Influence of pH and $NaHCO_3$ on Effectiveness of Imazalil to Inhibit Germination of *Penicillium digitatum* and to Control Postharvest Green Mold on Citrus Fruit

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ABSTRACT

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In vitro, spores of *Penicillium digitatum* germinated without inhibition between pH 4 and 7, but were inhibited at higher pH. Estimated concentrations of imazalil (IMZ) in potato-dextrose broth–Tris that caused 50% reduction in the germination of spores (ED_{50}) of an IMZ-sensitive isolate M6R at pH 4, 5, 6, and 7 were 0.16, 0.11, 0.015, and 0.006 µg/ml, respectively. ED_{50} IMZ concentrations of an IMZ-resistant isolate D201 at pH 4, 5, 6, and 7 were 5.9, 1.4, 0.26, and 0.07 µg/ml, respectively. The natural pH within 2-mm-deep wounds on lemon was 5.6 to 5.1 and decreased with fruit age. IMZ effectiveness to control green mold and its residues increased with pH. The pH in wounds on lemon fruit 24 h after immersion in 1, 2, or 3% $NaHCO_3$ increased from pH 5.3 to 6.0, 6.3, and 6.7, respectively. $NaHCO_3$ dramatically improved IMZ performance. Green mold incidence among lemon fruit inoculated with M6R and treated 24 h later with IMZ at 10 µg/ml, 1% $NaHCO_3$, or their combination was 92, 55, and 22%, respectively. Green mold among lemon fruit inoculated with D201 and treated 24 h later with water, IMZ at 500 µg/ml, 3% $NaHCO_3$, or their combination was 96.3, 63.0, 44.4, and 6.5%, respectively. $NaHCO_3$ did not influence IMZ fruit residue levels.

Citrus is the world's premiere fruit crop, grown in over 100 countries on six continents (33). Worldwide production of citrus reached nearly 100 million tons in 2002 (16). Citrus fruit are enjoyed around the world for their taste, nutritional value, and relatively low price. Control of postharvest diseases of citrus is vital for maintaining quality and shelf life in a market where transport from producer to consumer may take several weeks. Green mold of citrus fruit, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is one of the most economically important postharvest diseases of citrus in arid growing regions of the world. The primary infection courts of this pathogen are wounds on fruit where nutrients are available to stimulate spore germination. Wounds can be inflicted during harvest and handling, and the resulting infections must be eradicated to achieve acceptable levels of control. Fungicides such as thiabendazole, sodium o-phenylphenate, and imazalil are used worldwide to minimize postharvest decay (5,16,41).

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Many issues make the development of new postharvest decay control treatments for citrus fruit important, including concerns about the dietary and environmental safety of fungicides, as well as the occurrences of fungicide resistance in pathogens (14). Several aspects of pH are important for the control of postharvest disease because it (i) directly affects the germination of conidia (27), (ii) influences the virulence of pathogens through their colonization of host tissue (30,31), and (iii) affects the toxicity of fungicides used to control these diseases (6).

The growth of *P. digitatum* is optimal under conditions of low pH (27). In the case of postharvest pathogenic fungi, ambient pH is important because it contributes to the ability of the pathogen to successfully colonize the targeted host tissue (31). Pathogens may enhance their virulence by locally modulating the host's ambient pH either up or down. *Penicillium* spp. colonization acidified citrus and apple tissues and was enhanced by low pH (28–30). The increase in ambient pH was shown to increase gene expression of hydrolases in *Colletotrichum gloeosporioides* (29) and of glucanases in *Alternaria alternata* (7).

The influence of pH on the activity of the fungicide sodium o-phenylphenate, commonly used on citrus fruit after harvest, has been studied thoroughly. Proper control of pH of solutions of the fungicide when it is applied commercially is critical to optimize its effectiveness, control fruit residue levels, and minimize phytotoxicity caused by this fungicide (6). Although it long has been known that pH affects imazalil activity, the imazalil solution pH is not controlled when used in packinghouses. Siegel et al. (35) and Guan et al. (11) both observed that imazalil inhibited *P. italicum* growth more at pH 7 than at pH 5.2 or 5.3. Holmes and Eckert (14) observed that imazalil inhibited *P. digitatum* growth more at pH 5.9 than at pH 5.1.

Our objectives were to quantify the influence of pH on the toxicity of imazalil to spores of an imazalil sensitive- and a resistant-isolate of *P. digitatum,* to determine the effect of alkaline buffers on improving imazalil effectiveness in controlling green mold, to measure the pH of wounds within lemon fruit after some of these treatments, and to determine the influence of an effective buffer–imazalil combination on residues of the fungicide within fruit. To control pH in alkaline solutions, we included $NaHCO_3$ (sodium bicarbonate) because, in addition to being a common buffer and food additive, it also controls many plant pathogens (32,43) including postharvest green mold of citrus (21,26,38). $NaHCO_3$ has become popular in California citrus packinghouses in recent years to control green mold; however, its influence on imazalil activity has not been studied.

MATERIALS AND METHODS

Pathogen culture. Two *P. digitatum* isolates (imazalil-sensitive M6R and imazalil-resistant D201) were cultured for 1 to 2 weeks on potato dextrose agar (PDA, Difco Laboratories, Detroit) at 25ºC. Both were isolated from infected lemon fruit from citrus packinghouses in California. Spores of isolate M6R do not germinate on imazalil-amended PDA containing the fungicide at 0.01 µg/ml, whereas those of isolate D201 germinate on imazalil in PDA at 2 µg/ml. The level of imazalil resistance of this isolate is similar to the level of imazalil resistance observed among 50 isolates collected in 2004 from six packinghouses. Isolate M6R is controlled by typical commercial imazalil applications in California packinghouses, whereas isolate D201 is not controlled. Spores were harvested by adding 5 ml of sterile, de-ionized water

(diH_2O) containing 0.05% Triton X-100 to the petri dish. Spores then were rubbed with a sterile glass rod, and spore suspension was passed through two layers of cheese cloth. The suspension was diluted with water to an absorbance of 0.1 at 425 nm as measured with a spectrophotometer; a density that contained about 1×10^6 spores/ml (5).

Spore germination assay. Sterile dishes with 24 microwells (Nunclon Surface; Nalge Nunc, Int., Roskilde, Denmark) with a capacity of 4 ml/well were used. A germination medium was prepared by placing 96 g of potato-dextrose broth (PDB; Difco Laboratories) and 12.14 g of Tris buffer (hydroxymethyl aminomethane, Polysciences, Inc. Warrington, PA) in 1 liter of diH_2O and adjusting the pH of the medium to 4, 5, 6, 7, 8, or 9 with concentrated H_2SO_4, H_3PO_4, or NaOH. In each well, 0.5 ml of PDB-Tris, 0.5 ml of spore suspension, imazalil (IMZ; 44.6% imazalil, Fungaflor 500EC; Janssen Pharmaceutica, Beerse, Belgium) at various concentrations, and diH_2O were added to a final volume of 2.0 ml and mixed. Actual IMZ concentrations were 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, and 0.4 µg/ml for the M6R isolate, and 0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 µg/ml for the D201 isolate. After 24 h of incubation at 24°C, germinated and ungerminated spores in each well were counted by observation using an inverted compound microscope (×200). Within each replicate, 100 to 150 spores were examined and the percentage of germinated spores was calculated. Determination of the influence of $NaHCO_3$ on spore germination was similar to tests with IMZ, except only isolate M6R was used in PDB alone. When the pH was not controlled, it was 6.2 to 9.4 depending on the $NaHCO_3$ content. In the experiment where the pH was controlled to 7.2, the solution contained phosphate buffer at 100 mM. Each treatment included three replicates, and each test was repeated three times.

Lemon albedo pH measurements. Wounds (1 mm in diameter and 2 mm deep) were made on fruit by puncturing the rind with a steel rod. To determine the pH inside wounds on fruit, 15 µl of diH_2O was placed inside each wound with a pipette; then, a microelectrode (model MI-410; Microelectrodes, Inc., Bedford, NH) with a tip 0.5 mm in diameter was inserted to a depth of 1.5 mm and the pH recorded after the reading stabilized. In order to determine the natural pH of lemon albedo tissue, we harvested lemon fruit from three different groves in Ventura County, CA. Fruit from each location were divided into four categories according to their color (an indication of maturity): (i) dark green, (ii) light green, (iii) green-yellow, and (iv) yellow. Two wounds were made on each fruit and the pH inside the wounds was measured on eight lemon fruit within each color category from each grove.

Inoculation and treatment procedures. Commercially harvested 'Eureka' lemon fruit were randomized for use in these experiments. Fruit were inoculated 24 h before treatments were applied, using *P. digitatum* isolates M6R or D201, by dipping a steel rod with a 1-mm-wide and 2-mm-long tip into the spore suspension and making a single wound per fruit with the rod (5). Fruit were immersed in 15 liters of each solution contained within 22-liter-capacity stainless-steel tanks. The temperature of the solutions was maintained at 25°C and constantly stirred with a 5-cm-diameter propeller. After treatment, all fruit were left unwaxed and stored for 2 weeks at 20°C and 95% relative humidity (RH), after which the number of decayed fruit was counted.

IMZ effectiveness and solution pH. Lemon fruit were inoculated as previously described with isolate M6R 24 h before IMZ treatment. IMZ was prepared in pH 4.0 or 7.5 buffers at concentrations of 0, 10, and 30 µg/ml. The buffers were 100 mM K_2HPO_4 and 10 mM citric acid for the pH 4.0 IMZ solutions and 100 mM K_3PO_4 and 10 mM citric acid for pH 7.5 IMZ solutions. The pH was adjusted with concentrated H_2SO_4, H_3PO_4, and KOH. The fruit were immersed for 1 min in the treatment solution and not rinsed. The pH within noninoculated wounds of eight fruit per treatment was measured after the fruit had dried. Each treatment consisted of four replicates with 25 lemon fruit each. The experiment was done twice.

In another test, solutions at pH 3, 5, 7, or 9 containing IMZ at 0, 10, 20, or 200 µg/ml were evaluated for the effectiveness of IMZ to control green mold incidence on inoculated orange fruit. Solutions were buffered using 10 mM Trisma, 10 mM citric acid, and either 100 mM NaH_2PO_4 (for pH 3 and 5) or 100 mM Na_2HPO_4 (for pH 7 and 9). Solution pH was adjusted using sulfuric acid and sodium hydroxide. Fruit were inoculated with IMZ-sensitive isolate M6R 24 h before immersion for 30 s in one of the solutions. Each treatment was applied to four replicates of 20 orange fruit each. The number of infected fruit was counted after storage for 2 weeks at 20°C and 90 to 95% RH. An additional treatment containing IMZ at 500 µg/ml at each pH also was applied to five replicates of six noninoculated orange fruit each to determine residues of IMZ. Residues were determined by gas chromatography (39). The test was repeated three times, once with Valencia and twice with navel orange fruit (depending upon availability of fresh fruit throughout the season).

NaOH influence on IMZ effectiveness. Lemon fruit were inoculated as previously described with isolate M6R. They were sprayed for 3 s each using a compressed air sprayer 1 to 2 h later, with either water or 1 N NaOH (pH about 14). One hour later, an unbuffered aqueous IMZ solution, about pH 6.5, containing 0, 100, 200, or 300 µg/ml, was applied similarly. In one experiment, residual droplets of NaOH were removed by blotting the fruit surface with tissue paper before IMZ treatment or storage. Fruit were stored at 20°C for 2 weeks. Each treatment was applied to three replicates with 27 fruit per replicate. The experiment was conducted twice.

Effectiveness of IMZ and $NaHCO_3$ mixtures. Lemon fruit were inoculated as described previously using isolates M6R or D201 and treated 18 to 24 h later. Treatments applied to inoculated lemon fruit were (i) $NaHCO_3$ at 0, 1, or 3%; (ii) IMZ at 200 or 500 µg/ml; and (iii) combinations of 1 and 3% $NaHCO_3$ and IMZ at 200 or 500 µg/ml. Fruit were immersed for 30 s in treatment solution, not rinsed, and then stored at 20°C for 2 weeks. In each experiment, each treatment was applied to four replicates of 27 lemon fruit each. The treatment containing IMZ at 500 µg/ml and 3% $NaHCO_3$ also was applied to three replicates of eight noninoculated lemon fruit each to determine residues of IMZ. Residues were determined by gas chromatography (39). The experiment was conducted twice.

A similar experiment was done to determine the $NaHCO_3$ and IMZ effectiveness, alone or in combination, immediately and 1 week after their preparation. Lemon fruit were inoculated as previously described using isolate D201 24 h before treatment. Treatments applied were $NaHCO_3$ at 1, 2, or 3% (wt/vol), alone or combined with IMZ at 500 µg/ml. Four replicates of 27 lemon fruit each were immersed for 60 s, not rinsed after treatment, and then stored at 20°C for 2 weeks. The solutions were in open-top tanks at 25°C and the experiment was repeated with the same solutions 1 week later. The pH within the noninoculated wound site on lemon fruit was determined 5 to 6 h after treatment. Three replicates of 10 healthy, unwounded fruit per treatment also were treated and stored for 3 weeks to determine the influence of the treatment on fruit weight loss and appearance. Weight loss was determined by weighing each fruit weekly during storage. Appearance was recorded by a subjective visual rating, where fruit without injuries were classified as 1, those with minor injuries were 2, those with moderate injuries that would reduce their inspection rating were classified as 3, and those with severe injuries and unmarketable were 4. Additional observations included the pH of fresh and 1-week-old treatment solutions.

Statistical analysis. The concentrations of IMZ in PDB-Tris that caused 50% reduction in the germination of spores (ED_{50}) were estimated by probit analysis (ver. 10.0; SPSS Inc., Chicago). The incidence of green mold was analyzed by an analysis of variance applied to the arcsin of the square root of the proportion of in-

fected fruit, followed by Fisher's protected least significant difference test ($P \leq 0.05$) to separate means. Actual values are shown. The lemon appearance ratings and weight loss were analyzed by analysis of variance followed by Fisher's protected least significant difference test ($P \leq 0.05$) to separate means. A paired *t*-test was applied to separate means in an experiment that compared the effectiveness of fresh and 1-week-old IMZ solutions.

RESULTS

Spore germination assay. Germination of spores of both isolates of *P. digitatum* was more than 98% in PDB-Tris from pH 4 to 7 (Fig. 1). At higher pH, germination was inhibited. It was about 70 and 10% at pH 8 and 9, respectively. The pH of the solutions did not change during incubation.

IMZ effectiveness to inhibit spore germination of both isolates profoundly improved as pH of the medium increased (Fig. 2). The ED_{50} IMZ concentration, estimated by probit analysis, to inhibit spores of isolate M6R was IMZ at 0.006 µg/ml at pH 7; whereas, at pH 4, the ED_{50} was IMZ at 0.16 µg/ml (about a 27-fold higher concentration; Fig. 3). Similarly, the ED_{50} of isolate D201 spores was IMZ at 0.07 µg/ml at pH 7; whereas, at pH 4, the ED_{50} was IMZ at 5.9 µg/ml (about an 84-fold higher concentration).

$NaHCO_3$ inhibited the germination of spores of isolate M6R whether the pH was controlled or not, although more $NaHCO_3$ was required when the pH was controlled (Fig. 4). The ED_{50} without pH control was 14.1 mM $NaHCO_3$, whereas the ED_{50} when the pH was controlled to pH 7 (±0.2) was 37.2 mM $NaHCO_3$. Without pH control, the solution pH increased as $NaHCO_3$ concentration increased; it was 6.1 and 9.4 when $NaHCO_3$ concentration was 2.5 and 50 mM, respectively (Fig. 4).

Lemon albedo pH measurements. The natural pH of lemon albedo varied with fruit color class (an indicator of maturity and fruit age). The highest pH, 5.6, occurred within the albedo of dark green (least mature) lemon fruit; whereas, among the yellow (most mature) fruit, the lowest pH, 5.1, occurred (Fig. 5). The albedo pH of the other color classes was between these values.

IMZ effectiveness and solution pH. Green mold incidences on lemon fruit treated with pH 4.0 or 7.5 buffer solutions were 99 and 100%, respectively (Fig. 6). As IMZ was added and its concentration increased, green mold incidence decreased. IMZ effectiveness was significantly better at pH 7.5 compared with pH 4.0. The pH within wounds treated with pH 4.0 or 7.5 buffer solutions is shown (Fig. 7). The pH 1 h after treatment averaged 4.7 after pH 4 buffer treatment and 6.5 after pH 7.5 buffer treatment, and the pH was not influenced by the presence of IMZ. After an additional 24 h, the pH within all the wounds treated with either buffer declined significantly, except those treated with pH 4.0 buffer containing IMZ at 30 µg/ml, which did not significantly decline.

On orange fruit (Fig. 8), the percentage of infected control fruit treated with buffer solutions alone was slightly but significantly lower at pH 9 than at lower pH. The effectiveness of low IMZ rates (10 or 20 µg/ml) to control green mold was similar

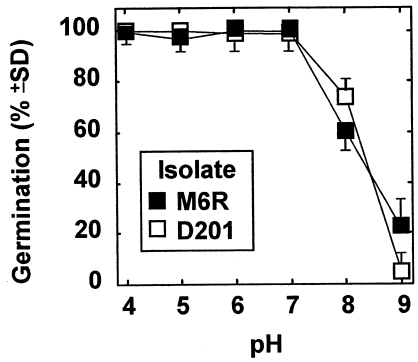

Fig. 1. Germination of spores of *Penicillium digitatum* isolates M6R (imazalil sensitive) and D201 (imazalil resistant) after incubation at 24°C for 24 h in buffered potato dextrose broth adjusted to pH 4 to 9.

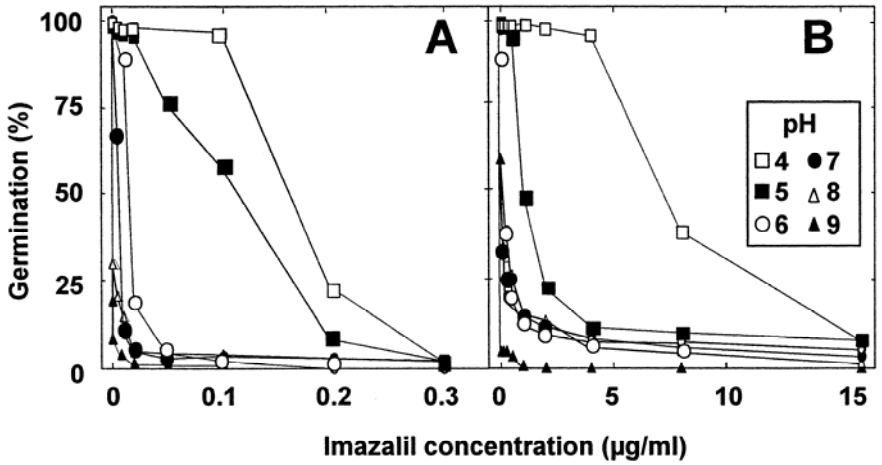

Fig. 2. Germination of spores of *Penicillium digitatum* isolates **A,** M6R (imazalil sensitive) and **B,** D201 (imazalil resistant) after incubation at 24°C for 24 h in solutions of buffered potato dextrose broth containing imazalil at pH 4, 5, 6, 7, 8, or 9.

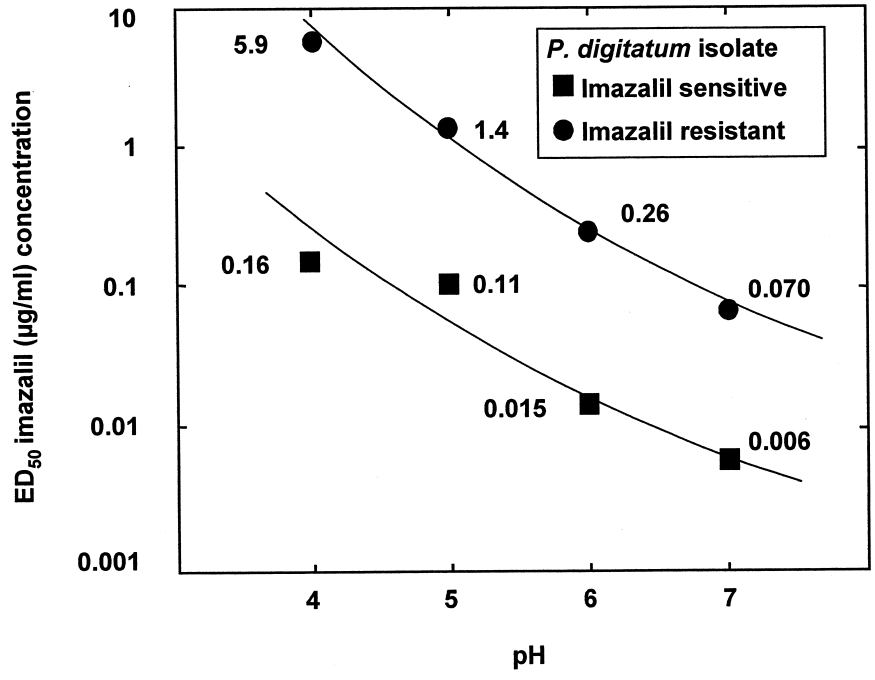

Fig. 3. Concentrations of imazalil in buffered potato dextrose broth at pH 4, 5, 6, or 7 that inhibited the germination of one half of the spores (ED_{50}) of *Penicillium digitatum* isolates M6R (imazalil sensitive) and D201 (imazalil resistant) exposed for 24 h at 24°C. The ED_{50} imazalil concentration for M6R = $1328.920(pH)^{-6.256}$ ($r^2 = 0.916$). The ED_{50} imazalil concentration for D201 = $444545.578(pH)^{-8.007}$ ($r^2 = 0.994$).

at pH 5, 7, and 9. At pH 3, IMZ effectiveness applied at low rates was slightly but significantly inferior. However, at 200 µg/ml, IMZ effectiveness was high and similar at each pH and reduced green mold by about 99%. IMZ residues on orange fruit treated with IMZ at 500 µg/ml were about double at pH 7 and 9 compared with pH 3 and 5.

NaOH influence on IMZ effectiveness. The application of NaOH (1 N) alone to inoculated lemon fruit reduced green mold incidence from 100% in untreated fruit to 4.6% in treated fruit (Fig. 9). The application of IMZ alone reduced green mold incidence. Treatment with NaOH before treatment with IMZ at 100 or 200 µg/ml reduced green mold incidence to 2.3 and 1.2%, respectively. Although green mold was controlled, injuries (numerous brown lesions 1 to 2 mm in size) developed during storage on all of the lemon fruit treated with NaOH. If residual droplets of NaOH were removed by blotting the fruit surface with tissue paper before IMZ treatment or storage, these injuries did not develop.

Effectiveness of IMZ and $NaHCO_3$ mixtures. IMZ did not control green mold caused by the IMZ-resistant isolate of *P. digitatum*, whereas green mold caused by the sensitive isolate was controlled effectively (Table 1). Combinations of IMZ and $NaHCO_3$ controlled green mold caused by both isolates. Treatment with either 3% $NaHCO_3$ or IMZ alone at 500 µg/ml reduced green mold incidence from 96.3% among the water-treated fruit to 44.4 and 63.0%, respectively. Treatment with their combination reduced green mold incidence to 6.5%.

In a similar experiment with the IMZ-resistant isolate alone, treatment with $NaHCO_3$ particularly reduced the incidence of green mold when 3% $NaHCO_3$ was used, whereas the effectiveness of treatment with IMZ at 500 µg/ml was poor (Fig. 10). The effectiveness of the combination of IMZ and $NaHCO_3$, specifically when 2 or 3% $NaHCO_3$ was used, was comparatively greater than any of these alone. The pH within lemon wounds treated with $NaHCO_3$, alone or combined with IMZ, were not statistically different from each other (Fig. 11). The pH increased when the $NaHCO_3$ concentration in the treatment solution increased and was not influenced by the presence of IMZ.

One week after their preparation, the pH of solutions containing $NaHCO_3$ increased from 8.4 to 9.3 (Table 2). The 1-week-old solution effectively controlled green mold after inoculation of fruit with the IMZ-resistant isolate D201, although its effectiveness had decreased slightly and significantly (mean incidence of green mold after each treatment in a paired *t*-test, $P = 0.0292$) compared with that of the fresh solution.

Residues of IMZ in lemon fruit immersed for 30 s in IMZ alone at 500 µg/ml or with 3% $NaHCO_3$ were 2.59 (±0.45) and 2.56 (±0.28) µg/g of fruit fresh weight, respectively, and did not differ significantly. Residues (± standard deviation) of IMZ in lemon fruit immersed for 60 s in IMZ alone at 500 µg/ml or with 3% $NaHCO_3$ were 3.58 (±0.07) and 3.02 (±0.45) µg/g of fruit fresh weight, respectively, and did not differ significantly.

The appearance of lemon fruit treated with $NaHCO_3$ was not visibly changed, although a thin white residue of the salt was present on the fruit. Weight loss among water-treated lemon fruit was 4.71% after 3 weeks of storage at 25°C. Among lemon fruit treated with 1, 2, or 3% $NaHCO_3$ alone, weight loss averaged 7.02%; whereas, among those treated with IMZ alone at 500 µg/ml, weight loss averaged 6.20%. Weight loss among lemon fruit treated with the combination of IMZ at 500 µg/ml and 1, 2, or 3% $NaHCO_3$ averaged 7.32%. Weight loss rates were similar after $NaHCO_3$, IMZ, or combination treatments and significantly increased compared with water-treated controls.

DISCUSSION

The combined use of IMZ and $NaHCO_3$ inhibited spore germination and controlled green mold decay, even that caused by an IMZ-resistant isolate. Germination of spores of both *P. digitatum* isolates (M6R and D201) was inhibited in vitro at elevated pH and with increasing IMZ or $NaHCO_3$ concentrations, particularly when these solutions were used at higher pH (8 or 9).

The growth of most *Penicillium* spp. is inhibited by high pH. In addition to direct toxicity, Griffin (10) stated that the influence of pH on fungal growth is complex and dependent upon the ionization of acids or bases in the medium in which the fungus resides, and that pH can alter membrane potentials that change the permeability of fungal membranes to many substances, including toxic compounds.

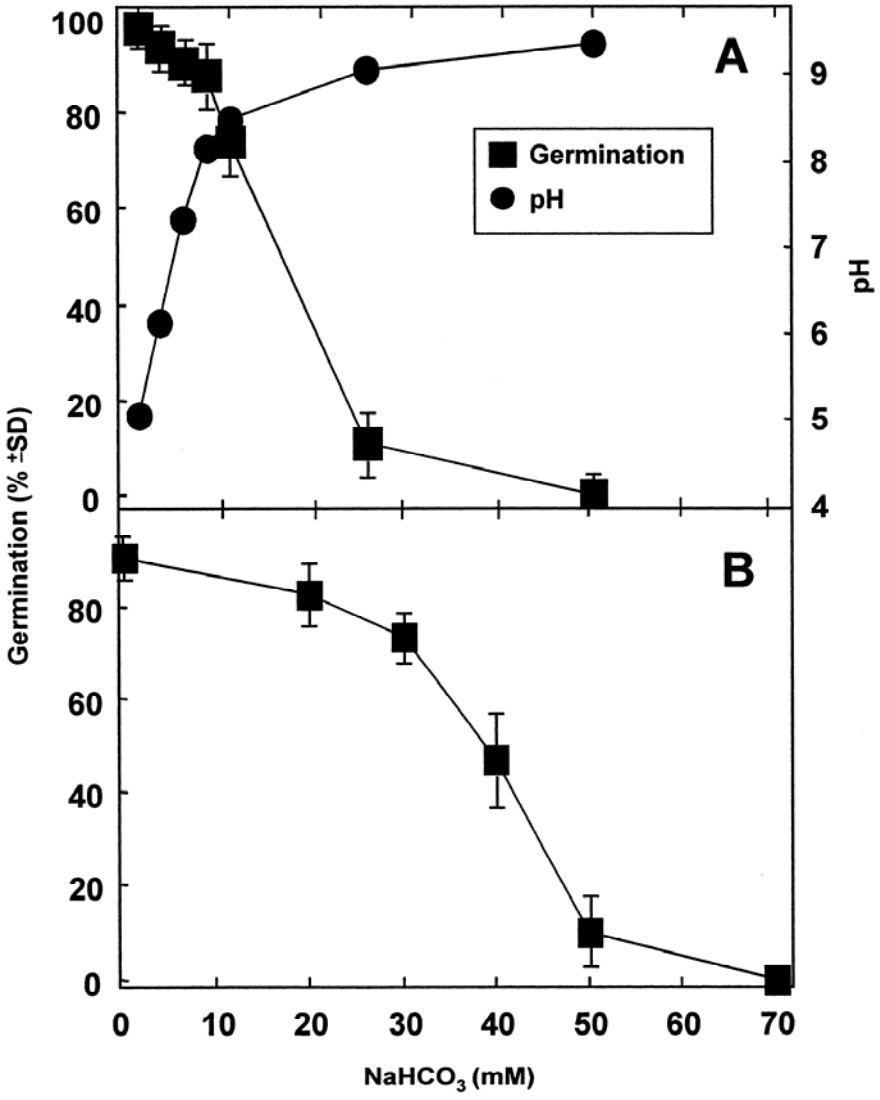

Fig. 4. Germination of spores of *Penicillium digitatum* isolate M6R (imazalil sensitive) after incubation at 24°C for 24 h in potato dextrose broth containing $NaHCO_3$. **A,** The pH of the solution was not buffered and it increased with $NaHCO_3$ concentration; **B,** the pH was controlled with phosphate buffers to about pH 7.

Hwang and Klotz (15) stated that hydrogen and hydroxyl ion concentrations were important factors in the inhibitory or lethal activity of many compounds to fungal spores, although they reported that brief exposures of spores of *P. digitatum* and *P. italicum* to media of pH 2.4 to10.0 did not result in mortality and that they could germinate afterward. Marloth (21) reported that the maximum germination of spores was between pH 2.8 and 3.1.

IMZ interferes with ergosterol synthesis by inhibition of C-14 demethylation of lanosterol or 24-methylene-24, 25-dihydrolanosterol, resulting in accumulation of C-14 methyl sterol precursors (18,36). The fungitoxicity of demethylation inhibitors (DMIs) has been attributed to depletion of ergosterol and to accumulation of sterol precursors in fungal membranes. This abnormal sterol content causes membrane hyperfluidity, leading to changes in membrane permeability and activity of membrane-bound enzymes (17). IMZ alters membrane activity; Griffin (10) stated that, at low concentrations of IMZ, selective changes in membrane permeability may occur; leakage of potassium ions is the first detectable event and, at higher concentrations, leakage of amino acids and other metabolites follow. The target enzyme (cytochrome P-450_{14DM}) for DMIs is present in both IMZ-sensitive and IMZ-resistant isolates of *P. italicum*; its inhibition occurred at higher extracellular concentrations of IMZ in resistant isolates (11). Resistance to IMZ in *P. digitatum* occurred by efflux of the fungicide by an ATP-binding cassette (12), which also affected resistance to other fungicides (23).

Our results indicated that the toxicity of IMZ was pH dependent, expressed as inhibition of either the germination of spores or its effectiveness to control green mold on fruit, and increased with increasing pH. Lukens (20) reported that neutral forms of fungicides penetrated membranes and were more toxic than charged forms. Siegel et al. (35) showed the fungicide was more toxic to *P. italicum* at pH 7 than at pH 5, and observed that little IMZ entered the mycelium at pH 5 compared with pH 7. They ascribed this difference in potency to the charge present on the molecule and concluded that reduced toxicity of IMZ at lower pH, when the molecule is charged, happens because it is not incorporated by the mycelium. Holmes and Eckert (14) reported an increase in IMZ activity against *P. digitatum* when the pH of the assay medium was in the range of pH 5.1 to 5.9, which reflected the greater concentrations of dissociated IMZ at the higher pH value. They found that the EC_{50} for an IMZ-resistant *P. digitatum* isolate was 4.66 µg/ml at pH 5.1; however, it was 0.88 µg/ml at pH 5.7, which corroborates our findings with wider pH range. A slight difference in the pH could have a significant effect on the concentration of the neutral (dissociated) form of IMZ, which is largely responsible for the antifungal activity of this compound (11,35). Above the pK_a of IMZ (pH 6.53), the imide nitrogen of the molecule is primarily undissociated, the molecule is not charged, and it is more lipophilic and soluble in membranes. At pH below the pK_a, the imide nitrogen is primarily protonated, the molecule is charged, and it has less lipid solubility and poor penetration into membranes.

Our results showed that $NaHCO_3$ inhibited germination of spores of *P. digitatum* and it was more effective at higher pH. Toxicity of $NaHCO_3$ to spores of *P. digitatum* has been reported previously (21,38). Punja and Grogan (32) also found that carbonate and bicarbonate anions consistently inhibited *Sclerotium rolfsii,* irrespective of their associated cations, and were only fungicidal at 30 or 50 mM concentrations. In practical experiments to control green mold on citrus fruit, $NaHCO_3$ was more effective than its potassium ($KHCO_3$) or ammonium (NH_4HCO_3) salts (38).

Palmer et al. (25) showed that the bicarbonate ion concentration in solution is directly related to the pH of that solution. Bicarbonates are ineffective under acidic conditions because carbonic acid predominates in solutions at lower pH, and it is unstable and readily decomposes to carbon dioxide gas and water. As the pH of the solution increases, the concentration of bicarbonate ion increases. Above pH 8.5, bicarbonate ion concentration decreases and that of carbonate ions increases. Bicarbonates may have several modes of action against fungi, including an elevated pH environment and increased osmotic stress. In addition, it was demonstrated that sodium bicarbonate inactivated extracellular enzymes from *Penicillium* spp. and may directly interact with membranes to alter their normal activities and disrupt cellular physiology (4,8,24,25). Sears and Eisenberg (34) concluded that the bicarbonate would cause an increase in the membrane permeability to ionic species. Fallik et al. (8) reported that $KHCO_3$ inhibited in vitro mycelial growth, spore germination, and germ tube elongation of *Botrytis cinerea* and *A. alternata.* Germ tube elongation was found to be more sensitive

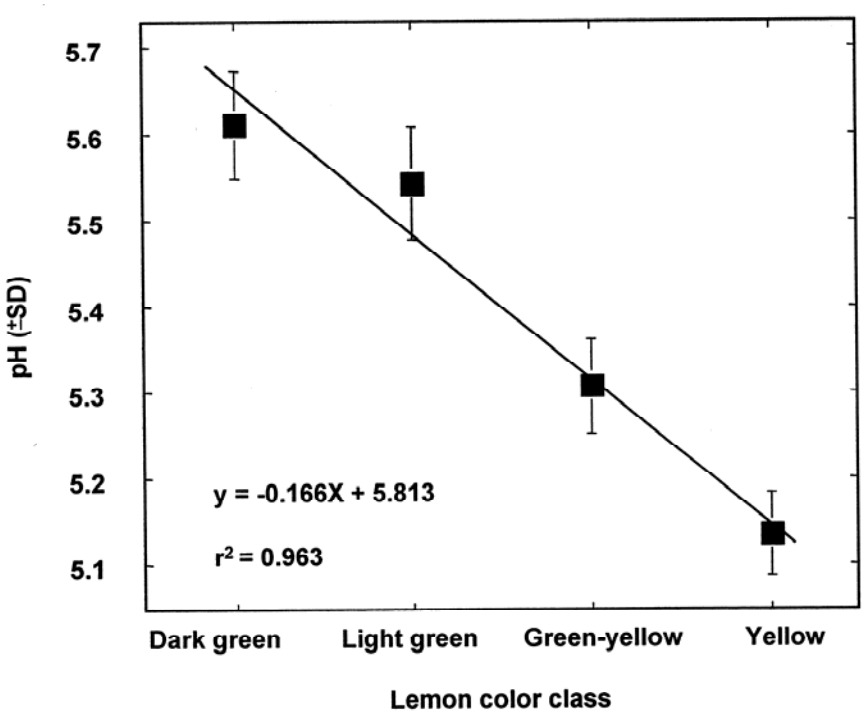

Fig. 5. Natural albedo pH in relation to lemon color. The pH was measured at a depth of 1.5 mm in wounds 1 mm wide and 2 mm deep.

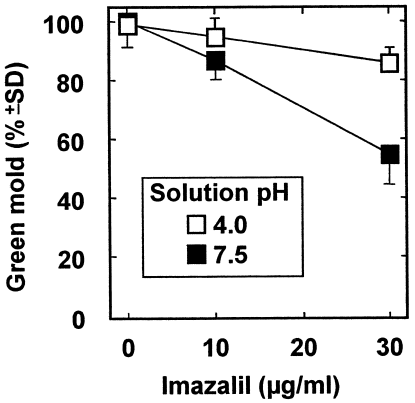

Fig. 6. Green mold incidence on lemon fruit as affected by imazalil concentration and pH of the treatment solution. Fruit were inoculated with *Penicillium digitatum* imazalil-sensitive isolate M6R 24 h before they were immersed in the solutions for 60 s, followed by storage for 2 weeks at 20°C.

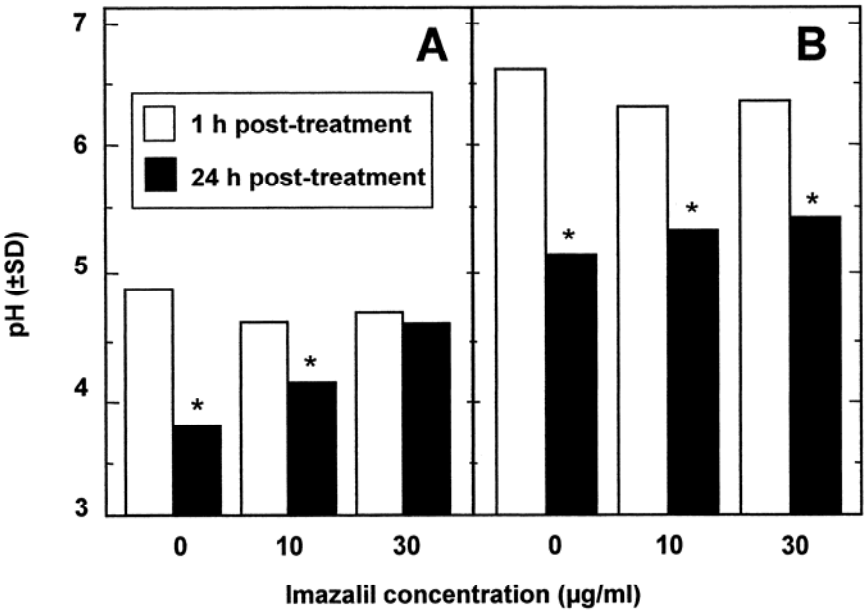

Fig. 7. pH in lemon wounds 1 and 24 h after immersion for 1 min in imazalil solution at pH **A,** 4.0 or **B,** 7.5. The pH was measured at a depth of 1.5 mm in wounds 1 mm wide and 2 mm deep. Presence of asterisk indicates pH after 24 h was significantly lower than that after 1 h.

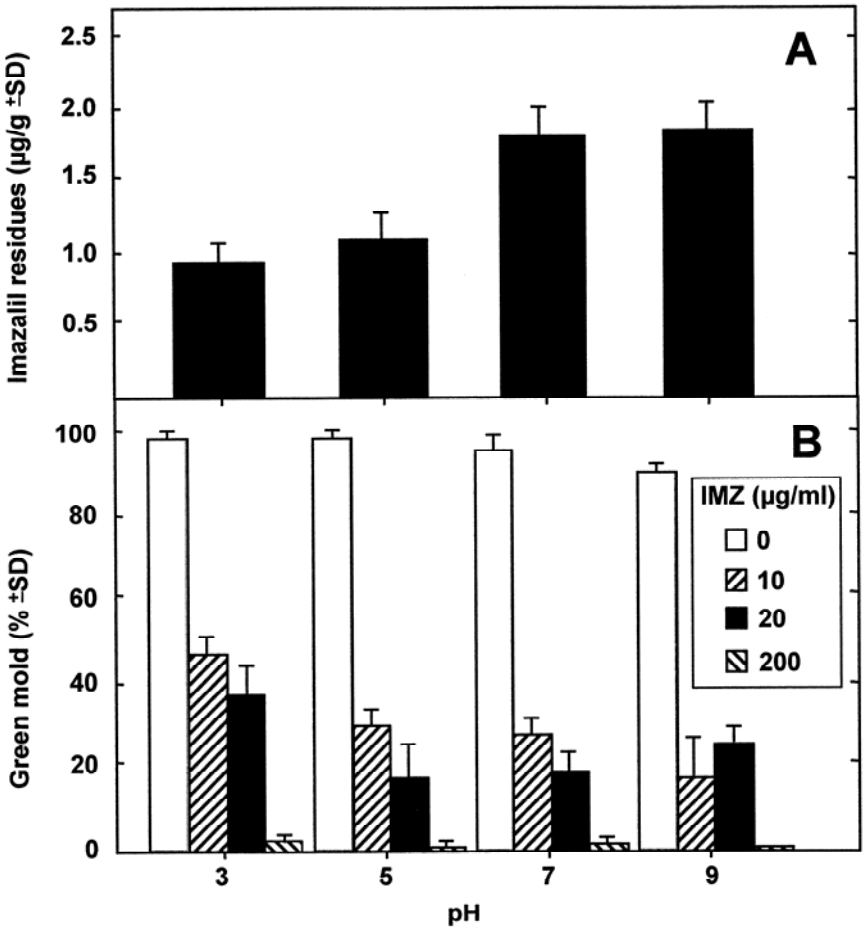

Fig. 8. A, Effect of solution pH on imazalil (IMZ) residues on orange fruit (µg/g fresh weight) immersed for 30 s in solutions of IMZ at 500 µg/ml. **B,** Green mold incidence on orange fruit inoculated with IMZ-sensitive isolate M6R after 30 s of immersion in solutions of IMZ at 0, 10, 20, or 200 µg/ml as affected by solution pH.

than mycelial growth and spore germination to elevated $KHCO_3$ concentrations. They concluded that inhibitory effect of $KHCO_3$ on the two fungi probably was due to a reduction in fungal cell turgor pressure which resulted in collapse and shrinkage of hyphae and spores.

Our observations showed a relationship between lemon color, which indicates fruit age and maturation, and pH within the albedo. The greenest lemon fruit were highest in pH, whereas the pH of the albedo of yellow and oldest fruit was lowest. Although the pH decline was not large, it could contribute to an increased susceptibility of the fruit to green mold infection as they age (6). The natural pH within the wounds was relatively low and would not promote IMZ activity. The susceptibility of harvested fruit and vegetables to decay agents depends mainly on their ripening stage at the time of picking and increases as ripening progresses. Various tissue characteristics, such as the acidity level, tissue turgor, and nutrient availability, change throughout the senescing and ripening stages and may, separately or in combination, enhance the susceptibility to disease (1).

Spores of *P. digitatum* do not germinate in water on the surface of citrus fruit unless the peel is injured (42). However, if the peel is wounded during harvesting or subsequent handling and processing, *P. digitatum* spores germinate and initiate infection, and eventually develop into typical green mold lesions. Low pH (between

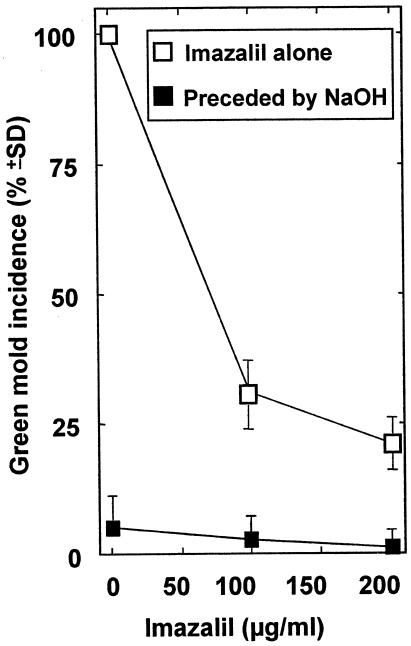

Fig. 9. Green mold incidence on lemon fruit after treatment with imazalil at different concentrations either alone or preceded by 1 N NaOH, followed by storage for 2 weeks at 20°C. Fruit were inoculated with *Penicillium digitatum* imazalil-sensitive isolate M6R 24 h before treatments.

4 to 5.5), in addition to other compounds, stimulates the germination of spores and the mycelial growth of *P. digitatum*, which is a pathogen specific to citrus fruit (9,27). At higher pH (6 to 8), conidial germination and germ tube growth rates of *P. digitatum* were lower than those at lower pH (4 to 5.5).

In our work, green mold incidence was affected only modestly by the pH of the IMZ solution. When measured 1 h after treatment with the pH 7.5 buffer solution, the pH within wounds on lemon fruit increased. The pH within these wounds (about 6.5) was not sufficient to inhibit growth of the pathogen alone, but it did improve IMZ effectiveness slightly. The extent of the pH change in wounds in the fruit rind tissue is influenced by the buffering capacity of the tissue. Lemon albedo apparently has large buffering capacity, because we were able to raise the pH only modestly within wounds on fruit, about 1.5 to 2 units, by alkaline buffers or $NaHCO_3$ solutions, and the pH declined sharply afterward. Because the pH in the wounds was increased only slightly, IMZ activity was improved only modestly by raising the pH of the treatment solution. The extremely high pH of NaOH, although it could injure the fruit, reduced green mold incidence alone most probably by inhibiting spore germination or germ tube growth while also increasing IMZ effectiveness. Treatments that combined IMZ with $NaHCO_3$ or other buffers that increased wound pH were more effective, even with an IMZ-resistant isolate (D201). This could be partially explained, as previously mentioned, by an increase in the pathogen membrane permeability or an increase in mobility and toxicity of the fungicide when in the neutral molecular form.

The increase in IMZ residues we observed on orange fruit at pH 7 or higher probably is the result of an increase in solubility of the neutral form of IMZ into the fruit cuticle and natural waxes. Although the higher-pH IMZ solutions were only slightly more effective than the low-pH solutions for the control of green mold, the added residue that resulted from the high-pH solutions should improve the suppression of sporulation and provide superior protection against post-treatment infections later in storage. Application of IMZ at higher pH would help achieve fruit residues of 2 µg/g or more that are needed to control sporulation from *P. digitatum* lesions; control of sporulation is an important aspect of sanitation in the management of this disease (6).

P. digitatum prefers a low-pH environment for infection and reduces the pH of the tissue within lesions on fruit as it develops (30). Bateman and Beer (2) were the first to suggest the close relationship between pH and pathogenicity. They claimed that acidification of the tissue during pathogen attack was necessary in order to adjust the apoplastic pH to values that would be better suited for enzymatic degradation of plant cell walls by the pathogen. Similar influence of pH later was shown for postharvest pathogens (29–31). Prusky et al. (30) reported that tissue acidification during *Penicillium* spp. pathogenesis is enhanced by the accumulation of organic acids or H^+ excretion, which influences cell wall integrity by changes in calcium chelation activities and solubility (3,22). Prusky et al. (30) reported that *P. digitatum* and *P. italicum* isolates caused the decay symptoms 5 to 6 days after inoculation of citrus fruit and decreased the pH of tissue from about 4.7 in the healthy tissue to about 3.1 in the decayed lesion. In addition, treatment of Golden Delicious apple fruit with a 140-mM $NaHCO_3$ solution increased the pH to 7.1 from 4.4 and reduced *P. expansum*-induced decay. Its colonization could be enhanced by treatments with exogenous citric and gluconic acids, or suppressed by $NaHCO_3$ treatment, and they suggested that tissue acidification is a significant factor in determining virulence. In our experiments, exogenous treatments with $NaHCO_3$ or strong alkaline solutions applied to lemon fruit raised the pH within wounds and subsequently reduced green mold incidence.

In our study, we found that 1-week-old solutions of IMZ and $NaHCO_3$ were only

Table 1. Green mold incidence on lemon fruit after treatment with imazalil (IMZ; 200 or 500 µg/ml) and $NaHCO_3$ at 1 or 3% (wt/vol), alone or in combination, followed by storage for 2 weeks at 20°C[z]

	Green mold incidence (%)	
Treatment	**Isolate M6R**	**Isolate D201**
Control	100.0 a	96.3 a
1% $NaHCO_3$	62.0 b	43.5 c
3% $NaHCO_3$	38.0 c	44.4 bc
IMZ at 200 µg/ml	14.8 d	85.2 a
IMZ at 500 µg/ml	10.2 de	63.0 b
IMZ at 200 µg/ml + 1% $NaHCO_3$	6.5 de	35.2 c
IMZ at 500 µg/ml + 1% $NaHCO_3$	10.2 de	15.7 d
IMZ at 200 µg/ml + 3% $NaHCO_3$	4.6 de	16.7 d
IMZ at 500 µg/ml + 3% $NaHCO_3$	5.6 e	6.5 d

[z] Lemon fruit were inoculated with *Penicillium digitatum* IMZ-sensitive isolate M6R or IMZ-resistant isolate D201 24 h before treatments. Each value is the mean of four replicates of 27 fruit each. Values within columns followed by unlike letters differ significantly by Fisher's protected least significant difference ($P < 0.05$).

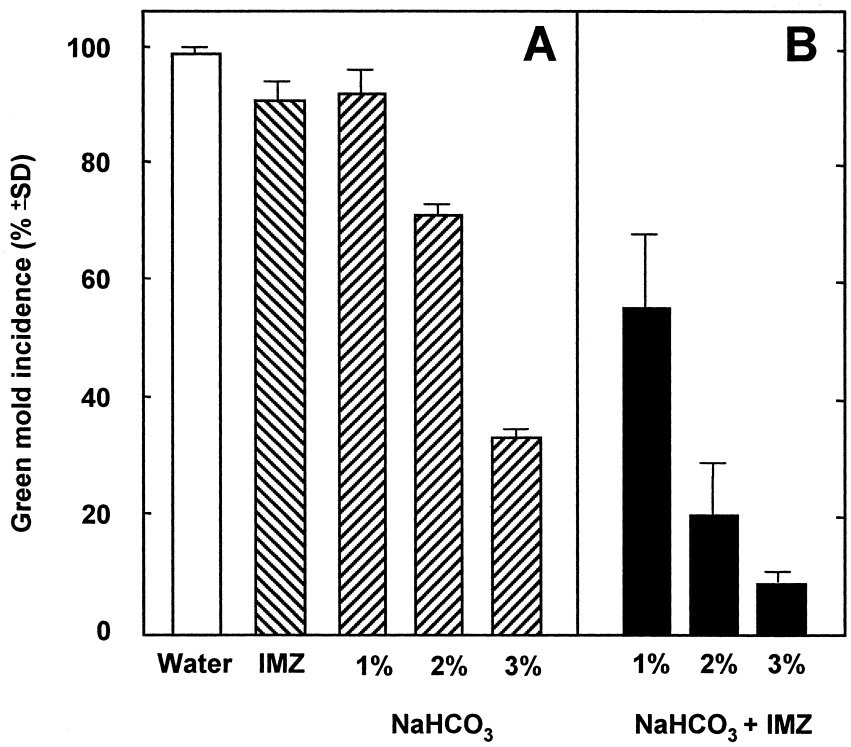

Fig. 10. Green mold incidence on lemon fruit after immersion for 60 s in **A,** water; sodium bicarbonate at 1, 2, or 3% (wt/vol); or imazalil (IMZ) at 500 µg/ml; or **B,** a combination of IMZ at 500 µg/ml with 1, 2, or 3% (wt/vol) sodium bicarbonate. Fruit were inoculated with *Penicillium digitatum* imazalil-resistant isolate D201 24 h before treatments, followed by the storage for 2 weeks at 20°C.

slightly less effective in controlling green mold, in spite of the increase in pH which presumably was due to a conversion of bicarbonate into carbonate ions. Hall (13), comparing fresh and 3-week-old IMZ solutions at 250 µg/ml at various pH levels (5, 6, 7, 8, 9, and 10) to control citrus green mold decay, found some loss of sporulation control at pH 5 whereas, at other pH values, suppression of sporulation was retained in freshly prepared solutions. However, in the aged solutions, there was a marked loss of sporulation control at pH 5, 6, and 7, whereas the treatments at pH 8, 9, and 10 were somewhat better. He concluded that low-pH solutions should be avoided. From our work, we believe the superior control of sporulation he observed probably was due to the higher residues present when the fruit were treated with IMZ at higher pH.

Combination of IMZ and $NaHCO_3$ resulted in greater effectiveness than when the solution pH was adjusted using other alkaline buffers whose mode of action would be due solely to elevated pH. The improved control of green mold with the combination of IMZ and $NaHCO_3$ could be explained by increased toxicity of the combination to the pathogen. This improvement could be a result of one or more of the following: (i) enhancement of IMZ toxicity due to high pH; (ii) inhibition of *P. digitatum* by high pH, although this is less likely because the pH within lemon wounds was increased by $NaHCO_3$ or other buffers to only between pH 6 and 7, which was insufficient to inhibit fungal growth; (iii) both $NaHCO_3$ and IMZ alter fungal membranes and their combination might elicit a synergistic action; and (iv) the toxicity of $NaHCO_3$, which has considerable potency in a neutral pH solution. In addition to direct toxicity to *P. digitatum*, $NaHCO_3$ may have a mode of action that includes interference with the infection process within wounds. Enzymes secreted by *Penicillium* spp. are instrumental for these pathogens to colonize fruit tissue. The bicarbonate ion could inhibit the activity of these enzymes in the host tissue by elevation of the tissue pH above their optima, or it could inhibit the secretion of these enzymes into the tissue by the pathogen.

The practical relevance of this work regarding the use of IMZ in citrus packinghouses includes the following points. (i) The combination of IMZ and $NaHCO_3$ was effective in controlling green mold even with fruit inoculated with an IMZ-resistant isolate of *P. digitatum.* (ii) The IMZ–$NaHCO_3$ solution was stable and long lasting. However, IMZ solubility declines at high pH and fine particles of it can be present; therefore, agitation of the solution is important. Filtration of the tank or drencher solution should be avoided because the particles become entrapped; therefore, heat pasteurization could be used instead. (iii) In our limited work on fruit quality, $NaHCO_3$ could be used up to 3% mixed with IMZ without harm to lemon appearance or excessive weight loss. However, in our work (37,38) and that of others (19), $NaHCO_3$ does increase weight loss; therefore, for long-term storage, the $NaHCO_3$ rate could be reduced to 1 or 2% and the application of $NaHCO_3$-compatible waxes could be used to minimize weight lost. (iv) $NaHCO_3$ will provide some control of sour rot, caused by *Geotricum citri-aurantii,* which is not controlled by IMZ and occasionally is the cause of significant decay losses in California (40). Finally, (v) the addition of $NaHCO_3$ to IMZ raises the solution pH and salt content, which can be significant disposal issues in some locations.

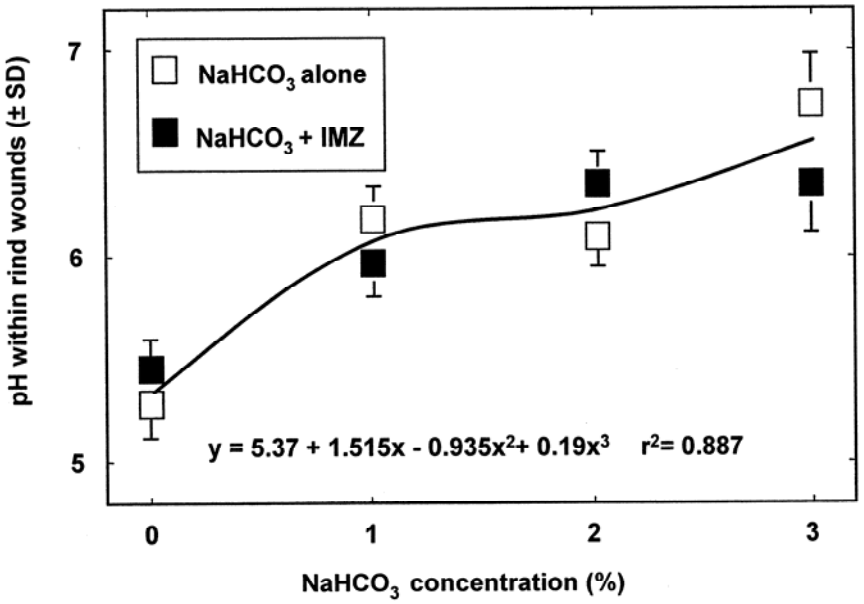

Fig. 11. Effect of $NaHCO_3$ concentration applied alone or with imazalil (IMZ) at 500 µg/ml on the pH within wounds on lemon fruit. Wounds (1 mm wide and 2 mm deep) were made before fruit were immersed for 60 s in treatment solutions. The pH was measured 5 to 6 h after treatment at a depth of 1.5 mm. The curve depicted is fitted to pH values within the wounds from both treatments.

Table 2. Green mold incidence on lemon fruit and pH of treatment solutions, fresh or 1 week old, that contained imazalil (IMZ; 500 µg/ml) or $NaHCO_3$ at 1, 2, or 3% (wt/vol) either alone or in combination[z]

	Solution age			
	Fresh		1 week old	
Treatment	pH	Green mold (%)	pH	Green mold (%)
Control	7.6	99.0 a	7.6	100.0 a
1% $NaHCO_3$	8.4	89.0 b	9.3	98.0 a
2% $NaHCO_3$	8.4	68.0 c	9.3	85.0 b
3% $NaHCO_3$	8.4	34.0 d	9.3	42.0 cd
IMZ (500 µg/ml)	7.6	90.0 b	7.1	92.0 ab
IMZ + 1% $NaHCO_3$	8.4	54.0 cd	9.3	54.0 c
IMZ + 2% $NaHCO_3$	8.4	16.0 e	9.3	36.0 d
IMZ + 3% $NaHCO_3$	8.4	7.0 e	9.2	9.0 e

[z] Lemon fruit were inoculated with *Penicillium digitatum* IMZ-resistant isolate D201 24 h before they were immersed in the solutions for 30 s, followed by the storage for 2 weeks at 20°C. Each value is the mean of four replicates of 27 fruit each. Values within columns followed by unlike letters differ significantly by Fisher's protected least significant difference ($P < 0.05$).

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