Antifungal activity of sodium propylparaben alone or in combination with low doses of imazalil against Penicillium decay on citrus fruit

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Concise title

Sodium propylparaben against citrus postharvest Penicillium molds

Abstract

The performance of postharvest treatments with sodium propylparaben (SPP), alone or combined with low doses of the fungicide imazalil (IMZ), against citrus green (GM) and blue (BM) molds was evaluated on several citrus species and cultivars artificially inoculated with *Penicillium digitatum* and *P. italicum*, respectively, and incubated at 20 °C or cold-stored at 5 °C. Effectiveness of 100 mM SPP dips at 20 °C for 60 s was higher on oranges than on mandarins, with GM and BM incidence reductions of up to 60-90 % after 7 days at 20 °C. Irrespective of citrus cultivar and storage condition, SPP generally improved the curative action of 25 µl l^{-1} IMZ to control Penicillium molds. In additional tests, 100 mM SPP dips at 20 °C for 60 s only prevented GM on 'Valencia' oranges inoculated 24 h after treatment when combined with IMZ. It can be concluded that postharvest SPP treatments show promise as an effective alternative to be considered in citrus postharvest disease control programs.

Key words: citrus; green mold; blue mold; *Penicillium digitatum*; *P. italicum*; fungicide alternatives; postharvest disease control; imazalil

INTRODUCTION

Significant losses can occur after harvest during storage and marketing of citrus fruit primarily due to green mold (GM), caused by the pathogen *Penicillium digitatum* (Pers.:Fr.) Sacc. and secondarily by blue mold (BM) caused by *P. italicum* Wehmer (Eckert and Eaks 1989). Currently, these diseases are primarily controlled by application of conventional synthetic fungicides such as imazalil or thiabendazole (Holmes and Eckert 1999; Palou et al. 2002b). However, resistance development to fungicides by plant pathogens is a factor that restricts the fruit production worldwide due to the decrease in efficacy of fungicides (Brent and Hollomon 2007). Also, in the development and use of chemical fungicides for postharvest decay control, considerable attention must be given to the preservation of the global environment. Thus, alternative methods are needed for the control of postharvest diseases, such as biological (antagonistic microorganisms), physical (i. e. heat or radiations) or low-toxicity chemical methods and also their combination (Lu et al. 2013; Palou et al. 2002b, 2008; Valencia-Chamorro et al. 2009).

Parabens, the alkyl esters of p-hydroxybenzoic acid, and their sodium salts are a class of antimicrobial agents particularly useful against molds and yeasts, with a broad-spectrum antimicrobial activity, and commonly used as food preservatives, cosmetic products, pharmaceuticals, or cleaning products (Arslan et al. 2009; Soni et al. 2001). There is some controversy on the potential health hazards associated with parabens, especially when used in cosmetic products, which is the most important route of exposure for parabens in humans. The status as food additives of propylparaben (E-number 216) and the salt sodium propylparaben (SPP; E-217) has been recently revisited in the European Union (EU) due to recent studies demonstrating the effects of certain negative reproductive parameters in rats. The Panel was unable to recommend

an acceptable daily intake (ADI) specification, leading to the exclusion of E-216 and E-217 from the EU list of food additives. However, as in the case of other parabens and their salts authorized for use in processed fruits and vegetables (E-214, E-215, E-218, E-219), the use of these compounds in unprocessed (entire fresh) horticultural products is not established (CR EU, 2011) and, after a proper registration procedure, they could be used to treat non-edible parts of produce such as the peel of fruits like citrus, bananas, pomegranates, etc. Advantages of the potential use of parabens as antimicrobial preservatives include a broad spectrum of modes of action, stability over the pH range, and high solubility in water to produce the effective concentration in aqueous phase (Soni et al. 2001, 2005).

Previous studies with SPP have demonstrated potential to control postharvest pathogens on some fruit species like strawberry or citrus (Valencia-Chamorro et al. 2009; Yildirm and Yapici 2007). However, in these research works, SPP was tested in *in vitro* conditions or incorporated to edible coatings as additional ingredients. Recently, the food additives sodium methylparaben (SMP) (Moscoso-Ramírez et al. 2013a) and sodium ethylparaben (SEP) (Moscoso-Ramírez et al. 2013b), applied *in vivo* to citrus fruits as postharvest aqueous dips, have demonstrated significant curative antifungal activity against citrus GM and BM. Since no information is available about the use of aqueous solutions of SPP as potential postharvest antifungal treatments, particularly against Penicillium molds of citrus fruit, the objectives of this study were to: (i) preliminarily evaluate the curative activity of SPP against GM and BM, (ii) optimize dip treatment conditions, (iii) determine the compatibility with imazalil treatments, (iv) determine the effectiveness on economically important citrus cultivars, (v) evaluate the curative activity on cold-stored fruit, and (vi) evaluate the preventive activity.

MATERIALS AND METHODS

Fruit

The experiments were conducted with 'Valencia' and 'Lanelate' oranges (*Citrus sinensis* (L.) Osbeck), 'Clemenules' (synonyms: 'Nules', 'Clementina de Nules') clementine mandarins (*Citrus reticulata* Blanco), and 'Nadorcott' (*C. reticulata* x *C. sinensis*; synonyms: 'Afourer', 'W. Murcott') and 'Ortanique' [*C. reticulata* x (*Citrus sinensis* x *C. reticulata*); synonym: 'Topaz'] hybrid mandarins. Fruit were collected from commercial orchards in the Valencia area (Spain) and used the same day or stored for up to 1 week at 5 °C and 90 % relative humidity (RH) before use. Before each experiment, fruit were selected, randomized, washed with tap water and allowed to air dry at room temperature.

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 glass rod and transferred to a sterile aqueous solution of 0.5 g kg^{-1} 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 conidia ml^{-1} using a haemocytometer. With the exception of the in vivo primary screening experiments, 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 fruit rind afterwards. With the exceptions of the experiments to evaluate the antifungal preventive action, in which only *P. digitatum* was the target pathogen, and in vivo primary screenings, in which different lots of fruit were used for each fungus, fruit

were inoculated at two opposite points in the equatorial zone, one with *P. digitatum* and the opposite with *P. italicum.* Inoculated fruit were kept in a temperature-controlled room at 20 ºC for 24 h, until treatment.

Antifungal curative action

In vivo primary screenings

In vivo primary screenings were conducted to establish the best SPP concentration for curative activity against GM and BM. SPP (Propyl 4-hydroxybenzoate sodium salt; Merck KgaA, Darmstadt, Germany; Table 1; Fig. 1) was tested at six concentrations to control citrus postharvest GM and BM on fruit previously inoculated with the pathogens: 0.1, 1, 4, 7, 10 and 100 mM SPP. Inoculum preparation was performed following the procedure described above. For fruit inoculation, 30 µL of 10^6 conidia ml^{-1} suspension of *P. digitatum* or *P. italicum* were placed, using a micropipette, in rind wounds made with the stainless steel rod that affected both peel flavedo and albedo. Each pathogen was inoculated in different sets of fruit. About 24 h after the inoculation of the pathogen, 30 µL of SPP solution at the above mentioned concentrations 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 concentration of SPP and pathogen, 4 replicates of 5 'Valencia' oranges each were used. Primary screening experiments were conducted once. Treated fruit were incubated at 20 ºC and 90 % RH for 6 days, at which time disease incidence (% of infected wounds) and severity (lesion diameter) and pathogen sporulation (% of lesions showing spores) were determined.

Determination of dip treatment conditions

Small-scale laboratory trials were conducted using 'Valencia' oranges to establish the best dip treatment conditions. Fungal inoculation at a concentration of 10^6 conidia ml^{-1}

was carried out with both pathogens in the same fruit (opposite equatorial sides) by immersing the stainless steel rod into the conidial suspension and puncturing the peel once.

Stainless steel buckets containing 10 l aqueous solution of 100 mM (18.02 g l^{-1}; 1.8 % (w/v)) SPP were used. This concentration of SPP was selected according to previous results obtained in the *in vivo* primary screenings. 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 30, 60 or 150 s at 20, 50 or 62 °C. After treatment, fruit were rinsed for 5 s with tap water at low pressure in order to eliminate paraben salt residues. Control fruit were dipped in water alone at 20 °C. Sixty fruit per treatment (3 replicates of 20 fruit each) 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. Potential fruit phytotoxicity caused by SPP or heat was visually assessed after 3 days at 20 °C. For this purpose, fruit were classified into one of four categories, depending on rind appearance: 0 = no rind damage; 1 = slight brownish blemishes present (<10 % fruit surface); 2 = moderate brownish blemishes present (>10 % and <25 % fruit surface) and 3 = severe rind injury (>25 % fruit surface). A ponderate rind pitting index (0–3 scale) was calculated for each treatment (Moscoso-Ramírez et al., 2013a). The experiment was performed once.

Combination with low doses of imazalil

To determine the effect of the combination of the paraben salt with low doses of the chemical fungicide imazalil (IMZ; (±)-1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy)

ethyl)-1H-imidazole; Fecundal-S 7.5 % EC; Fomesa Fruitech S.L., Valencia, Spain) to control GM and BM, the following treatments were considered: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µl l^{-1} IMZ (IMZ 25), (4) 50 µl l^{-1} IMZ (IMZ 50), and (5) combination of 100 mM SPP with 25 µl l^{-1} IMZ (SPP + IMZ 25). 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} IMZ). This experiment was conducted using 'Valencia' oranges. Fungal inoculation and dip treatments were performed following the procedure mentioned above. Dip conditions were temperature of 20 ºC and immersion time of 60 s. After treatment, only fruit treated with SPP were rinsed with tap water for 5 s. Each treatment was applied to 3 replicates of 20 fruit each. Disease incidence and pathogen sporulation were determined after 7 days of incubation at 20 ºC and 90 % RH. The experiment was conducted twice.

Effectiveness on major citrus species and cultivars

To assess whether the effectiveness of curative treatments was dependent on the host fruit, different citrus species and cultivars were subjected to the following treatments: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µl l^{-1} IMZ (IMZ 25), or (4) 100 mM SPP + 25 µl l^{-1} IMZ (SPP + IMZ 25). All treatments were applied as dips at 20 ºC for 60 s. The experimental design, fungal inoculation and dip treatments followed the same procedures previously described. Treated fruit were not rinsed with tap water, with the exception of fruit treated with SPP alone. Treated fruit were incubated at 20 ºC and 90 % RH for 7 days, at which time disease incidence and pathogen sporulation were assessed. For each fungus, the effectiveness of the treatments on each cultivar was assessed once.

Effectiveness on inoculated and long-term cold-stored citrus fruit

To evaluate the curative capability of SPP against GM and BM on 'Valencia' oranges subjected to long-term cold storage, an experiment was conducted using inoculated fruit treated with: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µl l^{-1} IMZ (IMZ 25) and (4) 100 mM SPP + 25 µl l^{-1} IMZ (SPP + IMZ 25). All treatments were applied as dips at 20 ºC for 60 s. Treated fruit were not rinsed with tap water, with exception of fruit treated only with SPP. Treated fruit were stored up to 8 weeks at 5 ºC and 90 % RH. Following the refrigeration period, the fruit were subjected to 7 days of shelf-life at 20 ºC and 70-80 % RH. Disease incidence and severity and pathogen sporulation were assessed after 2, 4, 6, and 8 weeks at 5 ºC and 8 weeks at 5 ºC plus 7 days at 20 ºC.

Antifungal preventive action

To evaluate whether SPP treatments or combinations showed a preventive effect on the control of GM and BM, an experiment was conducted with 'Valencia' oranges in which a 1 mm wide, 2 mm deep wound was made with a stainless steel rod on the equatorial region of each fruit to simulate natural wounds. Then, the following treatments were applied by dipping the fruit in aqueous solutions at 20 ºC for 60 s: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µl l^{-1} IMZ (IMZ 25) and (4) 100 mM SPP + 25 µl l^{-1} IMZ (SPP + IMZ 25). About 24 h later, fruit were inoculated with the tip of a stainless steel rod, 1 mm wide and 2 mm in length that had been immersed in a 10^5 conidia ml^{-1} suspension of *P. digitatum* and inserted in a new adjacent wound (about 2 mm of separation between wounds). This methodology and inoculum concentration have been previously recommended for assessment of antifungal preventive action against Penicillium molds on citrus fruits (Moscoso-Ramírez et al. 2013a,b; Youssef et al. 2014). 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, at which time disease

incidence and severity and pathogen sporulation were assessed. The experiment was conducted twice.

Statistical analysis

Data were analyzed by an analysis of variance (ANOVA) with Statgraphics software (Statgraphics Plus version 4.1; Manugistics Inc., Rockville, Maryland, USA). Data on disease incidence and pathogen sporulation were transformed to the arcsine of the square root of the proportion of infected or sporulated fruit to assure the homogeneity of variances. In some cases, reductions with respect to the controls were calculated as percentages. Statistical significance was judged at the level $P = 0.05$. When appropriated, the Fisher's Protected Least Significant Difference (LSD) test was used to separate means. Since experiment was a non-significant factor in the ANOVA, shown values are non-transformed means from repeated experiments.

RESULTS

Antifungal curative action

In vivo primary screenings

Among the concentrations of SPP evaluated in this set of experiments, the concentration of 100 mM completely inhibited the development of BM and reduced the incidence of GM by 94 % on 'Valencia' oranges after incubation at 20 ºC for 6 days (Fig. 2). Moreover, the concentrations of 7 and 10 mM SPP significantly reduced the incidence of GM and BM by 94 and 89 %, and 70 and 88 %, respectively, after 6 days of incubation at 20 °C. In contrast, the concentrations of 0.1 and 1 mM SPP were not effective to reduce the incidence of the molds on 'Valencia' oranges (Fig. 2). Treatments with 100 mM SPP effectively reduced the severity of GM and BM by 38

and 100 %, respectively, but concentrations of 0.1, 1, 4 and 7 mM SPP did not significantly reduce the severity of both molds (Fig. 2).

Determination of dip treatment conditions

A concentration of 100 mM SPP was selected in the previous *in vivo* primary screenings as the most appropriate for use in subsequent trials. SPP at 100 mM applied at 50 °C for 60 or 150 s significantly reduced the incidence of GM in comparison with fruit dipped for 30 s at the same temperature (Fig. 3). On fruit dipped at 20 or 62 °C for 30, 60 or 150 s, GM incidence reductions did not differ from each other after 7 days at 20 °C. On the other hand, SPP at 100 mM applied at 50 °C for 150 s, significantly reduced the incidence of BM when compared with fruit dipped for 30 or 60 s at the same temperature, with incidence reductions of 97, 81 and 78 %, respectively. Likewise, on fruit dipped at 62 °C for 30, 60 or 150 s, SPP at 100 mM significantly reduced the incidence of BM, with reductions of 83, 93 and 100 %, respectively (Fig. 3). Irrespective of treatment duration, temperatures of 62 °C improved the effectiveness of SPP dips against BM with respect to that obtained at 20 °C, but temperatures of 50 °C did not improve the performance of dips at 20 °C (Fig. 3). However, up to 60 % of oranges dipped at 62 °C showed slight phytotoxic injuries on the rind (superficial brownish blemishes of irregular size, rind pitting index = 1). Thus, according to overall results from this set of trials, SPP dips at 20 °C for 60 s were selected for use in further experiments.

Combination with low doses of imazalil

Dips of 'Valencia' oranges at 20 °C for 60 s with the combination SPP + IMZ 25 significantly enhanced the control of GM when compared to the rest of the treatments, with a GM incidence reduction after 7 days at 20 °C of 96 %. SPP, IMZ 25 and IMZ 50 reduced GM incidence by 72, 50 and 68 %, respectively, and did not significantly differ

from each other (Fig. 4). In contrast, SPP + IMZ 25 treatment did not improve the control of BM in comparison to SPP and IMZ 50 treatments. IMZ 25 was the least effective treatment against BM, and reduced its incidence by 51 %. On the other hand, all treatments exerted a high anti-sporulant activity against both *P. digitatum* and *P. italicum*, with sporulation reductions with respect to the control treatments ranging from 70 to 95 % (Fig. 4). No phytotoxicities were observed on treated fruit (rind pitting index = 0).

Effectiveness on major citrus species and cultivars

Overall, the effectiveness of 100 mM SPP aqueous treatment applied alone at 20 °C for 60 s to control GM and BM was significantly higher on oranges than on mandarins. After incubation at 20 °C for 7 days, SPP applied alone significantly reduced the incidence of GM and BM by 72 and 58 % and 90 and 54 % on 'Valencia' and 'Lanelate' oranges, respectively (Fig. 5). Conversely, SPP reduced the incidence of GM and BM by less than 36 % on mandarins. Similarly, IMZ 25 and the combined treatments were more effective to reduce the incidence of GM and BM on oranges than on mandarins. IMZ 25 and combined treatments reduced the incidence of the molds, especially on 'Lanelate' and 'Valencia' oranges, respectively, and were much less effective on 'Clemenules' mandarins (Fig. 5). On the other hand, all three treatments presented an important anti-sporulant activity on most of the cultivars after 7 days at 20 °C. Irrespective of the dip treatment, sporulation of both *P. digitatum* and *P. italicum* was higher on 'Clemenules' mandarins than on the rest of citrus cultivars (Fig. 5).

Effectiveness on inoculated and long-term cold-stored citrus fruit

The incidence of both GM and BM on 'Valencia' oranges stored up to 8 weeks at 5 °C and 90 % RH followed by 7 days of shelf-life at 20 °C were effectively reduced by the application of SPP, IMZ 25 and SPP + IMZ 25 as 60 s dips at 20 °C (Fig. 6). The SPP

and combined treatments were significantly more effective than the IMZ 25 treatment (reductions of GM and BM incidence after 8 weeks at 5 °C of about 80-97 % and 48-57 %, respectively). After 8 weeks at 5 °C plus 7 days of shelf-life at 20 °C, the incidence of GM and BM on fruit treated with SPP + IMZ 25, SPP, and IMZ 25 was 9, 17 and 44 %, and 14, 20 and 53 %, respectively, while decay was 98 % on control fruit (Fig. 6).

In general, all three treatments significantly reduced the severity of GM and BM during the entire cold storage period at 5 °C, but the SPP and combined treatments were more effective than IMZ 25. While GM- and BM-infected control oranges were completely rotten, with maximum lesion diameters of 130 and 72 mm after 4 weeks at 5 °C, respectively, GM and BM lesion diameters on treated fruit at this time were 0-26 and 2-15 mm, respectively (Fig. 6). Similarly, all three treatments significantly prevented pathogen sporulation on decayed 'Valencia' oranges stored for 8 weeks at 5 °C plus 7 days of shelf-life at 20 °C, being the SPP and combined treatments superior to IMZ 25 alone (Fig. 6).

Antifungal preventive action

The treatments IMZ 25 and SPP + IMZ 25 effectively prevented GM on 'Valencia' oranges inoculated with *P. digitatum* after treatment and incubated at 20 °C for 7 days. In contrast, SPP treatment showed no preventive activity and did not reduce the incidence of GM with respect to the control fruit (Fig. 7). Likewise, similar results were obtained for disease severity and pathogen sporulation (Fig. 7).

DISCUSSION

The antifungal action of postharvest SPP treatments against citrus GM and BM was evaluated in this research work in order to set the basis for potential commercial treatments alternative or complementary to conventional chemical fungicides. *In vivo*

preliminary tests to select the most effective concentration of SPP to control GM and BM on previously inoculated oranges (curative action) were first conducted. Among the wide range of concentrations tested, a concentration of 100 mM SPP was selected to be used in small-scale trials to determine the most appropriate dip treatment conditions. Since dips in SPP aqueous solutions at 20 °C for 60 s were selected instead of dips in hot solutions, it seems that the synergy between heat (water heated at non-phytotoxic temperatures) and SPP for disease control is lower than that between heat and other compounds also tested as alternative control means (Montesinos-Herrero et al. 2009; Palou et al. 2001). Similar lack of complementary activity was observed when aqueous solutions of the paraben salts SMP and SEP (Moscoso-Ramírez et al., 2013a, b) were heated to 50 ºC. As in the case of SPP, heating these solutions to 62 ºC also resulted in production of slight superficial browning on the citrus fruit rind. The elevated disease control level obtained with SPP dips at room temperature is a factor that might facilitate the commercial adoption of postharvest SPP treatments in citrus packinghouses, since implementation and application costs of non-heated solutions would be considerably lower. Nevertheless, the effectiveness of 100 mM SPP dips at 20 ºC for 1 min was lower than that obtained in *in vivo* primary screenings (incidence reductions of 94 and 100% for GM and BM, respectively; Fig. 2), possibly because of the longer contact time of the SPP drop with the rind wound inoculated with *P. digitatum* or *P. italicum* compared with the dip contact time.

Potential antifungal activity of SPP against postharvest pathogens causing fruit decay has been demonstrated in some previous studies. For instance, SPP showed a strong inhibitory effect on mycelial growth and conidia germination of *Botrytis cinerea* at the high dose of 1000 μg ml^{-1} (Yildirm and Yapici 2007) and films containing SPP at concentrations very close to those tested here inhibited the growth of *P. digitatum* and

P. italicum in dichloran rose-bengal chloramphenicol agar (DRBC) (Valencia-Chamorro et al. 2008). Furthermore, in *in vivo* research, edible fruit coatings containing SPP as an ingredient significantly reduced the incidence of GM on 'Clemenules' mandarins artificially inoculated with *P. digitatum* 24 h before treatment and incubated at 20 °C for 7 days (Valencia-Chamorro et al. 2009). In this work, the antifungal curative action against GM and BM of SPP applied alone at the same dip and incubation conditions to 'Valencia' oranges or 'Clemenules' mandarins was equiparable or even higher than that of sodium carbonate, potassium benzoate, sodium bicarbonate (disease incidence reductions of about 50 %), sodium benzoate or potassium sorbate (reductions of up to 70%) (Palou et al. 2001, 2002a, b). Moreover, when compared to similar postharvest treatments with other sodium paraben salts to 'Valencia' and 'Lanelate' oranges, the performance of SPP dips at 1.8 % (w/v) was superior to that of SEP dips at 1.3 % and equiparable to that of SMP dips at 3 % (Moscoso-Ramírez et al. 2013a, b). As it has been reported (Giordano et al. 1999), the general antimicrobial activity of parabens increases as the chain length of the alkyl group increases. In this work, satisfactory disease control was obtained with a SPP concentration of 100 mM, while a concentration of 200 mM SMP was needed in previous research (Moscoso-Ramírez et al. 2013a).

Overall, our results suggest that SPP applications were compatible with the fungicide IMZ at low doses, and consistently improved its performance for the control of GM. Nevertheless, the control of BM on 'Valencia' oranges incubated at 20 °C for 7 days was not improved by the combination of both active ingredients. Furthermore, the combined treatment (SPP + IMZ 25) did not improve the performance of SPP alone for the control of both GM and BM on 'Valencia' oranges stored at 5 °C for 8 weeks plus 7 days of shelf-life at 20 °C. From this point of view, the behavior of SPP clearly differed

from that of other paraben salts like SEP or SMP, which not only were compatible with the fungicide IMZ, but also significantly improved their performance against GM and BM when combined with the treatment IMZ 25 under similar experimental conditions (Moscoso-Ramírez et al. 2013a, b). In terms of disease reduction, the effectiveness of the combination SPP + IMZ 25 and SPP applied alone to oranges were comparable to that obtained in previous research (Montesinos-Herrero et al. 2009; Moscoso-Ramírez et al. 2013a, b; Smilanick et al. 2008) with potassium sorbate, SMP and SEP, all combined with 25 $\mu l\ l^{-1}$ IMZ.

Regarding the effectiveness of SPP to control GM and BM on major citrus species and cultivars, we have generally observed that the treatments were more effective on oranges than on mandarins. These differences on treatment effectiveness clearly show the strong influence that intrinsic fruit characteristics may have on either fruit susceptibility to infection by *P. digitatum* and *P. italicum* or on fruit response to SPP application. In fact, the influence of the type of fruit on the performance of food additives or GRAS substances was reported in previous studies (Palou et al. 2001, 2002a). They found that treatments with aqueous solutions of sodium bicarbonate or sodium carbonate for 150 s were significantly less effective against GM and BM on mandarins than on oranges. Similar observations were reported by Montesinos-Herrero et al. (2009) and Moscoso-Ramírez et al. (2013a, b) regarding potassium sorbate, SMP and SEP treatments. In general, the inhibitory ability of low toxicity antifungal compounds such as SPP or other food additives depends on the presence of residues of the compound within the wound infection courts occupied by the fungus and on interactions between this residue and constituents of the rind (Palou et al. 2001, 2002a; Smilanick et al. 1999). Apparently, the nature of such interactions would be different according to the citrus species and cultivars as a consequence of different flavedo and

albedo characteristics or presence of different constitutive antifungal compounds in the rind. Additionally, such constituents and their concentration in the rind would be determined by not only the genotype but also the fruit physical and physiological condition. On the one hand, these factors determine the natural fruit susceptibility to decay; mature citrus fruits are typically more susceptible to decay than immature ones because, among other possible causes, their level of preformed antifungal compounds is lower. On the other hand, the biosynthesis and/or accumulation of antifungal compounds as a response to different postharvest treatments is also lower in mature fruit (Ben-Yehoshua and Porat 2005; Del Río and Ortuño 2004). It is known that an indirect mechanism of action of certain postharvest treatments such as heat (Ben-Yehoshua and Porat 2005) or solutions of some GRAS compounds (Venditti et al. 2005; Youssef et al. 2014) is the induction in the treated fruit tissues of disease resistance. According to these considerations, the relatively poor performance of SPP and the other tested treatments on 'Clemenules', and in some cases on 'Nadorcott' mandarins, could be explained by the weak physical condition of their rind and perhaps a reduced ability to synthesize antifungal compounds. At commercial maturity, the rind of these mandarin cultivars is typically soft and thin and after harvest they can evolve to senescence or overmature stages easier and faster than other citrus species and cultivars such as 'Valencia' or 'Lanelate' oranges.

Some aspects of the potential mode of action of SPP against fungal pathogens are common for different paraben sodium salts such as SMP or SEP (Moscoso-Ramírez et al. 2013a, b). In general, they can interfere on microbial development through the inhibition of membrane transport and mitochondrial functions. The undissociated form of SPP passes by diffusion through the plasma membrane; then, once inside the cell in a higher pH environment, the acid dissociates causing an accumulation of protons and

anions that cannot pass back across the plasma membrane and cause the pH to low with a consequent inhibition of cell metabolism (Brul and Coote 1999). In general, SPP is effective over a wide pH range of 4-8 (Aalto et al. 1953; Thompson 1994). Thus, the toxicity of SPP aqueous solutions at its natural pH of 10.0 is low. However, when applied to citrus fruit they become more active within the wounds in the albedo tissue because of the relatively low pH in these wounds. Rind pH of citrus fruit ranges from 4 to 6 depending on the species and cultivars (Prusky et al. 2004; Smilanick et al. 1999), and according to this, SPP would be more effective against *P. digitatum* or *P. italicum* on fruit with lower rind pH. However, such rind pH differences cannot explain alone the different effectiveness of the treatments among different citrus species and cultivars. In fact, previous research with some GRAS compounds showed that pH of salt solutions did not have a dominant role in their activity against postharvest pathogens (Nigro et al. 2006; Youssef et al. 2012b).

In this research, the three treatments SPP applied alone, IMZ 25, and SPP + IMZ 25 were all effective to reduce the incidence of GM and BM on inoculated and long-time cold-stored 'Valencia' oranges, although SPP alone and the combination were significantly superior to IMZ 25 at the end of the shelf-life period at 20 °C. The effect of these treatments on disease severity and pathogen sporulation showed a very similar trend. It can be concluded that, in general, citrus fruit treated with SPP alone or combined with IMZ can be exposed to commercial cold storage temperatures for relatively long periods of time.

It is clear from this study that SPP, applied alone at 100 mM in dips at room temperature for 60 s about 24 h prior to fungal inoculation, showed no preventive effect to protect 'Valencia' oranges from infections by *P. digitatum*. In contrast, in these experimental conditions, preventive treatments with IMZ (alone or combined with SPP)

greatly reduced GM after 7 days of incubation at 20 °C. This result indicates that SPP, unlike IMZ, is not able to spread itself through flavedo and albedo tissues, hence providing protective effect against further wound fungal infections. Similar results have been reported for other reduced-risk chemicals tested as postharvest antifungal treatments such as sodium carbonate, sodium bicarbonate or potassium sorbate (Montesinos-Herrero et al. 2009; Usall et al. 2008). This is a clear disadvantage of these alternative control means with respect to conventional fungicides. Although less cost-effective, an option to overcome this weakness might be the integration of alternative treatments with different modes of action (Palou et al. 2008; Youssef et al. 2012a).

It can be concluded from this research work that postharvest SPP treatments may be effective enough to be potentially included in integrated disease management programs as novel tools for a satisfactory control of citrus GM and BM. This is especially important in citrus growing areas with high levels of fungicide resistant strains of *Penicillium* spp. In addition, they could be used in combination with the conventional fungicide IMZ to effectively reduce its doses, which is currently an important issue for citrus exporters to EU or other worldwide markets. Before these good results on laboratory and small-scale trials with citrus fruit artificially inoculated with *P. digitatum* and *P. italicum*, postharvest treatments with SPP show promise as a novel postharvest antifungal treatment for citrus fruit. As the next research step before pursuing a registration of this compound for postharvest commercial use, confirmatory large-scale trials in citrus packinghouses with naturally infected fruit should be conducted.

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Table 1. Physicochemical properties of sodium propylparaben

Characteristics	Description
Formula	$C_{10}H_{11}NaO_3$ (Fig. 1)
Molecular weight	202.18
Synonyms	Sodium propyl *p*-hydroxybenzoate
	Sodium 4-propoxycarbonylphenolate
	Sodium 4-propoxycarbonylphenoxide
	4-hydroxybenzoic acid propyl ester sodium salt
Physical state	White crystalline powder
Melting point (°C)	105
Boiling point (°C)	294.3
Solubility in water	Soluble
pH	9.5-10.5 (concentration w/w: 0.1 %)
Stability	Stable

Source: Merck-Millipore, MA, USA, and EMD Chemicals Inc., NJ, USA.

Fig. 1. Chemical structure of sodium propylparaben

Fig. 2. Curative activity of sodium propylparaben at different concentrations against green (GM) and blue (BM) molds in *in vivo* primary screenings with 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum*, treated 24 h later, and incubated for 6 days at 20 °C and 90 % RH. Reductions of disease incidence and severity were determined with respect to control fruit treated with water (incidence of 95 and 75-85 % for GM and BM, respectively, and severity of 104-112 mm and 39-52 mm for GM and BM, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Incidence values were arcsine-transformed. Non-transformed means are shown.

Fig. 3. Effect of dip temperature and length on the effectiveness of 100 mM sodium propylparaben to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum*, treated 24 h later, and incubated for 7 days at 20 °C and 90 % RH. Reductions of disease incidence were determined with respect to control fruit treated with water (incidence of 100 and 98 % for GM and BM, respectively, for all temperatures and times). For each mold, columns with different lowercase and capital letters indicate significantly different dip length and dip temperature, respectively, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown. "P" indicates appearance of slight phytotoxicities on the fruit rind (pitting index = 1).

Fig. 4. Effectiveness of 100 mM sodium propylparaben alone (SPP), 25 µl l^{-1} imazalil (IMZ 25), 50 µl l^{-1} imazalil (IMZ 50) and combination of 100 mM SPP and 25 µl l^{-1} imazalil (SPP + IMZ 25) to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum*, treated 24 h later for 60 s at 20 °C, and incubated for 7 days at 20 °C and 90 % RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 100 and 98 % for GM and BM, respectively, and pathogen sporulation of 100 and 97-98 % for *P. digitatum* and *P. italicum*, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means from two experiments are shown.

Fig. 5. Incidence and sporulation of green (GM) and blue (BM) molds on citrus species and cultivars artificially inoculated with *Penicillium digitatum* or *P. italicum*, dipped 24 h later in water (control), 100 mM sodium propylparaben alone (SPP), 25 µl l^{-1} imazalil (IMZ 25), or 100 mM SPP combined with 25 µl l^{-1} imazalil (SPP + IMZ 25) for 60 s at 20 °C, and incubated for 7 days at 20 °C and 90 % RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 93-100 and 95-100 % for GM and BM, respectively, and pathogen sporulation of 80-100 and 65-100 % for *P. digitatum* and *P. italicum*, respectively, for all cultivars). For each mold and dip treatment, columns with different letters are significantly different, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown.

Fig. 6. Incidence and severity of green (GM) and blue (BM) molds, and sporulation of *Penicillium digitatum* and *P. italicum* on 'Valencia' oranges artificially inoculated with the pathogens, dipped 24 h later in water (control), 100 mM sodium propylparaben alone (SPP), 25 µl l^{-1} imazalil (IMZ 25), or 100 mM SPP combined with 25 µl l^{-1} imazalil (SPP + IMZ 25) for 60 s at 20 °C, and cold stored at 5 °C and 90 % RH for 8 weeks followed by 7 days of shelf-life at 20 °C. For each mold and evaluation date, means with different letters are significantly different, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means are shown.

Fig. 7. Preventive activity of sodium propylparaben (SPP) at 100 mM, 25 µl l^{-1} imazalil (IMZ 25), or 100 mM SPP combined with 25 µl l^{-1} imazalil (SPP + IMZ 25) against green mold on 'Valencia' oranges treated, artificially inoculated 24 h later with *Penicillium digitatum*, and incubated for 7 days at 20 °C and 90 % RH. Control fruit were treated with water and inoculated. Columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means from two experiments are shown.