Assessment of optimal postharvest treatment conditions to control green mold of oranges with sodium benzoate

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Abstract

Green mold, caused by the pathogenic fungus *Penicillium digitatum*, is the most important citrus postharvest disease in Mediterranean climate areas such as Spain. Due to health and environmental issues, alternative methods to synthetic chemical fungicides are needed to control this disease. The most effective concentration of the food additive and GRAS (generally regarded as safe) compound sodium benzoate (SB) was 3% (w v^{-1}), as determined in *in vivo* primary screenings with 'Valencia' oranges. Optimal postharvest treatment conditions for maximum curative activity of SB against green mold were assessed on 'Valencia' oranges artificially inoculated with *P. digitatum* and dipped 24 h later in 3% SB aqueous solutions. Tested dip temperatures were 20, 50, 53, 58, and 62ºC. Dips at each of these temperatures were performed for two or more of the following immersion times: 5, 15, 30, 60, and 150 s. Dip treatments at 50 or 53ºC for 30 or 60 s resulted in reductions of green mold incidence of 60-80% on oranges incubated at 20ºC for 7 days. Furthermore, SB treatment at 50ºC for 60 s reduced by about 85% the incidence of green mold on 'Valencia' oranges inoculated, treated, and stored for 2 months at 5°C and 90% RH. Therefore, heated SB aqueous solutions may be an interesting additional tool for integrated control of green mold in citrus packinghouses, especially for markets with zero tolerance to fungicide residues.

Keywords: *Citrus sinensis*, *Penicillium digitatum*, GRAS compounds, alternative postharvest decay control

INTRODUCTION

Green mold, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is one of the major 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 synthetic fungicides such as imazalil (IMZ), thiabendazole (TBZ), sodium-o-phenylphenate (SOPP), or other active ingredients. Nevertheless, consumer trends and legislation changes are increasingly favoring a continuous reduction in the amount of these substances allowed by authorities to be present on fruit. Further, 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, dip treatments with low toxicity substances with antimicrobial properties are interesting because the replacement of

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synthetic fungicides by these products would not require substantial changes in the industrial procedures followed in citrus packinghouses. Inorganic or organic salts listed as food additives or generally regarded as safe (GRAS) compounds are good candidates because they are allowed for many industrial and agricultural applications by regulations worldwide. Among them, carbonates and bicarbonates (Smilanick et al., 1999; Palou et al., 2001; Youssef et al., 2014), potassium sorbate (Smilanick et al., 2008; Montesinos-Herrero et al., 2009), or sodium parabens (Moscoso-Ramírez et al., 2013) have provided satisfactory control of green mold in laboratory or semicommercial trials. According to Hall (1988), other GRAS salts with antifungal activity like sodium benzoate (SB; EU food additive number E-211) may have similar inhibitory activity against *P. digitatum*, but they have not been extensively assayed in postharvest applications for control of citrus postharvest diseases.

Therefore, the objectives of this research work were to evaluate the curative activity of postharvest treatments with SB for green mold control on artificially inoculated oranges and optimize the conditions for SB dip applications. The effectiveness of SB dips was also evaluated on long-term cold-stored fruit.

MATERIALS AND METHODS

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 25ºC. 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 or 10^6 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 'Valencia' oranges. Fruit were inoculated with *P. digitatum* at one point in the equatorial zone of the fruit rind. Treatments were always applied after incubation of inoculated fruit at 20ºC for 24 h, so curative activity was assessed.

SB concentration

Solutions of the organic salt SB ($NaC_7H_5NaO_2$; Guinama S.L., Alboraia, València, Spain) were prepared at concentrations of 100 and 200 mM by diluting with sterile water. The most effective concentration was determined in in vivo primary screening tests. About 24 h after the inoculation of the pathogen at 10^5 spores mL^{-1}, 30 µL of SB solution at the corresponding concentration and room temperature were placed, using a micropipette, in the same inoculation rind wound. Control fruit were treated with 30 µL of sterile distilled water at room temperature. For each treatment, 4 replicates of 5 'Valencia' oranges each were used. Treated fruit were incubated at 20ºC and 90% RH for 6 days, at which time disease incidence (% of infected fruit) was determined. The experiment was conducted 3 times.

Assessment of dip treatment conditions

Small-scale trials were conducted using 'Valencia' oranges to establish the best dip treatment conditions to resemble potential commercial applications in citrus packinghouses. Dips of oranges artificially inoculated 24 h before with *P. digitatum* at 10^6 spores mL^{-1}, were performed in stainless steel buckets containing 10 L of 3% (w v^{-1}) aqueous solution of SB. This concentration was used according to the results of previous in vivo primary screenings, in which the concentration of 200 mM (2.9%) was selected. For heated treatments, SB solution was heated by placing the buckets in a 250-L stainless steel water tank fitted with two electrical resistances (4.5 kW each), a thermostat, and an

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automatic water-recirculating system. Fruit were placed into 18 L multi-perforated wall stainless steel containers, exactly fitting in the above-mentioned buckets, and completely immersed in the SB solution for 15, 30 or 60 s at temperatures of 20, 50, 53, 58, or 62°C, although not all these combinations were tested (Table 1). Control fruit were dipped in water at 20°C for 60 s. SB-treated fruit were rinsed for 5 s with tap water at low pressure to eliminate salt residues from the fruit rind. Each treatment was applied to 3 replicates of 25 fruit each. 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 was assessed.

Effectiveness on long-term cold-stored oranges

The curative activity of postharvest SB dips against green mold was evaluated on 'Valencia' oranges artificially inoculated 24 h before with *P. digitatum* at 10^6 spores mL^{-1}, dipped in 3% SB aqueous solution at 50ºC for 60 s and stored in a cold room for up to 2 months at 5ºC and 90% RH followed by a shelf life period of 7 days at 20ºC and 70% RH. These dip conditions were selected as the most effective and practical in previous experiments. Control fruit were dipped in water alone at 20°C for 60 s. SB-treated fruit were rinsed with water as described above. Three replicates of 20 fruit each were used per treatment. Disease incidence was assessed after 1 and 2 months at 5ºC and after shelf life.

Statistical analysis

Data were analyzed by an analysis of variance (ANOVA) with statistical software (Statgraphics Plus v. 5.1; Statpoint Technologies Inc., Warrenton, VA, USA). 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

SB concentration

The most effective SB concentration for green mold control in in vivo primary screenings with 'Valencia' oranges was 200 mM (29 g L^{-1}, 2.9%), with an average disease reduction close to 100% in all three repeated experiments (data not shown). For practical reasons, a concentration of 3% (w v^{-1}) was selected for use in subsequent trials.

Dip treatment conditions

Irrespective of immersion time and dip temperature, dips with 3% SB significantly reduced the incidence of green mold with respect to control fruit (treated with water at 20ºC for 60 s) on 'Valencia' oranges inoculated 24 h before treatment and incubated for 7 days at 20°C (Table 1). While disease incidence was 95% on control fruit, it was 18, 18, and 46% on fruit dipped at 20ºC for 15, 30, and 60 s, respectively. Depending on the experiment, heating SB solutions to 50, 53, 58, or 62ºC significantly improved the effectiveness of SB dips, irrespective of immersion time. The highest disease reductions were obtained with solutions heated to 58 or 62ºC, but these temperatures were too high from a practical point of view of potential industrial application and risks of rind phytotoxicities associated with heat. Therefore, dips at 50ºC for 30 or 60 s were selected as the most practical treatments and these conditions were applied on the subsequent trials with long-term cold-stored oranges.

Interestingly, the general effectiveness of 3% SB applied as aqueous dips was considerably lower than that obtained in in vivo primary screenings (green mold reductions close to 100%). This was possibly due to the increased contact time of the SB drop with the

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rind wound inoculated with *P. digitatum* with respect to the contact time of dip treatments (up to 60 s), and also to the fact that SB-treated oranges were rinsed with water after dip treatment. These results are in agreement with those from research with other commodities such as stone fruit (Palou et al., 2009) or longan fruit (Yahia, 2011), in which a noticeable antimicrobial effect of SB postharvest treatments was observed. In the present work, the combination of SB with heat was synergistic, similarly to what have been reported in previous studies with other GRAS salts such as sodium carbonates (Palou et al., 2001) or potassium sorbate (Montesinos-Herrero et al., 2009).

Regarding the mode of action of SB against *P. digitatum*, it is probable that in acidic medium such as the citrus rind, the undissociated form of benzoic acid is able to enter the fungal cell and the neutralization of this form causes an acidification responsible for cell growth inhibition (Krebs et al., 1983). The observed synergism between heat and SB treatments seems to indicate that high temperatures increase the cell membrane permeability, facilitating the entrance of SB, the internal pH reduction, and the formation of benzoic acid.

Table 1. Green mold incidence on 'Valencia' oranges artificially inoculated with *Penicillium digitatum*, dipped 24 h later in water at 20ºC for 60 s (Control) or in 3% sodium benzoate (SB) aqueous solutions at different temperatures for 15, 30, or 60 s, and incubated at 20ºC for 7 days.

	Green mold incidence (% ± SE)[1]		
	Immersion time (s)		
SB dip temperature (ºC)	15	30	60
Water-20 (Control)	95.0 ± 2.9 a	95.0 ± 0.0 a	95.0 ± 2.9 a
20	18.3 ± 3.3 bc	18.3 ± 1.7 b	45.9 ± 3.7 b
50	-[2]	-	30.0 ± 2.9 c
53	28.3 ± 8.3 b	15.0 ± 2.9 b	-
58	11.6 ± 1.7 c	6.6 ± 1.7 c	-
62	8.3 ± 3.3 c	5.0 ± 2.9 c	6.7 ± 2.7 d

[1]For each immersion time, 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]Not applied.

Effectiveness on long-term cold-stored oranges

'Valencia' oranges inoculated with *P. digitatum* and treated with water at 20ºC (control) showed very high levels of decay (more than 98%) after 1 month of storage at 5ºC. At this time, the incidence of green mold was only around 7% on fruit dipped in 3% SB at 50ºC for 60 s (Table 2). However, the incidence of green mold on treated fruit gradually increased along storage time and even more after 1 additional week of shelf life at 20ºC, when disease incidence reached 26%. This fact indicates that, in contrast to conventional fungicides and similarly to other food additives (Palou et al., 2008), SB is a poor eradicant unable to kill all the spores in rind wounds; hence, its inhibitory action is more fungistatic than fungicidal and not very persistent.

CONCLUSION

In the present research, SB postharvest dips showed considerable efficacy for the control of green mold on 'Valencia' oranges. This kind of alternative treatments is currently very demanded worldwide, particularly for organic markets or conventional markets with zero or very low tolerance to fungicide residues. Heated SB solutions could be of application

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at the commercial level in many modern citrus packinghouses and, as an allowed food additive or GRAS compound, SB may have the same level of allowance by authorities than potassium sorbate, which is currently accepted as postharvest treatment for citrus fruit with a maximum residue level (MRL) of 20 mg kg^{-1}. Therefore, postharvest heated SB treatments may be an interesting additional tool for integrated control of green mold in citrus packinghouses.

Table 2. Green mold incidence on 'Valencia' oranges artificially inoculated with *Penicillium digitatum*, dipped 24 h later for 60 s in water at 20ºC (Control) or in 3% sodium benzoate aqueous solutions at 50ºC (SB), and stored for 2 months at 5ºC followed by 1 wk of shelf life (SL) at 20ºC.

	Green mold incidence (% ± SE)[1]	
	Dip treatment	
Storage time (months)	Control	SB
1	98.3 ± 1.7 a	6.8 ± 4.4 b
2	98.3 ± 1.7 a	17.5 ± 4.3 b
2+1 wk SL	100.0 ± 0.0 a	26.2 ± 4.6 b

[1]For each storage time, values in rows 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.

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