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Sodium Bicarbonate Induces Crystalline Wax Generation, Activates Host-Resistance, and Increases Imazalil Level in Rind Wounds of Oranges, Improving the Control of Green Mold During Storage

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Imazalil (IMZ) was quantified in the flavedo and albedo (*Citrus* fruits outer and inner tissue of the exocarp) of wounded and unwounded Valencia L. Olinda oranges following a 2 min immersion at 25 °C in 50, 100, or 250 μg mL^{-1} of the fungicide mixture with or without 3% sodium bicarbonate (SBC). The addition of SBC significantly reduced the decay incidence throughout 30 d of storage at 10 °C with 95% RH and 6 d of simulated marketing period at 25 °C and 75% RH. In unwounded oranges, IMZ uptake was not changed by the coapplication of SBC, and the fungicide was predominantly recovered in the flavedo. To the contrary, in the albedo of wounded fruit, the residue level increased by about 6-fold when the fungicide was applied with SBC. When SBC was coapplied to wounded fruit, the phytoalexin scoparone was induced in the albedo and the accumulation was not affected by IMZ. When fruit was treated with SBC, scanning electron microscopy observations evidenced a production of crystalline wax patches with branched stripes and the magnitude was positively correlated to the salt concentration in the mixture. The generation as fast as 24 h post-treatment, and the different morphology of the new wax suggests a displacement of intracuticular waxes which can affect the fungicide sorption and diffusion coefficient into the rind.

KEYWORDS: Combined treatment; scoparone; *Citrus sinensis*; *Penicillium digitatum*; cuticle membrane

INTRODUCTION

During the postharvest stage of *Citrus* fruits, the use of synthetic fungicides is necessary to prevent the development of green and blue mold caused by *Penicillium digitatum* Pers.: Fr. Sacc. and *Penicillium italicum* Wehmer, respectively. Unfortunately, the restricted number of registered postharvest chemicals (*1*, *2*), often in conjunction with an inappropriate use of the allowed ones (*3*), led to the outbreak of resistant biotypes (R-biotypes). The resistant pathogenic fungi impair severe economic losses to the citrus industry. To face the problem, two different routes were followed: the synthesis of new generation pesticides and the optimization of the traditional ones (*4*).

In the first case, molecules with a novel biochemical mechanism of action were prepared and tested; in the latter, new modes of application for the conventional fungicides were developed (*5*). In this direction, several researches have been carried out leading to the so-called "combined treatments". Within the latter, conventional chemicals are used in the presence of physical, biological, or chemical agents that, operating directly or indirectly, increase their effectiveness. From the safety, environmental, and efficiency point of view, good results were obtained by applying traditional fungicides as heated mixtures (*6*, *7*) by using them with biocontrol organisms (*8*) or with food additives (*9*, *10*). A significant improvement in controlling citrus green mold was attained with carbonic acid salts applied as heated solutions or combined with low doses of conventional postharvest pesticides (*9*, *10*). Compared to the fungicide alone, Smilanick et al. (*9*) observed a considerable reduction of decay caused by *P. digitatum* R-biotypes when sodium bicarbonate (SBC) was used with imazalil (IMZ). Because no rise of IMZ residue in the rind occurred nor the augment of pH could explain the increase of effectiveness, the enhanced control of the pathogen was attributed to the direct effect of SBC on the R-biotype (*9*). Furthermore, when thiabendazole (TBZ) was used, Schirra et al. (*10*) evidenced a greater amount of TBZ into the epicuticular waxes when it was applied with SBC. This different distribution of the fungicide within the rind was believed to be a factor contributing to increase the effectiveness in the SBC-fungicide coapplication treatment (*10*). Indeed, the distribution and concentration of the pesticide within the different tissues of the rind plays an important role in preventing infections for wound pathogens such as *P. digitatum* and *P. italicum* (*11*−*13*).

The fungicide movement, allocation, and concentration on/into the rind is strictly dependent on several factors: the physicochemical characteristic of the compound, the properties and

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integrity of the cuticle (*14*), and the mode of application. The effect of the operating conditions in determining the residue allocation in *Citrus* fruits rind was clearly demonstrated when fungicides were applied as heated mixtures (*15*, *16*). Heat affects the cuticle permeability and integrity as well as the epicuticular wax physical properties and, accordingly, promotes the sorption and the encapsulation of the fungicide into the wax layer (*10*, *13*, *17*−*20*). Studies on polar ion penetration across astomatous cuticular membranes evidenced the presence of "polar paths" (*21*). The rate of penetration across the cuticle for salts was independent from temperature and use of plasticizers, while it was dependent upon humidity and the point of deliquescence of the salt (*22*−*24*). Thus, SBC should penetrate the cuticle membrane via the "polar pathway", while IMZ or TBZ move along the "lipophilic pathway". During fruit growth, the resistance provided by the cuticle and the epicuticular wax layer against different stresses is efficient, while after harvest and during storage these structures undergo cracking and lose their mechanical barrier role, offering entry points for wound pathogens (*25*, *26*).

The induction of resistance in the rind was shown to be triggered by biotic and abiotic agents and, among them, carbonic acid salts have been reported to induce the accumulation of scoparone, a phytoalexin, into the albedo of wounded *Citrus* fruits (*27*−*31*). Scoparone buildup, following treatments with increasing concentrations of sodium carbonate or SBC, was positively correlated to the control of *P. digitatum* rots in stored lemon fruit (*31*). According to these findings, it is likely that beyond the fungistatic effect of the salt (*9*, *32*−*35*) and the differential partition of the fungicide in the flavedo (*10*), also the induced resistance may contribute to improve the efficacy of the coapplication treatments.

On the basis of these previous reports, the aims of this investigation were to: (1) evaluate the contribution of the induced natural resistance in the control of *P. digitatum*, (2) examine the IMZ diffusion within the rind wounds following the coapplication, and (3) shed light on the effects of SBC on the cuticular membrane properties.

MATERIALS AND METHODS

Chemicals. Anhydrous sodium bicarbonate [S6297] was from Sigma Aldrich, Inc. (St. Louis, MO). Imazalil mixtures employed for treatments were obtained by diluting in deionized water a commercially available fungicide (Decozil 50; 44.66% imazalil, Elf Atochem, Paris, France).

Fruit. Oranges [*Citrus sinensis* (L) Osbeck. cv Valencia L. Olinda] were harvested twice between May and July when commercially mature. Fruit were picked from an experimental orchard located in central Sardinia, Italy, receiving standard cultural practices. The same day of harvest, fruit was selected, labeled, randomized into boxes (40 or 60 fruit each), and conditioned at 20 °C and 90% relative humidity (RH) for 24 h. Then, before treatments, fruit were either artificially wounded, wounded and inoculated, or left unwounded according to the experimental design.

Wounding and Inoculation. Following a 2 min immersion in a 0.2% NaOCl (pH 7.0) solution and a rinse with sterile water (SW), fruit was wounded with a sterile steel rod, impairing at the equatorial area four circular (Ø 3 mm) wounds (3 mm deep). The *P. digitatum* (isolate Pd-02) conidial suspension was obtained by adding SW with 0.05% (w/v) surfactant (Tween 80) to Petri dishes containing 7 d old actively sporulating cultures. The concentration was determined with a hemocytometer and adjusted to 10^5 conidia mL^{-1} by diluting with SW. In the experiments where wounded fruit were artificially inoculated, 20 μL of a freshly prepared conidial suspension was injected with a micropipet into each injury 1 h after wounding. Then fruit were left for 24 h at room temperature (20 °C and 75% RH) before treatment and subsequent incubated (25 °C and 90% RH) for 7 d. On the basis of preliminary inoculation trials with different conidial concentrations, we employed the concentration providing a decay percentage between 85 and 90% in untreated fruit incubated for 6 d.

Treatment. Wounded, unwounded, and wound-inoculated oranges were treated, if not otherwise specified, by a 2 min immersion at 25 °C as follows: (1) in water (control), (2) in 1000 $\mu g\ mL^{-1}$ imazalil (IMZ, fungicide control), (3) in 1, 2 or 3% (w:v) $NaHCO_3$ (SBC), (4) in 50, 100, or 250 $\mu g\ mL^{-1}$IMZ, (5) in SBC−IMZ mixture. For each IMZ concentration (50, 100, or 250 $\mu g\ mL^{-1}$), three levels of SBC (1, 2, or 3% w/v) were used. The immersion tank was thermoregulated by a heating-recirculating device (DL30, Haake instruments, Inc., Paramus, NJ). The highest concentration of IMZ (1000 $\mu g\ mL^{-1}$) was employed to simulate a commercial treatment. Following immersion fruit were left to dry at room temperature before storage or incubation.

Storage. Fruit used for the residue determination, monitoring of natural decay and scoparone analysis were stored at 10 °C and 95 ± 3% RH for 30 d, followed by a simulated marketing period (SMP) of 6 d at 20 °C and 75% RH.

Decay Monitoring. For the experiments performed with wound-inoculated fruit kept under incubation conditions, the amount of infected wounds was counted 7 d post-inoculation. Wounds with actively growing *P. digitatum* were considered as infected, independently from the expansion area or sporulation degree. When oranges were stored, the degree of natural infection was checked 14 and 30 d post-treatment and following SMP. Decay magnitude is expressed as a percentage of the total amount of fruit employed or artificially wounded.

Analysis of IMZ. Sample preparation, extraction, cleanup, recovery assays, and instrumental analysis were performed according to Dore et al. (*17*). The recoveries ranged from 94 to 103%.

Identification and Quantitative Assay of Scoparone. Exocarp tissue (flavedo + albedo) of unwounded and wounded fruit was removed with a cork-borer (0.8 cm Ø), trimmed with a scalpel to attain 0.8 cm thick cylinders, and then immediately stored at −20 °C. Extraction, cleanup, and instrumental analysis were performed according to Venditti et al. (*31*).

Assessment of IMZ Residue in the Flavedo and Albedo of Wounded and Unwounded Fruit According to Treatment. The experiment aiming to quantify IMZ residue in the flavedo and albedo of wounded fruit compared to unwounded ones was performed using 840 oranges. Half of the fruit was wounded as described and left for 1 h at 25 °C and 75% RH. Then, for wounded and unwounded fruit, seven sets of 60 fruit each were prepared according to the following treatments: sets 1−4 were immersed in a IMZ mixture with 50, 100, 250, or 1000 $\mu g\ mL^{-1}$, respectively; sets 5−7 were immersed in a mixture of 3% (w/v) SBC with 50, 100, or 250 $\mu g\ mL^{-1}$ IMZ, respectively. The fungicide residue was quantified 1, 14, and 30 d post-treatment and at the end of the SMP.

Decay in Stored Fruit as Affected by Rind Wounding, IMZ or SBC Concentration, and IMZ−SBC Co-Application. The decay control efficacy of IMZ (0, 50, 100, or 250 $\mu g\ mL^{-1}$) applied with SBC (0, 1, 2 or 3%) was evaluated during storage on artificially wounded and unwounded oranges. Half of the fruit (2040 oranges) were wounded 1 h before treatment (group a) and half remained unwounded (group b). Then, within each group, 17 sets of 120 fruit (3 replicates of 40 fruit) were prepared according to the following treatments: sets 1−5 were immersed in 0, 50, 100, 250, or 1000 $\mu g\ mL^{-1}$ of IMZ, respectively; sets 6−8, 9−11, 12−14, and 15−17 were immersed in 0, 50, 100, or 250 $\mu g\ mL^{-1}$ of IMZ combining each concentration of fungicide, with 1, 2, or 3% SBC, respectively. Following treatments, fruit was left to dry and then placed into storage and the occurrence of decay was monitored as described.

Decay in Wound-Inoculated Fruit as Affected by IMZ or SBC Concentration and IMZ−SBC Co-Application. An experiment to evaluate the efficacy of the coapplication treatments was repeated twice using 24 h old *P. digitatum* wound-inoculated "Valencia" oranges. Imazalil was either applied alone at different concentrations (0, 50, 100, 250, or 1000 $\mu g\ mL^{-1}$ IMZ) or applied with 1, 2, or 3% SBC. To carry out each experiment, 510 oranges were divided into 17 sets of 30 fruit (3 replicates of 10 fruit). Fruit from sets 1−5 were immersed in 0, 50, 100, 250, or 1000 $\mu g\ mL^{-1}$ mixtures of IMZ, respectively; fruit from sets 6−9, from 10−13, and from 14−17 in mixtures of 0, 1, 2, or 3% SBC combined with 0, 50, 100, or 250 $\mu g\ mL^{-1}$ IMZ, respectively. Then, when dried, all fruit was stored under incubation conditions until decay was quantified.

Scoparone Induction and IMZ Accumulation in Wounded Rind Following Separate or Combined Application with SBC. Oranges were wounded as described and separated into nine sets of 75 fruit. One h after wounding, fruit were treated by immersion as follows: (1) water

Table 1. Imazalil Residue (μg g^{-1} fresh weight) in the Flavedo and Albedo of Wounded and Unwounded Exocarp of "Valencia Olinda" Oranges Following a 2 min Immersion in Rising Concentrations of Imazalil with (+) or without (−) 3% (wt/vol) $NaHCO_3$, Followed by 30 d of Storage at 10 °C with 90% RH and 6 d at 25 °C and 75% (RH)[a]

	Imazalil concentration (μg g^{-1})						
	50		100		250		1000
time post-treatment (days)	−SBC[b]	+SBC	−SBC	+SBC	−SBC	+SBC	−SBC
			Flavedo				
1	2.29 Ab	2.84 Ab	4.11 Bb	4.30 Bb	6.26 Cb	6.45 Cb	24.16 Ea
14	1.42 Aa	1.65 Aa	3.37 Bab	3.20 Bab	6.03 Cb	6.36 Cb	24.18 Ea
30	1.29 Aa	1.25 Aa	2.10 Ba	2.10 Bb	5.05 Ca	5.59 Ca	20.55 Ea
30 + 6[c]	1.28 Aa	1.52 Aa	2.22 Ba	2.29 Bb	5.68 Ca	5.06 Ca	34.65 Eb
			Albedo (Unwounded Exocarp)				
1	0.58 Ab	0.60 Ab	0.83 Ab	0.62 Aab	1.06 Ba	0.98 Ba	2.88 Cb
14	0.54 Ab	0.42 Aa	0.62 Aa	0.69 Ab	1.07 Ba	1.00 Ba	2.16 Ca
30	0.41 Aa	0.45 Aa	0.53 Aa	0.59Aab	1.08 Ba	0.88 Ba	2.00 Ca
30 + 6	0.39 Aa	0.47 Aa	0.55 Aa	0.50 Aa	0.96 Ba	0.85 Ba	3.13 Cb
			Albedo (Wounded Exocarp)				
1	1.04 Ab	1.88 Ab	1.86 Ab	3.20 Ba	3.75 Bb	6.97 Ca	13.68 Da
14	1.04 Ab	1.45 Ab	1.75 Aab	3.11 Ba	3.43 Bb	6.68 Ca	12.61 Da
30	0.78 Aa	0.82 Aa	1.34 ABa	3.08 Ca	2.28 Ba	6.06 Da	12.86 Ea
30 + 6	0.52 Aa	0.65 Aa	1.10 Aa	3.70 Bb	2.39 Ba	8.46 Cb	17.18 Db

[a] Residue means ($N = 5$), capital letters relate to comparisons within rows, lower case letters to comparisons within columns, different letters indicate differences at $P \leq 0.05$ according to Newman−Keuls test. [b] SBC = $NaHCO_3$. [c] Six day of simulated marketing period at 25 °C and 75% relative humidity (RH).

(control), (2) 100 μg mL^{-1} IMZ, (3) 1, 2, or 3% SBC, (4) 1, 2, or 3% SBC with 100 μg mL^{-1} IMZ. Following immersion, fruit were left to dry and then stored. Scoparone quantification was performed on 60 wounds excised from 15 fruit (3 replicates of 20 wounds randomly gathered), after 1, 3, 7, 14, and 30 d of storage and after the 6 d of SMP, whereas IMZ was quantified as described before from the same excised wounds of fruit treated with 100 μg mL^{-1} IMZ . The experiment was repeated twice, and the concentrations of scoparone reported are means of the two experiments ± SD.

Scanning Electron Microscopy (SEM). Oranges subjected to the coapplication treatment with 0, 1, 2, or 3% SBC and 100 μg mL^{-1} IMZ and stored at 10 °C were used to study the effect of the treatment on the cuticle and epicuticular waxes by means of SEM. Observations were performed on replica of the fruit rind adapting the methodology of Scherrer et al. (*36*). In short, a ultralow-viscosity hydrophilic siloxane (ULVHS) (Dentsply De Trey GmbH, D-78467 Konstanz, Germany) was put onto a trapezoidal stub and positioned on a randomly marked out area (trapezoidal shaped) of the pericarp. Replicas of the same marked area were made on dry fruit before and after the treatments (1 and 30 d post-treatment). After 1 min, the stub was carefully removed, obtaining the negative replica of the rind. Then, the positive replica was prepared by pouring a resin onto the ULVHS negative. The positive resin replica was then gold−palladium coated with a sputter coater (S-150; Edward, West Sussex, U.K.) and used for SEM observation. Samples were observed with a SEM (DSM 962; ZEISS, Oberkochen, Germany) at 20 kV at different magnifications.

Statistical Analysis. All data were subjected to ANOVA using the statistical program OpenStat and where appropriate mean separation was performed according to the Newman−Keuls Test. The decay inhibition data were transformed to angular values before ANOVA. Synergy testing of the combined treatments was performed according to Plascencia-Jatomea et al. (*37*), calculating the expected and registered efficacy by Limpel's equation: $E_e = X + Y - (XY/100)$, where E_e was the expected additive response to imazalil and $NaHCO_3$, and X and Y were the percentages of inhibition of either imazalil or $NaHCO_3$ when applied alone.

RESULTS

Assessment of IMZ Residue in the Flavedo and Albedo of Wounded and Unwounded Fruit According to Treatment. As IMZ concentration increased in the treatment mixture, the residue in the flavedo significantly augmented, reaching 1 d post-treatment 2.3, 4.1, and 6.3 μg g^{-1} fresh weight (FW) with 50, 100, and 250 μg mL^{-1}, respectively. The application with SBC did not increase the residue level (**Table 1**). With 50 or 100 μg mL^{-1} IMZ, a significant decrease of residue occurred already 14 d post-treatment, and the application with SBC did not impeded this tendency. Conversely, during storage, losses were minimal when treated with 250 μg mL^{-1}, and again the coapplication did not influence residue persistence. The treatment with 1000 μg mL^{-1} provided 1 d post-treatment a residue of 24.2 μg g^{-1} FW, which decreased slightly during storage (20.6 μg g^{-1} FW) and increased significantly during the SMP, reaching 34.7 μg g^{-1} FW (**Table 1**). One d post-treatment, the residue in the albedo of unwounded fruit was much lower compared to that of the flavedo and after SMP the residue loss was about 33, 67 and 19% when treated with 50, 100, or 250 μg mL^{-1}, respectively. As for the flavedo, the application with SBC neither increased nor contained the loss of fungicide. When fruit was immersed into a IMZ mixture with 1000 μg mL^{-1}, the residue in the albedo 1 d post-treatment resulted 2.9 μg g^{-1} FW, it decreased during storage to 2.0 μg g^{-1}, and increased to 3.1 μg g^{-1} FW following SMP. The fungicide residue in the albedo of wounded fruit was greater compared to unwounded ones, and with 50 or 100 μg mL^{-1} IMZ it doubled (increase from 0.6 to 1.0 μg g^{-1} FW and from 0.83 to 1.9 μg g^{-1} FW, respectively), while with 250 μg mL^{-1} it raised 3-folds (from 1.1 to 3.8 μg g^{-1} FW). With time, the residue in the albedo decreased, but at the end of the SMP, all concentrations were still significantly higher compared to unwounded fruit (**Table 1**). During storage, the drop of IMZ in the albedo of wounded fruit treated with 100 and 250 μg mL^{-1} was minimal and an increase occurred during SMP. The trend was different for 50 μg mL^{-1}, and after storage the residue was halved (0.8 μg g^{-1} FW). Residue in the albedo increased twice when SBC was coapplied with 100 and 250 μg mL^{-1} IMZ, and again no loss occurred throughout storage and a significant increase took place during the SMP. When wounded fruit was immersed in the mixture containing 1000 μg mL^{-1} IMZ, the residue resulted six times (13.7 μg g^{-1} FW) the amount found in

the albedo of unwounded fruit and a significant increase of residue occurred after SMP (**Table 1**).

Decay in Stored Fruit as Affected by Rind Wounding, IMZ or SBC Concentration, and IMZ−SBC Co-Application. Concerning unwounded oranges, most fruit was lost by *P. digitatum* mold (83%) and nearly 50% of the untreated fruit rotted during the experiment, while when using 1000 μg mL^{-1} IMZ a complete control of decay was achieved up to 30 d post-treatment and 5% rotted during the SMP (**Figure 1**). Two weeks post-treatment, 18, 12, and 9% of the fruit treated with 50, 100, and 250 μg mL^{-1} IMZ rotted, respectively, and following storage, an additional 7, 16, and 12% was lost mainly by *P. digitatum*, and after the SMP the total loss was about 38, 31, and 33%, respectively (**Figure 1**). Oranges treated with 1, 2, or 3% SBC were mainly infected by *P. italicum* (75%) and less by *P. digitatum* (10%), and after SMP, 37, 35, and 30% of the fruit were lost, respectively. The SBC−IMZ coapplication improved the treatment efficacy, which was positively correlated to the salt and IMZ concentrations (data of 1 and 2% SBC are not reported). Compared to the fungicide alone, a minimal improvement occurred when 3% SBC was applied with 50 μg mL^{-1} IMZ, while with 100 or 250 μg mL^{-1}, the decay was lowered by 18 and 21%, respectively. Concerning the artificially wounded fruit left untreated, all wounds rotted within the first two weeks of storage. With 1000 μg mL^{-1} IMZ, a complete control was achieved up to 14 d post-treatment, while 3% and 6% of the wounds resulted infected after storage and SMP, respectively (**Figure 2A**). During storage, the control of decay by SBC was dependent upon concentration, while after SMP, no differences were found and about 35−40% of the wounds resulted infected mainly by *P. italicum* (60%) (**Figure 2A**). In employing 50 μg mL^{-1} IMZ, about 51% and 55% of the wounds rotted (77% by *P. digitatum* and 33% by *P. italicum*) during storage and

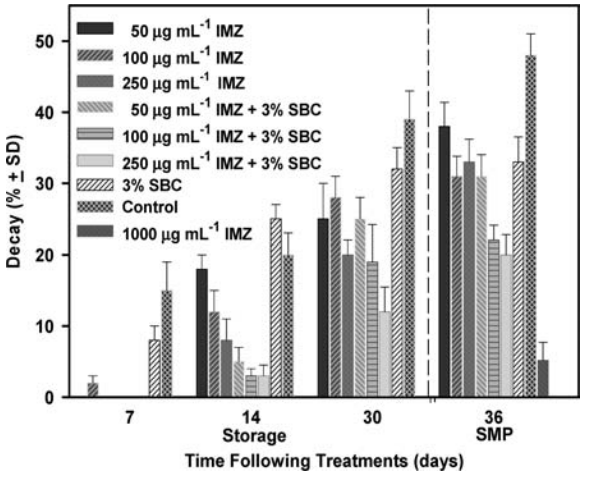

Figure 1. Percentage ± SD of unwounded "Valencia Olinda" oranges rotted after 7, 14, and 30 d of storage at 10 °C and 90% RH and after 6 d at 25 °C and 70% RH when treated with 0 (control), 50, 100, 250, or 1000 μg mL^{-1} imazalil (IMZ), 3% $NaHCO_3$ (SBC), or when coapplied.

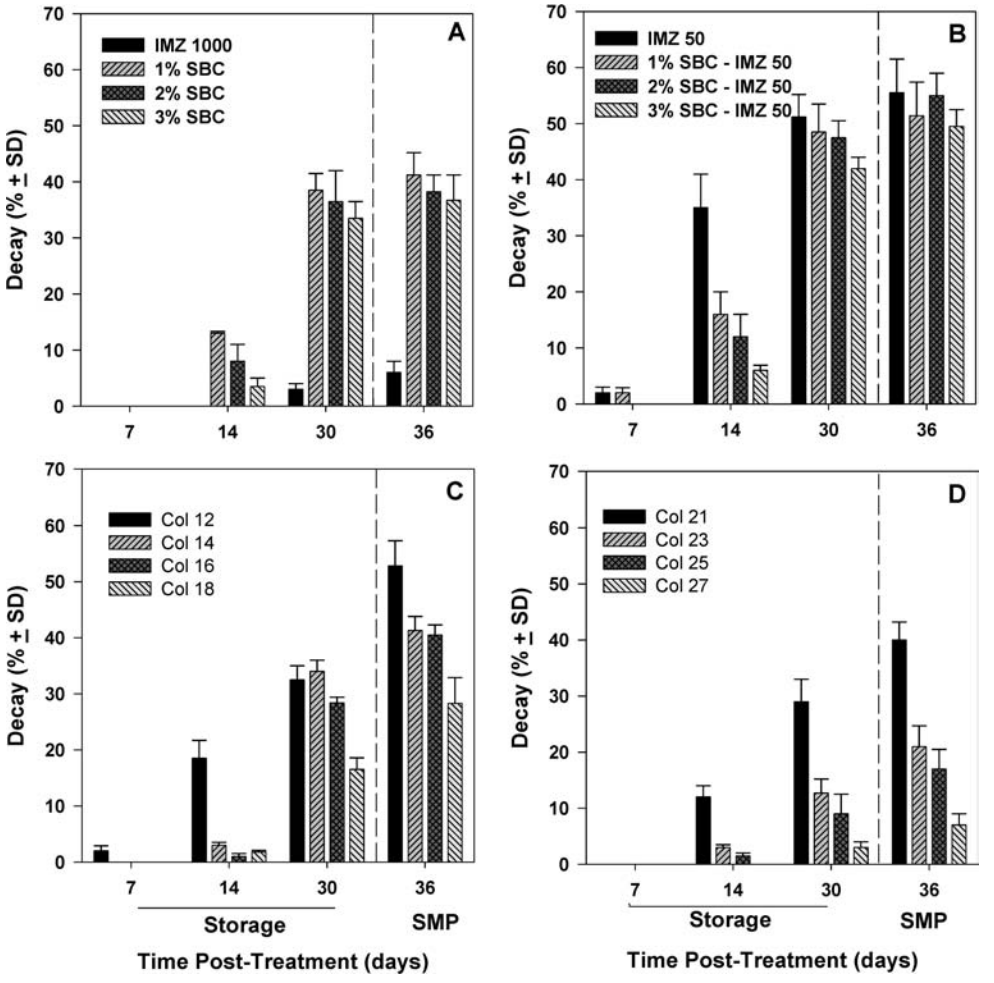

Figure 2. Percentage ± SD of wounds infected in "Valencia Olinda" oranges after 7, 14, and 30 d of storage at 10 °C and 90% RH and after 6 d of a simulated marketing period (SMP) at 25 °C and 70% RH when treated with 1000 μg mL^{-1} imazalil (IMZ) or 1, 2, and 3% $NaHCO_3$ (SBC) (**A**), or when 1, 2, or 3% SBC was coapplied with 50 (**B**), 100 (**C**) or 250 μg mL^{-1} IMZ (**D**).

Table 2. Expected (E_e) and Registered Efficacy (R_e) Calculated According to Limpel's Equation for the Control of Decay after 7, 14, and 30 d of Storage at 10 °C and 90% RH and After 6 d of a Simulated Marketing Period at 25 °C and 70% RH in Artificially Wounded "Valencia Olinda" Oranges Immersed 2 min in Combined Imazalil and $NaHCO_3$ Mixtures at Different Concentrations. Synergistic Interactions ($E_e < R_e$) are Evidenced in Bold[a]

		time of decay control following treatment (days)							
chemicals and concentration		7		14		30		30 + 6	
imazalil (μg mL^{-1})	$NaHCO_3$ (%)	E_e	R_e	E_e	R_e	E_e	R_e	E_e	R_e
50	1	99	95	86	83	79	51	71	49
	2	99	**100**	89	87	81	52	73	45
	3	99	**100**	92	**93**	85	58	79	50
100	1	99	**100**	92	**97**	87	66	72	59
	2	99	**100**	94	**99**	88	72	74	60
	3	99	**100**	96	**98**	91	83	80	72
250	1	100	100	95	**97**	88	88	79	79
	2	100	100	96	**99**	91	**96**	91	83
	3	100	100	97	**100**	92	**97**	85	**93**

[a] Limpel's equation: $E_e = X + Y - (XY/100)$, where E_e is the expected additive response to the combined treatments where X and Y are the percentages of decay inhibition relative to imazalil (50, 100, or 250 (μg mL^{-1}) or $NaHCO_3$ (1, 2, or 3%; wt/vol) employed alone.

SMP, respectively (**Figure 2B**). The application with 1, 2, or 3%, SBC significantly reduced the infections up to 14 d post-treatment, whereas at 30 d post-treatment, it was significant only with 3% SBC, and following SMP, no differences were found (**Figure 2B**). During storage, the control of decay with 100 and 250 μg mL^{-1} IMZ was similar (32% and 29%, respectively), while after SMP, differences became significant (53% and 40%, respectively) (**Figure 2C**,**D**). When 3% SBC was applied with 250 μg mL^{-1} IMZ, the efficacy became similar to that of 1000 μg mL^{-1} IMZ, with the only difference that *P. italicum* was the predominant cause of rots (**Figure 2A**,**D**). The comparison between the expected (E_e) and registered (R_e) efficacy in controlling decay of wounded fruit evidenced synergic interactions. The E_e of 250 μg mL^{-1} IMZ combined with 3% SBC resulted lower of the R_e during the whole experiment indicating a durable synergic interaction. With 100 μg mL^{-1} IMZ, the E_e values were lower for the first two weeks of storage while with 50 μg mL^{-1} only for the first week (**Table 2**). The lasting of the synergistic interactions was clearly dependent upon SBC and IMZ concentrations.

Decay in Wound-Inoculated Fruit as Affected by IMZ or SBC Concentration and IMZ−SBC Co-Application. The mean percentage of infected wounds in fruit used as control was 93 ± 3%, while with 1000 μg mL^{-1} IMZ, only 5% of the inoculated wounds rotted (**Figure 3A**). With respect to 1000 μg mL^{-1} IMZ, a significantly lower efficacy occurred by using 250 and 100 μg mL^{-1}, meanwhile, differences between them were negligible (5%). With 50 μg mL^{-1} IMZ, almost 57% of the inoculated wounds rotted, resulting in being less effective than 2 or 3% SBC (40 and 33%, respectively) (**Figure 3A**). With 1% SBC, about 62% of the wounds resulted infected. Compared to the treatment with 50 μg mL^{-1} IMZ alone, the application with 1, 2, or 3% SBC increased the efficacy by 8, 19, and 24%, respectively (**Figure 3A**,**B**). The beneficial effect of the coapplication was greater for 100 or 250 μg mL^{-1} IMZ, and a comparable control of decay to 1000 μg mL^{-1} IMZ was achieved when 3% SBC was applied with 250 μg mL^{-1} IMZ (**Figure 3A**,**B**).

Scoparone Induction and Accumulation in Wounded Rind Following Separate or IMZ−SBC Co-Application. Scoparone was not induced in the flavedo and albedo of unwounded fruit

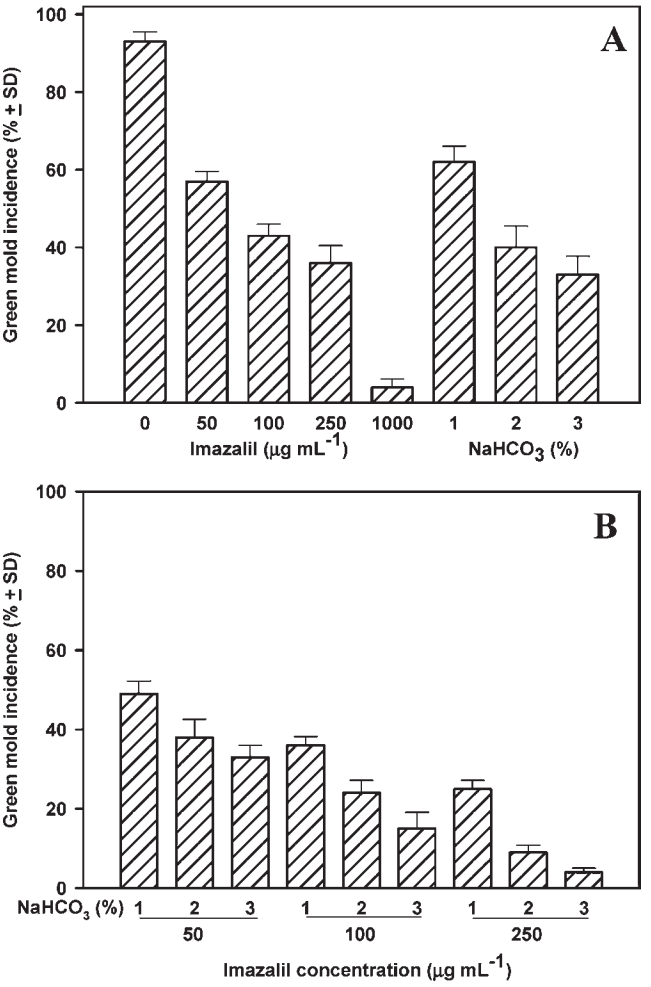

Figure 3. Percentage ± SD of wounds infected following a 7 d incubation at 25 °C and 90% RH of "Valencia Olinda" oranges inoculated with a 10^5 mL^{-1} conidial suspension of *P. digitatum* 24 h before single application of $NaHCO_3$ (1, 2, or 3%) or imazalil (0, 50, 100, 250, and 1000 μg mL^{-1}) (**A**) or coapplication (**B**).

following all applications (data not shown). In wounded fruit, a negligible amount of scoparone was found in the albedo, either following immersion in water (control) or in all IMZ mixtures throughout the storage at 10 °C and 95% RH (**Table 3**). When 3% SBC was used alone or applied with IMZ, a clear induction of the phytoalexin in the albedo took place. Scoparone accumulation was positively correlated to SBC concentration but not to IMZ ones. The amount of scoparone in wounded tissue increased by time and reached its peak accumulation after 2 weeks of storage. At the end of storage and SMP, the phytoalexin scoparone was significantly decreased and resulted below the ED_{50} (29 μg g^{-1}) for *P. digitatum* germ-tube elongation (*38*) (**Table 3**).

Scanning Electron Microscopy (SEM) Observation. The rind replica of the same area before treatment and 1 d post-treatment, evidenced changes of the epicuticular wax layer when treated with SBC (**Figure 4C−H**). Crystalline wax patches appeared on the rind surface, size and amount depending on the concentration of SBC (**Figure 4E−H**). With 3% SBC, patches expanded and branches became evident. At the end of the experiment, wax patches were not found on the replica and the surface was only in part covered with wax platelets, and several cracks were visible and differences according to SBC concentrations were negligible (data not shown).

Table 3. Scoparone Accumulation After 3, 7, 14, and 30 d of Storage at 10 °C and 95% RH and After a 6 d Simulated Marketing Period at 25 °C and 70% RH in the Albedo of Wounded "Valencia Olinda" Oranges Immersed for 2 min at 25 °C in Imazalil Mixtures of 0 (control), 50, 100, or 250 μg mL^{-1} with (+) or without (−) 3% (wt/vol) $NaHCO_3$[a]

	scoparone accumulation in the albedo (μg g^{-1}fresh weight)							
	imazalil concentration (μg mL^{-1})							
	0		50		100		250	
time (days)	+SBC	−SBC	+SBC	−SBC	+SBC	−SBC	+SBC	−SBC
3	30.7 a	7.4 b	20.5 a	10.3 b	25.8 a	10.5 b	28.7 a	6.2 b
7	86.4 a	48.0 b	80.2 a	40.6 b	88.5 a	48.6 b	82.4 a	35.8 b
14	193.7 a	5.2 b	188.5 a	8.3 b	201.8 a	10.2 b	181.6 a	8.5 b
30	22.5	nd[b]	17.2	nd	27.4	nd	11.0	nd
30 + 6	7.0	nd	5.4	nd	6.4	nd	6.0	nd

[a] Scoparone values are means (N = 15) of two experiments, storage was performed at 10 °C and 95% RH. Different letters within rows indicates differences at $P \leq 0.05$ according to Newman−Keuls test. [b] nd = not detected.

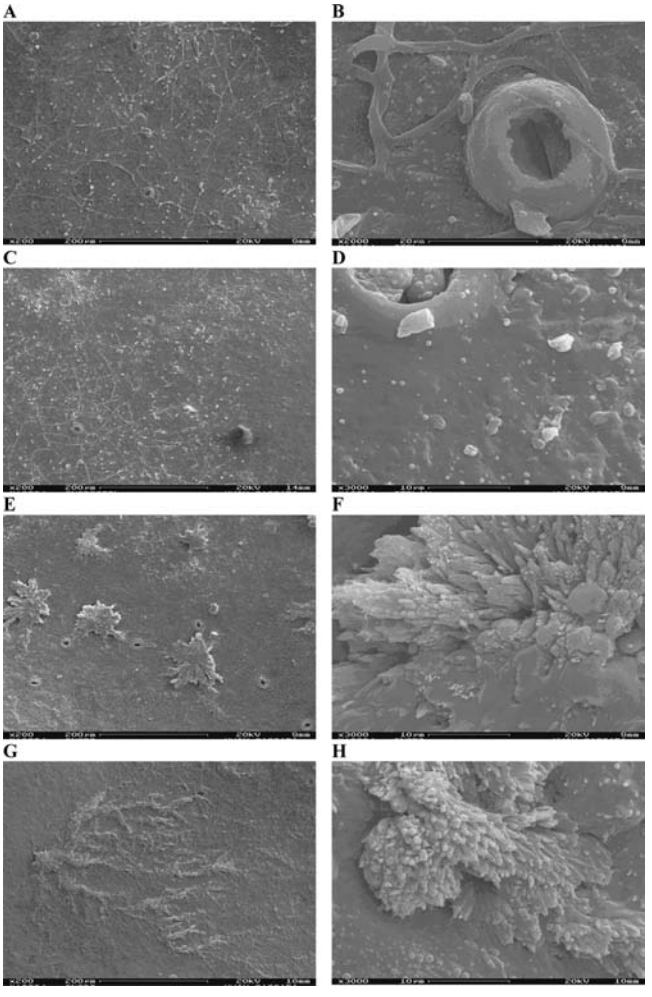

Figure 4. SEM micrographs of "Valencia Olinda" oranges rind before a 2 min immersion in water at 25 °C (**A**) and 24 h after immersion in water (**B**) or in solutions of 1 (**C**,**D**), 2 (**E**,**F**), and 3% $NaHCO_3$ (**G**,**H**).

DISCUSSION

Imazalil sorption and diffusion in the rind (flavedo + albedo) of unwounded "Valencia L. Olinda" oranges immersed in SBC−IMZ mixtures matched the results reported for lemon fruit by Smilanick et al. (*9*). Indeed, the SBC−IMZ coapplication did not increase the residues level in the rind (**Table 1**) but significantly improved the control of decay (**Figure 1**). In our experiments with oranges, fungicide residue in the rind was greater compared to that reported for lemon fruit (*9*), but it must be pointed out that, beside the fungicide concentration in the dipping mixture, the deposition is greatly influenced by species, variety, maturation stage, and by the treatment conditions (*38*, *39*). Most of the residue was retained in the flavedo (cuticle membrane, epidermal cells, and oil glands), corroborating previous findings on the feeble transcuticular diffusion and long half-life of this fungicide in citrus rind (*17*, *18*). In the albedo of unwounded fruit, only traces of IMZ were found and the residue concentration was lower than the ED_{50} ($\cong$1.4 μg mL^{-1}) for resistant *P. digitatum* biotypes (*3*, *9*). IMZ level increased as concentration was raised from 50 to 250 μg mL^{-1}, reaching the toxic threshold only with 1000 μg mL^{-1}.

A different situation was observed in wounded fruit. Indeed, in the albedo of the latter treated with IMZ alone, a 2−3-fold increase in residue concentration occurred, raising up to around 6-fold when the fungicide was applied with SBC (**Table 1**). In all cases, IMZ toxic levels for resistant *P. digitatum* biotypes were reached. When fruit was moved from 10 °C and 95% RH to 25 °C with 75% RH, a notable increase of residue occurred in the flavedo and albedo of wounded and unwounded fruit treated with 1000 μg mL^{-1} IMZ, and the same trend occurred in the albedo of wounded fruit when 3% SBC was coapplied with 100 and 250 μg mL^{-1} IMZ. This augmentation of residue has been reported also by other authors, and it is likely the result of the significant increase of transpiration when fruit were moved from storage conditions to simulated marketing ones. A greater IMZ deposition in the albedo of wounded fruit is consistent with previous reports (*12*, *13*, *17*), but its significant increase in the presence of SBC was unexpected. This latter augmentation can not be explained either by changes of the partition coefficient (the treatment temperature was unchanged) or by the occurrence of cuticular fracturing, as observed when fungicides were applied as heated mixtures (*6*, *13*, *16*, *17*). In our trials, the fast augmentation of fungicide levels within the albedo suggests the presence of a trapping mechanism, similar to that reported for Na^+ by Venditti et al. (*31*).

Indeed, the increase of residue and immovability in the albedo can only be explained by the embedding of IMZ within the alkaline hydrolysate of parenchyma pectins. It is reported that IMZ efficacy in controlling incipient and latent infections is related upon its concentration, half-life, and toxicity in the infection point (*1*, *11*−*14*), while carbonic acids salts affect spore germination, mycelial growth, increase albedo pH, promote alkaline hydrolysis of pectins, and induce phytoalexin biosyntesis in the albedo of *Citrus* fruits (*9*, *31*, *34*, *35*, *39*). Thus, in our inoculation experiment where pathogenesis was favored, the increase of efficacy by the coapplication should be attributed to the higher concentration and augmented toxicity of IMZ in the infection point rather than to the direct effect of the salt, even if a possible increase of the effective salt concentration in the wound sites should also be considered.

According to literature, the fungistatic activity of the salt in vitro inhibits the pathogenesis only for a short period (*33*). This may explain the consistent lost by decay within the first week of storage when unwounded fruit was treated with SBC alone (**Figure 1**). To the contrary, when fruit was wounded before SBC application, no wound infection was monitored during the same period of storage. This different behavior clearly indicates that other issues than the fungistatic activity contribute to control pathogenesis when SBC performs at the wound site. It is accepted that host-resistance plays a role in the decay control efficacy during storage (*27*−*30*), and it was reported that carbonic

acid salts induce the phytoalexin "scoparone" in the albedo of wounds (*31*).

The induction was positively correlated to the salt concentration and fungi toxic concentrations were kept from 7 up to 14 d post-treatment (*38*). In addition, we found that following the SBC−IMZ coapplication, scoparone was induced at considerable levels only in the albedo of wounded fruit and IMZ did not interfere on its buildup (Table. **3**). The decline of scoparone and fall of its toxic levels within the albedo of wounded fruit harmonized with the loss of efficacy observed during storage for the coapplication treatments. Thus, a lower or delayed biosynthesis of scoparone within the infection point may account for the lower efficacy attained by the coapplication on wounds artificially inoculated compared to wounds naturally infected during storage (**Figure 3B**; **2A−C**). Indeed, the fungistatic activity of SBC and the increase of IMZ toxicity within existing wounds are transitory but fundamental in the early stage of infection (first 3−4 d of storage), as such to jeopardize pathogenesis and to endorse the buildup of natural resistance, as clearly evidenced by comparing the results of the application with 50 and 100 μg mL^{-1}IMZ (**Table 2**; **Figure 2B−C**). The synergic effect gained up to 14 d of storage by the application of 3% SBC with 50 and 100 μg mL^{-1}IMZ is the result of scoparone accumulation at the infection point. While, with 250 μg mL^{-1}IMZ, the significant increase of residue in the albedo attained by the coapplication, especially with 3% SBC, became the dominant factor of the increased efficacy as is evidenced by the extension of the synergic interaction until after SMP.

The micrographs of rind off-prints one day post-treatment show a clear generation of wax patches in fruit treated with SBC, and the amount of patches was positively correlated to the salt concentration. As a general rule, after harvest, the amorphous epicuticular wax layer undergoes cracking and a platelets structure becomes evident, while a small regeneration of amorphous wax occurs underneath (*40*). Wax regeneration in *Citrus* fruits after harvest occurs slowly, and it was shown to be induced by ethylene (*26*). Fast regeneration was not yet reported for *Citrus* fruits, but it was reported for young leaves or following wax removal (*41*, *42*). Wax biosynthesis takes place in the epidermal cells and moves through the cell wall and the subcuticular pectinaceous layer by a lipid transferase protein into the inner cuticle structure, from where it is transported outside under the driving force of the cuticular transpiration (*24*). The rate of water loss is a key factor determining the keeping quality of horticultural commodities after harvest, and in most crops it is greatly dependent upon cuticular permeability. Diluted solutions of carbonic acid salts (especially K_2CO_3) increase cuticular water permeability and is currently employed commercially to fasten raisins' dehydration (*43*). Also Schirra et al. (*10*) monitored in oranges an increase of weight loss following the treatment with SBC, corroborating the results reported for astomatous cuticles (*44*).

Because SBC does not affect wax fluidity and, under normal conditions, the wax transport through the cuticle takes place by transpiration (*41*,*42*), it is likely that the wax patches observed are produced by a transpiration burst occurring within the first day post-treatment. The patches are morphologically different from the amorphous wax covering the rind, and the new crystalline wax appeared directly on the amorphous epicuticular wax and grows independently; therefore, it may be concluded that the regenerated wax is chemically different from the existing one (*42*). Because these patches were generated within 24 h post-treatment, we agree with the statement of Koch et al. (*42*) that under certain stress conditions new wax is not generated by the epidermal cells but is the result of a displacement of intracuticular wax. This idea is supported by the results of Cajuste et al. (*26*), which found a slow regeneration of amorphous wax following ethylene treatments during storage. Because the epicuticular waxes affect the retention (sorption) of the fungicide but rarely affect a compound's rate of penetration into the tissue, the diffusion is not dependent on the cuticle thickness but upon the intracuticular waxes nature. Thus, a displacement of the intracuticular wax will drastically change the barrier properties of the transport limiting layer, becoming more hydrophilic and increasing the diffusion coefficient for water but reducing it for lipophilic compounds such as IMZ and TBZ. The decrease of the diffusion coefficient avoids fungicides to move into the intracuticular waxes, which along with the augmentation of the epicuticular waxes (amorphous and new crystalline waxes), increase sorption and partition coefficient into the epicuticular wax. This may explain the results obtained by Schirra et al. (*10*), where, following the coapplication of SBC with TBZ, almost all the fungicide was found in the chloroform-wax extract. The patches on the surface appeared at different spots and from there the wax started to expand and branched stripes arose (**Figure 4G**). The number of spots was positively correlated to the concentration of SBC, while the appearance of the branched stripes were evident only with 3% SBC, indicating that the concentration not only influences the number of patches but also the degree of regeneration (*42*). These observations may explain the increase of fruit weight loss during storage as SBC concentration was raised (personal observations).

The results reported here suggest that, beyond the fungistatic activity of SBC and the increased toxicity of IMZ, the induction of natural resistance in the infection point effectively contributes to the synergistic effect obtained by the SBC−IMZ coapplication. In addition, the increase of residue within existing wounds is a key factor to jeopardize incipient infections, allowing the host to build up natural resistance. The freezing of IMZ within the epicuticular waxes seems the result of a drastic increase of transpiration induced by SBC, which promotes the intracuticular waxes displacement changing the diffusion coefficient of the limiting barrier.

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LITERATURE CITED

(1) Holmes, G. J.; Eckert, J. W. Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. *Phytopathology* **1999**, *89*, 716–721.

(2) Kanetis, L.; Förster, H.; Adaskaveg, J. E. Comparative efficacy of the new postharvest fungicide azoxystrobin, fludioxonil and Pyrimethanil for managing citrus green mold. *Plant Dis.* **2007**, *91*, 1502–1511.

(3) Kinay, P.; Mansour, M. F.; Mlikota, Gabler, F.; Margosan, D. A.; Smilanick, J. L. Characterisation of fungicide-resistant isolates of *Penicillium digitatum* collected in California. *Crop Prot*. **2007**, *26*, 647–656.

(4) Schirra, M.; D'hallewin, G.; Ben-Yehoshua, S.; Fallik, E. Host-pathogen interactions modulated by heat treatment. *Postharvest Biol. Technol*. **2000**, *21*, 71–85.

(5) Palou, L.; Smilanick, J. L. Droby, S. Alternatives to conventional fungicides for the control of citrus postharvest green and blue moulds. *Stewart Postharvest Rev*. **2008**, 2:2 doi: 10.2212/spr.2008.2.2.

(6) Smilanick, J. L.; Michael, I. F.; Mansour, M. F.; Mackey, B. E.; Margosan, D. A.; Weist, C. F. Improved control of green mold of citrus with imazalil in warm water compared with its use in wax. *Plant Dis*. **1997**, *81*, 1299–1304.

(7) D'Aquino, S.; Schirra, M.; Palma, A.; Angioni, A.; Cabras, P.; Migheli, Q. Residue levels and effectiveness of Pyrimethanil *vs* imazalil when using heated postharvest dip treatments for control

of *Penicillium* decay on citrus fruit. *J. Agric. Food Chem.* **2006**, *54*, 4721–4726.
(8) Sugar, D.; Basile, S. R. Timing and sequence of postharvest fungicide and biocontrol agent applications for control of pear decay. *Postharvest Biol. Tecnol.* **2008**, *49*, 107–112.
(9) Smilanick, J. L.; Mansour, M. F.; Margosan, D. A.; Mlikota Gabler, F. Influence of pH and $NaHCO_3$ on effectiveness of imazalil to inhibit germination of *Penicillium digitatum* and to control postharvest green mold on citrus fruit. *Plant Dis.* **2005**, *89*, 640–648.
(10) Schirra, M.; D'Aquino, S.; Palma, A.; Angioni, A.; Cabras, P. Factors affecting the synergy of thiabendazole, sodium bicarbonate, and heat to control postharvest green mold of citrus fruit. *J. Agric. Food Chem.* **2008**, *59*, 10793–10798.
(11) Brown, G. E. Efficacy of citrus postharvest fungicides applied in water or resin solution water wax. *Plant Dis.* **1984**, *68*, 415–418.
(12) Brown, E. G.; Nagy, S.; Maraulja, M. Residues from postharvest non recovery spray applications of imazalil to oranges and effects on green mold caused by *Penicillium digitatum*. *Plant Dis.* **1983**, *67*, 954–957.
(13) Brown, G. E.; Dezman, D. J. Uptake of imazalil by citrus fruit after postharvest application and the effect of residue distribution on sporulation of *Penicillium digitatum*. *Plant Dis.* **1990**, *74*, 927–930.
(14) Buchholz, A. Characterization of the diffusion of non-electrolytes across plant cuticle: properties of the lipophilic pathway. *J. Exp. Bot.* **2006**, *57*, 2501–2513.
(15) Schreiber, L. Review of sorption and diffusion of lipophilic molecules in cuticular waxes and the effects of accelerators on solute mobilities. *J. Exp. Bot.* **2006**, *57*, 2515–2523.
(16) Satchivi, N. M.; Stoller, E. W.; Wax, L. M.; Briskin, D. P. A nonlinear dynamic simulation model for xenobiotic transport and whole plant allocation following foliar application. III. Influence of chemical properties, plant characteristics and environmental parameters on xenobiotic absorption and translocation. *Pestic. Biochem. Physiol.* **2001**, *71*, 77–87.
(17) Dore, A.; Molinu, M. G.; Venditti, T.; D'hallewin, G. Postharvest immersions at 50 °C altered lemon fruit cuticle and promote permeation of imazalil into rind wounds. *J. Agric. Food Chem.* **2009**, *57*, 623–631.
(18) Cabras, P.; Schirra, M.; Pirisi, F. M.; Garau, V. L.; Angioni, A. Factors affecting imazalil and thiabendazole uptake and persistence in citrus fruits following dip treatments. *J. Agric. Food Chem.* **1999**, *47*, 3352–3354.
(19) Schirra, M.; Cabras, P.; Angioni, A.; D'hallewin, G.; Pala, M. Residue uptake and storage responses of Tarocco oranges after preharvest thiabendazole and postharvest heat treatment. *J. Agric. Food Chem.* **2002**, *50*, 2293–2296.
(20) Baur, P.; Schönherr, J. Temperature dependence of the diffusion of organic compounds across plant cuticles. *Chemosphere* **1995**, *7*, 1331–1340.
(21) Schonherr, J. Calcium chloride penetrates plant cuticles via aqueous pores. *Planta* **2000**, *212*, 112–118.
(22) Schonherr, J.; Luber, M. Cuticular penetration of potassium salts: effects of humidity, anions, and temperature. *Plant Soil* **2001**, *236*, 117–122.
(23) Schonherr, J.; Schreiber, L. Size selectivity of aqueous pores in astomatous cuticular membranes isolated from *Populus canescens* (Aiton) Sm. Leaves. *Planta* **2004**, *219*, 405–411.
(24) Schreiber, L. Polar paths of diffusion across plant cuticles new evidence for an old hypothesis. *Ann. Bot.* **2005**, *95*, 1069–1073.
(25) Storey, R.; Price, W. E. Microstructure of the skin of d'Agen plums. *Sci. Hortic.* **1999**, *81*, 279–286.
(26) Cajuste, J. F.; González-Candelas, L.; Veyrat, A.; García-Breijo, F. J.; Reig-Armiñana, J.; Lafuente, M. T. Epicuticular wax content and morphology as related to ethylene and storage performance of "Navelate" orange fruit. *Postharvest Biol. Technol.* **2010**, *55*, 29–35.
(27) Rodov, V.; Ben-Yehoshua, S.; Fang, D. Q.; D'hallewin, G.; Castia, T. Accumulation of phytoalexins scoparone and scopoletin in citrus fruits subjected to various postharvest treatments. *Acta Hortic.* **1994**, *381*, 517–523.
(28) Porat, R.; Pavoncello, D.; Peretz, Y.; Weiss, B.; Cohen, L.; Ben-Yehoshua, S.; Fallik, E.; Droby, S.; Lurie, S. Induction of resistance against *Penicillium digitatum* and chilling injury in Star Ruby grapefruit by short hot water brushing treatment. *J. Hort. Sci. Biotechnol.* **2000**, *75*, 428–432.
(29) D'hallewin, G.; Schirra, M.; Pala, M.; Ben-Yehoshua, S. Ultraviolet-C irradiation at 0.5 kJ m^{-2} reduces decay without causing damage or affecting postharvest quality of "Star Ruby" grapefruit (*C. paradisi* Macf.). *J. Agric. Food Chem.* **2000**, *48*, 4571–4575.
(30) Rodov, V.; Ben-Yehoshua, S.; Kim, J. J.; Shapiro, B.; Ittah, Y. Ultraviolet illumination induces scoparone production in kumquat and orange fruit and improves decay resistance. *J. Am. Soc. Hortic. Sci.* **1992**, *117*, 788–92.
(31) Venditti, T.; Molinu, M. G.; Dore, A.; Agabbio, M.; D'hallewin, G. Sodium carbonate treatment induces scoparone accumulation, structural changes and alkalinization in the albedo of wounded *Citrus* fruits. *J. Agric. Food Chem.* **2005**, *53*, 3510–3518.
(32) Smilanick, J. L.; Margosan, D. A.; Mlikota Gabler, F.; Usall, J.; Michael, I. F. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. *Plant Dis.* **1999**, *83*, 139–145.
(33) Palmer, C. L.; Horst, R. K.; Langhans, R. W. Use of bicarbonates to inhibit in vitro colony growth of *Botrytis cinerea*. *Plant Dis.* **1997**, *81*, 1432–1438.
(34) Aharoni, Y.; Fallik, E.; Copel, A.; Gil, M.; Grimberg, S.; Klein, J. D. Sodium bicarbonate reduces postharvest decay development on melons. *Postharvest Biol. Technol.* **1997**, *10*, 201–206.
(35) Palou, L.; Smilanick, J. L.; Usall, J.; Viñas, I. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate, and sodium bicarbonate. *Plant Dis.* **2001**, *85*, 371–376.
(36) Scherrer, S. S.; Quinn, B. J.; Quinn, D. G.; Wiskott, H. W. A. Fractographic ceramic failure analysis using the replica technique. *Dent. Mater.* **2007**, *23*, 1397–1404.
(37) Plascencia-Jatomea, M.; Viniegra, G.; Olayo, R.; Ortega-Castillo, M. M.; Shirai, K. Effect of chitosan and temperature on spore germination of *Aspergillus niger*. *Macromol. Biosci.* **2003**, *3*, 582–586.
(38) Kim, J. J.; Ben-Yehoshua, S.; Shapiro, B.; Henis, Y.; Carmeli, S. Accumulation of scoparone in heat-treated lemon fruit inoculated with *Penicillium digitatum* Sacc. *Plant Physiol.* **1991**, *97*, 880–885.
(39) Prusky, D.; McEvory, J. L.; Saftner, R.; Conway, W. S.; Jones, R. Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. *Phytopathol.* **2004**, *94*, 44–51.
(40) Sala, J. M. Content, chemical composition and morphology of epicuticular wax of Fortune mandarin fruits in relation to peel pitting. *J. Sci. Food Agric.* **2000**, *80*, 1887–1894.
(41) Neinhuis, C.; Koch, K.; Barthlott, W. Movement and regeneration of epicuticular waxes through plant cuticles. *Planta* **2001**, *213*, 427–434.
(42) Koch, K.; Neinhuis, C.; Ensikat, H. J.; Barthlott, W. Self assembly of epicuticular waxes on living plant surfaces imaged by atomic force microscopy (AFM). *J. Exp. Bot.* **2004**, *397*, 711–718.
(43) Uhlig, B. A.; Walker, R. R. Mode of action of the drying emulsion used dried vine fruit production. II. The effect of emulsion pH. *Austral. J. Grape Wine Res.* **1996**, *2*, 91–96.
(44) Schönherr, J. Water permeability of isolated cuticular membranes: The effect of pH and cations on diffusion, hydrodynamic permeability and size of polar pores in the cutin matrix. *Planta* **1976**, *128*, 113–126.

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