JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Residue Level, Persistence, and Storage Performance of Citrus Fruit Treated with Fludioxonil

MARIO SCHIRRA,*,† SALVATORE D'AQUINO,† AMEDEO PALMA,† SALVATORE MARCEDDU,† ALBERTO ANGIONI,‡ PAOLO CABRAS,‡ BARBARA SCHERM,§ AND QUIRICO MIGHELI§

C.N.R. Istituto di Scienze delle Produzioni Alimentari, Sezione di Sassari, via dei Mille, 07100 Sassari, Italy, Dipartimento di Tossicologia, Università di Cagliari, via Ospedale 72, 09124 Cagliari, Italy, and Dipartimento di Protezione delle Piante−Centro interdisciplinare di eccellenza per lo sviluppo della ricerca biotecnologica e per lo studio della biodiversità della Sardegna e dell'area mediterranea, Università di Sassari, Via Enrico De Nicola 9, 07100 Sassari, Italy

The potential of postharvest dip treatments with fludioxonil (FLU) (a synthetic analogue of the bacterial metabolite of pyrrolnitrin), in controlling postharvest decay caused by *Penicillium digitatum* and *Penicillium italicum* of citrus fruit was investigated in comparison with the conventional fungicide imazalil (IMZ). The ultrastructural changes of fruit epicuticular wax was investigated as a function of water dip temperature, and the possible role of these changes was related to residue accumulation under FLU treatment. Residues retained by fruit were determined as a function of fungicide concentration, dip temperature, and fruit storage conditions. Scanning electron microscopy analysis revealed that fruit dipping in water at 30 or 40 °C did not cause differences in cuticular wax's ultrastructure in comparison to control fruit, while treatments at 50, 55, or 60 °C caused the disappearance of wax platelets, resulting in relatively homogeneous skin surface, due to partial "melting" of epicuticular wax. Residues of FLU in fruit treated at 20 or 50 °C were significantly correlated with the doses of fungicide applied. When equal amounts of fungicide were employed, the residue concentrations were notably higher (from 2.6- to 4-fold) in fruit treated at 50 °C than in fruit treated at 20 °C. The dissipation rate of FLU in "Salustiana" and "Tarocco" oranges was lower in fruit subjected to treatment at 50 °C. The minimal FLU concentration for almost complete decay control in artificially wounded fruit during 7-d storage at 20 °C was 400 mg/L active ingredient (ai) in fruit treated at 20 °C and 100 mg/L ai in fruit treated at 50 °C. Results on nonwounded Tarocco oranges subjected to 3 weeks of simulated quarantine conditions at 1 °C, plus 6 weeks of standard storage at 8 °C and an additional two weeks of simulated marketing period (SMP) at 20 °C revealed that almost complete decay control with FLU applications of 100 mg/L at 50 °C and 400 mg/L at 20 °C resulted in ca. 0.8 mg/kg FLU fruit residues, in agreement with results on wounded citrus fruit. When equal concentrations and temperatures were applied, FLU treatments were as effective as IMZ. In vitro trials showed a low sensitivity to FLU against *P. digitatum* and *P. italicum* isolates. MIC values for the complete inhibition of mycelium growth were $\geq$100 μg/mL, while ED_{50} values ranged from 0.1 to 1 μg/mL for *P. digitatum* and from 1 to >100 μg/mL for *P. italicum.* The latter result suggests that care should be taken to avoid exclusive application of FLU in a sustainable program for management of fruit decay. However, integrating fungicide application and hot water dip may reduce the possibility of selecting fungicide-resistant populations of the pathogen, by increasing the effectiveness of the treatment.

KEYWORDS: Citrus fruit; disease control; hot water dip; fungicide treatments; fludioxonil; imazalil; residues; fungicide resistance, phenylpyrrole

INTRODUCTION

The increased interest in naturally occurring compounds as a source of antimicrobial agents or as starting points for chemical synthesis of novel active ingredients together with the knowledge of the structures and physical properties of some of these compounds has prompted the synthesis of novel, broad-spectrum, "natural mimetic" fungicides with different mecha-

* Corresponding author permanent address: C.N.R. ISPA, Sezione di Sassari, Unità staccata di Oristano, Località Palloni, 09170 Oristano, Italy. Telephone: +39 0783 33224. Fax: +39 0783 33959. E-mail: mario.schirra@ispa.cnr.it.

† Istituto di Scienze delle Produzioni Alimentari.
‡ Università di Cagliari.
§ Università di Sassari.

10.1021/jf051004w CCC: $30.25
Published on Web 08/04/2005

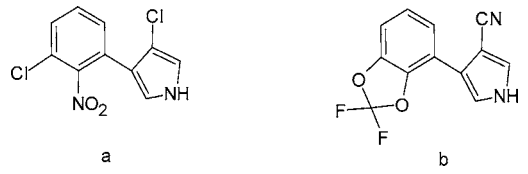

Figure 1. Chemical structure of pyrrolnitrin (**a**) and fludioxonil (**b**).

nism of action compared to other currently registered pesticides for a given crop group (*1*, *2*). Pyrrolnitrin (**Figure 1a**), for example, a secondary metabolite of *Pseudomonas pyrrocinia* (*3*), produced by various *Pseudomonas* spp. with a role in some biocontrol systems, especially in the control of strawberry gray mold (*4*, *5*), is effective against a wide range of Mitosporic, Ascomycete and Basidomycete fungi. However, pyrrolnitrin is not suitable for use as an agricultural fungicide, because it is highly photodegradable (*6*). By contrast, the phenylpyrrole fludioxonil (FLU) (**Figure 1b**), a synthetic analogue of pyrrolnitrin developed for chemical control of *Botrytis cinerea* in viticulture (*7*), is more resistant to sunlight than the naturally occurring compound pyrrolnitrin. FLU is rated as "reduced risk" toxicology by the US Environmental Protection Agency (*8*). It inhibits spore germination, germ tube elongation, and mycelium growth of *B. cinerea* and induces morphological alterations of germ tubes (*7*, *9*). It is also active against other fungi, such as *Monilinia* spp., *Sclerotinia* spp. (*10*), and *Penicillium expansum* (*11*, *12*).

The degradation trend of FLU has been investigated in grape after standard field treatments (*13*) and in lettuce and table grape under field conditions and during refrigeration (*14*). Using model systems, Garau et al. (*15*) elucidated the mechanism of disappearance of FLU residues from tomatoes grown in a commercial greenhouse.

The present study aimed at estimating (a) the influence of fungicide concentration and temperature on the pattern of active-ingredient uptake and persistence in oranges subjected to postharvest dip treatment with FLU; (b) the ultrastructural changes of fruit epicuticular wax as a function of water dip temperature and the potential significance of these changes for fruit residue accumulation under FLU treatment; and (c) the effectiveness of postharvest dip treatments with FLU in comparison to the fungicide imazalil (IMZ), which is widely used on a commercial scale to control green and blue molds of citrus fruit.

MATERIALS AND METHODS

Fruit. Oranges [*Citrus sinensis* (L.) Osbek] cvs. Salustiana and Tarocco, lemons [*Citrus limon* (L.) Burm.] cv. Eureka, grapefruits [*Citrus paradisi* (Marcf.)] cv. Marsh Seedless, clementine mandarins [*Citrus reticulata* (Blanco)] cv. Monreal and mandarins cv. Palazzelli and Tardivo di Ciaculli were hand-harvested in the middle of the harvest season from an experimental orchard located in central western Sardinia (Italy), receiving standard horticultural practices. Fruits were placed in plastic trays and delivered to the laboratory immediately after harvest. Then, 40 fruits of medium size and free from rind defects were selected, numbered, returned to each box and left overnight at 20 °C.

Scanning Electron Microscopy (SEM) Analysis. SEM analysis was performed on oranges cv. Salustiana subjected to 3-min dipping in water at 20, 30, 40, 50, 55, and 60 °C. Samples of five fruits per treatment were used for SEM analysis. Two rind samples (2 × 2 cm) were excised with a new razor blade from the equatorial zone of each fruit and immediately fixed in a phosphate buffer (pH 7.4) containing 3% gluteraldehyde. They were then kept at 4 °C until further preparation. The fixed tissue was rinsed three times in phosphate buffer (pH 7.4) and then three times in deionized water**.** They were dried by washing with increasing concentrations of ethanol (20, 50, 70, 80, 95, and 3 × 100%), the samples being left for 20 min before each wash. The dried samples were placed on aluminum stubs using silver conductive glue, and a gold−palladium coating was applied with an Edwards S-150 A sputter coater. Until observation, samples were stored in a vacuum-dryer. SEM was carried out with a ZEISS DSM 962 microscope at 30 kV and 50−5000× magnifications.

Influence of Fungicide Concentration and Treatment Temperature on Residue Levels. Oranges cv. Salustiana were subjected to the following 3-min dip treatments: (a) treatment with a FLU/water mixture containing 25, 50, 100, 200, or 400 mg/L active ingredient (ai) at 20 or 50 °C or (b) treatment with FLU/water mixture containing 150 mg/L ai at 20, 40, 50, or 60 °C. Following treatments, fruit were left to dry at room temperature for approximately 5 h and then peel samples were taken and kept frozen at −18 °C until analysis. All treatments were performed on four replicates of six fruits per treatment.

Fludioxonil and Imazalil Analysis. *Chemicals.* Acetone and hexane were of GC grade (Merck, Milan, Italy). Sodium chloride was analytical grade (Carlo Erba, Milan, Italy). The active ingredients standards, FLU (at 95%) and IMZ (at 97%), were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions of the ai (500 mg/kg) were prepared in acetone. Working standard solutions of ai were prepared by diluting the stock solutions with the extract from the peel of untreated fruit.

Extraction Procedure. Five fruits per replication were weighed, and their peel was removed. The peel was weighed and its percentage with respect to the whole fruit was calculated. It was then minced with a mincing knife and homogenized. The samples were stored in a freezer at −20 °C until analysis. For IMZ extraction, a 2.5-g aliquot of peel sample was weighed in a 40-mL screw-capped tube; 20 mL acetone/hexane mixture (1/1, v/v) and 6 g of NaCl were added. For FLU extraction, 5 g of homogenized sample was weighed in a 40-mL screw-capped flask to which 10 mL of acetone/hexane (1/1) and 6 g of NaCl were added. Then, the mixtures were agitated in a rotary shaker for 20 min. Subsequently, the phases were allowed to separate and the organic layer was injected in GC for the analysis without any cleanup step.

Apparatus. A TQ Trace gas chromatograph, coupled with a NPD 80 detector, a split/splitless injector, and a AS200 autosampler (Termo Quest, Milan, Italy) was used. The column was a fused silica capillary DB 5 MS (30 m × 0.25 mm i.d.; 0.25 μm, J & W Scientific, Folsom, CA). The injector and detector temperatures were set at 200 and 300 °C, respectively. The sample (2 μL) was injected in splitless mode (30 s). The oven temperature was programmed as follows: 110 °C (1 min), raised to 310 °C (at 20 °C/min). Helium was the carrier gas and N_2 the make up at 1.1 and 15 mL/min, respectively. NPD conditions were as follows: source current, 2.8; polarization voltage, 3.5. Oxygen and H_2 flows were at 60 and 2.3 mL/min, respectively.

Recovery Assays. Samples of untreated citrus peel were fortified with appropriate volumes of stock standard solutions to reach concentrations of 0.1, 0.5, and 1 mg/kg. The samples were allowed to settle for 30 min prior to extraction and then processed according to the above procedure. Average recovery from four replicates showed values of FLU ranging from 84 to 96% with a RDS of 6% while IMZ ranged between 87 and 102% with a RDS of 7%.

Storage Response to Fludioxonil and Imazalil Treatments. *Assays with Wounded Fruit.* Trials on wounded fruit were performed to favor the development of green and blue mold pathogens. Fruits were wounded by four slits (3 × 3 mm) equatorially, at the four opposite sides, and kept at 20 °C for 24 h before treatments. To assess the minimal concentration of fungicide for controlling decay, Monreal and Palazzelli mandarins were subdivided into 10 treatment groups corresponding to 3-min dipping treatment in water containing 0, 50, 100, 200, or 400 mg/L ai FLU at 20 or 50 °C. On the basis of results on Monreal and Palazzelli mandarins, treatment groups for wounded Tarocco oranges, Eureka lemons, Tardivo di Ciaculli mandarins, and Marsh Seedless grapefruits were six, corresponding to the following treatments: water treatment at 20 °C or at 50 °C, FLU or IMZ at 400 mg/L and 20 °C, FLU or IMZ at 100 mg/L and 50 °C. Following treatments, fruit were left to dry at room temperature. Finally, the fruits were moved to a storage room and kept at 20 °C and ca. 90% relative humidity (RH), and the percentage of rotten fruit was determined after

1 week. Dip treatments were performed as described elsewhere in detail (*16*). Each treatment was performed on three replicates of 40 fruit.

Assays with Sound Fruit. Oranges cv. Tarocco were grouped into six treatment groups (five fruit boxes per treatment, forty fruits per box), corresponding to the following 3-min dip treatments: (I) H_2O at 20 °C (control), (II) FLU at 400 mg/L and 20 °C, (III) IMZ at 400 mg/L and 20 °C, (IV) H_2O at 50 °C, (V) FLU at 100 mg/L and 50 °C, (VI) IMZ at 100 mg/L and 50 °C. Fungicide concentrations refer to active ingredient. Treatment groups for Salustiana oranges were four (two fruit boxes per treatment), not including water treatments at 20 or 50 °C. Following treatment, fruit were left to dry at room temperature for approximately 5 h. Salustiana oranges were used only for FLU residue analysis, carried out after treatment (time 0) and after 3 weeks at 17 °C. Each treatment group of Tarocco oranges was divided into two subgroups. Three replicate fruit boxes from the first subgroup were used for visual assessment, which included chilling injury (CI), decay, treatment damage, and external fruit quality (*16*). The fruits of the remaining group were used for FLU and IMZ residue analysis. Finally, the fruits were moved to a ventilated room and kept at cold quarantine conditions at 1 °C for 3 weeks, applied to comply with quarantine regulations to prevent the spread of fruit flies within flesh fruit (*17*). After quarantine, fruit were stored for 6 weeks at 8 °C. Fruits were then held at 20 °C for 2 weeks to simulate a 2-week marketing period (SMP). Relative humidity during quarantine, storage, and SMP was set at 90%.

In Vitro Assays. Monosporic isolates of *P. digitatum* (Saccardo: Fries) (coded PD-8, PD-5, and PD-a) and *P. italicum* Wehmer (coded PI-4, PI-7, and PI-b) were obtained from rotten oranges cv. Tarocco harvested in an orchard located in central western Sardinia (Italy) and cultured on potato dextrose agar (PDA, Merck) amended with streptomycin sulfate and oxytetracycline hydrochloride (100 + 100 μg/mL) to prevent growth of bacterial contaminants. Sensitivity to FLU (Savior Flowable 20, 20% ai, Syngenta) and IMZ (Deccozil 50, 44.66% ai, Elf Atochem, Janssen Pharmaceutica N. V., Beerse, Belgium) was tested at the final concentration of 0.1, 1, 10, and 100 μg/mL ai. Fungicides were added to molten PDA at 45 °C, as 1000-fold concentrated aqueous suspension. Plugs (8 mm diameter) of PDA were cut with a sterile cork borer from actively growing cultures of the different isolates and transferred to fungicide-containing media (three plugs per plate). The radial growth of the fungus was counted after 4−8 d of incubation at 25 °C. The results are expressed as 50 % effective dosage (ED_{50}) and minimum inhibitory concentration (MIC, minimum concentration of the antimicrobial agent in a given culture medium that completely inhibits microbial growth) values in μg/mL ai after 8 d of growth at 25 °C. The experiment included three replicate plates for each isolate/substrate combination and was repeated twice.

Statistical Analysis. Analysis of variance (ANOVA) was performed by Statgraphics (Manugistics, version 5 Professional, 2000) statistical program, according to a single factor, randomized complete block design. Percentages were subjected to arcsin-square-root or square-root transformation before the ANOVA, depending on the range of variation of data (*18*). Mean comparisons of the effects of treatments were calculated, where applicable, by Tukey's test, $P \leq 0.05$. To determine the pattern of ai accumulation in fruit, plots of residue uptake against dip temperature or fungicide concentration were computed for each data set, and the maximum squares of correlation coefficients found were used to determine the equation of best fit curve.

RESULTS AND DISCUSSION

Scanning Electron Microscopy (SEM) Analysis. The ultrastructure of the epicuticular wax of control fruit exhibited the typical wax layers in the form of rough platelets (**Figure 2A**). Fruit dipping in water at 30 or 40 °C did not cause noticeable differences in cuticular wax's ultrastructure (**Figure 2B,C**). By contrast, treatment at 50, 55, or 60 °C caused the complete disappearance of wax platelets as a result of partial "melting" of the wax and a skin surface that appeared relatively homogeneous (**Figure 2D−F**). Similar changes in epicuticular wax structure have been observed in "Fortune" mandarins subjected to hot water dipping at temperatures ranging from 50 to 54 °C, while higher temperatures (56−58 °C) caused an apparent removal of epicuticular wax (*19*).

Influence of Fungicide Concentration and Treatment Temperature on Residue Level and Persistence. After FLU treatment with 25 mg/L at 20 °C, the residue uptake was 0.17 mg/kg (ai, on a whole-fruit basis) (**Figure 3**). A double fungicide concentration (50 mg/L FLU) did not produce a significant increase in residue uptake compared to the treatment with 25 mg/L FLU, whereas significant increases of residues were recorded when the application rate increased from 100 to 400 mg/L. A nonlinear (cubic) relationship regression equation ($R^2 = 0.98059$, $P \leq 0.0001$) was found between the residue level in the fruits and the amount of fungicide employed at 20 °C. Accordingly, residues of FLU in fruit treated at 50 °C were significantly correlated ($R^2 = 0.93654$, $P \leq 0.0001$) to the doses of fungicide applied and described a nonlinear (cubic) regression equation, but residue levels were remarkably higher (from 2.6- to 4-fold) than those left by the treatments at 20 °C.

It has been reported that following dip treatment with IMZ or thiabendazole (TBZ) fungicides at temperatures ranging from 20 to 50 °C residue levels in oranges is closely related to dip temperatures. (*20*). Treatment at 50 °C produced a deposition of IMZ and TBZ ca. 8- and 2.5-fold higher than that of treatments performed at 20 °C (*20*). Results of this study showed that no significant differences in residue levels were recorded after FLU treatments at 20 and 40 °C (**Figure 4**). Conversely, FLU treatment at 50 and 60 °C produced a deposition that was approximately 2.5- and 5-fold higher than deposition from treatment at 20 °C. Residues of FLU were significantly correlated ($R^2 = 0.90050$, $P \leq 0.0001$) with dip temperature and described a nonlinear (cubic) regression equation. It is possible that physiological and structural changes in the rind may have contributed to the increase in FLU residues in fruit treated at 50 and 60 °C.

Marin et al. (*14*) reported that when FLU is applied as a preharvest spray, dissipation rates in lettuce and grapes are notably higher in the field than under storage conditions at 4 °C in dark. Our results reveal that the dissipation rate of FLU in Salustiana oranges was dependent on treatment temperature and was notably lower in fruit subjected to treatment at 50 °C. Following treatments with 400 mg/L FLU at 20 °C and 100 mg/L FLU at 50 °C, mean values of residue levels in fruit were 1.30 and 1.53 mg/kg, respectively. After 3 weeks of storage at 17 °C residues in fruit treated at 20 and 50 °C were about 38 and 57% of their initial concentrations, respectively (**Figure 5**). Following treatment with 400 mg/L FLU at 20 °C, Tarocco oranges had similar residue levels than fruit treated with 100 mg/L FLU at 50 °C (**Table 1**). Conversely, fruit treated with 100 mg/L IMZ at 50 °C resulted in significantly higher residues than in fruit treated with 400 mg/L IMZ at 20 °C. Both fungicides had a greater persistence when they were applied at 50 °C. The lower dissipation rate in fruit treated at 50 °C than in fruit treated at 20 °C may be ascribed to the better encapsulation of the ai by epicuticular wax as a result of the melting of the wax platelets during treatment with heated fungicide, thereby providing better protection against the chemical.

Storage Response to Fludioxonil and Imazalil Treatments. *Assays with Wounded Fruit.* The predominant fungal pathogen responsible for decay in wounded fruit was green mold (*P. digitatum*). Fruit infected by *P. italicum* were observed only occasionally, and no decay due to other pathogens was detected

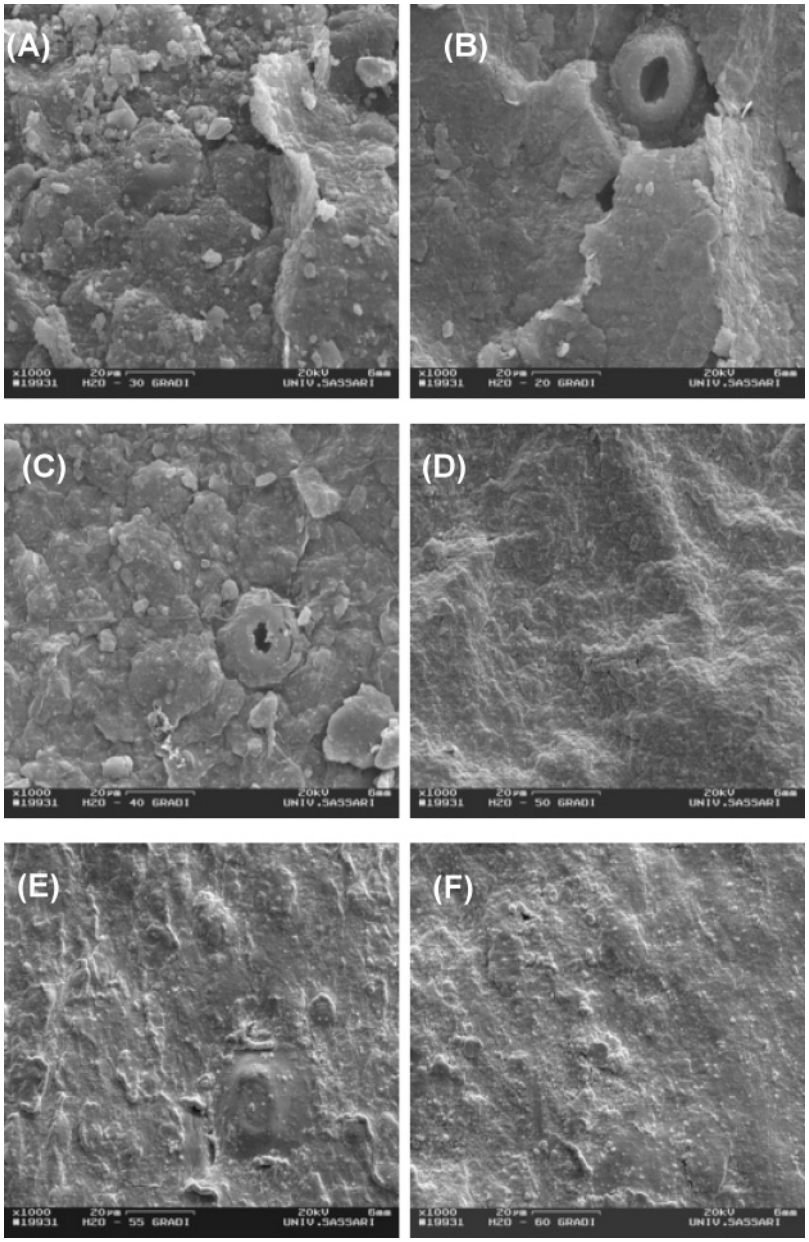

Figure 2. Scanning electron microscopy of epicuticular wax: of Salustiana oranges following 3 min dipping in water at 20 °C (**A**), 30 °C (**B**), 40 °C (**C**), 50 °C (**D**), 55 °C (**E**), or 60 °C (**F**).

(data not shown) The effectiveness of FLU against decay in wounded Palazzelli (**Figure 6**) and Monreal mandarins (**Figure 7**) increased with increasing fungicide concentrations. With respect to fruit dipped in water at 20 °C, treatment with FLU at 50 mg/L and 20 °C provided approximately 62 and 64% decay control in Monreal and Palazzelli, respectively. When higher concentrations of fungicide were supplied, the percentage of rotten fruit decreased as the fungicide level increased: FLU treatment at 400 mg/L and 20 °C resulted in approximately 93% decay control in Palazzelli and in almost complete control of decay in Monreal mandarins. When equal fungicide concentrations were used, treatments at 50 °C were more effective than at 20 °C. The increased efficacy of FLU treatment at 50 °C compared to treatments at room temperature was related to the synergistic effect of heat, enhanced active ingredient uptake, and diffusion of ai in the cuticular wax (*20*−*23*). The minimal concentration of 100 mg/ L ai FLU at 50 °C provided almost complete control of decay, i.e., 4-fold less than treatment at 20 °C.

It has been reported (*12*) that FLU, at a concentration of 100 μg/mL ai, effectively controlled blue mold caused by TBZ-sensitive and -resistant isolates of *P. expansum* (Link) in the cotreatment and postinoculation treatment on "Empire" and "Gala" apples. Later studies (*24*) have proven that FLU treatments have both curative and protective activity against blue mold caused by *P. expansum* on apples. A concentration of 300 mg/L completely suppressed TBZ-resistant and -sensitive strains of *P. expansum* in Empire apples kept for 105 d under controlled atmosphere at 2 °C or 42 d in standard storage conditions at 4 °C, whereas 450 mg/L were required to achieve complete control of blue mold during the further 6 d of simulated shelf-life conditions at 20 °C (*24*). Results of this study on wounded citrus fruit confirmed those results and revealed that FLU and IMZ treatments similarly suppressed decay development upon application of equal active ingredients and temperatures. In comparison with fruit dipped in water at 20 °C, hot water treatment provided some measure of decay control, depending on species: it was nonsignificant in Palazzelli mandarins, but

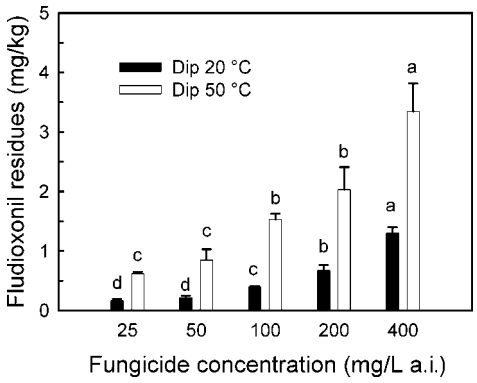

Figure 3. Influence of fludioxonil (FLU) concentration on residue levels (mg/kg on a whole-fruit basis) in Salustiana oranges following treatment with FLU-based commercially available fungicide mixture at 20 or 50 °C. The fungicide concentrations refer to active ingredient (ai). Treatments are 3-min dip followed by air-drying of dipped fruit. Vertical bars indicate standard deviation ($n = 4$). Within each treatment temperature, different letters indicate statistical differences as per Tukey's test, $P \leq 0.05$.

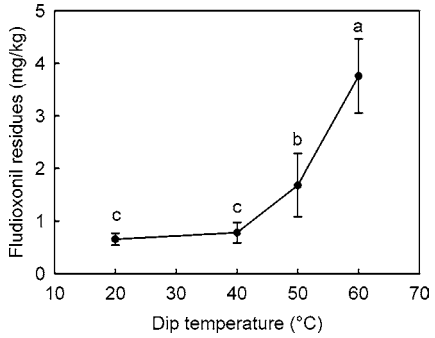

Figure 4. Influence of dip temperature on fludioxonil (FLU) residues (mg/kg on a whole-fruit basis) in Salustiana oranges, following treatment with 150 mg/L FLU-based commercially available fungicide mixture. The fungicide concentrations refer to active ingredient (a.i.). Treatments are 3-min dip followed by air-drying of dipped fruit. Vertical bars indicate standard deviation ($n = 4$). Different letters indicate statistical differences as per Tukey's test, $P \leq 0.05$.

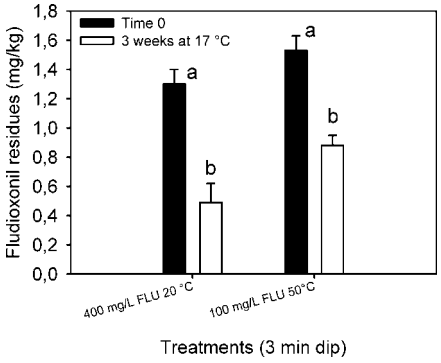

Figure 5. Fludioxonil residues (on a whole-fruit basis) in Salustiana oranges following 3-min dip treatment (time 0) and 3-week simulated marketing period at 17 °C. Vertical bars indicate standard deviation ($n = 4$). Within each treatment, different letters indicate statistical differences as per Tukey's test, $P \leq 0.05$.

it was effective in the other fruit samples (**Figures 6** and **7**, **Table 2**).

Assays with Sound Fruit. No decay development was observed during cold quarantine and there was very little rotting at the end of storage, the percentage rates being 4.2, 2.5, and 0% in fruit subjected to treatment in water at 20 and 50 °C or

Table 1. Fludioxonil and Imazalil Residues (on a whole fruit basis) in Tarocco Oranges following 3-min Dip Treatment (time 0) and after Cold Quarantine for 3 Weeks at 1 °C, Subsequent Standard Storage for 6 Weeks at 8 °C, and a 2-Week Simulated Marketing Period (SMP) at 20 °C

	fungicide residues (mg/kg)[b]	
treatments[a]	time 0	SMP
400 mg/L fludioxonil at 20 °C	0.57 a(a)	0.25 a(b)
100 mg/L fludioxonil at 50 °C	0.77 a(a)	0.49 a(b)
400 mg/L imazalil at 20 °C	0.76 a(a)	0.42 a(b)
100 mg/L imazalil at 50 °C	1.10 b(a)	0.75 b(b)

[a] Treatments are 3-min dips followed by air-drying of dipped fruit. [b] In each row or column grouping, mean separation was by Tukey's test, $P \leq 0.05$. Letters without parentheses relate to comparisons of the effects of treatments, within each storage time. Letters in parentheses relate to comparisons of the influence of storage time, within each treatment.

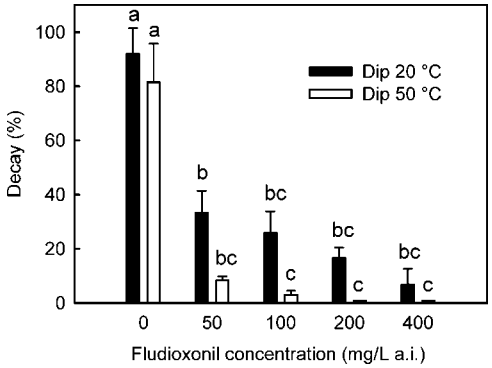

Figure 6. Effect of postharvest treatment in water or FLU-based commercially available fungicide mixture, at 20 or 50 °C, on decay development in artificially wounded Palazzelli mandarins after 1 week of storage at 20 °C. The fungicide concentrations refer to active ingredient (ai). Treatments are 3-min dip followed by air-drying of dipped fruit. Vertical bars indicate standard deviation ($n = 4$). Within each treatment temperature, different letters indicate statistical differences as per Tukey's test, $P \leq 0.05$.

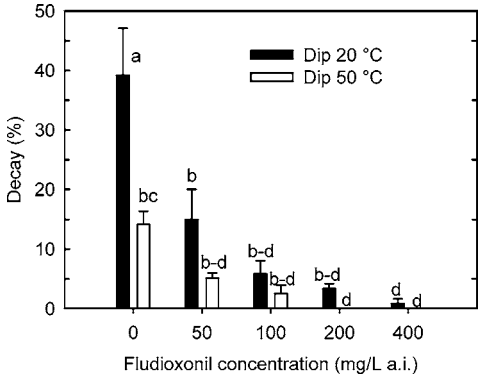

Figure 7. Effect of postharvest treatment in water or FLU-based commercially available fungicide mixture, at 20 or 50 °C, on decay development in artificially wounded Monreal clementines after 1 week of storage at 20 °C. The fungicide concentrations refer to active ingredient (ai). Treatments are 3-min dip followed by air-drying of dipped fruit. Vertical bars indicate standard deviation ($n = 3$). Within each treatment temperature, different letters indicate statistical differences as per Tukey's test, $P \leq 0.05$.

with fungicides, respectively (data not shown). After the subsequent 1 and 2 weeks of SMP, the decay incidence in control fruit (mainly caused by *P. digitatum*) was 10% and 16.7%, respectively (**Table 3**); after 1 week of SMP, there were

Table 2. Influence of 3-min Dip Treatments with Water, Fludioxonil, or Imazalil on Decay Incidence in Artificially Wounded Tarocco Oranges, Eureka Lemons, Tardivo di Ciaculli Mandarins, and Marsh Seedless Grapefruits after 7 d at 20 °C

	decay (%)[b]			
treatments[a]	Tarocco oranges	Eureka lemons	Tardivo di Ciaculli mandarins	Marsh Seedless grapefruits
H_2O 20 °C	80.5 a	19.3 a	99.3 a	38.9 a
H_2O 50 °C	12.7 b	12.5 b	38.7 b	13.2 b
400 mg/L fludioxonil 20 °C	0.0 c	0.2 c	4.0 c	3.3 bc
100 mg/L fludioxonil 50 °C	0.0 c	0.2 c	3.3 c	0.4 c
400 mg/L imazalil 20 °C	0.0 c	1.0 c	1.3 c	2.2 c
100 mg/L imazalil 50 °C	0.0 c	0.0 c	2.7 c	0.4 c

[a] Treatments are 3-minute dips followed by air-drying of dipped fruit. The fungicide concentrations relate to active ingredient. [b] Different letters denote significant differences within a column group at $P \leq 0.05$ by Tukey's test.

Table 3. Influence of 3-min Dip Treatments with Water, Fludioxonil, or Imazalil at 20 or 50 °C on Decay Incidence in Tarocco Oranges Subjected to Cold Quarantine for 3 Weeks at 1 °C, Subsequent Standard Storage for 6 Weeks at 8 °C, and Simulated Marketing Period at 20 °C for 1 Week (SMP1) or 2 Weeks (SMP2)

	decay (%)[b]	
treatments[a]	SMP1	SMP2
H_2O 20 °C	10.0 a	16.7 a
H_2O 50 °C	7.5 ab	10.8 a
400 mg/L fludioxonil at 20 °C	7.5 ab	10.0 ab
100 mg/L fludioxonil at 50 °C	0.8 b	3.3 bc
400 mg/L imazalil at 20 °C	0.8 b	2.5 c
100 mg/L imazalil at 50 °C	0.8 b	3.3 bc

[a] Treatments are 3-min dips followed by air-drying of dipped fruit. [b] Within each storage period different letters denote significant differences by Tukey's test, $P \leq 0.05$.

no significant differences between hot water dipping and fungicide treatment. However, after the second week of SMP, treatments with 400 mg/L IMZ at 20 °C or with heated FLU and IMZ at 50 °C were more effective, with only 2.5−3.3% decay incidence, than either hot water or FLU treatment at 20 °C. Thus, in agreement with the results observed on wounded fruit, almost complete control of decay was achieved with 100 mg/L FLU at 50 °C, with a residue level, following treatment, of ca. 0.8 mg/kg (**Table 1**).

After 2-week SMP, the percentage of fruit with slight, moderate, and severe chilling injury (CI) in control fruit was 2.5, 4.2, and 15%, respectively (data not shown). FLU and IMZ applied at 20 °C did not significantly affect the expression of CI and were as effective as hot water when applied at 50 °C (data not shown). No treatment-dependent effects were recorded on fruit external appearance and organoleptic (flavor and taste) acceptance during storage and SMP (data not shown). Therefore, optimum FLU treatment conditions, 400 mg/L a.i. at 20 °C or 100 mg/L a.i. at 50 °C, resulted in considerable decay control without compromising fruit quality during storage and SMP.

In Vitro Assays. Despite the effectiveness demonstrated by FLU under in vivo trials, all the tested *P. digitatum* and *P. italicum* isolates presented a low sensitivity to this fungicide when tested in vitro. MIC values determining complete inhibition of mycelium growth were ≥100 μg/mL, while ED_{50} values ranged from 0.1 to 1 μg/mL for *P. digitatum* and from >1 to >100 μg/mL for *P. italicum* (**Table 4**). After 8 d of incubation on FLU-amended PDA, resistant sectors of mycelial growth arose frequently from the colonized plugs, and fungal development or sporulation was not inhibited when mycelial fragments or conidia were transferred to PDA containing 100 μg/mL FLU. Moreover, 10 randomly chosen FLU-insensitive mutants of *P. italicum* and *P. digitatum*, respectively, did not exhibit reduced aggressiveness toward artificially inoculated orange fruit (data not shown). The in vitro selection of FLU-insensitive mutants has been described in different fungi, such as *Aspergillus nidulans* and *B. cinerea* (*25*), and field isolates of *Alternaria* spp. exhibiting high levels of resistance to both dicarboximide and phenylpyrrole fungicides were recently reported (*26*). Further characterization of *Penicillium* spp. FLU-insensitive mutants is now being carried out to ascertain the exact nature of this feature and shall be published elsewhere.

Table 4. Effectiveness of Fludioxonil and Imazalil Fungicides against Colony Growth of Three Isolates of *Penicillium digitatum* (PD) and Three Isolates of *Penicillium italicum* (PI)[a]

	fludioxonil		imazalil	
isolate	ED_{50}	MIC	ED_{50}	MIC
PD-8	1	>100	>0.1 to <1	1
PD-5	1	100	>0.1 to <1	1
PD-a	0.1	>100	>0.1 to <1	1
PI-4	100	>100	0.1	1
PI-7	>1 to <10	>100	>0.1 to <1	10
PI-b	>100	>100	>0.1 to <1	10

[a] Results are expressed as ED_{50} and MIC values (μg/mL) after 8 d of incubation at 25 °C on fungicide-amended potato dextrose agar.

The high effectiveness of IMZ was confirmed by in vitro tests, where ED_{50} was 0.1−1 μg/mL and MIC values were included between 1 and 10 μg/mL for the tested isolates of both pathogens (**Table 4**).

Conclusions. The present study depicts the effect of FLU concentration and temperature on the pattern of active-ingredient uptake and persistence in citrus fruit subjected to postharvest dip treatment and demonstrates that FLU applied at 50 °C is effective in controlling green and blue mold in citrus fruit. The lowest threshold of FLU residue concentration in fruit capable of achieving an effective control decay was ca. 0.8 mg/kg, which is notably lower than the maximum residue limit (10 mg/kg) fixed in the United States for citrus fruit (*27*), and was achieved by treating with 400 mg/L FLU at 20 °C or with 100 mg/L FLU at 50 °C.

Given the favorable toxicological profile of FLU and the low effective dose (and residues) needed to control green and blue mold in citrus fruit, this natural mimetic compound may represent a useful option in reducing postharvest losses of citrus fruit when the use of previously registered fungicides such as TBZ and IMZ is off-set by the selections of TBZ- and IMZ-resistant strains. However, the frequent appearance of insensitive mutants of both *P. italicum* and *P. digitatum* on FLU-amended substrate suggests that care should be taken to avoid exclusive application of FLU in a sustainable program for management of fruit decay. Integrating fungicide application and hot water dip may reduce the possibility of selecting fungicide-resistant populations of the pathogen, by increasing the effectiveness of the treatment.

ACKNOWLEDGMENT

The authors gratefully acknowledge the Syngenta Crop Protection, Italy for kindly supplying the fungicide Fludioxonil. Research was partially supported by MiPAF Progetto: "Ricerche e Sperimentazioni nel Settore dell'Agrumicoltura Italiana" Paper no. 144.

LITERATURE CITED

(1) Gullino, M. L.; Leroux, P.; Smith, C. M. Use and challenges of novel compounds for plant disease control. *Crop Prot.* **2000**, *19*, 1−11.

(2) Leroux, P. Modes d'action des produits phytosanitaires sur les organismes pathogèns des plantes. *C. R. Biol.* **2003**, *326*, 9−21.

(3) Arima, K.; Imanaka, H.; Kousaka, M.; Fukuda, A.; Tamura, C. Pyrrolnitrin, a new antibiotic substance produced by *Pseudomonas*. *Agric. Biol. Chem.* **1964**, *28*, 575−576.

(4) Gerth Trowitzsch, W.; Wray VHoffle, G.; Irskic H.; Reichenbach, H. Pyrrolnitrin from *Myxococcus fulvus* (mixobacterales) *J. Antibiot.* **1982**, *35*, 1001−1003.

(5) Roitman, J. N.; Mahoney, N. E.; Janisiewicz, W. J.; Benson, M. A new chlorinated phenylpyrrole antibiotic produced by the antifungal bacterium *Pseudomonas capacia*. *J. Agric. Food Chem.* **1990**, *38*, 538−541.

(6) Umio, S.; Kamimura, T.; Kamishita, T.; Mine, Y. Antifungal composition employing pyrrolnitrin in combination with as imidazol compound. U.S. Patent 4,636,520. **1987**.

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

(8) Environmental Protection Agency (EPA). Fludioxonil: Pesticide tolerances. *Federal Register* **2004**, *69*, 58084−58091.

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

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

(11) Rosenberger, D. A.; Meyer, F. W.; Ahlers, C. A.; Van Camp, R. L. Controlling *Penicillium expansum* in apples in postharvest applications of fludioxonil. *Phytopathology* **2002**, *92* S145 (Abstract).

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

(13) Cabras, P.; Angioni, A.; Garau, V. L.; Melis, M.; Pirisi, F.; Minelli, E. V.; Cabitza, F.; Cubeddu, M. Fate of some new fungicides (Cyprodinil, Fludioxonil, Pyrimethanil, and Tebuconazole) from vine to wine. *J. Agric. Food Chem.* **1997**, *48*, 2708−2710.

(14) Marin, A.; Oliva J.; Garcia C.; Navarro S.; Barba A. Dissipation rates of cyprodinill and fludioxonil in lettuce and table grape in the field and under cold storage conditions. *J. Agric. Food Chem.* **2003**, *51*, 4708−4711.

(15) Garau, V. L.; Angioni, A.; Aguilera Del Real, A.; Russo, M. T.; Cabras P. Disappearance of Azoxystrobin, Cyprodinil, and Fludioxonil on tomato in a greenhouse. *J. Agric. Food Chem.* **2002**, *50*, 1929−1932.

(16) Schirra, M.; Cabras, P.; Angioni, A.; Brandolini, V. Residue levels and storage decay control in 'Star Ruby' grapefruit after dip treatments with azoxystrobin *J. Agric. Food Chem*. **2002**, *50*, 1461−1465.

(17) Armstrong, J. W. Heat and cold treatment, in *Insect Pests and Fresh Horticultural Products: Treatments and* responses, Paull R. E.; Armstrong J. W. Eds.; CAB International, Wallingford U.K. 1994; pp 103−119.

(18) Gomez, K. A.; Gomez, A. A. Statistical procedures for agricultural research. Wiley: New York, 1984; p 680.

(19) Schirra, M.; D'hallewin, G. Storage performance of Fortune mandarins following hot water dips. *Postharvest Biol. Technol.* **1997**, *10*, 229−237.

(20) Cabras, P.; Schirra, M.; Pirisi, F. M.; Garau, V. L.; Angioni, A. Factors affecting imazalil and thiabendazole uptake and persistence in citrus fruits following dip treatments. *J. Agric. Food Chem*. **1999**, *47*, 3352−3354.

(21) Baurr, P.; Schönhen, J. Temperature dependence of the diffusion of organic compounds across plant cuticles. *Chemosphere* **1995**, *30*, 1331−1340.

(22) Schönherr, J.; Baurr, P. Effects of temperature, surfactants and other adjuvants on rates of uptake of organic compounds. In *Plant cuticles: An integrated functional approach*; Kerstiens, G., Ed.; BIOS Scientific Publishers: Oxford, U.K., 1996; pp 135−155.

(23) Schirra, M.; D'hallewin, G.; Ben-Yehoshua, S.; Fallik, E. Host pathogen interaction modulated by heat treatment. *Postharvest Biol. Technol*. **2000**, *21*, 71−85.

(24) Errampalli, D.; Northover, J.; Skog, L.; Brubacher, N. R.; Collucci, C. A. Control of blue mold (*Penicillium* expansum) by fludioxonil in apples (cv Empire) under controlled atmosphere and cold storage conditions. *Pest Manag. Sci.* **2005**, *61*, 591−596.

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

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

(27) USDA Foreign Agricultural Services MRL Data Base, 2005, www.mmrldatabase.com.

Received for review May 2, 2005. Revised manuscript received July 7, 2005. Accepted July 7, 2005.

JF051004W