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

Clara Montesinos-Herrero[1,2], Pedro A. Moscoso-Ramírez[1,3], Lluís Palou[1]

[1]*Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), 46113 Montcada, València, Spain.*

[2]*Decco Ibérica Post-Cosecha S.A.U., 46988 Paterna, València, Spain.*

[3]*Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México.*

*** Corresponding author:** palou_llu@gva.es; Tel. +34 963424117;

Fax: +34 963424001

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 *P. 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 ºC 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 μL 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.

Keywords: *Penicillium digitatum*; *P. italicum;* sodium benzoate; dehydroacetic acid; dimethyl dicarbonate; ethylene diamine tetracetic acid; sodium acetate

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 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,b, 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 x (*C. sinensis* x *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 ºC. 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 ºC 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 ºC 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 were treated with water alone at 20 °C for 60 s. Each treatment was applied to 3 replicates of 25 fruit, which were arranged in plastic cavity sockets on cardboard trays. Treated fruit were incubated at 20 °C and 90% RH for 7 days, at which time disease incidence was assessed. Three days after treatment, fruit were inspected for potential rind injuries caused by the treatments.

The same procedure was followed in further experiments to test the treatments on 'Clemenules' mandarins, 'Fino' lemons, and 'Ortanique' hybrid mandarins. Treatment conditions tested on these cultivars were chosen according to results previously obtained with 'Valencia' oranges.

2.5. Combination with low doses of imazalil

Following the same procedure previously described for dip treatments, 'Valencia' oranges were inoculated with 10^6 spores mL^{-1} *P. digitatum* or *P. italicum*, held for 24 h at 20 ºC and 90% RH, and treated for 60 s dips in deionized water (control), 3% SB, 25 μL L^{-1} active ingredient (a.i.) of IMZ (IMZ 25; (±)-1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl)-1H-imidazole; Fecundal 7.5% EC; Fomesa Fruitech S.L., Valencia, Spain), 50 μL L^{-1} a.i. IMZ (IMZ 50), or the combination of SB with each of the two concentrations of IMZ. For the combinations, aqueous solutions of both chemicals were mixed into 10 L buckets and manually stirred with a clean plastic rod. IMZ was used at two doses considerably lower than those recommended for commercial applications (500-1000 μL

L^{-1} a.i.). Tested temperatures were 20 ºC (except for the combination treatments) and 50 ºC. Fruit treated with SB were rinsed with tap water and left to dry before incubation at 20 ºC and 90% RH. Each treatment was applied to 3 replicates of 20 fruit each. Disease incidence was determined after 7 days of incubation. Fruit phytotoxicities were assessed after 3 days at 20 ºC. The experiment was performed twice.

2.5.1. Effectiveness on major citrus species and cultivars

Time and temperature conditions selected from the previous trials with 'Valencia' oranges were tested on other commercially important citrus species and cultivars. On the one hand, 'Clemenules' and 'Ortanique' mandarins were treated with water or 3% SB for 60 s at 20, 53, 58 or 62 °C, and at 20 or 60 °C, respectively. Likewise, 'Fino' lemons were treated with water at 20 ºC for 30 s (control treatment) or 3% SB at 20 or 62 °C, both for 15 or 30 s. On the other hand, in order to study the compatibility with common postharvest treatments, 'Lanelate' oranges and 'Ortanique' mandarins were treated for 60 s with water or 25 μL L^{-1} IMZ at 20 °C, or 25 μL L^{-1} IMZ, 3% SB, or 3% SB followed by 25 μL L^{-1} IMZ at 50 °C. Only fruit treated with SB alone was rinsed with tap water after treatment. Green and blue mold incidence was assessed after 7 days of incubation at 20 ºC and 90% RH.

2.5.2. Effectiveness of treatments during cold storage

'Valencia' oranges were artificially inoculated with *P. digitatum* and *P. italicum* and then dip-treated for 60 s with water or aqueous solutions of 25 μL L^{-1} IMZ at 20 °C, or 25 μL L^{-1} IMZ, 3% SB, or 3% SB followed by 25 μL L^{-1} IMZ at 50 °C. Only fruit treated with SB alone was rinsed with tap water after treatment. Treated fruit were stored for 8 weeks at 5 ºC and 90% RH, then moved to a chamber at 20 ºC and 70-80% RH and further held there for 7 days, simulating shelf-life. Green and blue mold incidence was assessed after 2, 4, 6, and 8 weeks at 5 ºC and at the end of shelf-life.

2.6. Preventive activity

The efficacy of treatments with SB in the prevention of green mold was assessed on 'Valencia' oranges. Fruit were first dip treated with 3% SB at 50 ºC for 60 s, and then inoculated, after 24 h, with the tip of a stainless steel rod, 1 mm wide and 2 mm in length that had been immersed in a 10^5 spores mL^{-1} conidial suspension of *P. digitatum*. Each treatment consisted of 3 replicates of 20 fruit each. After inoculation, treated fruit were incubated at 20 ºC and 90% RH for 7 days and then disease incidence was evaluated. The trial was performed twice.

2.7. Statistical analysis

Data on disease incidence (%) were transformed to the arcsine of the square root of the proportion of infected fruit to assure the homogeneity of variances. In some cases, reductions with respect to control treatments were calculated as percentages. Transformed data were analyzed by analysis of variance (ANOVA) with Statgraphics software (v. 5.1, Statpoint Technologies Inc., Warrenton, VA, USA). The level of significance was established at $P = 0.05$. When appropriate, Fisher's Protected Least Significant Difference (LSD) test was used for means separation. Values displayed in graphs are non-transformed means. The term synergy was applied as defined by Richer (1987), where the effectiveness of a combination of treatments exceeds the prediction of the effectiveness of their additive action estimated by Limpel's formula ($Ee = X + Y - (XY/100)$).

3. Results

3.1. *In vivo* primary screenings

Among the variety of food additives and concentrations tested in primary screenings, NaDHA at concentrations above 40 mM and SB at concentrations of 100 or 200 mM were the most effective treatments to reduce the incidence of both green and blue molds after both 3 and 6 days (Fig. 1) of incubation at 20 ºC and 90% RH. Treatments with NaAc were slightly effective, even at very high

doses of 600 to 1000 mM. Treatments with DMDC showed very poor efficacy against both pathogens, with disease reductions lower than 40% at concentrations as high as 600 mM. Similarly, treatments with EDTA were not generally effective, except when applied at 100 mM against green mold, which was reduced by over 90% (Fig. 1). Rind phytotoxicity (dark blemishes or pitting on the rind area surrounding the inoculation site) was not observed for any of these treatments.

3.2. Dip treatment conditions

In accordance to the previous results, SB at 3% (~200 mM) was selected to test the efficacy of this treatment in further dip applications. In tests with 'Valencia' oranges, treatments with SB were always more effective against green and blue molds than those with water alone applied at the same temperature for the same time (Fig. 2). SB dip treatment for only 5 s required very high application temperatures to be effective. Treatments with water applied for longer than 5 s reduced the incidence of green and blue molds proportionally to the dip temperature. This trend, although less marked, was also observed on fruit treated with SB. Thus, decay was strongly reduced on fruit treated with SB for 15 or 30 s, even at dip temperatures of 20 °C, and rising the treatment temperature to 62 °C only increased the efficacy of the treatments slightly. The lowest green and blue mold incidence was observed on fruit treated with SB at 62 °C for 60 s, with incidence values as low as 6 and 1%, respectively, at the end of the incubation period. Increasing the duration of SB treatments at this temperature to 150 s did not substantially improve their efficacy. In general, SB efficacy was highly influenced by treatment temperature, showing a synergistic effect between these two factors (see asterisks on Fig. 2). With the exception of slight blemishes on the rind of 'Valencia' oranges treated at 62 ºC for 150 s, no rind phytotoxicities were observed in these trials.

3.3. Combination with low doses of imazalil

Dip treatments with 3% (w/v) SB at 20 ºC for 60 s reduced green and blue mold incidence from 100% on control fruit to less than 50 and 60%, respectively (Fig. 3). Treatments with IMZ at concentrations of 25 or 50 μL L^{-1} were not more effective than SB treatments applied at the same temperature for the same time. Heating dip solutions to 50 ºC generally improved the efficacy of all treatments, including water alone, against both target diseases, although the differences were not always statistically significant. The incidence of both green and blue molds was greatly reduced on 'Valencia' oranges treated with 3% SB combined with 25 or 50 μL L^{-1} IMZ, with no significant differences between treatments. In all cases, the efficacy of combined treatments was higher than the expected by the mere addition of individual efficacies, showing a synergistic effect according to Limpel's formula (Fig. 3). None of the tested treatments at any temperature was visibly phytotoxic to 'Valencia' oranges.

3.3.1. Effectiveness on major citrus species and cultivars

On 'Clemenules' mandarins, treatments with 3% SB for 60 s were not effective for the control of green and blue molds when applied at 20 ºC (Fig. 4). Regardless of treatment temperature, all treatments with water were ineffective to control green and blue molds, being disease incidence always close to 100%. Heating solutions to 53 ºC increased the efficacy of the dips for blue mold, but not for green mold. Disease incidence was also significantly lower on fruit treated at 58 or 62 ºC, but with reductions of both molds of only around 30%. On 'Fino' lemons, the incidence of green and blue molds was not highly reduced after treatments with 3% SB at 20 ºC irrespective of dip duration, and most fruit showed disease incidence of around 75%, while decay on fruit of the unique control treatment performed with this citrus species (water at 20 ºC for 30 s) was close to 100%. Increasing the temperature of SB aqueous solutions to 62 ºC reduced green and blue mold incidence on treated lemons to approximately 30 and 50%, respectively, with respect to the control treatment of water at 20 ºC. For these SB solutions heated to 62 ºC, no significant differences were observed between treatment durations of 15 and 30 s. Likewise, on 'Ortanique' mandarins, treatments with SB at 20 ºC

for 60 s reduced green and blue molds incidence by 40 and 30%, respectively. The efficacy of the treatments increased when applied at 62 ºC, reducing the incidence of green and blue molds to 20 and 30%, respectively. According to Limpel's formula, the two factors, temperature and SB concentration, were synergistic in mandarins (Fig. 4). Synergy was not analyzed in lemons since treatments with heated water alone were not performed. The tested dip conditions did not cause any visible phytotoxicity on the fruit rind.

SB at 3% in combination with low doses of IMZ (25 μL L^{-1}) was studied on 'Lanelate' oranges and 'Ortanique' mandarins. Incidence of both green and blue molds on 'Lanelate' oranges treated with water at 20 ºC for 60 s (control) was 100% after 7 days of incubation (Fig. 5). Incidence of green and blue molds was reduced by around 80 and 70%, respectively, on oranges treated with either IMZ alone at 20 or 50 ºC or SB at 50 ºC. Decay incidence on oranges treated with the combination of SB and IMZ at 50 ºC was as low as 5%, showing a synergistic effect between these two treatments, according to Limpel's formula. The same pattern was observed when 'Ortanique' mandarins were treated following identical procedures. In this case, treatments with either IMZ or SB alone were less effective than on 'Lanelate' oranges, although the combined treatment reduced disease incidence to a lower extent (2 and 4% incidence of green and blue molds, respectively), showing a stronger synergism between SB and IMZ (Fig. 5).

3.3.2. Effectiveness of treatments during cold storage

'Valencia' oranges inoculated with *P. digitatum* or *P. italicum* and treated with water at 20 ºC (control) showed high levels of decay after the first two weeks of storage at 5 ºC, being the incidence of green and blue molds around 96 and 88%, respectively (Fig. 6). At this time, the incidence of both green and blue molds was around 10% on fruit treated with 25 μL L^{-1} IMZ at 20 ºC, and close to zero on oranges treated with 25 μL L^{-1} IMZ, 3% SB, or the combination of both chemicals at 50 ºC. On fruit treated with IMZ or SB alone, the incidence of green and blue molds gradually increased along storage time and even more after 1 additional week at room temperature, when final incidence of

green and blue molds was respectively 45 and 55% for IMZ at 20 ºC, 27 and 42% for SB at 50 ºC, and both 28% for IMZ at 50 ºC. Nevertheless, the incidence of both diseases remained close to zero on oranges treated with the combination of SB and IMZ at 50 ºC at the end of the cold storage period and shelf-life. Again, this latter treatment showed a high synergism between IMZ and SB according to Limpel's formula (Fig. 6).

3.4. Preventive activity

SB treatments were not effective in reducing green mold incidence when applied about 24 h before wound inoculation with *P. digitatum* (data not shown).

4. Discussion

In this study, the antifungal properties of five food additives were tested at a wide range of concentrations in primary screening tests on citrus fruit inoculated with *P. digitatum* or *P. italicum*. The importance of using *in vivo* tests instead of *in vitro* tests is illustrated by the fact that, as a pathogenicity mechanism, the secretion of organic acids by the pathogens varies when it is produced *in vitro* and *in vivo* (Prusky et al., 2004), which could presumably alter the toxicity of antifungal treatments with food additives or other compounds. For instance, notable differences in efficacy between *in vivo* and *in vitro* tests have been observed in experiments with carbonates against citrus green mold (Smilanick et al., 1999). Therefore, *in vivo* primary screenings with citrus fruit instead of *in vitro* assays were selected in this study to preliminary assess the efficacy of different food additives and concentrations. Among the tested substances (SB, NaDHA, NaAc, DMDC, and EDTA), SB at 100 and 200 mM, NaDHA at 70 and 100 mM, and NaAc at 800 and 1000 mM reduced green and blue molds by 80% or higher. Required NaAc concentrations were too high and unpractical, and even less effective than lower concentrations of SB or NaDHA. SB parental chemical benzoic acid and the related compound benzyl benzoate are registered by US EPA as

insecticides and fungicides. Moreover, while SB is approved in the EU for use in foods, the status of NaDHA has been revisited due to its more toxic profile (Kegley et al., 2014). These facts accounted for the choice of SB to be used in subsequent trials to assess the efficacy of dip treatments. Although the differences between 100 and 200 mM SB were not significant in the primary screenings, blue mold incidence was slightly lower after SB treatment at 200 than at 100 mM. Further, previous studies showed that SB at 200 mM was an effective treatment against postharvest pathogens of stone fruit (Palou et al., 2009). For these reasons, this concentration of approximately 3% SB was selected to be tested in further trials.

In general in the present study, SB dip treatments controlled green and blue molds more effectively on oranges than on mandarins after identical treatments applied at the same conditions. In all tests, decay on control fruit was high and similar on both oranges and mandarins, so differences in efficacy were not caused by mere differences in fruit susceptibility to decay. Disease development results from complex interactions between fruit, pathogen and environment. Efficacy of antifungal food additives depends on the amount of salt residue present within wound infection courts occupied by the fungus and on interactions between this residue and rind constituents. Apparently, such interactions may be different in oranges and mandarins as a consequence of different rind characteristics or composition. In prior research, the efficacy of treatments with hot water, sodium carbonate, sodium bicarbonate (Palou et al., 2001, 2002), potassium sorbate (Montesinos-Herrero et al., 2009) or sodium parabens (Moscoso-Ramírez et al., 2013a,b, 2014) against green and blue molds was also lower on mandarins than oranges. On the other hand, the effectiveness of 3% SB applied as aqueous dips was considerably lower than that obtained in *in vivo* primary screenings, in which the incidence reductions were of 100%. This was possibly due to the increased contact time of the SB drop with the rind wound inoculated with *P. digitatum* or *P. italicum* with respect to the contact time of dip treatments. In fact, contact time or dip duration was an influencing factor on the effectiveness of SB treatments applied on ‘Valencia’ oranges.

The antimicrobial effect of SB has been widely reviewed and, in particular, several studies showed its efficacy as antifungal postharvest treatment on citrus (Hall, 1988), stone fruits (Palou et al., 2009), or longan fruit (Yahia, 2011). In the present work, different dip conditions (duration and temperature) were tested in order to optimize dip applications of SB. High temperatures tested ranged from 50 to 62 °C because synergistic effects between these temperatures and alternative chemicals such as sodium carbonate or potassium sorbate had been previously observed; lower temperatures were less effective and higher temperatures led to visible phytotoxicities on the fruit rind (Smilanick et al., 1999; Montesinos-Herrero et al., 2009). The combination of SB with heat was synergistic and SB treatments at higher temperatures were more effective against green and blue molds in all cultivars tested, although the most aggressive treatment combination of 62 ºC for 150 s resulted in slight rind phytotoxicity in some oranges. This kind of synergy was observed in previous studies with other food additives such as carbonates (Palou et al., 2001) or potassium sorbate (Montesinos-Herrero et al., 2009), but not with other compounds such as potassium silicate (Moscoso-Ramírez and Palou, 2014) or sodium methyl- (Moscoso-Ramírez et al., 2013a), ethyl- (Moscoso-Ramírez et al., 2013b) or propyl- (Moscoso-Ramírez et al., 2014) parabens. It was concluded in these cases that the potential benefits from heating the solutions did not compensate for the potential commercial costs. Synergistic effects between SB and heat were also dependent on the citrus cultivar. The efficacy of heated SB solutions was not as high on 'Clemenules' mandarins than on other citrus species and cultivars, probably due to a higher sensitivity of this cultivar to hot water dips, which may adversely affect the skin of the fruit and made it more susceptible to fungal development. It is actually found in the literature that hot water dip treatments were less effective as antifungal treatments on mandarins than on oranges (Schirra et al., 1998; Palou et al., 2001, 2002). Immersion time is an important factor influencing efficacy, with higher control achieved by longer treatments. However, commercial conditions during fruit manipulation in citrus packinghouses do not allow prolonged treatment times and thus, times from 5 to 150 s were chosen. As expected,

longer treatments yielded higher efficacies against green and blue molds. However, the efficacy of SB treatments applied for 60 or 150 s was not different enough to select the longest immersion time. It is clear that, at the commercial level, longer dip treatments lead to an increase in energy costs and risks of phytotoxicity in sensitive cultivars.

Dip treatments with 3% SB reduced green and blue mold incidence to a similar extent than dip treatments with low doses of IMZ. The combinations of SB with IMZ consistently improved the efficacy of these treatments and always showed a synergistic effect. This effect was particularly marked when treated fruit were stored at low temperatures. The observed synergism between SB and IMZ is presumably due to the different modes of action of these chemicals, which are more lethal when acting together. In addition, both substances were more effective when applied at higher temperatures, which favored their synergism. It is known that IMZ, like the rest of fungicides in the imidazole family, inhibits ergosterol synthesis in fungi by blocking C-14 demethylation (Van den Bossche et al., 1978; Siegel and Ragsdale, 1978; Henry and Sisler, 1979). This results in accumulation of C-14 methyl sterol intermediates, such as lanosterol, and a decrease in ergosterol, with the subsequent deterioration of the cell membrane. Moreover, heat increases the level of IMZ residues in the fruit rind, probably due to partial melting and remodeling of the wax layer by heat, resulting in the disappearance of cracks in this layer and in trapping of the active ingredient (Schirra et al., 1997). On the other hand, it has been demonstrated that in acidic medium such as the wounded rind of citrus fruit, the undissociated form of the benzoic acid (to which the yeast cell membrane is permeable) enters the cell until the concentration equals inside and outside the cell (Restaino et al., 1981; Krebs et al., 1983). The neutralization of the undissociated form by cell buffers causes an acidification inside the cell that ultimately inhibits cell growth. The fall in intracellular pH caused by the accumulation of benzoic acid at low external pH inhibits glycolysis at the stage of phosphofructokinase, thus depleting the cell of ATP and in consequence restricting its growth (Krebs et al., 1983). The observed synergism between heat and SB treatments seems to indicate that high

temperatures improve the mode of action of SB by increasing the permeability of the membrane, facilitating the entrance of SB, a pH reduction and the formation of benzoic acid (Rushing and Senn, 1963). Different and complementary modes of action of IMZ and SB may account for their synergism when applied together against green and blue molds.

In contrast to other works in which the preventive activity of postharvest treatments with food additives such as sodium carbonates against citrus green or blue molds has been demonstrated (Venditti et al., 2005; Youssef et al., 2014), no preventive effect of SB treatments was observed in this research. We believe that the use of different experimental procedures may account as the main reason to explain these differences. In this work, SB was applied to intact unwounded oranges that were wound inoculated with *P. digitatum* about 24 h later. Therefore, the active ingredient remained on the rind surface and, in contrast to conventional fungicides such as IMZ, it was not able to penetrate directly into the rind to protect the fruit from potential fungal infections that could take place later in new wounds inflicted to the fruit. Nevertheless, using this methodology nothing can be concluded about the ability of SB to protect from future infections rind wounds already inflicted to the fruit at the time of the treatment.

The use of SB in postharvest applications for fresh horticultural produce may have the same level of allowance by authorities than potassium sorbate, since SB is also a food additive authorized by EU and USA regulations. Potassium sorbate is currently accepted as postharvest treatment for citrus fruit, with an MRL of 20 mg kg^{-1} (EC, 2010). At present, SB, benzoic acid, potassium benzoate and calcium benzoate are authorized food additives in the EU. They can be used up to 150 mg kg^{-1} in traditional Swedish and Finnish fruit syrups and flavored drinks excluding dairy-based drinks; up to 200 mg kg^{-1} in Danish juice sweet, alcohol-free wine and beer, and aspic; up to 500 mg kg^{-1} in olives and olive-based preparations, seaweed preparations, low-sugar and similar low calorie or sugar-free marmalades and other fruit-based spreads; up to 1000 mg kg^{-1} quince paste and cooked crustaceans and mollusks; up to 1500 mg kg^{-1} in cooked shrimps in brine, and up to 2000 mg kg^{-1} in

cooked red beet (EC, 2015). In the USA, SB and benzoic acid are included in the Food and Drug Administration (FDA) list of GRAS substances. Both may be used as antimicrobial agents, flavoring agents, and as adjuvants with a current maximum level of 0.1% in food (US FDA, 2001).

In the present research, SB alone or combined with low doses of IMZ showed very high efficacy for the control of citrus green and blue molds. This kind of treatments with low or no use of synthetic fungicides is very demanded by general consumers and especially by most fruit distributors in the EU and worldwide. In addition, the different mode of action of SB could be an important additional tool to hinder the proliferation of IMZ resistant strains of *P. digitatum* and *P. italicum*, which is at present a common important issue in most citrus packinghouses. In this sense, the use of SB would allow the fight against resistances without the addition of other synthetic fungicides in the packinghouse disease management program. Treatments tested in this study, including those using high temperatures, are currently feasible to be applied at the commercial level in many modern Spanish and worldwide citrus packinghouses. Heated solutions of SB alone satisfactorily controlled green and blue mold on oranges, even when treated fruit were cold-stored. However, on mandarins and lemons, the combination of SB with low doses of IMZ was necessary to reduce disease incidence to commercially acceptable levels. In this case, combinations with control methods other than synthetic fungicides should be tested in order to improve SB efficacy and offer alternative treatments for markets with zero tolerance to fungicide residues. After these laboratory and small-scale trials with artificially inoculated fruit, large-scale trials in citrus packinghouses with naturally infected fruit should be conducted to confirm the potential of SB treatments against green and blue molds and justify the registration of this compound for postharvest commercial use.

Acknowledgements

Federico Izquierdo and Fontestad S.A. (Montcada, València) are gratefully acknowledged for providing fruit. This work was partially funded by the Spanish 'Ministerio de Educación y Ciencia'

(MEC; project AGL2004-05271) and the European Union (FEDER Program). Clara Montesinos-Herrero's doctorate program was supported by the IVIA. Pedro Antonio Moscoso-Ramírez's doctorate program is supported by a scholarship by the "Consejo Nacional de Ciencia y Tecnología de México" (CONACYT-124182-México). In memory of Dr. Miguel Ángel del Río, for his guidance and support and for his unconditional friendship.

References

Askarne, L., Talibi, I., Boubaker, H., Serghin, M.A., Boudyach, E.H., Ait Ben Aoumar, A., 2011. Effects of organic acids and salts on the development of *Penicillium italicum*: The causal agent of citrus blue mold. Plant Pathology Journal 10, 99-107.

Bautista-Baños, S. (ed.), 2014. Postharvest Decay. Control Strategies. 1st ed. Academic Press, Elsevier. London, UK.

Brown, G.E., 1985. Effectiveness of postharvest fungicides for the control of citrus fruit decays. Proceedings of the Florida State Horticultural Society 98, 208-211.

Bus, V.G., Bongers, A.J., Risse, L.A., 1991. Occurrence of *Penicillium digitatum* and *P. italicum* resistant to benomyl, thiabendazole, and imazalil on citrus fruit from different geographical origins. Plant Disease 75, 1098-1100.

Cunningham, N., 2010. Combinations of treatments to replace the use of conventional fungicides for the commercial control of postharvest diseases of citrus fruit. Stewart Postharvest Review 1:2, 1-8.

D'Aquino, S., Palma, A., Angioni, A., Schirra, M., 2013. Residue levels and efficacy of fludioxonil and thiabendazole in controlling postharvest green mold decay in citrus fruit when applied in combination with sodium bicarbonate. Journal of Agricultural and Food Chemistry 61, 296-306.

EC (European Commission), 2010. Commission Directive No 2010/69/EC modifying annexes of Directive 95/2/EC relative to food additives other than colorants and sweeteners. Official Journal No. L279, 22-31.

EC (European Commission), 2015. Database on Food Additives. Annex II of Regulation (EC) No 1333/2008. Online: https://webgate.ec.europa.eu/sanco_foods/main/index.cfm?sector=FAD&auth=SANCAS

Eckert, J.W., Eaks, I.L., 1989. Postharvest disorders and diseases of citrus fruits. In: Reuter, W., Calavan, E.C., Carman, G.E. (eds.), The Citrus Industry. Division of Agriculture and Natural Resources, University of California Press, Berkeley, CA, USA.

Eckert, J.W., Sievert, J.R., Ratnayake, M., 1994. Reduction of imazalil effectiveness against citrus green mold in California packinghouses by resistant biotypes of *Penicillium digitatum*. Plant Disease 78, 971-974.

Erasmus, A., Lennox, C.L., Smilanick, J.L., Lesar, K., Fourie, P.H., 2013. Imazalil residue loading and green mould control on citrus fruit as affected by formulation, solution pH and exposure time in aqueous dip treatments. Postharvest Biology and Technology 77, 43-49.

Hall, D.J., 1988. Comparative activity of selected food preservatives as citrus postharvest fungicides. Proceedings of the Florida State Horticultural Society 101, 184-187.

Henry, M.J., Sisler, H.D., 1979. Effects of miconazole and dodecylimidazole on sterol biosybthesis in *Ustilago maydis*. Antimicrobial Agents and Chemotherapy 15, 603-607.

Holmes, G.J., Eckert, J.W., 1995. Relative fitness of imazalil-resistant and -sensitive biotypes of *Penicillium digitatum*. Plant Disease 79, 1068-1073.

Ippolito, A., Schena, L., Pentimone, I., Nigro, F., 2005. Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pulllulans* in combination with calcium chloride or sodium bicarbonate. Postharvest Biology and Technology 36, 245-252.

Janisiewicz, W.J., Conway, W.S., 2010. Combining biological control with physical and chemical treatments to control fruit decay after harvest. Stewart Postharvest Review 1:3, 1-16.

Krebs, H.A., Wiggins, D., Stubbs, M., Sols, A., Bedoya, F., 1983. Studies on the mechanism of the antifungal action of benzoate. Biochemistry Journal 214, 657- 663.

Kegley, S.E., Hill, B.R., Orme, S., Choi, A.H., 2014. PAN Pesticide Database, Pesticide Action Network, North America, Oakland, CA, USA. Online: http://www.pesticideinfo.org/

Kinay, P., Mansour, M.F., Mlikota-Gabler, F., Margosan, D.A., Smilanick, J.L., 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Protection 26, 647-656.

Montesinos-Herrero, C., Palou, L., 2010. Combination of physical and low-toxicity chemical postharvest treatments for integrated disease management of citrus fruit: a review. Stewart Postharvest Review 1:1, 1-11.

Montesinos-Herrero, C., del Río, M.A., Pastor, C., Brunetti, O., Palou, L., 2009. Evaluation of brief potassium sorbate dips to control postharvest penicillium decay on major citrus species and cultivars. Postharvest Biology and Technology 52, 117-125.

Moscoso-Ramírez, P.A., Montesinos-Herrero, C., Palou, L., 2013a. Characterization of postharvest treatments with sodium methylparaben to control citrus green and blue molds. Postharvest Biology and Technology 77, 128-137.

Moscoso-Ramírez, P.A., Montesinos-Herrero, C., Palou, L., 2013b. Control of citrus postharvest penicillium molds with sodium ethylparaben. Crop Protection 46, 44-51.

Moscoso-Ramírez, P.A., Montesinos-Herrero, C., Palou, L., 2014. Antifungal activity of sodium propylparaben alone or in combination with low doses of imazalil against Penicillium decay on citrus fruit. European Journal of Plant Pathology 140, 145-157.

Moscoso-Ramírez, P.A., Palou, L., 2014. Preventive and curative activity of postharvest potassium silicate treatments to control green and blue molds on orange fruit. European Journal of Plant Pathology 138, 721-732.

Palou, L., 2014. *Penicillium digitatum, Penicillium italicum* (Green Mold, Blue mold). In: Bautista-Baños, S. (ed.), Postharvest Decay. Control Strategies. 1st ed. Academic Press, Elsevier. London, UK. pp. 45-102.

Palou, L., Smilanick, J.L., Crisosto, C.H., 2009. Evaluation of food additives as alternative or complementary chemicals to conventional fungicides for the control of major postharvest diseases of stone fruit. Journal of Food Protection 72, 1037-1046.

Palou, L., Smilanick, J.L., Droby, S., 2008. Alternatives to conventional fungicides for the control of citrus postharvest green and blue moulds. Stewart Postharvest Review 4:2, 1-16.

Palou, L., Smilanick, J.L., Usall, J., Viñas, I., 2001. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate, and sodium bicarbonate. Plant Disease 85, 371-376.

Palou, L., Usall, J., Muñoz, J.A., Smilanick, J.L., Viñas, I., 2002. Hot water, sodium carbonate, and sodium bicarbonate for the control of postharvest green and blue molds of clementine mandarins. Postharvest Biology and Technology 24, 93-96.

Plaza, P., Usall, J., Torres, R., Abadías, M., Smilanick, J.L., Viñas, I. 2004. The use of sodium carbonate to improve curing treatments against green and blue moulds on citrus fruits. Pest Management Science 60, 815-821.

Prusky, D., McEvoy, J.L., Saftner, R.A., Conway, W.S., Jones, R., 2004. Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. Phytopathology 94, 44-51.

Restaino, L.K., Komatsu, K., Syracuse, M.J., 1981. Effects of acids on potassium sorbate inhibition on food-related microorganisms in culture media. Journal of Food Science 47, 134-143.

Richer, D.L., 1987. Synergism - a patent view. Pesticide Science 19, 309-315.

Romanazzi, G., Lichter, A., Mlikota-Gabler, F., Smilanick, J.L., 2012. Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. Postharvest Biology and Technology 63, 141-147.

Rushing, N.B, Senn, V.J., 1963. The effect of benzoic, sorbic, and dehydroacetic acids on the growth of citrus products spoilage organisms. Proceedings of the Florida State Horticultural Society 76, 271-276.

Sánchez-Torres, P., Tuset, J.J., 2011. Molecular insights into fungicide resistance in sensitive and resistant *Penicillium digitatum* strains infecting citrus. Postharvest Biology and Technology 59, 159-165.

Schirra, M., D'hallewin, G., Cabras, P., Angioni, A., Garau, V.L., 1998. Seasonal susceptibility of Tarocco oranges to chilling injury as affected by hot water and thiabendazole postharvest dip treatments. Journal of Agricultural and Food Chemistry 46, 1177-1180.

Schirra, M., Cabras, P., Angioni, A., D'hallewin, G., Ruggiu, R., Minelli, E.V., 1997. Effect of heated solutions on decay control and residues of imazalil in lemons. Journal of Agricultural and Food Chemistry 45, 4127-4130.

Siegel, M.R., Ragsdale, N.N., 1978. Antifungal mode of action of imazalil. Pesticide Biochemistry and Physiology 9, 48-56.

Smilanick, J.L., Mansour, M.F., Mlikota-Gabler, F., Sorenson, D., 2008. Control of citrus postharvest green mold and sour rot by potassium sorbate combined with heat and fungicides. Postharvest Biology and Technology 47, 226-238.

Smilanick, J.L., Margosan, D.A., Mlikota-Gabler, F., Usall, J., Michael, I.F., 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. Plant Disease 83, 139-145.

Smith, W.L., 1962. Chemical treatments to reduce postharvest spoilage of fruits and vegetables. The Botanical Review 28, 411-445.

Sorenson, D., Smilanick, J.L., Margosan, D.A., 1999. Postharvest high pressure washing of citrus fruit with sodium bicarbonate to control green mold. Phytopathology 89, S74.

US FDA (United States Federal Drug Administration), 2015. Direct Food Substances Affirmed as Generally Recognized as Safe. Code of Federal Regulations, 21 CFR 184. Online: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=184&showFR=1.

Van den Bossche, H., Willemsens, G., Cools, W., Lauwers, W.F.J., Le Jeune, L., 1978. Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. Chemico-Biological Interactions 21, 59-78.

Venditti, T., Molinu, M.G., Dore, A., Agabbio, M., D'hallewin, G., 2005. Sodium carbonate treatment induces scoparone accumulation, structural changes, and alkalinization in the albedo of wounded *Citrus* fruits. Journal of Agricultural and Food Chemistry 53, 3510-3518.

Wang, C., Chen, Y., Xu, Y., Wu, J., Xiao, G., Zhang, Y., Liu, Z., 2012. Effect of dimethyl dicarbonate as disinfectant on the quality of fresh-cut carrot (*Daucus carota* L.). Journal of Food Processing and Preservation 37, 751-758.

Yahia, E.M. (ed.), 2011. Postharvest Biology and Technology of Tropical and Subtropical Fruits. Volume 3: Cocona to Mango. Woodhead Publishing Ltd. Cambridge, UK.

Youssef, K., Sanzani, S.M., Ligorio, A., Ippolito, A., Terry, L.A., 2014. Sodium carbonate and bicarbonate treatments induce resistance to postharvest green mould on citrus fruit. Postharvest Biology and Technology 87, 61-69.

Chen, Y., Wang, H., Xu, Y., Wu, J., Xiao, G., 2013. Effect of treatment with dimethyl dicarbonate on microorganisms and quality of Chinese cabbage. Postharvest Biology and Technology 76, 139-144.

Zhang, J., Swingle, P.P., 2003. Control of green mold on Florida citrus fruit using bicarbonate salts. Proceedings of the Florida State Horticultural Society 116, 375-378.

Zhu, J., Xie, Q., Li, H., 2006. Occurrence of imazalil-resistant biotype of *Penicillium digitatum* in China and the resistant molecular mechanism. Journal of Zhejiang University Science A 7 (Suppl. II), 362-365.

Figure captions

Fig. 1. Reduction of the incidence of green and blue molds on 'Valencia' oranges wound inoculated with *Penicillium digitatum* and *P. italicum*, respectively, treated 24 h later by placing in the wound 30 µL of aqueous solution at different concentrations of sodium benzoate (SB), ethylendiaminetetraacetic acid (EDTA), dehydroacetic acid sodium salt (NaDHA), sodium acetate (NaAc), or dimethyl dicarbonate (DMDC), and incubated at 20 ºC and 90% RH for 6 days. Each combination of pathogen, substance, and concentration was applied to 4 replicates of 5 oranges and repeated twice (n=12).

Fig. 2. Incidence of green (GM) and blue (BM) molds on 'Valencia' oranges wound inoculated with *Penicillium digitatum* and *P. italicum*, respectively, immersed 24 h later for different time periods in 3% (w/v) sodium benzoate (SB) at different temperatures, and stored for 7 days at 20 °C and 90% RH. For each immersion time and pathogen, columns with unlike letters (*a-d* for GM and *m-p* for BM) differ significantly according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to arcsine-transformed values. Non-transformed means are shown. Each treatment was applied to 3 replicates of 25 fruit (n=3). (*) Synergistic effect between temperature and SB according to Limpel's formula.

Fig. 3. Incidence of green (GM) and blue (BM) molds on 'Valencia' oranges wound inoculated with *Penicillium digitatum* and *P. italicum*, respectively, immersed 24 h later in water (control), 3% (w/v) sodium benzoate (SB), 25 or 50 µL L^{-1} imazalil (IMZ), or SB combined with IMZ at the indicated temperature, and stored for 7 days at 20 °C and 90% RH. For each pathogen, columns with unlike letters differ significantly according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to arcsine-transformed values. Non-transformed means are shown. Each treatment was

applied to 3 replicates of 20 fruit and repeated once (n=6). (*) Synergistic effect between SB and IMZ according to Limpel's formula.

Fig. 4. Incidence of green (GM) and blue (BM) molds on 'Clemenules' and 'Ortanique' mandarins and 'Fino' lemons inoculated with *Penicillium digitatum* and *P. italicum*, respectively, immersed in water (control), or 3% (w/v) sodium benzoate (SB), at the indicated temperature for the indicated time, and stored for 7 days at 20 °C and 90% RH. For each cultivar and pathogen, columns with unlike letters (*a-d* for GM and *m-p* for BM) differ significantly according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to arcsine-transformed values. Non-transformed means are shown. Each treatment was applied to 3 replicates of 25 fruit (n=3). (*) Synergistic effect between temperature and SB according to Limpel's formula.

Fig. 5. Incidence of green (GM) and blue (BM) molds on 'Ortanique' mandarins and 'Lanelate' oranges inoculated with of *Penicillium digitatum* and *P. italicum*, respectively, immersed for 60 s in water (control), 3% (w/v) sodium benzoate (SB), 25 μL L^{-1} imazalil (IMZ), or SB combined with IMZ at the indicated temperature, and stored for 7 days at 20 °C and 90% RH. For each pathogen, columns with unlike letters differ significantly according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to arcsine-transformed values. Non-transformed means are shown. Each treatment was applied to 3 replicates of 20 fruit (n=3). (*) Synergistic effect between SB and IMZ according to Limpel's formula.

Fig. 6. Incidence of green (GM) and blue (BM) molds on 'Valencia' oranges inoculated with *Penicillium digitatum* and *P. italicum*, respectively, immersed for 60 s in water (control) or 25 μL L^{-1} imazalil (IMZ) at 20 °C, or 3% (w/v) sodium benzoate (SB), 25 μL L^{-1} IMZ, or the mixture of SB and IMZ at 50 °C, and stored for 8 weeks at 5 °C and 90% RH followed by 7 days of shelf-life at 20

°C and 70-80% RH. For each pathogen, columns with unlike letters differ significantly according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to arcsine-transformed values. Non-transformed means are shown. Each treatment was applied to 3 replicates of 20 fruit (n=3). (*) Synergistic effect between SB and IMZ according to Limpel's formula.

Figure 1

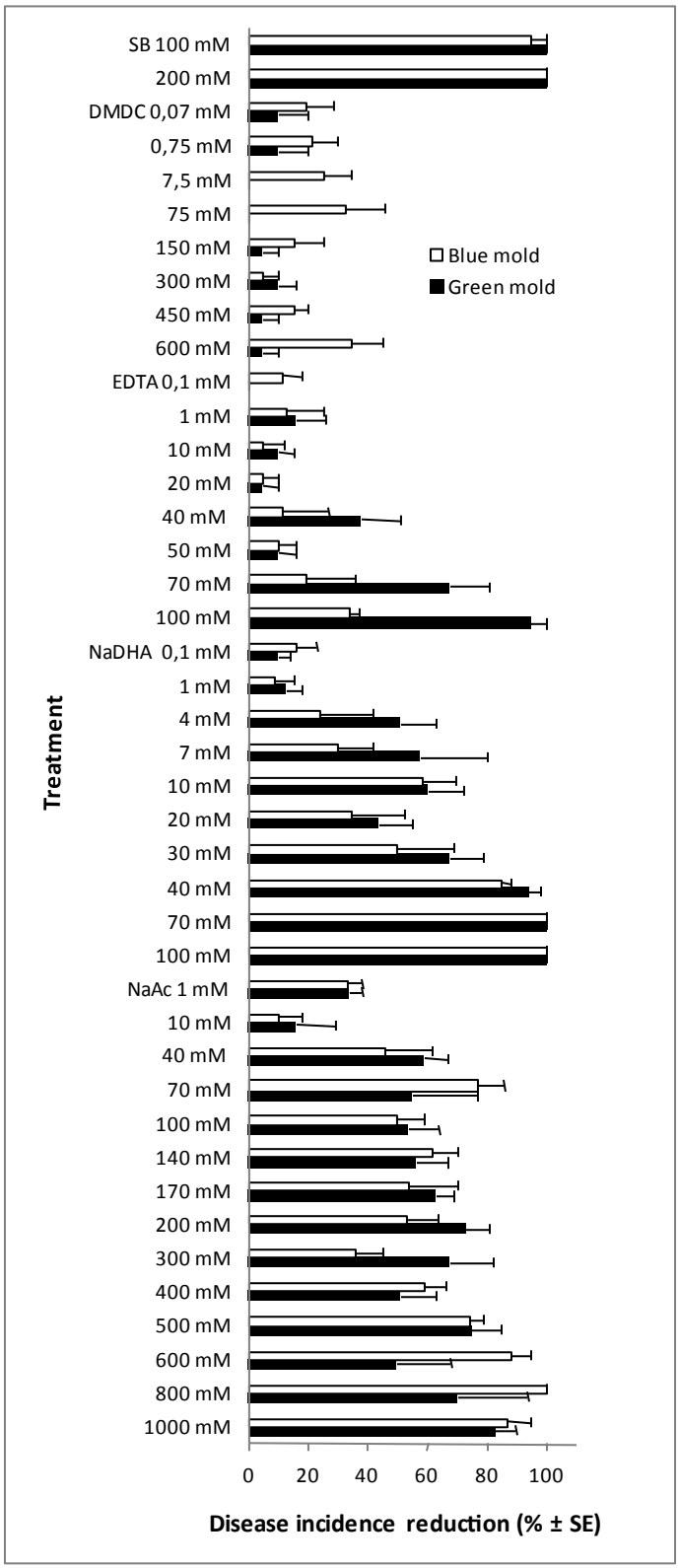

SB 100 mM
200 mM
DMDC 0,07 mM
0,75 mM
7,5 mM
75 mM
150 mM
300 mM
450 mM
600 mM
EDTA 0,1 mM
1 mM
10 mM
20 mM
40 mM
50 mM
70 mM
100 mM
NaDHA 0,1 mM
1 mM
4 mM
7 mM
10 mM
20 mM
30 mM
40 mM
70 mM
100 mM
NaAc 1 mM
10 mM
40 mM
70 mM
100 mM
140 mM
170 mM
200 mM
300 mM
400 mM
500 mM
600 mM
800 mM
1000 mM
Blue mold
Green mold
Treatment
0
20
40
60
80
100
Disease incidence reduction (% ± SE)

Figure 2

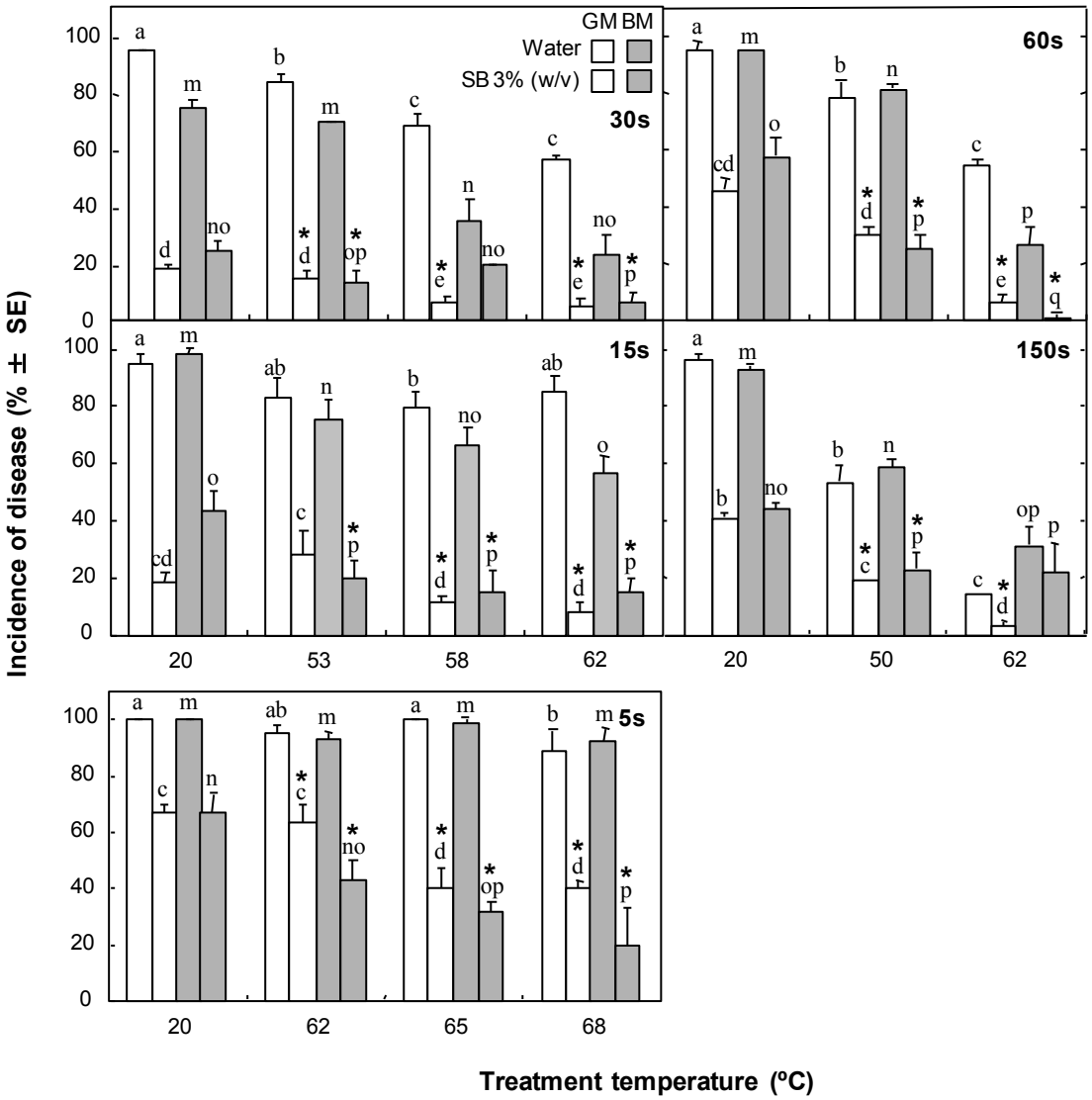

GM BM
Water
SB 3% (w/v)
30s
60s
15s
150s
5s
Incidence of disease (% ± SE)
Treatment temperature (ºC)
20
53
58
62
50
65
68

Figure 3

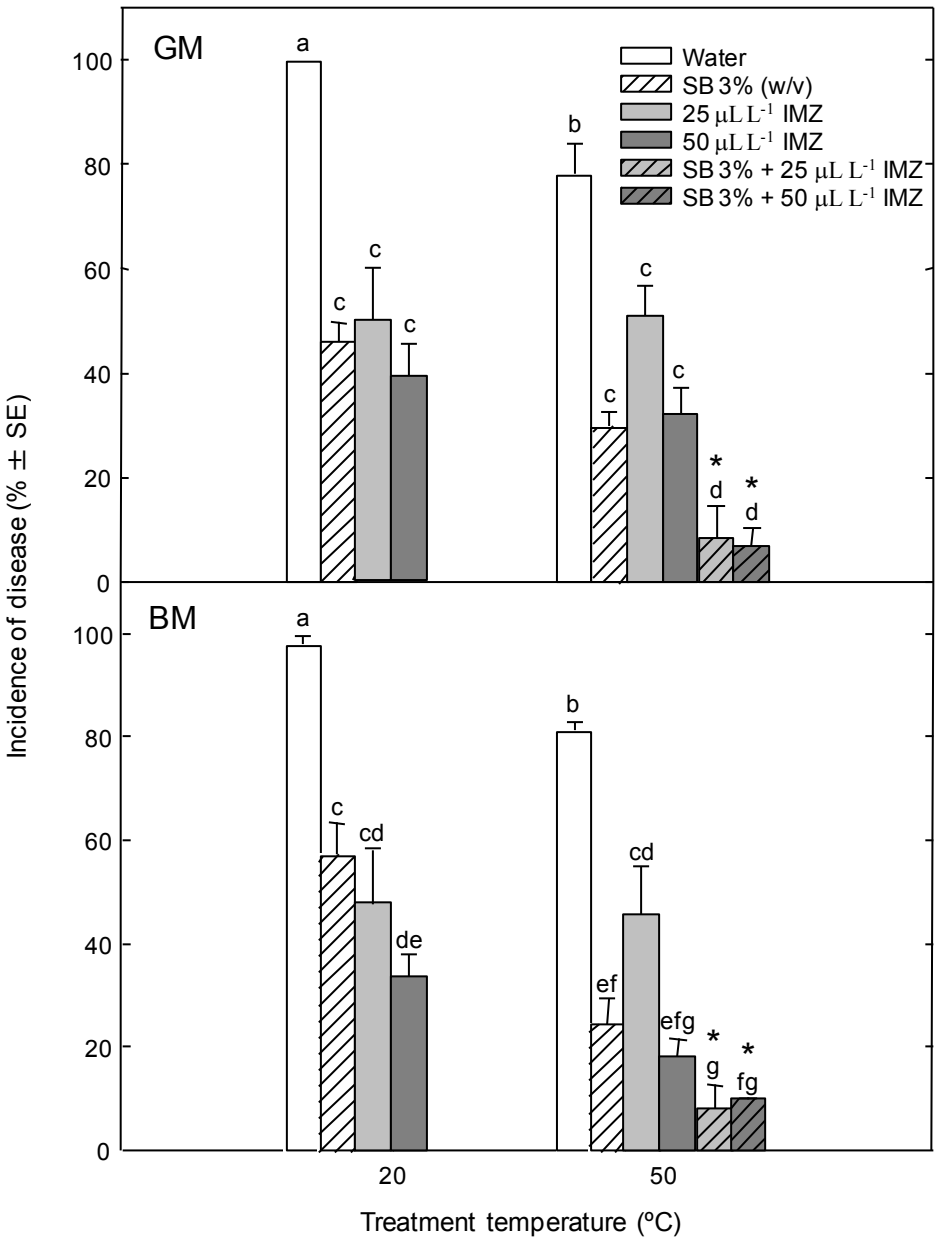

GM
BM
Water
SB 3% (w/v)
25 µL L-1 IMZ
50 µL L-1 IMZ
SB 3% + 25 µL L-1 IMZ
SB 3% + 50 µL L-1 IMZ
Incidence of disease (% ± SE)
Treatment temperature (ºC)
20
50
0
20
40
60
80
100
a
b
c
cd
d
de
ef
efg
g
fg
*

Figure 4

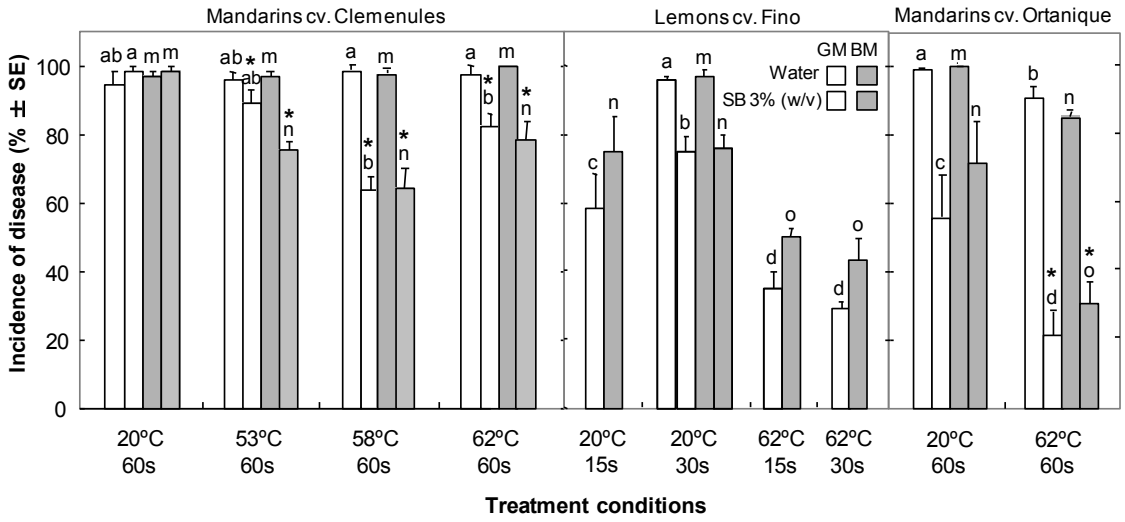

Mandarins cv. Clemenules
Lemons cv. Fino
Mandarins cv. Ortanique
GM BM
Water
SB 3% (w/v)
Incidence of disease (% ± SE)
0
20
40
60
80
100
20ºC 60s
53ºC 60s
58ºC 60s
62ºC 60s
20ºC 15s
20ºC 30s
62ºC 15s
62ºC 30s
20ºC 60s
62ºC 60s
Treatment conditions

Figure 5

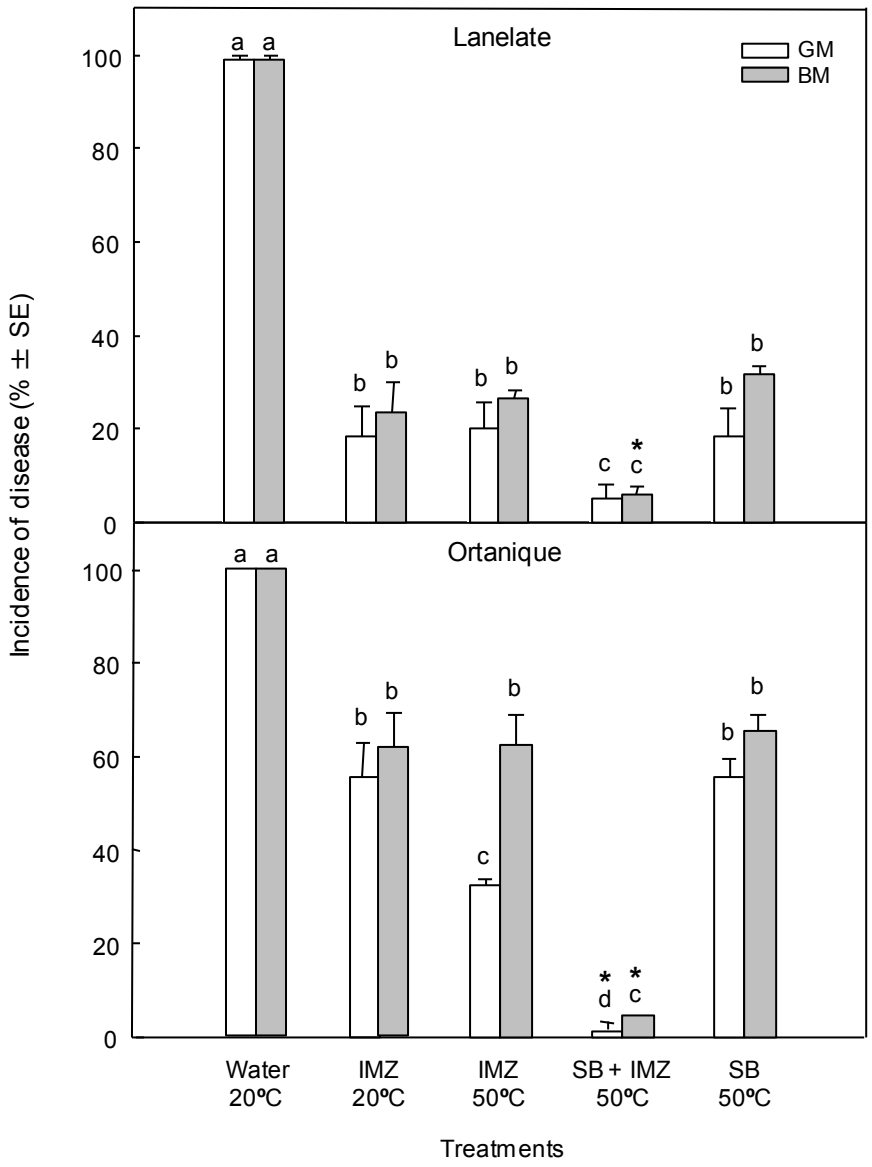
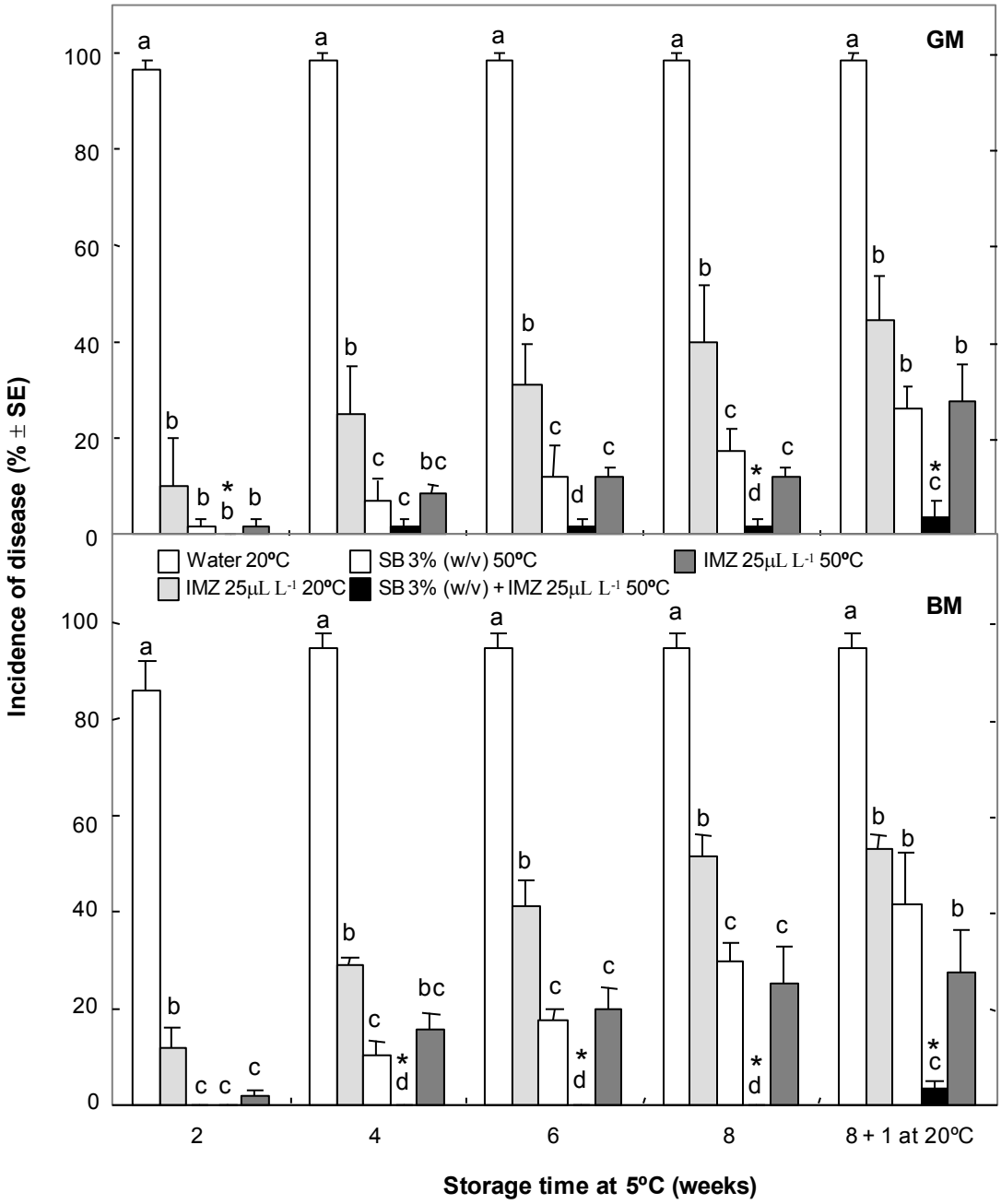

Lanelate
GM
BM
Ortanique
Incidence of disease (% ± SE)
Treatments
Water 20ºC
IMZ 20ºC
IMZ 50ºC
SB + IMZ 50ºC
SB 50ºC

Figure 6


GM
BM
Incidence of disease (% ± SE)
Storage time at 5ºC (weeks)
2
4
6
8
8 + 1 at 20ºC
Water 20ºC
IMZ 25µL L-1 20ºC
SB 3% (w/v) 50ºC
SB 3% (w/v) + IMZ 25µL L-1 50ºC
IMZ 25µL L-1 50ºC