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Research Paper

Physical postharvest treatments combined with antagonistic yeast on the control of orange green mold

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ABSTRACT

In order to control the green mold on orange, the effect of physical postharvest treatments, using hot water brushing (HWB) and ultraviolet C irradiation (UVC), alone or in combination with antagonistic yeast (*Candida membranifaciens* CMAA-1112) was studied. The mechanisms involved in the biocontrol and the effects of these treatments on postharvest quality of fruit were also investigated. The results showed that HWB at 55 °C for 30 s and UVC at 2 kJ m^{-2} stand-alone were capable of reducing the decay progress in around 70%. *C. membranifaciens* was effective in reducing the disease severity, and the main mechanism of control was by inducing systemic resistance on fruit peel. The combination of physical treatments and *C. membranifaciens* presented an additive effect increasing the efficacy in controlling the disease, and extended the fruit shef-life. Our data suggest that the integration of physical treatments combined with *C. membranifaciens* could be an alternative to fungicides use in postharvest treatment for the control of the green mold on orange.

1. Introduction

Global leader in the production and exportation of orange juice, Brazil exports 98% of its production, representing in 85% of the world market. Three out of every five glasses of orange juice consumed in the world are produced in Brazilian factories (Neves, 2016). According to MDIC/SECEX (2016), in 2015, Brazil exported 2 million tons of orange juice, which corresponds to US$ 1.8 billion (FOB) and around 120,000 tons of fresh citrus. This corresponds to a revenue of US$ 87 million.

Brazilian exportation shipment of citrus fresh fruit normally follows maritime routes, taking more than two weeks to reach its destination, therefore, the storage life of the product is a relevant factor to be considered, once the fruit susceptibility to postharvest diseases increases during long term storage due to physiological changes, favoring quiescent fungi development (Schirra et al., 2000). Green mold caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. causes the major losses when considering postharvest diseases in citrus, which are the main limiting factor in the storage life of this fruit.

The use of fungicides is still the most widely used method to control this disease, however, the continuous use of the same active principle has induced the development of resistant races, and the public concern over the impact of fungicides on human health and on the environment (Droby et al., 2009) has stimulated the search for non-chemical means of control.

Physical postharvest treatments such as short hot water brushing (HWB), and low dose of ultraviolet light irradiation (UV-C) has demonstrated efficient control of postharvest diseases in several species of fruits by the direct inhibition of the pathogen and by the stimulation of certain host-defense responses (Schirra et al., 2000; Terao et al., 2015).

The biocontrol using yeast is another promising strategy to control postharvest disease, due to the genetic stability, the effectiveness at low concentration, the ability to act on a broad spectrum of pathogens, and the fact that, generally, the production of toxic secondary metabolites is not the main mechanism of control involved. Moreover, controlled temperature and relative humidity in the environment during the postharvest stage create suitable conditions for the development of the biocontrol agents (Janisiewicz and Korsten, 2002; Wisniewski et al., 2007).

The combination of different methods of control has been proven to be more effective in reducing postharvest decay of fruit, compensating for the limitations of their individual use, showing to be promising means to minimize the use of agrochemicals in postharvest treatments (Wisniewski, 2016).

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The present study aimed at evaluating the efficacy of physical treatments, HWB and UV-C, used separately or in combination with biocontrol treatment using yeast to control green mold decay on orange.

2. Materials and methods

2.1. Fruit

Fresh-cut oranges (*Citrus sinensis* L. Osbeck) cultivar 'Pera', from a commercial property located in Engenheiro Coelho, SP, Brazil, were harvested and selected by size, maturation, and absence of physical injuries or evidence of infection by pathogens. Fruit were disinfected with 0.1% sodium hypochlorite for 1 min, washed with tap water and air-dried, prior to wounding and inoculation.

2.2. Pathogen inoculum and inoculation

Penicillium digitatum (Pers.) Sacc. was isolated from decayed orange and maintained on potato-dextrose-agar medium (PDA) at 4 °C, and fresh cultures were grown on PDA plates at 23 °C before use. The inoculation was done by depositing 5 μL of conidia suspension of *P. digitatum* (5×10^5 conidia mL^{-1}) on wounds previously done on the equatorial region of the fruit using a sterile nail with 3-mm diameter and 2-mm depth. After inoculation, fruit were stored in a humid chamber at 23 ± 2 °C for 6 h.

2.3. Effect of heat treatment and UVC-irradiation on spore germination of pathogen

For the evaluation of the effect of heat treatment on inhibition of spore germination, 100 μL of a spore suspension of *P. digitatum* (1×10^4 spores mL^{-1}) were added to 900 μL of distilled sterile water (DSW) in glass tube immersed in a circulating water bath, adjusted to evaluate the temperatures: 50 °C, 55 °C, 60 °C for 15 or 30 s. After the treatment, the glass tubes were immersed in a water bath at 20 ± 3 °C to stop the heating effect. The UV-C irradiation treatment were applied in a UV-C prototype consisted of an acrylic box with a reflecting surface at the top, using unfiltered Osram Puritec HNS 36-W germicidal lamp, with light emission concentrated on the UV-C wave-lenght of 253.7 nm, with an average power of 370 μW cm^{-2} at a distance of 46 cm. Light intensity was kept constant and the applied doses varied by modifying the exposure time. The dose of 1.0 kJ m^{-2} corresponded to 4 min and 40 s of exposition time. The *in vitro* study of UVC-irradiation was done by irradiating 3 mL of spore suspension of *P. digitatum* (1×10^3 spores mL^{-1}) in 5 cm diameter Petri dishes, at the doses of 0.25, 0.5, 1.0, 1.5, 2.0 kJ m^{-2}. After the heat treatment and UVC-irradiation, 0.1 mL aliquots of the treated spore suspensions were transferred and spread on PDA medium in 9-cm-diameter Petri dishes. After 72 h incubation at 23 ± 2 °C, the percentage of spore germination was determined. The trials were laid out in a completely randomized design with three replications.

2.4. Evaluation of physical postharvest treatments on fruit

The physical treatments, hot water brushing (HWB) and ultraviolet light C irradiation (UV-C), were first evaluated individually. HWB treatment consisted of spraying hot water at 55, 60, 65 and 70 °C for 30 s on fruit, as they move along brush-rollers. After the heat treatment, the fruit were cooled immediately by rinsing them with tap water at 20 °C for 2 min. As control, fruit were only rinsed in tap water at 20 °C for 2 min. For comparative reason, one other treatment was prepared with the use of fungicide thiabendazole (485 g a.i. 100 L^{-1}) exclusively.

The UV-C irradiation treatment on fruit were applied in a UV-C prototype, as previously described. The orange samples received doses of 0.25, 0.5, 0.75, 1.5 and 2.0 kJ m^{-2} of UV-C light. Fruit were rotated twice during the irradiation to homogenize the treatment. After exposure to UV-C irradiation, fruit were packed in cardboard boxes, protected from light and stored for one hour under refrigeration (10 ± 2 °C and 80 ± 2% of the relative humidity).

On the next step the best combination of temperature and time, and the UVC-irradiation dose was selected, and those treatments were applied combined.

Treated fruit were stored under refrigeration (10 ± 2 °C and 80 ± 2% of the relative humidity), for 15 days and then at room temperature (23 ± 2 °C) for 7 additional days. The severity of the disease was evaluated daily by measuring the diameter of the lesions. The experiment was laid out in a completely randomized design with eight treatments and 30 replications, considering one fruit as an experimental unit. The experiments were repeated twice.

2.5. Biological control

2.5.1. Screening of yeasts species

Yeast strains were obtained from the Collection of Microorganisms of Agricultural and Environmental Importance at Embrapa Environment in Jaguariúna, São Paulo, Brazil. The following yeasts species originally isolated from vineyards of Brazilian production areas were evaluated: *Sporidiobolus pararoseus* (CMAA-1106), *Candida membranifaciens* (CMAA-1108), *Candida membranifaciens* (CMAA-1110), *Meyerozyma guilliermondii* (CMAA-1111), *Candida membranifaciens* (CMAA-1112), *Candida* sp. (CMAA-1113) and *Meyerozyma guilliermondii* (CMAA-1109). Yeasts were applied on the inoculated wound by spraying the cell suspension (10^8 CFU mL^{-1}). Fruit treated with sterile distilled water served as control and the fungicide thiabendazole (485 g 100 L^{-1} of active ingredient) was used for comparison. After that, fruit were stored at room temperature (23 ± 2 °C) during 18 days, and disease severity was evaluated daily by measuring the diameter of the lesions. The experiment was laid out in a completely randomized design with nine treatments and 30 replications, considering one fruit as an experimental unit.

2.5.2. Study of antibiosis and lytic enzyme production involved in biocontrol

2.5.2.1. Inhibitory volatile compounds. In polystyrene bipartite Petri dishes with Potato Dextrose Agar (PDA) medium, 50 μL of yeast suspension (10^8 cells per mL) were deposited on one side of the culture media, whereas, a mycelial disk of 0.5 cm diameter of *P. digitatum* was placed on the other side. As control, Petri dishes with single mycelial disks of *P. digitatum* alone were used. Dishes were sealed with parafilm and incubated at 23 ± 2 °C with 12 h photoperiod. Six repetitions were used for each treatment. Evaluations were done by measuring the colony diameters on a daily basis until the colony on control treatment reached the edge of the plate, then comparing the growth rate among treatments.

2.5.2.2. Inhibitory diffusible compounds. Yeasts were grown on PDA at 23 ± 2 °C at the center of a 9-cm-diameter Petri dish. After incubation for 7 days, 2 mL of chloroform were placed inside each dishes lid. Dishes with lids were then stored in the laminar flow cabinet upside down for about 2 h. Thereafter, all Petri dishes were opened for chloroform volatilization and 15 mL of semi-solid PDA (7.5 g agar per liter) with 150-embedded conidia of *P. digitatum* were added to each plate as overlayer (Halfeld-Vieira et al., 2015). The cultures were incubated at 26 °C with 12 h photoperiod. After *P. digitatum* mycelial growth on the overlayer, the absence or presence of inhibition haloes was recorded. Three replicates were carried out for each yeast vs. *P. digitatum* combination.

2.5.2.3. Chitinase production. The chitinase production capability of yeasts was evaluated based on Renwick et al. (1991) assay. Each yeast