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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

colony was transferred to the center of three dishes with the 0.5% chitin medium as the sole carbon source (Renwick et al., 1991) and incubated at 23 ± 2 °C for 15 days. Chitinase production was evaluated by comparing the results with *Burkholderia pyrrocina* strain, as positive control, that uses chitin as sole carbon source evidencing a transparent halo around colony.

2.5.2.4. Killer toxins activity against P. digitatum. Yeasts from stock cultures were cultivated in 50 mL YNB-D minimal growth medium [1% (w/v) glucose and 0.67% (w/v) yeast nitrogen base] in 125-mL Erlenmeyer flasks, with pH values 3.0, 4.0, and 5.0 adjusted with 0.2 M citrate/phosphate buffer, incubated for 3 days at 180 rpm shaking rate at 18 °C. After growth, cultures were centrifuged at 10,000 rpm per 10 min and filtered through a 0.22 µm pore size Millipore membrane (Santos and Marquina, 2004). Inhibitory activity for *P. digitatum* was evaluated on Potato Dextrose Agar (PDA) by the addition of 100 µL of one filtrate to each of the four 0.5-cm-diameter wells placed diametrically opposed to each other 1 cm off plate border (Woods and Bevan, 1968). As control, only the YNB-D culture media with each buffered pH was added to the wells. At the center of each plate, a 0.5-cm *P. digitatum* active mycelium plug was placed and the materials were incubated at 20 °C. The experiment entailed three repetitions per treatment. Inhibition was considered positive if inhibition zones were apparent near the wells that received filtrates, compared to respective control plates.

2.6. Integration of physical postharvest treatments with yeast antagonist

2.6.1. Effect of postharvest treatments combining HWB, UV-C light, and yeast antagonist on the control of green mold decay on orange

Oranges previously inoculated were randomly grouped into eight lots according to the combination of the treatments: hot water brushing (HWB) treatment at 55 °C for 30s, ultraviolet C light irradiation (UV-C) at 1.5 kJ m^{-2} and biological control (*C. membranifaciens CMAA-1112*). The treatments were applied individually, combined two by two, or combining all of them. As control, fruit were only rinsed in tap water at 20 °C for 2 min. Treated fruit were stored under refrigeration (10 ± 2 °C and 80 ± 2% of the relative humidity), during 15 days and then at room condition (23 ± 2 °C) for 7 additional days. The severity of the disease was evaluated by measuring the diameter of the lesions on a daily basis. The experiment was laid out in a completely randomized design with eight treatments and 30 replications, considering one fruit as an experimental unit. The experiment was conducted twice.

2.6.2. Effect of postharvest treatments on defense-related enzymes

Samples from non-inoculated fruit peel were collected at 1, 2, 3, 4 and 5 days after the exposition of fruit to the physical and *C. membranifaciens* CMAA-1112 treatments individually, combined two by two, or combining all of them as described on item 2.6. Fruit rinsed in tap water at 20 °C for 2 min were considered the control treatment. The samples were wrapped in aluminum foil and immediately stored at −80 °C until further analysis. Three samples were collected per treatment. To determine the activities of polyphenoloxidase (PPO) and phenylalanine ammonia-lyase (PAL), one gram of peel was ground with a pestle in a mortar containing liquid nitrogen. The resulting powder was macerated for 30 s in 6 mL of 50 mM sodium phosphate buffer, pH 6.5, containing 1% polyvinylpyrrolidone and 1 mM phenylmethylsulfonyl (PMSF). The extract was centrifuged at 20,000*g* for 20 min at 4 °C and the supernatant was used as a crude enzyme extract (Baracat-Pereira et al., 2001).

The PPO activity was estimated according to Duangmal and Apenten (1999). Twenty µL of crude extract was added to 200 µL pyrocatechol (20 mmol L^{-1}). The mixture was incubated at 30 °C and absorbance (420 nm) readings were taken every 60 s for 8 min. PPO activity was expressed as changes in absorbance units per minute per mg of fresh tissue. The PAL activity was determined following the method of Pascholati et al. (1986). The reaction mixture consisted of 10 µL of crude extract, 500 µL of 100 mM borate buffer (pH 8.8) and 500 µL of 12 mM L-phenylalanine. After incubation at 37 °C for 20 min, the reaction was interrupted by the addition of 37 µL 6N HCl and the absorbance was measured at 290 nm. The PAL activity was expressed as µg of *trans*-cinnamic acid per minute per mg of fresh tissue. The conversion of L-phenylalanine to *trans*-cinnamic acid was determined using a standard curve of *trans*-cinnamic acid.

2.7. Effects of physical treatments isolated and in combination with biocontrol on postharvest quality of oranges

The effects of biocontrol, physical treatments, and their combination on postharvest quality of orange were measured before treatment and during storage. Quality measurements were made on three replicates of five oranges each. A CR300 colorimeter® (Minolta Co. Osaka, Japan) was used for instrumental color evaluation. The CIE Lab color scale (L*C*H*) was used with a D65 illuminant (standard day-light) and 2° angle. The L*C*H* parameters were determined according to the International Commission on Illumination (CIE, 1996). The total soluble solids were determined with a RF Sensor SR 400 refractometer with temperature compensation at 20 °C. Results were expressed as Brix degree. The total titratable acidity (TA) was determined by titrating 100 g of orange juice to a pH of 8.1. The content of TA was calculated as citric acid (mg 100 g^{-1} FW). The pH was measured by using measured by using a Digital pH meter (Genaka, São Paulo, Brazil). Firmness was measured using the TA-XT2i Texture Analyser by fruit compression using the probe P35 mm at 2.0 mm s^{-1}.

The sensory acceptability evaluation was carried out with 20 untrained evaluators using nine-point hedonic scale, where the degree in which they 'like' or 'dislike' the product is expressed. General fruit appearance aspects, flavor, texture and the purchase intent of consumer were evaluated.

2.8. Statistical analysis

The severity of the disease data was transformed into area under the disease progress curve (AUDPC) (Campbell and Madden, 1990) considering 22 evaluation days. The parameters AUDPC, PPO and PAL were analyzed by ANOVA and means compared by using Fisher's protected LSD ($p \leq 0.05$). The comparisons of means of *in vitro* tests and fruit quality parameters were done using Tukeý́s test. The Dunnett's test was used to compare the efficacy of yeast antagonist species in controlling green mold decay. The statistical analyses were performed using the SAS 9.2 GLM procedure (SAS Institute Inc., Cary, NC).

3. Results

3.1. Effect of heat treatment and UVC-irradiation on spore of P. digitatum germination

The *in vitro* evaluations revealed that *P. digitatum* spore germination is completely inhibited by hot water treatment from at least 55 °C for 15 s, and did not differ from treatment at 50 °C for 30 s (Table 1).

Regarding to UVC light irradiation, the dose of 2.0 kJ m^{-2} was sufficient to a complete inhibition of *P. digitatum* spores germination, not differing statistically from the dose of 1.0 kJ m^{-2} (Table 2).

3.2. Physical postharvest treatments

According to Fig. 1, the hot water brushing treatment (HWB), in general, reduced significantly the development of the green mold decay presenting a superior performance when compared to the fungicide thiabendazole.

The HWB treatment at 55 °C for 30 s reduced the disease progress in

Table 1
Inhibition of spore germination of *Penicillium digitatum* by using different temperature combinations and heat treatment period.

Treatments	Inhibition of spore germination (%)	
50 °C/15s	89.74	c
50 °C/20s	93.33	b
50 °C/30s	99.48	a
55 °C/15s	100	a

Means followed by the same letter are not significantly different according to Tukey's test ($p \leq 0.05$).

Table 2
Inhibition of spore germination of *Penicillium digitatum* by using different doses of ultraviolet-C irradiation.

Treatments	Inhibition of spore germination (%)	
0.25 kj m^{-2}	85.59	c
0.5 kj m^{-2}	93.48	b
1.0 kj m^{-2}	98.33	a
1.5 kj m^{-2}	99.24	a
2.0 kj m^{-2}	100	a

Means followed by the same letter are not significantly different according to Tukey's test ($p \leq 0.05$).

about 70%, and did not differ from the other higher temperature treatments.

Regarding the treatment with UVC light, it can be observed in Fig. 2 that from the dose of 1.5 kJ m^{-2}, the irradiation with UVC light was effective in controlling green mold decay in Pera orange, not differing from the dose of 2.0 kJ m^{-2}.

3.3. Biological control

3.3.1. Screening of yeast species on control of green mold decay on orange

Yeast strains CMAA 1106, CMAA 1109, CMAA 1111, and CMAA 1112 were able to reduce the severity of green mold decay on orange fruit. However, *C. membranifaciens* CMAA-1112 showed a significant

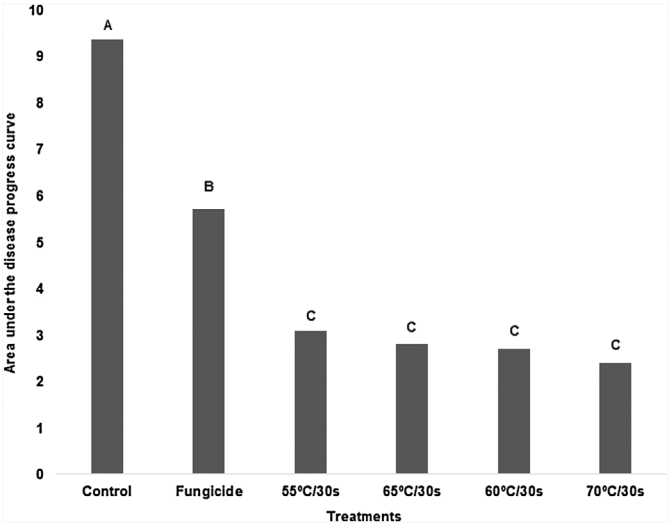

Fig. 1. Area under the disease progress curve of green mold decay percentage of infection, caused by *Penicillium digitatum*, during 22 days of storage, by using hot water brushing treatment, comparing with fungicide thiabendazole (485 g a.i. 100 L^{-1}) and control (sterile distilled water). Bars are means of 30 fruits per treatment. Means followed by different letters are significantly different according to Fisher's protected LSD ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

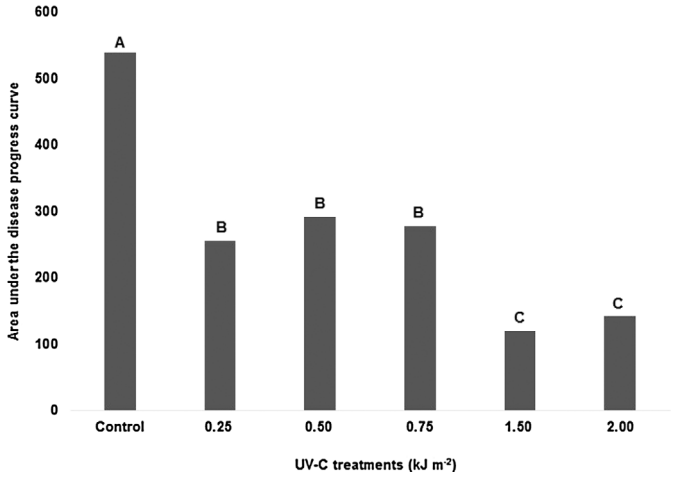

Fig. 2. Area under the disease progress curve of green mold decay severity, caused by *Penicillium digitatum*, during 22 days of storage, by using ultraviolet-C irradiation treatment, comparing with the control (sterile distilled water). Bars are means of 30 fruits per treatment. Means followed by different letters are significantly different according to Fisher's protected LSD ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

earlier and continued ability to control the disease compared to the others. The fungicide thiabendazole was not effective on the control of the green mold (Table 3).

3.3.2. Mechanisms of action involved in biocontrol

No mycelial growth inhibition was observed in any antibiosis and lytic enzyme production assays, and none of the yeast strains were capable of producing chitinase, resulting in no evidence of inhibitory substances or hydrolytic enzymes production by yeasts against *P. digitatum* (Data not shown).

3.4. The combination of physical postharvest treatments and biological control

During a twenty-two-day storage, the combination of the three treatments (T1UVC1Y1), HWB (T1), UVC (UVC1) and the antagonist yeast (Y1) *C. membranifaciens* CMAA-1112 presented the best control of disease capability, exhibiting lower severity, differing significantly from the control, and not differing from the treatment combining HWB and UVC (T1VCU1Y0), and from the HWB alone (T1UVC0Y0) (Fig. 3).

Table 3
Green mold decay severity in orange fruit up to 18 evaluation-day period. The yeast strains *Meyerozyma guilliermondii* (CMAA 1109), *Candida membranifaciens* (CMAA 1110), *Meyerozyma guilliermondii* (CMAA 1111), *Candida membranifaciens* (CMAA 1108), *Candida membranifaciens* (CMAA 1112), *Candida* sp. (CMAA 1113), *Sporodiobolus pararoseus* (CMAA 1106) and the fungicide thiabendazole (485 g a.i. 100 L^{-1}) were compared to the control (sterile distilled water).

Treatment	Lesion diameter (mm)		
	8 days	13 days	15 days
CMAA 1109	18.54 ± 5.36	32.22 ± 6.47	38.95 ± 6.45
CMAA 1110	18.55 ± 5.31	40.83 ± 6.24	43.34 ± 6.18
CMAA 1111	15.13 ± 4.79	34.70 ± 6.19	39.37 ± 6.14
CMAA 1108	30.47 ± 6.00	47.41 ± 6.10	49.95 ± 6.13
CMAA 1112	10.32 ± 4.37	26.29* ± 5.86	29.75* ± 6.38
CMAA 1113	28.43 ± 5.81	40.94 ± ± 6.01	46.44 ± 5.74
CMAA 1106	13.94 ± 5.19	27.37* ± 6.02	31.58* ± 6.31
Fungicide	27.04 ± 5.92	44.64 ± 5.41	50.91 ± 5.81
Control	29.88 ± 5.31	51.52 ± 5.41	56.93 ± 5.37
d.m.s.	20.26	22.81	22.91

* Treatment reduced significantly the lesion diameter compared to the control by Dunnett's test ($P \leq 0.05$).

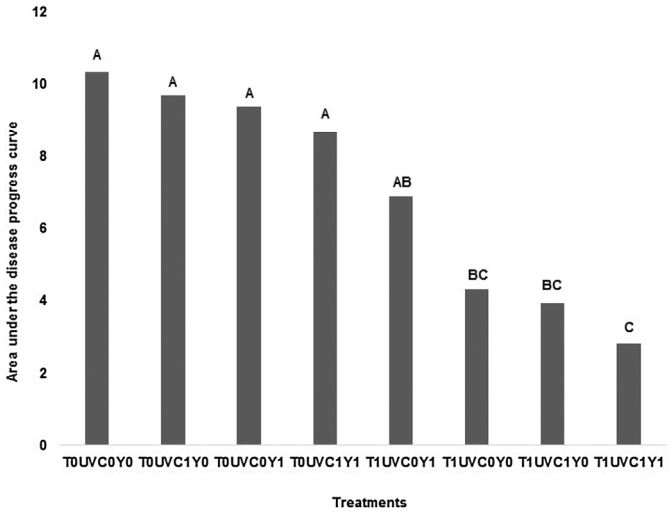

Fig. 3. Area under the disease progress curve of green mold decay percentage of incidence, caused by *Penicillium digitatum*, during 22 days of storage, by using the combination of hot water brushing (T1: with and T0: without); ultraviolet-C irradiation (UVC1: with and UVC0: without) and cells suspension of *Candida membranifaciens CMAA-1112* (Y1: with and Y0: without) comparing with the control (T0UVC0Y0). Bars are means of 30 fruits per treatment. Means followed by different letters are significantly different according to Fisher's protected LSD ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Induction of defense-related enzymes

The activity of PPO was higher on fruit three and four days after they received the suspension of *C. membranifaciens* CMAA-1112 (T5) (Fig. 4A). Indeed, the biocontrol treatment promoted a significant increase of PAL activity on the fourth day of the experiment (Fig. 4B), indicating the capability of this yeast strain to trigger an induced resistance response on fruit. Overall, the PPO and PAL activities were similar among the physical treatments, regardless of their combination, and the control treatment. The only exception was the heat treatment individually, which promoted a significant increase of PAL activity only 1 day after spraying hot water at 55 °C for 30 s (Fig. 4 B).

3.6. Effects of physical treatment and yeast antagonist on postharvest quality of oranges

Concerning to postharvest quality parameters, after 15 days of storage at 10 ± 2 °C, no significant difference between oranges from control treatment and treated with HWB, UVC light irradiation and yeast antagonist, alone or in combination was observed (data not shown).

On the other hand, after 22 days of storage (15 days at 10 ± 2 °C plus 7 days at 23 ± 2 °C), fruit treated with a combined treatment of HWB and UVC light irradiation (T7) presented significantly ($p \leq 0.05$) lower tritable acidity than the control fruit. For color parameters, fruit treated with HWB, in combination with yeast (T6) or with UVC and yeast antagonist (T8) showed significantly ($p \leq 0.05$) lower L (lightness) and C (chroma), and the combination of HWB and UVC higher H (hue angle) than the control fruit (Table 4).

The consumer acceptance evaluation indicated that, after 15 days of cold storage, there was no significant difference between treatments, however seven days after withdrawal from refrigeration, fruit submitted to physical treatment by HWB or UVC, alone or in combination with yeast antagonist, received higher grades of acceptance from the consumers, differing significantly ($p \leq 0.05$) from the control (Table 5).

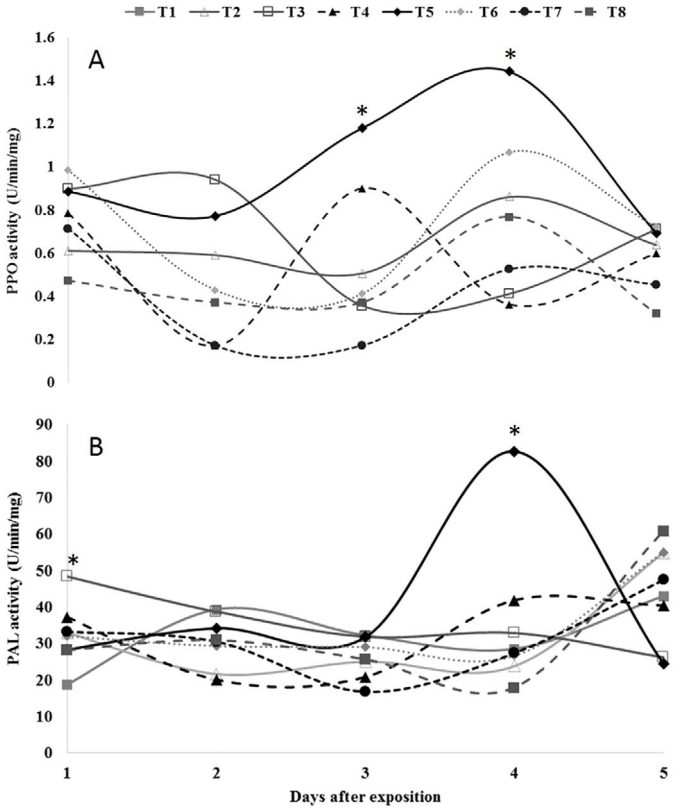

Fig. 4. Activities of polyphenol oxidase (PPO) (A) and phenylalanine ammonia-lyase (PAL) (B) in orange fruit cultivar "Pera" exposed to the treatments: Control (T1); ultraviolet-C irradiation (T2); hot water brushing (T3); combined T2 and T3 (T4); cells suspension of *Candida membranifaciens* CMAA-1112 (T5); combined T2 and T5 (T6); combined T3 and T5 (T7) and combined T2, T3 and T5 (T8) at different sampling time. Means marked with an asterisk (*) for each sampling time presented a higher significantly activity than all other treatments according to Fisher's protected LSD ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The *in vitro* tests evaluating the effect of the heat treatment and UVC-irradiation on spore germination revealed that *P. digitatum* conidia is relatively sensitive to heat and UVC light, since the exposure to hot water at 55 °C for 15 s and to a dose of 1.5 kJm^{-2} inactivated the spore germination. This result indicated that short exposure to hot water and UVC irradiation treatments are efficient to cause direct inhibition of *P. digitatum* conidial germination present on the surface of oranges. This information presents a practical importance since a reduction on the period of postharvest physical treatments increases the interest to commercial use of this technology (Usall et al., 2016).

Hot water brushing (HWB) and UVC irradiation treatments, in general, demonstrated efficiency in controlling green mold decay in orange. Although the *in vitro* tests indicated that the binomial 55 °C/15 s was enough to inhibit the *P. digitatum* spore germination, the binomial 55 °C for 30 s was used on *in vivo* HWB tests, since the longer interval time allows greater contact of the fruit with water, providing more homogeneous application of the heat treatment.

On the other hand, the fungicide thiabendazole (485 g 100 L^{-1} of active ingredient) was ineffective in controlling the disease, probably due to the selection of resistant races to the product. This resistance is caused by the continued use of the same active ingredient of systemic fungicides (Kinay et al., 2007; Erasmus et al., 2015).

The low dose of UVC light as 1.5 kj m^{-2} shown to be effective on the control of green mold. Furthermore, the direct germicidal effects of UVC light on the fruit surface, low dose of UVC irradiation can induce

Table 4
Quality parameters and peel color index of orange 'Pera' at 22 storage days submitted to hot water and brushing (HWB) (55 °C for 30 s), UVC light irradiation (1.5 kJ m^{-2}), and yeast antagonist (*Candida membranifaciens CMAA-1112*) alone or in combination. T1: Control; T2: yeast; T3: UVC; T4: UVC + yeast; T5: HWB; T6: HWB + yeast; T7: HWB + UVC; T8: HWB + UVC + yeast.

Treatments	pH	TSS (%)	Tritable acidity (% citric acidity)	Firmness (N)	Peel Color Index (PCI)		
					L	C	H
T1	3.73 a	12.17 a	1.06 a	69.57 a	72.16 a	54.89 a	85.54 b
T2	3.87 a	11.02 a	0.84 ab	54.98 a	69.33 ab	51.48 ab	86.03 b
T3	3.85 a	10.95 a	0.81 ab	48.81 a	69.30 ab	48.84 abc	88.77 ab
T4	3.79 a	10.96 a	0.90 ab	50.98 a	70.59 ab	49.96 ab	88.61 ab
T5	3.90 a	12.25 a	0.78 ab	51.13 a	66.61 bc	45.08 bcd	88.86 ab
T6	3.84 a	11.27 a	078 ab	46.47 a	64.53 c	42.61 cd	91.76 ab
T7	3.99 a	12.52 a	0.64 b	47.37 a	66.47 bc	44.75 bcd	92.48 a
T8	3.94 a	11.05 a	0.79 ab	47.07 a	62.66 c	38.49 d	90.19 ab

Values followed by the same letter in the column are not significantly different according to Tukey's test ($P \leq 0.05$).

Table 5
Consumer acceptance evaluation of orange 'Pera' at 15 and 22 days after postharvest treatment with hot water and brushing (HWB) (55 °C for 30 s), UVC light irradiation (1.5 kJ m^{-2}), and yeast antagonist (*Candida membranifaciens CMAA-1112*) alone or in combination. T1: Control; T2: yeast; T3: UVC; T4: UVC + yeast; T5: HWB; T6: HWB + yeast; T7: HWB + UVC; T8: HWB + UVC + yeast.

Treatments	Consumer acceptance grade	
	15 d	22 d
T1	2.20 a	1.18 b
T2	1.70 a	3.27 a
T3	1.50 a	3.54 a
T4	2.00 a	3.09 a
T5	1.90 a	2.90 a
T6	1.90 a	2.90 a
T7	2.00 a	3.09 a
T8	1.40 a	3.36 a

Values followed by the same letter in the column are not significantly different according to Tukey's test ($P \leq 0.05$).

the *hormesis* in fruit, delaying fungal growth and senescence in fresh fruits, reducing the decay development. High doses of UVC light can cause damage to fruit peel, increasing the severity of postharvest diseases (Terao et al., 2015; Romanazzi et al., 2016b).

The fruit submitted to the treatment with combination of HWB at 55 °C for 30s, the dose of 1.5 kJm^{-2} of UVC irradiation and the application of the yeast antagonist *C. membranifaciens CMAA-1112* presented very low severity of the decay during refrigerated storage, with the majority of fruit with no symptoms, during 15 evaluation days increasing slowly during the room storage period. In contrast, fruit from the control presented high severity of the disease since the beginning of the evaluations on the 11th day, increasing significantly when withdrawn from refrigeration.

Our results indicated that yeast *C. membranifaciens* CMAA-1112 promoted a significant contribution on the severity reduction of the disease not by producing inhibitory substances. The absence of production of substances against *P. digitatum* can configure an advantage to consumer safety, once secondary metabolite molecules could have an undesirable effect implying in use limitations of the biocontrol agent. On the other hand, this strain was able to induce resistance on fruit peel, increasing the PPO and PAL activity remarkably 4 days after fruit exposition to yeast suspension. These enzymes have been associated to the induced systemic resistance and elicit plant defense responses to abiotic and biotic stress (Shoresh et al., 2010). Numerous reports have indicated that yeast treatments can induce systemic resistance in several pathosystems (Romanazzi et al., 2016a). Among defense-related enzymes, PAL is considered the main enzyme that increases the fruit's resistance to *P. digitatum* (Ballester et al., 2010). The expression of phenylalanine metabolism defense-associated genes on orange fruit by the yeast *Kloeckera apiculata* was also previously reported to be involved on its efficacy to control the green mold on orange (Liu et al., 2016). PPO oxidizes the phenolic to quinones, which are toxic to invading pathogens (Mayer, 1987). Our results demonstrated that yeast *C. membranifaciens* CMAA-1112 induced significantly higher activities of both enzymes within the same storage period, resulting in increased resistance response against *P. digitatum*.

The hot water brushing also increased the PAL activity. However, this increase was restricted to the first-day storage. On the other hand, the yeast treatment after HWB and UVC irradiation decreased the capability of *C. membranifaciens* CMAA-1112 to increase PPO and PAL activity on fruit, so it is postulated that these physical treatments promote a physiological change that leads to a reduction of the capability to the peel tissues to have triggered the resistance induction response by the yeast.

It was observed an additive effect between physical and biological treatments that increased the efficacy in controlling green mold, reducing the incidence and the severity of the disease. This synergistic effect was previously reported considering the combined effects of *P. membranifaciens* with salicylic acid (Zhou et al., 2014b) and *P. membranifaciens* with hot water treatment (Zhou et al., 2014a), improving the control of postharvest diseases in citrus fruit.

Regarding the quality attributes of the fruit, according to the coloring index of the peel, at 22 days of storage, control fruit presented higher values of Lightness (L*), Chroma (C*), and lower hue angle (H*) than those treated with HWB, isolated or combining with UVC irradiation or antagonistic yeast, corresponding to a more intense yellow color, close to orange, indicating a higher maturation degree index. Possibly, those treatments preserved fruit quality by slowing down the maturation process, increasing the shelf life. Heat treatment was reported to induce beneficial effects on fruit physiology such as the reduction of yellowing, delaying the ripening-senescence process, reducing the susceptibility to decay (Usall et al., 2016). This observation corroborated the consumer acceptance test, in which evaluators classified the oranges of control which not acceptable for purchase, for the presence of stains on the peel and symptoms of rot, in contrast to those that received the combined alternative postharvest treatment that was well preserved with better appearance. During room temperature storage, there was a rapid onset of the symptoms of the green mold on oranges of the control which was perceived by the evaluators.

According to Wisniewski et al. (2016), for safe postharvest approaches, as physical and biological control methods, to be effective alternatives to synthetic fungicides, it is necessary to move from simplicity to complexity. The fruit postharvest disease must be seen as a process where multiple interventions, based on cost-effective, and reliable controls, may be a requirement in postharvest diseases control.

5. Conclusions

The systemic approach integrating the hot water brushing at 55 °C for 30s, 1.5 kJm^{-2} UVC irradiation, and the yeast *C. membranifaciens* CMAA-1112, is effective in reducing the green mold decay caused by *P. digitatum* on orange and delay the maturation process, preserving the fruit quality. The combination of physical treatments and biological control could be a reliable, safe and sustainable mean to control green mold on orange. Future research will be done for this technology to be used in commercial applications.

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References

Ballester, A.R., Izquierdo, A., Lafuente, M.T., González-Candelas, L., 2010. Biochemical and molecular characterization of induced resistance against *Penicillium digitatum* in citrus fruit. Postharvest Biol. Tecnol. 56, 31–38.

Baracat-Pereira, M.C., Oliveira, M.G.A., Barros, E.G., Moreira, M.A., Santoro, M.M., 2001. Biochemical properties of soybean leaf lypoxigenases: presence of soluble and membrane-bound forms. Plant Physiol. Biochem. 39, 91–98.

CIE, 1996. Proceedings of the CIE Expert Symposium'96 Colour Standards for Image Technology. Central Bureau of CIE, Vienna, Austria CIE Publ N°. x010–1996.

Campbell, C.L., Madden, L.V., 1990. Introduction to plant disease epidemiology. Wiley, New York.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: is it time for new paradigm? Postharvest Biol. Technol. 52, 137–145.

Duangmal, K., Apenten, R.K.O., 1999. A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum* var. Romano). Food Chem. 64, 351–359.

Erasmus, A., Lennox, C.L., Korsten, L., Lesar, K., Fourie, P.H., 2015. Imazalil resistance in *Penicillium digitatum* and *P. italicum* causing citrus postharvest green and blue mould: impacts and options. Postharvest Biol. Technol. 107, 66–76.

Halfeld-Vieira, B.A., Silva, W.L.M., Schurt, D.A., Ishida, A.K.N., Souza, G.R., Nechet, K.L., 2015. Understanding the mechanism of biological control of passionfruit bacterial blight promoted by autochthonous phylloplane bacteria. Biol. Control 80, 40–49.

Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest disease of fruits. Annu. Rev. Phytopathol. 40, 411–441.

Kinay, P., Mansour, M.F., Gabler, F.M., Margosan, D.A., Smilanick, J.L., 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Prot. 26, 647–656.

Liu, P., Chen, K., Li, G., Yang, X., Long, C., 2016. Comparative transcriptional profiling of orange fruit in response to the biocontrol yeast *Kloeckera apiculata* and its active compounds. BMC Genomics 17, 17–30.

Ministério do Desenvolvimento, 2016. Indústria e Comércio Exterior (MDIC). Secretaria de Comércio Exterior (SECEX). http://www.mdic.gov.br. (Accessed 16 April 2016).

Mayer, A.M., 1987. Polyphenol oxidase in plants: recent progress. Phytochemistry 26, 11–20.

Neves, M.F., 2016. (Coord). [S.l.]: Markestra. O retrato da citricultura brasileira. http://citrusbr.com/download/Retrato_Citricultura_Brasileira_MarcosFava.pdf. (Accessed 16 April 2016).

Pascholati, S.F., Nicholson, R.L., Butler, L.G., 1986. Phenylalanine ammonia-lyase activity and anthocyanin accumulation in wounded maize mesocotyls. J. Phytopathol. 115, 165–172.

Renwick, A., Campbell, R., Coe, S., 1991. Assessment of *in vitro* screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. Plant Pathol. 40, 524–532.

Romanazzi, G., Sanzanib, S.M., Bic, Y., Tiand, S., Martíneze, P.G., Alkanf, N., 2016a. Induced resistance to control postharvest decay of fruit and vegetables. Postharvest Biol. Technol. 122, 82–94.

Romanazzi, G., Smilanick, J.L., Feliziani, E., Droby, S., 2016b. Integrated management of postharvest gray mold on fruit crops. Postharvest Biol. Technol. 113, 69–76.

Santos, A., Marquina, D., 2004. Killer toxin of *Pichia membranifaciens* and its possible use as a biocontrol agent against grey mould disease of grapevine. Microbiology 150, 2527–2534.

Schirra, M., Ďhallewin, G., Ben-Yehoshua, S., Fallik, E., 2000. Host-pathogen interactions modulated by heat treatment. Postharvest Biol. Technol. 21, 71–85.

Shoresh, M., Harman, G.E., Mastouri, F., 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. Annu. Rev. Phytopathol. 48, 21–43.

Terao, D., Campos, J.S.C., Benato, E.A., Hashimoto, J.M., 2015. Alternative strategy on control of postharvest disease of mango (*Mangifera indica* L.) by use of low dose of Ultraviolet irradiation. Food Eng. Rev. 7, 171–175.

Usall, J., Ippolito, A., Sisquella, M., Neri, F., 2016. Physical treatments to control postharvest pathogens. Postharvest Biol. Technol. 122, 30–40.

Wisniewski, M., Wilson, C., Droby, S., Chalutz, E., El Ghaouth, A., Stevens, C., 2007. Postharvest biocontrol: new concepts and applications. In: Vicent, C., Goettel, M.S., Lazarovits, G. (Eds.), Biocontrol: a Global Perspective. Cabi, Wallingford, Oxfordshire, pp. 262–273.

Wisniewski, M., Droby, S., Norelli, J., Liu, J., Schena, L., 2016. Alternative management technologies for postharvest disease control: the journey from simplicity to complexity. Postharvest Biol. Technol. 122, 3–10.

Woods, D.R., Bevan, E.A., 1968. Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. J. Gen. Microbiol. 51, 115–126.

Zhou, Y., Ming, J., Deng, L., Zeng, K., 2014a. Effect of *Pichia membranaefaciens* in combination with salicylic acid on postharvest blue and green mold decay in citrus fruits. Biol. Control 74, 21–29.

Zhou, Y., Deng, L., Zeng, K., 2014b. Enhancement of biocontrol efficacy of *Pichia membranaefaciens* by hot water treatment in postharvest diseases of citrus fruit. Crop Prot. 63, 89–96.