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# Sodium Carbonate Treatment Induces Scoparone Accumulation, Structural Changes, and Alkalinization in the Albedo of Wounded *Citrus* Fruits

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Following sodium carbonate treatment, accumulation of scoparone (6,7-dimethoxycoumarin) but not scopoletin (6-methoxy-7-hydroxycoumarin) was found in the albedo of wounded fruit from different *Citrus* sp. and cultivars. Treating wounded mandarin fruit cv. Fairchild with 5% $Na_2CO_3$ (SC) lead to a scoparone accumulation in the albedo of 310, 361, and 382 $\mu$g $g^{-1}$ fresh weight after 7, 10, and 15 days, respectively. Scoparone accumulation was associated with a decrease in decay severity. When oranges cv. Biondo comune wounded and treated with 5% SC were inoculated with *Penicillium digitatum* or *Penicillium italicum* conidia 3 days posttreatment, the decay percentage as compared to untreated wounds was reduced by 97.2 and 93.9%, respectively. Observations by scanning electron microscopy of wounded *Citrus* fruits treated at 20 °C with 2, 3, 4, or 5% (w/v) solutions of sodium carbonate showed structural modifications to the albedo as well as damage to 24−48 h old mycelia of *P. digitatum*, the cause of citrus green mold. Modifications were more evident in orange, lemon, and grapefruit as compared to mandarin fruit. The efficacy of the treatment was strictly related to the SC interaction with the albedo tissue that, in addition to structural changes, significantly increased tissue pH, affecting *P. digitatum* pathogenicity. The SC remaining as a film on unwounded flavedo had no effect in preventing contact infection by the *Penicillia*.

**KEYWORDS: Scoparone; green mold; blue mold; GRAS compounds; induced resistance; host−pathogen interaction**

## INTRODUCTION

Concerns for human health and safety as well as environmental considerations linked to the use of most synthetic pesticides have constrained their use, creating a void in the technology for pest control (*1*). This trend, combined with the proliferation of resistant strains of pathogens and the necessity to limit food losses, has encouraged the development and implementation of safe, effective, and economical treatment alternative approaches. Compounds classified by the U.S. Food and Drug Administration as generally recognized as safe meet most environment and safety requirements and are now largely used for preventing microbial spoilage in the food industry (*2*). Among these compounds, sodium carbonate (SC, $Na_2CO_3$) and sodium bicarbonate (SBC, $NaHCO_3$) used to control *Penicillium digitatum* (Pers. Fr.) Sacc. (green mold) and *Penicillium italicum* Wehmer (blue mold) on fruits of *Citrus* sp. were reported at the beginning of the 20th century (*3*, *4*). Marloth (*4*) studied the in vivo effects of different carbonates and bicarbonates on spores and 24 h old germlings of the *Penicillia*. He found that increasing the SC concentration or lengthening the treatment duration at constant pH (10+) extended the time needed for *P. digitatum* spore germination relative to the *P. italicum* germination time, while mortality rates were increased equally. Germinated spores appeared to be more sensitive to the SC treatment, and a plasmolytic effect was observed. However, the reduction in germination was not sufficient to explain the degree of decay control obtained in tests of the commercial process. Recent investigations were carried out to determine the influence of SC concentration, temperature, and immersion duration on the control of *P. digitatum* (*5*). All studied parameters affected the efficacy, with SC concentration having the highest impact. On the basis of the laboratory results, a predictive model was developed for commercial conditions and tested with orange fruits cvs. Valencia and Washington navel. The large scale trails showed a superior control of green mold relative to that predicted by the model. This difference was attributed to a less thorough water rinse in the packinghouse than in their experiments, which allowed more of the SC to remain within inoculated wounds on the rind. The primary finding of this work

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was that SC controlled 24 h old green mold infections. The same author (*6*) determined the effective dose ($ED_{50}$) concentration of SC and SBC needed to inhibit the germination of *P. digitatum* spores in vitro. Despite a large difference of effectiveness found in vitro between the two salts, similar control of green mold was achieved in vivo. As a result, the in vitro toxicities did not indicate the efficacy of SC and SBC in controlling natural decay. Furthermore, the treatment effect was shown to be fungistatic and not persistent. In the same paper, trials on orange and lemon fruit showed that posttreatment rinsing with water at low pressure did not reduce the efficacy while high pressure washes did. Palou et al. (*7*) provided further evidence on the effectiveness of these two salts in controlling *P. digitatum* on orange fruit (cv. Navelate), reporting efficacy similar to that of hot water dips at 50−55 °C. Attempts to control *P. italicum* on oranges (cvs. Washington n., Navelate, Lanelate, and Valencia) were made using 2, 3, or 4% SC treatments at 20 or 45 °C for 60 or 150 s (*8*). For *P. italicum*, it was demonstrated that the treatment temperature influenced the effectiveness more than did SC concentration or treatment duration. With short-term storage (20 °C for 7 days), the control of blue mold and green mold on artificially inoculated orange fruit was similar, while blue mold control was inferior for long-term cold storage. The two salts were also employed to treat artificially inoculated Clementine mandarins (cv. Clemenules) stored at 20 °C for 7 days (*9*). From this study, it was clear that treatment conditions required to control green or blue mold on orange and lemon fruits were significantly less harsh than those required to contain the same molds on mandarin fruits.

While from the technical point of view these reports provided guidance on how to use these salts, not much was revealed about the host−pathogen interactions influenced by these treatments. It is likely that, in vivo, some additional factors related to the wound area affect the pathogenicity of the *Penicillia*. The differences in treatment efficacy in vitro and in vivo as well as between SC and SBC have been suggested to depend on the presence of salt residues within the wound site and on interactions between the residue and the constituents of the rind. However, to our knowledge, no evidence supporting this idea has been reported. Recently, alkalinization of the wound site was shown to modulate the host−pathogen interactions and to interfere with the *Penicillia* pathogenicity (*10*). Barmore and Brown (*11*) reported that when intact rind of citrus fruit was treated with spores of *P. digitatum* or *P. italicum* contained in a nutrient solution, infection occurred only after adding galacturonic acid. They suggested that this acidic compound had a key role in the pathogenicity related to its ability to damage the healthy rind. Arimoto et al. (*12*) observed that acidic treatments, below pH 5, enhanced green mold infection, whereas treatments at pH 7 or higher inhibited the infection. These observations may explain the fungistatic effect of alkaline treatments; however, it is not clear why *P. digitatum* is differently affected in vivo as compared to in vitro by the SC treatment. In addition, the lower efficacy in controlling *Penicillia* molds on mandarin fruit as compared to orange and lemon fruit should also be clarified. On the basis of these points, the objective of the present study was to describe host behavior as affected by SC treatment and to correlate host responses with *Penicillia* pathogenicity. To study these phenomena, experiments were carried out using fruits of different *Citrus* sp. and cultivars.

## MATERIAL AND METHODS

**Fungal Isolates, Cultural Conditions, and Inoculum Preparation.** Green mold isolate "PD-02" (*P. digitatum*) and blue mold isolate "PI-04" (*P. italicum*) wild types were isolated from naturally infected oranges cv. Washington navel and lemons cv. Eureka, respectively. After achieving a monoconidial culture, the fungi were maintained in 150 mm × 22 mm glass culture tubes on potato dextrose agar (PDA) [Difco, 213400] slants. Seven days before inoculation, spores from both fungi were transferred to Petri dishes (9 cm diameter, Ø) containing PDA and put in a thermoregulated cabinet at 25 °C with a 12 h light/dark cycle. The conidial suspension of the two fungi was obtained by filtering a scrubbed sporulating 7 day old culture submersed in sterile water containing 0.05% (w/v) Tween 80 [Sigma, P 1754] through six layers of cheesecloth. The conidial concentration was adjusted to a $10^7$ spores $mL^{-1}$ (based on hemocytometric count), and the suspension was used within a few hours for the in vitro and in vivo tests.

**Effect of SC on *P. digitatum* and *P. italicum* Growth on PDA.** PDA prepared as recommended by the manufacturer was added to 40 Petri dishes (9 cm Ø), left overnight in a hood under ultraviolet light and laminar air flow. Then, for each pathogen, four conidial suspensions were prepared. The first, used as a control, was obtained by adding 1 mL of the conidial suspension ($10^7$ spores $mL^{-1}$) to 9 mL of sterile water, and the remaining suspensions, called salt−conidial suspensions, were prepared in order to contain the same spore concentration ($10^6$ spores $mL^{-1}$) and 0.357, 0.476, or 0.595 M $Na_2CO_3$ [Aldrich, 22353-0]; that is, 37.8, 50.4, or 63.1 g/L, respectively. All suspensions were vortexed 1 min to break up spore aggregates before use. Suspensions, with or without SC, had pH 11 or pH 7, respectively. Each of the four salt−conidial suspensions of the two pathogens was introduced in 10 replicate dishes by pipetting at the center an aliquot of 100 $\mu$L (100000 spores). Dishes were then stored at 20 °C with 12 h light/dark cycles, and the increase in radial growth was measured daily for 5 days. This experiment was carried out in November, January, and April.

**Fruit.** The investigation was carried out with commercially mature mandarins (*Citrus reticulata* Blanco, cvs. Fairchild and Fremont), oranges [*Citrus sinensis* (L.) Osbeck, cvs. Salustiana, Hamlin, and Biondo comune], grapefruits (*Citrus paradisi* Macf., cvs. Marsh and hybrid Oroblanco), and lemon [*Citrus limon* (L.) Burm., cv. Verna]. To perform all experiments, fruit was harvested twice based on a 2 week schedule, in an experimental grove (southwest Sardinia, Italy, 39° 55′ N) managed using standard horticultural practices. At each harvest, fruits were transported the same day to the laboratory, surface disinfected by a 2 min immersion in a sodium hypochlorite solution (2% NaOCl) [Aldrich, 42504-4], rinsed with distilled water (DW), and allowed to dry. Then, two-thirds of the fruits were used to check the treatment efficacy by wound inoculation or contact inoculation tests and the remaining fruits were used for scanning electron microscopy (SEM) observations or studies involving the detection, isolation, and accumulation of induced antifungal compounds by thin-layer chromatography (TLC) and gas chromatography−mass spectrometry (GC-MS) analyses.

**Inoculation by Wounding.** At each time, fruits were wounded within 24 h following harvest by injuring the flavedo (oil gland containing tissue) and albedo (inner white tissue) at four equatorial points with a steel rod (3 mm × 3 mm deep) and then held on the lab bench at 25 °C (± 2) for 1 h prior inoculation. Thereafter, fruits were divided into two groups of 160 fruits each. The first was inoculated with the four suspensions of *P. digitatum* and the second with *P. italicum*. Each group was subsequently divided into four subgroups of 40 fruits (four replicates of 10 fruits). Wound inoculation of all fruits within each subgroup was performed by injecting 20 $\mu$L (20000 spores) of the appropriate salt−conidial suspension into all wounds with a micropipet. Inoculated fruits were then placed in boxes with a wet filter paper on the bottom to ensure near 100% relative humidity (RH), covered, and held at 25 °C for 5 days before monitoring decay.

**Inoculation by Contact.** In mid-March, 288 oranges cv. Salustiana were harvested, selected, disinfected and left to dry. Half were inoculated with a conidial suspension containing $2 \times 10^6$ conidia $mL^{-1}$ once on the equator with *P. digitatum* or *P. italicum*. Then, they were stored at 25 °C and 90% RH for 5 days, when the lesions were 4−7 cm in diameter and sporulation was evident. At that time, an additional 144 oranges were harvested, disinfected as described, and divided into four groups, matching the treatments with 0, 3, 4, or 5% (w/v) SC, respectively. Fruits of each group were dipped for 2 min in the SC solution at 20 °C and left to dry. Within groups, half of the healthy

treated oranges were placed between two *P. digitatum*-infected fruits and the remainder between *P. italicum*-infected fruits. Healthy fruits were placed in boxes so that they touched the injury-infected and sporulating point on the infected fruits. Boxes were stored for 3 days at 12 °C and near 100% RH. Then, inoculated fruits were discarded and the contact-inoculated fruits were placed at 25 °C and high RH for an additional 3 days before contact infection was monitored.

**Posttreatment Inoculation.** Orange fruit cv. Biondo comune (400 fruits) was harvested in mid-April, surface disinfected, rinsed, left to dry, and wounded as described. After 1 h, 20 $\mu$L of DW (control) was added to half of the lesions and a 5% SC solution to the rest, and then, fruits were stored at 20 ± 2 °C in boxes containing wet paper on the bottom. The wounds of separate 30 fruit samples were then inoculated with 20 $\mu$L of a spore suspension ($2 \times 10^6$ spores $mL^{-1}$, *P. digitatum* or *P. italicum*) 2, 3, 5, 7, 10, or 15 days post-SC-treatment. Following inoculation, fruits were returned to the above-mentioned conditions and the infection percentage was monitored for 7 days postinoculation.

**Decay Monitoring.** The degree of infection was monitored and expressed as a percentage of the total number of wounds inoculated. In all experiments, a wound or contact site was considered infected if there was a visible sign of pathogen growth, irrespective of the symptom diameter. Decay was monitored 7 or 10 days postinoculation.

**pH of Albedo Following SC Treatment.** Fruits of orange cv. Biondo comune, grapefruit hybrid cv. Oroblanco, lemon cv. Verna, and mandarin cv. Clementine were surface sterilized as described, and the flavedo was carefully removed with a scalpel, avoiding oil leakage. Then, half of the fruits were dipped for 2 min in a water solution containing 5% SC and the remainder in sole DW. One hour after treatment, the outer albedo (5−7 mm) was removed with a scalpel and three replicates of 20 g each were put into 100 mL of DW, homogenized (Ultra Turrax T25, Janke&Kunkel KG, Germany), and centrifuged (6000*g*) for 10 min. The supernatant was collected and filtered through Whatman #1 paper (Whatman, Maidstone, England), and the filtrate pH was determined.

**SEM Observation and Sodium Localization.** At the second harvest, four wounds per fruit on three fruits were inoculated with *P. digitatum* and *P. italicum* conidia ($10^6$ conidia $mL^{-1}$), as described. Three additional fruits were inoculated with DW (control). After 24 h of incubation at 25 °C, all fruits were dipped for 2 min in a 5% SC solution at 20 °C and one dipped fruit was then gently rinsed with DW. Two wounds of each fruit were used for SEM observations, and the remaining wounds were subjected to SEM topographic ion microanalyses. All wounded rind samples were excised with a cork borer (0.8 cm Ø) and trimmed to 4 mm thickness with a scalpel. For SEM observations, samples were fixed in phosphate buffer (pH 7.4) [Sigma, P3813] containing 3% (v/v) glutaraldehyde [Sigma, G5882] and stored at 4 °C for later use. Fixed tissue was rinsed three times in phosphate buffer (pH 7.4) followed by three DW washes. Dehydration was carried out in an increasing ethanol concentration series (20, 50, 70, 80, and 95% and 3× at 100%) with samples held at each ethanol concentration step for 20 min. After completion of the dehydration, samples were dried by critical point and placed on aluminum stubs using silver conductive glue. Gold−palladium coating was performed with a sputter coater (S-150; Edward, West Sussex, United Kingdom). Samples were stored in a vacuum desiccator until examination with SEM (DSM 962; ZEISS, Oberkochen, Germany) at 20 kV at 80−500×. To perform the SEM topographic ion localization of $Na^+$ after treatment, fresh unfixed rind samples were immediately placed on aluminum stubs with contact glue and observed using a low vacuum (50 Pa) SEM (JSM-6460LV; JEOL-USA, Inc., Peabrody, MA) equipped with a field emission electron probe microanalyzer (JXA-8500F; JEOL-USA, Inc.) at 15 kV and 35× magnification. Because tissue dehydration was observed in preliminary studies, samples were left within the observation chamber not longer than 10 min.

**Identification and Quantitative Assay of Scoparone.** *Preparation of Crude Extracts.* Fruits of the studied cvs. were disinfected and inoculated with the four salt−conidial suspensions of both fungi, as described. The analysis of each combination included 30 fruits with three replications. Rind tissue, including the inoculated wounds, was removed daily for 7 days with a cork borer (0.8 cm Ø, 0.4 cm thick) and immediately stored at −20 °C. Two grams of frozen tissue was then homogenized for 3 × 2 min at medium speed with methanol as the extraction solvent at a ratio of 1/7.5 (w/v). Homogenates were centrifuged (5 min at 6000*g*), and the organic extracts were passed through filter paper (Whatman #1). For GC analyses, the filtrates were brought to a final volume of 50 mL and an aliquot of 10 mL was washed with 2 mL of a 2/3 (v/v) diethyl ether/*n*-hexane solution, the hexane/ether layer was removed, and the methanol phase was evaporated under a gentle stream of nitrogen until dry. The dried residue was immediately dissolved in 1 mL of methanol containing an internal standard (5 ppm of 5,7-dimethoxycoumarin) [Aldrich, 116238-1G] and analyzed by GC-MS. To perform the TLC analyses, extracts were pooled, dried with anhydrous $MgSO_4$ (Carlo Erba, 375709), filtered and concentrated in vacuo with a rotovapor (RE 121; Büchi, CH-9230 Flawil, Switzerland), and the residue was dissolved in 5 mL of methanol. All samples were kept in the dark capped vials stored at −20 °C until use.

*TLC and Detection of Antifungal Activity.* Ten microliters of each extract obtained 5 days posttreatment was subjected to TLC on silica gel 60 $WF_{254}$, 0.2 mm thick [Merck, 16484] with ethyl-acetate/petroleum ether (40−60) (2/1; v/v) as the carrier solvent. The plates were dried and exposed to UV light (366 and 254 nm), novel bands were marked, and the $R_f$ values were calculated. Duplicate plates were prepared with 6,7-dimethoxycoumarin, 6-methoxy-7-hydroxycoumarin (scopoletin) [Sigma, S-2500], and 7-hydroxycoumarin (umbelliferone) [Aldrich, H2400-3] used as $R_f$ reference standards. The antifungal activity of the novel compound was checked on TLC plates by a bioassay using *Cladosporium cladosporioides* G. A. De Vries as the test organism (*13*).

*Identification and Quantitative Assay of Scoparone by GC-MS.* The methanolic extracts were analyzed by GC (6890N; Agilent, Karlsruhe, Germany) equipped with a mass detector (MSD 5973N; Agilent). A 1 $\mu$L sample was injected onto a splitless injector held at 220 °C. The column was a 30 m × 0.25 mm i.d., 0.25 $\mu$m film thickness capillary column (HP-5 MS; Agilent), and the oven temperature was programmed with a 5 °C/min rise from 150 to 210 °C. The carrier gas was ultrahigh purity helium at a flow rate of 2.1 mL/min. The transfer line, ion source, and quadrupole temperatures were 250, 230, and 150 °C, respectively. The electron impact was kept at 70 eV, and the runs were carried out in selected ion monitoring mode.

*Spiked Sample Extraction.* Scoparone-free rind samples (2.0 g) were removed from untreated fruit as described, added to a known amount of a methanolic solution containing 0, 2, 10, or 40 ppm of scoparone standard and held in the dark for 30 min. Then, fortified samples were extracted and analyzed as above. Recovery values ± SE, obtained as means of at least five measurements for each concentration, were 0, 96 (±4.5), 107 (±2.5), and 98% (±5.4), respectively.

**Induction of Scoparone as a Function of SC Concentration.** At the beginning of March, mandarin fruits cv. Fairchild were harvested, divided into four groups (each having three replications of 10 fruits), disinfected, and wounded, as described. According to grouping, after 1 h, fruits were treated with 2, 3, 4, or 5% SC solutions for 2 min. Treated fruits were then transferred to boxes stored at 20 °C and 90% RH for 5 days, when wounds were removed, extracted, and analyzed by GC-MS for scoparone content.

**Data Analysis.** Analysis of variance (ANOVA) of all data was performed using the MSTAT-C software (Michigan State University, East Lansing, 1995), and when appropriate, mean separations were performed according to the Duncan's multiple range test at $P \leq 0.05$ or 0.01. Angular transformations of decay percentage values were performed prior to statistical analysis.

**Chemicals.** Diethyl ether [31690] and ethyl acetate [45760] were obtained from Fluka Chemie, AG (CH-9470 Buchs, Switzerland); methanol [6018] and *n*-hexane [4367] were obtained from Merck KGaA (Darmstadt, Germany); ethanol [414632] and petroleum ether (40−60) [447832] were obtained from Carlo Erba Reagenti SpA (Rodano, MI-Italy); Sigma-Aldrich products were from Sigma-Aldrich, Inc. (St. Louis, MO); Difco products were from Difco (Detroit, MI). All chemicals were of analytical grade and used without further purification.

## RESULTS

**Effect of SC on Pathogen Growth on PDA.** No significant differences were found among the three experiments across the

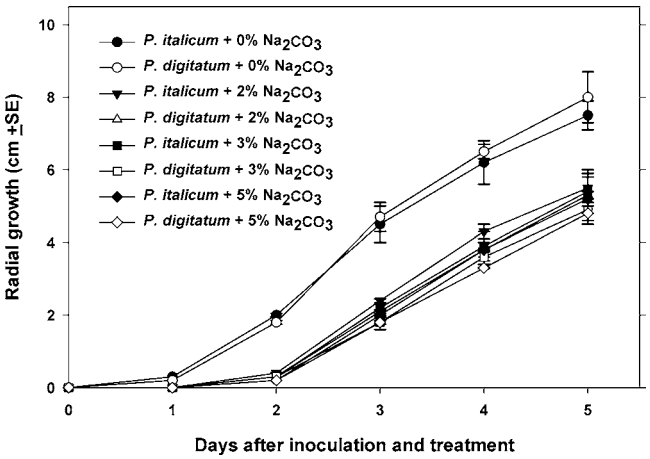


**Figure 1.** Radial growth (cm) of *P. digitatum* (white symbols) and *P. italicum* (black symbols) inoculated onto PDA as salt conidial solutions containing 0 (●, ○), 2 (▼, △), 3 (■, □), or 5% (◆, ◇) $Na_2CO_3$ and $10^5$ conidia $mL^{-1}$, cultured at 20 °C for 5 days. Each point is the mean of 30 replicates; bars indicate SE.

**Table 1.** ANOVA Table of *P. digitatum* or *P. italicum* Infection Percentage in Artificially Inoculated Fremont, Fairchild, Hamlin, Salustiana, Verna, and Marsh Fruits Treated with 0, 3, 4, or 5% SC Solutions and Stored at 20 °C and 90% RH for 5 Days

| source[a] | df | MS | F | *P* > *F* |
|---|---|---|---|---|
| experiment (A) | 1 | 19.84 | 4.59 | 0.0330 |
| species—cultivars (B) | 5 | 2097.14 | 485.35 | <0.000 |
| pathogen (C) | 1 | 4063.48 | 940.43 | <0.000 |
| concentration (D) | 3 | 51960.99 | 12025.54 | <0.000 |
| B × C | 5 | 198.93 | 46.04 | <0.000 |
| A × B × D | 15 | 374.68 | 86.72 | <0.000 |
| A × C × D | 3 | 1874.77 | 433.89 | <0.000 |
| A × B × C × D | 15 | 137.91 | 31.92 | <0.000 |
| error | | 4.23 | | |

[a] ANOVA was applied to the arcsine of the square root of the percentage of decayed fruit.

harvest period (**Figure 1**, relates to the means ± SE of all experiments). A clear delay in colonies initiation was inflicted to both pathogens by all SC solutions, while the expansion rate was similar to the control (**Figure 1**). The main effect of SC was observed during the first 48 h of incubation when no visible growth was monitored. The growth pattern and specific growth rates were similar for both pathogens (**Figure 1**).

**Inoculation by Wounding.** The efficacy of SC in reducing the development of infections was strictly dependent on concentration, on the *Citrus* sp. tested, and on the pathogen inoculated (**Table 1**). The influence of SC concentration was greater than that of *Citrus* sp. in reducing the two molds (**Table 1**). As a general rule, as SC concentration increased the infection magnitude of both pathogens decreased. The best control was attained with the highest concentration. Among the *Citrus* species investigated, a clear gap of efficacy was found between mandarins and other *Citrus* fruits. Treating mandarins with 5% SC solutions resulted in a reduction of *P. digitatum* infections by 80−87%. For the other species, the reduction was over 90%. *P. italicum* was less affected by SC treatments, and only high concentrations were effective, especially in the case of mandarin fruit.

**Inoculation by Contact.** The contact inoculation trial confirmed earlier results reported by Barmore and Brown (*11*), showing that *P. italicum* spread by contact more readily than *P. digitatum*. The spread from infected to untreated fruits was

**Table 2.** Scoparone Content and Infection Percentage of Wounded Orange Fruit Cv. Biondo Comune Treated with 0 or 5% $Na_2CO_3$ and Inoculated 0, 2, 3, 5, 7, 10, or 15 Days Posttreatment with *P. digitatum* or *P. italicum* ($10^5$ Conidia $mL^{-1}$) and Stored at 20 °C and 95% RH[a,b]

| | scoparone ($\mu g^{-1}$ FW) | | *P. digitatum* (infection %) | | *P. italicum* (infection %) | |
|---|---|---|---|---|---|---|
| inoculation time (days after treatment) | $Na_2CO_3$ (0%) (control) | $Na_2CO_3$ (5%) | $Na_2CO_3$ (0%) | $Na_2CO_3$ (5%) | $Na_2CO_3$ (0%) | $Na_2CO_3$ (5%) |
| 0 | | | 94 B | 8.5 C | 91 B | 7.3 C |
| 2 | 11.0 A | 43.5 A | 89 A | 6.0 C | 85 A | 10.0 D |
| 3 | 18.2 A | 78.0 A | 87 A | 2.8 B | 85 A | 6.1 C |
| 5 | 35.0 B | 187.3 C | 87 A | 2.0 B | 83 A | 3.3 B |
| 7 | 47.5 B | 310.0 D | 82 A | 0.0 A | 84 A | 1.0 B |
| 10 | 42.2 B | 361.7 E | 80 A | 0.0 A | 82 A | 0.0 A |
| 15 | 38.4 B | 382.0 E | 83 A | 0.0 A | 83 A | 0.0 A |

[a] In each column grouping, values followed by unlike letters are significantly different ($P \leq 0.01$) by Duncan's multiple range test; ANOVA analysis of decay percentage was performed with arcsine transformed values, and actual percentage values are reported in the table. [b] Decay percentage and scoparone content were monitored 7 days postinoculation (7, 9, 12, 14, 17, and 22 days posttreatment).

92 and 65% for *P. italicum* and *P. digitatum*, respectively. When treated fruits were subjected to contact inoculation, the percentages of infected sites were not significantly decreased at any of the concentrations tested as compared to the control.

**Posttreatment Inoculation.** Following SC application, the decay reduction was evident at all inoculation times (**Table 2**). Three days after SC application, the infection percentage was reduced by 97.2 and 93.9% for *P. digitatum* and *P. italicum*, respectively. A complete inhibition was obtained when inoculation took place 7 or 15 days posttreatment. The wounds treated with DW (control) showed a tendency toward reduced decay percentages after 2 days (**Table 2**), from when no differences were found and the decay percentage ranged between 80 and 87% for both pathogens. In all wounds treated with SC, the accumulation of scoparone increased with time and was considerably great between the 5th and the 7th day posttreatment (**Table 2**). Following this substantial increase, no decay occurred by either mold and scoparone accumulation was 382 $\mu g\ g^{-1}$ fresh weight (FW) after 15 days. In wounds treated with DW, a feeble accumulation occurred with a peak of 47.5 $\mu g\ g^{-1}$FW at the 7th day.

**pH of Albedo Following SC Treatment.** The albedo of orange fruit cv. Biondo comune, grapefruit hybrid cv. Orblanco, lemon cv. Verna, and mandarin cv. Clementine when treated with DW for 2 min had a pH (±SE) of 5.97 (±0.20), 6.20 (±0.10), 5.35 (±0.35), and 5.79 (±0.12), respectively. Following SC treatment, these values increased significantly to 9.58 (±0.50), 10.09 (±0.50), 8.95 (±0.10), and 8.90 (±0.30), respectively. The pH of the SC-treated tissue did not change significantly up to 6 days posttreatment.

**SEM Observation and Sodium Localization.** SEM observations of SC-treated samples showed clear changes of the albedo within the wounds, this being more evident in orange, lemon, and grapefruit than in mandarin. **Figure 2a** (87×) and **b** (413×) shows mandarin cv. Fairchild treated at 20 °C with 5% SC. Albedo treated with DW is shown in panel **c** (94×). Arrows in **Figure 2a** show albedo tissue with structural "melting" and the marked area is enlarged in panel **b**. The albedo tissue that interacted with the SC changed from rough porous to flat unporous. No such modifications were found in albedo of fruit treated with DW (panel **c**). No flavedo alterations arose in any SC-treated sample. The SC treatment affected the albedo

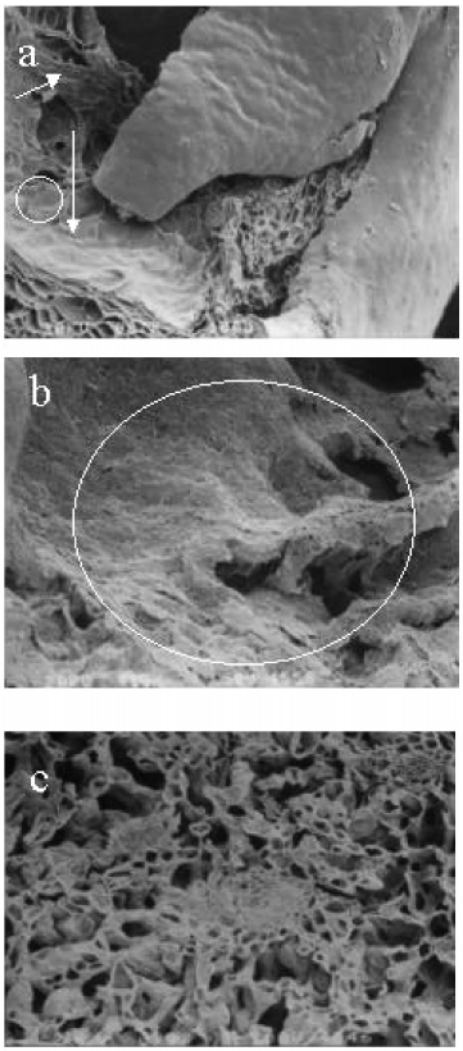


**Figure 2.** SEM image (87×) of mandarin cv. Fairchild wounded rind (flavedo and albedo) treated with 5% $Na_2CO_3$ (**a**). Arrows indicate the effect of $Na_2CO_3$ on the albedo tissue. The circled area is enlarged (**b**) (413×) and shows structural alterations. No modification is visible in the deionized water-treated albedo (**c**) (94×).

tissues of orange (**Figure 3a**,**b**; 87× and 484×, respectively), grapefruit (not shown), and lemon (not shown), more extensively than that of mandarin (**Figure 2a**,**b**). In all observations of fruit treated 24−48 h postinoculation, damaged hyphae were found (arrows in **Figure 4b**; 422×). In addition, the tissue softened by the invading pathogen was also affected by the SC treatment (**Figure 4a**; 91×). Rinsing of treated wounds did not affect the structural changes induced by the SC treatment (not shown). **Figure 5** shows SEM images of the SC-treated (panel **A**) and DW-treated (panel **B**) albedo. The corresponding SEM field emission electron localizations are shown in panels **C** and **D**. The white points in panels **C** and **D** indicate the distribution of $Na^+$. Greater amounts of $Na^+$ are present in albedo of a fruit treated with SC (panel **C**) as compared to the DW-treated one (panel **D**). Flavedo samples from SC-treated fruit (with and without posttreatment rinsing) showed low amounts of $Na^+$ as compared to the albedo (not shown). Images of the field emission electron microanalyses are of Oroblanco grapefruit hybrid, and similar results were obtained with the other *Citrus* species.

**Detection of Novel Compounds and Antifungal Activity by TLC.** TLC of the crude extracts of the studied species showed one newly formed compound when subjected to UV

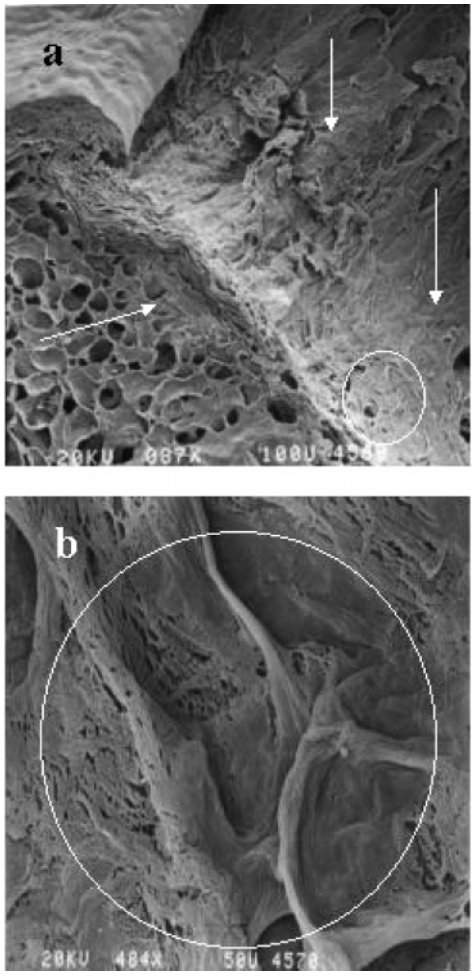


**Figure 3.** SEM image (96×) of orange cv. Biondo comune wounded rind (flavedo and albedo) treated with 5% $Na_2CO_3$ (**a**). Arrows indicate structural modifications of the albedo tissue. The circled area is enlarged (**b**) (484×), evidence of an homogeneous modification.

(366 nm) light. The $R_f$ of the compound accumulated in response to SC treatment ranged between 0.56 and 0.65. This compound was found to have antifungal properties by the TLC bioassay. TLC runs with the three standards indicated that no compounds had $R_f$ values corresponding to scopoletin or umbelliferone, while scoparone resulted having $R_f$ 0.65.

**GC-MS Identification and Quantitative Assay of Scoparone.** GC-MS analyses indicated that the active antifungal compound extracted and purified from wound SC-treated albedo was scoparone. The retention time for the internal standard was 10.10 min, while scoparone present in the extracts was detected at 9.70 min. The concentration of scoparone in the samples was calculated by comparison with a standard curve.

**Content of Scoparone in *Penicillium*-Inoculated Wounds with or without SC Treatment at 20 °C.** Following wounding and dipping in water at 20 °C for 2 min, the accumulation of scoparone in the albedo of the studied *Citrus* sp. reached detectable levels 2 days posttreatment. Patterns of scoparone accumulation were similar in the different cultivars, but the quantities detected differed. Here, we report the scoparone accumulation pattern of mandarin cv. Fairchild (**Figure 6**). The accumulation of scoparone increased in the injured albedo, and 5 days posttreatment with DW (control) or SC, the amount detected was 46 and 161 $\mu g\ g^{-1}FW$, respectively. If wounds were inoculated with sole conidia of *P. italicum* or *P. digitatum*, scoparone was 99 and 89 $\mu g\ g^{-1}FW$, respectively. When

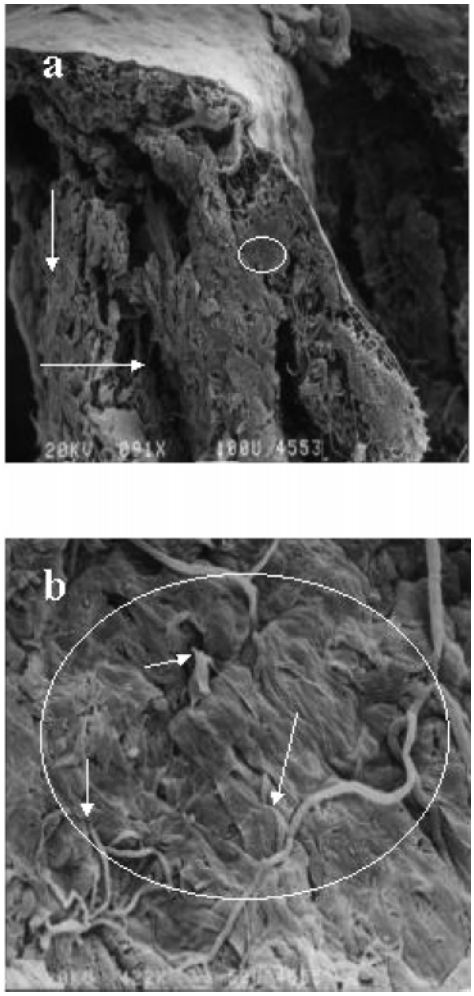


**Figure 4.** SEM image (91×) of orange cv. Biondo comune wounded rind (flavedo and albedo) inoculated with *P. digitatum* 24 h before treatment with 5% $Na_2CO_3$ (**a**). Arrows indicate structural modifications of the portion of albedo invaded by the pathogen. The circled area is enlarged (**b**) (422×), evidence of hyphae of *P. digitatum* damaged by the treatment.

inoculated as salt−conidial suspensions, there was a significant increase of scoparone, reaching 141 and 127 $\mu$g $g^{-1}$FW with *P. italicum* and *P. digitatum*, respectively. Wounding the albedo, alone, was sufficient to induce a low accumulation of scoparone that was significantly enhanced by SC treatment.

**Accumulation of Scoparone in Albedo as a Function of SC Concentration.** Samples of mandarin cv. Fairchild analyzed 5 days posttreatment showed a positive correlation between the accumulation of scoparone in the albedo and the SC concentrations (**Figure 7**). The SC dose−response of the albedo in terms of scoparone production is reported as a linear regression with a $r^2$ value of 0.926.

## DISCUSSION

Inoculation experiments were conducted using SC conidial suspensions because it was reported that spores of *P. digitatum* survived treatments of up to 5 min in 10% SC (*4*, *14*). Thus, spores are more difficult to eradicate and are likely the source of infection during long-term storage. In addition, this approach assured in all experiments the presence of the salt within the infection area and an equal contact time between conidia and SC. Because the span of SC concentration practically employable is dictated by efficacy, environmental concerns, and physiological damage (*6*), in all inoculation experiments, the maximum SC concentration used was 5% (w/v). On the basis of preliminary tests aimed at determining the pH of the PDA following the application of the salt conidial suspension (not reported data), it was found that the pH of the media at the

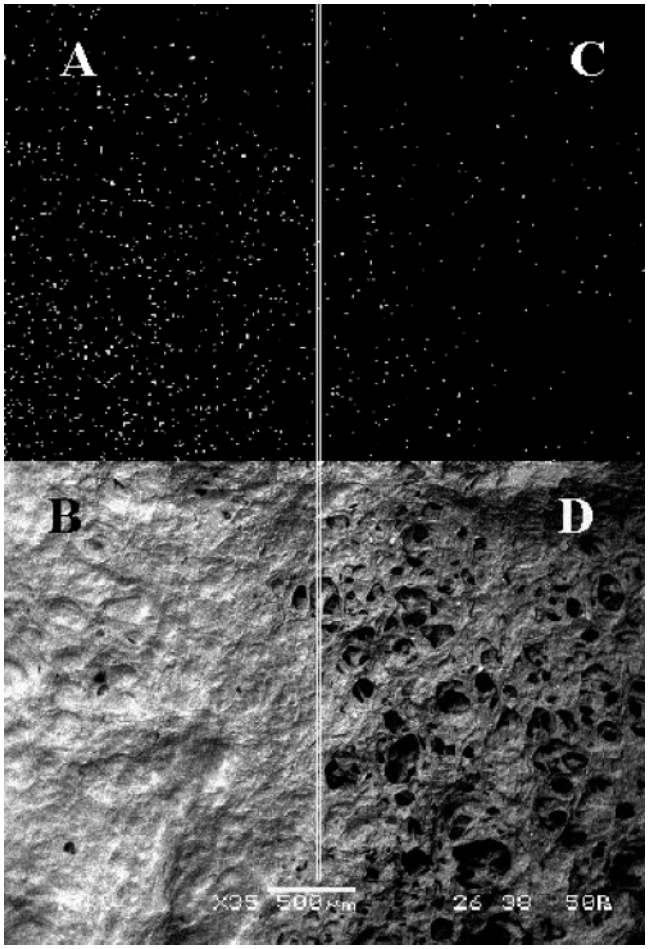


**Figure 5.** SEM images (35×) of the albedo of grapefruit hybrid Oroblanco treated with 5% $Na_2CO_3$ (**a**) or with deionized water (**b**) and its relative field emission localization of the $Na^+$ ions (white dots) (**c**,**d**), respectively.

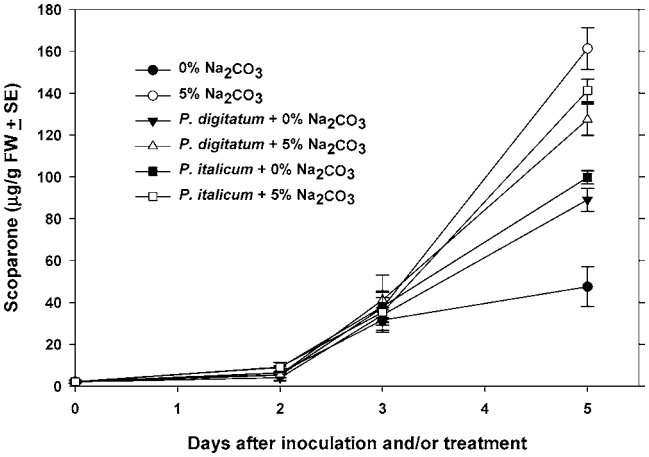


**Figure 6.** Scoparone accumulation over a 5 day period in the albedo of mandarin fruit cv. Fairchild wounded and treated with 0 or 5% $Na_2CO_3$ (● and ○, respectively), inoculated with *P. digitatum* (▼) or *P. italicum* (△) ($10^6$ $mL^{-1}$ conidia), or inoculated and treated with 5% $Na_2CO_3$ (■ and □, respectively). Fruits were stored at 20 °C and 95% RH. Each point is the mean of three replicates ± SE (bars). Each replicate was obtained by extracting 2.5 g of albedo (fresh weight) collected from 15 wounds.

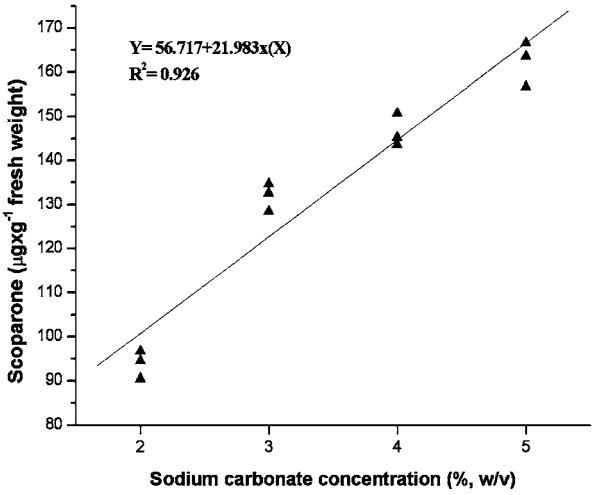


**Figure 7.** Linear regression of scoparone ($\mu$g g$^{-1}$ FW) detected in the albedo of wounded mandarin fruit cv. Fairchild 5 days posttreatment with 2, 3, 4, or 5% (w/v) $Na_2CO_3$ solutions. Each point is the mean of four replicates.

inoculation point ranged between 8.5 and 9, similar to pH values found in SC-treated albedo of the studied species. These results led to use the same SC concentrations for the in vitro growth rate experiments. The in vitro trials were designed to replicate the pH environment of the wounded sites following SC treatment, while avoiding the impact of activated host−defense mechanisms. As compared to untreated conidia, a delayed initiation of colony growth was observed for both fungi within the first 48 h following inoculation. According to Marloth (*4*), this effect should be linked to the high pH and was shown to be lost when mycelial plugs from SBC-amended medium were transferred to an unamended medium, indicating the fungistatic effect of SBC (*15*). This was also the case for the data reported in **Figure 1** where an initial growth delay affected the final radial size expansion. Recent findings of Prusky et al. (*10*) on the relationship between host acidification and virulence of *Penicillia* sp. evidenced a positive correlation between tissue alkalinization with $NaHCO_3$ and decay reduction in apple and citrus fruit (*10*). These findings may explain the fungistatic effect of postharvest SC treatments. Our data on the pH of SC-treated albedo from different *Citrus* sp. showed pH values between 8 and 10, out of the optimal growth range for *P. digitatum* and *P. italicum* (*4*), suggesting that acidification must occur before pathogenesis takes place. Currie and Thom (*16*) found a significant ex novo production of oxalic acid by *P. italicum* when grown in media with sucrose, while Prusky et al. (*10*) reported that *P. digitatum* mainly produced gluconic, citric, and fumaric acids. These released acids may modulate, in SC-treated fruit, the wound court pH, thus paving the way for active infection. In addition, when infection proceeds, we must take into account the acidification of the infection area by galacturonic acid-containing oligomers and monosaccharide produced after enzyme degradation of albedo pectins and shown to play a key role in contact infection (*11*). The initial delay of fungal growth (fungiststic phase) in vitro is likely to reflect the time required to acidify the alkalinized PDA layer, and once the mycelium bypasses or overgrows the alkaline area, the relative growth rate equals that for untreated conidia (**Figure 1**). Following the inhibition period, a slight but not significant difference in radial growth was found between the two fungi, *P. digitatum* showing the least increase. This is in agreement with previous reports demonstrating that *P. italicum* grows under a broader range of pH values (*4*, *14*).

The artificial inoculation trials with various *Citrus* sp. confirmed earlier findings (*5*, *6*) stating that SC treatment efficacy augmented as the concentration increased. In addition, our data provide clear evidence for a differential efficacy according to *Citrus* sp. Palou et al. (*8*) working with mandarin fruit reported that the SC treatment was rather ineffective in controlling losses, and high concentrations were required to achieve satisfactory control of *Penicillia* rots, especially for *P. italicum*. Data on the percentage of wounds infected by *P. digitatum* presented here agree with the findings of Palou et al. (*8*). When treatments were performed with 5% SC, infection was significantly lower in orange, lemon, and grapefruit as compared to mandarin. Following SC treatment, pH values of albedo were slightly different according to species. Even if small pH differences may make great differences in pathogenicity, we believe that differences in treatment efficacy between mandarin fruit and other *Citrus* species must be caused by other mechanisms in addition to tissue alkalinization. This conclusion was also reached by Homma et al. (*17*) and Palmer et al. (*18*) based solely on in vitro results and by Smilanick et al. (*6*) who reported great differences of efficacy in controlling *P. digitatum* infection in vivo between $Na_2CO_3$ and $K_2CO_3$ (both having pH 11). Most studies have considered the effects of the alkali salts only from the point of view of direct physiological and behavioral alterations of the fungi, without taking into account the complex host−pathogen interactions potentially modified by the treatment.

Albedo modification, shown by SEM, may be one of the additional factors modulating the efficacy of treatments when applied to *Citrus* sp. Mandarin fruit has, on average, a thin irregular layer of albedo, and SEM observations showed that its structural changes following SC treatment were less pronounced as compared to orange, lemon, and grapefruit. It is likely that the albedo "melting", following the SC treatment, is due to alkaline cleavage of albedo pectins, a process leading to demethylation of the methoxyl groups or by the break up of polygalacturonic chains via $\beta$-elimination reactions (*19*). Studying the composition of albedo, Liu Yan et al. (*20*) found significant differences in pectin and galacturonic acid content among *Citrus* sp. Thus, differences in structural changes suggested by SEM of mandarin and other *Citrus* sp. may be indicative of changes in albedo structural constituents. The weak, as demonstrated by the thickness of modified tissue, and irregular mandarin albedo tissue may result in a less effective mechanical and pH-modified barrier, more easily invaded by fungi. The direct damage to hyphae reported by Fallik et al. (*21*) was observed only when alkaline treatment was performed 24−48 h postinoculation (shown in **Figure 4**) and was more evident for *P. digitatum* than *P. italicum*. Additionally, it is clear from **Figure 4** that the SC interacts also with the enzyme-cleaved tissue, likely nullifying the acidification that may be caused by the accumulation of pectin polymer breakdown products. When treatment was performed before or together with inoculation, no such damaged hyphae were found. The effect of postinoculation treatment was more evident in orange, grapefruit, and lemon fruit, where the damaged albedo traps in several conidia and hyphae, significantly decreasing the inoculum potential. Whether conidia are killed or not by this structural alteration is not clear; however, hyphae were clearly damaged or killed, as reported earlier (*21*). The increase of efficacy obtained by Smilanick et al. (*6*) when SC treatment was followed by imazalil application (1000 $\mu$g mL$^{-1}$) may be related to

improved uptake of the active compound in the "melted" albedo, as suggested by the results of Schirra et al. (*22*) and Cabras et al. (*23*) when pesticides were applied as hot water solutions.

Quantification of $Na^+$ by SEM field emission electron microanalyses of wounded SC-dipped fruit showed that high concentrations of $Na^+$ were present only in the wounded albedo area, while insignificant amounts were found on the rind, especially after low or high pressure water rinsing (data not shown). The loss of efficacy due to the high pressure water rinsing reported by Smilanick et al. (*6*) may be caused by the removal from the wound site of resistance elicitor molecules (*24*, *25*) formed after the alkaline pH impact is initiated or to a decrease of pH that prevents SC's elicitation of defenses (rather than to the removal of SC). This latter statement is based on the findings that considerable amounts of $Na^+$ are still present in the wounds after heavy rinsing, whereas scoparone accumulation in the same wounds was negligible (data not shown). The contact inoculation trial with SC-treated, unrinsed fruit that retained a salt film on the surface (flavedo) did not indicate prevention of the spread of both fungi, supporting the idea that, in addition to the pH effect, the interaction between SC and albedo is fundamental for gaining control of the invading fungi. Rodov et al. (*26*) reported on the elicitation of phytoalexins (scoparone and scopoletin) in citrus mesocarp (albedo) and demonstrated their involvement in disease resistance. The results reported in **Figures 6** and **7** show that SC treatment induces scoparone accumulation in the albedo and, from the posttreatment inoculation experiment (**Table 2**), that there is a positive correlation between scoparone accumulation and treatment efficacy. No scopoletin was detected in the albedo in either of our experiments. Infection was completely contained only when scoparone levels were high (starting 7 days post-SC-treatment) notwithstanding that high $Na^+$ levels were detected in the wounds at all inoculation times by SEM field emission electron microanalyses. Still, it is not clear why in vivo such high levels of scoparone are required to control decay since in vitro (*27*) the $ED_{50}$ values for *P. digitatum* and *P. italicum* are 64 and 60 $\mu$g $mL^{-1}$, respectively.

The in vivo inoculation experiments on citrus fruit reported previously (*5*, *8*) involved treating 24 h postinoculated wounds, a time when many conidia have germinated. Under these conditions, which are similar to those found in packinghouses, the reduction of decay following SC treatment is probably caused by SC's direct killing effect on germlings, as observed by SEM and reported by other authors (*21*) and to suppression of the activity of lytic enzymes secreted during pathogenesis (*28*). The build-up of natural resistance, induced by the SC treatment, at the albedo level is crucial for restraining late-germinating conidia, as shown by the in vivo experiment. The alkalinization and the structural changes of the albedo followed by the induction of scoparone may account for the differences between the predicted and the actual efficacies of soda ash reported by Smilanick et al. (*5*). The correlation found between the SC concentrations and the accumulation of scoparone may explain the increasing efficacy reported by Smilanick et al. (*5*) and Palou et al. (*8*) when the SC concentration was increased. In addition, Palou et al. (*8*) reported that the efficacy of the SC treatment decreased when fruit susceptibility to decay was high (indicated by higher decay levels measured for control fruit) suggesting that under those conditions the natural defense responses may have been weaker. Studies are under way to correlate *P. italicum* and *P. digitatum* acidification capability with the differential efficacy of the SC treatment and to explain why SC and SBC have similar efficacies in vivo.

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