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Immersion of Lemons into Imazalil Mixtures Heated at 50 °C Alters the Cuticle and Promotes Permeation of Imazalil into Rind Wounds

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The influence of the immersion of lemon fruit in aqueous imazalil (IMZ) mixtures at 25 or 50 °C on the deposition and persistence of IMZ within the fruit rind and its effectiveness in controlling postharvest green mold, caused by *Penicillium digitatum*, was investigated. Concentrations of IMZ in the albedo tissue surrounding deep wounds in the rind were higher than the mean EC_{50} values for IMZ-resistant strains (1.0 $\mu g \cdot mL^{-1}$) after immersion of fruit into IMZ mixtures containing 25, 50, or 75 $mg \cdot L^{-1}$ heated to 50 °C, but not those at 25 °C, where fungicide deposition was insufficient. IMZ residue, one day after treatment with 25, 50, or 75 $mg \cdot L^{-1}$, was 3, 5, or 7 times higher after treatment at 50 °C compared to treatment at 25 °C. IMZ residues within the albedo of unwounded fruit treated with 25, 50, or 75 $mg \cdot L^{-1}$ at 50 °C were 0.8, 1.3, or 2.0 $\mu g \cdot g^{-1}$, respectively, while those similarly treated at 25 °C had negligible residues. Residues following treatment at 50 °C in the albedo did not decline during storage at 10 °C after 60 days. IMZ residues in the flavedo were higher than those in the albedo: after treatment with 25, 50, or 75 $mg \cdot L^{-1}$ IMZ, they averaged 1.0, 1.2, or 2.7 $\mu g \cdot g^{-1}$, respectively, after treatment at 25 °C, and 5.5, 7.9, or 16.2 $\mu g \cdot g^{-1}$, respectively, after treatment at 50 °C. IMZ effectiveness as an eradicant to control green mold improved when it was heated. Green mold was reduced by 22 or 95% after treatment of lemons inoculated 1 h before immersion of lemons in IMZ at 50 $mg \cdot L^{-1}$ at 25 or 50 °C, respectively. Green mold was reduced by 18 or 61% after treatment of lemons inoculated 1 day after immersion of lemons in IMZ at 50 $mg \cdot L^{-1}$ at 25 or 50 °C, respectively. Scanning electron microscopy of the rind surface indicated that treatment at 50 °C for 2 min also caused ruptures in the cuticle.

KEYWORDS: ***Citrus limon*** **(L.) Burm; postharvest decay; residues**

INTRODUCTION

Imazalil is an N-substituted imidazole fungicide that inhibits 1,4 α-demethylation in the ergosterol biosynthesis in fungal cells (*1*), and it has been widely employed to control pre- and postharvest fungal diseases (*2*). Its curative, eradicative, and antisporulant activities have made it a key fungicide for the control of citrus green mold caused by *Penicillium digitatum* (Pers.:Fr) (Pd) and citrus blue mold caused by *Penicillium italicum* Wehmer (Pi). However, its sustained use to overcome the occurrence of benzimidazole and *o*-phenylphenol resistance (*3, 4*) has favored the selection of single and cross-resistant biotypes (*5*), raising significantly the baseline sensitivity of Pd isolates (*6*). Holmes and Eckert (*7*) collected Pd and Pi biotypes from groves and evidenced a stepwise selection of IMZ-resistant strains (R-strains) in packing houses where, under field conditions, resistance to high doses was absent. Recently, fungicide-resistant isolates of *P. digitatum* were characterized (*8*). Studies on resistance molecular mechanisms to sterol demethylation-inhibitor fungicides attributed the reason for IMZ resistance to a tandem repeat of four additional copies of a unique 126-bp sequence in the promoter region of the *CYP51* gene (*9, 10*). The great economic losses imparted to the citrus industry by the outbreak of R-strains has evidenced the need to (1) develop alternative approaches; (2) maximize the efficiency of the treatments; and (3) evaluate new differently acting class fungicides. Recently, in California, new active ingredients have been registered (pyrimethanil, fludioxonil, and azoxystrobin) as *Citrus* postharvest fungicides, and others are pending (*11*). These compounds have been reported to control effectively the current, being R-strains (*12, 13*). However, new fungicides have turned out to be not always a panacea while significant improvements have been achieved by using protocols aimed to promote the fungicide absorption and translocation or to modulate its degradation (*14*). In this direction, successful results were

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obtained in the citrus industry by applying the fungicides as heated solutions (*12, 13, 15−18*). Compared to cold treatments, heated ones, increased IMZ and TBZ deposition on fruit, respectively by 8 and 2.5 fold, allowing reduction of the dose applied without losing efficacy (*19*). Investigations on postharvest fungicide residue *in vivo* have primarily focused on the fungicide−commodity interaction with respect to health and environment safety. The more complex fungicide−commodity interaction with respect to pathogenesis has been lesser investigated. For *Citrus* fruit, residue quantization has been performed predominantly on the rind (flavedo + albedo) and pulp expressing the active ingredient concentration on a whole-fruit basis. These studies evidenced that nearly all of the fungicide resided in the rind, either when applied as nonrecovery spray (NRS) in water-based resin solution wax (*20*) or by immersing fruit in heated mixtures (*15*). Transcuticular movement of IMZ was shown to be less than 1% during a 3 min wetting period at 20 °C, and the removal of the epicuticular wax did not affect the motion, evidencing a hindrance role of the cuticle (*21*). The fungicide deposition on the exocarp (epicuticular wax, cuticle, and flavedo) increased significantly by heating the IMZ mixtures (*15*). The limited migration through intact exocarp into the albedo may account for the outbreak of infections following long storage and subsequent shelf life when new entry gates arise as observed by Schirra et al. (*22*). The fungicide concentration inside the novel cuticular ruptures may be long below the EC_{50} threshold for citrus green and blue mold (1.0 and 0.5 $\mu g \cdot mL^{-1}$, respectively), especially for R-strains (*5−7, 23*). Considering the wound-obligate nature of Pd and Pi, it is clear that cuticular ruptures are fundamental for pathogenesis, thus wound healing and/or fungicide deposition within these sites is a prerequisite to contain late mold development. In this direction only Brown et al. (*24, 25*) studied the deposition of IMZ in wounded fruit and found a higher active ingredient accumulation in the parenchyma when IMZ was applied as a NRS in a water-based resin solution wax. To our knowledge, so far, no studies have been carried out to quantify IMZ within wounds following hot water immersion or to shed light on the differences between treatment temperatures with respect to the fungicide allocation in intact and wounded rind. With this aim, lemon fruit was used to investigate the allocation and persistence of IMZ in deep wounds according to treatment temperature (25 or 50 °C), active ingredient concentration, and storage duration. In addition, scanning electron microscopy observations were carried out in order to follow and quantify the natural occurrence of cuticular ruptures during fruit storage and understand their role in pathogenesis.

MATERIALS AND METHODS

Fruit. The first crop of lemon fruit (*Citrus limon* L. Burm 'Verna') was harvested when commercially mature from April to July of 2006 and 2007 from an experimental orchard located in central Sardinia, Italy (39°55′N). The same day fruit were selected, labeled, randomized into boxes, and conditioned at 20 °C and 90% relative humidity (RH) for 24 h. Fruit employed were either wounded or not before treatment and, when dry, stored according to the experimental plan.

Wounding. In all experiments where wounded fruit were employed, disinfection (2 min/0.2% NaOCl, pH 7.0) took place before making at the equatorial area four circular 3 mm wide and 3 mm deep wounds with a steel rod, simulating mechanical injuries caused by stems during handling.

Inoculation. Artificial inoculation took place 1 h after wounding by injecting with a micropipet 20 μL of a freshly prepared Pd conidial suspension into each injury. Conidia were obtained from 7 day (d) old actively sporulating cultures of Pd (isolate Pd-02) grown on potato dextrose agar (PDA) in the dark at 27 °C. To prepare the suspension, 5 mL of sterile water (SW) with 0.05% (w/v) surfactant (Tween 80) were added to the Petri dishes (9 cm diameter) and conidia were scrubbed off with a loop. Then, the suspended conidia were filtered on a two layer cheesecloth and concentration was adjusted with a hemocytometer to 2×10^5 spores$\cdot mL^{-1}$ by diluting with SW. Following inoculation, fruit were left for an additional 24 h (h) at room conditions (20 °C and 75% RH) before treatment and subsequent incubation (25 °C and 90% RH). Conidial concentration was established in order to attain between 80 and 90% decay in untreated fruit.

Treatment. Boxes containing lemon fruit (30 each) were immersed in sole water or in IMZ mixtures at 25 or 50 °C for 2 min. The immersion tank was thermoregulated by a heating-recirculating devise (DL30, Haake Instruments, Inc., Paramus, NJ). A commercial formulate having 446 $g \cdot L^{-1}$ of IMZ was employed and 25, 50, or 75 $mg \cdot L^{-1}$ were used in the mixtures. Fruit were left to dry at room conditions before storage or incubation.

Storage. Lemons used for the quantization of residues, decay monitoring, and scanning electron microscopy (SEM) observations were stored at 10 °C and 95 ± 3% RH for 30 or 60 d, followed by 6 d of a simulated marketing period (SMP) at 20 °C and 75% RH.

Decay Monitoring. Decay was evaluated 7 d postinoculation when experiments were performed under incubation conditions. While, when carried out under storage conditions, molds were checked at the end of storage and SMP, wounds affected by actively growing pathogen were considered as infected independently from the expansion area or sporulation degree. Results given as inhibition percentage of decay are obtained according to Brown (*23*) and are relative to fruit immersed in sole water at 25 or 50 °C (control) for 2 min.

Analysis of IMZ. *Standard Solutions.* Stock standard solutions of imazalil and triadimefon (used as an internal standard, i.s.) were obtained by dissolving 50 mg of powder in 50 mL of ethyl acetate. Working IMZ solutions, containing 5 $mg \cdot L^{-1}$ of the i.s., were prepared by subsequent dilutions with ethyl acetate. All the mixtures were stored in the dark at −20 °C until use.

Sample Preparation. Rind tissue was removed from the fruit with a cork borer (8 mm Ø, 4 mm high), and the flavedo was separated from the albedo with a scalpel. The tissue was homogenized and immediately stored at −20 °C until analysis.

Extraction and Cleanup Procedure. The procedure for IMZ extraction and cleanup was based on a protocol developed by Garrido et al. (*26*) for the determination of imazalil and carbendazim in fruits. In order to minimize the time of analysis and reduce the amount of extraction solvent, the original method was changed as follows: 3 g of frozen homogenized tissue (albedo or flavedo) was accurately weighed, mixed with 5 g of anhydrous sodium sulfate, and extracted with ethyl acetate (3 × 15 mL) with a high speed blender (Ultra Turrax T25; Janke & Kunkel Gmbh & Co., Stoufen, Germany) at 3000 rpm for 3 min. The organic extracts were collected and filtered over a porous septum, washing the solid residue with ethyl acetate (10 mL). The solvent was concentrated to about 2 mL by evaporation under reduced pressure at 45 °C (rotovapor RE 121; Buchi, CH-9230 Flawil, Switzerland) and then quantitatively transferred in 10 mL tubes. Two mL of sulfuric acid 0.0025 M were added and mixed vigorously by shaking with a vortex for 30 s, and then the separated organic layer was discarded. Two milliliters of the internal standard solution (5 $mg \cdot L^{-1}$ concentration) together with 2 mL of sodium bicarbonate 1 M were added, and the mixture was vigorously shaken again for 30 s. Once the two phases were separated, the aqueous layer was discarded and the organic solvent was dried over anhydrous sodium sulfate. The sodium sulfate was filtered off, and the ethyl acetate solution was transferred into a vial for GC analysis.

Instrumental Analysis. The organic extracts were analyzed by GC (6890N, Agilent, Karlsruhe, Germany) equipped with a mass detector (MSD 5973N; Agilent) and a 30 m × 0.25 mm i.d. × 0.25 μm film thickness capillary column (HP5-MS; Agilent). The GC conditions were as follows: injection volume 1 μL; injection mode splitless; injector temperature 300 °C; oven program temperature from 150 (0 min) to 210 °C at a rate of 10 °C$\cdot min^{-1}$, then from 210 to 250 °C at a rate of 30 °C$\cdot min^{-1}$ with a hold time of 5 min. The carrier gas was ultrahigh purity helium at a flow rate of 2.1 mL$\cdot min^{-1}$. The transfer line, ion source, and quadrupole temperatures were 250, 230, and 150 °C,

Table 1. Imazalil Residue ($\mu g \cdot g^{-1}$) in Different Tissue of Wounded or Unwounded Lemon Rind Following a 2 min Immersion and Storage at 10 °C and 90% RH for 1 or 60 Days Followed by a 6 Day Simulated Marketing Period (SMP) at 20 °C and 75% RH[a]

	imazalil conc and immersion temp					
	25 mg·L^{-1}		50 mg·L^{-1}		75 mg·L^{-1}	
rind tissue	25 °C	50 °C	25 °C	50 °C	25 °C	50 °C
			1 Day of Storage			
flavedo	1.70 Da	7.08 Ba	2.00 Da	7.80 Ba	3.13 Ca	16.30 Aa
albedo	0.35 Db	0.79 Cc	0.34 Dc	1.26 Bc	0.35 Db	1.98 Ac
albedo of wounds	0.43 Db	1.25 Cb	0.60 Db	3.12 Bb	0.69 Db	4.63 Ab
			60 Days of Storage			
flavedo	0.97 Da	5.30 Ba	0.89 Da	7.49 Ba	3.08 Ca	15.72 Aa
albedo	0.10 Db	0.63 Cc	0.12 Db	1.14 Bc	0.18 Dc	1.33 Ac
albedo of wounds	0.12 Eb	1.03 Cb	0.40 Db	2.88 Bb	0.49 Db	3.74 Ab
			60 Days of Storage + 6 Days SMP			
flavedo	0.45 Ea	4.14 Ca	0.63 Ea	8.32 Ba	1.86 Da	16.54 Aa
albedo	0.07 Dc	0.50 Cb	0.19 Db	1.47 Bc	0.23 Dc	2.76 Ac
albedo of wounds	0.18 Db	0.76 Bb	0.43 Cb	3.14 Ab	0.50 Cb	4.74 Ab

[a] Residue means ($N = 3$); capital letters relate to comparisons within rows, lower case letters to comparisons within each column at the considered time, different letters indicate differences at $P \leq 0.01$ according to the Newman−Keuls test.

respectively. The electron impact was kept at 70 eV, and all the runs were carried out in selected ion monitoring mode.

Recovery Assays. IMZ-free albedo or flavedo tissue was removed from untreated fruit as described and homogenized with a known amount of IMZ−ethyl acetate solution. Fortified samples were kept in the dark for 30 min and then extracted and analyzed as described. IMZ concentrations in spiked samples were 0.5, 5, 10, and 20 mg·L^{-1}. Recoveries ranged from 94 to 103%.

Scanning Electron Microscopy (SEM) Observation. In order to estimate the development of naturally occurring rind wounds and to shed light on the influence of dip temperatures on their healing, SEM observations were performed. At harvest, after 1 month of storage and following SMP, two rind specimens (4 × 3 × 1 cm^3; L × L × T) from the equatorial area of three randomly picked lemons were removed by means of a scalpel. Specimens were immediately fixed into a phosphate buffer (pH 7.4) containing 3% (v/v) gluteraldehyde and stored in glass vials at 4 °C until the subsequent dehydration step was carried out. Immediately before dehydration, samples were soaked three times in fresh phosphate buffer (pH 7.4) followed by 3 more times in deionized water. Increasing ethanol concentration series (20, 50, 70, 80, 95, and 3 × 100%) were used to dehydrate the specimens by keeping them at each ethanol concentration step 20 min. After completion of the 100% ethanol step, samples were dried by critical-point and fixed on aluminum stubs with silver conductive glue. Gold−palladium-coating was performed with a sputter coater (S-150; Edward, West Sussex, U.K). Samples were stored in vacuum desiccators until scanning electron microscope (DSM 962; ZEISS, Oberkochen, Germany) observations were performed at different magnifications at 20 kV. On each specimen (magnified ×100), 5 surface areas (0.8 mm^2) were randomly scanned, acquiring for each treatment and time 30 scans. Each scan was subjected to image analysis, monitoring the amount and mean size of cuticular ruptures. The percentage of wounded rind surface per square millimeter was calculated.

Assessment of IMZ Residue in the Flavedo and Albedo of Wounded and Unwounded Fruit. The experiment aimed to quantify the IMZ accumulation in the flavedo and albedo of wounded fruit compared to unwounded ones was performed using 1440 lemons. Half of the fruit (720 lemons) were wounded as described and left for 1 h at 25 °C and 75% RH (room conditions) together with the remaining fruit. Then, 8 sets of 90 fruit were prepared according to the following treatments: sets 1 to 4 immersion at 25 °C with 0, 25, 50, or 75 mg·L^{-1} fungicide, respectively; sets 5 to 8 immersion at 50 °C with 0, 25, 50, or 75 mg·L^{-1} IMZ, respectively. The residues were quantified 24 h following treatment, after 60 d of storage, and at the end of the SMP.

Effect of Treatment Temperatures, IMZ Concentration, and Rind Wounds on Decay. The combined effects of treatment temperatures (25 or 50 °C) and IMZ concentrations (0, 25, 50, or 75 mg·L^{-1}) were evaluated during storage on wounded and unwounded lemon fruit. Half of the fruit (720 lemons) were wounded 1 h before treatment (group a) and half-remained unwounded (group b). Then, within each group, 8 sets of 90 fruit were prepared according to the following treatments: sets 1 to 4 immersion at 25 °C with 0, 25, 50, or 75 mg·L^{-1} IMZ, respectively; sets 5 to 8 immersion at 50 °C with 0, 25, 50, or 75 mg·L^{-1} fungicide, respectively. Following treatments, fruit were kept at room conditions until dry and transferred to storage. Natural occurrence of decay was monitored as described after one month of storage and at the end of the SMP.

Effect of Treatment Temperatures, IMZ, and Inoculation Time on Decay. An experiment was performed in order to evaluate the influence of IMZ (0 or 50 mg·L^{-1}), treatment temperatures (25 or 50 °C), and time of wound inoculation (24 h *pre-* or *post*-treatment) on decay occurrence. For this purpose, 720 lemons were divided into 8 sets of 90 fruit (3 replicates of 30 fruit). Four sets (group a) were wound-inoculated (time 0) as described and treated 24 h later; the other four sets (group b) were first treated (time 0) and wound-inoculated after 24 h. Within each group, 2 sets were treated at 25 °C and 2 at 50 °C. For each temperature, 1 set was treated in water alone and the second one in the IMZ mixture. Then, when dry, all fruit were stored under incubation conditions until decay was quantified.

Statistical Analysis. All data were subjected to ANOVA using the statistical program OpenStat (2007), and where appropriate, mean separation was performed according to the Newman-Keuls Test. For inhibition data, ANOVA was applied to angular transformed values. The SEM data were elaborated with image analysis software (Image-Pro Plus 6.0, Media Cybernetics Inc., Bethesda, MD).

Chemicals. Ethyl acetate [45760] (pesticide analysis grade) and sulfuric acid [84721] were obtained from Fluka Chemie, AG (CH-9470 Buchs, Switzerland). Anhydrous sodium sulfate [71958] and anhydrous sodium bicarbonate [S6297] were from Sigma Aldrich, Inc. (St. Louis, MO). Imazalil [36130, 99.8% purity] and triadimefon [45693, 99.8% purity] were obtained from Riedel de Haen (Hanover, Germany). Imazalil mixtures employed for treatments were obtained by diluting in deionized water commercially available Deccozil 50 (44.66% active ingredient, Elf Atochem, Paris, France).

RESULTS

Assessment of IMZ Residue in the Flavedo and Albedo of Wounded and Unwounded Fruit. One day following the treatment at 25 °C, the quantity of IMZ detected in the flavedo of unwounded fruit was 1.7, 2.0, and 3.1 $\mu g \cdot g^{-1}$, as mixture concentrations were raised from 25 to 50 and 75 mg·L^{-1}, respectively (**Table 1**). During storage, the fungicide in the flavedo decreased, and after the SMP, residues were 0.4, 0.6, and 1.9 $\mu g \cdot g^{-1}$. In fruit treated at 25 °C, a small but significant augment of IMZ residue in the flavedo took place as concentra-

tion increased in the mixtures. Significantly higher quantities of fungicide were detected in the flavedo of fruit immersed at 50 °C, 4 to 5 times greater if compared to those at 25 °C one day *post*-treatment. At the end of storage, 96% of the active ingredient was still detected in the flavedo of fruit treated with 50 or 75 mg·L^{-1} IMZ while only 75% of the fungicide remained on fruit immersed in the mixture containing 25 mg·L^{-1} IMZ. During the SMP, a further reduction occurred for fruit treated with 25 mg·L^{-1} IMZ while small increases occurred for 50 or 75 mg·L^{-1} (**Table 1**). One day following treatment at 25 °C, residues in the albedo of unwounded lemons were negligible (about 0.3 μg·g^{-1}) and no increase was observed by raising the fungicide in the mixture (**Table 1**). With 25, 50, or 75 mg·L^{-1}, IMZ residues were significantly decreased after storage, and at the end of the SMP, the loss of active ingredient was about 80, 44, and 36%, respectively. In the albedo of fruit immersed in the IMZ mixture at 50 °C, the fungicide residue was 2, 4, and 6 times greater if compared to the cases at 25 °C employing 25, 50, or 75 mg·L^{-1} IMZ, respectively. The loss of IMZ in the albedo of unwounded fruit treated at 50 °C with 25, 50, or 75 mg·L^{-1} IMZ mixture was about 20, 10, and 33%, respectively. At 25 °C, IMZ residue in the albedo of wounds increased as mixture concentration raised. When immersion was performed with 50 or 75 mg·L^{-1} fungicide, the residues in the albedo of wounded fruit were greater compared to unwounded ones. The quantities of IMZ detected in the albedo of the wounds, 24 h post-treatment at 50 °C, were 1.2, 3.1, and 4.6 μg·g^{-1} by using 25, 50, and 75 mg·L^{-1}, respectively. These residues resulted to be 3, 5, and 7 times greater compared to those attained at 25 °C. IMZ residue in the albedo of wounded fruit treated at 50 °C decreased slightly during storage while with 50 and 75 mg·L^{-1} IMZ increased significantly during the SMP. Residues in the albedo of the wounds of fruit immersed at 50 °C were comparable or higher to those found in the flavedo of fruit dipped at 25 °C (**Table 1**).

Effect of Treatment Temperatures, IMZ Concentrations, and Rind Wounds on Decay. Compared to immersion in sole water at 25 °C, the addition of 25, 50, or 75 mg·L^{-1} IMZ exerted, on unwounded fruit, a weak but noticeable effect in controlling natural infections during storage. The same treatments performed with artificially wounded fruit were ineffective, and most rots developed within the first week of storage (**Figure 1A**). The increase of IMZ concentration did not improve decay control either in unwounded or wounded fruit when treatments were performed at 25 °C. During the SMP, a lower decay percentage occurred in unwounded compared to wounded fruit, but differences were not significant (**Figure 1A**). Throughout storage, unwounded and wounded fruit immersed in sole water at 50 °C were significantly less affected by decay compared to immersion at 25 °C (**Figure 1A**, **B**). The decay was additionally reduced compared to controls when IMZ was added, and for unwounded fruit, the decay control improved by augmenting the fungicide concentration in the mixture (**Figure 1B**). On the other hand, the same treatments performed on wounded fruit showed no differences of efficacy according to IMZ concentration (**Figure 1B**). After SMP, 22, 10, 9, and 6% of the unwounded lemons and 33, 9, 5, and 4% of the wounded ones rotted following treatments with 0, 25, 50, or 75 mg·L^{-1} IMZ, respectively (**Figure 1B**). When wounded fruit were immersed in water at 50 °C, infections started after 2 weeks of storage, and by the end, 45% of the lemons rotted. After SMP in the control sets, the percentages of rotted fruit were close to those for fruits treated at 25 °C.

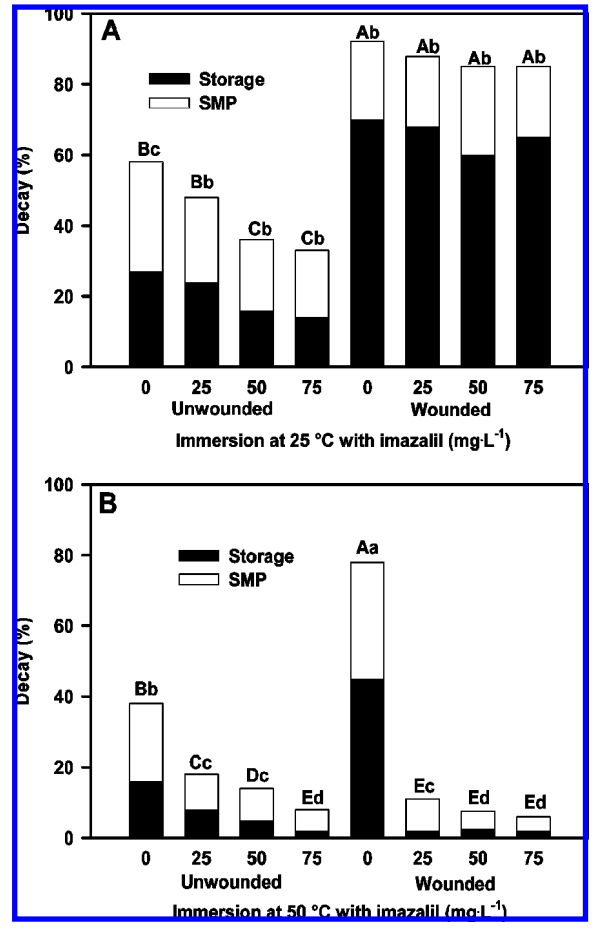

Figure 1. (**A**, **B**) Percentage of rotted lemons (wounded or unwounded) after 4 weeks of storage at 10 °C and 95% relative humidity (RH) (■) and during a 6 day simulated marketing period (SMP) at 20 °C and 75% RH (□) when fruit were treated at 25 (A) or 50 °C (B) with 0 (control), 25, 50, or 75 mg·L^{-1} IMZ mixtures for 2 min. Capital letters are related to storage (■) and lower case letters to the SMP (□). Different letters indicate differences at $P \geq 0.05$ according to the Scheffe test.

Effect of Treatment Temperatures, IMZ, and Inoculation Time on Decay. Treatments performed in a 50 mg·L^{-1} IMZ mixture at 25 or 50 °C 24 h before wound inoculation reduced the decay compared to the control by 18 and 61%, respectively (**Figure 2**). The effectiveness compared to controls was improved when the treatments were performed 24 h after wound inoculation, achieving at 25 and 50 °C a reduction of 22 and 95%, respectively (**Figure 2**). Differences between the two inoculation times were significant only by treating at 50 °C. It was noted that treatments with IMZ performed at 25 °C occasionally inhibited sporulation, while at 50 °C it was always complete.

Scanning Electron Microscopy Observation. Before immersion, besides mechanical damages caused at harvest (macroinjuries), the specimens observed evidenced several minor injuries caused by insects, and cuticular fractures near the stomata. Samples observed 24 h post-treatment evidenced clear differences between the two temperatures, while no differences were found between samples treated with or without IMZ and unimmersed compared to immersed at 25 °C. Following immersion at 25 °C, wax platelets, cuticular fractures near the stomata, and insect damages observed at harvest were still evident (**Figure 3A**). Treatments at 50 °C clearly melted the epicuticular wax covering most of the fruit surface, including stomata and cuticular damages (**Figure 3C**, **D**). After 1 month

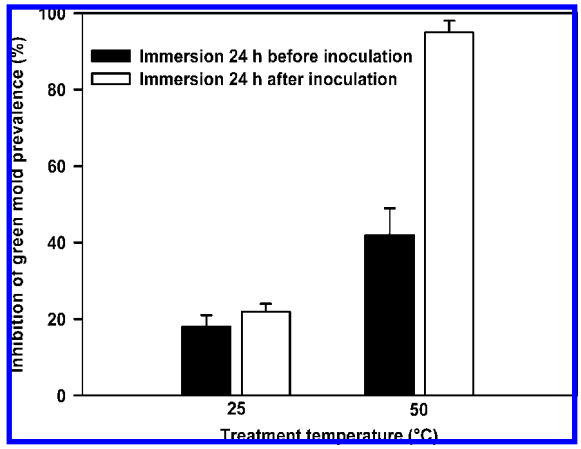

Figure 2. Percentage of *P. digitatum* decay inhibition on lemon fruit wound-inoculated 24 h before (■) or 24 h after (□) immersion in 0 (control) or 50 mg·L^{-1} IMZ mixture at 25 or 50 °C for 2 min. Decay was monitored after 7 days of incubation at 25 °C with 95% RH, and inhibition percentage was calculated with respect to controls (95 and 82% actual decay for 25 and 50 °C treated fruit, respectively).

of storage, fruit treated at 25 °C lost most of the epicuticular wax (60−70%) and the residual was heavily cracked (**Figure 3B**). Conversely, when fruit was treated at 50 °C, the epicuticular wax layer remained uniform on about 70% of the surface and in the remaining part it was missing or lightly cracked (**Figure 3E**, **F**). IMZ mixture at 25 or 50 °C did not influence the wax structure and distribution, while the occurrence and magnitude of cuticular fractures were significantly different at the end of storage and SMP (**Table 2**). Compared to the first day of storage, a significant reduction of cuticular fractures was observed on samples of unimmersed or immersed fruit at 25 °C with or without IMZ (**Figure 3B**). On the other hand, samples of fruit treated at 50 °C had an amount of fractures comparable to those observed one day *post*-immersion (**Table 2**). At the end of storage, the average size of fractures significantly increased only in samples of fruit treated with IMZ mixtures at 50 °C (from 500 to 900 μm^2) (**Table 2**) (**Figure 3E**, **F**). As a general rule in the remaining samples, there was a reduction of fractures per square millimeter in addition to a significant reduction of their size (**Table 2**). Following SMP, a marked loss of wax was observed and cuticular fractures were hardly found on samples of unimmersed or immersed fruit at 25 °C with or without IMZ (**Figure 3B**). Samples from fruit treated at 50 °C kept still 40% of the surface with uncracked wax, and when treated with IMZ, the size of cuticular fractures was increased while the amount was steady (**Table 2**).

DISCUSSION

It is well documented that several postharvest fungicides applied as heated solutions or mixtures increase considerably the decay control efficiency, allowing reduction of the healing concentration (*15*−*17*, *19*). Direct and indirect factors contribute to this outlet, suggesting a new hypothesis on the mechanism of uptake, persistence, and action of pesticides in fruit (*18*, *22*, *27*). In *Citrus* spp., the improved decay control efficiency, following immersion in heated IMZ mixtures, was attributed mainly to the thermally promoted "trapping action" of the epicuticular wax on the fungicide. This statement clearly points out the correlation between temperature and rind structure on the pesticide permeation in fruit and, as a consequence, on the decay control efficiency. Nevertheless, information available on the thermally promoted movement and distribution of IMZ in the different tissues of citrus fruit rind is incomplete. Up until now, quantification of IMZ in the albedo according to the application temperature and rind wounding was not investigated. As a consequence, the role played by the fungicide allocation and concentration in the different tissues of citrus rind on pathogenesis and sporulation is not clear. Results reported herewith refer to the thermally induced accumulation and persistence of IMZ in the flavedo and albedo of wounded and unwounded fruit rind, adding further knowledge to the complex xenobiotic−host−pathogen interaction. IMZ has been accounted to have systemic properties, and migration occurs via permeation and by the xylematic−transpiration flux (*28*−*30*). Nevertheless, the degree of systemicity and allocation depends upon the plant organ involved (stem, leaf, fruit, seed, etc.), treatment protocol (injection, nonrecovery spray, drenching, immersion), application temperature, ionization state (at physiological pH, IMZ is protonated), and concentration. All these factors by interfering IMZ motion, modulate the decay control efficiency (*19*, *23*, *25*, *30*). Under fully systemic conditions, the considerable difference of active ingredient concentration between citrus fruit exocarp and endocarp following immersion at 25 °C in IMZ mixtures, should favor an equilibrium translocation. Accordingly, the fungicide amount in the albedo (mesocarp) should increase rather than decrease, as was observed (**Table 1**). Indeed, our results pointed out a significant difference between the IMZ residue in the flavedo (outer 2 mm of the rind) and the albedo (parenchyma tissue below the flavedo) (**Table 1**), indicating a restricted inward motion of the active ingredient similar to what was found in seeds (*31*). In addition, differences between residue detected 24 h post-immersion and after 2 months of storage prove permeation to occur mostly during and narrowly after immersion, when fruit remains wet, as reported also by Brown and Dezman (*21*). Our results (**Table 1**) assert inward mobility during storage to be feeble or nearly inexistent, and this statement is furthermore supported if considering the slow degradation rate of IMZ (half-life 12−20 weeks) (*32*) and the simultaneous decrease of residue in the exocarp and mesocarp during storage (**Table 1**). The IMZ permeation process in unwounded rind may be jeopardized by the epicuticular waxes, the cuticle, and the essential oil glands. The latter ones, considering the lipophilic nature of IMZ, on one hand, may improve permeation, and the other one may favor local retention, as observed in *Citrus* rind for volatile organic compounds by Ligor and Buszewski (*33*). The highly hydrophilic nature of the airy-parenchyma albedo cells, containing the vascular bundles providing water and solutes to rind and vesicles, may further hold up permeation due to changes in the hydrophilic−lipophilic balancing. An additional drawback to the inward motion arises considering the xylematic-transpiration flux displacement of IMZ, which in fruit has a centrifugal direction from the vascular bundles in the albedo toward the flavedo (*29*). All these factors hamper inward permeation and may account for the high quantity (between 30 and 50%) of fungicide remaining on the rind and being removable by rinsing fruit, as reported by different authors (*19*, *21*, *34*). These thoughts, along with the results presented, indicate a not truly systemic behavior of IMZ when applied to *Citrus* fruit as a postharvest immersion treatment. Additional useful information comes from the IMZ residue allocated in the albedo of wounded fruit. When treatments were performed at 25 °C, the concentration of IMZ in the flavedo reached the EC_{50} threshold (mean EC_{50} value 0.87−0.92 μg·mL^{-1}) for R-strains of Pd (*7*), with all employed fungicide concentrations (**Table 1**). However, during storage, the treatment efficacy was equal only for 50

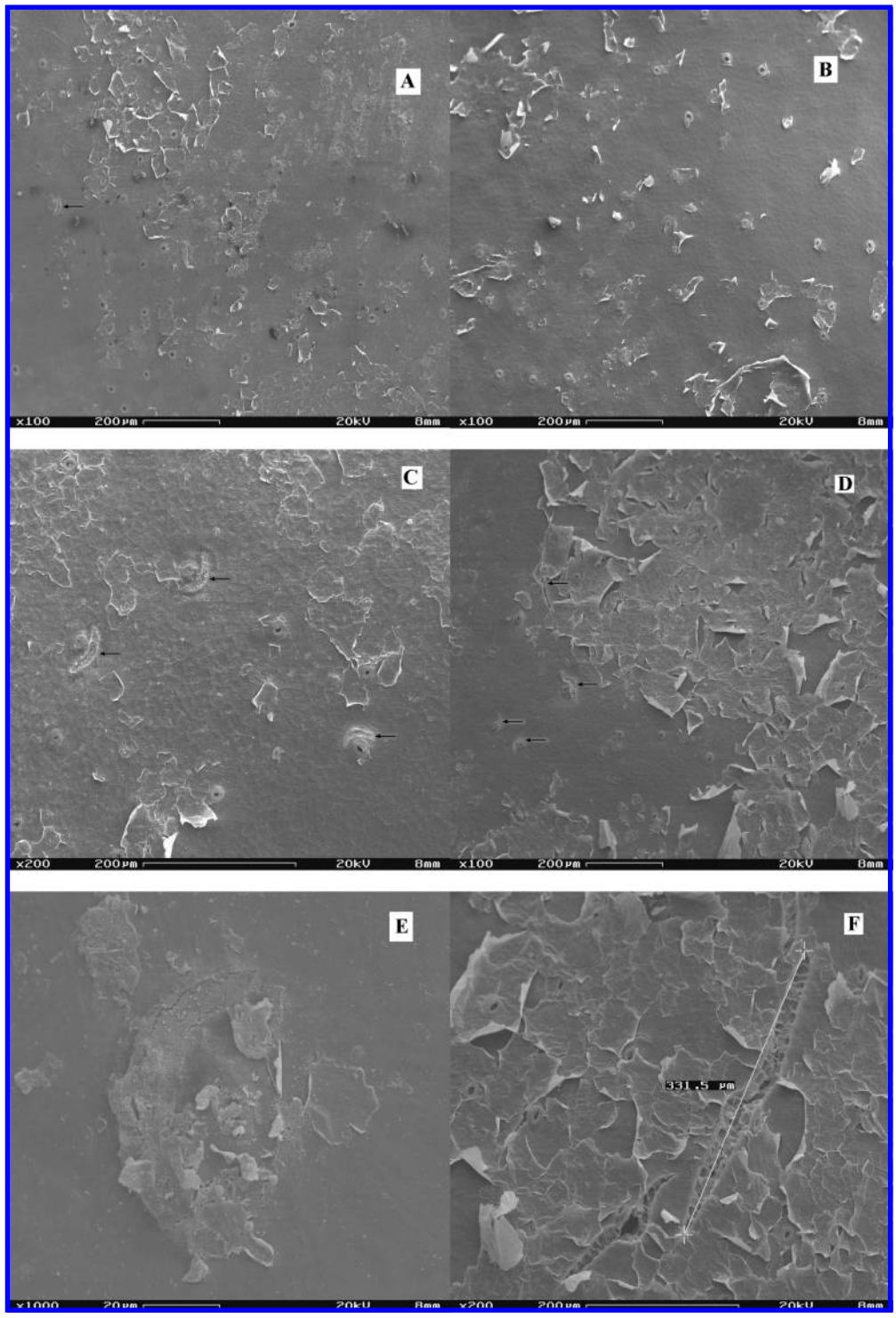

Figure 3. SEM micrograph of lemon fruit exocarp following immersed in a 50 mg·L^{-1} IMZ mixture at 25 or 50 °C after 1 day (**A** and **C**, respectively) and after 30 days of storage at 10 °C and 95% RH (**B** and **D**, respectively). Arrows indicate cuticular fractures, and 1 day following immersion at 50 °C, fractures were closed by melted wax (**E**), while after storage they were uncovered and enlarged (**F**).

and 75 mg·L^{-1} (**Figure 1A**). These results may find an explanation if we take into consideration the IMZ allocated in the albedo of wounded rind (**Table 1**). Indeed, comparable quantities of fungicide were found in the albedo of injured fruit immersed in water at 25 °C with 50 or 75 mg·L^{-1}, while residues were significantly lower using 25 mg·L^{-1} IMZ (**Table 1**). These differences, in addition to the lack of decay control when fruit were wound-inoculated after IMZ application, as the fungicide did not move inside the wounds (**Figure 2**), indicate a key role to the allocation and concentration of the active ingredient within the potential infection courts. Different authors evidenced the uptake and decay control efficiency of IMZ, following NRS at room temperature (20−25 °C), to depend greatly upon fungicide concentration, treatment duration, formulation, and rind integrity (*20*, *21*). In addition to these factors, by using concentrations about 40 to 120 times lower, here we evidenced that treatment efficiency depends primarily upon IMZ allocation. Indeed, decay control was clearly correlated to the fungicide allocation in the inner parenchyma and was strictly dependent upon the time of wound inoculation for both treatment temperatures (**Figure 2**). The fungicide allocated in the flavedo affected mainly sporulation, providing a feeble inhibition for

Table 2. Number, Size, and Percentage of Fruit Surface Affected by Cuticular Fractures As Observed by SEM on Lemon Fruit after 1 and 30 Days of Storage at 10 °C with 90% RH and Following a 6 Day Simulated Marketing Period (SMP) at 20 °C with 75% RH[a]

time (days)	treatment	no. of wounds (per mm^2)	size of wounds (μm^2)	wounded surface (% per mm^2)
1 (storage)	control	0.31 B	351.5 B	0.011 B
	25 °C	0.32 B	334.1 B	0.011 B
	50 °C	0.55 A	599.4 A	0.033 A
30 (storage)	control	0.18 B	112.7 C	0.0020 C
	25 °C	0.29 B	105.9 C	0.0030 C
	25 °C + IMZ	0.31 B	174.8 B	0.0054 BC
	50 °C	0.49 AB	201.8 B	0.0098 B
	50 °C + IMZ	0.57 A	892.0 A	0.050 A
30 + 6 (SMP)	control	0.02 B	106.1 B	0.00021 C
	25 °C	0.03 B	88.5 B	0.00026 C
	25 °C + IMZ	0.09 B	111.5 B	0.0010 B
	50 °C	0.09 B	123.0 B	0.0011 B
	50 °C + IMZ	0.57 A	1101.8 A	0.063 A

[a] $N = 30$; unimmersed fruit is referred to as control; different letters within each time group indicate differences at $P \leq 0.01$ according to the Scheffe test; records and measurements of wounds were performed with the software Image-Pro Plus 6.

low IMZ residues, and increased significantly when the amount of IMZ residue was raised. In addition, sporulation magnitude was not affected by the time of wound inoculation. As earlier reported on a whole fruit base (*15*, *16*, *19*), treatments carried out in heated mixtures provide a higher active ingredient residue level in the rind (flavedo + albedo). This significant increase was attributed primarily to the fungicide encapsulation within the remodeled epicuticular wax and to an expected higher transcuticular mobility (*15*, *16*, *35*, *36*). In agreement with these reports, we additionally found that the increase of the amount of residue occurred not solely at the flavedo level but also in the albedo, especially when the cuticle was damaged (**Table 1**). During storage, the decrease of IMZ in both tissues was negligible and a small augment was found after SMP (**Table 1**). According to other reports (*15*, *16*), it should be attributed to the considerable weight loss occurring at the rind level during this period and not to a permeation process. It is likely that the high temperature promoting the fungicide "trapping" effect also involves the cutin and the embedded waxes in the cuticle as well as the oil glands (*33*). These occurrences promote IMZ accumulation in the flavedo, affecting probably the inward permeation, as observed by the lack of residue increase in the albedo with time (**Table 1**). A comparable mechanism was suggested by other authors when decay control was lost following NRS application of IMZ or TBZ in water-based resin wax mixtures (*21*, *25*, *37*). Indeed, under these operative conditions, resin wax acting similarly to the epicuticular wax encapsulated the fungicides and made them less available at the infection sites. Heating the mixture promoted also an augment of IMZ in the albedo, especially when the rind was wounded. Indeed, 24 h post-immersion, the amount of residue in the albedo near the wounds was 2–7 times greater compared to the cases of immersions at 25 °C and nearly twice those found in unwounded fruit (**Table 1**). As heated mixtures considerably increased the fungicide allocation in the albedo near the artificial wounds, this may account for the significantly improved decay control obtained when wound inoculation took place before immersion (**Figure 2**). IMZ mixtures applied at 25 °C had a negligible diffusion into the wounds, inadequate to provide an effective active ingredient accumulation and permeation inside the injuries. Thus, when treating at 25 °C, the time of wound inoculation did not influence the decay control efficiency (**Figure 2**). These results and statements indicate a fast motion of the IMZ mixture into the wounds only when immersion was performed at 50 °C. From the residue data (**Table 1**), it is likely that IMZ is acquired in the albedo mainly at the time of treatment, while permeation from the flavedo during storage seems to be negligible, independent of the immersion temperature. Also, Brown (*25*) studied IMZ translocation into NRS treated fruit at 20 °C by using a destructive approach with a food dye. In agreement with our results, he found a restricted diffusion in intact rind but did not quantify IMZ residue in the albedo of wounded fruit. Furthermore, high IMZ concentration in the flavedo failed to control infections during long storage and SMP (**Figure 1B**), as observed also by other authors (*15*, *24*). Albeit the IMZ concentration in the flavedo remained higher than the EC_{50} threshold for R-strains, the restricted mobility may explain the infections occurring during long storage and SMP when new fractures appear and/or the existing ones enlarged (**Figure 3F**) (*38*). The development of cuticular cracks is a well-known phenomenon reported by several authors for various species and linked to the tissue extension process, to senescence, or to biotic or abiotic stress (*39*−*41*). Cuticular cracks occurring during fruit growth in the orchard are continuously repaired, whereas during storage an opposite trend seems to take place (**Table 2**). One day post-immersion, a significant increase of the number and size of cuticular fractures on the rind of fruit immersed at 50 °C for 2 min was observed. A comparable effect, involving the oil glands, was reported at the end of a 6 week storage period of "Valencia" oranges subjected to a hot water regime (45 °C core temperature for 42 min) to disinfect against Queensland fruit fly (*42*). Similarly, Baur and Schönherr (*35*), while studying the diffusion of pesticides in citrus leaves, observed irreversible damage to the cuticle when temperature was kept at 40 °C for 12 h. Thus, it is likely that the increase of cuticular fractures was induced by the short thermal stress, since this trend was not observed on unimmersed or immersed samples at 25 °C (**Table 2**). Most cuticular fractures that normally develop throughout the storage are induced by the immersion in heated mixtures and in this way protected by the fungicide. The observed increase of fracture size, in samples immersed at 50 °C, was made possible by the prompt IMZ motion into the lesions and the subsequent permeation into the bordering parenchyma cells. In this fruit, decay development from existing and heat-induced fractures was prevented and size could further enlarge during storage and SMP. The higher amount of IMZ allocated in the albedo surrounding artificial wounds and the lower decay percentage when wound inoculation was performed before immersion in IMZ mixtures at 50 °C, compared to results at 25 °C, support the above statements (**Figure 2**). When lemons were immersed in water at 50 °C, cuticular lesions increased and were temporarily healed (**Table 2**), but during storage and SMP new fractures and especially the increase of size provided fresh unprotected entry gates to pathogens, and decay took place. The increase of cuticular fracture size was not possible in fruit treated at 25 °C, most likely due to the minimal uptake and mobility of IMZ and to the rapid infection of the unprotected cuticular wounds. Up until now, these SEM observations have not been reported for citrus fruit. Further work is underway by employing low concentrations of IMZ mixtures in order to increase the

pesticide allocation within potential entry points for postharvest wound pathogens.

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