Curative activity against citrus postharvest green mold of composite hydroxypropyl methylcellulose-beeswax edible coatings with zeolites containing Ag-nanoparticles

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Abstract

Two types of commercial Na-zeolites, linde type A (LTA) and faujasite (FAU) were prepared with different percentage of silver that was incorporated by ion exchange using solutions of $AgNO_3$. The physico-chemical properties of the samples were established using different techniques such as atomic emission spectroscopy with inductively coupled plasma (ICP-OES), X-ray diffraction (XRD), electronic microscopy (SEM and EDX), and temperature-programmed reduction (TPR). The curative activity of these materials was first evaluated as water dispersions in in vivo primary screenings by placing a drop (30 µL) in a rind wound of 'Valencia' oranges that had been inoculated 24 h before with a conidial suspension of *Penicillium digitatum*. Selected Ag-zeolites were incorporated as antifungal ingredients at different concentrations into composite hydroxypropyl methylcellulose (HPMC)-beeswax (BW) edible emulsions prepared with 8% total solid content with stearic acid as emulsifier and glycerol as plasticizer. Depending on the experiment, these coatings were applied 24 h after fungal inoculation to 'Valencia' oranges as drops in rind wounds or covering the entire fruit. Controls included inoculated but untreated fruit and inoculated fruit treated with HPMC-BW without Ag-zeolites. After incubation of treated fruit at 20°C for 7 days, coatings amended with Ag-zeolites reduced the incidence and severity of green mold by more than 80%, showing high potential for effective disease control. Curative activity depended on Ag concentration and FAU zeolites were more effective than LTA zeolites, probably because of their larger pore apertures and higher Si/Al ratio that favored silver diffusion. However, coatings with the highest silver content were phytotoxic causing dark blemishes on the fruit rind. Further work is needed to optimize non-phytotoxic coatings with the highest curative activity.

Keywords: *Penicillium digitatum;* alternative postharvest disease control; nanotechnology

INTRODUCTION

Green mold, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is one of the most important diseases responsible for postharvest losses of citrus fruit worldwide (Palou, 2014). Economic losses due to this fungal disease have been reduced to commercially acceptable levels by the use of conventional fungicides such as imazalil (IMZ), thiabendazole (TBZ), sodium-o-phenylphenate (SOPP), or other synthetic agrochemicals. Nevertheless, consumer trends and legislation changes are increasingly favoring a continuous reduction in the amount of these substances allowed by authorities to be present in/on fruit.

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Furthermore, at present, large citrus distributors and major supermarket chains are even demanding particular and more restrictive fungicide usage. In addition, rising populations of *P. digitatum* strains resistant to these fungicides are compromising the efficacy of the treatments in many citrus packinghouses (Kinay et al., 2007). Therefore, it is necessary to implement alternative approaches and novel technologies for a cost-effective integrated control of green mold (Palou et al., 2008).

Among different alternative means that have been evaluated, the use of edible coatings formulated with food grade antifungal ingredients is gaining importance, especially because they can be an appropriate technology to substitute the commercial citrus waxes amended with chemical fungicides that are often used in citrus packinghouses. In general, matrix components of edible coatings are hydrocolloids (proteins or polysaccharides) and lipids. Composite coatings contain a combination of polysaccharides or proteins with lipids. Cellulose and derivatives such as hydroxypropyl methylcellulose (HPMC) or carboxyl methylcellulose (CMC) are among the polysaccharide materials most widely used to formulate edible coatings for fresh fruit (Valencia-Chamorro et al., 2011). According to their nature, the antifungal compounds that can be added as additional ingredients to edible coatings can be classified into three categories (Palou et al., 2015): i) synthetic food preservatives or GRAS (generally regarded as safe) compounds with antimicrobial activity, ii) natural compounds such us essential oils or other natural plant extracts, and iii) antimicrobial antagonists as biological control agents (mainly yeasts or bacteria).

Provided that they are used in concentrations safe for consumption, metal-based nanoparticles could be a suitable option for inclusion in the first group. Several metal cations such as Ag^+, Zn^{++} or Cu^+ have received a lot of attention as potential antimicrobial agents. Among them, silver is an interesting option due to the broad spectrum of its antimicrobial properties. Silver can be incorporated into different carrier support materials such as polymers, clays, or zeolites, which may behave as silver reservoirs (Ferreira et al., 2012). In general, for food applications, zeolites are among the most widely used materials to incorporate silver as antimicrobial agent. In the USA, zeolite-based technologies are listed under the US FDA Inventory of Effective Food Contact Substance Notifications for use in food-contact polymers (US FDA, 2012). In Europe, the EFSA also released a positive opinion concerning the use of two zeolites containing Ag ions in food contact surfaces, with silver migration into food matrices being restricted to 50 μg Ag per kg of food (Corrales et al., 2014).

The objectives of this research work were to prepare and characterize the physico-chemical properties of two different zeolites containing silver nanoparticles and use them as antifungal ingredients of novel HPMC-lipid edible coatings for citrus fruit. The curative activity against green mold of the zeolites alone or incorporated into the coatings was evaluated on oranges artificially inoculated with *P. digitatum*.

MATERIALS AND METHODS

Preparation and characterization of Ag-zeolites

Two Na-zeolites, a linde type A (LTA) and a faujasite (FAU) were used as parent zeolites. FAU zeolite was supplied by Zeolyst (CBV100) and LTA zeolite from Sigma-Aldrich (4A). These Na-zeolites were ion exchanged using $AgNO_3$ solutions at different concentrations in order to obtain zeolites in which Na^+ was completely exchanged by Ag^+ (total exchange, te) and zeolites with around 1-2% (wt) of silver (partial exchange, pe). The ion exchange procedure was performed with a solid/liquid ratio of 1/100 at 25°C for 16 h, under mechanical stirring and in darkness to avoid reduction of Ag^+ to Ag^0. Then, solids were filtered, washed to remove excess of silver, and dried at 100ºC for 24 h.

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The zeolites chemical composition was measured by inductively coupled plasma (ICP-OES) in a Varian 715-ES ICP-Optical Emission Spectrometer. Scanning electron microscopy (SEM) images were obtained with a microscope JEOL JSM-6300 LINK, which was also used to quantify and evaluate the distribution of the elements in the zeolites with its energy dispersive X-ray equipment system (EDX). A Philips X'Pert (Cubix)-advance diffractometer coupled to a copper anode X-ray tube was used for the X-ray diffraction (XRD) characterization. Compounds were conventionally identified using the JCPDS files. Temperature-programmed reduction (TPR) studies with H_2 were carried out with a TPD-TPR Autochem 2910 equipment using a thermal conductivity detector (TCD).

Preparation of edible coatings containing Ag-zeolites

Edible composite antifungal emulsions with 8% total solid content were prepared by combining the HPMC (1.2% wet basis, wb) with the lipid beeswax (BW; 3.5% wb) and the Ag-zeolites (2.0% wb) suspended in water. Glycerol and stearic acid were used as plasticizer and emulsifier, respectively. Ratios of HPMC-glycerol and BW-stearic acid were 2:1 and 5:1 (dry basis, db), respectively. For emulsion preparation, an aqueous solution of HPMC (5%, w/w) was dispersed in hot water at 90°C and later hydrated at 20°C. The rest of ingredients were added to the HPMC solution and heated to 90°C. Once the lipid was melted, samples were homogenized with a highshear probe mixer (Ultra-Turrax Model T25, IKA-Werke) for 1 min at 13,000 rpm followed by 3 min at 22,000 rpm. Emulsions were cooled under agitation to a temperature lower than 25°C by placing them in an ice water bath. To ensure completed hydration of HPMC, emulsions were further agitated at 13,000 rpm for 1 min. HPMC-BW coatings without Ag-zeolites (6% total solid content) were also prepared to be used as controls.

Fruit inoculation

Penicillium digitatum, isolate NAV-7, from the fungal culture collection of the IVIA CTP, was cultured on potato dextrose agar (PDA) plates at 25°C. Conidia of the fungus from 7 to 14 day-old cultures were taken from the agar surface and transferred to a sterile aqueous solution of 0.05% Tween 80. Conidial suspensions were filtered through two layers of cheesecloth, and adjusted to a concentration of 10^5 spores mL^{-1} using a haemocytometer. For fruit inoculation, the tip of a stainless steel rod, 1 mm wide and 2 mm in length, was immersed in the conidial suspension and inserted in the rind of oranges (*Citrus sinensis* (L.) Osbeck) cv. Valencia. Fruit were inoculated with *P. digitatum* at one point in the equatorial area of the fruit rind. Treatments were always applied after incubation of inoculated fruit at 20°C for 24 h, so curative activity was assessed.

In vivo primary screenings

In a first screening, 30 μL of aqueous suspension containing 2, 4 or 8% (wt) of a LTA Ag-zeolite totally exchanged (LTA_Ag(te)) were placed, about 24 h after the inoculation of the pathogen, in the same inoculation rind wound using a micropipette. Control fruit were treated with 30 μL of sterile distilled water. For each treatment, 3 replicates of 10 oranges each were used. Treated fruit were arranged in plastic cavity sockets on cardboard trays and incubated at 20°C and 90% RH for 7 days, at which time disease incidence (% of infected fruit) and severity (lesion size, in mm) and visible phytotoxicities on the fruit rind were assessed.

In a second screening using exactly the same methodology, the treatments were drops of aqueous suspensions with 4% of LTA_Ag(te) or 4% of a FAU Ag-zeolite totally exchanged (FAU_Ag(te)).

Curative activity of edible coatings containing Ag-zeolites

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In accordance to the results of the previous primary screenings, edible composite HPMC-BW coatings were prepared as described above with a LTA Ag-zeolite partially exchanged (LTA_Ag(pe)) or a FAU Ag-zeolite partially exchanged (FAU_Ag(pe)). In this case, partial silver exchange was used in order to reduce the amount of silver to values between 1 and 2% (wt). Once the emulsions were obtained, they were applied to 'Valencia' oranges artificially inoculated with *P. digitatum* about 24 h before using two different application methods: as 30 µL drops applied to the rind inoculation wound or as coating over the entire orange rind surface. In every case, 3 replicates of 10 oranges each were used for each treatment and treated oranges were incubated at 20°C and 90% RH for 7 days before assessment of green mold incidence and severity and rind phytotoxicities. Control fruit included inoculated but untreated oranges and inoculated oranges treated with HPMC-BW without Ag-zeolites.

Statistical analysis

Decay data were analyzed by an analysis of variance (ANOVA) with statistical software (Statgraphics Plus v. 5.1). Prior to analysis, disease incidence data were transformed to the arcsine of the square root of the proportion of infected fruit to homogenize the variances. Statistical significance was judged at the level P=0.05. Fisher's Protected Least Significant Difference (LSD) test was used to separate means.

RESULTS AND DISCUSSION

Characterization of Ag-zeolites

Among the results obtained with the different procedures used to characterize the Ag-zeolites, only the chemical composition measured by ICP-OES is shown (Table 1). LTA and FAU Na-zeolites with different Si/Al ratio were used to obtain zeolites with different levels of silver ion exchange. While the materials used for in vivo primary screenings were FAU and LTA zeolites with an almost total silver ion exchange (te) that rendered final silver contents of 30-50% (wt), the materials used as ingredients of edible coatings were only partially silver ion exchanged (pe) and had final silver contents of 1-2% (wt). Although the ion exchange procedure used to incorporate the silver into both zeolites was analogous (same precursor, same contact time, same temperature and same liquid/solid ratio), the final silver content in both zeolites after complete ion exchange was different because LTA and FAU zeolites had different Si/Al ratio. While complete ion exchange in the LTA zeolite (Si/Al ratio = 1.0) resulted in silver content of 48.4%, complete ion exchange in the FAU zeolite (Si/Al = 2.5) resulted in silver content of 30.6%.

Table 1. Chemical composition of Ag-zeolites measured by ICP-OES.

Zeolite	Ag (% wt)	Si/Al molar ratio	Ion exchange (%)
LTA	-	1.0	-
LTA_Ag(te)[1]	48.4	0.9	95.9
LTA_Ag(pe)[2]	1.8	0.9	2.7
FAU	-	2.4	-
FAU_Ag(te)[1]	30.6	2.5	93.4
FAU_Ag(pe)[2]	1.3	2.4	3.4

[1]Almost total silver ion exchange.
[2]Partial silver ion exchange.

On the other hand, analyses by SEM coupled to EDX (mapping) showed homogeneous distribution of silver in the zeolite matrix (data not shown). X-ray diffraction (XRD)

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measurements showed that the zeolite structures were not modified during the ion exchange process and each Ag-zeolite, independently of the amount of exchanged silver, had high crystallinity and absence of amorphous phases (data not shown). SEM micrographies showed that the morphology and particle size of both Ag-exchanged zeolites were similar to the parent zeolites (data not shown). Thus, irrespective of the amount of exchanged silver, the procedure used for ion exchange did not modify the morphology of the zeolites. TPR measurements of completely ion exchanged Ag-zeolites showed that the formation of silver clusters was more feasible in FAU than in LTA zeolites, probably because of the former had larger cavities and pores than LTA (data not shown).

In vivo primary screenings

The incidence of green mold on 'Valencia' oranges inoculated with *P. digitatum*, treated with drops of aqueous suspensions with LTA_Ag(te) contents of 2, 4, and 8%, and incubated at 20°C and 90% RH for 7 days was reduced by about 65, 80, and 80%, respectively, with respect to control fruit treated with a water drop. Likewise, green mold severity was reduced by about 75, 80, and 80%, respectively (data not shown). Therefore, a Ag-zeolite content of 4% in the suspension was selected to compare the antifungal activity of different types of Ag-zeolites. These results indicated an outstanding activity of Ag-nanoparticles released from zeolite matrixes for the in vivo reduction of green mold on citrus fruit. The electrostatic attraction between the negatively charged cell membrane of the fungus and the Ag^+ has been proposed as a possible fungicidal mode of action of silver ions (Chiericatti et al., 2014). This potential activity would be favored by the ion exchange properties of the zeolites, which could facilitate the release of silver cations and their disruptive interaction with fungal cells (Pereyra et al., 2014).

In a subsequent experiment, the incidence of green mold on 'Valencia' oranges inoculated with *P. digitatum*, treated with drops of aqueous suspensions of LTA_Ag(te) or FAU_Ag(te), both at 4%, and incubated at 20°C and 90% RH for 7 days was reduced by about 80 and 100%, respectively, with respect to control fruit treated with water. Green mold severity on these fruits was reduced by about 80 and 95%, respectively (data not shown). Therefore, higher green mold control was achieved with the totally exchanged FAU zeolite, even though this zeolite contained a lower percentage of silver, as illustrated in Table 1. This might be due to the different topology and Si/Al ratio of the zeolites. The FAU zeolite has larger pore sizes than the LTA zeolite and could provide an easier release of silver cations. Furthermore, the FAU zeolite has a higher Si/Al ratio (Table 1), which has been related to greater proportions of Ag^+, the active antifungal agent (Ferreira et al., 2012).

However, in spite of the great curative activity against green mold, it was observed in these tests that the drop application of totally exchanged Ag-zeolites was phytotoxic causing apparent circular black spots in the application site in the orange rind. Moreover, the high silver content of these materials did not comply with current legislations for food applications. Hence, partially exchanged zeolites with considerably lower silver concentrations were prepared to be used as ingredients of HPMC-BW edible coatings.

Curative activity of edible coatings containing Ag-zeolites

Preliminary work showed that HPMC-BW coatings formulated without Ag-zeolites were completely ineffective in reducing the incidence and severity of green mold on fruit artificially inoculated with *P. digitatum* (data not shown). Drops of HPMC-BW coatings containing 2% (wb) LTA_Ag(pe) or FAU_Ag(pe), with silver contents of 1.8 and 1.3%, respectively, applied on the orange rind inoculation site about 24 h after inoculation with the pathogen, significantly reduced both the incidence and severity of green mold after 7 days of incubation at 20°C and 90% RH. While disease incidence and severity were 82% and 77 mm, respectively, on untreated control fruit, they were 50% and 41 mm on oranges

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treated with LTA_Ag(pe) and 36% and 21 mm on oranges treated with FAU_Ag(pe) (Table 2). Similarly to what was observed in the in vivo primary screenings, the better performance of coatings containing FAU Ag-zeolites should be attributed to their larger pore sizes and higher Si/Al ratio, both related to an increased availability of silver cations (Ferreira et al., 2012).

Inoculated oranges were also treated by completely covering the entire fruit surface with the FAU_Ag(pe) coating. In this case, green mold incidence and severity after 7 days of incubation were 50% and 30 mm, respectively, which were higher values than those obtained with this coating applied as a drop (Table 2). This could be due to a better coverage of the infection site provided by the drop, which could facilitate the interaction of silver cations with fungal cells. It has been reported in research with antifungal chemicals alternative to synthetic fungicides that some compounds applied as drops in the rind inoculation site of citrus fruit were more effective to control green mold than applied as brief dips that covered the entire fruit surface (Moscoso-Ramírez et al., 2013).

In these tests with HPMC-BW coatings formulated with partially exchanged Ag-zeolites, no induction of visible orange rind phytotoxicities was observed.

Table 2. Green mold incidence and severity on 'Valencia' oranges artificially inoculated with *Penicillium digitatum*, treated 24 h later with HPMC-BW coatings with Ag-zeolites partially exchanged (pe) applied as drops in the rind inoculation wound or as coating covering the entire fruit surface, and incubated at 20°C and 90% RH for 7 days.

HPMC-BW coating containing zeolites	Green mold[1]	
	Incidence (%± SE)	Severity (mm± SE)
Control (untreated)	82.1 ± 3.6 a	77.2 ± 3.8 a
LTA_Ag(pe)[2] Drop	50.0 ± 13.6 b	41.3 ± 12.6 b
FAU_Ag(pe)[3] Drop	35.7 ± 12.7 c	21.2 ± 4.3 c
FAU_Ag(pe)[3] Coating	50.0 ± 9.2 b	30.5 ± 3.9 bc

[1]Values in columns followed by unlike letters are significantly different according to Fisher's protected LSD test (P=0.05). Incidence data were arc-sine transformed. Non-transformed means are shown.
[2]Silver content in the zeolite is 1.8% (wt).
[3]Silver content in the zeolite is 1.3% (wt).

CONCLUSION

This research show the potential of edible HPMC-BW coatings formulated with zeolites containing silver nanoparticles to control existing infections of *P. digitatum* in citrus fruit. The curative activity of zeolites and coatings was higher for materials with greater amount of silver, larger pores, and higher Si/Al ratio. Coatings with FAU Ag-zeolites were superior to coatings with LTA Ag-zeolites. Totally exchanged zeolites had large amounts of silver and were phytotoxic causing dark blemishes on the orange rind. Partially exchanged zeolites containing 1-2% (wt) of silver were not phytotoxic and, according to current legislation, could be of use as antifungal ingredients of edible coatings.

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