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Evaluation of sodium benzoate and other food additives for the control of citrus postharvest green and blue molds

Clara Montesinos-Herrero[a,b], Pedro A. Moscoso-Ramírez[a,c], Lluís Palou[a,*]

[a] Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d'Investigacions Agràries (IVIA), 46113 Montcada, València, Spain
[b] Decco Ibérica Post-Cosecha S.A.U., 46988 Paterna, València, Spain
[c] Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México

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ABSTRACT

The curative activity of the food additives dehydroacetic acid, dimethyl dicarbonate, ethylene diamine tetracetic acid, sodium acetate, and sodium benzoate (SB) was tested in *in vivo* preliminary screenings against green and blue molds on citrus fruit artificially inoculated 24 h before with *Penicillium digitatum* and *Penicillium* italicum, respectively. SB was the most effective compound and it was further tested in trials simulating postharvest industrial applications. Dip treatments for 60 s with 3% (w/v) SB heated above 50 °C resulted in about 90% reduction of green and blue mold incidence on 'Valencia' oranges inoculated, treated, and incubated at 20 °C and 90% RH for 7 days. This treatment was also effective on 'Lanelate' oranges, 'Fino' lemons and 'Ortanique' mandarins, but not on 'Clemenules' mandarins. Heated solutions combining SB with low doses (25 or 50 $\mu L\,L^{-1}$) of the fungicide imazalil (IMZ) were synergistic and greatly improved the efficacy of stand-alone treatments. On 'Valencia' oranges stored for 8 weeks at 5 °C followed by 7 days of shelf-life at 20 °C, this combination reduced the incidence of green and blue molds almost by 100%. It was found in additional trials to test the preventive activity that 3% SB dips at 50 °C for 60 s did not reduce green mold on 'Valencia' oranges treated, inoculated with *P. digitatum* 24 h later, and incubated at 20 °C for 7 days. It can be concluded from this work that heated SB aqueous solutions might be in the future an interesting nonpolluting disease control alternative for the commercialization of citrus in markets with zero tolerance to fungicide residues.

1. Introduction

The most common citrus postharvest diseases in Mediterranean climate regions are green and blue molds, caused by *Penicillium digitatum* and *P. italicum*, respectively (Eckert and Eaks, 1989; Palou, 2014). Economic losses due to these diseases have been reduced to commercially acceptable levels by the use of synthetic fungicides such as imazalil (IMZ), thiabendazole (TBZ), sodium-*o*-phenylphenate, or others for more than 30 years (Brown, 1985; Erasmus et al., 2013; D'Aquino et al., 2013). Deeper knowledge about residue levels in fruit and the toxicology of these fungicides and, on the other hand, consumers trends to eat more natural food, are favoring a continuous reduction in the amount of these substances allowed by authorities to be present on fruit. Furthermore, at present, large citrus distributors and major supermarket chains are even demanding particular and more restrictive fungicide usage. In addition, rising populations of resistant strains of disease-causing pathogens to these fungicides are an important threat, which is compromising the efficacy of the treatments (Bus et al., 1991; Eckert et al., 1994; Holmes and Eckert, 1995; Zhu et al., 2006; Kinay et al., 2007; Sánchez-Torres and Tuset, 2011). Consequently, the citrus industry worldwide is increasingly demanding for alternatives to conventional fungicides to control postharvest diseases. In the last few years, many studies have been published and reviewed on alternatives to synthetic fungicides for the control of postharvest decay of fresh horticultural produce (Palou et al., 2008; Cunningham, 2010; Janisiewicz and Conway, 2010; Montesinos-Herrero and Palou, 2010; Romanazzi et al., 2012; Bautista-Baños, 2014). Among them, dip treatments with low toxicity substances with antimicrobial properties has been one of the first approaches (Hall, 1988), since the substitution of synthetic fungicides by these products would not require substantial changes in the industrial procedures followed in the packinghouses. These alternative compounds should be natural or

* Corresponding author. Fax: +34 963424001.
E-mail address: palou_llu@gva.es (L. Palou).

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synthetic substances with toxicity to humans and wildlife extensively evaluated and proven to be very low. Food additives, especially preservatives, and generally regarded as safe (GRAS) compounds, which are allowed with very few restrictions for many industrial and agricultural applications by regulations worldwide meet these conditions. A number of food additives have been successfully tested for this purpose against citrus postharvest diseases. These include carbonates and bicarbonates (Smilanick et al., 1999; Sorenson et al., 1999; Palou et al., 2001, 2002; Zhang and Swingle, 2003; Plaza et al., 2004; Venditti et al., 2005; Youssef et al., 2014), potassium sorbate (Smilanick et al., 2008; Montesinos-Herrero et al., 2009), or sodium parabens (Moscoso-Ramírez et al., 2013a, 2013b, 2014). Other food additives with antimicrobial activity, commonly used as preservatives, may have similar control ability when applied as postharvest treatments against citrus pathogens, but they have not been extensively assayed in postharvest applications. This is the case of sodium benzoate (SB; EU food additive number E-211), which was first identified as a potential citrus postharvest antifungal agent by Hall (1988). This worker found that the efficacy of treatments with 2% (w/v) SB in the control of green mold was similar to that of TBZ commercial treatments. More recently, Palou et al. (2009) tested *in vivo* several food additives against postharvest pathogens of stone fruit such as *Monilinia fructicola, Botrytis cinerea, Geotrichum candidum, Alternaria alternata,* or *Penicillium expansum* and found that treatments with 200 mM SB were among the most effective in the control of diseases caused by these pathogens. *In vitro* assays with ethylendiaminetetraacetic acid (EDTA, E-385) showed complete inhibition of *P. italicum* growth and sporulation (Askarne et al., 2011). Dehydroacetic acid sodium salt (NaDHA, E-265) was successfully tested in dip treatments to reduce postharvest spoilage of different fruit and vegetables (Smith, 1962). In preliminary tests, sodium acetate salts (NaAc, E-262) reduced by 70% the incidence of gray mold caused *B. cinerea* on sweet cherries compared to the water control treatment (Ippolito et al., 2005). Postharvest treatments with 200 mg L^{-1} of dimethyl dicarbonate (DMDC, E-242) significantly reduced the total mold count of the leaf and stalk of Chinesse cabbage and this substance was suggested as an alternative sanitation treatment (Chen et al., 2013). Likewise, count of total yeasts and molds in fresh-cut carrots treated with DMDC were significantly reduced by 3.01 and 3.43 log cfu g^{-1}, respectively, in comparison with water-treated controls (Wang et al., 2012). Therefore, according to such previous reports, the objective of the present work was to test the efficacy of postharvest treatments with SB, EDTA, NaDHA, NaAc, and DMDC against green and blue molds of citrus fruit, and to assess the feasibility of the application of selected compounds, *viz.* SB, as part of the commercial handling procedures followed in the packinghouses for decay control.

2. Materials and methods

2.1. Fruit

Fruit used in the experiments were 'Valencia' and 'Lanelate' oranges (*Citrus sinensis* (L.) Osbeck), 'Clemenules' (synonyms: 'Nules', 'Clementina de Nules') clementine mandarins (*Citrus clementina* Hort. ex Tanaka), 'Ortanique' [*Citrus reticulata* Blanco × (*C. sinensis* × *C. reticulata*); synonym: 'Topaz'] hybrid mandarins, and 'Fino' lemons (*Citrus limon* (L.) Burm.). Fruit were collected from commercial orchards in the Valencia area (Spain) and used the same day or stored up to 1 week at 5 °C and 90% relative humidity (RH) before use. Fruit used in the study were free from previous postharvest treatments or coatings. Before each experiment, fruit were selected, randomized, washed with tap water and allowed to air-dry at room temperature.

2.2. Fungal inoculation

Penicillium digitatum and *P. italicum*, isolates NAV-7 and MAV-1, respectively, from the fungal culture collection of the IVIA CTP, were cultured on potato dextrose agar (PDA, Sigma-Aldrich Chemical Co., St. Louis, MA, USA) plates at 25 °C. Conidia of each fungus from 7 to 14-day-old cultures were taken from the agar surface with a sterile rod and transferred to a sterile aqueous solution of 0.05% Tween® 80 (Panreac, S.A.U., Barcelona, Spain). Conidial suspensions were filtered through two layers of cheesecloth to separate hyphal fragments and adjusted to a concentration of 10^5 or 10^6 spores mL^{-1} using a haemocytometer. 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 fruit rind afterwards. Except for *in vivo* primary screening tests, fruit were inoculated at two opposite points in the fruit equatorial zone, one with *P. digitatum* and the other with *P. italicum.* Inoculated fruit were kept in a temperature-controlled room at 20 °C and 90% RH for 24 h, until treatment. In the case of *in vivo* primary screenings, each pathogen was inoculated in different sets of fruit.

2.3. In vivo *primary screenings*

Several substances, previously selected for their potential antifungal properties, were tested at different concentrations to assess their control ability of citrus postharvest green and blue molds on fruit previously inoculated with the pathogens. These concentrations and substances were 100 and 200 mM SB ($NaC_7H_5NaO_2$; Guinama S.L., Alboraia, València, Spain); 0.1, 1, 10, 20, 40, 50, 70, and 100 mM EDTA ($C_{10}H_{16}N_2O_8$); 0.1, 1, 4, 7, 10, 20, 30, 40, 70, and 100 mM NaDHA ($C_8H_8O_4$); 1, 10, 40, 70, 100, 140, 170, 200, 300, 400, 500, 600, 800, and 1000 mM NaAc ($NaC_2H_3O_2$); and 0.07, 0.75, 7.5, 75, 150, 300, 450, and 600 mM DMDC ($C_4H_6O_5$) (all purchased to Sigma–Aldrich Chemie Gmbh, Steinheim, Germany). Inoculation with *P. digitatum* or *P. italicum* was carried out following the procedure described above, with an inoculum concentration of 10^5 spores mL^{-1}. About 24 h after fungal inoculation, 30 μL of the solution to be tested at the specified concentration were placed, using a micropipette, in the same inoculation rind wound. Control fruit were treated with 30 μL of sterile distilled water. For each combination of pathogen, substance, and concentration, 4 replicates of 5 'Valencia' oranges each were used. Treated fruit were incubated at 20 °C and 90% RH for 3 and 6 days, at which time disease incidence (% of infected fruit) was determined. Trials were repeated three times, and average values were calculated.

2.4. Dip treatment conditions

Since SB at 200 mM (29 g L^{-1}; 2.9% w/v) was selected as the best among all treatments assayed in the previous *in vivo* primary screening tests, trials with 3% SB were conducted using 'Valencia' oranges to establish the best dip conditions for this treatment. Fruit were inoculated with *P. digitatum* and *P. italicum* at a concentration of 10^6 spores mL^{-1} following the procedure mentioned above, and then dip-treated using stainless steel buckets containing 10 L aqueous solution of 3% SB. When needed, solutions were 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 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 treatment solution for 5, 15, 30, 60, or 150 s at 20, 50, 53, 58, 62, 65, or 68 °C, although not all time-temperature combinations were tested. After treatment, the fruit were rinsed for 5 s with tap water at low pressure in order to eliminate SB salt residues. Control fruit