Residues from Postharvest Nonrecovery Spray Applications of Imazalil to Oranges and Effects on Green Mold Caused by *Penicillium digitatum*

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ABSTRACT

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Imazalil is an imidazole fungicide effective against green mold of citrus caused by *Penicillium digitatum*. A comparison of residues and efficacy was made by applying imazalil in either water or water-based resin solution wax using a nonrecovery spray application to oranges revolving on horsehair brushes saturated with the treating solutions. Applications of imazalil in water resulted in higher residues than comparable concentrations applied in water wax, and residues from water treatments were also enhanced by increased time on the brushes. Residues were not enhanced by degreening fruit with ethylene before fungicide treatment. Injured rind contained higher residues of imazalil than uninjured tissue and residues on fruit washed after imazalil treatment were reduced only slightly. Higher concentrations of imazalil were required in wax than in water applications for control of infection of posttreatment injuries and sporulation by *P. digitatum*.

Imazalil is a fungicide developed for postharvest application to citrus fruits for decay control. One of several closely related N-substituted imidazole fungicides, it is specifically characterized by two nitrogen atoms in the unsaturated heterocyclic ring (14). Imazalil inhibits fungal growth by preventing sterol demethylation in the biosynthesis of ergosterol (14,16), the major sterol used in membrane synthesis by the higher fungi.

Imazalil has shown a high degree of effectiveness against *Penicillium digitatum* (green mold) and *P. italicum* (blue mold) for decay and sporulation control (9–12) and control of benzimidazole-resistant strains of these two organisms (1,8–10,12). Currently, tolerances of 2–5 μg/g on whole fruit are permitted in many European countries and Canada, Australia, and South Africa. In the United States, imazalil has only been applied commercially under experimental use permits (9) for testing and emergency labels to combat benzimidazole-resistant *P. digitatum* (5).

Commercial postharvest citrus fungicides are applied frequently as nonrecovery sprays. Relatively small volumes of expensive fungicide formulations can be applied in this manner and problems with sanitation, pH control, and stability can be minimized (2). Fungicides are often applied with water-based waxes that are now replacing the more expensive petroleum-based solvent waxes (7).

The purpose of this study was to evaluate residues of imazalil retained by oranges from commercial-type nonrecovery spray applications. Residues from applications in water and water-based wax and their efficacy against *P. digitatum* were compared.

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MATERIALS AND METHODS

Fruit. Oranges (*Citrus sinensis* (L.) Osbeck 'Hamlin,' 'Pineapple,' or 'Valencia') were washed for about 30 sec on tumbler brushes at 200 rpm and dried with sponge eliminator rolls, polisher-drier brushes, and exposure to hot air (40 C) for 1 min. Fruit were graded for external color and blemishes and uniform size. When desired, unwashed fruit were degreened 72 hr at 30 C and 92–96% relative humidity (RH) in the presence of 5–10 μl ethylene per liter of air.

To study the effect of injuries on residues, washed fruit were rubbed against No. 60 grit coarse sandpaper (3M Company, No. 9003) causing an injury about 14 mm in diameter at the fruit equator.

Fungicide application. Imazalil (68% EC) was applied either in water or water-based resin solution wax (7), (Citrus Lustr 266, 3.8 cps and 15.5% solids). Water and water-wax treatments at 1,000 μg/ml, except when noted otherwise, were applied using a single traversing hollow-cone mist-spray nozzle (No. F-80, Monarch Manufacturing Works, Inc., Philadelphia, PA), 3.50 and 1.65 90° W, respectively. Fungicide atomized at 60 psi was applied through the nozzle traversing above the middle of a bed of six horsehair-polyvinyl (50/50) brushes rotating at 100 rpm. The fungicide was applied until brushes were saturated with the material before dried fruit were placed on a slat conveyor that transported fruit to the brush bed. The fruit were continually atomized with fungicide while rotating on the brushes to provide thorough coverage of fruit surfaces until runoff. Treatment time ranged from 10 to 20 sec except in studies specified otherwise. Fruit were dried for 3 min at 50–54 C with a slat conveyor dryer and stored at 21 C for 1–3 days except in the storage study. Samples were then removed and frozen for later extraction. Treatments were applied to three replicates, each with 30–50 fruit. Ten fruit were then usually selected to obtain tissue for whole-fruit, peel, exocarp, and mesocarp residues.

Inoculation. Control of sporulation was evaluated by injecting 1 ml of spores (1×10^6) with a 10-ml sterile hypodermic syringe into the central cavity of the fruit before treating them with imazalil. Each treatment contained three replicates, each with 10 fruit, which were rated (5 = heavy sporulation, 0 = negligible sporulation) (3) during a 2-wk period at 24 C, and 93% RH. Infected tissue was fixed in 3% glutaraldehyde, dehydrated with increasing concentrations of tertiary butyl alcohol to 100%, embedded in paraffin, and sectioned at a thickness of 10 μm. Sections were stained with analine blue for viewing and photography.

Infection of oranges by *P. digitatum* after treatment with imazalil was evaluated by injuring the fruit with the No. 60 grit coarse sandpaper using care that each injury was formed in unused areas of the sandpaper sheet to prevent contamination of injured tissue with imazalil. Injuries were dusted with dry spores. Fruit were then incubated at 24 C and 93% RH for mold development. Each treatment contained two replicates, each with 10 fruit.

Preparation of samples and residue analyses. Eight fruit were quartered and one quarter from each fruit was selected randomly for whole-fruit analysis. These eight quarters were weighed, placed in a Waring Blendor with distilled water (2 parts fruit: 1 part water), and homogenized for about 5 min. Sixty grams of homogenate (40 g fruit) was transferred to a 200-ml square bottle, and 50 g Na_2SO_4, 5 ml 5 *N* NaOH, and 75 ml

ethyl acetate were added. The mixture was sonicated and homogenized with a Polytron (Brinkman Instruments, Westbury, NY) for 5 min, after which the mixture was centrifuged to separate the ethyl acetate phase (containing imazalil). Twenty-five milliliters of ethyl acetate was removed, placed in a 125-ml separatory funnel, and extracted with two 50-ml 0.05 *N* H_2SO_4. The aqueous acidic extracts (containing imazalil) were collected, adjusted to pH 12 with saturated NaOH, and returned to a 125-ml separatory funnel. The basic mixture was extracted with 10 ml ethyl acetate, collected over Na_2SO_4, and kept under refrigeration until gas chromatographic analysis.

The peel of a fruit was quartered by carefully cutting through the exocarp (flavedo) and mesocarp (albedo) tissues with a surgical scalpel. The quartered peels were removed without damaging the juice vesicles. One peel quarter from each of five fruit was collected for peel analysis. The peel quarters were weighed, cut into small pieces, and placed in a Waring Blendor. To the cut peels, water was added (1 part peel:4 parts water) and the mixture homogenized for about 5 min. Twenty-five grams of homogenate (5 g whole peel) were transferred to a 200-ml square bottle, and 0.5 ml 5 *N* NaOH and 5 g Na_2SO_4 added. The mixture was stirred for 5 min, after which 30 ml ethyl acetate was added. The mixture was homogenized with a Polytron for 5 min and centrifuged to separate the ethyl acetate phase. Ten milliliters of ethyl acetate was removed, placed in a 125-ml separatory funnel, and washed twice with 20 ml of 0.05 *N* H_2SO_4. The combined acid washes were adjusted to pH 12 and extracted with 5 ml ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and stored.

The source of exocarp was one peel quarter from each of five fruit. Exocarp tissue was carefully separated from mesocarp tissue with a surgical knife. The exocarp was sliced into small pieces, placed in a Sorvall Omni Mixer, water added (1 part exocarp:4 parts water), and homogenized. Twenty-five grams of homogenate was processed for imazalil in a manner similar to that outlined for whole peel.

Imazalil extraction from mesocarp tissue differed only slightly from the procedure for exocarp tissue. Sliced mesocarp tissue was homogenized with water at a ratio of 1 part mesocarp:6 parts water. From this point, the procedure was similar to procedures outlined for exocarp and whole peel.

Gas chromatographic analysis. Imazalil (99.7%) in ethyl acetate was resolved on a column (1.22 m long and 2 mm i.d.) packed with 3% OV-17 on Gas Chrom Q (100–120 mesh) (Applied Science, State College, PA) in a Hewlett Packard Model 5880A gas chromatograph equipped with an electron capture detector and a level 4 computing system. The injection port was 250 C and the detector was 270 C. Samples of 2–6 μl were injected on-column at 230 C, held for an initial time of 6 min, programmed at 15 C/min up to 260 C, and held isothermally at 260 C for 3 min. Concentrations of imazalil in the unknown samples were determined by comparison with a standard imazalil plot as constructed by the 5880A level 4 computing system for external standards.

RESULTS

Recovery of residues of imazalil resulting from aqueous applications to Valencia oranges is shown in Figure 1, where fruit were stored at 21 C for 42 days. Some loss of imazalil on a whole-fruit basis was observed after 28 and 42 days of storage. When fruit were separated into the peel and pulp components and analyzed separately, however, no decrease in imazalil residue during storage was detected. Because most imazalil residue was restricted to the peel, with less than 1 μg within each gram of pulp, further studies were conducted with only the peel to compare residues from water and water-wax applications. Feasibly, residue levels could be determined more exactly by eliminating pulp containing only small amounts of imazalil.

Residues of imazalil recovered from orange peel were increased by increasing the treatment concentrations and by applying the imazalil in water rather than the water-based wax (Fig. 2). Residues from imazalil applied at 3,000 μg/ml were not much greater than those from applications at 2,000 μg/ml when applied in water. When applied in water wax, the higher treatment concentration did enhance resulting residues.

A major portion of the imazalil existed in the exocarp, where 94 and 91% of the residues were recovered from water and water-wax applications, respectively.

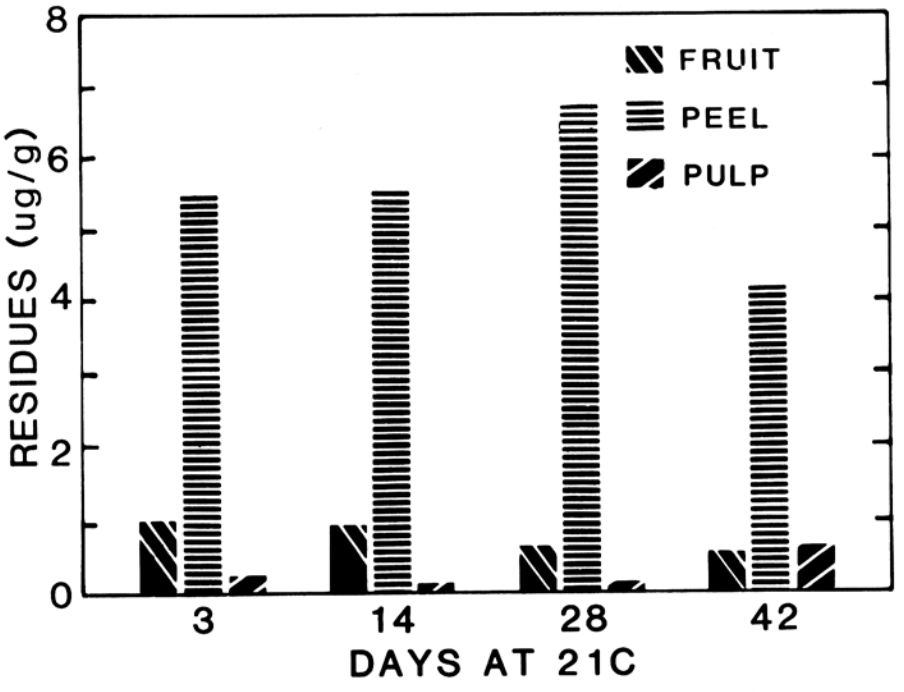

Fig. 1. Residues of imazalil recovered from Valencia oranges stored at 21 C for 42 days.

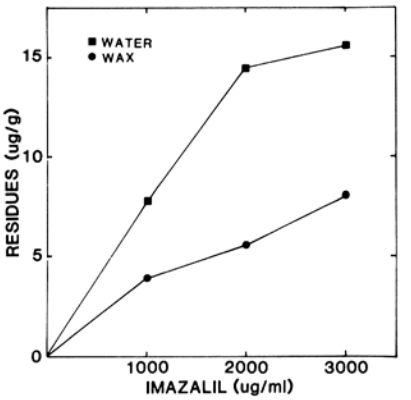

Fig. 2. Residues of imazalil recovered from the peel of Hamlin oranges treated with imazalil in water or water wax.

Table 1. Sporulation of *Penicillium digitatum* on inoculated Valencia oranges treated with imazalil in water or water wax

Imazalil (μg/ml)	Sporulation index[a]
Water	
0	5.00[b] ± 0.00
2,000	0.44 ± 0.06
3,000	0.24 ± 0.07
4,000	0.31 ± 0.10
Wax	
0	4.50 ± 0.19
2,000	1.03 ± 0.17
3,000	0.54 ± 0.09
4,000	0.35 ± 0.07

[a]Sporulation index: 5 = heavy sporulation, 0 = negligible sporulation.
[b]Values represent the mean sporulation index and standard error of 30 fruit.

Residues of imazalil in the exocarp and mesocarp from water applications to the fruit were 19.4 and 1.3 μg/g, respectively. Applications of imazalil in water wax resulted in lower residues of 4.2 and 0.4 μg/g in the exocarp and mesocarp.

Residues were not easily removed by washing fruit on tumbler brushes, whether the imazalil was applied in water or water wax. Only 3% of the residues from water applications were removed by washing, whereas 31% were removed from fruit treated with imazalil in the wax. Peel residues from water applications were 11.1 μg/g, and washing reduced these to 10.8 μg/g. Wax applications left residues of 3.6 μg/g of peel, which were reduced by washing to 2.5 μg/g.

Residues of imazalil in the peel of Hamlin oranges degreened before treatment were similar to those in nondegreened fruit. Injured peel tissue accumulated higher residues of imazalil than uninjured tissue. Residues in injured tissue were higher from applications of imazalil in water wax. Injured peel receiving water applications of imazalil contained 27.4 μg/g of peel, whereas 42.2 μg of imazalil was recovered from each gram of peel treated with the wax formulation.

Residues of imazalil within the peel of oranges were increased by treating the fruit on the brushes for an additional length of time. A water application of imazalil for 15 sec produced a residue of 7.6 μg/g of tissue. Residues were increased to 14.1 μg/g, nearly double, by increasing the time of brushing to 1 min. An additional 1 min of brushing, or a total of 2 min, did not enhance the residues any more than the 1-min treatment.

Higher incidences of green mold developed in fruit sprayed with applications of imazalil in wax rather than in water when inoculations with *P. digitatum* were made after fungicide treatment. An application of imazalil in water (1,000 μg/ml) prevented infection from subsequent inoculations. An application of 3,000 μg/ml was required for similar control when imazalil was applied in water wax. A similar response was also noted when the effect of water and wax applications was evaluated on sporulation by *P. digitatum*. Control of sporulation was better with 2,000 and 3,000 μg imazalil per milliliter of water than of water wax, but 4,000 μg/ml was equally effective whether applied in water or wax (Table 1). Fruit treated with aqueous imazalil applications (2,000 μg/ml) had decay with brownish discoloration of the peel but mycelium on the fruit surface was scant (Fig. 3A). Conversely, oranges treated with the same imazalil concentration in wax often showed surface mycelium that usually did not sporulate (Fig. 3B).

DISCUSSION

Applications of nonrecovery sprays containing imazalil in water apparently resulted in better penetration of the fungicide into the uninjured exocarp than when imazalil was applied with water-based wax. Fruit treated with imazalil in water were less susceptible to infection through posttreatment injuries than fruit treated with imazalil in wax. Such injuries removed the cuticle and epidermal cells and exposed the parenchyma cells. In treatments with imazalil in water, these cells contained higher levels of imazalil that were sufficient to inhibit growth of *P. digitatum*. Better penetration in water was also confirmed with the sporulation experiment. Finally, washing removed more imazalil from wax-imazalil combinations than water treatments, indicating better penetration of imazalil in the presence of water and therefore less removal during washing.

Resin solution waxes contain several alkali-soluble or resinlike materials, such as shellac, proteins, natural gums, tall oil, or wood resins, which are often modified with organic and mineral acids or glycerol (7). The formulas may also contain various organic acids, wetting agents, and oils that act as leveling agents and plasticizers. These waxes are also somewhat more viscous than water. Systemic movement of imazalil through the cuticle into the exocarp could be hindered by one or more of the constituents in the water-wax formulation. The pH of solutions containing imazalil determines lipophilicity and changes in the oil/water partition coefficient (15). The alkali of the wax (pH 9.5) may partition more imazalil into the wax, leaving less compound available in the aqueous phase to penetrate the peel.

The variation in residues from identical treatment concentrations used in the various experiments was disconcerting although such variation was also evident in other published work (6,9,13,17,18). Residues of imazalil were enhanced by increasing the time of fungicide application. Some differences of 5–10 sec in application time between treatments could have accounted for much of the variation observed between experiments because we had concluded that no additional residue was obtainable after complete coverage and runoff. Evidently, this is not so, and additional brushing action may remove or redistribute natural waxes on the fruit surface that are important barriers to movement of pesticides through the cuticle (4).

Injured tissue, which is required for infection by wound pathogens, absorbed higher levels of imazalil than intact peel. Penetration of injured cells apparently occurred more readily than movement through the intact cuticle into uninjured cells. Greater deposits of the more viscous wax probably accumulated on roughened injured surfaces, giving rise to greater imazalil residues than observed with water treatments.

Although variation in residues existed between experiments, levels of imazalil were consistently higher from nonrecovery water applications than from wax applications. The peel appeared nearly saturated with imazalil after aqueous applications of 2,000 and 3,000 μg/ml. Better movement and greater accumulation of imazalil within the exocarp probably occurred in the presence of water even though a thicker surface deposit remained from applications in water wax. Under commercial conditions, residues from nonrecovery water applications may be less than reported in this paper. Aqueous fungicide applications are often followed by brushing to enhance drying and fruit shine and by water-wax applications that reportedly reduce surface residues of commercial fungicides in current use (2). The removal, however, may be less with imazalil because washing was only

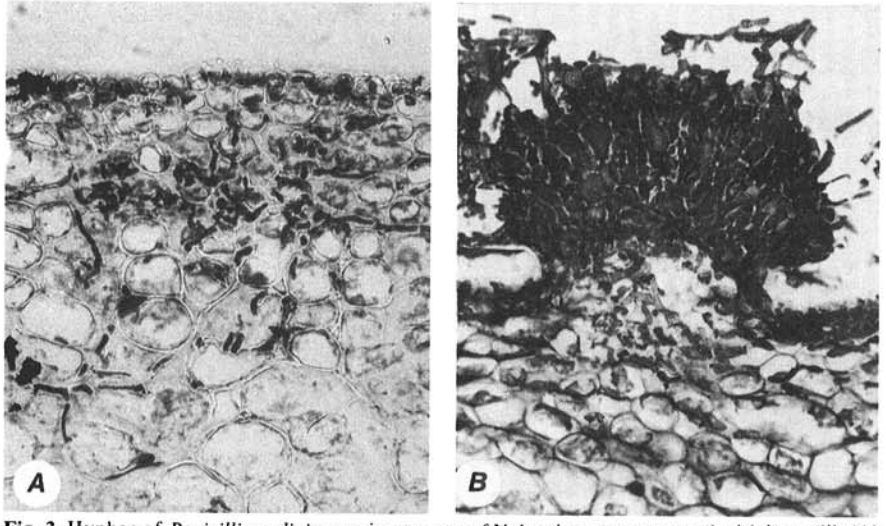

Fig. 3. Hyphae of *Penicillium digitatum* in exocarp of Valencia oranges treated with imazalil. **(A)** Restriction of hyphae within the exocarp of an orange treated with imazalil in water. **(B)** Eruption of sporophores through the epidermis of an orange treated with imazalil in water wax. Spores were not produced by these sporophores.

partially effective in removing surface residues in this and other studies (9).

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