowledgements

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