Food Microbiology xxx (2012) 1–4

Contents lists available at SciVerse ScienceDirect

Food Microbiology

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

Short communication

Inhibition of *Penicillium expansum* by an oxidative treatment

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ARTICLE INFO

Article history:
Received 5 June 2012
Accepted 22 September 2012
Available online xxx

Keywords:
Penicillium expansum
Apple
Hydrogen peroxide
Sodium hypochlorite

ABSTRACT

Several oxidizing compounds such as sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) are used to control postharvest decay in fresh fruit due to their antimicrobial effects. Here, we applied these compounds *in vitro*, in the presence of $CuSO_4$, against *Penicillium expansum*, causal agent of apple blue mold. MICs were 50 mg L^{-1} and 400 mmol L^{-1} for NaClO and H_2O_2, respectively, when these compounds were individually applied to conidia suspensions during 2 min. A combined oxidative treatment (OT) consisting on an incubation with 1 mg L^{-1} NaClO and 200 mmol L^{-1} H_2O_2, in the presence of 6 mmol L^{-1} $CuSO_4$, inhibited growth, conidial germination and fungal infectivity on apple. The fractional inhibitory concentration index for the interaction between NaClO and H_2O_2 in the OT was 0.52 indicating a synergistic effect of the oxidizing compounds. These results suggest that the OT could be an interesting alternative for apple diseases postharvest control.

1. Introduction

Penicillium expansum is a widely spread fungal pathogen, with a broad host range, that causes blue mold rot (Jones and Aldwinckle, 1991; Pitt and Hocking, 1997). It causes significant economic losses to the fruit industry and is also of potential public health concern because it produces toxic secondary metabolites, including patulin, citrinin, and chaetoglobosins (Andersen et al., 2004). Control of decay caused by *P. expansum* has become important to ensure the quality and safety of a variety of fruits, including apples, pears, grapes, peaches, and cherries. The use of synthetic chemicals as fungicides is a primary method of control of postharvest fungal decay of apple fruit (Eckert and Ogawa, 1988). However, concerns about public health and the development of fungicide resistance by pathogens have encouraged the search for alternative methods (Giraud and Fauré, 2000; Prusky et al., 1985; Qin and Tian, 2005).

Due to their low cost and availability, oxidizing biocides, such as chlorine and peroxides, are highly used for general sanitation. Chlorine and some hypochlorite salts were used for many years to sanitize drinking water, fruit, vegetables, and food processing equipment. Hydrogen peroxide was successfully applied in disinfection treatments of minimally processed fruit and vegetables (Smilanick et al., 1995; DeQueiroz and Day, 2007), and to control postharvest decay in fresh fruit (Cerioni et al., 2012; Sapers et al., 2001; Simmons et al., 1997).

In our laboratory, a sequential oxidative treatment (SOT) using NaClO and H_2O_2 in the presence of Cu (II) ions, inhibited conidial growth and germination of *Penicillium digitatum* (green mold), *Penicillium italicum* (blue mold), *Geotrichum citri-aurantii* (sour rot) and an IMZ-resistant *P. digitatum* isolate (Cerioni et al., 2009). The SOT mechanism of action 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).

Although the effectiveness of SOT on citrus was reported, it was not assayed on pathogens of pome fruit like *P. expansum* until now.

In this work, we studied the efficacy of NaClO and H_2O_2 in the presence of $CuSO_4$ to inhibit *P. expansum* growth. The determination of oxidant compounds minimal inhibitory concentration (MIC) and the evaluation of combined treatments were carried out.

2. Materials and methods

2.1. Fungal strain and preparation of conidial suspensions

An isolate of *P. expansum* was obtained from naturally infected apple, showing the typical symptoms of blue mold. This isolate was identified by phenotypic features according to Pitt and

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0740-0020/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2012.09.011

Hocking (1997). Fungus was grown on potato dextrose agar (PDA) for 7–10 d at 23 °C. Conidia suspensions were prepared with sterile distilled water containing 0.05% (v/v) Tween 80 (Fluka), vortexed for 5–10 s, and filtered through two layers of cheesecloth to remove hyphal fragments. The conidia concentration was determined by counting in a Neubauer chamber and adjusted with sterile distilled water at 10^6 conidia mL^{-1} unless stated otherwise. Isolates of *Penicillium* spp. or *Escherichia coli* were used for PCR analysis.

2.2. Detection of polygalacturonase gene of P. expansum by PCR

PCR was performed directly with conidial suspensions from *P. expansum* fungal isolate. *P. expansum* spores (10^8 conidia mL^{-1}) were disrupted by passing three times through a French press at 18,000 psi to prepare the conidial extract. A centrifugation at 16,000 × g was carried out to separate out insoluble cell debris. *P. expansum* isolate was identified by detection of polygalacturonase gene using PCR, following the protocol described by Marek et al. (2003). Amplified PCR products from each mold isolate were analyzed by 1.2% (w/v) agarose gel electrophoresis. The band corresponding to *P. expansum* were verified by DNA sequencing (CERELA-CONICET).

2.3. MIC determination

The MIC was defined as the lowest concentration of each compound that ensures the inhibition of mycelium growth for at least 14 d at 23 °C. Conidial suspension was incubated at 25 °C for 2 min with different concentrations of each oxidant compound (NaClO, $CuSO_4$ and H_2O_2). After incubations, samples were centrifuged at 3500 g for 1 min, and supernatants were discarded. Pellets were washed twice and resuspended to the original volume with sterile distilled water. Aliquots (5 μL) were taken from each sample and spot inoculated onto PDA plates.

2.4. Oxidative treatment on conidia and viability assay

Combinations of compounds were studied using a concentration range lower than the corresponding MIC. The conidia suspension was subjected to an incubation at 25 °C during 2 min, using 1, 3 or 5 mg L^{-1} of NaClO with different concentrations of H_2O_2 (100, 200, 300 mmol L^{-1}) in the presence of 6 mmol L^{-1} $CuSO_4$. After incubation, conidia suspensions were centrifuged, washed, resuspended and aliquots of 5 μL were placed on PDA plates for 7 d at 23 °C. As a control, conidial suspension was incubated with sterile water. To determine CFU, an aliquot of treated conidial suspension was spread on PDA plate and quantified after 4 d.

Interactions between different concentrations of NaClO and H_2O_2 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 is defined as synergistic when the FIC value was <1, additive when the value was 1, and antagonistic when the value was >1 (Berenbaum, 1978).

2.5. Conidial germination assay

After oxidative treatment described above, conidia suspension was centrifuged, washed with sterile distilled water, resuspended with potato dextrose broth (PDB) and then incubated at 23 °C for 7 d. Periodically, aliquots of conidial suspension were collected for microscopic analyses to determine conidia germination. About 200 conidia per replicate were examined under the microscope Olympus BX51TF (Olympus Co., Tokyo, Japan). Conidia were considered as germinated when germination tubes were at least twice as long as conidia diameters (Xiaoping et al., 2007).

2.6. Residual infectivity on apple assay

Red Delicious apples used for this study were collected from Tucumán province markets and stored at 5 °C and 90% of relative humidity (RH). Fruits were homogeneous in maturity and size, and wounds and rots free. Before use, they were rinsed with tap water and dried at room temperature.

To evaluate conidia infectivity, the apples surfaces were wounded at three different sites and 10 μL of the treated suspension were placed on each wound (Yu et al., 2009). Fruits were then stored at 20 °C and 95% RH for 14 d. Fruits were daily analyzed to evaluate the incidence of infection. The diameter of each lesion was measured to calculate the infection severity.

2.7. Statistical analysis

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 and discussion

3.1. Detection of P. expansum polygalacturonase gene

A 404-bp product, corresponding to an internal, conserved fragment of *polygalacturonase gene* (Pepg1) (Innis and Gelfand, 1990) was observed on *P. expansum* isolated from rot spotted apples (Fig. 1, lane 2). When other common foodborne *Penicillium* species and *E. coli* were tested, no amplification was observed (Fig. 1, lane 3–5). These results confirmed that the amplified DNA sequence is specific for *P. expansum*.

3.2. Oxidant compounds MIC and combined treatment effect on P. expansum growth

The antifungal activity of NaClO, $CuSO_4$ and H_2O_2 on *P. expansum* was studied (Fig. 2A and B). It was found that the NaClO MIC was 50 mg L^{-1} whereas for H_2O_2 was 400 mmol L^{-1}.

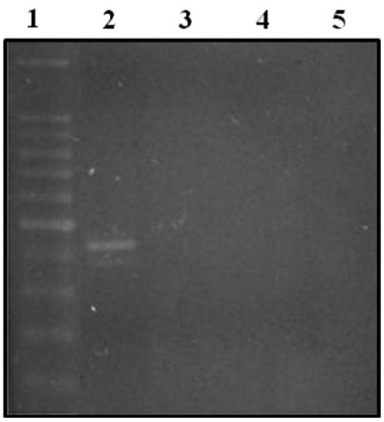

Fig. 1. Agarose gel electrophoresis of PCR products from *Penicillium* sp conidia suspensions and *E. coli*. Lane 1, 1 kb-ladder (Invitrogen); Lane 2, *P. expansum*; Lane 3, *P. digitatum*; Lane 4, *P. ulaiense*; Lane 5, *E. coli*.

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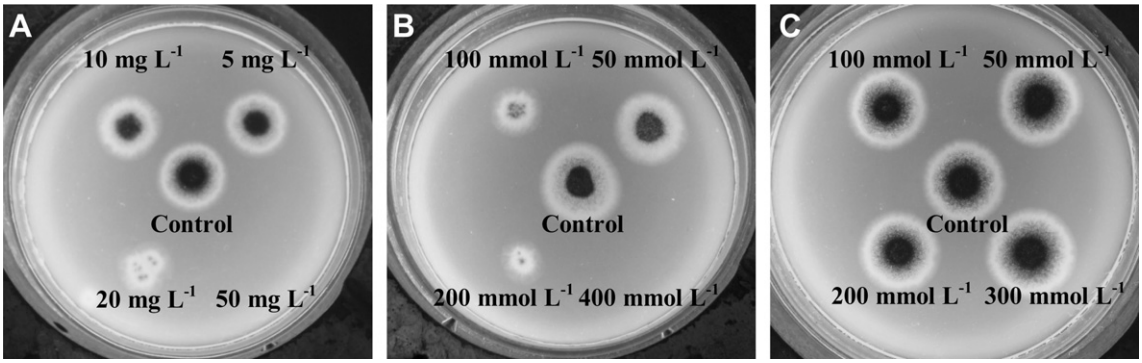

Fig. 2. Effect of oxidizing compounds on *P. expansum* grown. Conidia were treated with distilled sterile water (control) or with indicated concentration of NaClO (A), H_2O_2 (B) or $CuSO_4$ (C). Photographs correspond to 4 days of growth. Data are representative of four independent experiments.

Copper MIC could not be determined, since salt concentrations as high as 300 mmol L^{-1} did not affect conidia viability (Fig. 2C).

In order to study the synergism between the oxidizing compounds on *P. expansum*, we applied a one-step oxidative treatment (OT) that combined all the chemicals. This treatment consisted in a 2-min incubation with a sub-lethal NaClO concentration (1, 3 or 5 mg L^{-1}) and different H_2O_2 concentrations, in the presence of 6 mmol L^{-1} $CuSO_4$ (Table 1). It was observed that the OT with 1 mg L^{-1} NaClO, 200 mmol L^{-1} H_2O_2 and 6 mmol L^{-1} $CuSO_4$ produced a total inhibition of mycelia growth (Table 1). Also, the FIC index for the interaction between NaClO (1 mg L^{-1}) and H_2O_2 (200 mmol L^{-1}) in OT was 0.52. Together, these results would demonstrate the synergistic effect of the oxidizing compounds, previously studied on *P. digitatum* (Cerioni et al., 2009). It is worth to mention that NaClO and H_2O_2 concentrations necessary to prevent conidial growth were 50 and 2-fold lower than their MICs alone (Table 1). In fact, NaClO and H_2O_2 concentrations applied in packinghouses and fruit treatments are very high in respect to the concentrations used in the oxidative treatment (Junli et al., 1997; Annous et al., 2001; McWatters et al., 2002). This is important to minimize the probable negative impact that treatment could have on fruits. Besides, the OT with 3 or 5 mg L^{-1} NaClO, 100 mmol L^{-1} H_2O_2 and 6 mmol L^{-1} $CuSO_4$, were also a lethal treatment (Table 1). However, it is known that chlorine reacts with natural organic matter or contaminants in surface waters and produces a complex mixture of disinfection by-products (DBPs), some of which have been shown to be carcinogenic, mutagenic and/or teratogenic in animal studies (Ferraris et al., 2005). Due to this reason, the minimal assayed chlorine concentration was chosen in the oxidative treatment.

3.3. Inhibition of P. expansum conidial germination

We analyzed the ability of conidia to germinate after OT application. Fig. 3 shows that germination of conidia treated with 1 mg L^{-1} NaClO, 200 mmol L^{-1} H_2O_2 and 6 mmol L^{-1} CuSO4 (lethal OT) did not germinate until 96 h. With sub-lethal OTs, using 50 mmol L^{-1} or 100 mmol L^{-1} H_2O_2, germination started after 48 or 72 h, respectively. These results demonstrate a direct relationship between the increase in H_2O_2 concentration used in OT and conidial mortality (Table 1) and germination. Previously, we demonstrated in *P. digitatum* that the OT mechanism of action involved damage to different cellular levels by reactive oxygen species (ROS) generation (Cerioni et al., 2010). Other authors reported nitric oxide also caused an oxidative damage on conidia and inhibited the germination of *P. expansum* (Lai et al., 2010). Furthermore, Maneerat and Hayata (2006) showed a similar damage when UV-A, combined with TiO_2, was used to inhibit colony growth of *P. expansum*. These results suggest that *P. expansum* could be very susceptible to oxidizing compounds that generate ROS.

Table 1
Effect of oxidative treatment (OT) on *P. expansum* viability.

H_2O_2 (mmol L^{-1})	UFC mL^{-1}		
	NaClO 1 mg L^{-1}	NaClO 3 mg L^{-1}	NaClO 5 mg L^{-1}
0	1.13×10^6	7.2×10^3	4×10^2
50	9×10^4	2×10^2	ND
100	2×10^2	0	0
200	0	0	0

3.4. Conidial infectivity after sequential treatment

The disease incidence and severity on apples were used as indicators of conidial infectivity. Conidia treated with the OT using 100 mmol L^{-1} H_2O_2 were not able to infect apples (Fig. 4), although they maintained their viability under these conditions (Fig. 2A and Table 1). This effect may be due to the fact that the number of viable conidia is not enough to develop blue mold on fruit although a reduction on the *P. expansum* virulence cannot be discarded. Previous studies carried out in apples (Baert et al., 2008) and

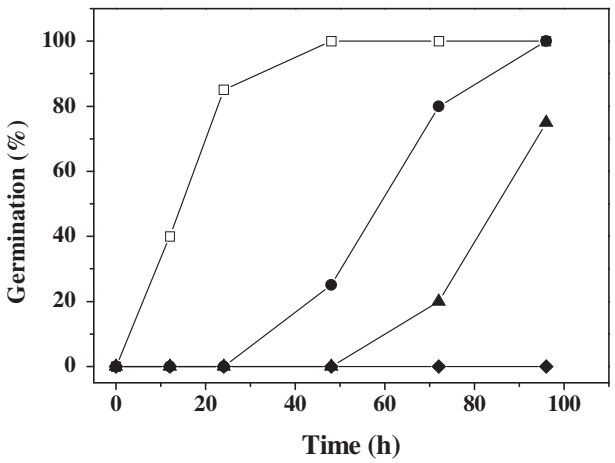

Fig. 3. Conidia germination after oxidative treatment (OT). *P. expansum* conidia were exposed to OT using 1 mg L^{-1} NaClO, 6 mmol L^{-1} $CuSO_4$ (-□- control) and different H_2O_2 concentrations (-●- 50 mmol L^{-1}; -▲- 100 mmol L^{-1}; -◆- 200 mmol L^{-1}). Percentages of germination were determined at the indicated times by microscopic examination. Data are representative of three independent experiments.

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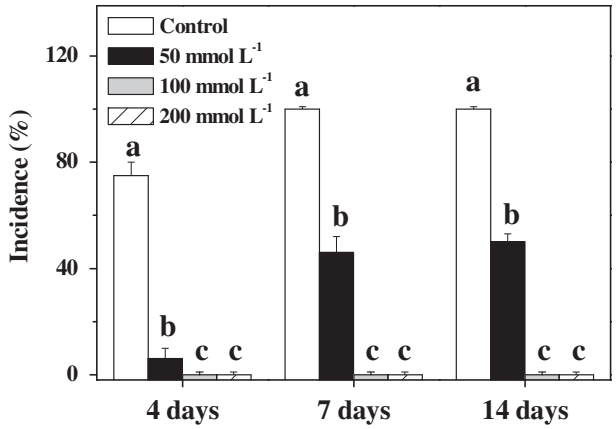

Fig. 4. Blue mold incidence in apple fruits inoculated with *P. expansum* conidia after oxidative treatments (OT). Percentages of incidence were calculated in respect to total inoculated fruits for each treatment. Fruits were inoculated using conidia suspensions treated with sterile water (control) or OT with 1 mg L^{-1} NaClO, 6 mmol L^{-1} $CuSO_4$ and H_2O_2 concentrations, as indicated. For each condition, values are the mean of 4 assays which consisted in 5 fruit with 3 wounds each. Different letters are significant differences among the treatment in each period of time (LSD, $p \leq 0.05$).

oranges (Vilanova et al., 2012) inoculated with *P. expansum* showed that a minimum inoculum concentration is necessary to infect the fruit. Furthermore, *P. expansum* was considered as non-host pathogen of oranges, but Vilanova et al. (2012) demonstrated that from the commercial harvest, an incompatible interaction can become compatible under favorable conditions. Since a previous work reported that a similar oxidative treatment was effective to control green mold in citrus fruit (Cerioni et al., 2012), we consider that the OT would have the same effect against *P. expansum*.

4. Conclusions

Based on our results, the oxidative treatment was established as 2 min-incubation with 1 mg L^{-1} NaClO, 200 mmol L^{-1} H_2O_2 and 6 mmol L^{-1} $CuSO_4$, which prevented 100% of conidial growth. The OT combines compounds that are safe to the environment and human health, thus it represents a potential alternative to synthetic fungicides for the integrated control of postharvest diseases on apples fruit.

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). L.C. and J. M. V are fellows from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

References

Andersen, B., Smedsgaard, J., Frisvad, J.C., 2004. *Penicillium expansum*: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. J. Agric. Food Chem. 52, 2421–2428.

Annous, B.A., Sapers, G.M., Mattrazzo, A.M., Riordan, D.C., 2001. Efficacy of washing with a commercial flatbed brush washer, using conventional and experimental washing agents, in reducing populations of *Escherichia coli* on artificially inoculated apples. J. Food Prot. 64, 159–163.

Baert, K., Devlieghere, F., Bo, L., Debevere, J., 2008. The effect of inoculum size on the growth of *Penicillium expansum* in apples. Food Microbiol. 25, 212–217.

Berenbaum, M.C., 1978. A method for testing for synergy with any number of agents. J. Infect. Dis. 137, 122–130.

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.

Cerioni, L., Rodríguez-Montelongo, L., Ramallo, J., Prado, F.E., Rapisarda, V.A., Volentini, S.I., 2012. Control of lemon green mold by a sequential oxidative treatment and sodium bicarbonate. Postharvest Biol. Technol. 63, 33–39.

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.

Eckert, J.W., Ogawa, J.M., 1988. The chemical control of postharvest diseases: deciduous fruits, berries, vegetables and roots/tuber crops. Annu. Rev. Phytopathol. 26, 433–469.

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.

Giraud, M., Fauré, J., 2000. Assessment of Penicillium Risk on Pome Fruit in Storage. In: Integrated Control of Pome Fruit Diseases, vol. 23. 10 BC WPRS Bulletin, pp. 153–158.

Innis, M.A., Gelfand, D.H., 1990. Optimization of PCR. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, New York, pp. 3–12.

Jones, A.L., Aldwinckle, H.S., 1991. Compendium of Apple and Pear Diseases. APS Press, pp. 53–55.

Junli, H., Li, W., Nenqi, R., Fang, M., Li, J., 1997. Disinfection effect of chlorine dioxide on bacteria in water. Water Res. 31, 607–613.

Lai, T., Li, B., Qin, G., Tian, S., 2010. Oxidative damage involves in the inhibitory effect of nitric oxide on spore germination of *Penicillium expansum*. Curr. Microbiol. 63, 229–234.

Maneerat, C., Hayata, Y., 2006. Antifungal activity of TiO_2 photocatalysis against *Penicillium expansum* "*in vitro*" and fruit tests. Int. J. Food Microbiol. 107, 99–103.

Marek, P., Annamalai, T., Venkitanarayanan, K., 2003. Detection of *Penicillium expansum* by polymerase chain reaction. Int. J. Food Microbiol. 89, 139–144.

McWatters, L.H., Chinnan, M.S., Walker, S.L., Doyle, M.P., Lin, C.M., 2002. Consumer acceptance of fresh-cut iceberg lettuce treated with 2% hydrogen peroxide and mild heat. J. Food Prot. 65, 1221–1226.

Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage, second ed. Blackie Academic & Professional, London.

Prusky, D., Bazak, M., Ben-Arie, R., 1985. Development, persistence, survival, and strategies for control of thiabendazole-resistant strains of *Penicillium expansum* on pome fruits. Phytopathology 75, 877–882.

Qin, G.Z., Tian, S.P., 2005. Enhancement of biocontrol activity of *Cryptococcus laurentii* by silicon and possible mechanisms involved. Phytopathology 1, 69–75.

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.

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.

Vilanova, L., Viñas, I., Torres, R., Usall, J., Jauset, A.M., Teixidó, N., 2012. Infection capacities in the orange-pathogen relationship: compatible (*Penicillium digitatum*) and incompatible (*Penicillium expansum*) interactions. Food Microbiol. 29, 56–66.

Xiaoping, L., Jiye, W., Ping, G., Cungui, M., Zeng-Rong, Z., Hongy, L., 2007. *In vitro* inhibition of postharvest pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin A. Int. J. Food Microbiol. 119, 223–229.

Yu, T., Chen, J., Lu, H., Zheng, X., 2009. Indole-3-acetic acid improves postharvest biological control of blue mold rot of apple by *Cryptococcus laurentii*. Phytopathology 99, 258–264.

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