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# Complexation of Imazalil with β-Cyclodextrin, Residue Uptake, Persistence, and Activity against Penicillium Decay in Citrus Fruit Following Postharvest Dip Treatments

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A method for the inclusion of imazalil (IMZ) in the β-cyclodextrin (βCD), structural characterization of the inclusion complex and its antifungal activity against *Penicillium digitatum* and *P. italicum* assessed by in vitro and in vivo tests are reported. According to the starting stoichiometry of βCD with respect to IMZ, an equimolar ratio β-cyclodextrin−IMZ (βCD−IMZ) was detected by $^1$H NMR. In vitro assays showed that the freshly prepared βCD−IMZ was as effective as IMZ, although 1- and 4-day-old βCD−IMZ mixtures were more effective. Studies on Star Ruby grapefruit showed no significant differences in residue uptake between treatments with an IMZ commercially available fungicide (Deccozil) or βCD−IMZ when equal active ingredient (a.i.) concentrations (250 mg/L) and dip temperatures (20 or 50 °C) were used. By contrast, treatments of Tarocco oranges and Di Massa lemons with 250 mg/L βCD−IMZ at 50 °C produced significant differences in residue uptake in comparison with 250 mg/L Deccozil treatments at 50 °C. The a.i. degradation rate in grapefruit during postquarantine and simulated marketing period (SMP) at 20 °C was not affected by the type of formulation used, whether at 20 or 50 °C. Conversely, IMZ in oranges and lemons had greater persistence when applied at 50 °C. All fungicide treatments showed a comparable efficacy against decay in grapefruit and oranges, whereas treatment in lemons at 250 mg/L a.i. of heated fungicides had higher suppressive effects against decay than unheated chemicals having equal a.i. concentrations and comparable activity at 1200 mg/L IMZ at 20 °C.

**KEYWORDS: Imazalil; cyclodextrins; inclusion complex; citrus; treatments; residue analysis; storage decay**

## INTRODUCTION

Current strategies for postharvest decay control of citrus fruit include proper harvesting, minimizing fungal spore contamination in the grove and packing house, sanitary procedures with chlorine and quaternary ammonium salts, treatments with a broad-spectrum fungicide during fruit washing, and treatment of cleaned fruit with selective fungicides such as benomyl, thiabendazole, and imazalil (*1*). In view of public concerns about human health and the environment, research efforts are focused on the enhancement of host resistance to diseases through chemical, physical, or biological inducers (*2, 3*) or controlled-release (CR) formulations from synthetic or naturally occurring polymers (*4, 5*) or both. The "molecular inclusion" of a pesticide may offer advantages with respect to conventional agrochemical formulates. This methodology entails controlled delivery of the active agent at a constant level for a specified period of time, which provides enhanced drug efficiency, reduced mammalian toxicity and pesticide contamination of the environment, reduced phytotoxic effects to plants, and, often, increased solubility of the water-insoluble compounds.

Despite important benefits in dispensing active agents, there are certain disadvantages related to the biopolymeric matrix, including cost, water solubility, and issues involved in molecular encapsulation of drugs and agrochemicals. In various industrial applications, cyclodextrins (CDs), of which the structure is a torus-like anphipathic macroring built up of glucopyranose units with a lipophilic cavity and an external hydrophilic surface, have been selected as the matrix of CR formulations because they

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can include hydrophobic molecules within their cavity (*6*). The inclusion complexes consist of electronic interactions between the torus-like macroring of cyclodextrin (host) and the active agent (guest), which fits well into the lipophilic cavity of the biopolymeric matrix. Three cyclodextrins producted by enzymatic degradation of starch are readily available: αCD (six glucose units), βCD (seven glucose units), and γCD (eight glucose units), which have glucose units connected through glycosidic α-1,4 bonds. All of them are water soluble and chemically and thermally stable. Both βCD and γCD are not toxic to mammalian and plant species.

Many reports and patents have dealt with the chemical and physical properties of inclusion complexes of cyclodextrins, as well as with agricultural applications of their inclusion complexes, especially β-cyclodextrin (βCD) (*5*−*9*). This is because βCD is commercially available in large quantities and at a price readily affordable for the agrifood, toiletry, and pharmaceutical industries. However, no specific studies have dealt with the feasibility of CR fungicides for postharvest decay control of citrus fruit.

The present study describes a methodology for the inclusion of technical imazalil with β-cyclodextrin, which involves no functional modification either in the active agent or in the β-cyclodextrin. Residue uptake, persistence, and efficacy of the novel inclusion complex in the penicillium decay control of citrus fruit were investigated at both 20 and 50 °C in comparison to an IMZ-based commercially available fungicide.

## MATERIALS AND METHODS

**Preparation of β-Cyclodextrin−Imazalil (βCD−IMZ) Inclusion Complex.** β-Cyclodextrin (CAVAMAX ®7 PHARMA) was obtained from Wacker − Chemie Italia SpA and was used. Imazalil (97%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany) and used without purification. $^1$H NMR spectra of β-cyclodextrin complex were recorded in $D_2O$ solution with a Varian VXR 5000 spectrometer at 299.94 MHz. Chemical shifts are given in ppm (δ), which were measured relative to the peak of the solvent $D_2O$ (4.74 ppm). Multiplicities are indicated by d (doublet), t (triplet), m (multiplet), or dd (double of doublets). All $^1$H NMR spectra were recorded with a 5 mm tube in $D_2O$ without degassing.

β-Cyclodextrin, analytic grade, was dissolved as a 15 mM solution in degassed distilled water under an inert atmosphere at 70 °C for 1 h. One equivalent of IMZ technical grade was added in one pot, and the reaction mixture was heated at 60 °C under stirring for 14 h. After this time, water was evaporated under vacuum, and the inclusion complex was recovered as a pale yellow solid. An equimolar ratio of β-cyclodextrin−IMZ was detected by $^1$H NMR spectrum of the solid under conditions described above. No significant amounts of free β-cyclodextrin was detected in the aqueous solution by $^1$H NMR analysis.

To determine possible release of the guest from the inclusion complex during treatments, we performed $^1$H NMR analysis 30 min after fruit dipping in the βCD−IMZ at 50 °C. A sample (1 L) of the treatment solution was taken, and water was evaporated at 40 °C for approximately 36 h in a thermoventilated oven. Then, the crude was analyzed by $^1$H NMR without any further treatment.

**In Vitro Assays.** Monosporic isolates of *Penicillium digitatum* cv. Sacc. (PHI-26) and *Penicillium italicum* cv. Wehmer (PHI-1), obtained from rotten citrus fruits, were cultured on potato dextrose agar (Difco, Detroit, MI) at 24 °C in the dark. Spore suspensions were obtained from 1-week-old cultures by scrapping the agar surface with a sterile spatula and suspending in sterile water. The suspension was filtered through glass wool, quantified with a hematocytometer and brought to a final concentration of $10^5$ mL$^{-1}$ in potato dextrose broth (PDB, Difco, Detroit, MI). Spore suspensions were inoculated into the different media within 3 h after collection. Growth assays were conducted in 96 well microtiter plates (NUNC, Roskilde, Denmark) in a final volume of 200 μl as described by López-García et al. (*10*). Wells contained 130 μl of PDB, 50 μl of spore suspension, and 20 μl of test compounds from 10-fold concentrated solutions. Plates were incubated at 24 °C without agitation. $A_{492}$ was periodically recorded during 3 days in a microplate reader (Titertek Multiskan Plus; Labsystems, Helsinki, Finland). Three replicates were prepared for each sample, and the mean and standard deviation were calculated after subtraction of background $A_{492}$ values from control noninoculated medium. Imazalil (IMZ, 99.9% active ingredient (a.i.); Riedel-de Haën GmbH, Germany) and βCD−IMZ stock solutions were prepared at 100 μg ml$^{-1}$ a.i. in sterile deionized water and heated at 65 °C for 2 h. βCD stock solution was prepared at 376 μg ml$^{-1}$, which corresponds to the amount of βCD present in the complex stock solution. To detect the controlled release of active IMZ from the βCD−IMZ, stocks solutions were prepared 4, 1, and 0 days in advance and maintained at room temperature until use. All assays were conducted simultaneously with the same spore solutions. The $ED_{50}$, the fungicide concentration that reduced the fungal growth rate by 50% compared to growth in nonamended medium, was determined by interpolation of the linear regression between the relative growth at 72 h of incubation versus the logarithm of the fungicide concentration. The minimal inhibitory concentration (MIC) at which no fungal growth was detected after 72 h of incubation was also recorded.

**Plant Material.** Red-fleshed grapefruit (*Citrus paradisi* var. *macf.*) cv. Star Ruby, blood oranges [*Citrus sinensis* var. (L.) *osbek*] cv. Tarocco and lemons [*Citrus limon* var. (L.) *burm*] cv. Di Massa were hand harvested in the mid-harvest season from an experimental orchard located in central western Sardinia (Italy), receiving standard horticultural care. Harvests involved a random sampling from 12 trees. The fruits were picked from the outside of the canopy of each tree, placed in plastic trays, delivered to the laboratory immediately after harvest, graded, sized, and left overnight at 20 °C.

**In Vivo Assays with Wounded and Artificially Inoculated Grapefruit**. Two-hundred grapefruits were surface sterilized by 5 min immersions in a 2% sodium hypochloride−water solution, rinsed in boiled water, and left to dry at ambient temperature. Fruits were wounded 1 h before inoculation. On each fruit, three slits (apical, central, and basal, 3 mm × 3 mm) at the four opposite sides were made. Wounds were inoculated with 25 μL aliquots of a *P. digitatum* conidia suspension ($10^4$ mL$^{-1}$) and kept at 20 °C for 24 h before treatments. For the artificial inoculation experiments, *P. digitatum* spore suspensions were obtained from 5-day-old cultures by suspending them in sterile distilled water containing 10 μL of surfactant (Twin 20, Merck Sharp and Dohme, Netherlands). Then, fruits were subdivided into 8 groups of 25 fruits (replicates) per treatment and subjected to the following 3 min dip treatments: (I) $H_2O$ 20 °C; (II) 1200 mg/L a.i. imazalil-based fungicide, Deccozil 50 (Deccozil) (Elf Atochem, Janssen Pharmaceutica N. V. Beerse, Belgium (44,66% a.i.) 20 °C; (III) 250 mg/L Deccozil 20 °C; (IV) 250 mg/L βCD−IMZ 20 °C; (V) $H_2O$ 50 °C; (VI) 250 mg/L Deccozil 50 °C; (VII) 250 mg/L βCD−IMZ 50 °C. Noninoculated fruits were used as control.

Dip treatments were performed using an apparatus described by Schirra and D′hallewin (*11*). Following treatment, fruits were left to dry at room temperature for approximately 5 h. Finally, the fruits were moved to a storage room and kept at cold quarantine conditions at 1 °C and ca. 85% relative humidity (RH) for 16 days (*12*). Then, fruits were stored for 3 weeks at 8 °C and for one additional week at 20 °C, 80% RH. The percentage of infected wounds was determined after quarantine, storage, and simulated marketing period (SMP).

**Storage Experiments with Sound Fruit.** Grapefruits and oranges were subdivided into 7 groups (9 trays containing 40 individually numbered fruits each) and subjected to treatment with water, Deccozil, or βCD−IMZ as were wounded and artificially inoculated fruit. Treatment groups for lemons were nine, that is two more treatment groups than grapefruit and oranges, including the β-cyclodextrin treatments at 20 and 50 °C. Each treatment group (9 trays containing 40 individually numbered fruits each) was then divided into three subgroups. Four replicate fruit boxes from the first subgroup were used for assessment, which included chilling injury, decay, treatment damage, and external fruit quality. Four replicate fruit boxes from the second subgroup were used for organoleptic acceptance and IMZ analysis. The fruits from the remaining box were individually weighed to determine the transpiration rate from fruit mass loss. Finally, the fruits were

subjected to cold quarantine as were wounded and artificially inoculated fruit. After quarantine grapefruits and oranges were stored for 3 weeks at 8 °C and ca. 85% RH, while lemons were stored for 8 weeks at 11 °C. Fruit were then stored at 20 °C and 80% RH for another 1 (grapefruits) or 2 weeks (oranges and lemons) to simulate 1 or 2 weeks of marketing period (SMP).

**Visual Assessments, Organoleptic Acceptance, and Fruit Weight Loss.** Visual assessments included chilling injury (CI), decay incidence, and external appearance. CI and rot incidence were evaluated at the end of quarantine, storage, and shelf life. CI (peel pitting and brown staining) was scored as slight when light-brown CI covered less than 5% of the rind surface and the damage was not objectionable and would not deter purchase by consumers, moderate when darker brown spots and depressions covered up to 25% of the rind surface (some consumers might reject these fruit unless they could be purchased at reduced prices), and severe when injury covered over 25% of the rind surface and the fruit would therefore be rejected. Then, the percentage of fruit in each rating was calculated. To obtain a weighted average for a CI index, the number of fruit in each CI rating was multiplied by the designated number and a weighted average was calculated using the following formula: $CI_x = [\{(\text{percent of fruit with slight CI} \times 1) + (\text{percent of fruit with moderate CI} \times 2) + (\text{percent of fruit with severe CI} \times 3)\}/100]$. Decay incidence was assessed as total rots caused by blue mould (*P. italicum*), green mold (*P. digitatum*), brown rot (*Phytophtora citrophthora*), or miscellaneous rots of other fungi. External appearance was rated subjectively into one of five categories (5 (excellent), 4 (good), 3 (fair), 2 (poor), and 1 (very poor)), and an average value was calculated. Organoleptic acceptance (fruit flavor and taste) was scored as 3 (good), 2 (fair), and 1 (poor). Fruit weight loss was determined at the end of cold quarantine, after storage, and subsequent to simulated shelf life conditions.

**IMZ Analysis**. *Chemicals*. Acetone and hexane were of analytical grade (Merck, Milan, Italy). Imazalil (97%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions of imazalil (500 mg/kg) were prepared in acetone. Working standard solutions of IMZ were prepared by diluting with extract from untreated matrix.

*Apparatus and Chromatography*. A TQ trace gas chromatograph, coupled with a NPD 80 detector and a AS2000 auto sampler (ThermoQuest, Milan, Italy), was used. The column was a fused silica capillary DB 35 (30 m, 0.25 mm; 0.25 $\mu$m). The injector and the detector were at 200 and 280 °C, respectively. The sample, 1 $\mu$L, was injected in the split (1/25) mode. The oven was programmed as follows: 110 °C (1 min), raised to 130 °C (5 °C/min) and then to 300 °C (15 °C/min), and hold for 5 min.

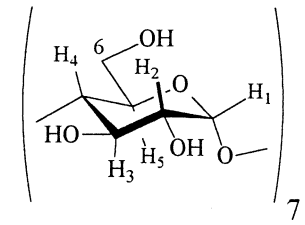
*Gas Chromatography (GC) Analysis of IMZ in βCD−IMZ Complex*. The $\beta$CD−IMZ complex (0.1 g) was dissolved with 100 mL of water−acetone (1:1 v/v). This solution was diluted in acetone up to 10 ppm IMZ concentration (a.i.) and then injected into the GC for quantitative analysis of IMZ. To avoid any matrix effect, the work solutions of IMZ were prepared in the same manner using equal concentration of $\beta$CD.

*Peel Sample Preparation and GC Analysis*. From each group subjected to treatment with IMZ or $\beta$CD−IMZ, five fruits per replication were weighed; their peel was removed and weighed, and its percentage with respect to the whole fruit was calculated. Samples of peel were then triturated with a mincing knife and homogenized. Five grams of sample was collected in a 40 mL screw cap flask. Ten milliliters of a mixture of hexane/acetone (1:1, v/v) and 2 g of NaCl were added. The mixture was agitated in a rotatory shaker (Stuart Scientific) for 20 min, and the organic solution was injected into the GC for analysis.

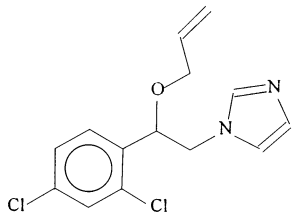
**Statistical Analysis**. Analysis of variance (ANOVA) was performed by MSTAT-C software (1991). Mean comparisons were performed by Tukey's test at $P \leq 0.05$ or 0.01, where appropriate.

## RESULTS

**NMR Properties of βCD−IMZ Inclusion Complex.** $^1$H NMR spectroscopy, which has proved to be a good diagnostic tool, as well as being useful in the study and characterization of $\beta$CD inclusion complexes, was used to prove the formation of the $\beta$CD−IMZ inclusion complex and to measure the host−guest molar ratio in water. Because of the water solubility of the $\beta$CD−IMZ complex at 20 °C at a reasonable concentration (>2 mM), $^1$H NMR experiments, performed in $D_2O$, provide further experimental evidence of the formation of this complex because free IMZ is soluble only in chlorinated organic solvents. According to $^1$H NMR data on $\beta$-cyclodextrin complexes reported in the literature (*6*, *13*, *14*), chemical shifts of $H^3$ and $H^5$ $\beta$CD protons, which point into the lipophilic cavity, are a useful probe to observe formation of inclusion complexes and, hence, to evaluate the structural modification of $\beta$CD (**Figure 1**).




β-Cyclodextrin




Imazalil

**Figure 1.** Structure of $\beta$-cyclodextrin (guest) and imazalil (host).

**Table 1.** $^1$H NMR Shifts (ppm) of $\beta$CD and $\beta$CD–IMZ in $D_2O$ and the Corresponding Complexation Shifts ($\Delta\delta = \delta_{complex} - \delta_{free}$, Hz)

| | $H^1$ | $H^2$ | $H^3$ | $H^4$ | $H^5$ | $H^6$ |
|---|---|---|---|---|---|---|
| free $\beta$CD | 4.85 (d) | 3.58 (dd) | 3.89 (t) | 3.51 (t) | 3.76 (m) | 3.80 (m) |
| $\beta$CD–IMZ | 4.81 (d) | 3.48 (dd) | 3.70 (t) | 3.46 (t) | 3.57 (m) | 3.70 (m) |
| $\Delta\delta$[a] | −12.0 | −30.0 | −56.9 | −15.0 | −56.9 | −30.0 |

[a] Negative value indicates shift to high field (Hz).

Chemical shift variations of $H^3$ and $H^5$ $\beta$CD protons reflect the formation of a complex between them. In fact, the entry of the apolar guest (IMZ) into the lipophilic cavity of the host ($\beta$CD) induces a shielding of $H^3$ and $H^5$ as reported in **Table 1**. These data indicate that the interaction between IMZ and $\beta$CD is a true inclusion.

Both $H^3$ and $H^5$ of $\beta$CD in the inclusion complex undergo a high-field shift. Small but significant chemical shift variations can be also observed for the other protons; a participation of the external proton of $\beta$CD (e.g., $H^1$, $H^2$, $H^4$) warrants further investigations.

The complex stoichiometry (host−guest 1:1) was determined by integration of the inclusion complex signals in the $^1$H NMR spectra. Equimolar ratio of $\beta$-cyclodextrin−imazalil was calculated in preparations carried out with the same ratio of the starting materials but in different amounts (starting from 1 g to 200 g of the guest compound), indicating that the preparation can be scaled up.

Three different approaches were applied to evaporate the solvent (water) from the inclusion complex after preparation. Rotoevaporation under vacuum, lyophilization of the cake, or evaporation of the water into a thermoventilated oven (40 °C

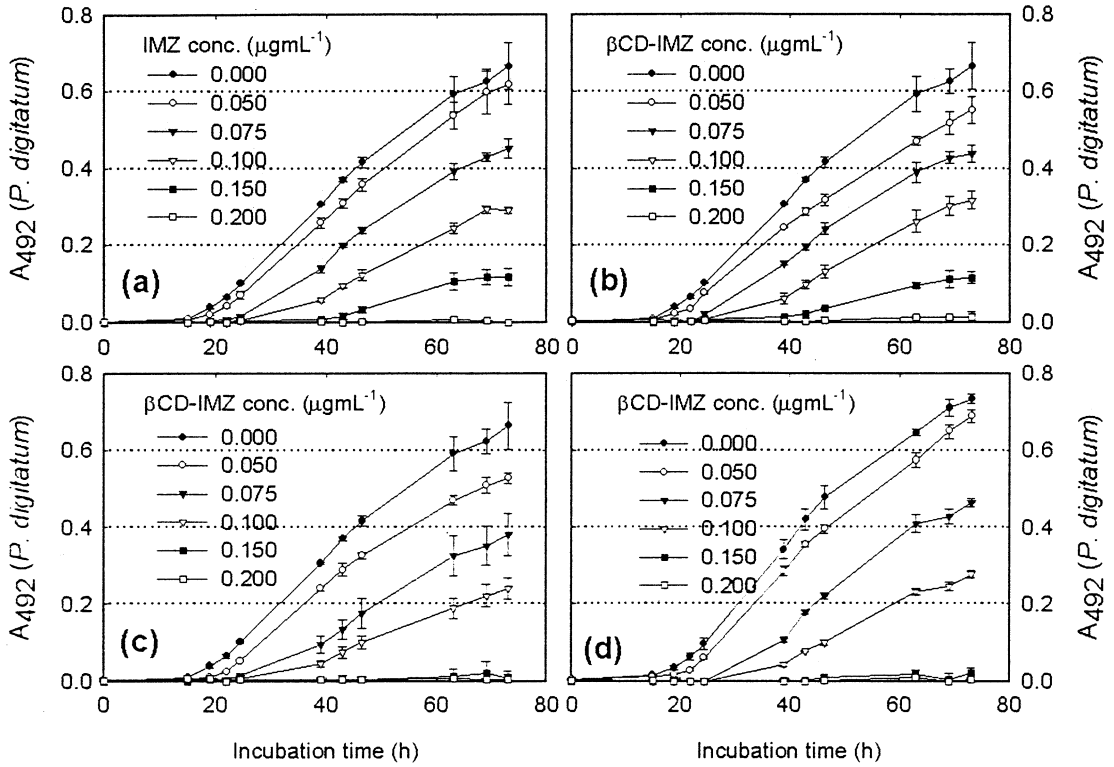



**Figure 2.** Effect of imazalil (IMZ) and β-cyclodextrin–IMZ inclusion complex (βCD–IMZ) on in vitro growth of *Penicillium digitatum* (PHI-26). Assays were performed with freshly prepared mixtures of (**a**) IMZ and (**b**) βCD–IMZ or with βCD–IMZ mixtures that were prepared (**c**) 1 day or (**d**) 4 days before use.

**Table 2.** Effects of Imazalil (IMZ) and β-Cyclodextrin–IMZ Inclusion Complex (βCD–IMZ) on in Vitro Growth of *Penicillium digitatum* (PHI-26) and *P. italicum* (PHI-1)[a]

| stock mixtures | elapsed time (days) | Monosporic isolates: *P. digitatum* (PHI-26) $ED_{50}$ | MIC | *P. italicum* (PHI-1) $ED_{50}$ | MIC |
|---|---|---|---|---|---|
| | | Active Ingredient Concentration (μg mL$^{-1}$) | | | |
| IMZ | 0[b] | 0.094 ± 0.002 | 0.20 | 0.135 ± 0.002 | >0.2 |
| βCD–IMZ | 0[b] | 0.091 ± 0.002 | 0.20 | 0.141 ± 0.005 | >0.2 |
| βCD–IMZ | 1 | 0.079 ± 0.002 | 0.15 | 0.113 ± 0.003 | 0.2 |
| βCD–IMZ | 4 | 0.086 ± 0.002 | 0.15 | 0.107 ± 0.001 | 0.2 |

[a] Mean values of three replicate measurements (±SD). [b] Freshly prepared mixtures.

**Table 3.** Imazalil (IMZ) Residues (on a Whole Fruit Basis) in Star Ruby Grapefruit Following a 3-min Dip Treatment (Time 0), after Cold Quarantine for 3 Weeks at 1 °C and Subsequent Storage for 3 Weeks at 8 °C (Quarantine + Storage), and after an Additional 1 Week of Simulated Marketing Period (SMP) at 20 °C

| treatments[a,b] | postharvest conditions: time 0 | quarantine + storage | SMP |
|---|---|---|---|
| | IMZ Residue (mg kg$^{-1}$ Active Ingredient)[c] | | |
| Deccozil (1200 mg/L a.i.), 20 °C | 5.38a | 4.56a | 4.48a |
| Deccozil (250 mg/L a.i.), 20 °C | 1.32b | 1.65b | 1.59b |
| βCD–IMZ (250 mg/L a.i.), 20 °C | 1.27b | 1.21b | 1.46b |
| Deccozil (250 mg/L a.i.), 50 °C | 5.31a | 4.57a | 4.12a |
| βCD–IMZ (250 mg/L a.i.), 50 °C | 5.35a | 3.44a | 3.91a |

[a] Treatments are 3-min dips followed by air-drying of dipped fruit. [b] Deccozil, imazalil-based commercially available fungicide; βCD–IMZ, β-cyclodextrin–imazalil inclusion complex. [c] Within each storage period, different letters denote significant differences by Tukey's test, $P = 0.01$.

for 24 h) gave the same results as host−guest ratio and $^1$H NMR spectrum. GC analysis of βCD−IMZ supported these results (data not shown). The β-cyclodextrin−imazalil complex is stable in the solid state; the equimolar host−guest ratio was observed after 6 months of storage at room temperature of the complex in a closed dark bottle. Nonsignificant release of the guest was detected after 30 min at 50 °C in the aqueous solution of fruit dip treatment.

**Effect of IMZ, βCD, and βCD−IMZ on *Penicillium digitatum* and *P. italicum* in Vitro.** IMZ effectively inhibited the growth of both isolates to an extent that was closely dependent on the amount of a.i. employed (**Figures 2** and **3**). IMZ did not change its antifungal activity for up to 4 days when water mixtures were stored at room temperature (data not shown). The freshly prepared βCD−IMZ mixture showed an efficacy similar to that of IMZ, within the range of applied concentrations (**Figures 2** and **3**; **Table 2**). However, 1- and 4-day-old βCD−IMZ mixtures had higher antifungal activity than freshly prepared IMZ. Both IMZ and βCD−IMZ were less effective against *P. italicum* than against *P. digitatum.* βCD alone at concentrations of 0.19, 0.28, 0.38, 0.56, and 0.75 μg mL$^{-1}$ did not inhibit the growth of *P. digitatum* or *P. italicum* (data not shown).

**Effect of Treatments on Residue Levels.** After treatment with 1200 mg/L Deccozil at 20 °C, residue levels in Star Ruby grapefruit (**Table 3**) were very similar to those found following treatment with 250 mg/L Deccozil at 50 °C (ca. 5.4 mg/kg a.i., whole-fruit basis), which were ca. 4.2-fold higher than those of fruit treated with 250 mg/L Deccozil at 20 °C. Similarly, equal residue uptakes were registered in oranges (ca 8.3 mg/kg) (**Table 4**) and lemons (ca 8.5 mg/kg) (**Table 5**) after Deccozil treatment with 1200 mg/L at 20 °C or 250 mg/L at 50 °C. The residue levels after treatment with 1200 mg/L Deccozil at 20 °C were ca. 3.5- and 4.0-fold higher than those of oranges and lemons treated with 250 mg/L Deccozil at 20 °C. There were no significant differences in residue uptake in Star Ruby grapefruit

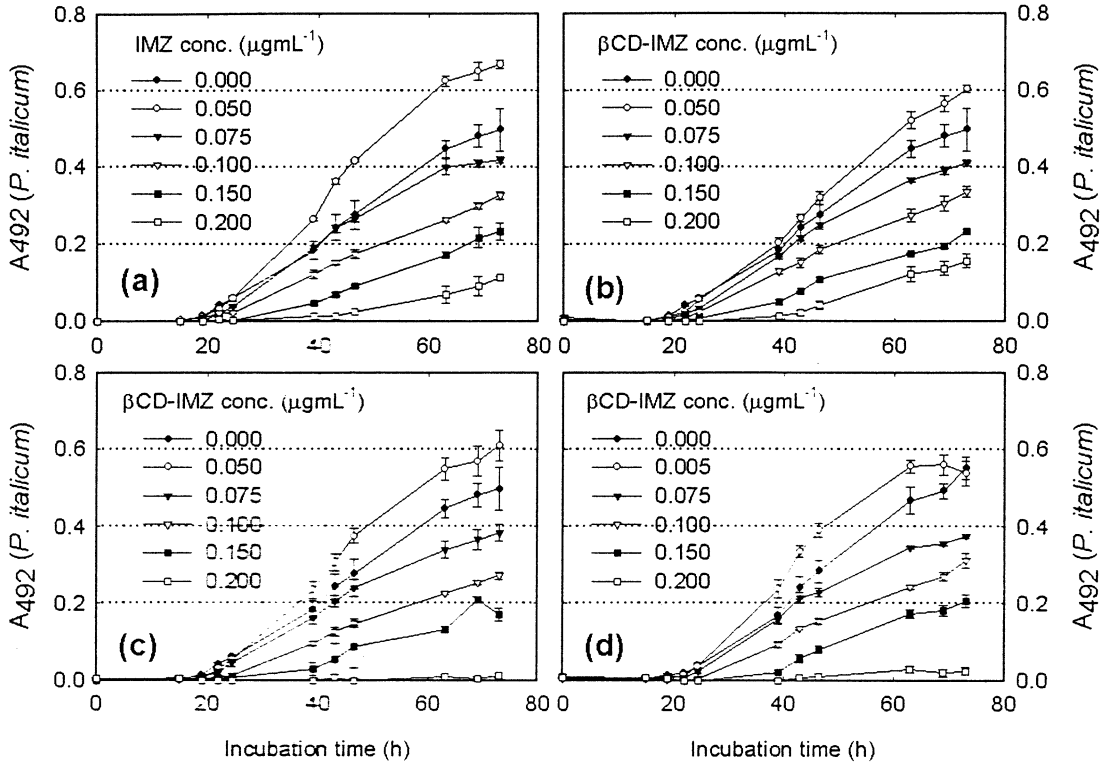



**Figure 3.** Effect of imazalil (IMZ) and $\beta$-cyclodextrin–IMZ inclusion complex ($\beta$CD–IMZ) on in vitro growth of *Penicillium italicum* (PHI-1). Assays were performed with freshly prepared mixtures of (**a**) IMZ and (**b**) $\beta$CD–IMZ or with $\beta$CD–IMZ mixtures that were prepared (**c**) 1 day or (**d**) 4 days before use.

**Table 4.** Imazalil (IMZ) Residues (on a Whole Fruit Basis) in Tarocco Oranges Following a 3-min Dip Treatment (Time 0), after Cold Quarantine for 3 Weeks at 1 °C and Subsequent Storage for 3 Weeks at 8 °C (Quarantine + Storage), and after an Additional 2 Weeks of Simulated Marketing Period (SMP) at 20 °C

| | postharvest conditions | | |
|---|---|---|---|
| treatments[a,b] | time 0 | quarantine + storage | SMP |
| | IMZ Residue (mg kg$^{-1}$ Active Ingredient)[c] | | |
| Deccozil (1200 mg/L a.i.), 20 °C | 8.46a | 3.73b | 2.93b |
| Deccozil (250 mg/L a.i.), 20 °C | 2.41c | 1.48c | 0.76c |
| $\beta$CD–IMZ (250 mg/L a.i.), 20 °C | 1.06c | 0.74c | 0.56c |
| Deccozil (250 mg/L a.i.), 50 °C | 8.47a | 6.19a | 4.84a |
| $\beta$CD–IMZ (250 mg/L a.i.), 50 °C | 6.02b | 6.67a | 3.10b |

[a] Treatments are 3-min dips followed by air-drying of dipped fruit. [b] Deccozil, imazalil-based commercially available fungicide; $\beta$CD–IMZ, $\beta$-cyclodextrin-imazalil inclusion complex. [c] Within each storage period, different letters denote significant differences by Tukey's test, $P = 0.01$.

**Table 5.** Imazalil (IMZ) Residues (on a Whole Fruit Basis) in Di Massa Lemons Following a 3-min Dip Treatment (Time 0), after Cold Quarantine for 3 Weeks at 1 °C and Subsequent Storage for 8 Weeks at 11 °C (quarantine + storage), and after an Additional 2 Weeks of Simulated Marketing Period (SMP) at 20 °C

| | postharvest conditions | | |
|---|---|---|---|
| treatments[a,b] | time 0 | quarantine + storage | SMP |
| | IMZ Residue (mg kg$^{-1}$ Active Ingredient)[c] | | |
| Deccozil (1200 mg/L a.i.), 20 °C | 8.33b | 6.51b | 3.64abc |
| Deccozil (250 mg/L a.i.), 20 °C | 2.04c | 1.81c | 2.44bc |
| $\beta$CD–IMZ (250 mg/L a.i.), 20 °C | 1.59c | 1.40c | 1.17c |
| Deccozil (250 mg/L a.i.), 50 °C | 8.21b | 7.98a | 5.76ab |
| $\beta$CD–IMZ (250 mg/L a.i.), 50 °C | 10.47a | 8.13a | 6.24a |

[a] Treatments are 3-min dips followed by air-drying of dipped fruit. [b] Deccozil, imazalil-based commercially available fungicide; $\beta$CD–IMZ, $\beta$-cyclodextrin–imazalil inclusion complex. [c] Within each storage period, different letters denote significant differences by Tukey's test, $P = 0.01$).

as a function of formulation type (Deccozil or $\beta$CD−IMZ) under equal a.i. concentrations and dip temperatures. By contrast, treatments of oranges and lemons with 250 mg/L $\beta$CD−IMZ at 50 °C produced significant ($P \leq 0.01$) differences in residue uptake (lower in oranges and higher in lemons) in comparison with 250 mg/L Deccozil treatments at 50 °C. The degradation rate of a.i. in grapefruit was not affected by the type of formulation used, whether at 20 or 50 °C. Conversely, in oranges and lemons, IMZ had greater persistence when it was applied at 50 °C.

**Effect of Treatments on Artificially Inoculated Grapefruit.** The incidence of inoculated wounds that developed green mould decay in fruit dipped in $H_2O$ at 20 °C was over 92% after quarantine and reached 100% at the end of storage (**Table 6**). Fungicide treatments significantly reduced decay development during quarantine and storage, differences due to treatment type being negligible. Hot-water dip effectively reduced the percentage of rotten wounds during quarantine but was ineffective after storage. After shelf life, fungicide treatments with 250 mg/L Deccozil or $\beta$CD−IMZ at 20 °C were less effective than treatments with 1200 mg/L Deccozil at 20 °C and 250 mg/L Deccozil at 50 °C; $\beta$CD−IMZ at 50 °C exhibited the highest inhibitory activity with a rot incidence reduction of 65% with respect to 250 mg/L Deccozil treatment at 50 °C.

**Fruit Storage Responses to Dip Treatments.** After quarantine there was very little rotting (<1%) in untreated fruit (data not shown). By the end of storage and, especially after SMP, the occurrence of decay increased to an extent depending on the fruit species (**Figure 4a**,**b**, **Table 7**). Hot-water dip effectively reduced the percentage of decay in grapefruit (**Figure 4a**) and oranges (**Figure 4b**). However, fungicide treatments gave better protection against decay, providing complete control

**Table 6.** Influence of 3-min Dip Treatments in Water, β-Cyclodextrin (βCD), Imazalil-Based Fungicide (Deccozil), or β-Cyclodextrin–Imazalil Inclusion Complex (βCD–IMZ) at 20 or 50 °C on Percentages of Infected Wounds after Artificial Inoculation with a $10^4$ $mL^{-1}$ *Penicillium Digitatum* Spore Suspension in Star Ruby Grapefruit after Cold Quarantine for 3 Weeks at 1 °C, Subsequent Storage for 3 Weeks at 8 °C (Quarantine + Storage), and an Additional 1 Week of Simulated Marketing Period (SMP)[a]

| | storage conditions | | |
|---|---|---|---|
| treatments[b] | quarantine | quarantine + storage | SMP |
| | Infected Wounds (%)[c] | | |
| $H_2O$ 20 °C | 92.7a | 100a | – |
| Deccozil (1200 mg/L a.i.), 20 °C | 11.0bc | 14.0c | 65.0b |
| Deccozil (250 mg/L a.i.), 20 °C | 20.0b | 24.0c | 85.0ab |
| βCD–IMZ (250 mg/L a.i.), 20 °C | 18.7b | 21.0c | 80.3ab |
| $H_2O$ 50 °C | 10.7bc | 92.7a | 100a |
| Deccozil (250 mg/L a.i.), 50 °C | 8.7bc | 16.7c | 65.3b |
| βCD–IMZ (250 mg/L a.i.), 50 °C | 8.0bc | 11.3c | 22.7c |
| Control[d] | 3.0c | 58.0b | 90.0ab |

[a] The concentrations of fungicides are referred on active ingredient (a.i.). [b] Three-minute dipping, air-dried. [c] Data are means of 25 replicate fruit samples per treatment (12 inoculated wounds per fruit, total 300 wounds). Within each storage period, different letters denote significant differences by Tukey's test, $P = 0.01$. [d] Wounded, noninoculated fruit (naturally infected wounds).

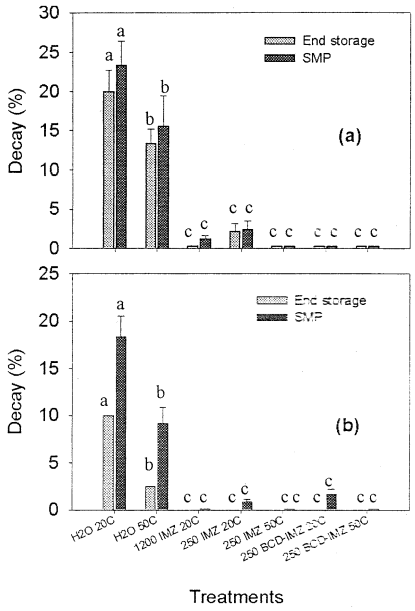



**Figure 4.** Influence of 3 min water-dip treatments with imazalil-based fungicide (Deccozil) or β-cyclodextrin–imazalil inclusion complex (βCD–IMZ) at 20 or 50 °C on decay incidence in (**a**) Star Ruby grapefruit and (**b**) Tarocco oranges after cold quarantine for 3 weeks at 1 °C and 3 additional weeks of storage at 8 °C (End storage). Subsequent simulated marketing period (SMP) at 20 °C was 1 week for Star Ruby grapefruit and 2 weeks for Tarocco oranges. The fungicide concentrations refer to active ingredient (a.i.). Within each storage period, different letters denote significant differences by Tukey's test, $P = 0.01$.

of rots during cold storage with negligible decay after SMP. Treatments of βCD−IMZ on lemon at 20 °C were as effective as those using Deccozil when a.i. was 250 mg/L (**Table 7**). The efficacy of Deccozil increased when the application rate was 1200 mg/L, providing complete control of decay during cold storage with negligible decay after SMP. Similar results were achieved when both fungicide formulations were employed at 50 °C. By contrast, decay development was unaffected by

**Table 7.** Influence of 3-min Dip Treatments in Water, β-Cyclodextrin (βCD), Imazalil-Based Fungicide (Deccozil), or β-Cyclodextrin–Imazalil Inclusion Complex (βCD–IMZ) at 20 or 50 °C on Decay Incidence in Di Massa Lemons after 3 Weeks of Quarantine at 1 °C and 8 Weeks of Storage at 11 °C (Quarantine + Storage) and after an Additional 2 Weeks of Simulated Marketing Conditions (SMP) at 20 °C

| treatments[a] | quarantine + storage[b] | SMP[b] |
|---|---|---|
| $H_2O$, 20 °C | 9.2ab | 17.5a |
| $H_2O$, 50 °C | 6.7bc | 14.2ab |
| βCD, 20 °C | 11.7a | 18.3a |
| βCD, 50 °C | 6.7bc | 10.8bc |
| Deccozil (1200 mg/L a.i.), 20 °C | 0.0e | 0.8d |
| Deccozil (250 mg/L a.i.), 20 °C | 5.0bcd | 10.0bc |
| Deccozil (250 mg/L a.i.), 50 °C | 0.0e | 3.3d |
| βCD–IMZ (250 mg/L a.i.), 20 °C | 3.3cde | 5.8cd |
| βCD–IMZ (250 mg/L a.i.), 50 °C | 0.8de | 1.7d |

[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, $P = 0.01$.

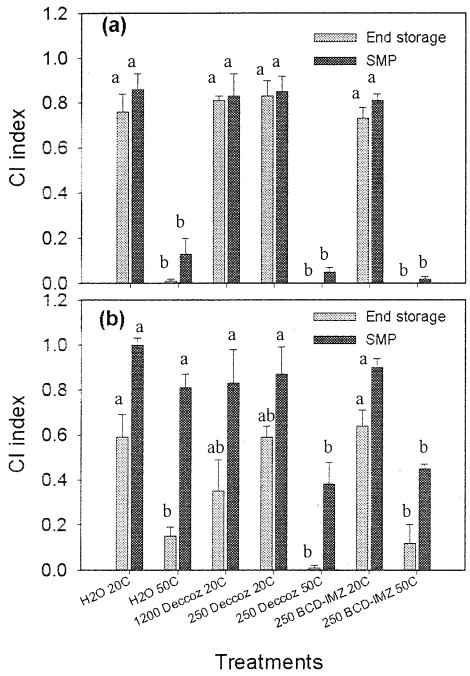



**Figure 5.** Influence of 3 min water-dip treatments with imazalil-based fungicide (Deccozil) or β-cyclodextrin–imazalil inclusion complex (βCD–IMZ) at 20 or 50 °C on chilling injury index in (**a**) Star Ruby grapefruit and (**b**) Tarocco oranges after cold quarantine for 3 weeks at 1 °C and 3 additional weeks of storage at 8 °C (End storage). Subsequent simulated marketing period (SMP) at 20 °C was 1 week for Star Ruby grapefruit and 2 weeks for Tarocco oranges. The fungicide concentrations refer to active ingredient (a.i.). Within each storage period, different letters denote significant differences by Tukey's test, $P = 0.01$.

treatment with βCD at 20 °C; the effect of water dips at 50 °C with or without βCD was nonsignificant.

All treatments at 50 °C considerably reduced CI in grapefruit both during storage and SMP (**Figure 5a**). Treatments with hot water and, to a lesser extent, with 1200 mg/L Deccozil at 20 °C reduced CI in oranges during storage, but their effect did not last during SMP (**Figure 5b**). Better results were achieved when hot water was employed in combination with Deccozil or βCD−IMZ, which notably reduced CI even during SMP. No visible symptoms of CI occurred in lemons over storage and SMP.

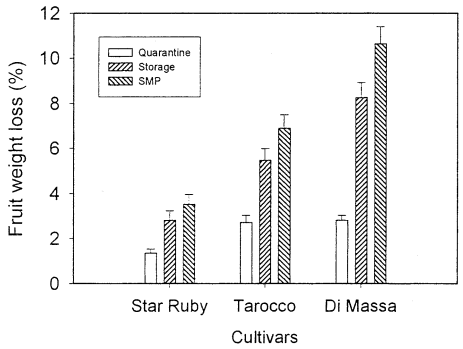



**Figure 6.** Percentages of fruit weight loss in Star Ruby grapefruit, Tarocco oranges, and Di Massa lemons after cold quarantine, subsequent storage, and an additional period of simulated marketing period. Quarantine conditions were 3 weeks at 1 °C, subsequent storage was 3 weeks at 8 °C for grapefruits and oranges and 8 weeks at 11 °C for lemons, and simulated marketing period (SMP) was 1 week at 20 °C for grapefruit and 2 weeks at 20 °C for oranges and lemons.

There were no treatment-dependent differences in fruit flavor and taste during storage and SMP (data not shown). Fruit weight loss was not significantly affected by treatment. Therefore, the data are presented as overall means (±SE, 99% conf) (**Figure 6**).

## DISCUSSION

Fungicide effectiveness is known to be dependent on its water solubility, and it can be increased by fungicide complexation with cyclodextrins (*6*). Recent studies (*9*) have demonstrated that the solubility of certain benzimidazole-type fungicides (thiabendazole, carbendazim, and fuberidazole), largely employed in pre- or postharvest treatments of horticultural crops, increase remarkably after complexation with $\beta$-cyclodextrin. Accordingly, this study showed that water solubility of IMZ increased after complexation with $\beta$-cyclodextrin.

Szejtli et al. (*7*) have shown that, when the $\beta$-cyclodextrin is applied in solid culture medium at a concentration ranging from 0.1 to 20 $\mu$g/mL, it exhibits fungistatic activity against *Alternaria tenuis*, *Sclerotinia sclerotiorum*, and *Rhizoctonia solani*. The fungistatic effects were manifested in the different morphology, structure, and color of colonies and in the quantity of dyes diffused into the nutrient media. When the $\beta$-cyclodextrin was applied at higher dosage, it led to a decrease in the diameter of the colonies and inhibited the formation of fungi propagative organs. Results reported herein show that the $\beta$-cyclodextrin did not affect the growth of *Penicillium digitatum* and *P. italicum* isolates at any of the concentrations assayed. Accordingly, its application to lemon did not influence decay development. By contrast, the growth of both isolates was affected by IMZ.

In vitro assays showed that the freshly prepared $\beta$CD−IMZ was as effective as IMZ, indicating that the IMZ present in the inclusion complex is as effective as the IMZ in the commercial formulation, although 1- and 4-day-old $\beta$CD−IMZ mixtures were more effective. At present, we are not able to explain why these particular $\beta$CD−IMZ mixtures are more effective than IMZ or the freshly prepared $\beta$CD−IMZ mixtures, especially because the a.i. concentration was the same in all assays. One assumption in this connection is that the concentration of IMZ present in the medium (potato dextrose broth) is increased when the IMZ included in the $\beta$-cyclodextrin is gradually released from the complex, thereby increasing its "bio-availability".

Previous studies on the baseline sensitivity of *P. digitatum* to IMZ found median $ED_{50}$ values of 0.055 and 0.026 mg/L (*15, 16*). The results of this study indicate that *P. digitatum* PHI-26 shows a 1.8- to 3.6-fold increase from the baseline sensitivities. The sensitivity of *P. italicum* PHI-1 to IMZ is also 4.4-fold higher than the median $ED_{50}$ described by Holmes and Eckert (*16*) for citrus grove isolates. These differences may be explained, at least in part, by the different methods employed for $ED_{50}$ calculations. Both IMZ formulations, commercial IMZ and $\beta$CD−IMZ, were slightly less effective against *P. italicum* than *P. digitatum*, the same response observed by Holmes and Eckert (*16*). The higher resistance of *P. italicum* to IMZ might be explained by the greater suppressive effect of the fungicide on *P. digitatum*, thereby creating more favorable conditions for *P. italicum* to develop and build up resistance.

The effectiveness of $\beta$CD−IMZ and Deccozil was generally similar when equal treatment temperature and a.i. concentrations were applied. Treatments with 250 mg/L a.i. of heated fungicides had higher suppressive effects against decay in lemon than unheated chemicals having equal active ingredient concentrations and comparable activity of 1200 mg/L IMZ at 20 °C. These effects make it possible to reduce the amount of fungicide needed to control postharvest decay, in agreement with previous studies (*17, 18*) thanks to the combined effect of heat and increased heated fungicide action due to the improved a.i. penetration and coverage of fruit (*19, 20*).

The a.i. degradation rate in grapefruit was not affected by the type of formulation used, whether at 20 or 50 °C. Conversely, in oranges and lemons, IMZ showed greater persistence when applied at 50 °C.

## CONCLUSIONS

The present paper represents the first application of the $\beta$-cyclodextrin inclusion complex in postharvest treatment of citrus fruit, and the reported data prompt a number of considerations. The $\beta$CD−IMZ inclusion complex is a promising candidate for application of $\beta$-cyclodextrin as a biopolymeric matrix in agricultural uses, including postharvest treatments, which do not require agrochemical inputs as large as those required in field treatments. Although treatments with $\beta$CD−IMZ complex had an efficacy comparable to the commercially available IMZ fungicide against *P. italicum* and *P. digitatum*, this novel biocide complex should also be considered for certain other advantages. $\beta$-cyclodextrin is recognized as nontoxic when used in oral or topical formulations; thus, the $\beta$CD−IMZ inclusion complex makes possible reduced mammalian toxicity of IMZ for technicians and greater ease of handling. Our preparation takes advantage of two key aspects: economic and environmental sustainability. In fact, we prepared the $\beta$CD−IMZ complex in water and can detect its formation by $^1$H NMR in $D_2O$ without the use of an organic solvent at any preparation step.

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