Essential oils and natural plant extracts as antifungal ingredients of pectin-based edible composite coatings to control green mold and maintain postharvest quality of `Valencia´ oranges

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Abstract

Pectin-beeswax edible coatings containing essential oils (EOs) and plant extracts as antifungal ingredients have been developed to reduce postharvest losses in 'Valencia' oranges. After in vitro evaluation of the antifungal activity of the ingredients against *Penicillium digitatum*, selected agents and concentrations (0.2-2%, w/w) were incorporated into the coating. The curative activity of antifungal edible coatings (AECs) to control green mold was tested on artificially inoculated oranges incubated 8 days at 20°C. The effects of selected AECs on green mold and fruit physicochemical and sensory quality were tested on oranges stored for up to 8 weeks at 5°C plus 1 week at 20°C. Commercial compounds evaluated in vitro were cinnamon (CN), lemongrass (LG), *Satureja montana* (SM), myrrh (MY), eugenol (EU), geraniol (GE), green tea extract (GT), propolis (PRO), and vanillin (VA). Mycelial growth inhibition of *P. digitatum* after 7-14 days of incubation at 25°C was evaluated in PDA media exposed to EOs volatiles or by direct contact with the extracts using the agar dilution method. CN, SM, EU and GE (at a dose of 20 µL) inhibited the fungus radial growth by 90-100%; whereas, VA, PRO and MY were effective at 0.125-0.5%. After 8 days of incubation at 20°C, AECs containing 0.2% GE, 0.8% EU or 1.5% MI reduced green mold incidence (infected fruit, %) on oranges by more than 40%, while the highest reduction in disease severity (lesion diameter, mm) was observed with 0.8% CN. After 4 weeks of cold storage, 0.2% GE and 0.8% EU-based coatings reduced disease incidence by more than 50%, and 0.8% EU-coating was the most effective to reduce severity. In addition, the 0.8% EU-based coating was the most effective to reduce weight loss and provided the highest gloss on coated oranges at the end of the storage, showing its potential to reduce citrus postharvest losses.

Keywords: citrus, *Penicillium digitatum*, disease control, natural antifungal agents, postharvest quality

INTRODUCTION

Citrus are the most widely produced fruits worldwide. They are grown in over a hundred countries, and Spain is the leading country for exports of fresh produce (Martínez-Blay et al., 2020). Decay caused by fungal pathogens and fruit weight loss are among the main factors contributing to postharvest citrus spoilage and quality deterioration, leading to significant economic losses (Palou et al., 2015). Green mold (GM) caused by *Penicillium*

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digitatum (Pers.: Fr.) Sacc. is one of the most important postharvest citrus diseases, particularly in Mediterranean climate regions. This fungus is a strict wound pathogen that infects citrus fruit through rind injuries caused during harvest, transportation, and postharvest handling in the packinghouse.

Treatments with synthetic fungicides applied as aqueous solutions or added to waxes have been traditionally used to reduce postharvest citrus decay to commercially acceptable levels. However, legislative restrictions and consumer trends have led to the search for effective and environmentally friendly alternatives (Palou et al., 2015). In this sense, a very active research field nowadays is the development of antifungal edible coatings (AECs) as a sustainable and safe technology intended to reduce citrus losses due to both physiological and pathological problems. AECs are biodegradable emulsions (based on protein, polysaccharides or lipids, alone or in combination) formulated to replace commercial waxes, thus avoiding the use of synthetic components such as polyethylene wax, ammonia, and morpholine. The antifungal activity of these coatings is accomplished by adding additional ingredients with antifungal properties, such as plant extracts, essential oils (EOs), food additives or low toxicity compounds classified as generally recognized as safe (GRAS), and antagonists as biocontrol agents (Palou et al., 2016). Among these ingredients, many EOs and natural extracts have proven antifungal activity against important postharvest fungal pathogens. However, only a few studies reported the application of biopolymer-based ECs functionalized by the incorporation of these type of natural antifungal compounds for controlling citrus decay and preserving quality (Chafer et al., 2013; Zeng et al., 2013; Shao et al., 2015; Kharchoufi et al., 2018).

Among many polysaccharides studied to develop ECs, pectin (PEC) is an excellent film-former, biodegradable, biocompatible, easily available, and low cost compound (Panahirad et al., 2021). In addition, PEC films are odorless, tasteless, and with low oxygen permeability, which makes this biopolymer a good candidate to coat fresh fruits and vegetables because the created modified atmosphere greatly contributes to an extension of the produce postharvest life. On the other hand, PEC-based coatings are suitable for gaining new functionalities through the incorporation into the formulations of additional ingredients. For example, PEC coatings formulated with active antifungal ingredients such as GRAS salts and EOs have been successfully applied to fresh-cut persimmon, peach, and strawberry, among others (Ayala-Zavala et al., 2013; Guerreiro et al, 2015; Treviño-Garza et al., 2015; Sanchís et al., 2016). However, no information is available regarding the utilization of plant extracts and EOs as ingredients of citrus PEC-based ECs for the control of major fungal postharvest diseases of citrus fruit. Therefore, the objectives of this work were to evaluate the in vitro antifungal activity of EOs and plant extracts against *P. digitatum* and to develop PEC-based edible coatings with selected antifungal agents to reduce postharvest losses of 'Valencia' oranges. The curative activity against GM of PEC-based ECs was evaluated on artificially inoculated oranges incubated at 20°C and during long-term storage at 5°C. The effect of selected AECs on postharvest quality of cold-stored fruit, followed by a 7-day shelf-life period at 20°C, was also evaluated.

MATERIALS AND METHODS

In vitro antifungal activity of natural agents against *P. digitatum*

In vitro mycelial growth inhibition was evaluated in PDA Petri dishes by exposure to the volatile compounds of EOs in the vapor phase, as described by Plaza et al. (2004), or by direct contact of the natural extracts using the agar dilution method, as described by Martínez-Blay et al. (2020). Commercial EOs and volatile compounds evaluated were cinnamon (CN), lemongrass (LG), *Satureja montana* (SM) and myrrh (MY) EOs, eugenol (EU), and geraniol (GE). Dry extracts included green tea (GT), propolis (PRO), and vanillin (VA). Volatiles were applied at doses of 10, 20, and 40 µL by soaking 55 mm sterile filter paper

discs placed in the lid of 90-mm diameter PDA Petri dishes inoculated with *P. digitatum*. The dry extracts were evaluated at concentrations of 0.5, 1.0, and 2.0% GT; 0.5 and 1.0% PRO; 0.062, 0.125, and 0.25% VA; and 0.125, 0.25, and 0.5% MY. For both methods, each plate was inoculated by adding 20 µL of a 10^6 spores mL^{-1} suspension of *P. digitatum* in the center of the agar and incubated for 7 days at 25°C in the dark. Radial mycelial growth was determined in each plate by calculating the mean of two perpendicular fungal colony diameter measurements. Five replicates were used per treatment and results were expressed as the percentage of mycelial growth inhibition compared to control plates (PDA without antifungal agents).

Preparation of antifungal edible coatings (AECs)

Edible composite coatings were prepared by combining citrus PEC (2% wet basis; DE 70–75%; CEAMSA, Pontevedra, Spain) with the lipid beeswax (BW; 0.7% wb) and the EOs and natural extracts, selected from the in vitro test, suspended in water. Thus, SM, CN, EU, and GE were added to the PEC-based ECs at 0.2, 0.4, and 0.8%; VA at 0.25, 0.50, and 1.0%; PRO at 0.5, 1.0, and 2.0%; and MY at 1.5%. Glycerol (Gly) was used as plasticizer and oleic acid and palmitic acid as emulsifiers at ratios of PEC:Gly and BW:emulsifier of 2:1 and 5:1, respectively. To prepare the emulsions, all the ingredients except for the EOs and extracts were heated above the melting point of BW (>66°C) and samples were homogenized with a high-shear probe mixer for 1 min at 12,000 rpm, followed by 3 min at 22,000 rpm. The antifungal agents were incorporated after cooling down the emulsions to a temperature lower than 25°C, with further homogenization for 2 min at 16,000 rpm.

Curative activity of PEC-based edible coatings

P. 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 adjusted to a concentration of 10^5 spores mL^{-1}. 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 equatorial area of the rind of 'Valencia' oranges harvested in commercial orchards in Valencia (Spain). Treatments were applied after incubation of inoculated fruit at 20°C for 24 h, so curative activity was assessed. After this period, coatings were applied by adding 400 µL of the coating emulsion onto each fruit and rubbing with gloved hands, as described by Karaca et al. (2014). Treated oranges were incubated at 20°C and 90% RH for up to 12 days for assessment of GM incidence and severity. During this period, disease incidence (% of infected fruits) and disease severity (mm, diameter of the lesion) were evaluated. Control fruit included inoculated but untreated oranges and inoculated oranges treated with PEC-BW without antifungal gents. In every case, 4 replicates of 5 oranges each were used for each treatment.

Effect of selected AECs on decay and quality of cold-stored oranges

AECs containing 0.8% CN, 0.8% EU, 0.2% GE, or 1.5% MY were selected from the previous experiment to be tested during fruit cold storage. GM control was evaluated on artificially inoculated oranges coated 24 h later as described above. Disease incidence (%) and severity (mm) were assessed after 2, 4, and 6 weeks of storage at 5°C and 90% RH in 4 replicates of 10 fruits each.

Fruit quality was assessed at harvest and after 4 and 8 weeks of cold storage at 5°C and 90% RH plus 1 week of shelf life at 20°C, following the methodology described by Valencia-Chamorro et al. (2009). Quality attributes included weight loss (% of initial weight loss; n=20 fruits per treatment), firmness (% of rind deformation related to initial fruit diameter after application of a load of 10 N to the equatorial region of the fruit, n=10 fruits per treatment), juice quality (titratable acidity (TA, g L^{-1} of citric acid) and soluble solids

content (SSC, °Brix); n=3 juice replicates of five fruit each per treatment), volatiles content in juice (ethanol and acetaldehyde content (mg L^{-1}) in the headspace of juice samples using a gas chromatograph; n=3), and sensory properties (overall taste, off-flavor, external appearance and ranking of fruit gloss; n=10 semi-trained judges).

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). Prior to analysis, disease incidence data were arcsine transformed. Friedman test ($P<0.05$) was used for ranking fruit gloss. Analyses were performed with the software Statgraphics Centurion XVII (Statgraphics Technologies Inc., The Plains, VA, USA).

RESULTS AND DISCUSSION

In vitro antifungal activity of natural agents against *P. digitatum*

According to the chemical nature of the antifungal compounds, two different methods were used for the in vitro antifungal activity assessment. The volatile exposure method was used to test the activity of commercial EOs and pure volatile compounds; whereas, the dry extracts and MY EO, which showed no activity when tested in the vapor phase due to its low volatility compared to the other EOs, were evaluated by the agar dilution method (Figure 1). Among the natural agents applied as volatiles, SM and EU (at a dose of 10 µL) and CN and GE (at a dose of 20 µL) were the most effective to inhibit the mycelial growth of *P. digitatum*, with 90-100% inhibition. Among the agents applied in agar, 0.125% VA and 0.5% PRO were the most effective, with 100% growth inhibition. GT (at 1 and 2%) and MY EO (at 0.125–0.5%) showed a moderate inhibitory effect against GM. In the case of MY EO, the results contrast with the null effect when applied as volatiles.

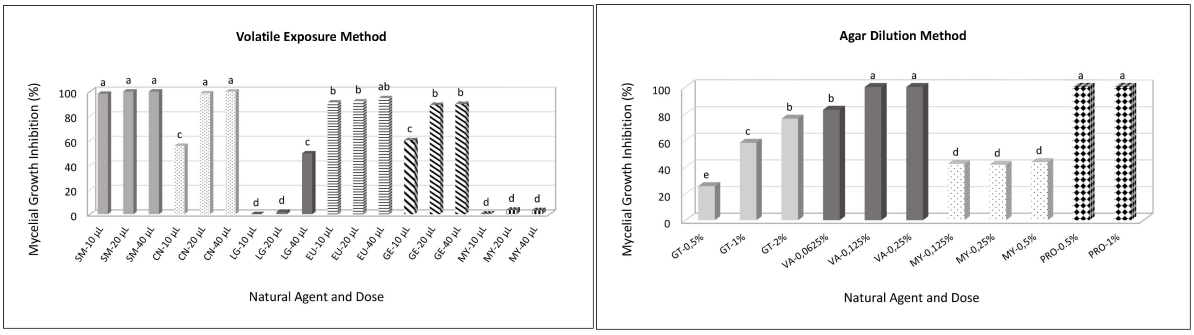

Figure 1. Mycelial growth inhibition of *Penicillium digitatum* exerted by essential oils (EOs) and natural extracts in in vitro tests after 7 days of incubation at 25°C. For each method, means with different letters indicate significant differences among treatments according to LSD test ($P<0.05$).

Similarly, several research works have reported the in vitro inhibitory effect of these natural agents against *P. digitatum* and other postharvest fungi (Plaza et al., 2004; Combrinck et al., 2011; Prakash et al., 2012; Dudoit et al., 2020; Moreno et al., 2020). In these works, the effectiveness depended on many factors such as the fungus, type of test, dose, and differences in the composition of the EOs and natural extracts used. Typically, the antifungal activity of these agents cannot be explained by a single specific mechanism, but rather by the combined effect of the different chemical constituents that include high contents of terpenes, terpenoids, esthers, aldehydes, polyphenolic compounds, phenolic acids, and other aromatic constituents (Kuorwel et al., 2011). Furthermore, in the case of

EOs, their effectiveness has also been attributed to their hydrophobic character, which might contribute to separate the lipids of the bacterial cell membranes, making them more permeable (Combrinck et al., 2011). This effect could explain the higher effectiveness of MY EO when tested by direct contact in the agar dilution method compared to the volatile exposure method.

Curative activity of PEC-based edible coatings

Based on the in vitro results, SM, CN, EU, GE, VA, PRO, and MY were incorporated into PEC-based formulations to evaluate their curative activity on 'Valencia' oranges inoculated with *P. digitatum*. Results of disease incidence and severity reduction compared to uncoated fruit after 8 days of incubation at 20°C are shown in Figure 2. As expected, the PEC coating without antifungal ingredient did not control GM after incubation at 20°C, confirming the need for additional active antifungal ingredients in the PEC matrix. Among the different active ingredients, 0.4% and 0.8% EU, 0.2% GE, and 1.5% MY significantly reduced the incidence of GM by more than 40%, while a reduction in disease severity above 45% was observed with 0.4% EU, 0.8% CN, and GE, at all tested concentrations (P<0.05). However, SM, VA, and PRO, which were very effective in the in vitro tests, did not reduce the disease incidence and severity on coated fruit. On the other hand, the effectiveness of these natural agents in reducing GM incidence and severity did not always increase with increasing concentrations.

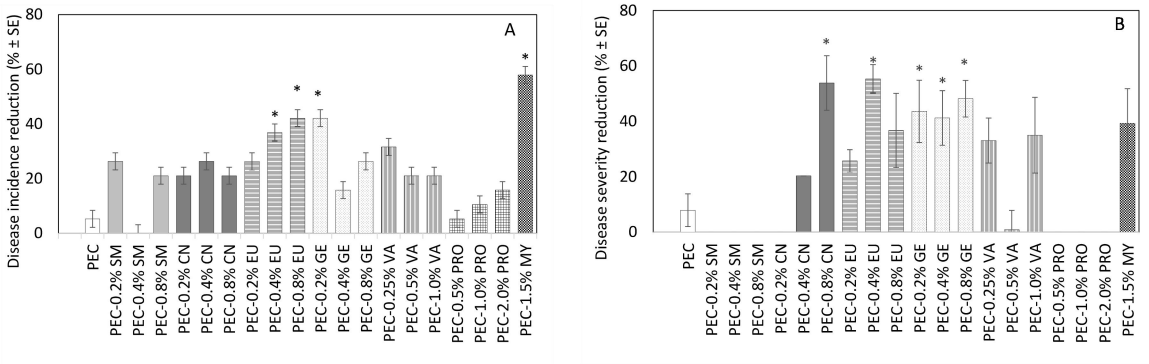

Figure 2. Reduction in green mold incidence (A) and severity (B) with respect to uncoated (control) fruit on 'Valencia' oranges artificially inoculated with *Penicillium digitatum*, coated 24 h later with pectin (PEC)–lipid edible composite coatings containing different concentrations of selected antifungal agents, and incubated for 8 days at 20°C and 90% RH. Mean incidence and severity on control fruit were 95.0 ± 5.0% and 90.7 ± 17.2 mm, respectively. * indicates means significantly lower than control and PEC-coated (without antifungal agent) samples (P<0.05).

In general, the complex interactions between host, pathogen, and environment that occur during disease development determine the in vivo disease control ability of antifungal agents (Palou et al., 2016). In addition, the success of AECs for fruits is not only determined by the selection of the most appropriate coating-forming constituents and active ingredients for each pathogen and fruit host, but also by other factors such as type of coating application, environmental storage conditions, and bioavailability of the active ingredients. Thus, emulsion composition and properties, possible interactions of the antifungal compound with the coating components, and volatility of the ingredient, can play an important role in the overall antifungal performance of the coating since they can affect the availability and release ability of the agent (Karaca et al., 2014).

Effect of selected AECs on GM control on cold-stored oranges

Based on the results of GM incidence and severity on coated fruits during incubation at 20°C, AECs containing 0.8% CN, 0.8% EU, 0.2% GE, or 1.5% MY were selected to be tested under commercial cold-storage conditions and the curative activity on inoculated 'Valencia' oranges is shown in Table 1. After 4 weeks at 5°C, AECs formulated with EU and GE significantly reduced GM incidence by more than 50%. At the end of the storage period, although EU and GE coatings induced lower disease incidence than the coating without antifungals, no significant differences were observed between coated and uncoated oranges. In the case of disease severity, all the AECs, except for PEC-1.5% MY, significantly inhibited fungal growth during the entire cold-storage period, with reductions that reached 40-50% after 6 weeks. These results confirm that the effect of these natural agents is rather fungistatic than fungicidal, as reported in many other works with citrus fruits where GRAS salts, EOs, or natural extracts have been incorporated to biopolymer-based ECs (Chafer et al., 2012; Shao et al., 2015; Soto-Muñoz et al., 2021).

Table 1. Incidence and severity of green mold on 'Valencia' oranges artificially inoculated with *Penicillium digitatum*, uncoated (control) or coated with selected pectin (PEC)–lipid edible composite coatings containing essential oils and stored at 5°C and 90% RH for up to 6 weeks.

Treatment	Incidence (% ± SE)			Severity (mm ± SE)		
	2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
Control	10.0 ± 0.0 a	62.5 ± 10.3 ab	75.0 ± 9.6 ab	0.6 ± 0.1 a	29.2 ± 8.2 ab	82.9 ± 17.6 ab
PEC	7.5 ± 0.0 ab	80.0 ± 10.8 a	87.5 ± 6.3 a	0.7 ± 0.5 a	30.5 ± 3.8 a	101.4 ± 7.3 a
PEC-0.8% CN	0.0 ± 0.0 b	40.0 ± 4.1 bc	60.0 ± 8.2 b	0.0 ± 0.0 a	13.5 ± 2.5 cd	56.7 ± 7.2 bc
PEC-0.8% EU	2.5 ± 2.5 b	27.5 ± 6.3 c	55.0 ± 6.5 b	0.3 ± 0.3 a	9.2 ± 2.9 d	44.4 ± 6.1 c
PEC-0.2% GE	2.5 ± 2.5 b	32.5 ± 7.5 c	57.5 ± 9.5 b	0.1 ± 0.1 a	15.6 ± 4.7 bcd	51.7 ± 7.7 bc
PEC-1.5% MY	2.5 ± 2.5 b	52.5 ± 8.5 bc	77.5 ± 7.5 ab	0.1 ± 0.1 a	26.6 ± 5.5 abc	91.3 ± 12.3 a

For each parameter and storage time, means with different letters are significantly different according to LSD test ($P<0.05$).

Effect of selected AECs on the physicochemical and sensory quality of cold-stored oranges

AECs containing EU, GE, or MY significantly reduced weight loss of 'Valencia' oranges at the end of the storage period (Table 2). The reduction in weight loss by these AECs did not translate into a maintenance of fruit firmness, which decreased during storage time from 2.4 to 4.0% rind deformation (data not shown). Similarly, no significant differences in TA and SSC were observed among coated and uncoated oranges during the entire storage period, with values that ranged from 7 to 9 mg L^{-1} and 10 to 11 °Brix, respectively (data not shown). On the other hand, the application of the AECs increased the content of ethanol and acetaldehyde in the juice, showing the ability of the coatings to modify the fruit internal gas composition by creating a barrier to gases (Table 2). However, sensory properties of oranges were not negatively affected by the application of the AECs ($P>0.05$). As expected, overall flavor decreased with storage period and, at the end of storage, it was rated as acceptable (from 5 to 6) and off-flavor scores were considered very slight (<2.0) for all the samples (data not shown). Furthermore, the PEC-0.8% EU coating improved fruit gloss after 4 weeks of cold storage plus the shelf-life period, probably due to differences in the optical properties of the coating according to its composition (data not shown).

Table 2. Weight loss and volatile content in the juice of 'Valencia' oranges uncoated (control) or coated with pectin (PEC)–lipid edible coatings containing essential oils after different periods of cold storage, followed by 1 week of shelf life at 20°C.

Treatment	Weight loss (% + SE)		Acetaldehyde (mg L^{-1} + SE)		Ethanol (mg L^{-1} + SE)	
	4 wks 5°C + 1 wk 20°C	8 wks 5°C + 1 wk 20°C	4 wks 5°C + 1 wk 20°C	8 wks 5°C + 1 wk 20°C	4 wks 5°C + 1 wk 20°C	8 wks 5°C + 1 wk 20°C
Control	2.25 ± 0.07 a	3.75 ± 0.12 a	10.5 ± 0.5 c	10.5 ± 1.4 b	316.1 ± 14.9 d	434.7 ± 86.9 d
PEC	2.26 ± 0.07 a	3.77 ± 0.15 a	13.2 ± 1.1 abc	18.4 ± 0.3 a	534.2 ± 91.5 bc	1039.7 ± 31.0 ab
PEC-0.8% CN	2.15 ± 0.10 ab	3.60 ± 0.13 ab	14.5 ± 0.9 a	20.5 ± 0.5 a	810.2 ± 105.2 a	1148.9 ± 78.0 a
PEC-0.8% EU	1.97 ± 0.09 bc	3.05 ± 0.16 c	15.0 ± 0.8 a	18.6 ± 1.5 a	712.3 ± 44.7 ab	905.9 ± 103.4 bc
PEC-0.2% GE	1.97 ± 0.08 bc	3.34 ± 0.13 bc	13.8 ± 0.7 ab	19.1 ± 0.4 a	544.0 ± 51.5 b	747.1 ± 11.6 c
PEC-1.5% MY	1.87 ± 0.08 c	2.94 ± 0.13 c	11.7 ± 1.2 bc	19.3 ± 0.5 a	328.5 ± 57.2 cd	1011.6 ± 33.8 ab

For each parameter and storage time, means with different letters are significantly different according to LSD test (P<0.05).

CONCLUSION

Among the different AECs evaluated, those formulated with EU or GE showed a significant curative activity against GM on artificially inoculated oranges either incubated at room temperature or stored at 5°C. Moreover, AECs containing EU or MY satisfactorily reduced weight loss and maintained the quality of cold-stored oranges, while ECs with EU improved fruit gloss. Therefore, the PEC-EU could be a promising commercial treatment to reduce GM decay and maintain postharvest quality of citrus fruits, providing a safe alternative to conventional waxes amended with synthetic chemical fungicides.

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