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Residue Levels and Efficacy of Fludioxonil and Thiabendazole in Controlling Postharvest Green Mold Decay in Citrus Fruit When Applied in Combination with Sodium Bicarbonate

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ABSTRACT: The curative and protective activity of sodium bicarbonate (SBC) at 1% alone or in combination with fludioxonil (FLU), thiabendazole (TBZ), or FLU and TBZ together, between 50 and 600 mg/L, was evaluated on fruit of different citrus species and cultivars. Fruits were either artificially inoculated with a resistant (TBZ-r) or sensitive (TBZ-s) strain of *Penicillium digitatum* or not inoculated and incubated at 20 °C and 90% relative humidity (RH) for 7 days (incubated fruit) or stored at 1.5 °C for 21 days plus 7 days of simulated marketing conditions at 20 °C and 60% RH. The effectiveness of these treatments was related with treatment-induced changes of epicuticular wax morphology, the mode of distribution of SBC, TBZ, and FLU on the fruit surface, and FLU and TBZ fruit residue levels. SBC alone showed a weak activity against both strains of *P. digitatum*. Both TBZ and FLU were very effective at all rates used, and their activity markedly increased when combined together or with SBC, even at concentrations of 50−150 mg/L. Fruit treated with SBC either alone or in combination with TBZ and/or FLU increased weight loss, although no treatment damage was detected. Residue levels of TBZ generally increased when TBZ was combined with FLU and/or SBC, whereas those of FLU slightly increased only in treatment where FLU was combined with TBZ and SBC. Initial residues of TBZ and FLU when applied at 600 mg/L were around 2 and 1 mg/kg, respectively, several times below the lowest MRLs set by most important citrus-producing countries. In treatments with SBC or SBC plus TBZ or FLU, SEM observation of the fruit surface showed a smoothing of cuticular wax platelets' surface, whereas ESEM micrographs showed irregular spots of salt deposits of roundish to irregular shape. The apparently uneven distribution of SBC or SBC plus TBZ or FLU or SBC plus TBZ and FLU on fruit might in part reduce the potential beneficial effects of SBC or of fungicide−SBC mixtures.

KEYWORDS: *Penicillium decay, citrus fruit, scanning electron microscopy, environmental scanning electron microscopy, storage*

INTRODUCTION

Green mold and blue mold caused by *Penicillium digitatum* and *Penicillium italicum* Pers.: Fr.) Sacc., respectively, are common postharvest rot diseases of citrus fruit worldwide and can result in severe economic losses during transport, storage, and marketing.[1] Wounds of the peel are the main ways for *Penicillium* spp. to infect citrus fruit. Thus, careful handling during harvesting and packinghouse operations is strongly recommended to reduce the risk of infection. For many years, sodium *o*-phenylphenate (SOPP), thiabendazole (TBZ), and imazalil (IMZ) have been applied for postharvest control of the main pathogens of citrus fruit in various citrus-producing countries.[2,3] However, treatment efficacy of these fungicides is often challenged by the buildup of SOPP-, TBZ-, and IMZ-resistant pathogen populations. In recent years, novel effective broad-spectrum fungicides have been registered in the United States for postharvest decay management of various horticultural crops, including citrus fruits.[4] Among them, the phenylpyrrole fludioxonil (FLU) is under registration in the European Union (EU) to control postharvest disease of citrus fruits. FLU, developed in the mid-1990s to control *Botrytis cinerea* in viticulture,[5] is also active against other fungi such as *Monilinia* spp., *Sclerotinia* spp.,[6] *Penicillium expansum*,[7] *Lasiodiplodia theobromae*,[8] *Dothiorella dominicana* and *Colletotrichum gloeosporioides*,[9] and *P. digitatum*, including isolates of the pathogens that are resistant to IMZ or TBZ.[10] FLU inhibits spore germination, germ tube elongation, and mycelium growth of *B. cinerea* and induces morphological alterations of germ tubes.[5,11] The genetic and biochemical mechanism of FLU resistance in field isolates of *P. digitatum* was investigated.[12]

The in vitro selection of FLU-insensitive mutants has been described in different fungi, such as *Aspergillus nidulans* and *B. cinerea*,[13] and field isolates of *Alternaria* spp. exhibiting high levels of resistance to both dicarboximide and phenylpyrrole fungicides were recently reported.[14] Field resistance of *Alternaria* spp. and *P. digitatum* of lemons to FLU has been reported.[14,15]

To reduce the risk of selection of resistant isolates, it is highly recommended to alternate or mix fungicides with different modes of action and maintain appropriate rates even when two or more active ingredients are combined, in that if sublethal rates of the mixtures' active ingredients are used, the selection process for isolates resistant to single fungicides may begin.[16] However, in contrast with the general worldwide tendency aimed at minimizing residues on produce, this would increase

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their levels, especially if various active ingredients would be mixed.

Numerous studies have also shown the potential application of natural products or generally recognized as safe compounds (GRAS) for controlling postharvest decay in different commodities and their advantages in optimizing the treatments' performance when used in combination with conventional or recently registered fungicides.[17] Among them, carbonate salts, which have been extensively used as sanitizing agents to reduce the level of *Penicillium* spp. and *Geotrichum candidum* Link ex Pers. inoculum,[1,18,19] combined with IMZ,[20] TBZ,[21] or pyrimethanil (PYR),[22] increased their activity to control green mold, even against isolates of *P. digitatum* resistant to IMZ or TBZ. However, because the risk of phytotoxicity by SBC at concentrations of 3−4% is high, at commercial level fungicides are not applied in combination with SBC, but after fruits are dipped in SBC solutions and subsequently rinsed with fresh water. An approach scarcely investigated is the combination of synthetic fungicides with reduced concentrations of SBC followed by no rinse with fresh water. This strategy could increase the protective activity of the treatments, thanks to the residues of SBC, while minimizing the risk of phytotoxicity. Indeed, Kanetis et al.[15] tested the effect of combined treatments of FLU with SBC in laboratory studies as well as in experimental packing line, but SBC was used at 3%.

Factors affecting SBC phytotoxicity, besides its own intrinsic caustic properties, are not well understood. An uneven distribution on the fruit surface that would lead to a localized accumulation of the salt might be an important cause of phytotoxicity. Research dealing with the distribution of carbonate salts on fruit surface is poor. Scanning electron microscope (SEM) examination of fruit surface treated with SBC may explain many aspects of the influence of carbonate salts on epicuticular wax, but gives little information on salts distribution on the fruit surface. The chemical fixation required to prepare the sample and the following operations of dehydration and coverage with electrically conductive materials may in part remove or alter the original pattern of salt deposit, even if the structure of the cuticular wax is essentially not altered.[23] Environmental scanning electron microscopy (ESEM), which allows a direct examination of fresh tissues as they are, without modifying the natural surface or creating artifacts by sample preparation,[24] may be a useful means to get new knowledge on deposits coverage of salt or fungicide mixtures on fruit surface.

In Italy, IMZ, TBZ, and SOPP fungicides are still effective in controlling *Penicillium* decay of citrus fruit, in part because no fungicide, active against *Penicillium* decay, is registered for field applications and because most of the fruit destined to the fresh national market are not subjected to postharvest treatments. Thus, the introduction of new active ingredients should not lead to the replacements of TBZ, IMZ, or SOPP but rather to an integration of the new fungicides with the older ones and with means other than synthetic fungicides in the perspective of an integrated management of postharvest diseases that, while optimizing the use of all available means, satisfies the requirements of different markets.

The present study was undertaken to (a) evaluate the performance of FLU, TBZ, and SBC alone or in combination in controlling postharvest green mold decay of citrus fruit; (b) evaluate the residue levels of FLU and TBZ as affected by combined treatments with themselves and/or SBC; (c) gain new information on fungicide distribution on the fruit surface by ESEM observation; and (d) investigate the changes of epicuticular wax morphology induced by treatments with SBC, TBZ, and FLU on the fruit surface applied alone or in combination.

MATERIALS AND METHODS

Fruit. Commercially mature oranges [*Citrus sinensis* (L.) Osbek cv. 'Washington' navel (3080 fruits) and 'Salustiana' (30 fruits)], lemons [*Citrus limon* (L.) Burm cv. 'Verna' (1608 fruits), 'Lisbon' (2840 fruits), and 'Femminello siracusano' (4800 fruits)], clementines [*Citrus reticulata* Blanco cv. 'Hernandina' (4200 fruits)], and fruit of 'Fortune' mandarin (60 fruits), a hybrid of 'Clementine' mandarin [*Citrus clementina* Hort (ex Tanaka)] and 'Dancy' mandarins (*C. reticulata* Blanco), were hand-harvested from the experimental orchard of the Institute of Sciences of Food Production, located in central-western Sardinia (latitude 39° 55′ 31″ N, longitude 8° 35′ 43″ E, Italy). The trees received standard horticultural practices. Fruits were placed in plastic trays and delivered to the laboratory the same day of harvest. Then, medium-size fruit free from rind defects were selected at random and left overnight at 20 °C.

Fungicides. The following fungicides were used in this study: TBZ [TECTO SC, 42.9%, active ingredient (ai), Decco Italia, Piano Tavola Belpasso, Catania, Italy]; FLU (Scholar, 20.4% ai, Syngenta Crop Protection S.p.A., Milan, Italy); and SBC (99.7% ai, Carlo Erba, Milan, Italy).

Total Epicuticular Wax Content. On the basis of the method of McDonald et al.,[25] wax extraction was carried out with chloroform from 4 replicates of 8 fruits each. 'Femminello siracusano' lemons were immersed and shaken for 2 min in two successive beakers, each containing 500 mL of solvent at ambient temperature (24 °C). The combined extracts were filtered and reduced to dryness on a rotary evaporator under reduced pressure. The total epicuticular wax content was determined gravimetrically and expressed as micrograms total wax per square centimeter of fruit surface area, which was calculated according to Turrell tables.[26]

Influence of SBC on the pH of Water and Water Mixtures Containing FLU or TBZ. The measurements of the pH of tap water and water mixture containing FLU or TBZ at 300 mg/L without or with SBC at 1% (w/v) were performed after 0 (freshly prepared mixtures), 3, 6, and 24 h. For each treatments the pH measurements were determined with a pH-meter (Orion, model 420A, Milan, Italy).

Fungal Strains and Growth Conditions. A single-spored isolate of TBZ-sensitive (TBZ-s) *P. digitatum*, collected from decayed oranges cv. 'Tarocco' harvested in an orchard located in central-western Sardinia, Italy (collection of Q. Migheli, University of Sassari, Italy), and a TBZ-resistant (TBZ-r) isolate of *P. digitatum* (MIC = 10−20 μg/mL), coded PD04-99 [collection of J. L. Smilanick, U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS), Parlier, CA], were cultured on potato dextrose agar (PDA, 39 g/L in distilled water, autoclaved; Merck and Co., Whitehouse Station, NY, USA), amended with streptomycin sulfate and oxytetracycline hydrochloride (100 + 100 μg/mL) to prevent growth of bacterial contaminants. A conidial suspension (1×10^8 conidia/mL) of *P. digitatum* was prepared as follows: fungal isolates were grown in Petri plates (90 mm in diameter) containing 15 mL of PDA under constant fluorescent light; after 2 weeks of incubation at 25 °C, spores were collected by scraping the colony surface with a sterile scalpel, resuspended in sterile Ringer's solution (NaCl, 8.6 g/L; KCl, 0.3 g/L; $CaCl_2$, 0.48 g/L), filtered through two layers of sterile cheesecloth, and counted with a hemocytometer.

Assays with Wounded and Artificially Inoculated Fruit. 'Washington' navel oranges, 'Lisbon' and 'Femminello siracusano' lemons, and 'Hernandina' clementines were used to determine the influence of treatments against TBZ-s and TBZ-r isolates of *P. digitatum*. Before inoculation, fruits were divided into 14 treatment groups, superficially disinfected by immersion for 30 s in household bleach (1 g/L sodium hypochlorite), rinsed with fresh water, and allowed to dry at ambient temperature. Then, each fruit was punctured twice equatorially on opposite sites with a 2 mm wide, 2 mm long

stainless steel rod and inoculated by dipping for 1 min into a 72 L high-density polyethylene tank that contained 50 L of conidial suspension of each pathogen (final concentration of 1×10^5 conidia/mL).

After inoculation, fruits were incubated at 20 °C and 90−95% relative humidity (RH) for 24 h before treatment to simulate commercial conditions, where the infection may occur at harvest in the field or during transit before treatment. For 'Washington' navel oranges, 'Lisbon' lemons, and 'Hernandina' clementines, treatments were performed by immersing the fruit for 1 min in (a) water or water mixtures at 20 °C containing SBC at 1%, TBZ at 100, 300, or 600 mg/L, and FLU at 100, 300, or 600 mg/L; (b) TBZ at 50, 150, or 300 mg/L in combination with FLU at 50, 150, or 300 mg/L, respectively, and TBZ or FLU at 100 mg/L in combination with SBC at 1%; or (c) TBZ at 50 mg/L, in combination with FLU at 50 mg/L and SBC at 1%.

'Femminello siracusano' lemons were dipped for 1 min in (a) water or water mixtures at 20 °C containing SBC at 1%, TBZ at 100 or 600 mg/L, and FLU at 100 or 600 mg/L; (b) TBZ at 100 or 600 mg/L in combination with FLU at 100 or 300 mg/L, respectively; or (c) TBZ or FLU at 100 or 300 mg/L in combination with SBC at 1% or TBZ at 100 or 300 mg/L in combination with FLU at 100 or 300 mg/L and SBC at 1%.

Treatments were performed by dipping the fruit in a bath fitted with 3.96 kW/h heating elements, an electronic recirculation pump (22 L/min water flow), and an electronic thermostat (OEM/HT, Carel, France).[27] Following treatments, fruits were left to dry at room temperature and subsequently stored at 20 °C and ca. 90−95% RH. Fruits were evaluated for decay incidence caused by green mold (percentage of fruit with decay lesions of any size compared to the total, within each replicate) after 7 days. Four replicates were used for each treatment. Each replicate contained 20 ('Washington' navel oranges and 'Lisbon' lemons) or 25 fruits ('Hernandina' clementines and 'Femminello siracusano' lemons).

Storage Response to Postharvest Treatments. Fruits of all cultivars were subjected to 1 min dip treatments with water or water mixtures containing SBC, TBZ, FLU, or their combination as described above. Each treatment was applied to 3 replicates of 20 fruits each for 'Washington' navel oranges and 'Verna' lemons and 4 replicates of 25 fruits for clementines.

After treatments, fruits were left to dry at room temperature for approximately 5 h, moved to a ventilated room, and kept for 21 days at 1.5 °C (cold quarantine conditions) followed by a subsequent 7 day period of simulated marketing conditions at 20 °C and 60% RH.

Decay incidence was assessed as total rots caused by blue mold (*P. italicum* Wehmer), green mold (*P. digitatum* Sacc.), and brown rot [*Phytophtora citrophthora* (Smith and Smith) Leon] or as miscellaneous rots of other fungi.

Influence of Treatments and Storage Conditions on Residues of FLU and TBZ. Twelve treatment groups were used for residue analysis. Treatments with water or SBC alone were used for matrix-matched calibration curves. 'Verna' lemons were used to determine the influence of the treatments on residues of TBZ or FLU. After treatments, fruits were stored at 17 °C and 90% RH (simulated shelf life conditions). Residue analyses were performed at time 0 (immediately after treatment) and after 12 days. All analyses were performed on four replicates per treatment.

Chemicals. Acetone and hexane were of GC grade (Merck, Milan, Italy). NaCl was of analytical grade (Carlo Erba, Milan, Italy). The analytical standards of TBZ (at 95%) and FLU (at 95%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock standard solutions of the active ingredient (500 mg/L) were prepared in acetone. Working standard solutions of ai were prepared by diluting the stock solution with the extract from untreated fruit.

Apparatus. A TQ Trace gas chromatograph, coupled with an NPD 80 detector, a split/splitless injector, and an AS200 autosampler (TermoQuest, Milan, Italy), was used. The column was a fused silica capillary DB 5 MS (30 m × 0.25 mm i.d.; film thickness = 0.10 μm, J&W Scientific, Folsom, CA, USA). The injector and detector temperatures were set at 220 and 300 °C, respectively. The sample (2 μL) was injected in splitless mode (30 s). The oven temperature was programmed as follows: 90 °C (1 min), raised to 310 °C (at 15 °C/min). He was the carrier gas and N_2 the makeup gas at 1.1 and 15 mL/min, respectively. NPD conditions were as follows: source current, 2.8; polarization voltage, 3.5. O_2 and H_2 flows were at 60 and 2.3 mL/min, respectively.

Extraction Procedure and Residue Analysis. Six lemons per replication were weighed and their peels removed. The peel was weighed and its percentage with respect to the whole fruit calculated. Then, peel samples were kept frozen at −40 °C until analysis. Five grams of minced and homogenized peel was weighed in a 40 mL screw-cap flask, and 10 mL of acetone/hexane mixture (1:1, v/v) and 4 g of NaCl were added. For recovery assay samples of untreated lemon peel were fortified with appropriate amounts of standard stock solutions to reach concentrations from 0.1 to 10 mg/kg. The samples were allowed to settle for 30 min prior to extraction and then processed according to the above procedure. The average recovery from four replicates showed values of TBZ ranging between 85 and 92% with a maximum coefficient of variation of 9%, whereas FLU ranged between 90 and 105% with a maximum coefficient of variation of 15%.

Environmental Scanning Electron Microscopy Observations. ESEM studies were performed on 'Salustiana' oranges following dip treatments for 60 s in water or water mixtures at 20 °C containing SBC at 1%, TBZ or FLU at 300 mg/L, and TBZ or FLU at 300 mg/L in combination with SBC at 1%. From each fruit two rind samples excised from the equatorial zone were stuck on aluminum stubs using ethyl cyanoacrylate-based glue (Loctite Super Attack, Henkel Italia S.p.A., Milano, Italy). ESEM analyses were performed with a Zeiss Evo LS10 environmental scanning electron microscope and energy-dispersive spectroscopy (EDS), using the following parameters: accelerating voltage, 20 kV; chamber pressure, 600 Pa. The parameters are visible in the picture bar of each image. For each treatment five fruits were used; however, images from only a single fruit are shown, because this sample most represented features seen in the others.

Scanning Electron Microscopy Analysis. SEM studies were performed on 'Fortune' mandarins following treatments (dip for 60 s in water or water mixtures at 20 °C containing SBC at 1%, TBZ or FLU at 300 mg/L, and TBZ or FLU at 300 mg/L in combination with SBC at 1%, respectively) or after 1 week at 20 °C and 60% RH. SEM studies were carried out with a Zeiss DSM 962 microscope at 30 kV. From each fruit two rind samples excised from the equatorial zone of each fruit were fixed in a phosphate buffer (pH 7.4) containing 3% gluteraldehyde. Before observation, fixed tissues were rinsed three times with phosphate buffer (pH 7.4), dried by washing with increasing concentration of ethanol, stuck on aluminum stubs, and coated with gold−palladium.[27] Samples from five fruits were examined during each evaluation; however, only images from a single fruit are shown, because this sample most represented features seen in the others.

Statistical Analysis. Statistical analysis was performed using Statgraphics software (Manugistics, version 5 Professional, 2000) statistical program. Analyses of variance (one-way ANOVA) and covariance (only for fruit weight loss) were conducted using a unifactorial complete randomized block design. For decay incidence, the percentages were not transformed or were transformed in $\arcsin\sqrt{x}$ or $\sqrt{x}$ before the ANOVA, depending on the range of variation of data prior to analysis, but no transformed values are given. Means comparisons of the effects of treatments were calculated by the Duncan's multiple-range test at $P \leq 0.05$. Limpel's formula, as described by Richter,[28] was used to determine synergistic interactions between SBC and TBZ mixtures. Limpel's formula is $E_e = X + Y - (XY/100)$, in which E_e is the expected effect from additive responses of two treatments and X and Y are the percentages of decay reduction relative to each agent used alone. Thus, if the combination of the two agents produces any value of decay reduction greater than E_e, then synergism exists.

RESULTS

Wax Content. The total wax extracted from lemons was unaffected by treatments (data not shown). Pooled data of wax content averaged 134.4 ± 20.8 $\mu g/cm^2$.

Influence of the Addition of SBC on the pH of Water and Water Mixture Containing FLU or TBZ. Water pH alone was 7.42; immediately after the addition of SBC to water, the value changed to 8.18, and similar values were detected in the mixture containing FLU or TBZ at 300 mg/L plus SBC (Table 1). The pH of water solutions of FLU or TBZ alone was similar to water pH. In all of the samples with SBC alone or in combination, the mean values of pH gradually but moderately increased over the 24 h monitoring (Table 1), whereas samples with TBZ and FLU alone did not show any changes during the monitoring period.

Table 1. Changes in pH of Water (Tap Water) or Water Mixtures Containing SBC at 1% (w/v), TBZ at 100 mg/L, TBZ plus SBC, FLU at 100 mg/L, or FLU plus SBC

	time			
solution	0 h	3 h	6 h	24 h
water	7.42			
1% SBC	8.18	8.24	8.30	8.48
100 mg/L TBZ	7.36	7.47	7.49	7.51
100 mg/L TBZ + 1% SBC	8.18	8.23	8.28	8.45
100 mg/L FLU	7.23	7.42	7.43	7.43
100 mg/L FLU + 1% SBC	8.16	8.22	8.30	8.45

Assays with Wounded and Artificially Inoculated Fruit. Postharvest treatment with SBC alone significantly ($P < 0.05$) but moderately reduced the incidence of green mold decay caused by both isolates (TBZ-s and TBZ-r) of *P. digitatum* in 'Lisbon' lemons and 'Washington' navel oranges (Table 2). TBZ applied at 100 mg/L to lemons effectively reduced (81% control) green mold caused by the TBZ-s isolate but had little (20.5% control), albeit significant ($P < 0.05$), effect in controlling decay incidence caused by the TBZ-r isolate. Superior control of decay was achieved when the concentration of TBZ was raised to 300 mg/L, whereas the efficacy of TBZ against both isolates (TBZ-s and TBZ-r) did not increase further when it was applied at 600 mg/L. However, when TBZ was applied to lemons at reduced rate (100 mg/L) in the presence of SBC, its efficacy against both isolates of *P. digitatum* was superior to that of TBZ at 600 mg/L.

When applied to oranges, concentrations of 100 and 300 mg/L TBZ were similar in efficacy against the TBZ-s isolate, but significantly improved at 600 mg/L. TBZ applied to oranges at 100 mg/L was not effective in controlling the TBZ-r isolate (100% decay incidence). The effectiveness of TBZ significantly but moderately improved when it was applied at 300−600 mg/L. Treatments carried out on clementines showed a similar behavior of the three TBZ treatments against the TBZ-s isolate even if they were less effective than the same treatments applied on lemons and oranges; moreover, they showed a reduced effect in controlling decay incidence caused by the TBZ-r isolate with only a small increase with treatments at 300 and 600 mg/L. By contrast, the combination of TBZ at 100 mg/L and SBC dramatically improved the efficacy of TBZ in controlling the TBZ-r isolate in all samples.

According to Limpel's formula, the combination of TBZ at 100 mg/L with SBC produced positive synergistic effects in controlling decay caused by the TBZ-r isolate as well as the TBZ-s isolate.

When equal concentrations of active ingredient were applied, FLU was more effective than TBZ in controlling the TBZ-s isolate in lemons and clementines and similarly effective in oranges. FLU treatments applied to lemons, oranges, and clementines provided excellent curative control against green

Table 2. Incidence of Postharvest Green Mold Caused by *Penicillium digitatum* on 'Lisbon' Lemons, 'Washington' Navel Oranges, and 'Hernandina' Clementines After They Were Dipped for 1 min in SBC, TBZ, or FLU, Alone or in Combination, Followed by Storage at 20 °C for 7 Days[a]

	green mold[b] (%)					
	lemons		oranges		clementines	
treatment[c]	TBZ-s	TBZ-r	TBZ-s	TBZ-r	TBZ-s	TBZ-r
water (control)	100 a	100 a	95.0 a	100 a	100 a	100 a
1% SBC	78.0 b	71.0 b	73.3 b	88.3 b	83 b	85 b
100 mg/L TBZ	19.0 c	79.5 b	41.7 c	100 a	21 cd	75 c
300 mg/L TBZ	7.0 d	63.5 c	30.0 cd	86.7 b	21 cd	68 cd
600 mg/L TBZ	9.5 d	45.5 c	13.3 ef	88.3 b	20 d	63 d
100 mg/L TBZ + 1% SBC	3.3 e*	14.5 d*	16.6 def*	28.3 f*	13 e*	23 e*
100 mg/L FLU	9.0 d	3.0 e	28.3 cd	35.0 ef	22 cd	22 e
300 mg/L FLU	2.5 e	1.5 ef	21.7 de	20.0 gh	18 d	13 f
600 mg/L FLU	1.5 ef	1.0 ef	11.7 ef	13.3 i	9 f	15 f
100 mg/L FLU + 1% SBC	2.7 e*	2.0 ef*	18.3 def*	16.7 i*	8 f*	10 f*
50 mg/L TBZ + 50 mg/L FLU	0.0 f	0.5 f	30.0 cd	43.3 e	26 c	27 e
50 mg/L TBZ + 50 mg/L FLU + 1% SBC	0.0 f	0.7 f	23.3 de	53.3 d	11 ef	12 f
150 mg/L TBZ + 150 mg/L FLU	0.5 ef	1.0 ef	21.7 de	26.7 fg	20 d	12 f
300 mg/L TBZ + 300 mg/L FLU	0.5 ef	0.5 f	8.3 f	18.3 hi	11 ef	12 f

[a]Two isolates of the pathogen were used: TBZ-sensitive isolate (TBZ-s) or TBZ-resistant isolate (TBZ-r). Fruit was inoculated 24 h before treatments. [b]Percentages were transformed in arcsin$\sqrt{x}$ before statistical analysis; means in columns followed by a common letter are not significantly different by Duncan's multiple-range test, $P \leq 0.05$. An asterisk (*) indicates that the combination was synergistic according to Limpel's formula. [c]Concentrations of SBC are expressed by w/v; each treatment was performed on 4 replicates of 20 fruit each for oranges and lemons and on 4 replicates of 25 fruit for clementines.

mold decay caused by the TBZ-s isolate as well as TBZ-r isolate, especially at the highest concentration. The application of Limpel's formula indicated a significant synergistic improvement in effectiveness to control green mold caused by both isolates of *P. digitatum* when FLU at 100 mg/L was applied in the presence of SBC.

In lemons and clementines, mixtures of TBZ-FLU at the rate of 50, 150, or 300 mg/L of each fungicide were more effective in controlling both (TBZ-s and TBZ-r) isolates than single treatments with TBZ or FLU at 100, 300, or 600 mg/L, respectively, whereas in oranges the TBZ-FLU mixture at 50 mg/L did not increase its activity after coapplication of SBC.

In 'Femminello siracusano' lemons treated 12 h after inoculation with the TBZ-s isolate, SBC reduced decay only by 26%, whereas all other treatments, including TBZ and FLU alone or in combination with SBC, gave almost a complete control of decay (Table 3). Moreover, in fruit inoculated with the TBZ-r isolate and treated 12 h after inoculation, FLU and SBC efficacy did not change with respect to fruit inoculated with the TBZ-s isolate. Decay control by TBZ at 100 or 600 mg/L was significant but modest, but synergistic effects were detected when TBZ was combined with SBC (Table 3).

Table 3. Incidence of Postharvest Green Mold Caused by *Penicillium digitatum* on 'Femminello siracusano' Lemons after They Were Dipped for 1 min in SBC, TBZ, or FLU, Alone or in Combination, Followed by Storage at 20 °C for 7 Days[a]

	green mold[b] (%)			
	12 h post inoculation		24 h post inoculation	
treatment[c]	TBZ-s	TBZ-r	TBZ-s	TBZ-r
water (control)	100 a	100 a	100 a	100 a
1% SBC	64 b	68 b	78 b	71 b
100 mg/L TBZ	1 c	75 b	7 d	80 b
600 mg/L TBZ	0 c	62 b	13 c	64 c
100 mg/L FLU	0 c	2 e	9 d	3 f
600 mg/L FLU	3 c	0 e	10 d	6 f
100 mg/L TBZ + 1% SBC	0 c	28 c	2 ef*	41 d*
600 mg/L TBZ + 1% SBC	0 c	15 d*	3 e*	32 e*
100 mg/L FLU + 1% SBC	1 c	0 e	1 ef*	1 f
600 mg/L FLU + 1% SBC	1 c	2 e	0 f*	1 f
100 mg/L TBZ + 100 mg/L FLU + 1% SBC	0 c	0 e	3 e	2 f
600 mg/L TBZ + 600 mg/L FLU + 1% SBC	0 c	0 e	0 ef	0 f

[a]Two isolates of the pathogen were used: TBZ-sensitive isolate (TBZ-s) or TBZ-resistant isolate (TBZ-r). Fruit was inoculated 12 or 24 h before treatments. [b]Means in columns followed by a common letter are not significantly different by Duncan's multiple-range test, $P \leq 0.05$. An asterisk (*) indicates that the combination was synergistic according to Limpel's formula. [c]Concentrations of SBC are expressed by w/v; each treatment was performed on 4 replicates of 25 fruits each.

Decay increased in all treatments as the lag time between inoculation and treatment increased to 24 h (Table 3); nevertheless, the activity of treatments in which FLU or TBZ was combined with SBC significantly improved and frequently showed synergistic effects (Table 3).

Storage Response to Postharvest Treatments. There was no decay in untreated fruit after 21 days of simulated quarantine conditions at 1.5 °C (data not shown), whereas after a subsequent 7 days of simulated marketing conditions, decay incidence (mainly caused by natural infections of *P. digitatum*) ranged from 12 to ca. 21.7%, depending on the cultivar (Table 4). Decay development was not affected by SBC application.

Table 4. Influence of Postharvest Dip Treatments with SBC, TBZ, or FLU, Alone or in Combination, on the Incidence of Decay in 'Washington' Navel Oranges, 'Verna' Lemons, and 'Hernandina' Clementines Subjected to Cold Quarantine for 21 Days at 1.5 °C and a Subsequent Simulated Marketing Period at 20 °C for 7 Days

	decay[a] (%)		
treatment[b]	lemons	oranges	clementines
water (control)	18.3 a	21.7 ab	12.0 a
1% SBC	15.0 a	23.3 a	9.0 ab
100 mg/L TBZ	8.3 b	15.0 abc	4.0 c
300 mg/L TBZ	1.7 de	11.7 cd	1.0 d
600 mg/L TBZ	0.0 e	3.3 ef	1.0 d
100 mg/L FLU	5.0 bcd	13.3 bcd	6.0 bc
300 mg/L FLU	1.7 de	6.7 de	0.0 d
600 mg/L FLU	0.0 e	1.7 fg	0.0 d
50 mg/L TBZ + 50 mg/L FLU	8.3 b	13.3 bcd	0.0 d
150 mg/L TBZ + 150 mg/L FLU	3.3 cde	8.3 cd	0.0 d
300 mg/L TBZ + 300 mg/L FLU	0.0 e	0.0 h	0.0 d
100 mg/L TBZ + 1% SBC	6.7 bc	8.3 cd	0.0 d
100 mg/L FLU + 1% SBC	3.3 abc	10.0 cd	1.0 d
50 mg/L TBZ + 50 mg/L FLU + 1% SBC	0.0 e	6.7 de	0.0 d

[a]Means in columns followed by a common letter are not significantly different by Duncan's multiple-range test, $P \leq 0.05$. [b]Concentrations of SBC are expressed by w/v; each treatment was performed on 3 replicates of 20 fruit each for oranges and lemons and on 4 replicates of 25 fruit for clementines.

TBZ and FLU at 100 mg/L provided a significantly better control of decay in lemons and clementines, but not in oranges. A significantly better decay control was achieved when TBZ or FLU was applied at higher rates (300−600 mg/L). Similar results were obtained with TBZ-FLU mixtures at rates of 150−300 mg/L. The SBC coapplication did not significantly affect the performance of TBZ, FLU, and TBZ + FLU.

No treatment damage was observed during quarantine and subsequent simulated marketing conditions, although weight losses were slightly but significantly higher in fruit treated with SBC. As a general trend, weight losses did not increase in treatments in which TBZ or FLU was used individually or in combination, but increased when applied in combination with SBC (Table 5).

Influence of Treatment and Storage Conditions on TBZ and FLU Residues. Following treatments with TBZ alone at 100, 300, or 600 mg/L or in combination with FLU at rates for each fungicide of 50, 150, or 300 mg/L, residue levels of TBZ were significantly correlated to application rates (Table 6). When equal active ingredient concentrations were used (300 mg/L), the coapplication of FLU significantly increased TBZ residues with respect to fruit treated with TBZ alone. The application of TBZ in the presence of SBC resulted in a deposition 2 times higher than TBZ alone at 100 mg/L.

The changes in TBZ residues after 12 days of fruit storage were strongly dependent on treatments, ranging between 18 and 64% of initial values (data not shown).

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Table 5. Influence of Postharvest Dip Treatments with SBC, TBZ, or FLU, Alone or in Combination, on Weight Loss of 'Washington' Navel Oranges, 'Verna' Lemons, and 'Hernandina' Clementines Subjected to Cold Quarantine for 21 Days at 1.5 °C and a Subsequent Simulated Marketing Period at 20 °C for 7 Days

treatment[b]	weight loss[a] (%)		
	lemons	oranges	clementines
water (control)	4.2 c	3.7 c	6.7 bc
1% SBC	4.7 b	4.1 b	7.0 b
100 mg/L TBZ	4.1 c	3.8 c	6.6 bc
300 mg/L TBZ	4.3 c	3.6 c	6.4 bc
600 mg/L TBZ	4.0 c	3.7 c	6.5 bc
100 mg/L FLU	4.1 c	3.5 c	7.0 b
300 mg/L FLU	4.2 c	3.9 bc	6.4 bc
600 mg/L FLU	4.1 c	3.7 c	6.0 c
50 mg/L TBZ + 50 mg/L FLU	4.4 bc	3.9 bc	6.5 bc
150 mg/L TBZ + 150 mg/L FLU	4.3 c	3.6 c	6.9 b
300 mg/L TBZ + 300 mg/L FLU	4.4 bc	3.7 c	6.6 bc
100 mg/L TBZ + 1% SBC	5.2 a	4.5 a	7.8 a
100 mg/L FLU + 1% SBC	5.0 a	4.4 a	7.7 a
50 mg/L TBZ + 50 mg/L FLU + 1% SBC	5.3 a	4.5 a	7.9 a

[a]Means in columns followed by a common letter are not significantly different by Duncan's multiple-range test, $P \leq 0.05$. [b]Concentrations of SBC are expressed by w/v; each treatment was performed on 3 replicates of 20 fruits each for oranges and lemons and on 4 replicates of 25 fruits for clementines.

Table 6. Influence of Postharvest Dip Treatments with a Water Mixture Containing TBZ, FLU, or Both (TBZ + FLU), Alone or in Combination with SBC, on TBZ and FLU Residues (on a Whole Fruit Basis) in 'Lisbon' Lemons after a 1 min Dip Treatment (Time 0) and after 12 Days of Storage at 17 °C

treatment	TBZ residues[a] (mg/kg)		FLU residues[a] (mg/kg)	
	at time 0	after 12 days	at time 0	after 12 days
100 mg/L TBZ alone or 100 mg/L FLU alone	0.20 d	0.13 e	0.18 cd	0.07 e
300 mg/L TBZ alone or 300 mg/L FLU alone	1.18 b	0.93 b	0.42 b	0.28 b
600 mg/L TBZ alone or 600 mg/L FLU alone	2.02 a	1.65 a	0.91 a	0.49 a
50 mg/L TBZ + 50 mg/L FLU	0.14 d	0.06 e	0.11 d	0.06 e
150 mg/L TBZ + 150 mg/L FLU	0.76 c	0.40 db	0.22 c	0.18 c
300 mg/L TBZ + 300 mg/L FLU	1.86 a	0.69 c	0.35 b	0.29 b
100 mg/L TBZ + 1% SBC or 100 mg/L FLU + 1% SBC	0.44 cd	0.16 e	0.19 cd	0.10 de
50 mg/L TBZ + 50 mg/L FLU + 1% SBC	0.41 d	0.23 de	0.20 cd	0.14 cd

[a]Means in columns followed by a common letter are not significantly different by Duncan's multiple range test, $P \leq 0.05$.

FLU residues showed a more homogeneous behavior, with residues strictly related to the doses of active ingredients used in the mixture and showing no influence by single combination with TBZ or SBC. In contrast, residue levels of FLU applied at 50 mg/L in simultaneous combination with TBZ and SBC were similar to those of treatments in which FLU was used at 100 mg/L (0.18−0.19 mg/kg). FLU residues recorded after treatments at 300 or 600 mg/L were ca. 2- and 5-fold higher than treatment at 100 mg/L, respectively (Table 6). After 12 days of storage, the degradation rate of FLU residues was remarkably dependent on treatment type, ranging from ca. 16 to 64% when compared to initial values.

Environmental Scanning Electron Microscopy Analysis. The surface of untreated (control) 'Salustiana' oranges appeared relatively homogeneous, without surface sculpturing, and various open stomata with ring-like caps were clearly visible on the cuticular surface (Figure 1A). The ESEM micrographs of fruit treated with FLU (Figure 1C) or TBZ (Figure 1E) alone at 300 mg/L were very similar to those of control fruit. By contrast, the surface of fruit treated with SBC, alone (Figure 1B) or in combination with FLU (Figure 1D) or TBZ (Figure 1F), revealed irregular spots of salt deposits of roundish to irregular shape.

Scanning Electron Microscopy Analysis. SEM micrographs of 'Fortune' control mandarins revealed the typical irregular and discontinuous rough structured cuticular ridges of roundish to irregular shape (Figure 2A). Treatments with FLU (Figure 2C) or TBZ (Figure 2E) alone at 300 mg/L did not cause appreciable changes in cuticular surface structure in comparison with control fruit, although details of epicuticular wax structure were less visible than control. After 1 week at 20 °C, irregular ridges and platelets showed a few changes in control fruit (Figure 2B), whereas in fruit treated with FLU (Figure 2D) or TBZ (Figure 2F), large spots of fruit surface appeared smooth or apparently without wax, as if pieces or whole wax platelets had been detached.

In contrast, treatments with SBC alone (Figure 3A) or in combination with FLU (Figure 3C) or TBZ (Figure 3E) produced appreciable changes in cuticular wax structure, by removing the ridges and the upward edges of platelet waxes and leaving the fruit surface smoother. After 1 week at 20 °C, the samples treated with SBC (Figure 3B), FLU + SBC (Figure 3D), or TBZ + SBC (Figure 3F) showed the appearance of large spots of fruit surface smooth and apparently without wax, as reported above.

DISCUSSION

Although in Italy IMZ, TBZ, and OPP fungicides are still active against *Penicillium* decay, recently a commercial formulation of pyrimethanil in combination with imazalil (Philabuster, Janssen PMP, Beerse, Belgium) was registered for use in citrus fruit, and FLU is under registration (personal communication from Syngenta Crop Protection, Milan, Italy). As new fungicides are being used, antiresistance strategies based on the combined use of new and old fungicides need to be adopted to prevent selection of resistant strains of pathogens. On the other hand, in contrast with antiresistant strategies, many buyers impose more restrictive food safety standards than those set by countries' regulations including lower "maximum residue limits" (MRLs) and the presence of residues of only one active ingredient. For these reasons in this study experiments to better understand the effectiveness of SBC mixed with TBZ and FLU were carried out. To prevent the possibility that overwhelming activity of TBZ or FLU used at higher concentrations would have masked possible interactions between them and/or SBC, treatments with very low concentrations of both TBZ and FLU were also included.

Results showed an overall good curative activity of FLU applied 24 h after inoculation with both isolates of *P. digitatum*, which increased as the applied concentrations increased. The degree of FLU effectiveness was influenced by cultivar, being

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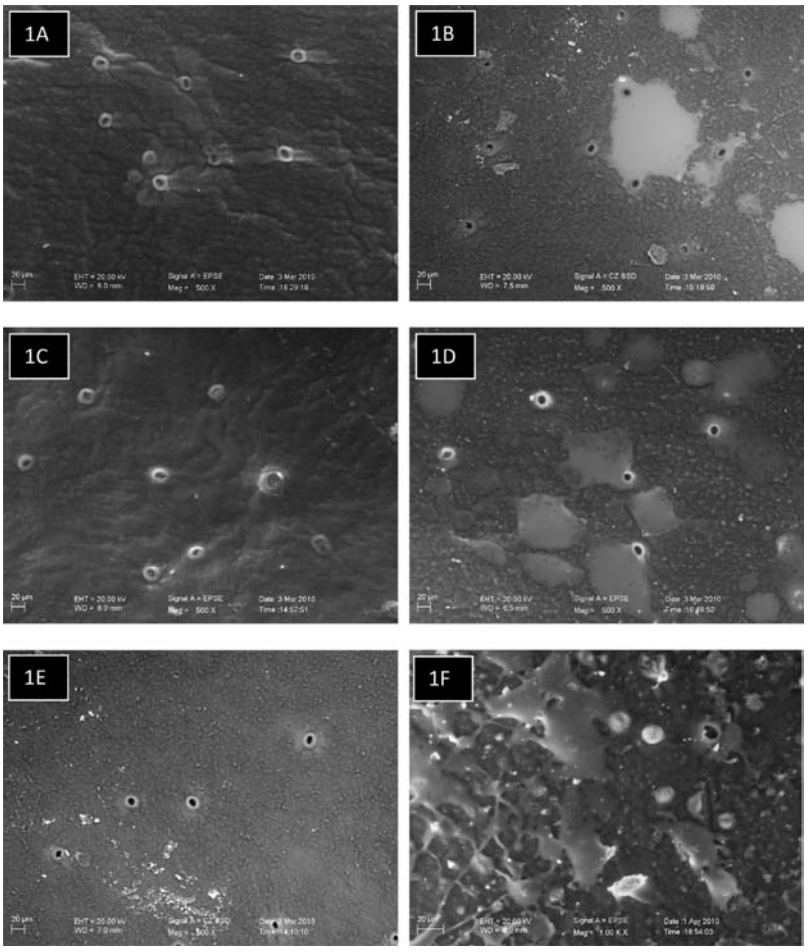

Figure 1. Environmental scanning electron microscopy micrographs of 'Salustiana' orange surface of untreated (control) fruit (A) or fruit treated with water mixtures containing sodium bicarbonate SBC (B), fludioxonil (FLU) (C), FLU + SBC (D), thiabendazole (TBZ) (E), or TBZ + SBC (F). Concentration of SBC was 1% (w/v), whereas FLU and TBZ were applied at 300 mg/L (active ingredient concentration).

higher in lemons than in oranges or clementines, but was not affected by the isolates.

Schirra et al.[27] found a high curative activity of FLU when wounded but not artificially inoculated 'Tarocco' oranges, 'Eureka' lemons, 'Tardivo di Ciaculli' mandarins, and 'Marsh seedless' grapefruits were dipped for 3 min in 400 mg/L FLU at 20 °C, but in that study infections were challenged by the natural presence of spores on fruit surfaces. In contrast, when 'Valencia' oranges artificially inoculated with a strain of *P. digitatum* sensitive to IMZ were dipped in 600 mg/L FLU at 20 °C for 30 or 60 s, the control of decay was modest.[29]

The effectiveness of a fungicide, besides its intrinsic toxicity, is also affected by host resistance and penetrability of the tissue to the chemical.[30] In this study a higher susceptibility of 'Washington' navel oranges and 'Hernandina' clementines to pathogens' growth with respect to 'Lisbon' lemons could reduce the ability of FLU to come in contact with established infections, thus reducing its effectiveness. It is likely that the 24 h lag time between inoculation and treatment was too long to allow FLU to be still highly active against established infections. This may be due to the fact that FLU as a nonsystemic fungicide is not able to move deep through the peel. Indeed, in 'Femminello siracusano' lemons treated for 12 h after inoculation with both TBZ-r and TBZ-s strains of *P. digitatum*, FLU almost completely suppressed green mold, and no significant difference was detected between treatments with 100 or 600 mg/L FLU or TBZ, either alone or in combination with each single fungicide or both in combination with SBC. These results are corroborated by findings of Kanetis et al.,[10] who reported a remarkable decreasing activity of FLU as time after inoculation increased from 9 to 21 h. In line with previous results,[8,10,29] FLU displayed the same level of activity to control disease caused by the TBZ-r and the TBZ-s *P. digitatum* isolates and confirmed that TBZ resistance does not confer cross-resistance to FLU.[29]

TBZ was slightly less effective than or equally effective as FLU against the TBZ-s strain of *P. digitatum* while showing only a weak activity against the TBZ-r strain when applied at 600 mg/L. In treatments in which TBZ and FLU were used in combination, with the concentration of each active ingredient halved with respect to treatments in which the same fungicides were used individually, disease control of both isolates was similar or better than when fungicides were used individually, revealing an additive effect of TBZ and FLU in combination. Kanetis et al.[10] found similar results, but at higher concentrations of TBZ + FLU (500 mg/L each) compared with 1000 mg/L FLU.

Carbonate salts have long been employed in the citrus industry to control various postharvest diseases since the beginning of the past century and have found renewed interest from the end of the last century when resistant strains of *P. digitatum* reduced the effectiveness of IMZ, TBZ, and SOPP.[17]

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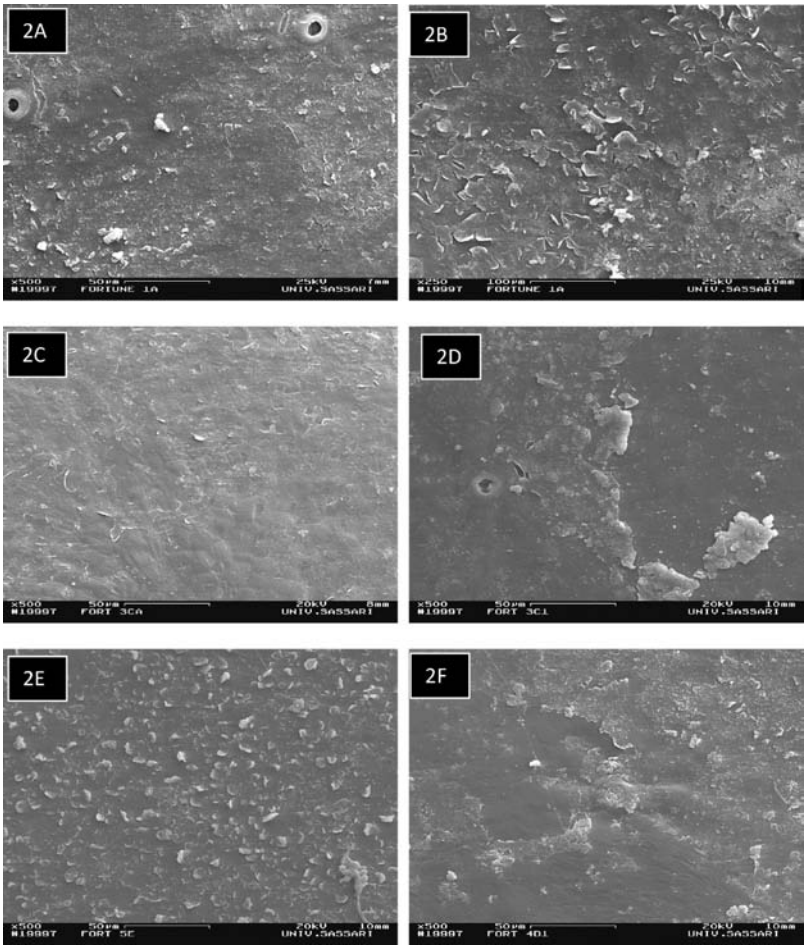

Figure 2. Scanning electron microscopy micrographs of 'Fortune' mandarin rind of untreated fruit (A, B) or fruit treated with water mixtures containing fludioxonil (FLU) (C, D) or thiabendazole (TBZ) (E, F) applied at 300 mg/L (active ingredient concentration), after the treatment (A, C, E) or after 1 week at 20 °C (B, D, F).

Carbonate salts efficacy is dependent on concentration, temperature, and duration of treatments. The best results are achieved with concentrations of 3−4% in solutions heated at 45−50 °C, but carbonate salts can cause fruit staining, rind dehydration, and excessive weight loss.[1,17] For this reason, in postharvest operations, carbonates are normally rinsed with fresh water after treatment, ensuring no residue remains on the fruit; thus, the potential protective activity of carbonate salts cannot be exploited. On the other hand, from the commercial point of view a 3−4 min treatment at a rate of 3−4% carbonate salts at 45−50 °C poses serious practical difficulties at packinghouses: these include long exposure time, cost of heating a large mass of water, and disposal of waste solutions, besides other drawbacks from specific managing procedures, which can markedly vary from one producing area to another.

For this reason, in this study the SBC concentration was reduced at 1% and used at 20 °C, both as single treatment or in combination with TBZ and/or FLU. Additionally, fruits were not rinsed after the treatment, to assess if at that rate the phytotoxicity of SBC could be reduced to the point of not negatively affecting fruit appearance. This would allow exploiting the potential protective activity of its residues either alone or in combination with TBZ and/or FLU and prevent the presence of SBC in water disposal.

Results showed a slight efficacy of SBC used alone (efficacy of 12−29%), but a marked increase in the activity of TBZ and FLU when SBC was mixed singularly with each fungicide and still more when it was combined with the two fungicides together in fruit treated 24 h after artificial inoculation. Moreover, the degree of control of disease of SBC in fruit inoculated with the TBZ-r of *P. digitatum* was very low and similar to that with TBZ, but when the two compounds were used in combination, the efficacy of the treatment was similar to that shown in fruit inoculated with the TBZ-s strain, thus resulting in a remarkably higher efficacy and synergy against the TBZ-r isolate.

Despite different studies reporting an improved performance of postharvest fungicides applied in combination with SBC, the mechanisms underlying this increased activity are still not well-known and may likely depend upon various factors. In IMZ one of the main elements underlying the synergy with SBC is the increase of pH, which strengthens the lipophilic properties of the fungicide and its penetrability through the pathogen's membranes.[21] In our study it is unlikely that the increase in pH induced by SBC could enhance lipophilic properties of TBZ or FLU. Rather, as the pH increases, TBZ solubility decreases,[21] whereas FLU has no dissociation within the range from pH 2 to 12 and its water solubility is not affected in the pH range from 4 to 10.[31] Nevertheless, the several modes of action of SBC might indirectly enhance the activity of both TBZ and FLU. Bicarbonates are known to inactivate extracellular enzymes from *Penicillium* spp. and to interact with membrane

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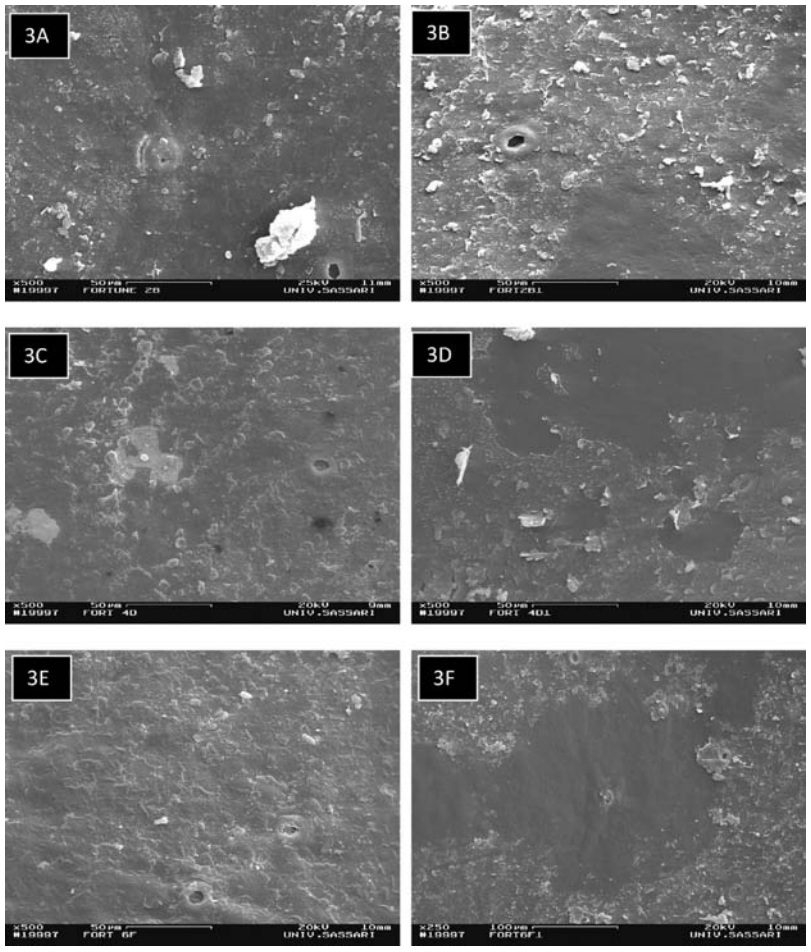

Figure 3. Scanning electron microscopy micrographs of 'Fortune' mandarin rind of fruit treated with water mixtures containing containing SBC (A, B), FLU + SBC (C, D), or TBZ + SBC (E, F) after the treatment (A, C, E) or after 1 week at 20 °C (B, D, F). Concentration of SBC was 1% (wt/vol), whereas FLU and TBZ were applied at 300 mg/L (active ingredient concentration).

permeability by reducing fungal cell turgor.[32] Venditti et al.[33] found alkalization and structural changes of the albedo and increased levels of scoparone in wounded tissue treated with sodium carbonate. Thus, even if 1% SBC was not able to effectively control decay, it might be that even a short delay of pathogens' growth may increase the activity of both fungicides, the effectiveness of which may markedly change with the growth stage of pathogens.

Residue levels of the active ingredient on the fruit surface and the way they interact with the fruit surface and cuticular wax are major factors in determining treatments' efficacy.[34] When two or more fungicides are combined in a single treatment, the residues of each may be enhanced, decreased, or not affected by the presence of the others. Residues of IMZ were significantly reduced in 'Valencia' oranges after IMZ was mixed with FLU, whereas FLU residues were not affected by the presence of IMZ.[35]

Wardowski et al.[36] found an increase in TBZ residues as the pH increased from 7.9 to 10.8. However, Schirra et al.[37] found that the addition of 0.5, 1, or 2% SBC to an aqueous solution of TBZ significantly reduced residues despite the increase in pH as compared to TBZ alone. In this study the rate of uptake of TBZ and FLU increased as their concentration of application increased from 100 to 300 mg/L, but residues of both fungicides were not affected in treatments in which the two active ingredients were used in combination. Similarly, SBC had no appreciable effect on residues of FLU in treatments in which it was combined singularly to FLU, but when it was added to the aqueous mixture of TBZ-FLU containing 50 mg/L of each active ingredient, residue levels of both fungicides were similar to or higher than treatments in which both fungicides were applied individually at 100 mg/L. Schirra et al.[37] found no enhancement of residues of TBZ when used in combination with SBC; however, the two compounds mixed in the same treatment improved the control of green mold, and SBC significantly raised the TBZ residue percentage that diffused through the cuticular wax, likely ameliorating the performance of the active ingredient. Thus, a better distribution of the active ingredient through the cuticular wax might be another factor affecting the observed synergistic effect between SBC and TBZ and/or FLU.

MRLs for both TBZ and FLU among the most important worldwide export countries vary from 5 to 10 mg/kg.[38] In this study initial residues of TBZ were 2.02 mg/kg when TBZ was used at 600 mg/L and <1 mg/kg when applied at concentrations of 150 mg/L or lower, whereas residues of FLU in all treatments were always <0.91 mg/kg.

Observation by ESEM showed that in treatments with SBC or SBC + FLU or SBC + TBZ, the distribution of the solutions on the fruit surface apparently was uneven. If this were true, wide areas of the skin might have been uncovered by the fungicidal solution and not protected against pathogen attacks.

On the other hand, SEM micrographs revealed a different framework of the surface of TBZ- and FLU-treated fruit, which showed a reduction of the platelet surface for the breakage and smoothing of the upward edges, and the surface of fruit treated with SBC either alone or in combination with TBZ or FLU, which apparently seemed to have a thin layer of wax for the lack of visible wax platelets, especially after 1 week of shelf life at 20 °C. Similar results were observed on 'Tarocco' oranges treated with TBZ.[39] The loss of detail of epicuticular wax fine structure was ascribed to selective solubilization of epicuticular wax by surfactant present in the commercial formulation of fungicide mixture.[40−42]

Further evidence of an altered functionality of cuticle and distribution of epicuticular wax on the fruit surface in fruits treated with SBC or SBC plus TBZ and/or FLU comes from the higher weight loss detected in fruit subjected to these treatments. Although considered as preliminary, our observations by ESEM indicate that fungicidal distribution on fruit surface might be uneven. As a consequence, the performance of synthetic fungicides when mixed with salts, such as carbonates, might be markedly reduced, for the increase of surface tension of the droplets of the fungicidal solution and the consequent reduction of their spreading properties on fruit surfaces.

From a practical point of view, our results are relevant for the citrus industry both in countries where TBZ is still very active and in those where resistant strains of *P. digitatum* to TBZ have developed. First, for the first time, an enhanced activity of TBZ and FLU when mixed with SBC either individually or in combination has been shown even at very low rates; second, synergism between TBZ and SBC against the TBZ-r strain of *P. digitatum* was demonstrated; and, third, these results were achieved by applying fungicidal compounds at ambient temperature and mixing SBC with TBZ and FLU, without rinsing SBC. Moreover, the high activity shown by TBZ and FLU when individually mixed with SBC could offer the possibility of rotating TBZ + SBC and FLU + SBC, which would satisfy at the same time the antiresistant principles of combining more than one active ingredient with different modes of action in the same treatment and of alternating active ingredients with different modes of action[43] while meeting increasing requirements from buyers and consumers for fresh fruit with residues of only one active ingredient. Yet, the results of this study show that even in treatments in which the highest concentrations of TBZ or FLU were used, the initial residue levels were always of different times below the MRLs set by governmental regulatory agencies of the most important citrus-producing countries.

Nevertheless, despite the improved performance of TBZ and FLU when combined with SBC, ESEM observations revealed an apparent uneven coverage of the fungicidal mixture on fruit surface. Further research aimed at testing the effect of surfactants on the complex fungicides−SBC might contribute to ameliorate the coverage of the fungicides−SBC solutions on the fruit surface and further improve treatments' performance.

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REFERENCES

(1) Eckert, J. W.; Eaks, I. L. Postharvest disorders and diseases of citrus fruit. In *The Citrus Industry*; Reuther, W., Calavan, E. C., Carman, G. E., Eds.; University of California, Division of Agricultural Science: Berkeley, CA, 1988; Vol. *5*, pp 79−260.

(2) Ismail, M.; Zhang, J. Post-harvest citrus diseases and their control. *Outlooks Pest Manage.* **2004**, *15*, 29−35.

(3) Ladaniya, M. S. *Citrus Fruit Biology, Technology and Evaluation*; Academic Press, Elsevier: San Diego, CA, 2008.

(4) Tomlin, C. *The Pesticide Manual*, 11th ed.; British Crop Protection Council: London, UK, 1997.

(5) Rosslenbroich, H. J.; Stuebler, D. *Botrytis cinerea* − history of chemical control and novel fungicides for its management. *Crop Prot.* **2000**, *19*, 557−561.

(6) Gehmann, K.; Nyfeler, R.; Leadbeater, A. J.; Nevil, D.; Sozzi, D. CGA173506: a new phenylpyrrole fungicide for broad spectrum disease control. In *Brighton Crop Conference − Pests and Diseases*; Brighton Crop Protection Conference: Thornton Heath, UK, 1990; pp 399-406.

(7) Errampalli, D. Effect of fludioxonil on germination and growth of *Penicillium expansum* and decay in apple cvs Empire and Gala. *Crop Prot.* **2004**, *23*, 811−817.

(8) Zhang, J. The potential of a new fungicide fludioxonil for stem-end rot and green mold control on Florida citrus fruit. *Postharvest Biol. Technol.* **2007**, *46*, 262−270.

(9) Swart, S. H.; Swart, G.; Labuscagne, C. Chemical control of post-harvest diseases of mango: the effect of fludioxonil and prochloraz on soft brown rot, stem-end rot and anthracnose. *Acta Hortic.* **2009**, *820*, 503−509.

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

(11) Leroux, P. Recent development in the mode of action of fungicides. *Pestic. Sci.* **1996**, *47*, 191−197.

(12) Kanetis, L.; Förster, H.; Jones, C. A.; Borkovick, K. A.; Adaskaveg, J. E. Characterization of biochemical and genetic mechanisms of fludioxonil and pyrimethanil resistance in field isolates of *Penicillium digitatum*. *Phytopathology* **2008**, *98*, 205−214.

(13) Ziogas, B. N.; Kalamarakis, A. E. Phenylpyrrole fungicides: mitotic instability in *Aspergillus nidulans* and resistance in *Botrytis cinerea*. *J. Phytopathol.* **2001**, *149*, 301−308.

(14) Iacomi-Vasilescu, B.; Avenot, H.; Bataille-Simoneau, N.; Laurent, E.; Guenard, M.; Simoneau, P. *In vitro* fungicide sensitivity of *Alternaria* species pathogenic to crucifers and identification of *Alternaria brassicicola* field isolates highly resistant to both dicarboximides and phenylpyrroles. *Crop Prot.* **2004**, *23*, 481−488.

(15) Kanetis, L.; Förster, H.; Adaskaveg, J. F. Optimizing efficacy of new postharvest fungicides and evaluation of sanitizing agents for managing citrus green mold. *Plant Dis.* **2008**, *92*, 261−269.

(16) Brent, K. J. Fungicide resistance in crop pathogens: how can it be managed? *FRAC Monograph 1*; Global Crop Protection Federation: Brussels, Belgium, 1995; ISBN 90-72398-07-6.

(17) 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*, 1−16.

(18) Smilanick, J. L.; Mackey, B. E.; Reese, R.; Usall, J.; Margosan, D. Influence of concentration of soda ash, temperature, and immersion

period on the control of postharvest green mold of oranges. *Plant Dis.* **1997**, *81*, 379−382.
(19) Plaza, P.; Usall, J.; Torres, R.; Abadias, M.; Smilanick, J. L.; Viñas, I. The use of sodium carbonate to improve curing treatments against green and blue moulds on citrus fruits. *Pest Manag. Sci* **2004**, *60*, 815−821.
(20) Smilanick, J. L.; Mansour, M. F.; Margosan, D. A.; Mlikota Gabler, F.; Goodwine, W. R. Influence of pH and $NaHCO_2$ on effectiveness of imazalil to inhibit germination of *Penicillium digitatum* and to control postharvest green mold of citrus fruit. *Plant Dis.* **2005**, *89*, 640−648.
(21) Smilanick, J. L.; Mansour, M. F.; Sorenson, D. Pre- and postharvest treatments to control green mold of citrus fruit during ethylene degreening. *Plant Dis.* **2006**, *90*, 89−96.
(22) Smilanick, J. L.; Mansour, M. F.; Mlikota Gabler, F.; Goodwine, W. R. The effectiveness of pyrimethanil to inhibit germination of *Penicillium digitatum* and to control citrus green mold after harvest. *Postharvest Biol. Technol.* **2006**, *42*, 75−85.
(23) Echlin, P. *Handbook of Sample Preparation for Scanning Electron Microscopy and X-ray Microanalysis*; Springer Science + Businesss Media: New York, 2009.
(24) Strokes, D. *Principles and Practice of Variable Pressure: Environmental Scanning Electron Microscopy (VP-ESEM)*; Wiley: Sothern Gate, UK, 2008.
(25) McDonald, R. E.; Nordby, H. E.; McCollum, T. G. Epicuticular wax morphology and composition are related to grapefruit chilling injury. *HortScience* **1993**, *28*, 311−312.
(26) Turrell, F. M. *Tables of Surfaces and Volumes of Spheres and of Prolate and Oblate Spheroids, And Spheroidal Coefficients*; University of California Press: Berkeley, CA, 1946.
(27) Schirra, M.; D'Aquino, S.; Palma, A.; Marceddu, S.; Angioni, A.; Cabras, P.; Scherm, B.; Migheli, Q. Residue level, persistence, and storage performance of citrus fruit treated with fludioxonil. *J. Agric. Food Chem.* **2005**, *53*, 6718−6724.
(28) Richter, D. L. Synergism: a patent point of view. *Pestic. Sci.* **1987**, *19*, 309−315.
(29) Smilanick, J. L.; Mansour, M. F.; Margosan, D. A.; Mlikota Gabler, F.; Sorenson, D. Control of postharvest green mold and sour rot by potassium sorbate combined with heat and fungicides. *Postharvest Biol. Technol.* **2008**, *47*, 226−238.
(30) Barkai-Golan, R. *Postharvest Diseases of Fruits and Vegetables: Development and Control*; Elsevier: Amsterdam, The Netherlands, 2001.
(31) U.S. EPA. Fludioxonil; http://www.fao.org/ag/AGP/AGPP/Pesticid/JMPR/Download/2004_eva/FLUDIOXONIL.pdf, 2004 (accessed July 2012).
(32) Fallik, E.; Grinberg, S.; Ziv, O. Use of bicarbonate salts to reduce decay development in harvested fruits and vegetables. *Phytoparasitica* **1996**, *54*, 153−154.
(33) 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.
(34) Faers, M. A.; Pontzen, R. Factors influencing the association between active ingredient and adjuvant in the leaf deposit of adjuvant-containing suspoemulsion formulations. *Pest Manag. Sci* **2008**, *64*, 820−833.
(35) Schirra, M.; Palma, A.; Barberis, A.; Angioni, A.; Garau, V. L.; Cabras, P.; D'Aquino, S. Postinfection activity, residue levels, and persistence of azoxystrobin, fludioxonil, and pyrimethanil applied alone or in combination with heat and imazalil for green mold control on inoculated oranges. *J. Agric. Food Chem.* **2010**, *58*, 3661−3666.
(36) Wardowski, W. F.; Hayward, F. W.; Dennis, J. D. A flood-recovery TBZ fungicide treatment system for citrus fruits. *Proc. Fla. State Hortic. Soc.* **1974**, *87*, 241−243.
(37) 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**, *56*, 10793−10798.
(38) Ritenour, M. A. Florida citrus pest management guide: fresh fruit residue limits; http://edis.ifas.ufl.edu/cg087, 2012 (accessed Oct 2012).
(39) Palma, A.; D'Aquino, S.; Vanadia, S.; Angioni, A.; Schirra, M. Cold quarantine responses of 'Tarocco' oranges to short hot water and thiabendazole postharvest dip treatments. *Postharvest Biol. Technol.* **2012**, DOI : 10.1016/j.postharvbio.2012.12.002.
(40) Kuzyc, I. J.; Meggitt, W. F. Alteration of epicuticular wax structure by surfactants. *Micron Microscopic Acta* **1983**, 279−280.
(41) Noga, G. J.; Knoke, M.; Wolter, M.; Barthlott, W. Changes in leaf micromorphology induced by surfactant application. *Angew. Bot.* **1987**, *61*, 521−528.
(42) Tamura, H.; Knoke, M.; Bukovak, M. J. Evidence for surfactant solubilization of plant epicuticular wax. *J. Agric. Food Chem.* **2001**, *49*, 1809−1816.
(43) Adaskaveg, J. E.; Förster, H. New developments in postharvest fungicide registrations for edible horticultural crops and use strategies in the United States. In *Postharvest Pathology, Plant Pathology in the 21st Century*; Prusky, D., Gullino, M. L., Eds.; Springer: Dordrecht, The Netherlands, 2008; Vol. *2*, pp 107−117.