

Synergistic Antifungal Activity of Sodium Hypochlorite, Hydrogen Peroxide, and Cupric Sulfate against *Penicillium digitatum*

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ABSTRACT

Oxidizing compounds such as sodium hypochlorite (NaClO) and hydrogen peroxide (H₂O₂) are widely used in food sanitization because of their antimicrobial effects. We applied these compounds and metals to analyze their antifungal activity against *Penicillium digitatum*, the causal agent of citrus green mold. The MICs were 300 ppm for NaClO and 300 mM for H₂O₂ when these compounds were individually applied for 2 min to conidia suspensions. To minimize the concentration of these compounds, we developed and standardized a sequential treatment for conidia that resulted in loss of viability on growth plates and loss of infectivity on lemons. The in vitro treatment consists of preincubation with 10 ppm of NaClO followed by incubation with 100 mM H₂O₂ and 6 mM CuSO₄ (cupric sulfate). The combination of NaClO and H₂O₂ in the presence of CuSO₄ produces a synergistic effect (fractional inhibitory concentration index of 0.36). The sequential treatment applied in situ on lemon peel 24 h after the fruit was inoculated with conidia produced a significant delay in the fungal infection. The in vitro treatment was effective on both imazalil-sensitive and imazalil-resistant strains of *P. digitatum* and *Geotrichum candidum*, the causal agent of citrus sour rot. However, this treatment inhibited 90% of mycelial growth for *Penicillium italicum* (citrus blue mold). These results indicate that sequential treatment may be useful for postharvest control of citrus fruit diseases.

Green mold, caused by *Penicillium digitatum* Sacc., is probably the most common worldwide postharvest disease of citrus fruits. During postharvest handling, about 90% of production loss is caused by this disease (5). Synthetic fungicides such as imazalil (IMZ) and thiabendazole are widely used to control green mold (17, 18, 31). With the appearance of strains resistant to fungicides, increased doses have been necessary to effectively protect fruits (3, 7, 11, 36). However, residues of these compounds in fruit may exceed the maximum residue limit allowed by importing countries. Thus, alternative methods for control of citrus fruit diseases are required to avoid environmental contamination (19, 34).

Oxidizing biocides such as chlorine and peroxides are nonspecific in their mode of action but are preferred for general sanitation because of their low cost and availability (4). Chlorine and some hypochlorite salts have been used for many years to sanitize drinking water, fruits, vegetables, and equipment for food processing. For treatment of fruits and vegetables, chlorine is commonly used at concentrations between 50 and 200 ppm with a contact time of 1 to 2 min (35). Hydrogen peroxide has been successfully used

for disinfection of minimally processed fruits and vegetables and has been experimentally applied for control of postharvest decay in fresh fruits (6, 23, 27–30).

Hydrogen peroxide and other peroxides produce the hydroxyl radical (HO•) through the Fenton and Haber-Weiss reactions. The transition metals (e.g., copper or iron) participate as catalysts in the formation of these free radicals (10). Excessive amounts of HO• and other reactive oxygen species have deleterious effects on cells, leading to damage in DNA, RNA, lipids, and proteins (20). In our laboratory, we demonstrated that copper acts as a mediator of hydroperoxide-induced damage in *Escherichia coli*. This process irreversibly affects the respiratory chain, with the consequent loss of bacterial viability (24–26). Thus, we hypothesized that a combination of sodium hypochlorite, hydrogen peroxide, and transition metals would induce irreversible oxidative damage in fungi. In this work, we assayed in vitro and in situ the effect of a sequential treatment with a combination of sodium hypochlorite, hydrogen peroxide, and cupric sulfate on *P. digitatum* conidia. The in vitro treatment also was applied to other postharvest pathogens of citrus such as *Penicillium italicum* Wehmer (causal agent of blue mold) and *Geotrichum candidum* Link (causal agent of sour rot). Our studies represent a first step toward finding a suitable method for prevention and control of postharvest diseases in citrus fruits.

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MATERIALS AND METHODS

Strains and preparation of conidia suspensions. An isolate of *P. digitatum* sensitive to IMZ was obtained from naturally infected citrus fruits. This strain was identified according to the key of Pitt and Hocking (22) and registered as PD-A in our laboratory collection. Isolates of *P. digitatum* resistant to IMZ, of *Penicillium italicum*, and of *Geotrichum candidum* (PD-1, PI-A, and GC-A, respectively) were provided by the Laboratory of Phytopathology (National Institute of Agricultural Technology, Tucumán, Argentina). PD-1 was resistant to immersion in 4 g liter⁻¹ IMZ for 30 s (33). The strains were grown on potato dextrose agar (PDA) containing 0.1 mg ml⁻¹ streptomycin (Sigma-Aldrich, St. Louis, MO), pH 5.5, at 28°C for 7 to 10 days. Suspensions of conidia and arthroconidia were prepared with sterile distilled water containing 0.05% Tween 80 (Sigma-Aldrich), vortexed for 5 to 10 s, and filtered through two layers of cheesecloth to remove hyphal fragments. The concentration of conidia and arthroconidia was determined by counting in a Neubauer chamber and was adjusted with sterile distilled water to 1×10^6 conidia ml⁻¹ (equivalent to $A_{420\text{ nm}} = 0.2$) and 2.5×10^6 arthroconidia ml⁻¹, respectively. The conidia concentration was recommended for evaluation tests of postharvest treatments for green mold control (31).

MIC determination. The MIC was defined as the lowest concentration of each compound that ensures the inhibition of mycelium growth for at least 14 days at 28°C. Aliquots of the conidia suspension (500 µl) were incubated at 25°C for 2, 5, or 10 min with different concentrations of sodium hypochlorite (NaClO) (10, 100, 200, or 300 ppm). After incubation, samples were centrifuged at $3,500 \times g$ for 1 min, and the supernatants were discarded. Pellets were washed twice with sterile distilled water and resuspended to the original volume with sterile distilled water. Aliquots (5 µl) were taken from each sample and spot inoculated onto PDA plates. Suspension pH was measured before the spot inoculation. Similar procedures were carried out with hydrogen peroxide (H₂O₂) (100, 200, or 300 mM), cupric sulfate (CuSO₄) (10, 100, 200, or 500 mM), and ferric chloride (FeCl₃) (10, 100, 200, or 500 mM).

Sequential treatment for conidia and assays of viability and infectivity. Combinations of compounds were studied using a concentration range lower than the corresponding MIC. The conidia suspension was subjected to two sequential incubation procedures (see scheme in Fig. 1). The first was performed in the presence of 10, 50, or 100 ppm of NaClO at 25°C for 2 min, and the second was performed with different concentrations of H₂O₂ (10, 50, 100, or 200 mM) in the presence of 6 mM CuSO₄ or FeCl₃ at 25°C for 2 min. After each incubation, conidia suspensions were centrifuged, washed, and resuspended up to the original volume. As a control, the conidia suspension was incubated twice with sterile water. For viability determination, aliquots of conidia suspension (5 µl) were placed on PDA and incubated at 28°C. To calculate the MIC of H₂O₂ in the sequential treatment, the diameter of the mycelium was measured daily for 7 days. Interactions between different concentrations of NaClO and H₂O₂ were determined by the fractional inhibitory concentration (FIC), which was calculated as follows: (MIC of compound A, tested in combination)/(MIC of compound A, tested alone) + (MIC of compound B, tested in combination)/(MIC of compound B, tested alone). This interaction was defined as synergistic when the FIC value was <1, additive when the value was 1, and antagonistic when the value was >1 (2).

To evaluate the infectivity of conidia, lemons (cv. Eureka) without commercial postharvest treatments were used for the as-

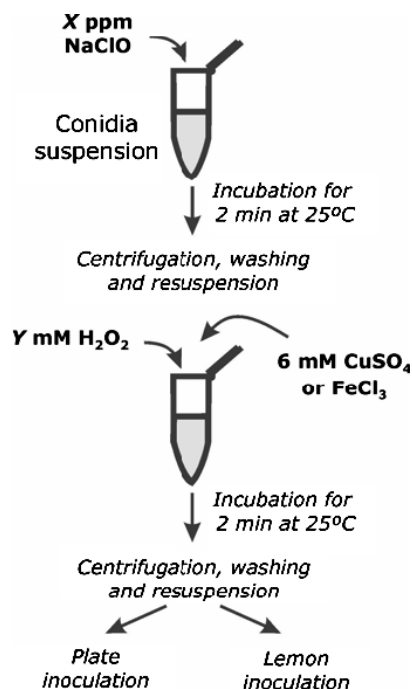


FIGURE 1. Scheme of the sequential treatments performed on *P. digitatum* conidia. (X) Different concentrations of NaClO; (Y) different concentrations of H₂O₂.

says. Aliquots (10 µl) of the conidia suspension were placed on wounds on the lemon peel. Wounds were created using a steel rod with a 1-mm-wide and 2-mm-long tip, penetrating both flavedo and albedo tissues but not juice sacs (5). Fruits were then stored at 20°C and 95% relative humidity for 14 days. Fruits were analyzed daily to evaluate the incidence of infection. The diameter of each lesion was measured to calculate the infection severity.

In situ sequential treatment on lemons previously inoculated with *P. digitatum*. Lemon fruits were washed with abundant fresh water and rinsed several times with distilled water. Randomized fruits were wounded in two equatorial opposed sites and then inoculated with 10 µl of *P. digitatum* conidia suspension on each wound. Fruits were stored at 20°C and 95% relative humidity. After 24 h, each wound was treated with 10 µl of 10 ppm of NaClO for 2 min, washed with sterile water, and treated with 20 µl of a mixture containing 6 mM CuSO₄ and 10, 50, or 100 mM H₂O₂. As a positive control, fruits inoculated with *P. digitatum* conidia were treated with sterile water. Treated fruits were stored at 20°C and 95% relative humidity for 14 days, and infection incidence was evaluated daily.

Statistical analysis. Lesion diameters and incidence percentages were analyzed by analysis of variance. When appropriate, means were separated with a least significant difference test (LSD, $P \leq 0.05$).

RESULTS

MICs of the compounds assayed. The antifungal activity of several chemicals against PD-A was studied as a function of time (Table 1). The MIC of NaClO was 300 ppm with 2 min of exposure, whereas it decreased to 50 ppm when conidia were exposed for 10 min. By contrast, H₂O₂ had only a slight decrease in the MIC with an increase of the incubation time (Table 1). In the presence of either copper or iron salts, the MIC could not be determined

TABLE 1. MICs of compounds at different incubation times

Incubation time (min)	MIC ^a			
	NaClO (ppm)	H ₂ O ₂ (mM)	CuSO ₄ (mM)	FeCl ₃ (mM)
2	300	300	>500	>500
5	100	200	>500	>500
10	50	200	>500	>500

^a For MIC determinations, suspensions of PD-A conidia were exposed to different concentrations of each compound during the indicated times.

because salt concentrations as high as 500 mM did not affect the viability of conidia. During the treatments, pH values were maintained between 5.5 and 6.

Effect of sequential treatment on mycelial growth.

To evaluate the potential synergistic effect of compounds and to minimize the concentration of the chemicals used, we analyzed different combinations. Strains PD-A (IMZ sensitive) and PD-1 (IMZ resistant) were preincubated with sublethal NaClO concentrations and then incubated with different H₂O₂ concentrations in the presence of 6 mM CuSO₄. Each incubation was applied for 2 min. No changes in pH (5.5 to 6) were observed in the suspensions during these treatments. The sequential treatment with 10 ppm of

TABLE 2. Effect of combination of NaClO and H₂O₂ on PD-A conidia viability^a

MIC (combined compounds) ^b		FIC ^c	Results
NaClO (ppm)	H ₂ O ₂ (mM)		
10	100	0.36	Synergism
50	50	0.33	Synergism
100	10	0.36	Synergism

^a MICs of compounds alone was 300 ppm for NaClO and 300 mM for H₂O₂.

^b Suspensions of conidia were exposed to sequential treatment in the presence of 6 mM CuSO₄ and the indicated concentrations of NaClO and H₂O₂.

^c FIC = (MIC of compound A, tested in combination)/(MIC of compound A, tested alone) + (MIC of compound B, tested in combination)/(MIC of compound B, tested alone).

NaClO and 100 mM H₂O₂ produced a total inhibition of mycelial growth (Fig. 2). The H₂O₂ concentration necessary to prevent conidial growth was threefold lower than the MIC for this compound alone as previously determined for PD-A (Table 1). The use of 50 mM H₂O₂ generated a delay in mycelial growth with a lag phase of 5 and 6 days for PD-A and PD-1, respectively. The FIC for the interaction between NaClO (10 ppm) and H₂O₂ (100 mM) was 0.36, indicating a synergistic effect for these compounds together. This effect also was obtained for the other combinations assayed (see Table 2).

Suspensions of conidia and arthroconidia of PI-A and GC-A, respectively, were treated with a sequential treatment standardized for *P. digitatum*, and the MIC of H₂O₂ was calculated as described above. For PI-A, the sequential treatment with 10 ppm of NaClO and 100 mM H₂O₂ produced 90% growth inhibition and the MIC for the H₂O₂ alone was 150 mM (data not shown). For GC-A, the MIC for H₂O₂ was 10 mM. GC-A was the most sensitive of the studied pathogens (data not shown).

Iron was used as alternative catalyst instead of copper. FeCl₃ was less effective than CuSO₄; in the sequential treatment that included FeCl₃, the viability of the PD-A isolate was maintained even with 100 mM H₂O₂ (Fig. 3). Similar

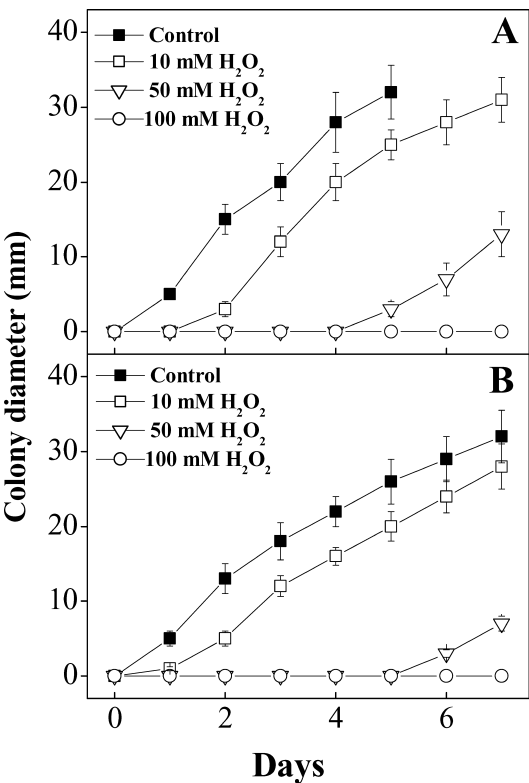


FIGURE 2. Growth of *P. digitatum* strains PD-A (A) and PD-1 (B) on PDA plates. Conidia suspensions were treated with sterile water (control) or exposed to sequential treatment shown in Figure 1, with 10 ppm of NaClO and the indicated H₂O₂ concentrations in the presence of 6 mM CuSO₄. Data are the mean of three independent experiments. Error bars indicate the standard deviation.

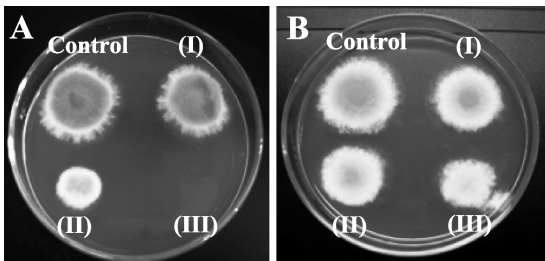


FIGURE 3. Effect of metals in the sequential treatment. Conidia of PD-A were treated with distilled sterile water (control) or with the sequential treatment shown in Figure 1 using 10 ppm of NaClO and 10, 50, or 100 mM H₂O₂ (I, II, and III, respectively) in the presence of CuSO₄ (A) or FeCl₃ (B). Photographs correspond to 4 days of growth on PDA plates. Data are representative of four independent experiments.

TABLE 3. Green mold incidence and severity in lemon fruits inoculated with *P. digitatum* conidia after sequential treatment^a

Treatments ^b	Green mold incidence (%) ^c			Green mold severity (%) ^d		
	4 days	7 days	14 days	4 days	7 days	14 days
Control	60 A	100 A	100 A	50 A	100 A	100 A
ST + CuSO ₄	0 B	0 B	0 B	0 B	0 B	0 B
ST + FeCl ₃	0 B	33 C	33 C	0 B	60 C	90 A

^a Values are the mean of three assays with 10 fruits for each condition. Within each column, means with different letters are significantly different (LSD, $P \leq 0.05$).

^b For the control, conidia suspensions were treated with sterile water. Sequential treatment (ST) included 10 ppm of NaClO, 10 mM H₂O₂, and 6 mM CuSO₄ or 6 mM FeCl₃.

^c Incidence percentage was calculated with respect to total inoculated fruits for each treatment.

^d Severity percentage was calculated on the basis of lesion diameter.

results were obtained with the PD-1 isolate (data not shown).

Infectivity of *P. digitatum* conidia after sequential treatment. As an indicator of conidial infectivity, the incidence and severity of the disease on lemon fruit were evaluated. In the sequential treatment applied to PD-A, infection was prevented with 10 mM H₂O₂ (Table 3), but under this condition, conidia were viable (Figs. 2A and 3A). In the presence of FeCl₃ plus 10 mM H₂O₂, the infection incidence was 33% and all infected lemons were completely damaged at 14 days (Table 3). Nevertheless, with 50 mM H₂O₂ no lemon disease was observed (data not shown). Therefore, the effectiveness of the treatment was higher with added CuSO₄ than with added FeCl₃. For PD-1, the disease incidence and severity were similar to those of PD-A with both the cupric and ferric salts (data not shown).

Based on all the above results, we propose an in vitro sequential treatment to completely prevent conidial growth of *P. digitatum*: 2 min of preincubation with 10 ppm of NaClO followed by a 2 min of incubation with 6 mM CuSO₄ and 100 mM H₂O₂.

In situ sequential treatment. Figure 4 shows the effect of the sequential treatment on lemon fruits previously inoculated with *P. digitatum* conidia (PD-A). Seven days after treatment, the disease incidence was almost 35% on fruits treated with 10 mM H₂O₂, whereas on water-treated fruits 67% were decayed. At 7 days, the disease was practically not observed on fruits treated with 100 mM H₂O₂. After 14 days, almost all control lemons were infected (95% incidence), whereas in lemons treated with 100 mM H₂O₂ the incidence was nearly 30%. According to these results, the development of green mold was significantly delayed with in situ treatment.

DISCUSSION

The combination of oxidizing agents (NaClO plus H₂O₂) in the presence of CuSO₄ as proposed here com-

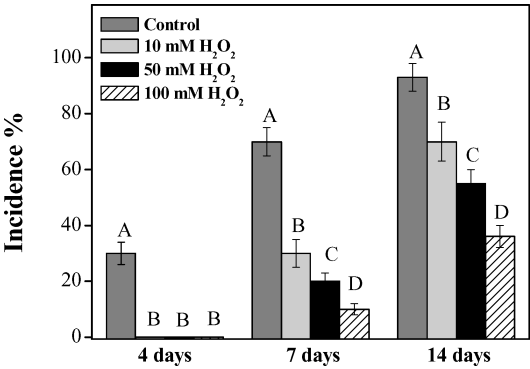


FIGURE 4. Green mold incidence on lemons inoculated and then treated in situ. Lemons were wounded and inoculated with conidia of strain PD-A. One day after inoculation, the treatment was performed. Control fruits were treated with only sterile distilled water. Incidence percentages were calculated based on total inoculated fruits for each condition at 4, 7, and 14 days after treatment. Data are the mean of four replicates ($n = 10$). Error bars indicate the standard deviation. Different letters represent significant differences between treatments for each period of time (LSD, $P \leq 0.01$).

pletely inhibited the growth of *P. digitatum* and *G. candidum* on PDA plates and produced a significant inhibition of mycelial growth of *P. italicum*. This treatment also provided effective control of citrus green mold on inoculated lemons. The combination of compounds at concentrations lower than the individual MICs is important to minimize the probable negative impact that treatment could have on fruits. To diminish residues of chemicals on the fruits, the minimal assayed chlorine concentration was chosen in the sequential treatment. The NaClO and H₂O₂ are broadly used in the food industry as disinfectants, but in the sequential treatment the NaClO concentration was 20-fold lower than those used in packinghouses, and the H₂O₂ concentration was 10-fold lower than those used in fruit treatments (1, 12, 16). Because both chemicals are quickly degraded, the amount of residue on the fruit would be practically negligible.

The individual MIC for CuSO₄ could not be determined. Therefore, a concentration of 6 mM was chosen for the sequential treatment because it is the concentration generally used for plant fumigation. Although both copper and iron are transition metals able to catalyze cyclic oxidoreduction processes generating reactive oxygen species and peroxides (8, 9), in our study Cu(II) was more effective for producing the loss of viability and infectivity of *P. digitatum* conidia. These results are in agreement with previous findings from our laboratory, which revealed that *E. coli* oxidative damage produced by the peroxide t-butyl hydroperoxide was mediated by Cu(II) but not by Fe(III) (24).

Plant-fungal pathogen interactions produce a fast reactive oxygen species generation (oxidative burst). This event occurs as an early plant response to nonpathogen or avirulent attack (14) and involves H₂O₂ production (13, 32). This effect inhibits the germination of fungal spores during plant-pathogen interactions (21). In a recent study, *P. digitatum* infection suppressed the H₂O₂ oxidative burst

in host cells, but nonpathogenic *Penicillium* strains triggered the production of H_2O_2 in citrus exocarp (15). Because H_2O_2 is a critical element in the natural defense of plants against pathogens, our treatment might lead to inhibition of the growth of *P. digitatum* in lemon fruits in a way similar to that occurring in the plant. In this context, the sequential oxidative treatment proposed in this study using a low concentration of sodium hypochlorite with a Cu(II) salt and H_2O_2 is a promising alternative for control of *P. digitatum* infection. The use of this treatment could replace or reduce the doses of fungicides currently used in fruit packinghouses. This treatment was effective against both IMZ-sensitive and IMZ-resistant strains of *P. digitatum*. However, further studies are needed to standardize the conditions and determine the effectiveness of the sequential treatment for application in packinghouses.

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