Environmentally Friendly and Safe Technologies for Quality of Fruits and Vegetables

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SECTION 4. ENVIRONMENTALLY FRIENDLY AND SAFE METHODS TO CONTROL POSTHARVEST LOSSES

25. INHIBITION OF POSTHARVEST *PENICILLIUM* MOLDS OF ORANGES BY ANTIFUNGAL HYDROXYPROPYL METHYLCELLULOSE-LIPID EDIBLE COMPOSITE FILMS AND COATINGS

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Abstract
New hydroxypropyl methylcellulose (HPMC)-lipid edible composite films and coatings containing low-toxicity chemicals with antifungal properties were developed. Tested antifungal chemicals were mainly salts of organic acids, salts of parabens, and other compounds, most of them classified as food additives or generally recognized as safe (GRAS) compounds. Stand-alone edible films were used for *in vitro* evaluation of their antifungal activity against the pathogens *Penicillium digitatum* and *Penicillium italicum* by disk diameter tests. Selected edible coatings containing food preservatives were tested *in vivo* on 'Valencia' oranges to determine their curative (coated after fungal inoculation) and preventive (coated before fungal inoculation) activity to control citrus postharvest green and blue molds, caused by *P. digitatum* and *P. italicum,* respectively. Film disks containing parabens and the organic acid salts potassium sorbate (PS) and sodium benzoate (SB) were the most effective to inhibit both *P. digitatum* and *P. italicum.* The use of mixtures of organic acid salts did not provide any additive or synergistic effect for *in vitro* pathogen inhibition when compared to the use of single chemicals. On 'Valencia' oranges, the curative activity of coatings with food preservatives was higher for blue mold than for green mold. Coatings containing the mixture SB + PS and SB and sodium propionate (SB + SP) reduced the incidence and severity of blue mold by 85 and 95%, respectively. PS- and SB-based coatings controlled green mold more effectively than coatings formulated with other food preservatives. Fruit coated before inoculation did not show any incidence or severity reduction of both green mold and blue mold (preventive activity). The antifungal curative action of the coatings was fungistatic rather than fungicidal.
Keywords: citrus, food additives, hydroxypropyl methylcellulose, *Penicillium digitatum*, *P. italicum*

Introduction
Postharvest diseases of citrus are mainly produced worldwide by the pathogens *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, which cause green and blue molds, respectively (Eckert & Eaks 1989). Consumer concerns about prolonged and extensive use of chemical fungicides such as imazalil, sodium ortho-phenyl phenate, or thiabendazole to control citrus postharvest decay are leading researches to look for alternative non-contaminant methods that do not generate harmful fruit residues and respect the environment. Several physical, chemical, or biological alternative methods and combinations have been assayed against both green and blue molds (Porat *et al*. 2002; Palou *et al*. 2008). Polysaccharides, proteins, lipids, and resins are mainly used to form edible coatings. Plasticizers and emulsifiers are usually added to increase the flexibility and surface tension between aqueous and lipid phases in the formulations that combine lipids and hydrocolloids (Nisperos-Carriedo 1994; Pérez-Gago & Krochta 2001). Edible films and coatings may incorporate food additives and other substances to enhance flavor, color, and texture, control microbial growth, and improve general coating performance (Cuppet 1994). Antimicrobials can be added to edible coatings to retard the growth of bacteria, yeasts, and molds during storage and distribution of fresh or minimally processed products. These compounds

include natural substances or generally recognized as safe (GRAS) compounds such as organic acids and their salts, parabens, bacteriocins, or chitosan (Matamoros-León *et al*. 1999; Chung *et al.* 2001; Min & Krochta 2005). For instance, it has been reported that the addition of potassium sorbate (PS) to edible coatings controlled microbial proliferation on strawberries (García *et al.* 1998; Park *et al.* 2005), the addition of acetic, citric, and sorbic acids effectively controlled the pathogen *Salmonella montevideo* on tomatoes (Zhuang *et al.* 1996), or the addition of parabens reduced coliform bacteria on citrus fruit (McGuire & Hagenmaier 2001). However, very few researches have focused on the development of new edible composite coatings with the addition of antifungal compounds as a new technique to control the major fungal postharvest diseases of fresh citrus fruits. The objectives of this study were to develop new hydroxypropyl methylcelullose-lipid edible composite films containing food additives with antifungal properties, evaluate the *in vitro* activity of selected films against *P. digitatum* and *P. italicum*, and evaluate the curative and preventive activity of selected HPMC-lipid edible composite coatings to control green and blue molds on artificially inoculated 'Valencia' oranges.

Table 1. Composition of HPMC-lipid edible composite films containing antifungal food additives and *in vitro* antifungal activity of films against *Penicillium digitatum* and *Penicillium italicum* at different inoculum concentrations.

HPMC-lipid films with food preservative	Molecular formula	Food preservative (%, wb)	Beeswax-shellac (%, db)	Length of inhibition zone (mm)[x]					
				P. digitatum inoculum concentration (spores mL^{-1})			*P. italicum* inoculum concentration (spores mL^{-1})		
				10^3	10^4	10^5	10^3	10^4	10^5
Organic acid salts									
Potassium sorbate	$C_6H_7O_2K$	2.0	25-25	16.8 e	15.8 e	17.3 ef	9.2 c	6.6 e	5.9 cd
Sodium benzoate	$C_7H_5O_2Na$	2.5	25-25	12.8 de	11.3 d	7.2 c	9.8 c	3.9 cd	2.9 b
Calcium propionate	$C_6H_{10}O_4Ca$	1.0	50-0	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Calcium formate	$C_2H_2O_4Ca$	1.0	50-0	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Organic acid salts (mixtures)									
Potassium sorbate (PS) + sodium propionate (6% SC)[y]		1.5 + 0.5	25-25	16.6 e	12.0 d	4.3 b	0.0 a	0.0 a	0.0 a
Sodium benzoate + potassium sorbate (8% SC)		2.0 + 0.5	25-25	13.6 de	10.6 cd	9.1 cd	11.0 c	4.5 cd	4.3 c
Sodium benzoate + sodium propionate (8% SC)		2.0 + 0.5	25-25	10.2 cde	7.8 b	0.7 ab	2.8 b	1.1 b	0.0 a
Parabens									
Sodium salt of methyl paraben	C_8H_7NaO3	1.5	50-0	22.1 f	24.2 f	21.1 f	18.3 d	18.5 g	19.9 f
Sodium salt of methyl paraben		1.0	50-0	22.3 f	21.9 f	17.4 ef	15.8 d	14.8 f	15.8 e
Other compounds									
2-deoxy-D-glucose	$C_6H_{12}O_5$	0.5	25-25	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Controls[z]									
Control (8% SC)			25-25	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC)			45-5	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC)			25-25	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC)			50-0	0.6 ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a

[x] Values are measurements of length (mm) of inhibitory zones around film disk (from the perimeter of the film disk until the edge of the inhibited area). Values within columns followed by unlike letters are different by the Fisher protected LSD test ($P<0.05$) applied after an analysis of variance of the square root of the inhibition zone plus 0.5. Nontransformed data are shown.

[y] SC = solid concentration.

[z] HPMC-lipid films without food preservatives at 6 or 8% SC and different percentages of beeswax-shellac.

Material & Methods

Preparation of Films and Coatings

To prepare emulsions, an aqueous solution of hydroxypropyl methylcellulose (HPMC, 5% w/w), the corresponding food preservative, beeswax (BW), glycerol, stearic acid, and water were mixed. Shellac solution was added to the HPMC dispersion. The samples were homogenized (90 °C) with a high-shear probe mixer and cooled. The emulsions were degassed and films were cast onto smooth plates and allowed to dry. For each emulsion, three to five films were prepared. Most of the antifungal chemicals tested were classified as food additives or GRAS compounds. The lipids (BW and shellac) were used at 50% (dry basis, db) and the ratios of HPMC-glycerol (2:1) (db) and lipids-stearic acid (5:1) (db) were kept constant throughout the study. The solid concentration (SC) in the samples was 6 or 8% (wet basis, wb). A large number of emulsion formulations were prepared, but only those capable of forming homogeneous films and coatings were used in this study (Table 1).

Determination of *in Vitro* Antifungal Activity. Disk Diameter Test

P. digitatum and *P. italicum* grown in Petri dishes on potato dextrose agar (PDA) for 7-10 d were used. High-density conidial suspensions were prepared by measuring the spore concentration with a haemacytometer. The antifungal activity of edible films was evaluated through the disk diameter test (adapted from that described by Min & Krochta (2005). Film disks (16 mm diameter) were aseptically transferred to dichloran rose-bengal chloramphenicol agar (DRBC) plates previously inoculated with 100 µL of conidial suspension of the corresponding pathogen. Plates were refrigerated at 4 °C for 3 h to allow diffusion of film ingredients and then incubated at 25 °C for 5 d. For each fungal species, inoculum densities of 10^3, 10^4 and 10^5 spores mL^{-1} were used. For each pathogen, inoculum density and film, three agar plates (replicates) were prepared. After incubation, the length of the inhibition zone around the film disk (from the perimeter of the film disk until the edge of the inhibited area) was measured with a digital caliper. Four measurements were performed for each plate.

Evaluation of the Curative and Preventive Activity of the Coatings

Oranges (*Citrus sinensis* [L.] Osbeck) 'Valencia' were used. For determination of curative activity, each fruit was inoculated with *P. digitatum* and *P. italicum* (inoculum density of 10^5 spores mL^{-1}) at opposite sides of the equator, incubated at 20 °C for 24 h, and coated by immersion (15 s at 20 °C) with the HPMC-lipid edible composite. To test preventive activity, the fruit were coated and inoculated with the pathogens about 24 h later. Inoculated but uncoated fruit were used as controls. Each treatment was applied to three replicates of 20 fruit each. Disease incidence (%) and severity (diameter of the infected area in mm) were determined after 7 d of incubation at 20 °C and 90% RH. In a second experiment, 'Valencia' oranges were treated with selected coatings and incubated at 20 °C for up to 21 d.

Statistical Analysis

Specific differences between means were determined by Fisher's protected least significant difference test (LSD, $P<0.05$) applied after an analysis of variance (ANOVA). For pathogen inhibition and disease incidence data, the ANOVA was applied to square root and arcsine transformed values, respectively.

Results & Discussion

In Vitro Antifungal Activity

Among all organic acid salts added into HPMC-lipid films, only films containing PS or sodium benzoate (SB) clearly inhibited the growth of both *P. digitatum* and *P. italicum* plated at 10^3 to 10^5 spores mL^{-1} on DRBC agar (disk diameter test, Table 1). In food systems, PS is one of the most widely used compounds to prevent the growth of molds and thus to extend produce shelf-life (Jarret *et al.* 2005). The antimicrobial activity of PS against *P. digitatum* and *P. italicum* has been observed in both *in vitro* and *in vivo* studies. For instance,

both pathogens were inhibited in PDA when PS was added at a concentration of 0.15-0.20 g L^{-1} (Matamoros-León *et al.* 1999). Films with a mixture of PS and sodium propionate (SP) significantly inhibited ($P<0.05$) the growth of *P. digitatum* on DRBC agar at all inoculum concentrations tested. As expected, *P. digitatum* at 10^3 spores mL^{-1} was more intensely inhibited (16.6 mm) than at 10^4 (12.0 mm) or 10^5 spores mL^{-1} (4.3 mm). The inhibition ability was therefore greatly dependent on the pathogenic inoculum density. In contrast, *P. italicum* was not inhibited at all by films formulated with this a mixture of PS and SP. Films containing a mixture of SB and PS significantly inhibited ($P<0.05$) the growth of *P. digitatum* and *P. italicum* on DRBC agar (Table 1). Jarret *et al.* (2005) observed that the combination of sorbates with either benzoates or propionates may be used to effectively inhibit microorganisms using lower concentrations of each preservative. In this work, however, the use of the mixtures PS + SP, SB + PS, and SB + SP incorporated to HPMC-lipid films was not additive or synergistic with respect to the use of these salts alone. On the other hand, films with sodium salt of methyl paraben (SMP) effectively inhibited both *P. digitatum* and *P. italicum* at all inoculum densities (Table 1). For both pathogens, an increase of SMP concentration in the film from 1.0 to 1.5% (wb) did not significantly increase the inhibition zone. Films containing 0.5% of 2-deoxy-D-glucose did not inhibit the growth of *P. digitatum* and *P. italicum* (Table 1).

***In Vivo* Curative and Preventive Activity**

HPMC-lipid edible composite coatings containing food preservatives showed an important curative activity on 'Valencia' oranges The reduction of incidence and severity of both *P. digitatum* and *P. italicum* with respect to the control treatment was significant ($P<0.05$) for most of the tested coatings. However, coatings containing PS, SB, or their mixtures controlled both *P. digitatum* and *P. italicum* more effectively than coatings prepared with the rest of organic acid salts or 2-deoxy-D-glucose. The SMP-based coating reduced the incidence and severity to values similar to those obtained with coatings formulated with organic acid salts (Fig 1). Some differences were observed between these results and those obtained in the *in vitro* tests, probably due to the complex interactions between host, pathogen and environment that occur during *in vivo* disease development. Likewise, it is probable that notable variations on the growth of green and blue molds *in vitro* and *in vivo* resulted from differences on the rate of release of food preservatives from films located on agar medium and coatings located on the fruit rind. PS and SB are generally recognized as safe compounds by regulations all over the world and they are therefore widely used as food preservatives with a broad-spectrum activity against a variety of yeasts and molds (Jarret *et al.* 2005). The effectiveness of aqueous solutions of PS or SB against postharvest green and blue molds of different citrus species and cultivars has been reviewed (Palou *et al.* 2008). An additional assay was conducted to study the performance of these coatings on 'Valencia' oranges incubated at 20 °C for up to 21 d. It was found that, after 7 d of incubation, these coatings greatly reduced the incidence and severity of green and blue molds if compared to uncoated fruit (Fig 2). However, these reductions were significantly lower ($P<0.05$) as the time of incubation increased, especially in the case of blue mold. After 14 and 21 d at 20 °C, the incidence of green mold on coated fruit was about 40 and 60%, respectively, and the incidence of blue mold was as high as 74 and 96%, respectively (Fig 2). Therefore, the inhibitory activity of the coatings was not very persistent on 'Valencia' oranges and their antifungal curative action was fungistatic rather than fungicidal. Coating the fruit 24 h before fungal inoculation did not significantly reduced the incidence and severity of both green and blue molds (data not shown). Thus, the tested coatings showed no preventive activity against the pathogens.

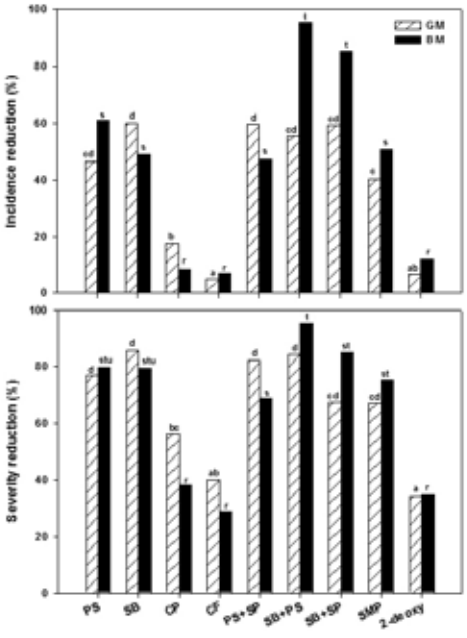

Fig 1. Incidence and severity reductions of green mold (GM) and blue mold (BM) with respect to control fruit (inoculated but uncoated) on 'Valencia' oranges artificially inoculated with the pathogens, coated 24 h later with HPMC-lipid edible composite coatings containing food preservatives, and incubated for 7 d at 20 ºC and 90% RH. For each mold, columns with different letters (a-d, r-u) are significantly different according to Fisher´s protected LSD test ($P<0.05$) applied after an ANOVA. Data on disease incidence reduction were arcsine-transformed; nontransformed means are shown. PS = potassium sorbate, SB = sodium benzoate, CP = calcium propionate, CF = calcium formate, SP = sodium propionate, SMP = sodium salt of methyl paraben, 2-deoxy = 2-deoxy-D-glucose.

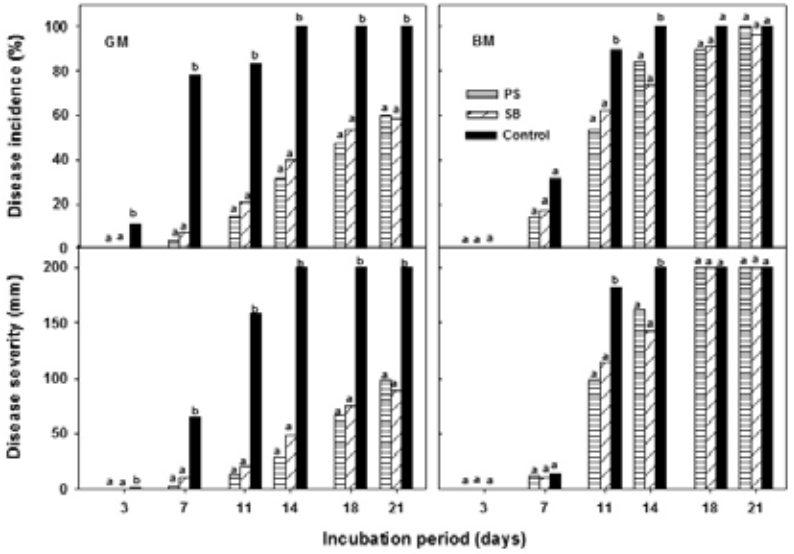

Fig 2. Incidence and severity of green mold (GM) and blue mold (BM) on 'Valencia' oranges artificially inoculated with the pathogens, uncoated (control) or coated 24 h later with HPMC-lipid edible composite coatings containing potassium sorbate (PS) or sodium benzoate (SB), and incubated up to 21 d at 20 ºC and 90% RH. For each incubation period, columns with different letters are significantly different according to Fisher´s protected LSD test ($P<0.05$) applied after an ANOVA. Data on disease incidence were arcsine-transformed; nontransformed means are shown.

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