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Factors Affecting the Synergy of Thiabendazole, Sodium Bicarbonate, and Heat To Control Postharvest Green Mold of Citrus Fruit

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The efficacy of thiabendazole (TBZ) to control postharvest decay caused by *Penicillium digitatum* of citrus fruit can be enhanced by co-application with sodium bicarbonate (SBC) and/or heat treatment. The impact of these treatments was investigated in citrus fruit, as a function of TBZ and SBC concentration and temperature, and were related to the amount of TBZ residues in fruit (total residues), in fruit surface, in the cuticular wax, and in the inner fruit. The residue levels of TBZ were determined in 'Valencia' oranges following a 1 min dip in an aqueous mixture of SBC at 0.5, 1, or 2 wt %/vol and TBZ at 600 or 400 mg/L (active ingredient, a.i.) at 20 or 40 °C and after 0 and 20 days at 17 °C and 90% relative humidity. The influence of SBC and heat on the TBZ residue concentration on the fruit surface, in cuticular wax, and on the inner cuticle tissue was determined in 'Salustiana' oranges after a 1 or 3 min dip in TBZ alone at 600 mg/L and 20 or 50 °C or for 1 min in TBZ at 600 mg/L and SBC at 2% and 20 °C. The efficacy of heat treatments with water, SBC, and TBZ, applied separately or in combination, was investigated on artificially inoculated 'Nova' mandarins and 'Valencia' oranges for the control of postharvest green mold caused by a TBZ-sensitive (TBZ-s) or TBZ-resistant (TBZ-r) isolate of *P. digitatum*. The residue levels of TBZ in fruit, evaluated as total residues, were not affected by the co-application of SBC in most samples. While TBZ residues in the fruit surface were not significantly affected by the dip temperature or by co-application of SBC, the rates of diffusion and penetration of TBZ into cuticular wax markedly increased in the presence of SBC or when TBZ was applied in combination with heat. TBZ residues in the inner tissue of fruits treated at 20 °C were not dependent upon the dip time or by the presence of SBC and were similar to those found in fruit treated with TBZ at 50 °C for 1 min, whereas significantly higher values were recorded in samples treated with TBZ at 50 °C for 3 min. When TBZ at 600 mg/L and 20 °C was applied in the presence of SBC at concentrations of 1−2 or 0.5−2%, it effectively reduced decay caused by the TBZ-resistant isolate of green mold in 'Nova' mandarins and 'Valencia' oranges. This treatment was also significantly more effective than TBZ alone to control green mold caused by a TBZ-s isolate in 'Valencia' oranges. The combination with SBC and mild heat (40 °C) and TBZ at 400 mg/L generally improved the control of a TBZ-r isolate of green mold with respect to the combined treatment at 20 °C. TBZ efficacy was also improved when applied at reduced rates (200 mg/L) and 50 °C, significantly suppressing green mold caused by a TBZ-s isolate of *P. digitatum* and effectively controlling a TBZ-r isolate. The rate of weight loss of 'Valencia' oranges was significantly increased by SBC treatment and was positively dependent upon the concentration of SBC used in the treatment, while the temperature of the treatment solution had little influence on later weight loss.

KEYWORDS: *Citrus*; *Penicillium* decay; hot water dip; fungicide treatments; thiabendazole; residues; sodium bicarbonate

INTRODUCTION

Of the various diseases that determine postharvest decay of citrus fruit, green mold (*Penicillium digitatum* Sacc.) and blue mold (*Penicillium italicum* Wehmer) are common worldwide and can determine severe losses. Careful handling during harvest and packinghouse operations and sanitation procedures with disinfectants are strongly recommended to reduce the risk of infections and postharvest losses. Citrus fruits after harvest are usually treated with sodium *o*-phenylphenate (SOPP), thiabendazole (TBZ), and imazalil (IMZ), the only fungicides registered for postharvest treatments of citrus fruit in most

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countries of the European Union. They are usually applied as a drench and/or spray treatment on the packing line before marketing or cold storage to control these fungi. Intensive and exclusive use of TBZ and IMZ has caused selection of TBZ- and IMZ-resistant isolates in most packinghouses in various citrus-producing areas (*1*−*3*). Efforts to manage fungicide resistance in citrus packinghouses have thus increased greatly.

In recent years, a wide range of effective broad-spectrum fungicides with novel biochemical modes of action have been developed and are currently in use for pre- or postharvest treatment of selected fruit crops. Among them, azoxystrobin, fludioxonil, and pyrimethanil, each belonging to a different chemical class, are active against *Penicillium* spp., including isolates of pathogens that are resistant to IMZ or TBZ (*4*). The activity of these fungicides may be improved when they are applied in combination with hot water because of the enhanced uptake and better encapsulation and diffusion of the active ingredient in the cuticular wax (*5*−*7*). However, because these formulations are not registered for postharvest applications in several countries, alternative solutions are needed.

Smilanick et al. (*8, 9*) have proven that, when TBZ or IMZ are applied in combination with sodium bicarbonate (SBC), a common buffer and food additive capable of controlling numerous plant pathogens, their effectiveness to control green mold consistently increased, even when fruits were inoculated with a TBZ- or IMZ-resistant isolate of *P. digitatum*. Similar synergistic effects were observed when SBC was applied in combination with pyrimethanil (*10*). While various factors affecting the mode of action of SBC are recognized (*11*−*14*), the synergy mechanism of SBC and TBZ has yet to be elucidated. The present paper provides evidence that SBC, hot water, or both positively affect the rate of absorption of TBZ by cuticular wax in oranges, thereby enhancing the activity against TBZ-resistant isolates of *P. digitatum*.

MATERIALS AND METHODS

Fruit. Oranges [*Citrus sinensis* (L.) Osbek] cvs. Valencia late and Salustiana and mandarins (*Citrus reticulata* Blanco) cv. Nova were hand-harvested when commercially mature from the experimental orchard of the Istituto di Scienze delle Produzioni Alimentari, CNR, located in central-western Sardinia, Italy, that received standard horticultural practices. Fruits were placed in plastic trays and delivered to the laboratory immediately after harvest. Then, fruits of medium size and free from rind defects were selected, returned to each box, and left overnight at 20 °C.

Treatment Device. A bath fitted with 3.96 kW/h heating elements and an electronic recirculation pump (22 L/min water flow) was used for treatments. A total of 200 L of water or fungicide mixtures was used for the treatments, dipping one box of fruit per run. The bath temperature was constantly maintained within ±0.5 °C of the set temperature by an electronic thermostat (OEM/HT, Carel, France) and probe (PTC 40, Carel, France).

Fungal Strains and Growth Conditions. A monosporic 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, United States 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), 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 hemeacytometer.

Assays with Wounded and Artificially Inoculated Fruit. 'Valencia' oranges and 'Nova' mandarins were used to determine the influence of treatments against TBZ-s and TBZ-r isolates of *P. digitatum*. Before inoculation, fruits were grouped into 18 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 at the opposite sites with a 2 mm wide and 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, fruit were incubated at 20 °C and 90% relative humidity (RH) for 20 h before treatment to simulate the commercial conditions, where the infection may have occurred at harvest in the field or during transit before treatment. Treatments were performed by immersing the fruit for 1 min in (1) water or water mixtures at 20 °C containing SBC at 0.5, 1, or 2% with or without TBZ at 600 mg/L, (2) water or water mixtures at 40 °C containing SBC at 0.5, 1, or 2% with or without TBZ at 400 mg/L, or (3) water or water mixture at 50 °C containing TBZ at 200 mg/L. The fungicide used was a commercial formulation of TBZ (TECTO SC, Syngenta Crop Protection S.p.A., Milan, Italy) containing 42.9% active ingredient (a.i.). Each treatment was repeated 3 times (replicates of 40 fruit each). Following treatments, fruits were left to dry at room temperature and then stored at 20 °C and ca. 90% RH. Fruits were evaluated for incidence of decay caused by green mold (percentage of fruit with decay lesions of any size compared to the total) after 5 days (mandarins) and 7 days (oranges).

Storage Response to Postharvest Treatments. 'Valencia' oranges were grouped into 18 treatment groups and subjected to 1 min dip treatments with water or water mixtures containing SBC, TBZ, or their combination as described above. Each treatment was applied to three fruit boxes (replicates) of 40 fruits each. After treatment, fruit were left to dry at room temperature for approximately 5 h, moved to a ventilated room, and kept 21 days at 17 °C.

Storage responses of fruits (decay incidence, treatment damage, external fruit appearance, and fruit weight loss) were determined as previously described (*15*). For each treatment, weight loss was determined on 40 individually numbered fruits and was expressed as a percent loss from the initial weight.

Influence of SBC on the pH of Water and Water Mixture Containing TBZ. The measurements of pH of water and water mixture at 20 or 40 °C containing TBZ at 600 mg/L without or with SBC at 0.5, 1, or 2% (wt/vol) were performed after 0 h (freshly prepared mixtures), after 6 h, and after 24 h. pH measurements were recorded by using a pH-meter (Orion, Model 420A, Milan, Italy.

Influence of Treatments and Storage Conditions on Residues of TBZ. 'Valencia' oranges were segregated into 18 treatment groups and subjected to 1 min dip treatments with water or water mixtures containing TBZ at 600 mg/L with or without SBC at 0.5, 1, or 2% (wt/vol) and 20 °C, TBZ at 400 mg/L with or without SBC at 0.5, 1, or 2% and 40 °C, or TBZ alone at 200 mg/L and 50 °C. Each treatment was repeated 3-fold (replications). After treatment, fruit were stored for 20 days at 17 °C and 90% RH (simulated shelf-life conditions). Analyses of TBZ were performed at time 0 (immediately after treatment) and after 10 and 20 days.

Influence of SBC on TBZ Residue in Fruit Surface, Absorption by Cuticular Wax, and Penetration into the Inner Fruit. 'Salustiana' oranges were washed with water, left to dry at room temperature, and subjected to 1 or 3 min dip treatments with 600 mg/L TBZ at 20 or 50 °C or to a 1 min treatment in a water mixture containing TBZ at 600 mg/L and SBC at 2% and 20 °C. Then, fruits were left to dry at 17 °C overnight in the dark. Each treatment was repeated 3-fold (replications).

Five fruits were randomly selected from each treatment group (three replications per group) and weighed. Then, fruits were first washed with water (to remove TBZ from rind surface), subsequently treated with chloroform (for TBZ extraction from cuticular wax), and finally used for residue analysis after extractions (inner TBZ).

To determine TBZ residues on the surface, five weighed oranges were immersed once in a 500 mL beaker with 400 mL of water and agitated for 5 min. A total of 5 mL of water solution was extracted in a 40 mL screw-capped flask with 10 mL of ethyl acetate/hexane (1/1) and 2 g of NaCl. The mixture was agitated in vortex for 1 min and in a rotatory shaker for 30 min. Subsequently, the phases were allowed to separate, and 2 mL of the organic solution were dried under a gentle nitrogen stream. The residues were then dissolved with 1 mL of acetone and injected in gas chromatography (GC) for the analysis.

To determine TBZ residues in the epicuticular wax, the same five oranges extracted with water were dipped in 400 mL of chloroform for 1 min. A total of 1 mL of chloroform solution was dried under a gentle nitrogen stream, and the residues were dissolved with 1 mL of acetone and injected in GC for the analysis.

After water and chloroform extractions, fruits were used for analysis of inner TBZ.

Total residues were determined in samples not subjected to extractions in water and chloroform.

Analysis of TBZ in Fruit. The peel of five oranges was taken, weighed, and kept frozen at −18 °C until TBZ analysis. A total of 5 g of homogenized peel was weighed in a 40 mL screw-capped flask, and 10 mL of ethyl acetate/hexane (1/1) and 2 g of NaCl were added. The mixtures were agitated in a rotatory shaker for 30 min. Subsequently, the phases were allowed to separate; 1 mL of the organic layer was dried under a gentle nitrogen stream; and the residues were dissolved with 1 mL of acetone and injected in GC for the analysis without any cleanup step. For recovery assays, samples from untreated oranges were fortified at 0.38, 1.52, 3.79, and 7.58 mg/kg of TBZ and subjected to the above extraction procedure. Three replicates of each concentration were analyzed. The recovery was 84.6−99.8%, with a maximum coefficient of variation (CV) of 6.7%. Good linearity was achieved in the 0.1−8.0 mg/kg range, with correlation coefficients between 0.9951 and 0.9998.

Statistical Analysis. Statistical analysis was performed using Statgraphics software (Manugistics, version 5 Professional, 2000) statistical program. Analysis of variance (one-way ANOVA) and covariance analysis (only for fruit weight loss) were conducted using a unifactorial complete randomized block design. Percentages were not transformed or transformed in $\arcsin\sqrt{x}$ or $\sqrt{x}$ before the ANOVA, depending upon the range of variation of data. Mean comparisons of the effects of treatments were calculated by the Fisher's least significant difference test at $p \leq 0.05$. Limpel's formula, as described by Richter (*16*), 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

Control of Green Mold. The application of SBC to 'Nova' mandarins at 0.5−2% and 20 °C or at 0.5% and 40 °C was not effective in controlling the TBZ-s isolate of *P. digitatum*, while it was effective when SBC was applied at 1−2% and 40 °C (**Table 1**). Superior control of green mold was achieved when SBC was applied to fruit inoculated with the TBZ-r isolate of *P. digitatum*, a result dependent upon the salt concentration and temperature. Indeed, while concentrations of 0.5 and 1% at 20 °C were similar in effectiveness, 2% was superior. At 40 °C, the efficacy of SBC increased progressively, with an increase in the SBC concentration. When applied alone, TBZ resulted in total or nearly total suppression of the TBZ-s isolate of *P. digitatum*; therefore, no synergistic effects could be found when TBZ was applied in combination with SBC. Conversely, TBZ at 600 mg/L and 20 °C was not effective in controlling green mold caused by the TBZ-r isolate of *P. digitatum*. The effectiveness of TBZ consistently but modestly improved when it was applied at 400 mg/L and 40 °C and was superior at 200 mg/L and 50 °C. The combination of TBZ and SBC at 20 or 40 °C effectively improved the efficacy of TBZ in controlling green mold caused by a TBZ-r isolate of *P. digitatum*. The application of Limpel's formula indicated a significant synergistic improvement in effectiveness to control green mold caused by a TBZ-r isolate of *P. digitatum* when TBZ at 600 mg/L and 20 °C was combined with SBC at 1 or 2% or when TBZ at 400 mg/L and 40 °C was combined with SBC at 0.5, 1, or 2%.

Table 1. Incidence of Postharvest Green Mold, Caused by *P. digitatum*, on 'Nova' Mandarins and 'Valencia' Oranges after They Were Dipped for 1 Min in SBC or TBZ, Alone or in Combination, Followed by Storage at 20 °C for 5 Days (Mandarins) or 7 Days (Oranges)[a]

	green mold incidence (%)[b]			
	'Nova' mandarins		'Valencia' oranges	
treatments[c]	TBZ-s	TBZ-r	TBZ-s	TBZ-r
control (water, 20 °C)	100 a	100 a	92.5 a	96.7 a
0.5% SBC, 20 °C	100 a	86.7 cde	64.2 b	85.8 bc
1% SBC, 20 °C	96.7 abc	83.3 def	30.8 de	54.2 d
2% SBC, 20 °C	96.7 abc	61.7 g	20.8 efg	40.0 e
600 mg/L TBZ, 20 °C	0.0 e	98.3 ab	31.7 de	78.3 bc
600 mg/L TBZ + 0.5% SBC, 20 °C	0.0 e	93.3 bcd	18.3 efg	38.3 e[d]
600 mg/L TBZ + 1% SBC, 20 °C	3.3 de	65.0 g[d]	9.2 hil	25 fg[d]
600 mg/L TBZ + 2% SBC, 20 °C	0.0 e	40.0 h[d]	2.5 l	21.7 fgh[d]
water, 40 °C	100 a	96.7 ab	66.7 b	90.0 b
0.5% SBC, 40 °C	100 a	83.3 def	42.5 cd	81.7 bc
1% SBC, 40 °C	93.3 bc	76.7 efg	29.2 def	60.8 d
2% SBC, 40 °C	88.3 c	61.7 g	14.2 ghi	60.8 d
400 mg/L TBZ, 40 °C	1.7 de	88.3 cde	32.5 de	55.8 d
400 mg/L TBZ + 0.5% SBC, 40 °C	0.0 e	70.0 fg[d]	16.7 fgh	14.2 h[d]
400 mg/L TBZ + 1% SBC, 40 °C	5.0 d	40.0 h[d]	5.0 hl	31.7 ef[d]
400 mg/L TBZ + 2% SBC, 40 °C	3.3 de	23.3 i[d]	12.5 ghi	20.0 gh[d]
water, 50 °C	98.3 ab	95.0 abc	53.3 bc	56.7 d
200 mg/L TBZ, 50 °C	3.3 de	65.0 g	5.8 hl	37.5 e

[a] Two isolates of the pathogen were used: TBZ-sensitive isolate (TBZ-s) or TBZ-resistant isolate (TBZ-r). Fruit were inoculated 20 h before treatments. [b] Each value is a mean of three replicates (40 fruit per replicate, 2 wounds per fruit). Percentages were transformed in $\arcsin\sqrt{x}$ before statistical analysis. Means in columns followed by a common letter are not significant different by Fisher's least significant difference (LSD) procedure, $p \leq 0.05$. [c] Treatments are 1 min dips followed by air drying of the dipped fruit. Concentrations of SBC are expressed by wt/vol. [d] Synergistic effect was present according to Limpel's formula.

In mandarins the incidence of green mold decay caused by both isolates (TBZ-s and TBZ-r) was unaffected by water dip at 40 or 50 °C.

On 'Valencia' oranges, SBC effectively reduced decay caused by both TBZ-r and TBZ-s isolates of *P. digitatum*. Concentrations of 1−2% were generally more effective than 0.5% at both 20 and 40 °C. Treatments with TBZ at 600 mg/L and 20 °C or TBZ at 400 mg/L and 40 °C were, in most samples, no more effective than treatments with SBC to control both TBZ-s and TBZ-r isolates of *P. digitatum*. According to Limpel's formula, the combination of TBZ at 600 mg/L and 20 °C or TBZ at 400 mg/L and 40 °C with SBC at 0.5, 1, or 2% produced synergistic improvement in effectiveness to control decay caused by the TBZ-r isolate of *P. digitatum*.

Treatment with 200 mg/L TBZ at 50 °C notably suppressed green mold decay caused by TBZ-s isolate of *P. digitatum* and effectively controlled TBZ-r isolate. The combination of bicarbonate and mild heat consistently improved the efficacy of TBZ in controlling green mold caused by the TBZ-s isolate of *P. digitatum*. Treatments with water at 40 or 50 °C had little, albeit significant, effect in controlling decay incidence caused by both isolates.

Storage Response to Postharvest Treatments. There were no treatment-dependent differences in the external appearance of oranges. Lacking visible symptoms of rotten fruit, the results

Table 2. Influence of Postharvest Treatments with TBZ Alone or in Combination with SBC on the Percentage of Fruit Weight Loss in 'Valencia' Oranges after 7, 14, and 21 Days of Storage at 17 °C

	fruit weight loss (%)		
	storage duration (days)[a]		
treatments[b]	7	14	21
water, 20 °C	3.13 lm	5.62 g	7.98 i
0.5% SBC, 20 °C	3.90 f	7.44 f	10.79 g
1% SBC, 20 °C	3.89 fgh	7.65 ef	11.20 fg
2% SBC, 20 °C	3.94 d−g	8.02 de	11.89 def
600 TBZ mg/L, 20 °C	3.59 hi	6.33 g	8.85 hi
600 mg/L TBZ + 0.5% SBC, 20 °C	4.28 bcd	8.15 def	11.84 def
600 mg/L TBZ + 1% SBC, 20 °C	4.33 bc	8.47 cd	12.35 cd
600 mg/L TBZ + 2% SBC, 20 °C	4.81 a	9.38 a	13.39 ab
water, 40 °C	3.42 il	6.24 g	8.83 hi
0.5% SBC, 40 °C	4.07 c−f	7.90 de	11.43 e−h
1% SBC, 40 °C	4.49 ab	8.68 bc	12.71 bc
2% SBC, 40 °C	4.70 a	9.20 ab	13.47 ab
400 mg/L TBZ, 40 °C	3.22 lm	6.26 g	8.96 h
400 mg/L TBZ + 0.5% SBC, 40 °C	4.23 b−e	8.39 cd	12.23 cd
400 mg/L TBZ + 1% SBC, 40 °C	3.98 c−f	8.11 cde	12.11 ce
400 mg/L TBZ + 2% SBC, 40 °C	4.55 ab	9.45 a	13.98 a
water, 50 °C	3.23 lm	6.18 g	8.83 hi
200 mg/L TBZ, 50 °C	2.98 m	5.80 g	8.54 hi

[a] Means in columns followed by a common letter are not significantly different by Fisher's least significant difference (LSD) procedure, $p \leq 0.05$. [b] Treatments are 1 min dips followed by air drying of the dipped fruit. Concentrations of SBC are expressed by wt/vol. Percentages were transformed in $\sqrt{x}$ before statistical analysis.

of this study do not enable evaluation of the influence of treatments on the decay control of naturally inoculated fruit (data not shown). During storage, all treatments with SBC significantly increased the rate of weight loss of 'Valencia' oranges with respect to control (**Table 2**). The weight loss rate was positively dependent upon the SBC concentration and dip temperature, increasing when SBC was increased from 0.5 to 2% and being generally higher in fruit treated at 40 °C than at 20 °C. When equal concentrations of SBC were used, combined treatments at 20 °C registered higher values than treatments applied alone, while when treatments were performed at 40 °C, differences were non-significant in most samples. The influence of treatments with water or TBZ at 20 or 40 °C on fruit weight loss was not significant.

Influence of the Addition of SBC on the pH of Water and Water Mixture Containing TBZ. Immediately after the addition of SBC at 0.5% to water or water mixture containing TBZ at 600 mg/L at 20 or 40 °C, pH values increased significantly; no consistent increases were recorded when the SBC concentration was raised to 1 and 2% (**Table 3**). After 6 and 24 h, mean values of pH were generally higher than those recorded initially.

Influence of Treatment and Storage Conditions on TBZ Residues. Treatment with a water mixture containing TBZ at 600 mg/L and 20 °C produced a deposition of 1.59 mg/kg TBZ, which was approximately 1.9- and 3.5-fold higher than deposition from treatments with TBZ at 400 mg/L and 40 °C and TBZ at 200 mg/L and 50 °C, respectively (**Table 4**). The application of TBZ at 600 mg/L plus SBC resulted in a small but significant decrease of the residue level with respect to TBZ treatment applied alone, with differences because of the SBC concentration being non-significant. There were no significant differences in initial residues between separate or combined treatments when TBZ was applied at 400 mg/L and 40 °C. The changes in residue levels during fruit storage were negligible in all samples and were not affected by the co-application of SBC.

Table 3. Changes in pH of Water or Water Mixtures Containing TBZ at 600 mg/L at 20 or 40 °C, after the Addition of SBC at 0.5, 1, or 2% (wt/vol)[a]

	temperature	solution	SBC concentration			
treatments	(°C)	age (h)	0.0%	0.5%	1%	2%
control (water)	20	0	6.96 ± 0.05	8.31 ± 0.02	8.26 ± 0.01	8.26 ± 0.02
		6	6.90 ± 0.16	8.50 ± 0.02	8.51 ± 0.01	8.51 ± 0.01
		24	7.16 ± 0.10	8.89 ± 0.07	8.83 ± 0.08	8.98 ± 0.04
	40	0	7.17 ± 0.10	8.45 ± 0.04	8.54 ± 0.03	8.55 ± 0.01
		6	7.27 ± 0.09	8.76 ± 0.03	8.84 ± 0.05	8.84 ± 0.04
		24	7.53 ± 0.51	8.98 ± 0.05	9.05 ± 0.02	9.05 ± 0.08
600 mg/L TBZ	20	0	7.04 ± 0.18	8.41 ± 0.03	8.48 ± 0.03	8.48 ± 0.02
		6	7.07 ± 0.04	8.47 ± 0.03	8.45 ± 0.05	8.53 ± 0.04
		24	7.96 ± 0.08	8.89 ± 0.03	8.79 ± 0.03	8.88 ± 0.03
	40	0	7.63 ± 0.13	8.52 ± 0.01	8.60 ± 0.08	8.46 ± 0.04
		6	7.95 ± 0.15	8.74 ± 0.03	8.93 ± 0.22	8.83 ± 0.03
		24	7.90 ± 0.37	9.02 ± 0.06	9.26 ± 0.18	8.99 ± 0.10

[a] Mean values ± standard deviation ($n = 3$). The pH was measured after 0 h (freshly prepared solutions) and after 6 and 24 h.

Table 4. Influence of Postharvest Treatments with TBZ Alone or in Combination with SBC on TBZ Residues (mg/kg, on a Whole Fruit Basis), in 'Valencia' Oranges after 1 Min Dip Treatment (Time 0) and after 10 and 20 Days of Storage at 17 °C

	TBZ residues (mg/kg)[a]		
	storage duration (days)		
treatments[b]	0	10	20
600 mg/L TBZ, 20 °C	1.59 a	1.57 a	1.54 a
600 mg/L TBZ + 0.5% SBC, 20 °C	1.25 b	1.40 ab	1.31 b
600 mg/L TBZ + 1% SBC, 20 °C	1.31 b	1.45 ab	1.40 bc
600 mg/L TBZ + 2% SBC, 20 °C	1.22 b	1.23 b	1.38 bc
400 mg/L TBZ, 40 °C	0.83 c	0.89 c	0.87 c
400 mg/L TBZ + 0.5% SBC, 40 °C	0.79 c	0.76 cd	0.79 c
400 mg/L TBZ + 1% SBC, 40 °C	0.75 c	0.70 cd	0.71 cd
400 mg/L TBZ + 2% SBC, 40 °C	0.74 c	0.65 d	0.58 d
200 mg/L TBZ, 50 °C	0.45 d	0.43 e	0.38 e

[a] Means in columns followed by a common letter are not significantly different by Fisher's least significant difference (LSD) procedure, $p \leq 0.05$. [b] Treatments are 1 min dips followed by air drying of the dipped fruit. Concentrations of SBC are expressed by wt/vol.

Table 5. TBZ Residues in Fruit Surface (mg/kg, on a Whole Fruit Basis), into Cuticular Wax, and into Inner Tissue, Total Residues (Calculated), and Total Residues in Control Fruit Following Dip Treatment 'Salustiana' Oranges with TBZ at 600 mg/L Alone at 20 or 50 °C for 1 or 3 Min or in Combination with 2% SBC at 20 °C for 1 Min

		TBZ residues (mg/kg)[a]				
treatments[b]	dip time (min)	fruit surface (A)	cuticular wax (B)	inner tissue (C)	total (A + B + C)	total (control)
TBZ, 20 °C	1	0.68 a	0.17 b	0.13 b	0.98 b	1.04 b
	3	0.69 a	0.11 b	0.13 b	0.92 b	1.10 b
TBZ, 50 °C	1	0.66 a	0.54 a	0.17 b	1.37 ab	1.12 b
	3	0.69 a	0.48 a	0.40 a	1.57 a	1.60 a
TBZ, 20 °C + 2% SBC	1	0.53 a	0.46 a	0.01b	1.00 b	0.91 b

[a] Means in columns followed by a common letter are not significant different by Fisher's least significant difference (LSD) procedure, $p \leq 0.05$. [b] Concentrations of SBC are expressed by wt/vol.

Influence of SBC on TBZ Residue in Fruit Surface, Absoption by Cuticular Wax, and Penetration into the Inner Fruit. After treatment with TBZ at 600 mg/L, the amount of TBZ residue deposition on fruit surface was not affected by dip time, temperature, or the presence of SBC (**Table 5**); significantly higher values in TBZ residues were recorded in

cuticular wax of fruit samples treated with TBZ for 1 or 3 min at 50 °C with respect to TBZ treatments at 20 °C. When SBC was applied in combination with TBZ at 20 °C, the rates of TBZ absorption by cuticular wax increased to a similar level found in fruit treated at 50 °C.

The amount of TBZ residue retained by fruit after water washing and chloroform extraction in samples treated at 20 °C was not dependent upon the treatment time or by the presence of SBC, and although similar to those found in fruit treated with TBZ at 50 °C for 1 min, it increased significantly when TBZ was applied at 50 °C for 3 min.

The amount of total residues in oranges, as calculated by adding TBZ on the fruit surface, to cuticular wax, and to the inner fruit was higher in oranges treated at 50 °C for 3 min, lower in those treated at 20 °C, and intermediate in samples treated at 50 °C for 1 min. These results were consistent with total residues determined on fruit samples not subjected to extraction.

DISCUSSION

The potential of SBC to improve the performance of TBZ to control *Penicillium* decay and to obtain some control of an isolate of *P. digitatum* with a high level of resistance to TBZ has been demonstrated by Smilanick et al. (*9*). The results of the present study confirm the ability of SBC to enhance TBZ efficacy against a TBZ-r isolate of green mold. Indeed, while TBZ alone at 600 mg/L and 20 °C had no or little effect in controlling green mold decay caused by a TBZ-r isolate in mandarins and oranges as compared to the control, it was effective when applied in the presence of SBC at concentrations of 1−2% (in mandarins) or 0.5−2% (in oranges). In these cases, the increase in the performance of TBZ was synergistic according to Limpel's formula. The combined treatments with TBZ at 400 mg/L, SBC, and mild heat (40 °C) were generally more effective than treatments at ambient temperature (20 °C) against the TBZ-r isolate of green mold, thus supporting previous findings on lemons (*9*). The efficacy of TBZ against the TBZ-r isolate of green mold also improved when applied alone at 400 mg/L and 40 °C or at 200 mg/L and 50 °C, especially in oranges. This finding provides for the first time an indication of the effectiveness of heated TBZ against an isolate of *P. digitatum* resistant to this fungicide.

The growth-inhibitor activity of SBC against various phytopathogenic fungi is well-documented by *in vitro* (*11*−*14*) and *in vivo* (*17, 18*) studies. While various factors affecting the mode of action of SBC (inhibition of spore germination, germ tube elongation, and production of pectinolytic enzymes) against several pathogens are well-recognized (*8*), the mechanism of synergy between SBC and TBZ has yet to be elucidated. SBC increases and buffers the pH of the TBZ mixtures; pH above 8 inhibits *P. digitatum* growth; and SBC inhibits *P. digitatum* growth even at neutral pH (*8*).

TBZ residues in citrus fruit were reported to be related to the pH of mixtures, increasing from 0.5 to 1.4 mg/kg when the pH was increased from 7.9 to 10.8 (*19*) to the fungicide concentration, and to the treatment temperature (*20*). Therefore, the improved performance of TBZ in combination with SBC might be ascribed, at least in part, to increases in TBZ residues and to inhibition of the fungus by SBC. In the present study, SBC generally increased the pH values of water mixtures containing TBZ, while decreasing or not affecting TBZ residues, when applied at 20 or 40 °C. Results on 'Valencia' oranges showed that the degradation rate of TBZ during fruit storage at 17 °C was unaffected by the presence of SBC. These results are important because TBZ residues are capable of controlling sporulation and protecting fruit from subsequent infection.

Thus far, the amount of residues on the fruit surface was not significantly affected by the dip temperature or by co-application of SBC, whereas the rates of diffusion and penetration of TBZ into cuticular wax notably increased (approximately 2-fold) in the presence of SBC or when TBZ was applied in combination with heat. These results, along with the evidence for the improved performance of combined treatments (TBZ plus SBC and TBZ plus heat) in controlling the resistant isolate of *P. digitatum*, indicate that the a.i. in the proper place (i.e., in the injuries, absorption into wax, uptake) may be more important for a.i. efficacy than the total amount of fungicide present on the fruit surface. In addition, our data show that TBZ residues in the inner tissue of fruits treated at 20 °C were not significantly affected by the treatment time or by the presence of SBC and were similar to those found in fruit treated with TBZ at 50 °C for 1 min, whereas significantly higher values were recorded in samples treated with TBZ at 50 °C for 3 min. These results suggest that when treatment is performed at 50 °C, the treatment time should be long enough to allow for the penetration of the active ingredient into the inner tissue and produce the heat-induced beneficial effects of treatment (*20*).

When applied alone, SBC and hot water treatment can control recently established (within 24 h) infections. SBC and hot water treatments, however, do not confer persistent protection of the fruit from re-infection (*12, 13*). In contrast, TBZ protects surface wounds from re-infection, and its efficacy is potentiated by co-application of SBC and/or hot water. However, both SBC and heat directly affected green mold development and both similarly improved the absorption of TBZ into cuticular wax, presumably by increasing the fluidity of waxes and the diffusion of the fungicide across the cuticle and, hence, increasing the efficiency of TBZ. We may thus indicate that the mechanism of synergy between SBC−TBZ and heat−TBZ appears, at least in part, similar. Heat treatment is known to affect the ultrastructure of fruit epicuticular wax (partial melting, rearrangement of wax distribution on the fruit surface, re-solidification, and occlusion of possible gaps for wound pathogens) (*21*), without causing relevant changes in water loss, as we show here on 'Valencia' oranges. Conversely, all treatments with water mixtures containing SBC notably increased fruit weight loss, which depended upon the SBC concentration (*22*). Thus, the impact of SBC on fruit cuticle was far different from that of heat. We may assume that SBC severely affected the interfacial properties of cuticle (chemical composition and/or spatial arrangement and assembly of its components, e.g., increasing the permeability of the cuticle, decreasing the tortuosity of the diffusion path in the amorphous wax fraction, and swelling of the polymer matrix of cuticles) (*23*−*26*), upon which water permeability of the cuticle is known to depend.

This is the first study on citrus fruit that provides information on the efficacy of heated TBZ applied alone against an isolate of *P. digitatum* resistant to this fungicide and that introduces new information on the influence of SBC, heat, or both on the rate of absorption of TBZ by cuticular wax or by the inner tissue. Our findings provide focus for future biochemical and molecular work needed to shed light on the mechanism of synergy between SBC and TBZ and to elucidate the relationships between chemical and physical modifications in cuticular wax and the enhanced transpiration rate induced by SBC.

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