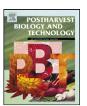
FISEVIER

Contents lists available at SciVerse ScienceDirect

### Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio



# Control of lemon green mold by a sequential oxidative treatment and sodium bicarbonate

Luciana Cerioni<sup>a</sup>, Luisa Rodríguez-Montelongo<sup>a</sup>, Jacqueline Ramallo<sup>b</sup>, Fernando Eduardo Prado<sup>c</sup>, Viviana Andrea Rapisarda<sup>a</sup>, Sabrina Inés Volentini<sup>a,\*</sup>

- <sup>a</sup> Instituto de Química Biológica, Facultad de Bioquímica, Química y Farmacia and Departamento Bioquímica de la Nutrición, Instituto Superior de Investigaciones Biológicas (CONICET), UNT, Chacabuco 461, CP T4000ILI, Tucumán-Argentina
- <sup>b</sup> Laboratorio de Desarrollo e Investigación, SA San Miguel, Lavalle 4001, CP T4000BAB, Tucumán-Argentina
- c Cátedra de Fisiología Vegetal, Facultad de Ciencias Naturales, UNT, Miguel Lillo 205, CP T4000JFE, Tucumán-Argentina

#### ARTICLE INFO

Article history: Received 26 May 2011 Received in revised form 26 July 2011 Accepted 21 August 2011

Keywords: Lemon green mold Hydrogen peroxide Sodium hypochlorite Sodium bicarbonate

#### ABSTRACT

A sequential oxidative treatment (SOT), using sodium hypochlorite (NaClO) and hydrogen peroxide  $(H_2O_2)$  in the presence of a cupric salt inhibited *in vitro* growth and germination of *Penicillium digitatum* conidia, causal agent of citrus green mold. Here, modifications of this SOT were evaluated *in vivo* to control this disease in inoculated lemons. The treatment that consisted of two sequential 2-min baths: one with 200 mg L<sup>-1</sup> NaClO followed by a second with 600 mmol L<sup>-1</sup>  $H_2O_2$  in the presence of 6 mmol L<sup>-1</sup>  $CuSO_4$ , resulted in 50% of disease control. When this treatment was combined with a third 2-min bath containing  $30 \, g \, L^{-1}$  NaHCO3 at  $37 \, ^{\circ}C$  (SOT-NaHCO3) and applied at 24 h post-inoculation, green mold incidence was reduced to  $\sim$ 5%. In non-inoculated lemons stored at  $5 \, ^{\circ}C$  for  $45 \, d$ , this treatment did not modify the appearance or weight compared to untreated lemons. Furthermore, phenolic content and the oxygen consumption rate in flavedo and albedo tissues were not affected by the SOT-NaHCO3. The malondialdehyde content in flavedo tissues increased immediately after treatment, but decreased to levels similar to control fruit 2 d later. The SOT-NaHCO3 combines compounds that are safe to the environment and human health, thus it represent a potential alternative to synthetic fungicides for the integrated control of postharvest diseases.

© 2011 Elsevier B.V. All rights reserved.

#### 1. Introduction

Green mold, caused by *Penicillium digitatum* Sacc., is one of the most important postharvest diseases of citrus (Eckert and Brown, 1986). It affects on average 5-8% of the total fruit handled (Eckert and Eaks, 1989), and results in a significant economic loss to both growers and packers. To maintain shelf-life and fruit quality in the market, control of postharvest diseases is crucial, because transport and marketing from producers to consumers requires a long time (weeks to months). Synthetic fungicides such as imazalil (IMZ) and thiabendazole are routinely used to control green mold (Holmes and Eckert, 1999; Smilanick et al., 1999). Appearance of fungicideresistant strains of P. digitatum has led to increases in fungicide doses in order to sustain their effectiveness as antifungal treatments (Bus et al., 1991; Holmes and Eckert, 1995). However, this practice can lead to an excess of fungicide residues in the fruit, which sometimes exceed maximum limits allowed by importing countries. Hence, there is an urgent need to develop alternative

technologies to control decay in citrus, which should be safe to consumers, workers, and the environment (Palou et al., 2002a, b; Venditti et al., 2005). Several alternatives show promise, but until now none alone is as effective as synthetic fungicides.

Carbonic acid salts, such as sodium carbonate ( $Na_2CO_3$ , soda ash) and sodium bicarbonate ( $NaHCO_3$ ), are common food additives permitted for many applications with no restrictions (Smilanick et al., 1997, 1999; Palou et al., 2001). In general, carbonic acid salts are considered to be good candidates to be used in combination with other chemical, physical or biological methods for the integrated control of postharvest diseases Narayanasamy (2006).

Oxidizing biocides, such as chlorine and peroxides, are preferred for general sanitation due to their low cost and availability. Chlorine and some hypochlorite salts have been used for many years to sanitize drinking water, fruit and vegetables, and food processing equipment. Hydrogen peroxide has been successfully used in disinfection treatments of minimally processed fruit and vegetables (Smilanick et al., 1995; DeQueiroz and Day, 2007), and has been experimentally applied for control of postharvest decay in fresh fruit (Simmons et al., 1997; Sapers et al., 2001). Moreover, chlorine reacts with natural organic matter or contaminants in surface waters and produces a complex mixture of disinfection by-products

<sup>\*</sup> Corresponding author. Tel.: +54 3814248921; fax: +54 3814248921. E-mail address: sabrina@fbqf.unt.edu.ar (S.I. Volentini).

(DBPs), some of which have been shown to be carcinogenic, mutagenic and/or teratogenic in animal studies (Ferraris et al., 2005).

Transition metals (e.g. copper and iron) act as catalysts in free radical formation (Halliwell and Gutteridge, 1992). By reacting with these metals, NaClO and peroxides can generate reactive oxygen species (ROS) (Candeias et al., 1993; Dukan and Touati, 1996). In cells, ROS can exert either beneficial effects by mediating several physiological processes (Tomankova et al., 2006; Yoshioka et al., 2009), or damage nucleic acids, lipids, carbohydrates and proteins that lead to cellular injury (Imlay and Linn, 1988; Halliwell and Gutteridge, 2006).

A sequential oxidative treatment (SOT), consisting of a combination of NaClO and  $H_2O_2$  in the presence of Cu(II) ions, inhibited conidial growth and germination of P. digitatum (Cerioni et al., 2009). This treatment was also effective against other citrus fungal pathogens, such as Penicillium italicum (blue mold), Geotrichum citri-aurantii (sour rot) and an IMZ-resistant P. digitatum strain. The mechanism of action of SOT on P. digitatum conidia was linked to an increase in intracellular oxidative stress markers and in membrane permeability, without apparent cell wall damage (Cerioni et al., 2010).

In this work, the *in vivo* effectiveness of SOT against green mold was studied in inoculated lemons. Furthermore, the physical and/or physiological properties in non-inoculated lemons peel were evaluated after treatment application.

#### 2. Materials and methods

#### 2.1. Fruit

Eureka lemons (Citrus limon (L.) Burm) were collected from commercial orchards in Tucumán (Argentina) and stored at 5 °C and 90% RH. Lemons used in the study were free from previous treatment or coating. Before each treatment, fruit were selected according to their size and color and were randomized, rinsed with tap water, and allowed to air-dry at room temperature.

#### 2.2. Preparation of conidial suspensions

An isolate of *P. digitatum* was obtained from naturally infected citrus fruit (Tucumán, Argentina) and deposited as PD-A in our laboratory collection (Cerioni et al., 2009). The *P. digitatum* isolate was grown on potato dextrose agar (PDA), pH 5.5, at 28 °C for 7 to 14 d. After that, conidia were suspended in sterile distilled water containing 0.05% (v/v) Tween 80, vortexed for 5–10 s and then filtered through two layers of cheesecloth to remove hyphal fragments. Conidia concentration was determined by counting in a Neubauer chamber and adjusted with sterile distilled water to  $10^6$  conidia mL $^{-1}$  as recommended for the evaluation of postharvest treatments for the control of green mold (Eckert and Brown, 1986).

#### 2.3. Inoculation, application of treatments and storage

For fruit inoculation, the tip of a stainless steel rod, 1 mm wide and 2 mm in length, was immersed in a conidial suspension of PD-A and then inserted in the lemon peel. Inoculated fruit were stored for 24 h at 20 °C and 90% RH. After that, inoculated lemons were treated as described below.

#### 2.3.1. SOT

Inoculated lemons were subjected to sequential oxidative treatments. In the first incubation, they were immersed in  $10\,L$  of either  $100\,$  or  $200\,$ mg  $L^{-1}\,$  NaClO solutions. After this, lemons were subjected to a second incubation in a  $10\,L$  mixture containing different concentrations of  $H_2O_2$  (100, 200, 300 and  $600\,$ mmol  $L^{-1}$ ) in the presence of  $6\,$ mmol  $L^{-1}\,$  CuSO $_4$  (see Fig. 1A). Both incubations were

for 2 min at 25 °C. The dip solutions were prepared using tap water. To avoid precipitation of  $\text{CuSO}_4$ , the water pH was adjusted to 5 by adding concentrated HCl. The temperature effect on disease control was also evaluated by changing it to 37 or 42 °C in the second incubation bath (H<sub>2</sub>O<sub>2</sub> and copper). In parallel, infected fruit were dipped in water alone as a control treatment.

#### 2.3.2. IMZ or NaHCO<sub>2</sub>

Inoculated lemons were treated with either 50–100 mg  $L^{-1}$  IMZ at 25  $^{\circ}C$  or 30 g  $L^{-1}$  NaHCO $_3$  at 37  $^{\circ}C$ , each for 2 min.

#### 2.3.3. SOT with IMZ or NaHCO<sub>3</sub>

Using inoculated lemons, a SOT with  $200\,\mathrm{mg}\,L^{-1}$  NaClO and  $600\,\mathrm{mmol}\,L^{-1}$   $H_2O_2$  in the presence of CuSO<sub>4</sub>, was evaluated in combination with a third incubation with either 50– $100\,\mathrm{mg}\,L^{-1}$  of the fungicide IMZ at  $25\,^\circ\mathrm{C}$  or  $30\,\mathrm{g}\,L^{-1}$  NaHCO<sub>3</sub> at  $37\,^\circ\mathrm{C}$ . All incubations were for  $2\,\mathrm{min}$ .

### 2.3.4. SOT with NaHCO<sub>3</sub> application at different post-inoculation times

Lemons inoculated with PD-A were stored at 20  $^{\circ}$ C and 90% RH for different times (0, 8, 18 or 24 h). After each time, SOT and 30 g L<sup>-1</sup> NaHCO<sub>3</sub> dips were applied (see Fig. 1B).

#### 2.3.5. Storage of fruit

After treatments, lemons were left to air-dry and placed in plastic commercial fruit trays. Treated fruit were stored at  $20\,^{\circ}$ C and 90% RH for 14 d. The incidence of disease was periodically evaluated and expressed as percentage of decayed fruit. For each treatment, 3 independent experiments in duplicate using n = 25 for each condition were tested.

# 2.4. Physical and physiological parameters of lemons after SOT-NaHCO $_3$

Non-inoculated lemons were treated with SOT-NaHCO $_3$  as described above (Fig. 1B), with water as the negative control or with 4.85 mol L $^{-1}$  H $_2$ O $_2$ , a concentration considered phytotoxic to the fruit, as a positive control (Smilanick et al., 1995). Lemons were incubated at 5 °C and 95% RH for 45 d. The physical and physiological parameters described below were determined immediately (0 h) and at 2, 7 and 45 d after treatments.

#### 2.4.1. Weight and external appearance determination

Periodically, lemons were weighed and their surfaces were visually analyzed (n = 10 for each condition, 3 independent experiments).

#### 2.4.2. Measurement of oxygen consumption

Respiration was measured polarographically with a Clark electrode (Yellow Springs Instrument Co. USA) and recorded in a Gilson oxygraph (Gilson Medical Electronics, Inc. USA), in a thermostatic cell for 30 min using 2 mL samples. Oxygen consumption rate was determined in flavedo and albedo samples of treated lemons. Flavedo (about 55 mg) and albedo (about 65 mg) samples were prepared from the lemon peel with a grater to obtain small uniform particles. Immediately, samples were placed in the cell oxygraph at 30  $^{\circ}$ C in 50 mmol L $^{-1}$  sodium phosphate buffer, pH 7.5. Oxygen consumption was recorded at least for 15 min. After normalizing oxygen consumption per mg of sample, the oxygen consumption rate was calculated as a percentage of consumption of the negative control at time zero (see Section 2.4).

#### 2.4.3. Degree of lipid peroxidation

The quantity of malondialdehyde (MDA) was measured by adapting the procedure previously described by Hodges et al.

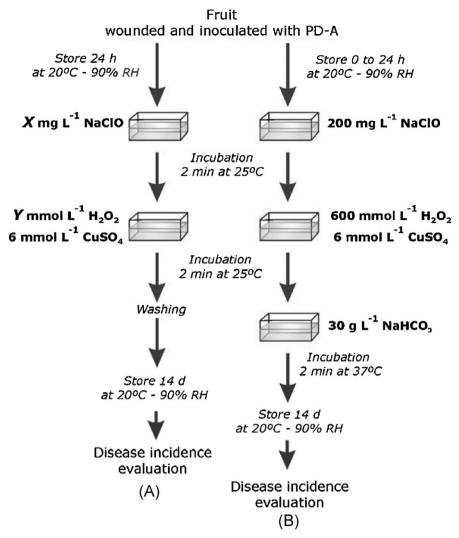


Fig. 1. Scheme of sequential treatments. X: different concentrations of NaClO, Y: different concentrations of H<sub>2</sub>O<sub>2</sub>.

(1999). Flavedo and albedo from treated lemons were separated using a scalpel and then homogenized in a mortar with inert sand in 1:25 (g FW/mL) 80:20 (v/v) ethanol:water, followed by centrifugation at 14,000 × g for 10 min. A 1 mL aliquot of the supernatant was added to a test tube with 1 mL of either (a) -TBA solution, comprised of 20% (w/v) trichloroacetic acid and 0.01% (w/v) butylated hydroxytoluene, or (b) +TBA solution, containing the above plus 0.65% (w/v) TBA. Samples were then mixed vigorously, heated at 95 °C in a block heater (Multiblok ZC 100, Zeltec, Argentina) for 25 min, cooled, and centrifuged at 14,000 × g for 10 min. Absorbance was read at 440, 532, and 600 nm. Malondialdehyde equivalents were calculated according to Hodges et al. (1999).

#### 2.4.4. Phenolic content

To determine the content of phenolic compounds, the technique of Swain and Hillis (1959) was used. Briefly, 200 mg of lemon flavedo or albedo were homogenized with 1 mL of 80% (v/v) ethanol and then centrifuged at  $14,000\times g$  for 10 min. A  $100~\mu L$  aliquot of supernatant was mixed with  $500~\mu L$  of 1~N Folin–Ciacalteau solution and 5~mL of  $2\%~(w/v)~Na_2CO_3$  and then incubated at room temperature for 10~min. After centrifugation at  $14,000\times g$  for 10~min, the absorbance of supernatant was measured at 724~nm. Phenolic concentration was determined from a standard curve with cinnamic acid.

#### 2.5. Statistical analysis

The experimental data shown are the mean of 3 replicate samples  $\pm$  SD. Data were subjected to analysis of variance (ANOVA) followed by Tukey's test with *Statitix 9.0* Analytical Software 2008 for Windows (USA). Differences at p < 0.05 were considered significant.

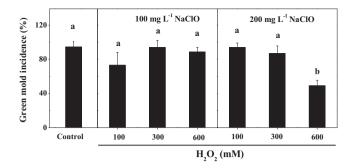
#### 3. Results

#### 3.1. In vivo application of SOT

SOT previously standardized *in vitro* was applied *in vivo* and was ineffective to control citrus green mold (data not shown). Therefore, we tested some variation in increasing the concentrations of oxidizing compounds. As shown in Fig. 2, SOT using 200 mg L $^{-1}$  NaClO plus 600 mmol L $^{-1}$  H $_2$ O $_2$  in the presence of 6 mmol L $^{-1}$  CuSO $_4$ , resulted in partial disease control, with an incidence of  $\sim$ 50%.

#### 3.2. Optimization of SOT

SOT (with the oxidizing compounds concentrations described in Section 2.3.1) was applied to lemons 24 h post-inoculation with *P. digitatum* conidia changing the following parameters: (a) increasing the temperature in the second bath, (b) adding a third bath with



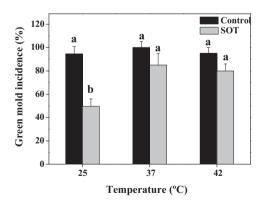
**Fig. 2.** Green mold incidence of lemons inoculated with PD-A after *in vivo* SOT applications. SOT was applied as was described in Fig. 1A, 24h post-inoculation with the indicated NaClO and  $H_2O_2$  concentrations and a fixed CuSO<sub>4</sub> concentration (6 mmol  $L^{-1}$ ). Percent incidence of decay at 14 d after treatment application was calculated considering the total number of inoculated fruit for each condition. Data are means of 3 independent experiments in duplicate using n = 25 for each condition tested. Different letters indicate significant differences among treatments according to Tukev's test with a p-value of 0.05.

the fungicide imazalil (IMZ) or (c) adding a third bath with NaHCO<sub>3</sub> at  $37\,^{\circ}\text{C}$  (see Fig. 1B). IMZ was used at 50 or  $100\,\text{mg}\,\text{L}^{-1}$ , which is 20--40 times lower than concentrations used in packinghouses. The NaHCO<sub>3</sub> concentration used in these assays was  $30\,\text{g}\,\text{L}^{-1}$ , as it is currently applied in packinghouses.

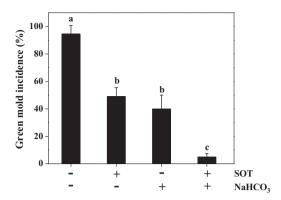
As shown in Fig. 3, the temperature rise to 37 or 42  $^{\circ}$ C in the second bath had a negative effect in controlling green mold, since the disease incidence was higher than that of SOT at 25  $^{\circ}$ C. In addition, SOT with IMZ 50 or 100 mg L $^{-1}$ , resulted in 40% and 20% of disease incidence, respectively, whereas IMZ alone completely controlled the disease (data not shown). Thus, a combination of SOT with IMZ resulted in an antagonistic effect. Conversely, a combination of SOT with 30 g L $^{-1}$  NaHCO $_3$  was effective in controlling the disease, since the incidence diminished to nearly 5% (Fig. 4).

## 3.3. Influence of post-inoculation time on SOT-NaHCO<sub>3</sub> effectiveness

To identify the optimal time for controlling infection of lemons by *P. digitatum* with SOT-NaHCO<sub>3</sub> we treated the fruit at different post-inoculation times. The combination of SOT with NaHCO<sub>3</sub> was effective at all times tested, but it had a maximum effect on green mold control when it was applied 18 or 24 h post-inoculation



**Fig. 3.** Green mold incidence of lemons inoculated with PD-A treated with SOT at different temperatures. Inoculated lemons were treated 24 h post-inoculation with SOT consisting of a first bath with  $200 \,\mathrm{mg} \,\mathrm{L}^{-1}$  NaClO followed by a second bath with  $600 \,\mathrm{mmol} \,\mathrm{L}^{-1} \,\mathrm{H}_2 \,\mathrm{O}_2$  in the presence of  $6 \,\mathrm{mmol} \,\mathrm{L}^{-1} \,\mathrm{CuSO}_4$  at each indicated temperature. Percent incidence of decay was calculated with respect to total inoculated fruit for each condition at 14 d after treatment. Data are means of 3 independent experiments in duplicate using n=25 for each condition tested. Different letters indicate significant differences among treatments according to Tukey's test with a p-value of 0.05.



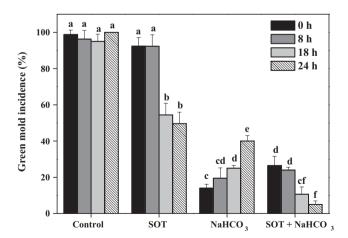
**Fig. 4.** Green mold incidence of infected lemons treated with SOT-NaHCO<sub>3</sub>. Each treatment was applied *in vivo* as was described in Section 2, 24 h post-inoculation. Percent incidence of decay was calculated with respect to total inoculated fruit for each condition at 14 d after treatment. Data are means of 3 independent experiments in duplicate using n = 25 for each condition tested. Different letters indicate significant differences among treatments according to Tukey's test with a p-value of 0.05.

(Fig. 5). Interestingly, the time between fruit harvest and arrival at packinghouses ranges between 15 and 24 h. Nevertheless, the effectiveness of treatments using SOT or NaHCO<sub>3</sub> alone, were dependent on post-inoculation time. In Fig. 5, it was observed that SOT only protected the fruit when applied after 18 h of inoculation, and the NaHCO<sub>3</sub> lost effectiveness with increasing time post-inoculation.

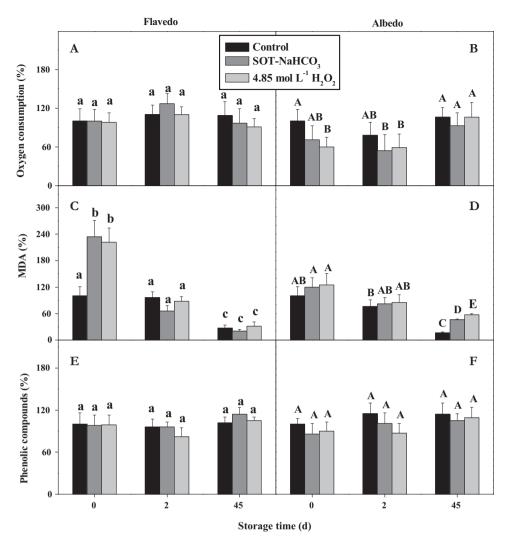
## 3.4. Effect of SOT-NaHCO<sub>3</sub> on physical and physiological parameters of lemon

Changes in treated lemon weight were not significant compared to control lemons, treated with water. Between 0 and 45 d of storage in a refrigerated room, the differences in weight were:  $6.6 \pm 2$  g for negative control,  $8.3 \pm 1.4$  g for NaHCO<sub>3</sub>-SOT and  $6.6 \pm 1.8$  g for positive control (4.85 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>).

As determined visually, surfaces of SOT-NaHCO $_3$  treated lemons did not show changes during the 45 d storage period. However, a red to bronze discoloration limited to the flavedo was observed in fruit treated with 4.85 mol L $^{-1}$  H $_2$ O $_2$  approximately 10 d after treatment (data not shown).



**Fig. 5.** Green mold incidence of infected lemons treated at different times after inoculation. Lemons inoculated with PD-A were treated with SOT alone, NaHCO<sub>3</sub> alone or SOT-NaHCO<sub>3</sub> (see Section 2 and Fig. 1B) at the indicated times after inoculation. Percent incidence of decay was calculated with respect to total inoculated fruit for each condition at 14 d after treatment. Data are means of 4 independent experiments in duplicate using n = 50 for each condition tested. Different letters indicate significant differences among treatments according to Tukey's test with a p-value of 0.05.



**Fig. 6.** Markers of oxidative stress in lemons peel. Control lemons or fruit subjected to oxidative treatments were analyzed for the following parameters: Oxygen consumption rate of flavedo (A) and albedo (B), MDA content in flavedo (C) and albedo (D), and total phenolic content in flavedo (E) and albedo (F). In all cases, percentages were calculated based on negative control at time zero. Data are means of 3 independent experiments in duplicate using n = 10 for each condition tested. Different letters in each tissue and parameters (small letters for flavedo, capital letters for albedo), indicate significant differences among treatments according to Tukey's test with a p-value of 0.05.

Oxygen consumption in peel samples of lemons treated with SOT-NaHCO $_3$  was similar to that of control lemons and was kept constant during the storage period (Fig. 6A and B). However, the consumption rates were different for each tissue. At time zero, the control oxygen consumption in albedo was  $135\pm43\,\mathrm{pmol/min\,mg\,FW}$  and in flavedo  $214\pm37\,\mathrm{pmol/min\,mg\,FW}$ .

The MDA content of control lemon peel was similar in flavedo and albedo, at time zero being  $45.5\pm10$  and  $37.6\pm8$  nmol/g FW, respectively. At the same time, MDA content in flavedo of lemons treated with the SOT-NaHCO $_3$  or  $4.85\,\mathrm{mol}\,L^{-1}\,H_2O_2$  showed a significant increase, but it was not observed in albedo tissue (Fig. 6C and D). The second day after treatments, the MDA content in flavedo decreased and remained as the negative control up to 45 d.

In addition, phenolic compound levels on lemons treated with SOT-NaHCO<sub>3</sub> or  $4.85 \, \text{mol} \, \text{L}^{-1} \, \text{H}_2\text{O}_2$  were similar to those observed in controls (Fig. 6E and F).

#### 4. Discussion

We have previously standardized in vitro a SOT, consisting of two sequential incubations, first with NaClO, and second with  $H_2O_2$  in

the presence of CuSO<sub>4</sub> (Cerioni et al., 2009). When this SOT was applied to lemons inoculated with P. digitatum conidia (data not shown), green mold was unsatisfactory controlled. Consequently, we increased the concentrations of oxidizing compounds in vivo by using 200 mg  $L^{-1}$  NaClO and 600 mmol  $L^{-1}$   $H_2O_2$  in the presence of 6 mmol L<sup>-1</sup> CuSO<sub>4</sub>. As a result, a decrease in disease incidence was achieved with these concentrations, but SOT alone did not effectively control green mold. It has been reported that an increase in temperature to 50 °C, improves disease control effectiveness with minimal increase in the risk of injury to the fruit (Smilanick et al., 1997, 2003; Palou et al., 2001). However, increases in temperature in the second bath did not improve SOT efficacy. This may be related to a temperature-induced instability of H<sub>2</sub>O<sub>2</sub> (Schumb, 1949). The SOT was also tested in combination with IMZ, the most widely used synthetic fungicide in citrus packinghouses. There was an antagonistic effect between the two compounds. In agreement with this finding, Kanetis et al. (2008a) reported that efficiency of IMZ decreases when combined with oxidizing compounds. Moreover, it is known that at low pH (as occurs in SOT), IMZ reduces its fungicide action on fungal pathogens (Smilanick et al., 2005).

Sodium bicarbonate has been widely evaluated for postharvest disease control and generally, it is used in association with other treatments (Smilanick et al., 1999; Palou et al., 2001). By using the

SOT-NaHCO<sub>3</sub>, we obtained an additive effect among compounds that reduced the incidence of green mold up to 95%. Since the effect of this treatment was optimal around 24 h post-inoculation (Fig. 5), we consider that the treatment is more effective when conidia start to germinate. This is in agreement with our previous results that indicated that fungi are more sensitive to the SOT in the mycelial stage (Cerioni et al., 2010).

The observed additive effect may be due to the different targets of the compounds included in the SOT-NaHCO<sub>3</sub> used. Whereas SOT generates oxidative stress and damages the pathogen directly (Cerioni et al., 2010), NaHCO<sub>3</sub> salt prevents infection by fungal pathogens, which need an injury on the fruit surface, by leaving a protective film against future infections (Venditti et al., 2005). According to the work by Usall et al. (2008), sodium bicarbonate treatment protects wounds present at the time of treatment from later infections, but it does not protect the uninjured rind from later infections. When applied alone, sodium carbonates are partially effective in controlling green mold on lemons (Smilanick et al., 1995), oranges (Smilanick et al., 1997, 1999; Palou et al., 2001) and, with less effectiveness, clementine mandarins (Palou et al., 2002a). Although, it has not been completely elucidated, their mode of action appears to be primarily fungistatic and in contrast to synthetic fungicides, these salts are not very persistent fungicides. Bicarbonates may act against fungi by buffering an elevated pH environment and by increasing osmotic levels on surfaces, both conditions being detrimental to fungal spores (Palmer et al., 1997). There is evidence that high pH alone cannot explain the inhibitory action of these salts (Homma et al., 1981). And that other mechanisms (e.g. inactivation of extracellular enzymes from Penicillium species and alteration membrane physiology by direct contact with the cellular membrane at concentration below saturation) may be involved (Palmer et al., 1997). Thereby, the SOT-NaHCO<sub>3</sub> protects lemons by at least a direct action on the fungus itself and by favoring wound healing. Further studies are needed to define if other mechanisms are also involved.

Synthetic chemical fungicides generally have a specific action site (Schmidt et al., 2006; Kanetis et al., 2008b). After repeated exposures to the fungicide, resistant mutants become progressively prevalent in the pathogen population. In contrast, oxidative stress produced by the SOT involves damage to different cellular levels. Thus, the emergence of resistant fungal strains will be relatively unlikely.

Lemons treated with SOT and sodium bicarbonate were periodically assessed during long refrigerated storage. Our results indicate that upon application of the oxidative treatment, the physiological state of fruit peel in stored lemons did not show significant differences compared to the control. The only parameter that changed was the level of MDA in lemon flavedo treated with oxidative treatments (SOT-NaHCO<sub>3</sub> and 4.85 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>) immediately after treatment (time zero), but it reached levels similar to the negative control two days after. In this context, it has been reported that stress (drought and salinity) and MDA appearance were linked to reduced activity of antioxidant enzymes and a decrease in polar antioxidant compounds (ascorbic acid and glutathione) (Hossain et al., 2009). In citrus plants exposed to abiotic stress (e.g. flooding, salinity), it also induced the synthesis of dehydrin proteins, free radical scavengers which prevent lipid peroxidation and chelates metals (Hara et al., 2004, 2005). The antioxidant compounds present in lemon peel (Alquezar et al., 2008; Guimaraes et al., 2010) may contribute to the rapid decline of MDA values to control levels.

In plants, many of the antioxidant compounds have a phenolic structure or are derived from phenolic compounds (Rice-Evans et al., 1996). For example rutin and quercetin are also effective antioxidants and chelating agents (Mira et al., 2002). However, in this work total phenolics in flavedo and albedo did not change after application of SOT-NaHCO<sub>3</sub>.

In summary, this study presents a promising strategy that combines methods to effectively control postharvest green mold of citrus fruit and offers an alternative to the use of conventional fungicides used now in citrus packinghouses. SOT-NaHCO3 application to P. digitatum infected lemons significantly reduced green mold incidence. This treatment consisted of three 2-min sequential baths: first 200 mg L<sup>-1</sup> NaClO, second 600 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> in the presence of  $6 \,\mathrm{mmol}\,\mathrm{L}^{-1}$  CuSO<sub>4</sub>, and third  $30 \,\mathrm{g}\,\mathrm{L}^{-1}$  NaHCO<sub>3</sub>. After this treatment, no changes in appearance or weight were observed in the fruit. In addition, phenolic contents and oxygen consumption rates in flavedo and albedo tissues were not affected. The SOT-NaHCO3 established in this study may be an efficient and alternative way for controlling of P. digitatum and other citrus postharvest pathogens, decreasing or replacing the application of synthetic fungicides. In addition, this treatment includes compounds that break down quickly and therefore, are harmless to lemons, the environment, and human health.

#### Acknowledgments

This work was supported by grants 26/D444 from CIUNT (Consejo de Investigaciones de la Universidad Nacional de Tucumán) and PFIP-ESPRO 016/07 from SeCyT (Secretaría de Ciencia, Tecnología e Innovación Productiva). We especially thank the lab colleague J.M. Villegas and PhD M.G. Vizoso Pinto for helpful discussions. We acknowledge SA SAN MIGUEL for providing equipment and material to conduct experiments. L.C. is a fellow from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

#### References

- Alquezar, B., Rodrigo, M.J., Zacarías, L., 2008. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed oranged mutant *Cara Cara*. Phytochemistry 10, 1997–2007.
- Bus, V.G., Bongers, A.J., Risse, L.A., 1991. Occurrence of *Penicillium digitatum* and *P. italicum* resistant to benomyl, thiabendazole, and imazalil on citrus fruit from different geographic origins. Plant Dis. 75, 1098–2001.
- Candeias, L.P., Patel, K.B., Stratford, M.R., Wardman, P., 1993. Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid. FEBS Lett. 333, 151–153.
- Cerioni, L., Rapisarda, V.A., Hilal, M., Prado, F.E., Rodríguez-Montelongo, L., 2009. Synergistic antifungal activity of sodium hypochlorite, hydrogen peroxide and cupric sulfate against *Penicillium digitatum*. J. Food Prot. 72, 1660–1665.
- Cerioni, L., Volentini, S.I., Prado, F.E., Rapisarda, V.A., Rodríguez-Montelongo, L., 2010. Cellular damage induced in *Penicillium digitatum* by a sequential oxidative treatment. J. Appl. Microbiol. 109, 1441–1449.
- DeQueiroz, G.A., Day, D.F., 2007. Antimicrobial activity and effectiveness of a combination of sodium hypochlorite and hydrogen peroxide in killing and removing *Pseudomonas aeruginosa* biofilms from surfaces. J. Appl. Microbiol. 103, 794–802.
- Dukan, S., Touati, D., 1996. Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. J. Bacteriol. 178, 6145–6150
- Eckert, J.W., Brown, G.E., 1986. Evaluation of postharvest treatments for citrus fruit. In: Hickey, K.D. (Ed.), Methods for Evaluating Pesticides for Control of Plant Pathogens. APS Press, St. Paul, MN, USA, pp. 92–97.
- Eckert, J.W., Eaks, I.L., 1989. Postharvest disorders and diseases of citrus fruit. In: Reuter, W., Calavan, E.C., Carman, G.E. (Eds.), The Citrus Industry, vol. 5. University of California Press, Berkeley, CA, USA, pp. 179–260.
- Ferraris, M., Chiesara, E., Radice, S., Giovara, A., Frigerio, S., Fumagalli, R., Marabini, L., 2005. Study of potential toxic effects on rainbow trout hepatocytes of surface water treated with chlorine or alternative disinfectants. Chemosphere 60, 65–73.
- Guimaraes, R., Barros, L., Barreira, J.L., Sousa, M.J., Cavalho, M.A., Ferreira, I.C., 2010.

  Targeting excessive free radicals with peels and juices of citrus fruit: grapefruit, lemon. lime and orange. Food Chem. Toxicol. 48. 99–106.
- Hara, M., Fujinaga, M., Kuboi, T., 2004. Radical scavenging activity and oxidative modification of citrus dehydrin. Plant Physiol. Biochem. 42, 262–265.
- Hara, M., Fujinaga, M., Kuboi, T., 2005. Metal binding by citrus dehydrin with histidine-rich domains. J. Exp. Bot. 56, 2695–2703.
- Halliwell, B., Gutteridge, J.M.C., 1992. Biologically relevant metal ion-dependent hydroxyl radical generation. FEBS Lett. 307, 108–112.
- Halliwell, B., Gutteridge, J.M.C., 2006. Free Radicals in Biology and Medicine, fourth ed. Oxford University Press, Oxford, UK.
- Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604–611.

- Holmes, G.J., Eckert, J.W., 1995. Relative fitness of imazalil-resistant and -sensitive biotypes of *Penicillium digitatum*. Plant Dis. 79, 1068–1073.
- Holmes, G.J., Eckert, J.W., 1999. Sensitivity of *Penicillium digitatum* and *Penicillium italicum* to postharvest citrus fungicides in California. Phytopathology 89, 716–721.
- Homma, Y., Arimoto, Y., Misato, T., 1981. Studies on the control of plant diseases by sodium bicarbonate formulation, 1. Effects of emulsifiers and surfactants on the protective values of sodium bicarbonate. J. Pestic. Sci. 6, 145–153.
- Hossain, Z., López-Climent, M.F., Arbona, V., Pérez-Clemente, R.M., Gómez-Cadenas, A., 2009. Modulation of the antioxidant system in *Citrus* under waterlogging and subsequent drainage. J. Plant Physiol. 166, 1391–1404.
- Imlay, J.A., Linn, S., 1988. DNA damage and oxygen radical toxicity. Science 240, 1302–1309.
- Kanetis, L., Förster, H., Adaskaveg, J.E., 2008a. Optimizing efficacy of new postharvest fungicides and evaluation of sanitizing agents for managing citrus green mold. Plant Dis. 92. 261–269.
- Kanetis, L., Förster, H., Jones, C.A., Borkovich, K.A., Adaskaveg, J.E., 2008b. Characterization of genetic and biochemical mechanisms of fludioxonil and pyrimethanil resistance in field isolates of *Penicillium digitatum*. Phytopathology 98, 205–214.
- Mira, L., Fernandez, M.T., Santos, M., Rocha, R., Florêncio, M.H., Jennings, K.R., 2002. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radic. Res. 36, 1199–1208.
- Narayanasamy, P., 2006. Integrated systems for the management of postharvest diseases. In: Hoboken, N.J. (Ed.), Postharvest Pathogens Disease Management. John Wiley Sons, Inc, USA, pp. 537–554.
- Palmer, C.L., Horst, R.K., Langhans, R.W., 1997. Use of bicarbonates to inhibit in vitro colony growth of Botrytis cinerea. Plant Dis. 81, 1432–1438.
- Palou, L., Smilanick, J.L., Usall, J., Viñas, I., 2001. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate, and sodium bicarbonate. Plant Dis. 85, 371–376.
- Palou, L., Usall, J., Munoz, J., Smilanick, J.L., Viñas, I., 2002a. Hot water, sodium bicarbonate and sodium carbonate for the control of green and blue mold of clementine mandarins. Postharvest Biol. Technol. 24, 93–96.
- Palou, L., Usall, J., Smilanick, J.L., Aguilar, M.J., Viñas, I., 2002b. Evaluation of food additives and low-toxicity compounds as alternative chemicals for the control of *Penicillium digitatum* and *Penicillium italicum* on citrus fruit. Pest Manage. Sci. 58, 459–466.
- Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20, 933–956.
- Sapers, G.M., Miller, R.L., Pilizota, V., Mattrazzo, A.M., 2001. Antimicrobial treatments for minimally processed cantaloupe melon. J. Food Sci. 66, 345–349.

- Schmidt, L.S., Ghosoph, J.M., Margosan, D.A., Smilanick, J.L., 2006. Mutation at  $\beta$ -tubulin codon 200 indicated thiabendazole resistance in *Penicillium digitatum* collected from California citrus packinghouses. Plant Dis. 90, 765–770.
- Schumb, W.C., 1949. Stability of concentrated hydrogen peroxide solutions. Ind. Eng. Chem. 41, 992–1003.
- Simmons, G.F., Smilanick, J.L., John, S., Margosan, D.A., 1997. Reduction of microbial populations on prunes by vapor-phase hydrogen peroxide. J. Food Prot. 60, 188-191.
- Smilanick, J.L., Margosan, D.A., Henson, D.J., 1995. Evaluation of heated solutions of sulfur dioxide, ethanol, and hydrogen peroxide to control postharvest green mold of lemons. Plant Dis. 79, 742–747.
- Smilanick, J.L., Mackey, B.E., Reese, R., Usall, J., Margosan, D.A., 1997. Influence of concentration of soda ash, temperature, and immersion period on the control of postharvest green mold of oranges. Plant Dis. 81, 379–382.
- Smilanick, J.L., Margosan, D.A., Mlikota, F., Usall, J., Michael, I.F., 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. Plant Dis. 83, 139–145.
- Smilanick, J.L., Sorenson, D., Mansour, M., Aieyabei, J., Plaza, P., 2003. Impact of a brief postharvest hot water drench treatment on decay, fruit appearance, and microbe populations of California lemons and oranges. HorTechnology 13, 333–338.
- Smilanick, J.L., Mansour, M.F., Margosan, D.A., Mlikota Gabler, F., 2005. Influence of pH and NaHCO<sub>3</sub> on effectiveness of imazalil to inhibit germination of *Penicillium digitatum* and to control postharvest green mold on citrus fruit. Plant Dis. 89, 640–648.
- Swain, T., Hillis, W.E., 1959. The phenolic constituents of *Prunus domestica*, The quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10, 63–68.
- Tomankova, K., Luhova, L., Petrivalsky, M., Pec, P., Lebeda, A., 2006. Biochemical aspects of reactive oxygen species formation in the interaction between *Lycopersicon* spp. and *Oidium neolycopersici*. Physiol. Mol. Plant Pathol. 68, 22–32.
- Usall, J., Smilanick, J.L., Palou, L., Denis-Arrue, N., Teixido, N., Torres, R., Vinas, I., 2008. Preventive and curative activity of combined treatments of sodium carbonates and *Pantoea agglomerans* CPA-2 to control postharvest green mold of citrus fruit. Postharvest Biol. Technol. 50, 1–7.
- Venditti, M.G., Molinu, A., Dore, M., D'Hallewin, G., 2005. Sodium carbonate treatment induces scoparone accumulation, structural changes, and alkalinization in the albedo of wounded citrus fruit. J. Agric. Food Chem. 53, 3510–3518.
- Yoshioka, H., Asai, S., Yoshioka, M., Kobayashi, M., 2009. Molecular mechanisms of generation for nitric oxide and reactive oxygen species, and role of the radical burst in plant immunity. Mol. Cells 28, 321–329.