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Combination of hot water, *Bacillus subtilis* CPA-8 and sodium bicarbonate treatments to control postharvest brown rot on peaches and nectarines

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Abstract The aim of this study was to evaluate the effect of hot water (HW), antagonists and sodium bicarbonate (SBC) treatments applied separately or in combination to control *Monilinia* spp. during the postharvest storage of stone fruit. Firstly, we investigated the effect of HW temperatures (55–70°C) and exposure times (20–60 s), seven antagonists at two concentrations (10^7 or 10^8 cfu ml^{-1}) and four SBC concentrations (1–4%). The selected treatments for brown rot control without affecting fruit quality were HW at 60°C for 40 s, SBC at 2% for 40 s and the antagonist CPA-8 (*Bacillus subtilis* species complex) at 10^7 cfu ml^{-1}. The combinations of these treatments were evaluated in three varieties of peaches and nectarines artificially inoculated with *M. laxa*. When fruit were incubated for 5 d at 20°C, a significant additional effect to control *M. laxa* was detected with the combination of HW followed by antagonist CPA-8. Only 8% of the fruit treated with this combination were infected, compared to 84%, 52% or 24% among the control, CPA-8, and HW treatments, respectively. However, the other combinations tested did not show a significant improvement in effectiveness to control brown rot in comparison with applying the treatments separately. When fruit were incubated for 21 d at 0°C plus 5 d at 20°C, the significant differences between separated or combined treatments were reduced and generally the incidence of brown rot was higher than when fruit were incubated for 5 d at 20°C. Similar results were observed testing fruit with natural inoculum.

Keywords *Bacillus subtilis* · Food additives · Heat treatments · Integrated disease management · *Monilinia* spp. · Stone fruit

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Introduction

Brown rot, caused by three species of the genus *Monilinia* namely, *M. laxa*, (Aderh et Rulh) Honey, *M. fructigena* Honey in Whetzel and *M. fructicola* (Wint.) Honey, is a serious disease in the European Mediterranean stone fruit production regions, including Spain (De Cal et al. 2009). The favourable conditions for *Monilinia* spp. infection that occur under field conditions favour the potential disease development after harvest where losses are typically

more severe reaching in some cases values of 80–90% (Hong et al. 1997). However, no chemical product is authorized to be applied after harvest of stone fruit in the E.U. and the spray programs in the field are the only available treatment to manage this disease. In addition, the consumer demands of low residues in fruit and environmental friendly treatments have created an urgent need to develop new and effective control methods.

In the search for alternative treatments to chemical products, other strategies have been considered. The use of physical treatments, as hot water (HW), is a simple technique that can be easily used in packing houses to reduce postharvest diseases of stone fruit. Previous studies have shown that it to be sufficiently effective in controlling postharvest diseases of peaches and nectarines (Margosan et al. 1997; Karabulut et al. 2002; Karabulut and Baykal 2004; Mari et al. 2007).

Biological control using microbial antagonists is also a potential alternative to control brown rot on postharvest of peaches and nectarines. Several studies have demonstrated the efficacy of different antagonists to control *Monilinia* spp. in stone fruit postharvest (Karabulut and Baykal 2003; Mari et al. 2007; Zhou et al. 2008); however, currently none of them are commercially available.

Sodium bicarbonate (SBC, $NaHCO_3$) has also been reported to play an important role in the inhibition of postharvest diseases in several fruits (Smilanick et al. 1999; Karabulut et al. 2001; Teixidó et al. 2001; Gamagae et al. 2003; Usall et al. 2008). It is widely used in the food industry as a food additive, which is allowed with no restrictions for many applications under European and North American regulations. Moreover, SBC is a very attractive alternative because it is readily available, inexpensive, and has little risk of phytotoxicity at the low concentrations (1–4%) used (Palou et al. 2001).

Generally, the efficacy of these alternative treatments has some limitations because their effectiveness is more influenced by environmental factors (Janisiewicz and Korsten 2002), sometimes the mode of action is fungistatic instead of fungicidal (Smilanick et al. 1999) or they do not provide persistent protection of fruit after treatment. For these reasons, recent research has focused on the enhancement of the efficacy of the postharvest alternative treatments by the combination of two or more postharvest treatments to control brown rot on stone fruit. For instance, hot water treatments were used in combination with SBC (2.5%) to control brown rot caused by *Monilinia* spp. on stone fruit (Mari et al. 2007). This combination resulted in better control of decay caused by *Monilinia* spp. than either hot water or SBC alone. Karabulut and Baykal (2004) showed similar results when they evaluated the efficacy of hot water, the use of a yeast antagonist and modified atmosphere packaging as single treatments or in various combinations to control the rots caused by *Botrytis cinerea* Pers.:Fr., and *Penicillium expansum* Link on peaches.

The research goal of our work was to combine several treatments to develop a strategy that could be applied after harvest to control brown rot on peaches and nectarines. Firstly, we determined the optimum HW temperature and exposure time, the best antagonist and its concentration, and the most effective SBC concentration to control *M. laxa* on artificially inoculated peaches and nectarines. Then, we evaluated the combination of the previously determined optimum treatments to control brown rot on fruit artificially inoculated and with natural inoculum.

Materials and methods

Fruit

Fruit used in this study were peaches ('Summer Rich', 'Rich Lady', 'Tardibelle', 'Elegant Lady' and 'Placido') and nectarines ('Big Top' and 'Venus'). Fruit were growth in Ribera d'Ebre, Segrià and Noguera areas of Catalonia following standard cultural practices and chemical spray programmes in the field for pest and disease control. Fruit without postharvest chemical treatments, without visible injuries, of similar size and visual maturity, were selected by hand from field bins immediately after harvest. Fruit not used immediately after harvest was stored at 0°C until required for experimentation.

Pathogen isolates

The isolate of *M. laxa* (CPML1) used in this study was from the collection of the Pathology Unit, Centre UdL-IRTA, Lleida, Catalonia and classified by the Department of Plant Protection, INIA, Madrid, Spain. *M. laxa* was isolated from brown rot infected fruit of

commercial orchards and were maintained in Petri dishes with potato dextrose agar (PDA, Biokar Diagnostics, 39 g l^{-1}) amended with acetone (J.T. Baker, 1%) and stored at 4°C in the dark until required.

Inoculum production and fruit inoculation

The pathogen used in this work was *M. laxa* since it is the main species affecting stone fruit in EU and other important stone fruit producing areas. The isolate of *M. laxa* was subcultured onto PDA Petri dishes amended with acetone and incubated in the darkness at 25°C for approximately 2 weeks. To ensure sufficient conidial production for experimentation, *M. laxa* was inoculated on superficially disinfected peaches or nectarines by wounding fruit with a sterilized steel rod (1 mm wide and 2 mm long) and transferring conidia and mycelium from the PDA culture to the wound site with a sterile pipette tip. Fruit were then incubated at 25°C and 85% RH in the dark for 7–10 d. Conidia were loop washed from the infected fruit surface and suspended in 5 ml of sterile distilled water containing one droplet of wetting agent per litre (Tween-80). Conidia concentration was measured by haemocytometer and diluted to 10^3 conidia ml^{-1}.

Fruit that were treated in tests to control brown rot were placed in single layer stone fruit boxes and wounded once per fruit using a sterilized steel rod (1 mm wide and 2 mm long) and inoculated with a 15 µl aliquot of the conidial suspension. The treatments were applied once the inoculum in the wound site had dried.

Effect of temperature and exposure time on hot water efficacy and fruit quality

In vitro studies

The culturability of *M. laxa* conidia was investigated in *in vitro* conditions. Sterile screw-capped glass tubes containing 1.8 ml of sterile distilled water were placed in water baths at 20°C, 55°C, 60°C and 70°C, and allowed to equilibrate for 30 min. Then 0.2 ml of concentrated *M. laxa* conidia suspension was added to the tubes, to achieve a final concentration of 10^5 conidia ml^{-1}. After 20 s, 40 s and 60 s of exposure time, tubes were removed from water baths and immediately placed on ice. There were three replicates (test tubes) for each treatment. Aliquots of 100 µl of each suspension were plated onto two PDA Petri dishes. After 72 h of incubation at 25°C, the colonies were counted and the results were expressed as percentage of culturable conidia.

In vivo studies

Peaches ('Rich Lady' and 'Elegant Lady') and nectarines ('Big Top' and 'Venus') were used to investigate the effect of different hot water temperatures for several exposure times to control brown rot caused by *M. laxa*. Stainless steel tank holding 15 l of tap water was heated at 20°C, 55°C, 60°C, 65°C and 70°C in a 172 l stainless steel water tank fitted with a 9 kW electric resistance heater and thermostat. Metallic grid baskets containing inoculated fruit with *M. laxa* as described above were submerged in the tank for 20 s, 40 s and 60 s. All treatments were carried out with four replicates and five fruit per replicate. Immediately after the treatment fruit were incubated for 7 d at 20°C and 85% RH. The number of brown rot infected fruit was recorded and expressed as percentage of brown rot (incidence).

The effect of hot water treatments on fruit quality was also investigated using peaches ('Rich Lady' and 'Elegant Lady') and nectarines ('Big Top' and 'Venus'). Fruit were dipped in the water bath at temperatures and exposure times described above and then incubated for 7 d at 20°C and 85% RH or for 21 d at 0°C and 85% RH plus 7 d at 20°C and 85% RH. All fruit were evaluated for visual fruit skin damage and fruit quality parameters including: colour development, fruit firmness, weight loss, soluble solids and acidity.

Surface colour measurements (*L*, *a* and *b*) were made at two equidistant points on the equator of each fruit with a Minolta chromameter (model CR-200, Osaka, Japan) using CIE illuminant C lighting conditions and an 8 mm-diameter measuring area.

Firmness was measured on two opposite peeled sides using a penetrometer (Effegi, Milan, Italy) fitted with an 8 mm diameter flat tip. Colour and firmness measurements were carried out on four replicates and five fruit per replicate.

The weight was measured by balance (SAC-62, SCALTEC) (±0.1 g) before the heat treatment (A) and after storing as described above (B), and the weight

loss was calculated as $100 \times [(A - B)/A]$. The analyses described above were applied to four replicates of five fruit each.

Soluble solids content was determined with a digital refractometer (Atago PR-100, Tokyo, Japan), by measuring the refractive index of juice and data was expressed as ºBrix. Acidity was measured by mixing 10 ml of juice with 10 ml distilled H_20 and three drops of phenolphthalein which was titrated with 0.1N NaOH. Acidity was expressed in grams of malic acid per l of juice (g m.a. l^{-1}). The analyses described above were applied to juice extracted from five fruits per replicate and four replicates.

Effect of hot water and storage temperature after treatment to control brown rot in fruit with natural inoculum

Metallic grid baskets containing 'Placido' peaches with natural inoculum were submerged in a 15 l tap water stainless steel tank heated at 20°C (control) or 60°C for 40 s, as descried previously. Once fruit were dried, each fruit were packaged with a transparent plastic bag to avoid contaminations between fruits during the storage period. After the hot water treatment, fruit were stored 21 d at 0°C and 85% RH plus 26 d at 20°C and 85% RH or incubated directly to 26 d at 20°C and 85% RH. All treatments were carried out with four replicates and 20 fruit per replicate. The number of brown rot infected fruit was recorded periodically during the incubation time at 20°C and data was expressed as percentage of reduction of brown rot.

Isolation of microorganisms and selection for antagonistic activity to control *Monilinia laxa*

Potential antagonists were isolated from peach and nectarine surfaces. The isolation of microorganisms and screening for antagonistic activity on peaches and nectarines to control brown rot caused by *M. laxa* were carried out as described by Viñas et al. (1998). A primary screening was carried out to select the most effective antagonists that were able to reduce disease development caused by *M. laxa*. Peaches were wounded twice and inoculated with *M. laxa*, as described previously. Once the inoculum side was dried, a 15 µl aliquot of each of the 200 isolated antagonists were applied. All treatments were carried out with three replicates and three fruit per replicated. Then, fruit were incubated 7 d at 20°C and 85% RH.

A secondary screening was conducted using the best isolates in order to select the best antagonist and its concentration to control *M. laxa* in peaches ('Rich Lady') and nectarines ('Big Top' and 'Venus'). Isolates were grown in 75 ml of nutritive broth (Biokar Diagnostics, 20 g l^{-1}) in 250 ml flasks at 30°C on a rotatory shaker at 150 rpm for 24 h. Cells were harvested by centrifugation at 9,820 g for 10 min, resuspended in buffer, and the concentration was adjusted to 10^7 (low dose) and 10^8 (high dose) cfu ml^{-1} measured by haemocytometer.

Fruit were inoculated with *M. laxa* as described above and once the inoculation site was dried, each antagonist was applied in the same wound site with 15 µl of antagonist cells at 10^7 or 10^8 cfu ml^{-1} or with buffer (untreated). All treatments were carried out with four replicates and ten fruit per replicate. Fruit were incubated at 20°C for 7 d and 85% RH and then the number of brown rot infected fruit was recorded and expressed as percentage of brown rot.

Effect of sodium bicarbonate to control *Monilinia laxa*

Metallic grid baskets containing inoculated peaches ('Summer Rich') and nectarines ('Big Top') with *M. laxa* as described above were submerged in a stainless steel tank holding 15 l of tap water at 20°C for 40 s with 0% (untreated), 1%, 2%, 3% or 4% (wt/vol) concentration of SBC. In order to test the effect of rinsing fruit, the experiment was conducted in duplicate with one set of fruit rinsed for 5 s with tap water and the other set not rinsed. All treatments were carried out with four replicates and ten fruit per replicate. Then, fruit were incubated for 5 d at 20°C and 85% RH and then the number of brown rot infected fruit was recorded and expressed as percentage of brown rot.

Combination of hot water, antagonist and sodium bicarbonate to control *Monilinia laxa*

Based on the results obtained in the above experiments, the selected treatments for combination were cells of CPA-8 antagonist at 10^7 cfu ml^{-1}, HW treatment at 60°C for 40 s and SBC bath at 2% for 40 s rinsing fruit after the treatment.

Peaches ('Elegant Lady' and 'Tardibelle') and nectarines ('Venus') were artificially inoculated with *M. laxa*, as described above, and the selected treatments described above were applied separately or combined. Single treatments were carried out as described above. The combination of HW plus SBC was conducted by dipping fruit in a 15 l HW bath at 60°C for 40 s containing SBC at 2%. For the combination of HW followed by antagonist, fruit were treated with HW at 60°C for 40 s and once fruit were dried, they were inoculated with CPA-8 antagonist at 10^7 cfu ml^{-1} in the wound site. The triple combination was conducted by, first, treating fruit as a double combination of HW plus SBC, as explained above, and once fruit were dried they were inoculated with CPA-8 antagonist at 10^7 cfu ml^{-1} in the wound site. All treatments were carried out with four replicates and ten fruit per replicate.

The same experiments were also applied in peaches ('Placido') with natural inoculum and were conducted as described above, except for the CPA-8 antagonist; in this case fruit were dipped in a 5 l bath containing 10^7 cfu ml^{-1} of CPA-8 antagonist for 1 min. All treatments were carried out with five replicates and twenty fruit per replicate.

For all experiments a set of fruit was dipped in a 15 l water bath at 20°C for 40 s as a control treatment. Fruit were incubated for 5 d at 20°C and 85% RH or 21 d at 0°C plus 5 d at 20°C and 85% RH. The number of brown rot infected fruit was recorded in both artificially inoculated and with natural inoculum fruit and data was expressed as percentage of brown rot.

Statistical analysis

The incidence of brown rot, reduction of brown rot incidence expressed as a percentage, percentage of culturable conidia, and quality parameters were analyzed by general linear model (GLM) analysis with SAS software (SAS Institute, version 8.01, Cary, NC, USA). Before analysis of data expressed as percentages, homogeneity of variance was tested by Barlett's test and when required data were transformed to the arcsine of the square root. Significance was judged at the level $P<0.05$. When the analysis was statistically significant, the least significant difference (LSD) test for separation of means was applied.

Results

Effect of temperature and exposure time on hot water efficacy and fruit quality

In vitro studies

The percentage of culturable conidia of *M. laxa* was significantly ($P<0.05$) reduced to 2.2% when conidial suspension was incubated at 55°C for 20 s (Table 1). A complete inhibition of *M. laxa* conidia was achieved increasing exposure time to 40 s and 60 s or temperature to 60°C and 70°C.

In vivo studies

The incidence of brown rot caused by *M. laxa* in 'Rich Lady' peaches was not statistically ($P<0.05$) affected by the exposure time (in the range 20–60 s) at any of the water bath temperatures investigated (20–70°C). However, the incidence of brown rot decreased significantly ($P<0.05$) when the bath temperature increased from 20°C to 70°C (Fig. 1). Brown rot incidence was 88%, 73%, 51%, 44% and 12% after water bath treatments of 20°C, 55°C, 60°C, 65°C and 70°C, respectively. Visual quality appearance of fruit after dipping at 70°C was drastically

Table 1 Percentage of culturable conidia of *Monilinia laxa* treated in a water bath at 20°C, 55°C, 60°C or 70°C for 20 s, 40 s or 60 s. Then conidia were plated on Petri dishes and incubated for 72 h at 25°C. The values are means of three replicates. Means followed by different letters are significantly ($P<0.05$) different according to LSD test

Temperature (°C)	20			55			60			70		
Exposure time (s)	20	40	60	20	40	60	20	40	60	20	40	60
Culturable conidia	1.1×10^4 ufc ml^{-1}	1.8×10^4 ufc ml^{-1}	5.7×10^4 ufc ml^{-1}	2.2%a	0%b	0%b	0%b	0%b	0%b	0%b	0%b	0%b

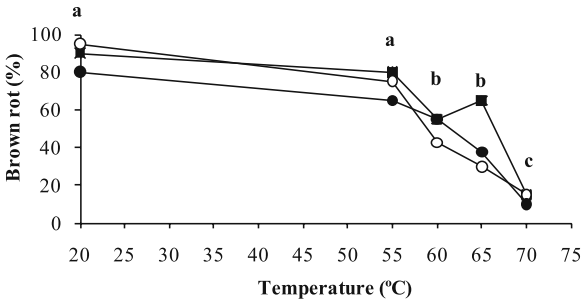

Fig. 1 Incidence of brown rot on 'Rich Lady' peaches artificially inoculated with *Monilinia laxa* at 10^3 conidia ml^{-1} and treated with hot water at 20°C, 55°C, 60°C, 65°C or 70°C for 20 (■), 40 (○) and 60 (●) s. Then fruit were incubated for 7 d at 20°C. The values are means of four replicates of five fruit. Means of the three exposure times for each temperature followed by different letters are significantly ($P<0.05$) different according to LSD test

affected at all exposure times. Decreasing the temperature to 65°C, the fruit skin was less affected and was dependent on the variety of stone fruit and exposure time. However, visual quality appearance of fruit and also the standard quality parameters including: firmness, acidity, total soluble solids, colour and percentage of lost weight were not affected when fruit were dipped in water baths at temperatures in the range 20–60°C. These results showed similar profiles in fruit incubated after the HW treatment for 7 d at 20°C or 21 d at 0°C plus 7 d at 20°C (data not shown).

The efficacy and quality results obtained at 60°C for 40 s were similar among 'Big Top', 'Elegant Lady' and 'Venus' varieties (data not shown). Based on the results described above, the HW treatment selected for controlling *M. laxa* without affecting fruit quality was 60°C for 40 s.

Effect of hot water and storage temperature after treatment to control brown rot in fruit with natural inoculum

Brown rot reduction after incubating 'Placido' peaches with natural inoculum for 5 d at 20°C was higher than 79% when the HW treatment was conducted at 60°C for 40 s being not significantly ($P>0.05$) different between fruit that were incubated previously at 0°C for 21 d, where the incidence of brown rot in control treatments stored firstly at 0°C or directly at 20°C, were 67% and 49%, respectively (Fig. 2). When the incubation time at 20°C was increased from 7 d to 26 d, reduction of brown rot incidence by the HW treatment was reduced from 77% to 21% or 50% to 1% in fruit incubated directly at 20°C or previously at 0°C, respectively. Over all this period, brown rot reduction was higher in fruit that were incubated immediately at 20°C than in fruit incubated previously at 0°C.

Isolation of microorganisms and selection for antagonistic activity to control *Monilinia laxa*

More than 200 microorganisms were isolated and tested in a primary screening to control *M. laxa* on peaches and nectarines (data not shown). The isolates with the best efficacy in reducing the number of infected fruit by *M. laxa* that were selected for the

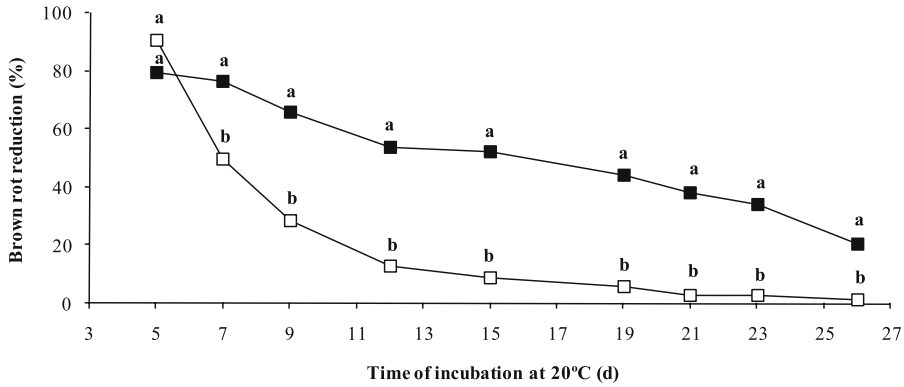

Fig. 2 Brown rot reduction on 'Placido' peaches with natural inoculum treated with hot water at 60°C for 40 s or untreated. Fruit were stored previously at 0°C for 21 d (□) or directly at 20°C (■), and then all fruit were incubated at 20°C. The values are means of four replicates of twenty fruit. Means for each time of incubation at 20°C followed by different letters are significantly ($P<0.05$) different according to LSD test

secondary screening were CPA-8, BFO-30, BFO-118, BFO-188, BFO-32b, BFO-30b and BFO-62.

In the secondary screening, the results indicated that the seven selected antagonists reduced significantly ($P<0.05$) the incidence of brown rot caused by *M. laxa* in artificially inoculated fruit compared to the untreated ones, for the studied varieties ('Big Top', 'Rich Lady' and 'Venus') (Table 2).

A complete control of *M. laxa* was observed in 'Big Top' nectarines with all the antagonists except for BFO-30 and BFO-30b, 2.5% and 10% of brown rot, respectively, in comparison with 35% in the untreated. In 'Rich Lady' peaches, disease development by *M. laxa* was completely controlled with the antagonists BFO-118, BFO-32b and BFO-30b at 10^8 cfu ml^{-1} and BFO-188 at 10^7 cfu ml^{-1}. For the remainder of the antagonists evaluated, the incidence of brown rot was always lower than 27.5% at 10^7 cfu ml^{-1} and 12.5% at 10^8 cfu ml^{-1} in comparison with the untreated (53.5% and 52.5% for 10^7 cfu ml^{-1} and 10^8 cfu ml^{-1}, respectively). In 'Venus' nectarines, complete control of brown rot was achieved testing the antagonists CPA-8, BFO-32b and BFO-30b at 10^7 cfu ml^{-1} and CPA-8, BFO-62, BFO-32b and BFO-30b at 10^8 cfu ml^{-1}. The incidence of brown rot for the rest of the treatments was lower than 12.5% at 10^7 cfu ml^{-1} and 2.5 at 10^8 cfu ml^{-1}, in comparison with 22.5% in the untreated.

The incidence of brown rot in general was not influenced by the concentration of antagonist applied (10^7 or 10^8 cfu ml^{-1}) except for BFO-30 and BFO-32b applied to 'Rich Lady' peaches and for BFO-30b applied to 'Big Top' nectarines, where brown rot incidence was significantly ($P<0.05$) lower at 10^8 than at 10^7 cfu ml^{-1}.

The isolate CPA-8 was selected to be combined with other alternative strategies and was identified by 16SrDNA partial analysis by the Netherlands Culture Collection of bacteria as a member of the *Bacillus subtilis* species complex.

Effect of sodium bicarbonate to control *Monilinia laxa*

The incidence of brown rot was not significantly ($P<0.05$) reduced by any of SBC concentrations tested in 'Big Top' nectarines artificially inoculated with *M. laxa* (Table 3). The same results were observed in 'Summer Rich' peaches, except when fruit were treated with 1% of SBC and then rinsed, where the incidence of brown rot was significantly ($P<0.05$) lower (73%) in comparison with the untreated (90%). The incidence of brown rot was not dependent on whether fruit were rinsed with water or not immediately after the treatment. SBC concentrations at 3% and 4% with or without rinsing produced phytotoxicity on fruit skin.

Combination of hot water, antagonist and sodium bicarbonate to control *Monilinia laxa*

According to the results obtained from the previous experiments, the best treatments controlling brown rot

Table 2 Incidence of brown rot on 'Rich Lady' peaches and 'Venus' and 'Big Top' nectarines artificially inoculated with *Monilinia laxa* at 10^3 conidia ml^{-1} and treated with the antagonists CPA-8, BFO-30, BFO-118, BFO-188, BFO-32b, BFO-30b, BFO-62 applied at 10^7 or 10^8 ucf ml^{-1} or untreated. Then fruit were incubated for 7 d at 20°C. The values are means of four replicates of ten fruit. Means for each variety followed by different letters are significantly ($P<0.05$) different according to LSD test

Treatment	'Big top'		'Rich lady'		'Venus'	
	10^7 cfu ml^{-1}	10^8 cfu ml^{-1}	10^7 cfu ml^{-1}	10^8 cfu ml^{-1}	10^7 cfu ml^{-1}	10^8 cfu ml^{-1}
Untreated	35.0a	35.0a	53.5a	52.5a	22.5a	22.5a
CPA-8	0c	0c	10.0cd	7.5cd	5.0bc	0c
BFO-30	0c	2.5c	27.5b	5.0cd	2.5bc	2.5bc
BFO-118	0c	0c	10.0cd	0d	12.5ab	2.5bc
BFO-188	0c	0c	0d	2.5d	5.0bc	2.5bc
BFO-32b	0c	0c	20.0bc	0d	0c	0c
BFO-30b	10b	0c	15.0bcd	0d	0c	0c
BFO-62	0c	0c	27.5b	12.5bcd	2.5bc	0c

Table 3 Incidence of brown rot on 'Summer Rich' peaches and 'Big Top' nectarines artificially inoculated *Monilinia laxa* at 10^3 conidia ml^{-1} and treated with sodium bicarbonate at 1%, 2%, 3%, 4% or untreated, for 40 s. Then fruit were incubated for 5 d at 20°C. The values are means of four replicates of ten fruit. Means for each variety followed by different letters are significantly ($P<0.05$) different according to LSD test

	Concentration of sodium bicarbonate								
	0%	1%		2%		3%		4%	
	Untreated	Rinsed	Not rinsed	Rinsed	Not rinsed	Rinsed	Not rinsed	Rinsed	Not rinsed
'Big Top'	64.4ab	73.6ab	69.2ab	74.4ab	62.8b	61.3b	72.2ab	88.4a	70.0ab
'Summer Rich'	89.7a	72.5b	85.0ab	81.4ab	97.5a	92.5a	97.5a	90.0a	92.2a

caused by *M. laxa* without affecting fruit quality that were selected to conduct the following treatments were: HW treatment at 60°C for 40 s and CPA-8 antagonist at 10^7 cfu ml^{-1}. Although, in general, SBC was not effective against *M. laxa* under the conditions evaluated, the treatment at 2% of SBC for 40 s followed by rinsing fruit, which was the maximum concentration of SBC without affecting fruit quality, was selected to test its combination with the other selected treatments, to study possible synergistic effects.

Brown rot incidence was significantly ($P<0.05$) reduced by the HW treatment in 'Tardibelle', 'Elegant Lady' and 'Venus' fruit incubated for 5 d at 20°C after the treatment, to 30%, 32% and 10%, compared with the control where the incidence was 100%, 100% and 54%, respectively (Fig. 3a). The treatment applied with the antagonist CPA-8 alone also decreased significantly ($P<0.05$) the incidence of brown rot to similar levels achieved by the HW treatment, except for 'Tardibelle' peaches where no brown rot control was observed in comparison with the control.

The combined treatment with HW and antagonist enhanced significantly ($P<0.05$) the efficacy of those treatments applied separately, reducing the incidence of brown rot to 7.5% and 10% in 'Tardibelle' and 'Elegant Lady' peaches, respectively. The use of SBC did not improve any synergistic effect of the studied combined treatments.

The incidence of brown rot on artificially infected fruit with *M. laxa* and incubated immediately after the treatments for 21 d at 0°C plus 5 d at 20°C is shown in Fig. 3b. Treatments with the antagonist strain CPA-8 (alone or in combination) showed that brown rot incidence was higher in fruit incubated for 21 d at 0°C plus 5 d at 20°C in comparison with fruit that were incubated only for 5 d at 20°C. The efficacy of CPA-8 antagonist applied alone was decreased when fruit were stored at 0°C after the treatment, where brown rot incidence was only reduced significantly ($P<0.05$) in 'Tardibelle' peaches to 73% in comparison with the control 95%. In general, the combinations reduced the incidence of brown rot in comparison with the control but did not improve the efficacy of the single treatments.

On 'Placido' peaches with natural inoculum, the strain CPA-8 and HW applied separately, and all the combinations that contained the HW treatment, reduced the incidence of brown rot to lower than 28% in comparison with the control (59% of brown rot) when fruit were incubated for 5 d at 20°C (Fig. 4). However, for SBC as a single treatment and combined with the CPA-8 antagonist, no statistically significant ($P<0.05$) differences were detected in comparison with the control. The efficacy level of the single treatments was not improved by any of the combinations evaluated.

When fruit were incubated for 21 d at 0°C plus 5 d at 20°C, HW treatment applied alone and its combination with SBC were the only treatments that reduced significantly ($P<0.05$) the incidence of brown rot to 70% and 63%, respectively in comparison with 94% in the control. However, the combined treatment did not improve the efficacy of both treatments applied separately.

Discussion

Alternative treatments to postharvest chemical products for brown rot control on stone fruit have been studied for many years, including hot water (Smith et al.

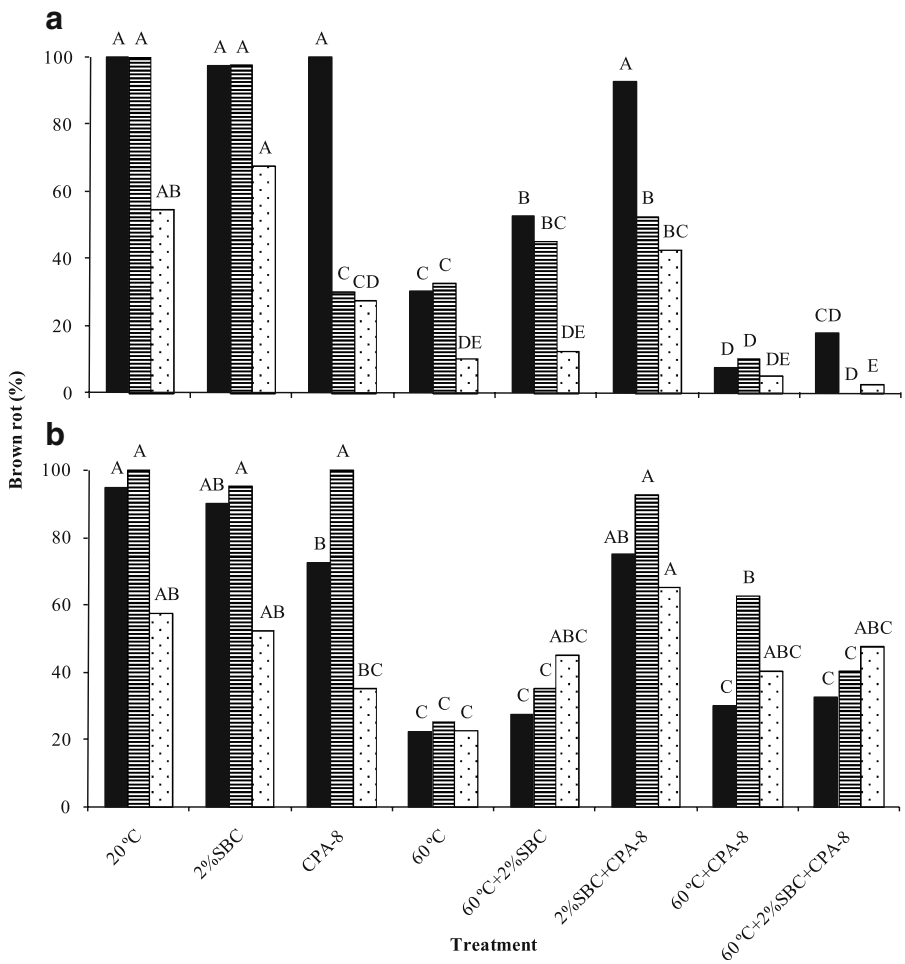

Fig. 3 Incidence of brown on 'Tardibelle' (■) and Elegant Lady (▤) peaches or Venus (□) nectarines artificially inoculated with *Monilinia laxa* at 10^3 conidia ml^{-1} and treated with hot water at 60°C for 40 s, sodium bicarbonate at 2% for 40 s or antagonist CPA-8 at 10^7 cfu ml^{-1} applied alone or in combination. Then fruit were stored for 5 d at 20°C (**a**) or 21 d at 0°C for plus 5 d at 20°C (**b**). The values are means of four replicates of ten fruit. Means for each variety followed by different letters are significantly ($P<0.05$) different according to LSD test

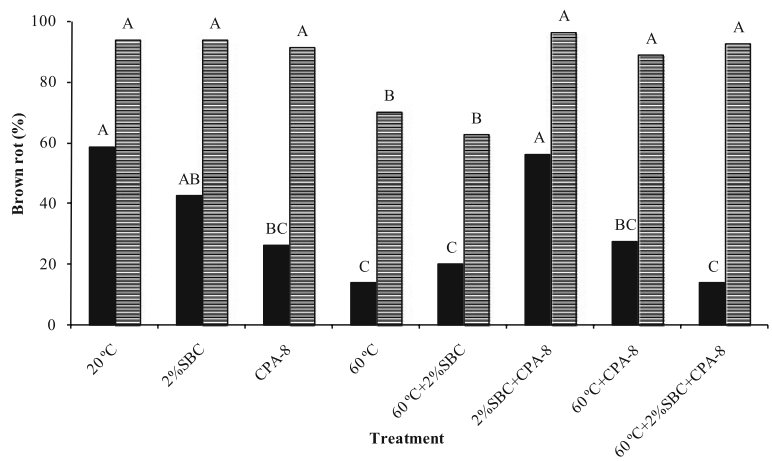

Fig. 4 Incidence of brown rot on 'Placido' peaches with natural inoculum and treated with hot water at 60°C for 40 s, sodium bicarbonate at 2% for 40 s or antagonist CPA-8 at 10^7 cfu ml^{-1} applied alone or in combination. Then fruit were stored for 5 d at 20°C (■) or 21 d at 0°C plus 5 d at 20°C (▤). The values are means of five replicates of twenty fruit. Means for each storage period by different letters are significantly ($P<0.05$) different according to LSD test

1964), biological control (Pusey and Wilson 1984) and sodium bicarbonate (Karabulut et al. 2001). Generally, these mentioned treatments have an inconsistent efficacy controlling postharvest diseases. However, recent studies have indicated that the integration of two or more alternative treatments might be a strategy to provide satisfactory control of brown rot disease on stone fruit (Droby et al. 2003; Karabulut and Baykal 2004; Mari et al. 2007). Therefore, our goal was to find a strategy to control brown rot on peaches and nectarines to provide consistent efficacy among cultivars. The present work is the most complete study evaluating all the possible combinations of *B. subtilis* CPA-8, HW and SBC treatments in a wide range of cultivars, artificially inoculated or with natural inoculum and incubated at 0°C or 20°C to control brown rot on peaches and nectarines. Also the HW efficacy was studied over a long shelf life period of fruit that could take place under the actual commercial handling of stone fruit.

Our findings indicated that under *in vitro* conditions, the percentage of culturable conidia of *M. laxa* was dramatically decreased by a HW bath at 55°C for only 20 s of exposure time. However, Karabulut et al. (2002) found that the culturability of *M. fructicola* conidia was completely reduced with lower temperature and shorter exposure time (50°C for 10 s), explained by differences between species tested. In the present study, HW treatments were also conducted under *in vivo* conditions and the results indicated that the incidence of brown rot by *M. laxa* was markedly reduced at the temperature range of 60–70°C. However, the most satisfactory HW treatment that reduces disease development without affecting fruit quality was 60°C, suggesting it as a potential postharvest treatment to control brown rot on nectarine and peach. Several previous studies agree with our finding, reporting similar HW treatment conditions to control brown rot on stone fruit. Smith et al. (1964) had shown that by dipping fruit in a HW bath at 55.5°C for 1.5 min, the reduction of decayed fruit by *M. fructicola* was 60%. Different results were reported by Karabulut et al. (2002) since they found greater efficacy with decreasing the temperature and exposure time to 55°C and 20 s, respectively; but treating fruit with a HW brushing achieved a reduction of decayed fruit by *M. fructicola* of 70%. This improvement in decay control in comparison with Smith et al. (1964) might be because the brush used at the same time that the HW treatment facilitated conidia removal from wounds and then HW acted directly on the conidia viability. Lower efficacy was reported by Margosan et al. (1997) who found that the decay reduction was only 47% when treating fruit at 50°C for 2.5 min. These studies suggest that HW treatment could be applied to control brown rot on peaches and nectarines by different strategies modifying the water bath temperature, exposure time or even using different techniques such as brushing. Moreover, our results indicated that, when peaches with natural inoculum, which could be placed inside fruit as pre-existing infections or as conidia on fruit surface, were incubated for 5 d at 20°C, the HW at 60°C for 40 s had also an important effect on brown rot reduction. However, when fruit were incubated for 21 d at 0°C prior to the shelf life period (for 5 d at 20°C), its efficacy was irregular depending on the experiment. A possible explanation is that at 0°C, *Monilinia* spp. infections in fruit are able to grow; moreover the germ tube lengths of latent infections may be dependent on the time that conidia started to germinate, affecting HW efficacy to reduce disease development. Karabulut et al. (2002) found that the same HW strategy could have an inconsistent effect depending on the pathogen and the time of the infection. Likewise, HW efficacy also will be dependent on several factors including variety and weather conditions during the growing season. In addition, our results showed that in fruit with natural inoculum, treated with HW and incubated further than our standardized commercial shelf life (5 d at 20°C) until 26 d at 20°C, brown rot reduction decreased progressively from higher than 79% to lower than 21%. This finding indicated that our HW treatment did not eliminate the natural inoculum, possibly, because HW would kill conidia *Monilinia* spp. placed on the fruit surface but not latent infections that probably are placed more deeply in fruit. When fruit were incubated at 0°C after HW treatment, HW was confirmed as a non-eradicative treatment; and in general brown rot reduction was lower for fruit incubated first for 21 d at 0°C plus 5 d at 20°C than for fruit incubated directly at 20°C for 5 d. A possibility is that culturable conidia resident on fruit after HW treatment were able to develop during the 21 d of storing at 0°C and for this reason the brown rot reduction was lower in comparison with fruit incubated directly at 20°C.

Our results showed that SBC had no potential to control *M. laxa* on peaches and nectarines in the concentration range that we tested (1–4%). Our findings agree with Palou et al. (2009) who found that SBC did not control brown rot caused by *M. fructicola*. However, previous experiments of Mari et al. (2007) indicated that SBC applied at 1% and 2.5% significantly reduced *M. laxa* decay on stone fruit compared with the control. Most studies in the use of SBC have focused on citrus fruit and reported SBC as an effective treatment to control postharvest diseases (Smilanick et al. 1999; Palou et al. 2001; Usall et al. 2008).

The variation in efficacy of the antagonist CPA-8 observed could be explained by the susceptibility of the varieties evaluated in relation to *M. laxa* infections. Hong et al. (1998) also found a variation in the control of decay among the peach cultivars evaluated. In our work, in general, the antagonist concentration applied (10^7 and 10^8 cfu ml^{-1}) had no effect on its efficacy and for this reason the lowest concentration was selected for the following experiments. From a commercial point of view, this concentration of the biocontrol agent that we found as effective (10^7 cfu ml^{-1}) is suitable for a biofungicide formulation. Previous studies related to biological control also showed that other antagonist isolates were able to inhibit the common postharvest stone fruit disease (Pusey and Wilson 1984, Karabulut and Baykal 2003; Zhou et al. 2008).

Under natural conditions *Monilinia* spp. infect fruit mainly in the field, before harvest. If the weather conditions has favoured infections, then for biological control agents lacking antibiosis it will be difficult to eradicate this kind of infection. In this work we complemented the biocontrol activity of the CPA-8 antagonist by applying it together with HW and SBC treatments. Generally, our results indicated that when fruit were incubated for 5 d at 20°C, the combined treatment with HW bath (at 60°C for 40 s) and the CPA-8 antagonist at 10^7 cfu ml^{-1} provided an additive effectiveness in the control of brown rot in comparison with applying the treatments separately. A possible explanation for these results is that the mode of action of both treatments may have complemented each other, whereby HW acts as a fungicidal treatment killing conidia and the CPA-8 antagonist protects fruit from conidia that have survived after HW treatment. In contrast, this additive effect was not observed in 'Venus' fruit. A possible explanation is that in this variety, the incidence of brown rot achieved in the control treatment was 46% and less than in the other varieties investigated, and then the HW and CPA-8 treatments applied separately reduced satisfactorily the brown rot incidence to 10% and 13%, respectively, probably too low a level to find an statistical improvement with the combined treatment.

It is noteworthy that while the incidence of brown rot observed by treating fruit with the CPA-8 antagonist varied significantly between varieties, its use in combination with HW always resulted in the same level of efficacy. Earlier studies by Karabulut et al. (2002) did not show an additive effect in controlling *M. fructicola* by combining HW brushing (at 60°C for 20 s) plus a *Candida* sp. antagonist. However, they found that this combination had an additive effect in comparison with the single treatments to control 24-h-old *P. expansum* infections on peaches and nectarines. Our results also showed that when fruit were incubated for 21 d at 0°C plus 5 d at 20°C, the additional effect to control *M. laxa* provided by the combined treatment with HW plus CPA-8 antagonist was not detected. The low efficacy of CPA-8 when fruit were incubated at 0°C could be explained because this antagonist grew poorly at 0°C. Although the mode of action of CPA-8 under our conditions is not known, at 0°C its physiological activity is delayed affecting all potential modes of action described for *B. subtilis*, including nutrient competition and production of antibiotics (e.g. iturin, surfactin, fengycin), enzymes that degrade fungal structural polymers (e.g. chitinase, β-1,3 glucanase), and antifungal volatiles (Fiddaman and Rossall 1993; Knox et al. 2000; Jiang et al. 2001; Pinchuk et al. 2002; Leelasuphakul et al. 2006). The result would be that CPA-8 would not inhibit *M. laxa* conidia that survive after the HW treatment which would be able to grow at 0°C. In contrast, *Cryptococcus informo-miniatus* and *C. laurentii* effectively controlled blue mold of apple at 5°C, but acceptable control was not achieved at 10°C or 20°C when the yeasts were used alone (Chand-Goyal and Spotts 1996). They overcome the lack of antagonist efficacy by combining them with a chemical fungicide. In our work, although SBC applied separately did not show any effect against *M. laxa*, we expected that it could complement the antagonist efficacy, especially when fruit were incubated at 0°C. However, the

combinations tested with SBC did not show an additive effect in controlling brown rot in peaches and nectarines. Nevertheless, Mari et al. (2007) observed a synergistic effect when they combined HW at 40°C for 150 s or at 60°C for 20 s and SBC (1–2.5%).

Combined treatments were also investigated in peaches with natural inoculum. In fruit incubated for 5 d at 20°C, the results obtained for HW treatment at 60°C for 40 s and CPA-8 were consistent with the experiments conducted with artificial infections, since in these there was also a satisfactory reduction in the incidence of brown rot. Moreover, the combinations that included the HW treatment also showed similar levels of efficacy. However, the synergistic effect provided in artificially inoculated fruit by applying the combined treatment with HW and the antagonist CPA-8 was not detected in fruit with natural inoculum. A possibility could be that in this case, inoculum can be located in both on the fruit surface and also inside fruit, and the inoculum development depends on the infection time. All of this makes more difficult the complementation of the control mechanisms of each treatment. However, when fruit were incubated previously for 21 d at 0°C, the efficacy of both treatments were drastically reduced and the HW treatment was the only one that had a slight effect in controlling brown rot. At both storage temperatures evaluated, fruit were too mature to find quality differences between them with the parameters determined in our work. However, after storing fruit for 21 d at 0°C, fruit senescence was probably at a higher advanced state because of pathogen development. These findings confirmed that HW is not an eradicative treatment against *M. laxa* and that the antagonist CPA-8 had no effect in terms of brown rot reduction when fruits were incubated at 0°C.

In conclusion, our results showed that the HW treatment at 60°C for 40 s plus strain CPA-8 of the *B. subtilis* species complex could be effective alternative treatments to control brown rot on peaches and nectarines incubated at 20°C, without affecting fruit quality, and that could be implemented in packing houses. However, when fruit were incubated for 21 d at 0°C and then transferred to 20°C, although HW treatment was still effective, the antagonist CPA-8 generally did not control disease development. Although under the commercial conditions in packing houses, peaches and nectarines are not widely incubated for long periods of time at 0°C, future studies will focus on the adaptation of the microorganisms to cold storage.

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