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Use of chitosan-based edible coatings in combination with other natural compounds, to control *Rhizopus stolonifer* and *Escherichia coli* DH5α in fresh tomatoes

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A B S T R A C T

During storage of tomatoes, *Rhizopus stolonifer* rapidly spreads towards adjacent fruits causing severe economic losses while *Escherichia coli* may cause serious even life threatening diseases. Chitosan-based materials can be used as edible films or coatings to avoid water loss and microbial spoilage. Waxes and essential oils may also be considered for use as antimicrobial agents in chitosan coating. In this study, various chitosan-based formulations (1%) mixed with beeswax (0.1%), oleic acid (1.0%), and lime or thyme essential oil (0.1%) were tested on tomato at three different maturity stages to control *R. stolonifer* and *E. coli* DH5α at storage temperatures of 12 °C and 25 °C. Control fruit were only dipped in water. Overall, fruit were wounded, coated and inoculated. Once the coatings were applied, 20 µl of *R. stolonifer* spore suspension at 10^5 spores ml^{-1} concentration and 35 µl of bacterial solution of *E. coli* DH5α, at 10^5 cfu μl^{-1}, concentration, were dispensed over the wounded surface. Experiments were carried out *in vitro*, at a small scale and at semi commercial level. Overall, the protection effect of coating applications was better against *E. coli* DH5α than *R. stolonifer*. For *in vitro* experiments the best coatings was that of chitosan (1%) + beeswax (0.1%) + lime essential oil (0.1%) since no growth of *R. stolonifer* and *E. coli* DH5α took place. Other coating that stopped *R. stolonifer* growth was that of chitosan (1%) + oleic acid (1%) + lime essential oil (0.1%) while for *E. coli* DH5α were chitosan (1%) + beeswax (0.1%) + thyme essential oil (1%) and chitosan (1%) + beeswax (0.1%). Observation with an electronic scanning microscope showed distorted mycelia and no development of *R. stolonifer* sporangiospores, and no growth of *E. coli* DH5α when both microorganisms were grown on the formulation of chitosan (1%) + beeswax (0.1%) + lime essential oil (0.1%). For *E. coli* DH5α, this same formulation applied on tomatoes at a small scale and in the semi commercial level completely controlled *E. coli* DH5α at both storage temperatures. The application of chitosan-based edible coating containing beeswax and lime essential oil is promising to follow since it is an environmentally-friendly alternative to control this important pathogenic microorganism. Export tomato producers might benefit from this nonchemical alternative.

1. Introduction

High quantities of tomato fruit are produced in Mexico, being one of the country's most important horticultural products for export purposes. Mexico's tomato exports to the United States and Canada make up 19.7% of world tomato exports. According to the United Nations International Trade Centre, for example, Mexican tomato is the world's 10th fresh vegetable export (Anonymous, 2010). During field production, tomatoes may be affected by microbial contamination (Serna et al., 2004; Delgado et al., 2010). Among others, the use of irrigation water, soil, field tools and containers are major sources of fungi and food-borne pathogens. In

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particular, throughout the distribution chain (harvest practices and handling operations), tomatoes may receive injuries that also provide new sites for colonization by pathogenic fungi and bacteria. *Rhizopus stolonifer* Ehrenb.:Fr. is the most predominant fungus of tomato fruits and is also acquired during harvest, handling and transportation (Bautista-Baños et al., 2008). This fungus can spread to adjacent fruit, often destroying the entire contents of the box within a few days. On the other hand, bacteria may induce serious ailments in humans when contaminated tomatoes are consumed (Castro-Rosas et al., 2006). Among the food-borne pathogens, various species of the genus *Escherichia* are the cause of food poisoning outbreaks. Overall, postharvest fungicides and sanitizers are the most frequently used methods to minimize pathogens levels in fruits and vegetables. For control of *R. stolonifer*, fungicides Iprodione (Rovral) and Dichloran are frequently used, although in some countries they are banned (Bautista-Baños et al., 2008). Chlorine, chlorine dioxide and ozone are commonly used to remove food-borne pathogens, however, there are some significant disadvantages involved in their use. For example, chlorine eventually forms by-products that may result in toxic compounds, while with ozone it is difficult to maintain a concentration over prolonged periods of time (Sapers, 2006).

Alternative methods to reduce postharvest fungi and food-borne pathogens have been proposed. Among them the antimicrobial properties of chitosan and essential oils from various plant species applied individually have proven to affect and arrest fungal and bacterial development in *in vitro* and *in vivo* experiments on various horticultural commodities (Bautista-Baños et al., 2006). Up until now, various chitosan-based edible coatings have been developed for fruit such as strawberries, grapefruits and litchi (El Ghaouth et al., 1992; Zhang and Quantick, 1997; Romanazzi et al., 2002). The beneficial effects of chitosan edible coating were seen in terms of extended storage life, quality maintenance and postharvest pathogen control. With respect to the essential oils, it has been demonstrated in different food types such as meat and bakery products that films containing essential oils of angelica, anise, cardamom, cinnamon, lime and thyme also inhibit various pathogenic moulds, bacteria and yeasts in various food products (Rabea et al., 2003; Plotto et al., 2003; Cagri et al., 2004). In *in vitro* evaluations, it was demonstrated that the combination of chitosan at 10 mg ml^{-1} and thyme essential oil at 300 μg ml^{-1} had a fungicidal effect on *R. stolonifer*, inhibiting mycelial growth, spore germination and sporulation of this fungus (Alvarado, 2009). In other studies, development of a papaya strain of *R. stolonifer* was seriously affected by thyme essential oil (Bosquez-Molina et al., 2010).

The synergistic combination of chitosan and essential oils for potential use in films, coatings, and packaging is under experimentation. In previous studies, tomato rot incidence and severity was reduced by 50% with chitosan at 10 mg ml^{-1} combined with different essential oils such as clove cinnamon and thyme at 300 μg ml^{-1} (Alvarado, 2009). For bacteria, the inhibitory effect of food coated with chitosan and rosemary essential oil at 1% on *Listeria monocytogenes* on the surface of pumpkins was confirmed in studies conducted by Ponce et al. (2008). It also inhibited halo bacteria growth by an additional 5 mm compared to the rest of the coatings tested, such as the chitosan-olive oil and chitosan-chile extract combinations. Another combination that produced good results was a chitosan and garlic oil-based coating studied by Pranoto et al. (2005), who found that it controlled the growth of *Staphylococcus aureus*, *L. monocytogenes* and *Bacillus cereus*. A coating of chitosan-oleic acid to cover strawberry fruits was produced by Vargas et al. (2006), who reported a reduced incidence of microorganisms of up to 80% by the end of the evaluation, in comparison with the untreated fruits. It was shown in this study as well that the chitosan-oleic acid combination had not only fungicidal and bactericidal effects, but it was also an alternative method to extend the shelf life of minimally processed carrots, reducing respiration and weight loss and maintaining fruit color.

The objective of this study was to evaluate the effectiveness of the combined treatments of different chitosan-based formulations with two essential oils, beeswax and oleic acid in controlling *R. stolonifer* and *Escherichia coli* in *in vitro* experiments and in tomatoes at a small scale and a semi commercial level.

2. Materials and methods

2.1. Microorganisms

2.1.1. Bacterial culture

E. coli DH5α strain used in this study was obtained from the culture collection of the Biotechnology Department of Morelos State Polytechnic University. The microorganism was inoculated into five test tubes containing 5 ml of nutrient lauryl tryptose broth (LT) and incubated for 24 h at 37 °C. The bacterial culture was subsequently diluted to obtain a final concentration of 10^5 cfu μl^{-1}. To confirm the viable cell concentration, spectrophotometer absorbance reading at 600 nm was carried out in a blank solution and nutrient agar.

2.1.2. Fungal culture

R. stolonifer was obtained from infected tomatoes. Fruit were placed in moist chambers at 25 ± 2 °C until symptoms appeared (*ca.* four days). To obtain pure cultures, portions of the infected tissue were placed on Petri plates containing potato dextrose agar (PDA). To obtain monosporic cultures, serial dilutions were prepared from pure cultures and individual spores were collected and grown on PDA at 25 ± 2 °C. To maintain pathogenicity of the fungus, periodic inoculations and re-isolations from infected tomatoes were carried out. The number (10^5) spore per ml of filtrate was determined using a Neubauer haemocytometer.

2.2. Materials

2.2.1. Chitosan preparation

Chitosan medium molecular weight (Mw = 2.38 × 10^4 Da, 75–85% degree of deacetylation; 200–800 cps) (Sigma Aldrich, St. Louis, M.O., USA) was prepared according to the methodology of El Ghaouth et al. (1992). The tested chitosan concentration of 1.0% was prepared by dissolving 10 g of chitosan in 1000 ml of distilled water, gradually adding 10 ml of acetic acid. The final pH was of 5.6. The solutions were heated and constantly agitated for 24 h. The final solution was adjusted to 1000 ml of distilled water.

2.2.2. Essential oil extraction by standard vacuum hydro distillation

Extraction of the essential oils was carried out following the methodology of Bosquez-Molina et al. (2010). Five kg of clean rinds of Mexican limes were blended in a proportion of 60% of rinds to 40% of water for 10 min. The mixture was heated at 60 °C for 2 h, while pressure was slowly decreased to 200 mmHg (1 mmHg = 133.322 Pa). In the case of thyme, fresh leaves were purchased in the Mexico City warehouse market; 40% of clean, grinded thyme plants to 60% of water were used for distillation under the same conditions as mentioned for Mexican lime.

2.3. Formulations

For *in vitro* experiments, six coatings containing the following formulations were tested to separately evaluate their effect on *R. stolonifer* and *E. coli* DH5α development: 1) chitosan 1% + beeswax 0.1% + lime essential oil 0.1%, 2) chitosan 1% + beeswax 0.1% + thyme