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Postharvest Treatment with Hydrogen Peroxide to Control Orange Fruit Decay Caused by *Penicillum digitatum* and *Penicillum italicum*

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Abstract: Imazalil and Thiabendazole chemical based fungicides are currently used to control citrus green/blue molds, which is mainly caused by *P. digitatum* and *P. italicum*. In order to find alternative methods for control of citrus fruit diseases to avoid fungicide caused health and environmental problems, current research was conducted to explore the antifungal effectiveness of H_2O_2-Ag^+ (Hydrogen peroxide stabilized with silver ions), which is a universally applicable and high effective disinfectant against pathogenic microorganisms, and has been used to control postharvest decay of fresh fruits in most developed countries, in the context of *in vitro* and *in vivo P. digitatum* and *P. italicum* development in the Newhall navel orange. H_2O_2-Ag^+ was found to be effective in inhibiting *in vitro* radial growth and *in vivo* inoculated lesion development of *Penicillum italicum* and *Penicillum digitatum.* Dipping fruit with H_2O_2 at concentrations of 1-2% before storage reduced the decay incidence of orange after 30 and 60 days cold storage following by 3 days shelf life, although it was less effective than the positive control of fungicide Imazalil (500ppm). H_2O_2 provided a disinfectant effect on the pericarp, as indicated by significant reduction of total bacterial, mold and yeast counts. After cold storage and shelf-life, no significant difference was found among all treatments in the total soluble solids (TSS), titratable acid (TA), while higher vitamin C content was found in the Imazalil treated fruit. This research suggest that H_2O_2 can be an alternative to chemical fungicides that, although more effective, pose problems due to their residue levels and health concerns, especially for the organic fruit industry.

Keywords: Antifungal, Decay Incidence, Disinfection, Hydrogen Peroxide, Postharvest Disease

1. Introduction

Because of sticky weather in south China, postharvest rotting and losses of perishable Newhall navel orange grown here are as high as 39%, which is mainly caused by *P. digitatum* and *P. italicum*, and are probably the most common postharvest disease of citrus fruits worldwide [1]. The current products used to control green/blue molds worldwide are Imazalil and Thiabendazole chemical based fungicides [2, 3]. Long-term use of chemical fungicides in China cause pathogen resistance problem, accompanied by fungicide doses increasing and excess of fungicides residues on fruits [4]. Additionally, there is increasing concerns about environmental hazards of chemical residues. Thus, alternative methods for control of citrus fruit diseases are required to avoid health and environmental problems.

As a sanitizer to treat the surface of fruits and vegetables against pathogenic microorganisms, Hydrogen peroxide (H_2O_2) has been experimentally applied for control of postharvest decay in fresh fruits [3, 5, 6]. H_2O_2 has also successfully been used on fresh-cut produce because it leads to an improved disinfectant efficiency and protection against decay [5, 7, 8]. However, there are efficiency issues when using a pure hydrogen peroxide solution because of its instability. Therefore, hydrogen peroxide is incorporated with stabilizers, such as acetic acid, silver ions, to produce a more

stable and powerful disinfectant [9, 10]. Cerioni et al. demonstrated that combination of H_2O_2 and copper sulfate produces a synergistic effect on *in vitro* and *in vivo P. digitatum* and *P. italicum* infection [3, 11]. Hydrogen peroxide supplemented with a low concentration of Ag^+ is a universally applicable disinfectant in most developed countries and has been approved for use in the food and beverage industry, in public water supplies, and in swimming pools as a disinfectant, because this form of H_2O_2 can remain in solution at low concentrations for extended periods of time, allowing for long-term disinfection and higher inhibiting potency on bacteria growth [12, 13]. Moreover, it is a non-carcinogenic substance and does not change or develop odors in treated foods and water. Nabizaden et al. reported that combination of 2% H_2O_2 and 0.05% Ag^+ can kill all target bacteria in 15 minutes [12].

H_2O_2-Ag^+ is a universally applicable and high effective disinfectant. Several kinds of products has successfully been used to control pathogen-induced decay of table grapes, melon, potato, eggplant, and pepper [14-16]. In addition, it was shown to control potato silver scurf, dry rot and soft rot; and can inhibit sprouting during storage [17, 18]. Our aim was to determine the antifungal effectiveness of H_2O_2-Ag^+ in the context of *in vitro* and *in vivo P. digitatum* and *P. italicum* development in the Newhall navel orange.

2. Materials and Methods

2.1. In Vitro Antifungal Effects of H_2O_2-Ag^+ on P. digitatum and P. italicum

Liquid H_2O_2-Ag^+ formulation containing 5% H_2O_2 and 100 ppm Ag^+ was supplied by Sanosil LLC., Beijing, China. *P. digitatum* and *P. italicum,* originally isolated from a naturally infected Shatang mandarin, were purified and routinely cultured and maintained on nutrient PDA media.

In vitro antifungal experiments were conducted as described by Meng et al. [19]. A liquid stock solution of H_2O_2-Ag^+ (pH=2.5) was supplemented aseptically with PDA media to make three concentrations of 0.25%, 0.5% and 1.0% H_2O_2. Controls consisted of negative PDA plates with water, and positive fungicide treatment was Imazalil (500ppm). Mycelial growth of *P. digitatum* and *P. italicum* was tested by placing a 5 mm-diameter culture disc cut from the periphery of a 7-day-old mycelial mat in the center of PDA plates supplemented with appropriate concentrations of H_2O_2. The cultures were incubated at 28°C, with 6 replicate plates per test. Growth was determined as the average diameter of the mycelial mat, and the inhibition rate was calculated on day 7 and day 10.

2.2. In vivo Inoculation Experiments

The fungal conidial suspensions at a concentration of 10^6 conidia·mL^{-1} were prepared for the *in vivo* inoculation experiments. Freshly harvested Newhall navel oranges from local orchards (Xingning city, Guangdong Province) were used for inoculations. Fruits were first rinsed with tap water, wounded by inflicting two 2 mm deep wounds using a sterile needle, and inoculated with *P. digitatum* and *P. italicum* by placing a 10-μL aliquot of a conidial suspension on each wound and then air-dried overnight. Fruits were then drenching in different concentrations of H_2O_2 for about 2 min and again air-dried. Then, the treated fruits were grouped into cartons and stored at 25°C and 90-95% relative humidity. Each dipping treatment consisted of 25 fruits, i.e., 50 inoculation replicates per treatment. At a corresponding storage time, fruits were imaged and their lesion diameters were measured using digital calipers. Disease severity index was defined as increasing disease severity from 0 to 7 (0-none, 7-highest lesion size). The experiment was repeated three times.

2.3. Postharvest Treatments and Storage

Oranges were harvested between November and January. Medium sized and damage-free fruits were selected for experiments. Fruits were dipped for 2 min in either H_2O_2 (1.0-2.0%, pH 3.3-3.7 respectively), or fungicide (500 ppm of Imazalil, positive control). Non-treated negative control fruits were dipped in water at the same time. After treatment, fruits were air-dried and packed in perforated shipping cartons. Fruit were placed in a storage room at 8°C and 95% relative humidity. The fruits were examined for decay development after 30 and 60 days of storage plus three days at shelf-life 25°C. The results are presented as percentage of disease incidence. Each treatment was performed with 10 replicate cartons, with approximately 10 kg of fruit in each carton, and was repeated over two growing seasons.

2.4. Microbiological Analysis

Samples were prepared by taking 10 g of orange pericarp cut from three fruits immediately after all treatments done. Pericarp cuts were placed aseptically in stomacher bags, diluted 1:10 in buffered peptone water and homogenized by using a stomacher. The homogenate was subjected to a ten-fold gradient dilution using sterile water and pipetted into a sterile petri dish, followed by mixing with the appropriate melted agar formulation. The total aerobic bacterial count was determined on Plate Count Agar (PCA) (30°C for 3 days). The mold and yeast counts were enumerated on Potato Dextrose Agar (PDA) (28°C for 5 days). Three samples per treatment were aseptically taken and analyzed after incubation. The results were expressed as log10 colony forming units per gram (log CFU/g).

2.5. Fruit Total Soluble Solids, Titratable Acidity and Vitamin C Content

Fruit were sampled for quality analyses during cold storage and shelf life. Total soluble solids (TSS) was determined by digital refractometer (PAL-1, ATAGO, Japan) on 10 fruits per treatment. The titratable acid (TA) content was determined by titrating fruit juice from each sample with 0.1N NaOH to pH 8.1. The vitamin C content was measured by the indophenol method as described in AOAC method 967.21 (AOAC International, 2000). Five milliliters of orange juice was added to 5mL of a metaphosphoric acid-acetic acid solution and

titrated with an indophenol dye solution until a pink color persisted in the solution.

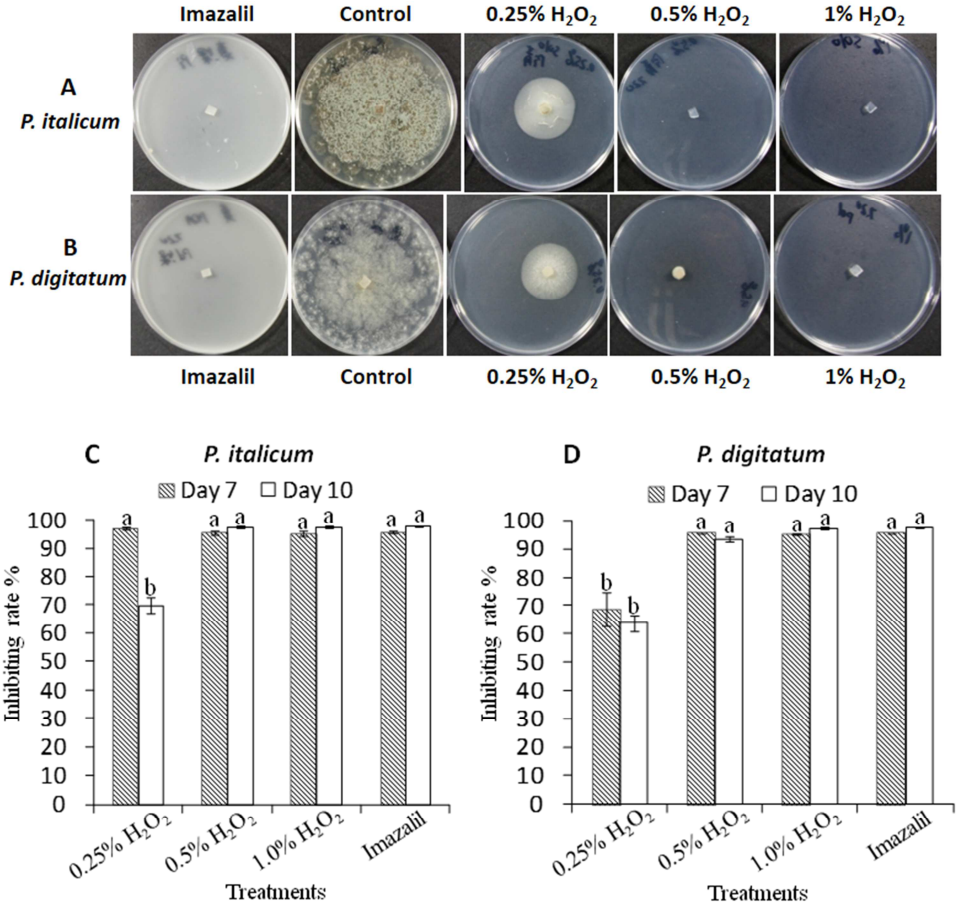
2.6. Statistical Analyses

All of the experiments were designed in a completely randomized fashion with three replicates. Data were subjected to analyses of variance (ANOVA) using SPSS software. Mean comparisons were analyzed using Duncan's multiple range test at P=0.05.

3. Results and Discussion

3.1. In Vitro Inhibitory Rate of H_2O_2 Against P. italicum and P. digitatum

The inhibition rate of H_2O_2, negative water control and positive Imazalil fungicide control, against the tested fungi are presented in Figure 1. After 7 days of incubation, *P. italicum* and *P. digitatum* showed normal radial growth on control PDA media, while the fungicide Imazalil (500ppm) completely inhibited their radial growth, and a nearly 100% inhibition rate of *P. italicum* and *P. digitatum* was observed with H_2O_2 at 0.5% and 1.0% (Figure 1). For a longer incubation time (10 days), there was a reduction of the inhibition rate by H_2O_2 (0.25%) on *P. Italicium* from close to 100% to 69.89%, while the inhibition rates at concentrations of 0.5% and 1.0% remained the same (Figure 1C). H_2O_2 at 0.5% and 1.0% showed the same controlling level on *P. digitatum* growth as on *P. italicum* (Figure 1C vs. Figure 1D). However, a lower inhibition rate was recorded on *P. digitatum* by 0.25% H_2O_2 after 7 and 10d of incubation (Figure 1D).



[+]Means followed by the same letters in the columns are not significantly different at P=0.05 according to Duncan's multiple range test. Bar=±S. E.

Figure 1. *In vitro culture of P. digitatum and P. italicum on PDA media supplemented with Imazalil (500ppm) or H_2O_2 (0.25%, 0.5% and 1.0%) (A, B) after 7 d incubation, and the inhibitory rate of colony growth after 7 and 10 days incubation (C, D).*

3.2. Effect of H_2O_2 Treatment on Disease Symptoms Caused by in Vivo Inoculation

As shown in Figure 2, no blue mold lesions developed in Imazalil-treated fruit on the wound site of inoculated fruit with *P. italicum* (Figure 2A). Imazalil treated fruit exhibited the smallest lesion diameter and the lowest disease severity index (Figure 2). For H_2O_2 treatments, it was found that the most effective results in controlling lesion development were obtained with H_2O_2 at concentrations between 1% and 2% (Figure 2A), with the same lesion diameter and disease index level as in the positive Imazalil treatment (Figure 2B and 2C). A lower inhibition rate was found for the 0.5% H_2O_2 treatment, where the lesion diameter and disease index were higher than with H_2O_2 treatment at 1.0% and 2.0% after 1 week at 25°C (Figure 2B and Figure 2C).

Decay lesions caused by *P. digitatum* develop on orange skins, as shown in Figure 3A, revealing that higher concentrations of H_2O_2 can suppress fungal proliferation of *P. digitatum*, as observed by a reduction in lesion size with increases of the H_2O_2 concentration. Significant differences in the lesion diameter and disease severity index were detected between the negative water and positive fungicide controls and H_2O_2 treatments (Figure 3B and 3C). H_2O_2 concentrations of 1% and 2% led to the most significant reductions in the lesion diameter and disease severity index, whereas the results obtained with H_2O_2 at a concentration of 0.5% showed that this concentration was much less effective. In general, similar inhibitory trends were observed in *P. digitatum* and *P. italicum* inoculated oranges, with more significant effects observed for H_2O_2 concentrations above 1.0%.

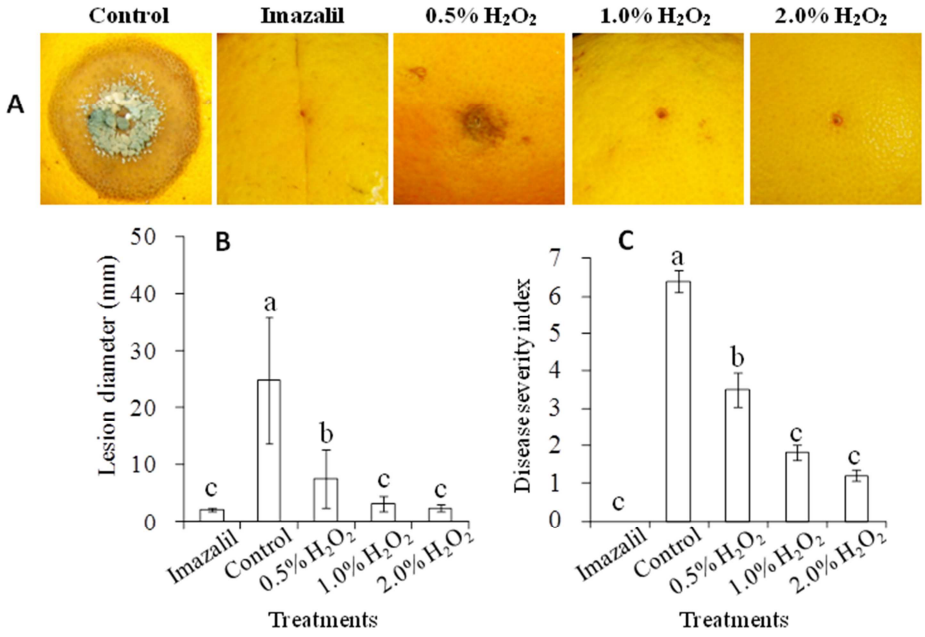


Figure 2. *Effects of H_2O_2 treatments on blue mold development in oranges inoculated with P. italicum.*

+After one week at 25°C, fruits were imaged (A) and the lesion diameter (B) and disease severity index (C) were measured and calculated. Means followed by the same letters in columns are not significantly different at *P*=0.05 according to Duncan's multiple range test. Bar=±S. E.

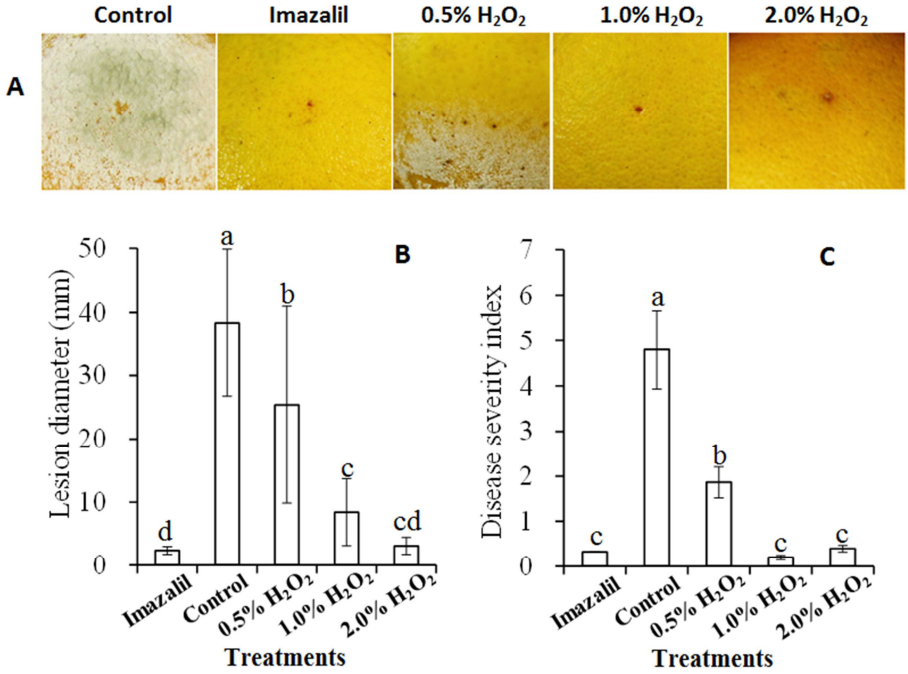


Figure 3. *Effects of H_2O_2 treatments on green mold development in oranges inoculated with P. digitatum.*

[+]After one week of storage, fruits were imaged (A) and the lesion diameter (B) and disease severity index (C) were measured and calculated. Means followed by the same letters in columns are not significantly different at *P*=0.05 according to Duncan's multiple range test. Bar=±S. E.

3.3. Effects of H_2O_2 on Total Bacteria, Mold and Yeast Counts on Orange Pericarp

As shown in Table 1, total bacterial, mold and yeast counts of oranges treated with positive Imazalil control and H_2O_2 were significantly reduced compared to those of the negative water control (Table 1). Compared to the Imazalil treatment, the 0.25% H_2O_2 treatment significantly decreased bacterial counts by 0.4 log CFU/g, although the two treatments had similar mold and yeast counts. However, 0.5% and 1.0% H_2O_2 treatment reduced the natural bacteria, mold and yeast populations on the surface of oranges to zero (Table 1).

Table 1. *Bacterial, mold and yeast counts on orange pericarp after treatment with different concentrations of H_2O_2.*

Treatments	Bacterial count (Log_{10}CFU/g)	Mold and yeast count (Log_{10}CFU/g)
Control	6.143±0.103 a	6.663±0.158 a
Imazalil	3.059±0.245 b	4.129±0.301 b
0.25% H_2O_2	2.662±0.096 c	3.758±0.187 b
0.50% H_2O_2	0±0 d	0±0 c
1.00% H_2O_2	0±0 d	0±0 c

[+]Colony counts were analyzed at the final incubation time which was on PCA (30°C for 3 days) and PDA (28°C for 5 days).
Means followed by different letters in rows are significantly different at P=0.05 according to Duncan's multiple range test. Bar=±S. E.

3.4. Effects of H_2O_2 on Disease Incidence of Orange Fruit During Cold Storage

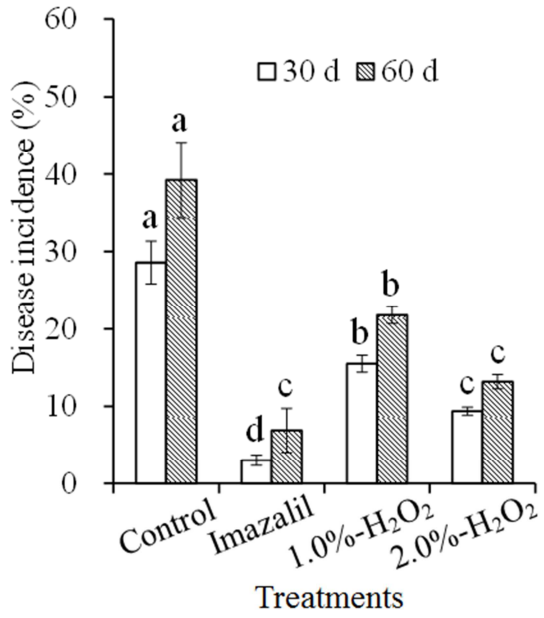


Figure 4. *Effects of H_2O_2 treatments on disease incidence mainly caused by P. digitatum and P. italicum which developed in oranges after 30 and 60 days at 8°C plus 3 d at 25°C.*

[+] Means followed by the same letters in columns are not significantly different at P=0.05 according to Duncan's multiple range test. Bar=±S. E.

After 30 days of cold storage plus 3d shelf life, a very low disease incidence was observed in positive fungicide Imazalil-treated fruit. While 1.0% H_2O_2 treatment reduced the decay incidence to 15.5%, compared to 28.6% of the water control (Figure 4). The disease incidence of fruit treated with H_2O_2 was progressively reduced when the concentration increased to 2.0%. After 60 days of cold storage plus 3 d shelf-life, there was a high rate of decay in all treatments. However, the positive fungicide treatment still exhibited the best controlling results. Fruit treated with 1.0% H_2O_2 had a 21.8% disease incidence; at the high concentration of 2.0%, the disease incidence was significantly reduced to 13.2% (Figure 4). These results are in agreement with others who showed the effectiveness of H_2O_2-Ag^+ on decay development of table grapes, melon, potato, eggplant, and pepper [14-18]. But still it was not given better protection than fungicide. It was shown that the combination of H_2O_2 with low doses of chemical fungicide can give good protection against decay development in citrus [9]. In the future, our research warrants further testing the synergistic effects of H_2O_2 with other postharvest disease controlling methods, in order to reduce completely the decay development in citrus during storage and marketing.

3.5. Effect of H_2O_2 Treatments on Orange Juice Quality Parameters During Storage and Shelf-life

As shown in Table 2. An increase in TSS from initial mean value 11.48% to 13.1-13.3% among all treatments was observed during 60 days of cold storage. No significant effect for any treatment on TSS at any length of storage time was observed. After 30 and 60 storage days followed by 3 shelf-life days, TA content decreased a little compared to the initial storage day, but was not different among all treatments. On the initial day of storage, the vitamin C content range was 436.2 mg•100 g^{-1}. After the first 30 days of storage, the vitamin C content of all of the treatments decreased and was less than that of harvest time. After 60 days of cold storage plus 3 days of shelf life, the significant higher vitamin C content was obtained in the positive Imazalil-treated fruit compare to the control and H_2O_2 treated fruit, which was correlated to the highest level of healthy fruit (Figure 4).

Table 2. *Changes in TSS, TA and Vitamin C content in oranges treated by H_2O_2 and stored at 8°C for 30 and 60 days plus 3 d at 25°C.*

	Initial value	30d+3d	60d+3d
	TSS (brix%)*		
Imazalil		12.85±0.62	13.20±0.80
Control		13.53±0.39	13.30±0.16
1% H_2O_2	11.48±0.30	13.07±0.10	13.10±0.24
2% H_2O_2		13.03±0.34	13.18±0.38
	TA content (g/100ml)*		

	Initial value	30d+3d	60d+3d
Imazalil	0.76±0.04	0.61±0.07	0.60±0.04
Control		0.66±0.05	0.62±0.04
1% H_2O_2		0.64±0.03	0.63±0.06
2% H_2O_2		0.67±0.03	0.68±0.01
	Vitamin C content (mg•100 g^{-1})*		
Imazalil	436.2±4.8	368.4±4.6	434.4±6.4*
Control		361.2±6.2	396.0±19.2
1% H_2O_2		370.8±10.2	352.8±9.6
2% H_2O_2		360.6±7.3	362.4±44.8

*Results are means of 10 fruits per treatment ± S. E.*Significant at P>0.05.

4. Conclusions

This current research show that H_2O_2 at a concentration above 0.5% completely inhibited *in vitro* growth of *P. digitatum* and *P. italicum*; and at a concentration above 0.25% can removed all bacteria, mold and yeast from the surface of fruit. However, to control decay development after long cold storage of Newhall navel orange, it was necessary to use higher concentration of H_2O_2 (2%), but still less effective than the chemical fungicide treatment. Therefore, we suggest that H_2O_2-Ag^+ can serve as promising sanitizer for short time storage decay prevention. For long storage, combing disinfection with H_2O_2-Ag^+ and cooling storage methods, is by now the potential safe alternatives to synthetic fungicides, which can be suitable even for the organic industry.

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