Uptake of Imazalil by Citrus Fruit After Postharvest Application and the Effect of Residue Distribution on Sporulation of *Penicillium digitatum*

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ABSTRACT

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Imazalil applied to citrus fruit after harvest moved into the rind while the fruit were wet from the treatment. Imazalil was absorbed by the epicuticular wax and cuticle within a wetness period lasting 1–3 min, but less than 1% of it moved through the cuticle during this period. However, 30–45 min after fruit were dipped in imazalil (1,000 μg/ml) for 15 sec, the fungicide was recovered from tissue deeper than 1 mm into the rind. About 40% of the applied imazalil remained on the fruit surface after the fruit dried. At comparable treatment concentrations and times, significantly more imazalil adhered to fruit that were dipped than to fruit treated with a nonrecovery spray over brushes saturated with the fungicide. Oranges absorbed more imazalil when treatment times were increased. Removal of the epicuticular wax from fruit before treatment with imazalil significantly reduced the control of sporulation of *Penicillium digitatum* and increased the rate of movement of the fungicide into the rind but did not significantly alter total residues.

Imazalil is a fungicide registered for postharvest application to citrus fruit for control of decay (8,10). The antisporulant action of imazalil against *Penicillium digitatum* (Pers.:Fr.) Sacc. (4) is a significant aspect of this treatment because it prevents the production of spores on infected fruit, which could soil healthy fruit packed in cartons.

Imazalil can be applied to citrus fruit after harvest in dips, wash tanks, or foam, in nonrecovery sprays (NRSs), or as drip treatments in water or water wax over brushes (8). Applications in water or water wax as NRSs or drip treatments are the preferred methods in Florida packinghouses. Because imazalil is less effective in water waxes than in water, treatment concentrations are usually increased to 2,000–3,000 mg/L to provide activity equivalent to that obtained with 1,000 mg/L in water (3,4).

Imazalil is systemic in citrus fruit when applied as aqueous formulations. It penetrated grapefruit rind to a depth of 2 mm and protected the fruit against green mold by preventing *P. digitatum* from invading fruit through injuries that occurred after fungicide treatment (3,4). Reported residues of imazalil from aqueous treatments of citrus fruit vary from less than 1 to more than 5 μg/g on a whole-fruit basis (6).

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In this study, we investigated factors responsible for the observed variation in residues from postharvest applications of aqueous imazalil, followed the movement of imazalil over time after application, and observed the effect of the distribution of imazalil in the rind on control of sporulation of *P. digitatum* following infection.

MATERIALS AND METHODS

Fruit treatments. Oranges (*Citrus sinensis* (L.) Osbeck 'Hamlin' or 'Valencia') were washed and prepared as reported previously (3). Imazalil (44.6% E) was applied either by submerging 10 fruits at a time in fresh fungicide suspensions to a depth of 2 cm or by treating five fruits at once with an NRS over fungicide-saturated brushes (4). Both treatments lasted 15 sec unless specified otherwise. Treatments were applied to 20 fruits, and individual fruits were randomly selected for residue analysis. All treatments were replicated three times.

To remove epicuticular wax, washed fruit were dipped in a 2:1 mixture of chloroform-methanol (25 C) for 1 min, wiped with a paper towel for 20 sec, rinsed in tap water for 2–3 min, and blotted dry with cheesecloth (5). Fruit surfaces were viewed with a scanning electron microscope (2) to judge the effectiveness of the wax removal treatment. To investigate whether the solvent extracted additional constituents of the rind, we tested the solvent by gas chromatography for the presence of limonene, the major component of peel oil located in the oil glands of the exocarp (9).

Fruit were inoculated with *P. digitatum* following reported methods (4) to evaluate sporulation control with imazalil.

Preparation of cuticles. Cuticles were obtained from washed oranges and lemons (*C. limon* (L.) Burm. 'Bearss') by making a circular cut 12 mm in diameter and 3 mm deep into the rind of each fruit with a cork borer while fruit were submerged in running tap water. The fruit were taken out of the water, and the cuticle disks, with a minimal amount of the exocarp, were removed with a scalpel.

Up to 60 disks were placed into 200 ml of an enzyme mixture of 1 g of cellulase (0.5–1.0 unit per milligram; Sigma), 12 ml of pectinase (59.8 units per milliliter; Sigma), and 13 mg of NaN_3 in 50 mM sodium citrate buffer at pH 4 (11). Peel disks were infiltrated under vacuum and incubated for 19–30 hr in the enzyme preparation at 36 C.

Cuticles were washed free from enzyme-degraded tissue with a gentle stream of deionized water and were floated on water. Cuticular disks, oriented with the outer surfaces upward, were floated and centered onto plastic supports prepared by using a paper punch to punch holes 7 mm in diameter in 2-cm squares of plastic cut from the base of polystyrene weighing dishes. The plastic supports were placed on folded filter paper, and the cuticles within the wells formed by the 7-mm-diam holes were inspected for holes and tears with a stereoscopic microscope. The supported cuticles were used immediately or were stored on folded filter paper at 4 C in a saturated atmosphere. Before use, free water was removed from the supports and cuticles with paper tissue, and the cuticles were again checked for physical damage.

Transcuticular fungicide movement. Imazalil movement through cuticles was measured with the bioassay of Edgington et al (7) using 5 ml of medium in 100 × 15-mm petri dishes and *P. digitatum* as the bioassay organism. Plastic supports with cuticles were placed on each of three bands of medium cut 10 mm wide in each dish, and imazalil was distributed evenly over the cuticle surface within the 7-mm-diam well in each support. The lower surface of the cuticle within each well was uniformly appressed against the medium. The supports and

cuticles were removed after specified intervals. Transcuticular movement of imazalil was determined by measuring the degree of inhibition of the growth of *P. digitatum* and comparing it to the inhibition caused by a known amount of imazalil in ethanol.

The bioassay was also used to estimate surface deposits of imazalil. The plastic support with its cuticle was held with forceps while 2 μg of imazalil was distributed on the cuticle surface. After 2 min, the deposit was dried with warm air from a hair drier. The cuticle surface was gently washed for 10 sec with a stream of deionized water, and the cuticle was placed in a culture tube (12 mm in width × 75 mm in height) with 2–3 ml of ethyl acetate. The tubes were covered and heated overnight at 75 C. Cuticles were then removed, and the ethyl acetate was evaporated at 87 C. The residue was dissolved in 0.2 ml of ethanol, and 5 μl was spotted on two thicknesses of 7-mm-diam filter paper disks placed in the center of each band of bioassay medium (7). Treated but unwashed cuticles were extracted as a control. Each treatment consisted of three or four cuticles.

Uptake of imazalil by the rind. A band of rind was cut and peeled from the fruit around the equator. Rind disks 6 mm in diameter were removed from this band with a cork borer at four equidistant locations. Disks were cut by placing two similarly treated pieces of rind back-to-back and forcing the cork borer through the mesocarp and exocarp of one of the two pieces, to prevent removal of surface residues of imazalil. The disk was then forced back out of the cork borer with a glass rod, and disks of exocarp and mesocarp about 1 mm thick were cut evenly with a sharp scalpel, using the cutting edge of the borer as a guide. The four disks of either exocarp or mesocarp from each fruit were placed in the culture tubes described earlier and dried at 53 C for 90 min. The disks were then removed, frozen with liquid nitrogen, and replaced in the tubes. The imazalil was then extracted with ethyl acetate as described for the cuticles and measured by bioassay with *P. digitatum* (7).

Chemical determination of residues. A method for extracting and detecting imazalil provided by Brogdex Co. (Pomona, CA) (*personal communication*) was modified for analysis with high-performance liquid chromatography (HPLC). Residues based on the removal of surface imazalil by stripping were determined by rolling two treated fruits in 250 ml of ethyl acetate in a 3.8-L glass jar for 15 min at 90 rpm. The fruit and jar were rinsed, and the volume was brought to 600 ml with additional ethyl acetate. An Extra-Sep diol disposable extraction column (Lida Manufacturing Corp., Bensenville, IL) was conditioned with three column volumes of ethyl acetate. With the vacuum off, the column was filled a fourth time, and a 25-ml reservoir was attached. Ten milliliters of the stripping solution containing imazalil was added to the reservoir, and the vacuum was applied. When the reservoir cleared, it was immediately flushed with 10 ml of ethyl acetate, and air was drawn through the column for at least 5 min. The imazalil was eluted from the column with 2 ml of a 50:50 (v/v) mixture of methanol and 112 mM aqueous H_3PO_4.

Whole fruit were homogenized, and 50–100 g of the homogenate was mixed with an equal weight of deionized water. Twenty grams of the puree was transferred to a 20 × 2.5-cm screw-cap test tube and treated with 10 ml of 1.0 N NaOH, 10 ml of heptane-isoamyl alcohol (95:5), and 2 g of anhydrous Na_2SO_4 powder. The tubes were sealed with foil-lined caps and shaken on a wrist-action shaker for 20 min. The samples were centrifuged at 3,000 *g* for 10 min. After centrifugation, the heptane-isoamyl alcohol layers were transferred to glass centrifuge tubes containing 5 ml of 0.1 N H_2SO_4. The tubes were shaken for 1 min, and when the phases separated, the organic phase was discarded. The puree was extracted twice, and each time the organic phase was transferred to the 5 ml of H_2SO_4. The acid was then washed with fresh heptane-isoamyl alcohol.

For HPLC analysis, all final samples from stripping or whole-fruit extraction were filtered through a 0.45-μm filter and injected into a Hewlett-Packard (Avondale, PA) column (2.1 × 100 mm) with Hypersil ODS (5 μm) packing material at an oven temperature of 40 C. A mobile-phase solvent of 75% methanol and 25% 0.2% aqueous ammonia at pH 7.5 was pumped through the system at 0.4 ml/min.

Weight determination of residues. Individual fruits were weighed to the nearest 0.01 g before and after treatment to determine the weight of the fungicide solution adhering to treated fruit. Any fungicide solution that ran off after fruit were treated was weighed, and the weight was subtracted from the final wet-fruit weight. All data were analyzed using SAS statistical procedures (12).

Table 1. Penetration of imazalil through detached cuticles of citrus fruit

Cultivar[w]	Epicuticular wax[x]	Transcuticular movement[y] 3 min (%)	Transcuticular movement[y] 20 hr (%)
Bearss lemon	+	0.11 a	6.4 a
	−	0.38 a	13.2 b
Hamlin orange	+	0.75	—[z]
Valencia orange	+	0.34 a	—
	−	0.91 a	—

[w] Cuticles of Bearss lemon were treated with 5.0 μg of imazalil for 3 min or with 0.25 μg for 20 hr. Cuticles of Hamlin and Valencia orange were treated with 5 and 2 μg, respectively.
[x] Intact (+) or removed (−).
[y] Each value is the mean of 9, 18, and 24 cuticles of Bearss lemon, Hamlin orange, and Valencia orange, respectively. Analysis of variance was used to compare movement through cuticles of each cultivar with intact or removed wax; values with unlike letters differ significantly ($P = 0.05$).
[z] Data not taken.

Table 2. Residues of imazalil (μg/g) determined by stripping surface residues or by macerating and extracting whole Valencia oranges treated with 1,000 μg/ml in dip or nonrecovery spray (NRS) applications

Analysis method	Application	Days after treatment[a] 0	1	2	7
Stripping	Dip	2.74	1.79	1.45	1.07
	NRS[b]	1.87	1.23	1.22	0.64
Whole fruit	Dip	2.15	2.39	2.30	2.40
	NRS[b]	1.57	1.60	1.31	1.34

Contrasts	$P > F$[c]
Stripping, day 0, dip vs. NRS	0.000
Whole fruit, day 0, dip vs. NRS	0.000
Dip, day 0, stripping vs. whole fruit	0.000
NRS, day 0, stripping vs. whole fruit	0.018
Stripping, dip, day 0 vs. days 1, 2, and 7	0.000
Stripping, NRS, day 0 vs. days 1, 2, and 7	0.000
Whole fruit, dip, day 0 vs. days 1, 2, and 7	0.040
Whole fruit, NRS, day 0 vs. days 1, 2, and 7	0.146

[a] Fruit were stored at 21 C and 88–92% relative humidity.
[b] Fruit were rotated on imazalil-saturated brushes.
[c] Probability, using orthogonal contrasts, that residues differ by chance.

RESULTS

Cuticular penetration. Movement of imazalil through cuticles of Hamlin and Valencia oranges was bioassayed at 30-sec intervals for 3 min (Table 1). Movement was quite variable, and differences among intervals were not significant in two tests with Hamlin cuticles and one test with Valencia cuticles. In all cultivars, less than 1% of the imazalil applied moved through the cuticle during 3 min of wetting. Removal of the

epicuticular wax from cuticle surfaces did not significantly enhance imazalil movement through the cuticle within 3 min in either Bearss lemon or Valencia orange, but removal of the wax from lemon cuticles exposed for 20 hr did improve movement (Table 1).

The quantity of imazalil remaining on the surface of detached cuticles was measured in four separate experiments. Of the original 2 μg of imazalil placed on each cuticle surface, 41, 55, 44, and 37% was removed when the dried deposits were gently washed with water.

Residues determined by chemical methods. Residues of imazalil remaining on Valencia oranges up to 7 days after dipping or NRS application were determined by stripping and by extracting whole fruit (Table 2). Residues were significantly affected by treatment method, analysis method, and number of days in storage. At day 0, residues recovered by stripping were greater than those recovered by extracting whole fruit; however, stripping removed significantly less residue than extraction after day 0, and by 7 days only 35–40% of the initial residue was recovered using this method. In contrast, residues from analysis of whole fruit did not change or increased slightly over time. Dip applications left significantly higher residues than NRS treatments.

Absorption of imazalil during fruit treatment. To detect active uptake of imazalil during treatment, residues of imazalil resulting from various application methods were compared by two methods of residue analysis (weighing the liquid imazalil adhering to the fruit, and extracting the imazalil from whole fruit and measuring) (Table 3). When residues were determined by the weighing method, more liquid remained on fruit from dip than from NRS treatments. However, dipping fruit for 15 sec deposited no more liquid than dipping for 2 sec. NRS treatment of individual fruits left more liquid imazalil on the fruit than treating samples of five fruits at a time, and the least liquid adhered to fruit treated only on imazalil-saturated brushes. Additional brushing after the NRS application to samples of five fruits did not remove a significant amount of the liquid imazalil suspension (Table 3).

Length of treatment was significant when residues were measured by extracting whole fruit. Greater residues were detected in fruit dipped for 15 sec than in fruit dipped for 2 sec and in fruit treated with NRS for 2 min than in fruit treated with NRS for 15 sec. The extraction method of residue analysis detected higher residues than the weight method at the longer treatment times for both types of treatment.

Residues measured by bioassay were also significantly higher in dipped fruit than in fruit that received NRS treatment, and more imazalil was present in the exocarp than in the mesocarp (Table 4). Imazalil was detected within the rind of dipped fruit immediately after the fruit had dried (30–45 min after treatment). Approximately 1% of the imazalil was recovered from rind tissue 1–2 mm beneath the rind surface. After 1 day, 10% of the imazalil had migrated to this depth, and by 1 wk, this percentage had more than doubled. No residues of imazalil from NRS applications were detected in tissue more than 1 mm beneath the surface until after 1 wk of storage. Recovery of imazalil from the 2-mm-thick surface portion of the rind with the bioassay procedure decreased over time for both types of treatment.

Role of epicuticular wax in imazalil uptake and sporulation control. Fruit surfaces observed with scanning electron microscopy after solvent treatment were virtually free of epicuticular wax. Limonene was not detected in extracts of epicuticular wax until the extract was concentrated 120 times. Removal of the epicuticular wax before fruit were dipped in imazalil interfered significantly with sporulation control activity but not with the quantity of imazalil deposited on the fruit (Table 5). Washing fruit immediately after treatment and drying removed more imazalil and reduced sporulation control more drastically when fruit were intact than when wax was removed from the fruit. When washing was delayed 6 hr, less residue was removed than when fruit were washed immediately after treatment and drying, and the residues were similar in intact and waxless fruit (1.33 and 1.37 μg/g, respectively); sporu-

Table 3. Imazalil residues (μg/g) on Valencia oranges exposed to 1,000 μg/ml for varying times, as determined by the weight of imazalil adhering to treated fruit or by extraction and analysis of whole fruit

Treatment	Analysis[v]		$P > t$[w]
	Weight	Whole fruit	
Dip, 2 sec	2.39 a	2.60 b	0.296
Dip, 15 sec	2.41 a	3.63 a	0.014
NRS, 15 sec[x]	1.10 c	1.13 cd	0.667
NRS, 15 sec, I[y]	1.58 b	1.40 c	0.080
NRS, 15 sec;[x] 105 sec on brushes[z]	0.84 cd	1.08 cd	0.205
NRS, 2 min[x]	1.10 c	2.40 b	0.001
Brushes, 2 min[z]	0.63 d	0.99 d	0.178

[v] Values in each column followed by unlike letters differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[w] Probability, by Student's t test, that residues determined with the two methods of analysis for each treatment differ by chance.
[x] Nonrecovery spray (NRS) application of imazalil to five fruits rotating on imazalil-saturated brushes.
[y] Fruit were treated individually with the NRS application.
[z] Fruit were rotated on brushes saturated with imazalil but were not sprayed with imazalil.

Table 4. Residues of imazalil from the rind of Hamlin oranges that received a dip or nonrecovery spray (NRS) application of imazalil at 1,000 μg/ml

Treatment	Days after treatment	Section[a]	Imazalil recovered[b]	
			Amount[c] (μg)	Percentage (%)
Dip	0	E	4.28	99.1
		M	0.04	0.9
	1	E	3.27	90.3
		M	0.35	9.7
	7	E	2.69	77.5
		M	0.78	22.5
NRS[d]	0	E	1.34	100.0
		M	0.00	0.0
	1	E	1.12	100.0
		M	0.00	0.0
	7	E	0.45	88.2
		M	0.06	11.8

Contrasts	$P > F$[e]
Dip vs. NRS	0.000
Exocarp vs. mesocarp	0.000
Dip, exocarp, day 0 vs. day 7	0.001
Dip, mesocarp, day 0 vs. day 7	0.056

[a] Sections were disks (6 mm in diameter, 1 mm thick) of surface exocarp (E) containing the epicuticular wax and cuticle and of the adjacent mesocarp (M).
[b] Residues were determined by bioassay with *Penicillium digitatum*.
[c] Total amount recovered from four disks.
[d] NRS application of imazalil to fruit rotating on imazalil-saturated brushes.
[e] Probability, using orthogonal contrasts, that residues differ by chance.

lation control, however, was significantly better for this sequence when the epicuticular wax was intact (Table 5).

DISCUSSION

The lipophilic nature of imazalil has been shown to be related to the unprotonated state of the molecule (13). At pH values above the pK of 6.53, the chemical is predominantly in the undissociated form, which is more lipophilic than the protonated or dissociated state. The aqueous preparations of imazalil used in these studies were at a pH of about 7.3, where about 85% of the material is undissociated.

About 40% of the imazalil applied in aqueous treatments to the fruit remained on the surface. This estimate was derived by stripping the fruit with solvent 1 wk after treatment and by gently washing detached cuticles with water immediately after imazalil deposits had dried. Although the solvent undoubtedly extracted some absorbed imazalil from the epicuticular wax and cuticle, imazalil recovered 1 wk after treatment may consist mostly of surface deposits, because absorbed imazalil would have penetrated more deeply into the rind. Gentle washing with water would also remove mostly surface residues.

Less than 1% of the imazalil penetrated the cuticle during exposures to liquid suspensions for 3 min. Because wetness during commercial treatments lasts for a similar amount of time or less, we conclude that most of the imazalil present on citrus fruit immediately after treatment is on and in the epicuticular wax and in the cuticle. A major portion of this imazalil continues to migrate deeper into the rind shortly after treatment. Presence of the epicuticular wax on the cuticle reduced the rate of imazalil penetration twofold to threefold. The effect was not noted after 3 min because of variability, but over a 20-hr period, the wax reduced penetration, as was observed with the penetration of apple cuticle by fungicides (14). Removal of the epicuticular wax before treatment with imazalil did not alter the quantity of imazalil absorbed by the fruit. However, without the epicuticular wax, the fungicide moved more rapidly into the rind. Increased dispersion of imazalil in the rind and lack of a concentrated deposit of imazalil at the surface in association with the epicuticular wax reduced the antisporulant activity of the fungicide. Effective control of sporulation of *P. digitatum* with imazalil depends partly on the presence of a concentrated deposit of imazalil at the fruit surface, where hyphae of the fungus sporulate after emerging from the infected tissue.

The quantity of epicuticular wax deposited on fruit surfaces differs among cultivars and geographic growing regions (1). These differences may be extreme enough to significantly influence the antisporulant activity of imazalil. Various packinghouse washing and polishing procedures before fungicide application may also influence the activity of imazalil, but perhaps not significantly because washing apparently removes little wax (1).

Absorption of imazalil by the fruit was quite evident when residues of imazalil determined by weighing the fungicide suspension adhering to the fruit were compared to those determined by extracting the fruit. The two methods of residue analysis gave different results as treatment times were increased because of the active uptake of imazalil by the fruit. Reported variations in imazalil residues on citrus fruit (6) could be related in part to differences in application methods and times of treatment used by individual investigators. Treatments similar in concentration but different in duration would leave significantly different residues.

The NRS treatment used in this study is more comparable to commercial application than is the dip treatment. Commercially, imazalil is sprayed or dripped on fruit for only 2–3 sec as they pass under the applicator while rotating on brushes saturated with fungicide. Most of the fungicide is transferred to the fruit during contact with the brushes, which normally lasts 8–10 sec. Thorough coverage of the fruit surface with imazalil is critical for reliable performance of the fungicide and can be expected to occur only if fruit tumble and all surfaces contact the brushes evenly during rotation. Higher residues can be attained by increasing treatment strength, provided label restrictions allow. Unfortunately, good contact between brushes and fruit is not achieved in many commercial packinghouses because the dump rate often exceeds the rate the equipment was designed for.

Table 5. Effect of removing the epicuticular wax from Valencia oranges before treatment with imazalil at 1,000 μg/ml on the uptake of imazalil and on the control of sporulation of *Penicillium digitatum*

Treatment[w]	Residue (μg/g)[x]		Sporulation index (SI)[y]	
	Wax intact	Wax removed	Wax intact	Wax removed
Control	0.00	0.00	5.0	4.8
Treated	2.32	2.17	0.0	3.0
Treated and washed, 0 hr	0.51	0.95	4.8	4.0
Treated and washed, 6 hr	1.33	1.37	1.2	3.2

Contrasts	$P > F$[z] Residues	SI
Treated, intact vs. removed	0.162	0.000
Treated and washed, 0 hr, intact vs. removed	0.001	0.004
Treated and washed, 6 hr, intact vs. removed	0.749	0.000
Treated and washed, intact, 0 hr vs. 6 hr	0.000	0.000
Treated and washed, removed, 0 hr vs. 6 hr	0.001	0.004

[w] Fruit were dipped for 15 sec, dried, and left unwashed or washed immediately or after 6 hr.
[x] Residues were determined by extraction and analysis of whole fruit.
[y] On a scale of 0–5, where 5 = fruit surface covered with green, sporulating mycelium, and 0 = fruit surface without mycelium or with white, nonsporulating mycelium.
[z] Probability, using orthogonal contrasts, that residues differ by chance.

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